KnipBio

Lawrence Feinberg, PhD
CEO
110 Canal street
Lowell, MA 01852

To the attention of
FDA
Division of Animal Feeds,
7519 Standish Place, HFV-220,
Rockville, MD 20855.

November 20, 2017

Re: GRAS notification submission for "***Methylobacterium extorquens* protein for use as a replacement for soybean or fishmeal at levels up to 10% of the diet of aquaculture species"**

Dear Colleagues,

Please find associated with this letter, a notification of GRAS status for a single cell protein derived from the leaf symbiont *Methylobacterium extorquens* for the use as an ingredient in aquaculture feeds for finfish or crustaceans. Having consulted with members of the CVM team throughout this process, the attached dossier represents a multi-year effort to collect data proving the efficacy for this material as a source of protein in animal diets for aquaculture.

KnipBio, a privately held company based in the Boston, MA area has developed a basis of understanding for the single cell protein derived from *M. extorquens,* and will refer to this ingredient within the dossier as KnipBio Meal, or KBM. KBM is a protein flour originating from a non-pathogenic, BSL1 leaf symbiont rich in protein and non-pigmenting carotenoids with decades of study documenting its physiology, genetics and other traits in the public record. The company's goal is to replace a portion of the expensive fishmeal or less digestible soybean meal in a given aquafeed by substituting KBM, which is possibly cheaper to produce, highly digestible and is comparable in its composition to alternatives.

Successful and consistent pilot scale fermentations demonstrated scalability and commercial manufacturing practices. The biomass generated from these campaigns were used for testing for safety and efficacy in various aquafeed diets according to standard recipes. The inclusion rate of up to 10% was proven to be an effective replace protein from soybean or fishmeal, while maintaining a significant gain in weight, FCR (feed conversion ratio) and overall digestibility. The target animals for use in aquaculture feeds include finfish and crustaceans based on studies with small mouth grunts, rainbow trout, Atlantic salmon and Pacific white shrimp.

KnipBio

M. extorquens is not a pathogen, does not produces toxins, is used at relatively low inclusion rates and did not show any harm in all our trials, KnipBio does not anticipate any safety issue for human consumption of fish or crustaceans fed KBM. We thank you in advance for your consideration of our notification.

For additional information or to ask follow-up questions, please do not hesitate to contact me using the information below.

Sincerely,

Lawrence Feinberg, CEO
KnipBio Inc
110 Canal Street, Lowell MA 01852
www.knipbio.com
lfeinberg@knipbio.com

GRAS Notice

Methylobacterium extorquens protein

for use as a replacement for soybean or fish meal al levels up to 10% of the diet of aquaculture species

Submitted by:
KnipBio, Inc.
110 Canal Street
Lowell, MA 01854
Phone: (978) 636-5647

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Part 1. Introductory Information

(1) The undersigned is hereby submitting a GRAS Notice in accordance with 21 CFR Subpart E, Section 570.

(2) The name and address of the organization is:

KnipBio, Inc.
110 Canal Street
Lowell, MA 01854
Phone: (978) 636-5647
info@knipbio.com

(3) Name of the notified substance:

Methylobacterium extorquens protein

(4) Intended conditions of use of the notified substance:

The substance will be used as a replacement for soybean or fish meal, to constitute up to 10% of the diet, in fish feed used for aquaculture.

(5) KnipBio, Inc. has concluded, through scientific procedures in accordance with §570.30(a) and (b), that the substance has GRAS status for the intended use.

(6) KnipBio, Inc. has concluded that the notified substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on the aforestated conclusion that the notified substance is GRAS under the conditions of its intended use.

(7) If the Center for Veterinary Medicine (CVM) asks to see the data and information that are the basis for this conclusion of GRAS status, either during or after its evaluation of this notice, KnipBio Inc. will:

(i) agree to make the data and information available to CVM; and
(ii) agree to both of the following procedures for making the data and information available to CVM:
(A) Upon CVM's request, KnipBio, Inc. will allow CVM to review and copy the data and information during customary business hours at the above-stated address, where these data and information will be available to CVM; and
(B) Upon CVM's request, KnipBio, Inc. will provide CVM with a complete copy of the data and information either in an electronic format that is accessible for its evaluation or on paper.

(8) Certain of the data and information in Parts 2 through 7 of this GRAS Notice are exempt from disclosure under the provisions of §552 (e.g., as trade secret or as commercial or

financial information that is privileged or confidential). Information claimed as confidential is shown in this document in black-bordered boxes.

(9) The undersigned hereby certifies that, to the best of KnipBio's knowledge, this GRAS Notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to KnipBio and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

(10) The name and title of the person who has signed this GRAS Notice is:

By: 

Name: Larry Feinberg

Title: Chief Executive Officer

Address: KnipBio, Inc.
110 Canal Street
Lowell, MA 01854

Part 2. Identity, method of manufacture, specifications, and physical or technical effect.

(a) Scientific data and information that identifies the notified substance.

The notified substance, *Methylobacterium extorquens* protein, is a Single Cell Protein preparation derived from *Methylobacterium extorquens*. Specifically, it is a spray-dried preparation derived from the biomass resulting from large-scale fermentation of strain KB203 of *M. extorquens*. The product is characterized by a protein content of greater than 50% and is suitable for use as a replacement for soybean or fish meal protein to constitute up to 10% of the total diet in aquaculture feed. Examples of species for which the notified substance can be used include shrimps (Pacific white shrimp, *Litopenaeus vannamei*), salmon (Atlantic salmon, *Salmo salar*) and trout (Rainbow trout, *Oncorhynchus mykiss*).

The α-proteobacterium *M. extorquens* (previously identified as a *Pseudomonas* sp.) has been studied for over 50 years (Large *et al.*, 1961). The prior species designation was first proposed to be changed to *Methylobacterium extorquens* by (Green and Bousfield, 1982), who reviewed 150 pink-pigmented facultatively methylotrophic bacteria (PPFMs) of the *'Pseudomonas extorquens'* type and recommended that they be reclassified as *Methylobacterium*. (Kelly, McDonald and Wood, 2014) say that the strain now known as *M. extorquens* is "possibly the earliest strain of a PPFM to be described was the oxalate-degrading organism isolated from earthworm excreta ("worm casts"), in the Botany Department garden of Basel University, in 1909 by Bassalik (1913)". *M. extorquens* is a facultative aerobic Gram-negative naturally occurring bacterium found in nature as a leaf symbiont (Balachandar *et al.*, 2008; Dourado *et al.*, 2013; Gourion *et al.*, 2006; Kutschera, 2007). It has the ability to use C1 compounds such as methanol as a carbon source and therefore has been the model system of choice for uncovering the genetic basis of growth on these compounds (i.e., methylotrophy) (Chistoserdov *et al.*, 1991: Chistoserdova *et al.*,2003; Gourion *et al.*, 2006; Marx *et al.*, 2003; Muller *et al.*, 2011; Šmejkalová *et al.*, 2010; Andersen, 2014; Ward *et al.*, 2004).

Strains of *M. extorquens* are available from the American Type Culture Collection, Accession Numbers ATCC_55366, ATCC_43645, ATCC_BAA-2500, ATCC_8457 and ATCC_14718 (ATCC, 2017). This species is designated as Biosafety Level 1 (ATCC, 2017). The complete genome sequences are now available for 7 strains of *M. extorquens* as well as about 20 other species in the genus (NCBI) (Marx *et al.*, 2012; Ward *et al.*, 2004; Peyraud *et al.*, 2011; Vuilleumier *et al.*, 2009). Reference sequences can be found on the following websites:
https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=419610
http://hamap.expasy.org/proteomes/METEP.html
http://www.ebi.ac.uk/Tools/dbfetch/expasyfetch?CP000908
https://www.ncbi.nlm.nih.gov/genome/?term=Methylobacterium%20extorquens%20PA1
https://biocyc.org/

Methylobacterium extorquens strain CM2730 (renamed KB201 in KnipBio's culture collection) was acquired from the laboratory of (b) (4), then located at

(b) (4) . This strain was described and characterized in Delaney *et al.* 2013 (Delaney *et al.*, 2013).

Following the gene deletion that is described below and which generated KB17, KnipBio streaked out a culture of that strain to determine if there was any contamination during in-house lab fermentation. One pink colony was selected from the culture and outsourced for 16S rRNA sequencing to (b) (4) who used standard in-house 16S bacterial forward and reverse primers. The sequence obtained was then compared to bacterial database using the BLAST program on the NCBI website. The results shown in **Appendix 2-1** confirm that strain KB203 is a *Methylobacterium extorquens* strain. That isolate was cultured and saved under the name KB203. Deletions of the (b) and (b) loci were verified by PCR. Moreover, the full genome sequence of the different strains described above was performed as explained in the "**Stability of Strain**" paragraph below.

(b) A description of the method of manufacture of the notified substance in sufficient detail to evaluate the safety of the notified substance as manufactured;

(b)(1) Construction and Characterization of the Production Microorganism (Pre-fermentation)

Overview. (b) (4)
(b) (4)

Strain Construction and Verification. (b) (4)
(b) (4)

(b) (4)

(b) (4)

(b) (4)

(b) (4)

Stability of Strain. The genetic stability of the strain has been shown through sequence analysis of samples as described in **Appendix 2-3**. As described in the **Appendix**, sequence analysis was conducted for the following *M. extorquens* strains:

- **KB201**: PA1 with a (b) (4) (CM2730 obtained from (b) (4))
- **KB17**: KB201 with a (b) (4) (obtained from (b) (4))
- **KB203**: the KB17 strain, as isolated and cultured at KnipBio
- **KB203-EF**: a culture aliquot obtained at the end of a (b) (4) fermentation of KB203 done in June 2016 (this strain was submitted to all sub-culturing events through serial growth in seed cultures and scale-up fermentation; approximately 20 generations)

As shown in **Appendix 2-3**, no difference could be detected in the (b) (4) between KB201, KB17, KB203, and KB203-EF. In addition, no difference could be detected in the (b) (4) deletion between strains KB17, KB203 and KB203-EF. **Appendix 2-2** also includes data showing that the deletion of the (b) (4) did not result in the creation of any toxin-encoding nucleic acid sequence. This shows that over time there is no variation in the deleted regions and that there is no new protein or toxin produced by the deletion. Therefore, KnipBio does not anticipate any spill-over effect from the modifications performed. The deletions described do not affect any properties of the strain that would be expected to be relevant to its safety status. KnipBio has sequenced the entire genome of its production strain, and this sequence can be made available to CVM for verification of the deletions and the stability of the strain.

(b)(2) Fermentation of the Production Microorganism to Manufacture the Notified Substance

Overview. (b) (4)
(b) (4)

(b) (4)

(b) (4)

(c) Specifications for material that is of appropriate grade for use in animal food.

The specifications for the notified substance are shown in **Appendix 2-8**. The composition of the material is summarized in the Data Sheet in the **Appendix** and is shown below in **Table 2-1(b)**, based on the batch analysis in **Table 2.1(a)**. Please note that the total of these components is not 100%. Other components such as RNA and carbohydrates are not measured but are known to be part of the notified substance (Peyraud *et al.*, 2011).

(*Table 2-1 begins on the next page. The rest of this page has been left blank*).

Table 2-1. Specifications of the Notified Substance

(a) Batch Analysis

Batch	Jun-15	Jun-16	Oct-16	Average
Analysis date	9/14/15 (%)	6/15/16 (%)	10/31/16 (%)	
Moisture	(b) (4)			4.82 +/- 0.8
Protein				51.82 +/- 1.5
Fat				0.48 +/- 0.3
Fiber				0.17 +/- 0.2
Ash				6.71 +/- 2.6
PHB				22.44 +/- 2.5

** = Not measured

Batch	Jun-15 (%)	Jun-16 (%)	Oct-16 (%)	Average
Analysis date	9/14/15	6/15/16	2/10/17	
Amino Acid				
Alanine	(b) (4)			4.21 +/- 0.40
Arginine				3.59 +/- 0.27
Aspartic Acid				4.32 +/- 0.18
Cysteine				0.36 +/- 0.04
Glutamic Aid				6.77 +/- 0.65
Glycine				2.66 +/- 0.16
Histidine				1.08 +/- 0.04
Isoleucine				1.87 +/- 0.18
Leucine				3.46 +/- 0.22
Lysine				2.87 +/- 0.41
Methionine				0.76 +/- 0.13
Phenylalanine				2.10 +/- 0.08
Proline				2.14 +/- 0.36
Serine				1.83 +/- 0.12
Threonine				2.25 +/- 0.26
Tryptophan				0.29
Tyrosine				1.47 +/- 0.04
Valine				2.90 +/- 0.20
TOTAL:				44.75 +/- 2.32

** = Not measured

(b) Average Analysis

Component	Percentage by Weight
Protein:	50% - 53%
Fat:	0.1% - 0.7%
Ash:	4.2% - 9.5%
Moisture Content:	4.0% - 5.6%
Polyhydroxybutyrates	ca. 20%
Salmonella:	Absent

Amino Acid	% (typical)	Amino Acid	% (typical)
Methionine	0.7-0.9	Glycine	2.5-2.8
Cysteine	0.3-0.4	Histidine	1.0-1.1
Lysine	2.5-3.3	Threonine	2.0-2.5
Tryptophan	~0.3	Valine	2.8-3.1
Arginine	3.3-3.8	Leucine	3.3-3.7
Isoleucine	1.7-2.1	Phenylalanine	2.0-2.2

The notified substance provides an excellent source of protein for the target fish species, and in particular includes high concentrations of specific amino acids including alanine, glycine, and valine (Cf. **Tables 2-2** and **2-3**). The ash content was seen to vary among the different analyses at different times, and since it is not possible for there to be any change in concentration during storage, we assume that the differences seen are the result of statistical variation of the analytical method, within the expected margin of error.

The following **Tables 2-2 and 2-3** compare the composition of *Methylobacterium extorquens* protein with that of other sources of protein provided by fishmeal or soybean meal. *Methylobacterium extorquens* protein is a comparable source of protein as compared to soybean meal and some fishmeal (i.e. tuna or local mixed species). Although the notified substance is low in certain amino acids, it is common practice for feed manufacturers to supplement any deficiencies in the feed with added amino acids.

(*Table 2-2 is shown on the next page. The rest of this page has been left blank*).

Table 2-2. Proximate composition of the ingredients used in the growth and digestibility trials.

The composition of the notified substance is as reported to KnipBio by (b) (4) (b) (4)

Composition	Notified Substance (KB0203)	Fish meal[1]	Soybean meal[2]
Crude protein	52.42	62.78	44.89
Moisture	3.50	7.99	10.97
Crude fat	1.29	10.56	3.78
Crude fiber	0.00	0.00	3.20
Ash	4.25	18.75	6.67
Phosphorus	1.00	3.15	0.66
Alanine	3.81	3.91	2.04
Arginine	3.23	3.68	3.35
Aspartic acid	3.72	5.34	5.10
Cysteine	0.30	0.47	0.62
Glutamic acid	6.13	7.47	8.24
Glycine	2.73	4.88	2.04
Histidine	0.80	1.63	1.20
Isoleucine	1.80	2.42	2.17
Leucine	3.24	4.21	3.57
Lysine	2.79	4.67	3.06
Methionine	0.86	1.61	0.66
Phenylalanine	2.04	2.39	2.35
Proline	2.25	3.08	2.39
Serine	1.21	2.11	1.90
Taurine	0.08	0.73	0.13
Threonine	2.00	2.41	1.75
Tryptophan	0.10	0.62	0.62
Tyrosine	1.34	1.67	1.64
Valine	2.84	2.99	2.34

Ingredients were analyzed at (b) (4) (b) (4)). All data is on an "as is" basis.

[1] (b) (4)

[2] De-hulled solvent extract soybean meal, (b) (4)

Table 2-3. Proximate composition of commonly used feed ingredients: Protein supplements.

Feedstuff	Dry Matter	Crude Protein	Crude Fat	Crude Fiber	Ash
Fish meal, Alaskan pollack	91.53	71.94	9.58	0.1	5.32
Fish meal, local, mixed species	91.12	52.89	5.78	3.11	21.9
Fish meal, Peruvian	92.83	67.39	6.97	0.89	18.84
Fish meal, tuna	93.24	52.04	10.37	1.56	24.15
Fish meal, white	93.63	68.68	6.78	1.46	20.16
Soybean meal, full fat	93.69	37.51	20.45	2.27	5.67
Soybean meal, defatted, undehulled, expeller process	89	42	3.5	6.5	6
Soybean meal, defatted, dehulled, solvent process	89.3	48	1	3	6
KnipBioMeal Range	94.22- 95.99	50.22- 53.15	none- 0.75	none- 0.26	4.09- 9.47

Data in %

http://www.fao.org/docrep/003/W6928E/w6928e1k.htm

The single cell protein preparation has low residual concentrations of viable *M. extorquens* organisms. Viable cell count (CFU/mL) in 3 batches of SCP flour was determined as described in KnipBio SOP #23 that is found in **Appendix 2-9**. Briefly summarized, 50 mg of SCP from (b) (4) fermentation batch was resuspended in 1 mL salt media and 0.1 mL of serial dilutions were plated in triplicate on agar media. Plates were incubated at 30°C and colonies were counted after 5 days. The results are shown in **Tables 2.4 (a) and (b).**

As shown in the **Tables**, a four-log killing occurred from the initial step of spray-drying the biomass. KnipBio expects that a further reduction in the viable cell count will occur during the downstream processing of the SCP into the feed pellets (e.g. through extrusion), as these processes are often carried out at high temperature.

Table 2-4. Viable Cell Counts in the notified substance.
(a) Cell count raw data

Colonies — Sample name

Dilution	Jun-15	Jun-16	Oct-16
1.00E+04	(b) (4)		
1.00E+04			
1.00E+04			
Average	203	46	190
std	9	15	19
1.00E+05	(b) (4)		
1.00E+05			
1.00E+05			
Average	19	8	18
std	7	3	8
1.00E+06	(b) (4)		
1.00E+06			
1.00E+06			
Average	1	1	2
std	1	1	1

cfu / mL

	Jun-15	Jun-16	Oct-16
	(b) (4)		
Average	2.03E+07	4.60E+06	1.90E+07
std	8.89E+05	1.47E+06	1.89E+06
	(b) (4)		
Average	1.90E+07	8.33E+06	1.77E+07
std	6.56E+06	2.52E+06	8.02E+06
	(b) (4)		
Average	6.67E+06	6.67E+06	2.00E+07
std	5.77E+06	1.15E+07	1.00E+07

(b) Calculation of estimated killing.

	Jun-15	Jun-16	Oct-16
final OD	(b) (4)		
total L			
g/L biomass			
CFU/ L			
CFU/ g			
g analyzed			
CFU/g if no cell death			
Average CFU/ g	1.57± 0.79 E+07	0.65± 0.62 E+07	1.89± 0.66 E+07

The stability of the single cell protein preparations has been studied in two ways. In one method, samples received from the fermentation runs conducted by (b) (4) are stored at KnipBio's laboratory in their initial package at room temperature (65-70° F, ambient humidity). This initial package consisted of 8-10 kg of Single Cell Protein stored in a double polypropylene bag stored in a black polypropylene pail. On a regular basis thereafter, samples from the package were sent to (b) (4) for proximate analysis. Some recent results are shown in **Table 2-5** below, and it can be seen that there is no significant change in the concentration of any of the assayed constituents. The analyses from (b) (4) underlying this data can be seen in **Appendix 2-8**.

Table 2-5. Changes in Composition of the Notified Substance During Room Temperature Storage.

Run date	Analysis date	Moisture	Protein	Fat	Fiber	Ash	PHB
Jun-15	7/24/17						(b)
	05/17/17	(b) (4)					
	10/31/16						(b)
	08/17/16	(b) (4)					
	09/14/15						
	Avg	**5.19**	**52.17**	**0.09**	**0.17**	**6.25**	
	std	**0.79**	**0.31**	**0.07**	**0.10**	**2.43**	
Jun-16	7/24/17						(b)
	05/17/17	(b) (4)					
	02/10/17	(b) (4)					
	10/31/16						
	09/09/16						
	06/15/16						(b) (4)
	Avg	**4.84**	**50.25**	**0.59**	**0.26**	**6.81**	
	std	**0.66**	**0.23**	**0.24**	**0.04**	**1.46**	
Oct-16	7/24/17						(b)
	05/17/17	(b) (4)					
	02/10/17						
	11/09/16						
	10/31/16						(b)
	Avg	**4.45**	**52.96**	**0.54**	**0.13**	**7.26**	
	std	**0.28**	**0.25**	**0.04**	**0.11**	**2.23**	

Additional stability testing has been performed by (b) (4), (b) (4). The aim of this study was to determine a theoretical 14 months shelf stability of the product under accelerated storage conditions from receipt of sample. Test samples from the October 2016 fermentation batch were aliquoted in small polypropylene bags. Once received by the (b) (4), these samples were stored immediately in controlled environmental conditions (45°C ± 2°C with ambient humidity for up to 12 weeks) and then tested at the time points 0, 3, 6, 9, 11 and 12 weeks to determine the optimum storage time for the products. A summary of the results is shown in **Table 2-6** below, and the data underlying these results are shown in **Appendix 2-10**.

Table 2-6. Stability of the Notified Substance Under Accelerated Storage Conditions.

Analysis	Method	Time 0	3 weeks	6 weeks	9 weeks	11 weeks	12 weeks
Total coliform (cfu/g)	MFHPB-34*	<5					
E. Coli (cfu/g)	MFHPB-34*	<5					
Standard Plate Count (cfu/g)	MFHPB-18*	1,800	87,000	180,000	57,000	120,000	120,000
Staph. Aureus (cfu/g)	MFLP-21*	<5					
Salmonella (cfu/g)	MFHBP-20*	-ve					
Yeast (cfu/g)	MFHPB-22*	<5	<5	<5	<5	<5	<5
Mold (cfu/g)	MFHPB-22*	5	<5	<5	<5	<5	<5
Protein (%)	AOAC2001.11*	49.4					
Fact Acidity (mg/KOH/100g)	AACC 02 01A (FFA-03)	5.00	5.75	13.5	7.74	18	9.23

The company also sent samples from each (b) (4) fermentation batch that had been stored at room temperature, ambient humidity, for analysis. These results are shown in **Table 2-7**. We note that the CFU count of yeast and mold in the sample (b) (4) is higher than the other samples in this analysis and higher than the counts seen in the time course experiment above. One possible explanation for this anomalous result is that sample (b) (4) was taken from a container that is stored at the KnipBio laboratory and that it is often opened and sampled for regular analysis in the laboratory, so that yeast and mold contamination may have occurred during such frequent access to this container. KnipBio does not anticipate this to happen during the manufacturing of the final feed.

Table 2-7. Stability of the Notified Substance in Storage at Room Temperature.

Product ID and Condition	Protein Flour 2016-10-81 Good, Intact	Protein Flour 2016-06 Good, Intact	Protein Flour 2015-06 Good, Intact	DL	Date Tested	Method Used
Lab ID Number	(b) (4)	(b) (4)	(b) (4)			
Analysis						
Total Coliforms (cfu/g) Presumptive	(b) (4)				5/8/17	MFHPB-34*
Total Coliforms (cfu/g) Confirmation						
E.coli (cfu/g) Presumptive					5/8/17	MFHPB-34*
E.coli (cfu/g) Confirmation						
Standard Plate Count (cfu/g)					5/8/17	MFHPB-33*
Staphylococcus aureus (cfu/g) Presumptive					5/8/17	MFLP-21*
Staphylococcus aureus (cfu/g) Confirmation						
Salmonella (Positive/ Negative) Presumptive					5/8/17	MFHPB-20*
Salmonella (Positive/ Negative) Confirmation						
Yeast & Mold (cfu/g)					5/8/17	MFHPB-22*
Crude Protein (%)					5/9/17	AOAC 2001.11 (PROT-01*)

Source: (b) (4) Certificate of analysis (b) (4); copy can be provided to CVM upon request.

(d) When necessary to demonstrate safety, relevant data and information bearing on the physical or other technical effect the notified substance is intended to produce, including the quantity of the notified substance required to produce such effect.

Methylobacterium extorquens single cell fermentation product is a valuable source of protein for use in aquaculture feeds when used at levels up to 10% of the feed. The analytical data demonstrate that the product will be at least 50% protein, with a balanced complement of amino acids. Part 2(c) of this Notice provides specific information on the composition of the product. There is a number of similar biomass products that are used as protein sources in animal (including aquaculture) feed listed as AAFCO defined ingredients 36.15 and 36.16 and assorted fermentation products (AAFCO 36.2-36.13). Consideration of microbial proteins as valuable for feeds have been covered in a number of publications (Nasseri, 2011; Matassa, 2016; Anupama and Ravindra, 2000; Goldberg, 1985; EFSA, 2005; National Research Council, 2011b).

KnipBio has corroborated the availability of the notified substance for use in aquaculture with a number of studies with various aquaculture species. Many of these studies have been published or are in the process of being published. But all are considered corroborative to support the analytical data demonstrating the nutritive value of the protein source.

The notified substance has utility in the use as a protein supplement for aquaculture. As demonstrated in (Tlusty *et al.*, 2017), the product provides available protein needed for the growth of fish in aquaculture. Tlusty *et al.* describes several animal feeding studies using the notified substance, including feeding studies on Pacific white shrimp (*Litopenaeus vannamei*) growth and consumer taste preference, a study for which FDA has reviewed the protocol. KnipBio has also conducted a 12-week feeding study on trout (*Oncorhynchus mykiss*), for which FDA also reviewed the protocol (results found in the Hardy *et al.* manuscript being prepared for submission, included in **Appendix 2-11**). In each of these studies, animals performed equivalently when fed diets containing *M. extorquens* as when fed a standard aquaculture diet. As the data supports the use of the substance as a valuable source of protein in a wide variety of aquaculture species (shrimp, trout, salmon, and small mouth grunt) KnipBio has requested the use in all aquaculture species.

Tlusty *et al.* (Tlusty *et al.*, 2017) includes a comprehensive summary of the methodology used in these studies. The results reported in the paper demonstrate the potential broad applicability of *Methylobacterium extorquens* protein, made from *Methylobacterium extorquens*, as a viable protein source for use in aquafeeds. When fed to fishes it resulted in equivalent performance in growth for grunts, and apparent digestibility coefficient for salmon as compared to traditionally formulated diets containing fishmeal. The salmon demonstrated higher digestibility for amino acids in diets containing the notified substance. The food conversion ratio of shrimp was best when there was 50% substitution with the notified substance, yet growth (weight gain and SGR) was greatest in the control diets. One possible reason for this discrepancy is that the consumption of feed between the different diets was not equivalent. As stated in the manuscript, there were air bubbles in the shrimp diet SHR-KH (100% KBM replacement) that did not sink as well as the other two diets that did not have air bubbles (SHR-LK and SHR-C). While all pellets were consumed by the shrimps, those fed SHR-HK would have needed to swim in the water column to retrieve some of the pellets, while those fed SHR-LK and SHR-C mostly fed off the tank floor, and such increased activity may account for the reduced weight gain and SGR. Another possibility is that the SHR-KH diet may have been less palatable, as the shrimps had a lower feed efficiency of this diet than the other diets. Whether this is an absence of an attractant not replaced in SHR-KH or the absence of a critical nutritional component like methionine (D.A. Davis, personal communication) in the diet, the exact cause is unknown at this time".

The Hardy *et al.* manuscript being prepared for submission, included in **Appendix 2-11**, shows that the notified substance, when fed to trout, resulted in equivalent performance for growth, feed utilization and nutrient retention, as compared to traditionally formulated diets containing fishmeal.

The data shown in Tlusty *et al.* (Tlusty *et al.*, 2017), as well as procedures and data for additional studies conducted by KnipBio to date, are shown in **Appendix 2-11. Table 2-8** below summarizes the results of the studies described in Tlusty *et al.*, as well as other studies that KnipBio has conducted or sponsored, in which aquatic species were fed the notified substance (in some cases, early formulations of the notified substance). In this Table, the studies published in the Tlusty paper are highlighted in blue and the study

described in the Hardy *et al.* manuscript highlighted in grey. The other studies have not been published, and in some cases, were not designed to produce publishable data, but they all show that the notified substance caused no harmful effects on the fish. Full study records for these experiments, particularly including the studies published in Tlusty *et al.*, and the Hardy *et al.* manuscript are available at the KnipBio offices should FDA be interested in reviewing them.

The data presented in this Section and its Appendices clearly show that the notified substance has utility in providing a source of protein for the target aquatic species: shrimp (Pacific white shrimps, *Litopenaeus vannamei*), Salmon (Atlantic salmon, *Salmo salar*) and trout (Rainbow trout, *Oncorhynchus mykiss*). Furthermore, for reasons explained in detail in the "**Narrative**" section below, KnipBio contends that the data from investigations support the broader use of the notified substance for all aquaculture feed, with the three species named above fulfilling all of the required criteria of covering a broad diversity of species that are well-studied, sensitive to testing, and commercially relevant.

(*Table 2-8 is shown on the next page. The rest of this page has been left blank*).

Table 2-8. Summary of Fish Feeding Studies with the Notified Substance.

Date and location	Goal	Animal	# of animals	% Notified Substance	Trial Length	Start size gm	Survival %	Conclusion
2014- Roger Williams University	Deposition	Clown fish	68	5 - 25	100 days	1.1 ± 0,03		No effect on coloration
2016-USDA-University of Idaho's Hagerman Fish Culture Experiment station	Fish meal replacement	Trout Lodge	75	5 - 10	12 weeks	15.6 ± 0.2	97- 100	No statistical difference w/ control diet
NEAq- Roger Williams University	FM replacement- Growth and survival	Shrimp	720	50-100	60-105 days			No effect on survival. Slight growth defect w/ 100% replacement. No difference in human taste trial.
2016- University of Auburn-Alabama	Soybean meal replacement. Growth and survival	Shrimp	96	6 - 12	6 weeks	~ 1.51	> 97	No statistical difference in weight gain compared to control diet
2016- University of Auburn-Alabama	Replacement of SBM. Retention efficiency of AA.	Shrimp	240	1 - 12	6 weeks	~ 0.98	92.5 - 100	No statistical difference up to 6% inclusion
2016- University of Auburn-Alabama	Replacement of SBM. Digestibility	Shrimp	240	6-26.6	6 weeks	~ 0.15	97.5 – 100	No statistical difference up to 12% inclusion
2016- Hawaii	Growth	Kampachi	66	5	12 weeks			Diet has produced statistically comparable growth to the control over the duration of the trial
Oregon State University	Taste							
2014- Roger Williams and NEAq	FM replacement. Growth and microbiome	Smallmouth grunts	120	5 - 25	41 days	1.37 ± 0.27		No statistical difference with the control diet. No significant effect on gut microbial community
2014- USDA Maine	Digestibility	Atlantic salmon	96	29.7	4 days	635±97		No significant difference w/ USDA control diet

Part 3. Target animal and human exposures.

In this Part 3 of the Notice, KnipBio provides data and information about exposure to the target animal and to humans consuming human food derived from food-producing animals.

(a) Exposure to the target animal.

> *(a)(1) The amount of the notified substance that different target animal species are likely to consume in the animal food (including drinking water) as part of the animal's total diet, including the intended use and all other sources in the total diet*

The notified substance is a Single Cell Protein preparation derived from *Methylobacterium extorquens*. The product is characterized by a protein content of 50-53% and is suitable for use as a replacement for soybean or fish meal protein to constitute up to 10% of the total diet in aquaculture feed, including feed for shrimps (Pacific white shrimp, *Litopenaeus vannamei*), salmon (Atlantic salmon, *Salmo salar*) and trout (Rainbow trout, *Oncorhynchus mykiss*).

> *(a)(2) When applicable, the amount of any other substance that is expected to be formed in or on food because of the use of the notified substance (e.g., hydrolytic products or reaction products)*

KnipBio believes that there will be no other substances formed in or on food because of the use of the notified substance, including any possible hydrolytic products, reaction products or other such substances.

> *(a)(3) When applicable, the amount of any other substance that is present with the notified substance either naturally or due to its manufacture (e.g., contaminants or by-products)*

The microbial strain on which the notified substance is based, *Methylobacterium extorquens*, is a natural producer of polyhydroxybutyrates (PHBs). The notified substance is therefore expected to contain certain amounts of PHBs, which might range from 15-25% depending on the fermentation conditions. As discussed below, KnipBio believes that the expected levels of PHBs in the notified substance will be well below the levels that might be expected to be harmful to the target species.

Other substances that might be present in the notified substance due to its manufacture are methanol (used as a feedstock in the fermentation) and formaldehyde. As discussed below, KnipBio believes that the expected levels of both compounds in the notified substance will be well below the levels that might be expected to be harmful to the target species.

M. extorquens is a natural producer of the C30 class of carotenoids, but these compounds, when ingested by aquatic animals, do not impart color to the flesh (Takaichi, 2009); (Torrissen *et al.*, 1989; Konovalova *et al.*, 2007; Delgado-Vargas, *et al.*, 2000). In addition, *M. extorquens* is supposedly also capable of producing the C40 carotenoid spirilloxanthin, but in the specific parental strain employed by KnipBio, it has recently been found that the spirilloxanthin pathway is inactive. Before learning this, the company deleted this pathway; however, deletion of the pathway does not affect the carotenoid profile of the strain, nor does it change the total amount of carotenoids synthesized by the strain, as detected by a full spectral analysis.

The data and information that is relied on to establish the amounts of these substances in the notified substance are presented in the following section.

> *(a)(4) The data and information you rely on to establish the amount of the notified substance and the amounts of any other substance in accordance with paragraphs (a)(1) through (a)(3) of this section that different target animal species are likely to consume in the animal food (including drinking water) as part of the animal's total diet*

Information has been presented in Part 2 above and its Appendices showing the composition of the notified substance, including its main components protein, moisture, fat and ash, as well as the analytical methods used to determine the composition. The animal will digest and absorb these nutrients as it would for other protein feed ingredients.

Concentration of PHBs in the notified substance. Experiments have been conducted to measure the concentration of PHBs that might occur in the notified substance. The results of a 2016 analysis by the National Research Council of Canada are shown in a March 30, 2016 report (see **Appendix 3-1**). *M. extorquens* cells grown in the DASGIP (1-liter) reactor were harvested and processed using the methods described in the **Appendix**. PHB content was then measured by GC using the methods of (Braunegg, *et al.* 1978) and (Bourque *et al.*,1992). As shown in the March 30, 2016 report, after 69 hours of fermentation in the 1-liter vessel (longer than the company's current production runs at larger scale fermentations; see below), PHB levels were found to be between 26.7 and 31.8 percent of the cell mass by weight.

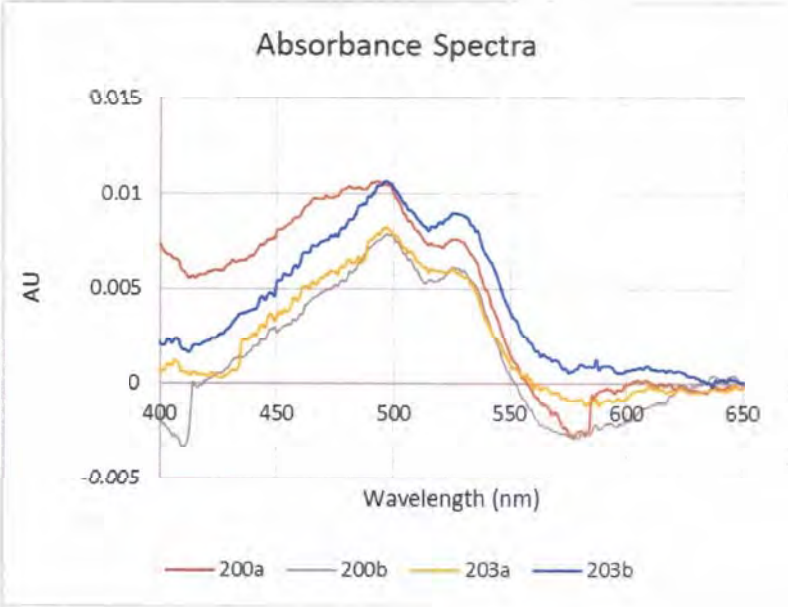
KnipBio has also developed a UPLC method for measuring PHB (described in **Appendix 3-2**), which the company has verified by comparison of results using this method to results of the Canadian NRC using published methods. Specifically, using the same end-of-fermentation samples, CNRC measured the PHB according to the published method shown in **Appendix 3-1**, and KnipBio extracted the PHBs from these samples as described in **Appendix 3-2**, and quantification was established by extrapolation with a standard-curve based on a known amount of PHB granules (Sigma, cat-363502 , USA). The SOP for the standard curve calibration can be provided to CVM upon request.

The company usually measures PHB either from the end of a culture (1L or 1500L) pellet that has been washed and frozen or from the end product (SCP) that has been spray-dried.

Recent measurements using this method on samples from 48-hour runs in the company's 1500L fermentations show PHB levels between 16 and 25% (Cf **Table 2-5**). Based on this data, it is the company's expectation that PHB levels in commercial fermentations will not exceed 25%, and that the higher concentrations seen in the 1-L fermentation runs discussed above were the result of the longer (69-hour) fermentation time.

Concentration of carotenoids in the notified substance. In order to detect if the deletion of the cellulose pathway and the spirilloxanthin pathway affected the production of carotenoids in KB203, the carotenoid profile of *Methylobacterium extorquens* PA1 (internally designated KB200) was compared to the related strain KB203 (KB200 Δ*celABC* Δ*crtCDF*). Cells of KB200 (PA1) and KB203 were grown in shake flasks with minimum medium supplemented with methanol and crude extracts were prepared as described in **Appendix 3-3**. These crude extracts were analyzed both by measuring the absorbance of samples by spectrophotometer from 350-800 nm wavelengths at 1 nm intervals (total carotenoid spectrum (**Figure 3-1**), and by Ultra Performance Liquid Chromatography (UPLC) to determine the specific carotenoids present (**Figures 3-2 and 3-3**).

Figure 3-1. Total Carotenoid Profile of Strains KB200 and KB203 Determined by Spectrophotometer Analysis



The spectrophotometric analysis shows that there is no difference in the total amount of carotenoids produced by the KB200 parent and strain KB203 (**Table 3-1** below). This strongly suggests that the deletion of the spirilloxanthin pathway does not influence the

total production of carotenoids. It also confirms that the production of C30 carotenoids by the KB203 strain is the same as production by the KB200 parent strain, and that in both strains, total C30 carotenoid concentrations are no greater than 60 ppm.

Table 3-1. Spectrophotometric Analysis of carotenoids produced by Strains KB200 and KB203.

Sample	Absorption	λmax	Total carotenoids (ug)	DCW content (ppm)
Blank	0.000000			
KB200a	0.010697	493	1.712	57.1
KB200b	0.007862	497	1.258	41.9
KB203a	0.008246	497	1.319	44.0
KB203b	0.010716	497	1.715	57.2

Carotenoid compounds were also analyzed by tunable UV (TUV) detector (470 nm wavelength). The TUV_470 nm spectra of strain KB200 and KB203 are presented in **Figure 3-2** below, and no significant difference was seen between the spectra of the two strains. This confirms that the deletion of the spirilloxanthin pathway does not affect the carotenoid profile of strain KB203. This strongly suggest that the parent strain KB200 does not produce spirilloxanthin under the experimental conditions.

These results were also confirmed by mass spectrometry (MS) analysis of the samples as represented in **Figure 3-3** below. The MS profiles of both strains show no significant difference between the two strains.

Concentration of formaldehyde in the notified substance. In the study described in the March 2016 report in **Appendix 3-1**, KnipBio's contractor included a measurement of formaldehyde levels arising from small-scale DASGIP fermentations of *M. extorquens*. In this study, formaldehyde concentrations ranged from undetectable to 908.7 µg/L. This is the only study in which KnipBio conducted measurements of formaldehyde levels in the fermentation cultures during the course of fermentation. Formaldehyde levels have not been measured at any of the company's subsequent (b) (4) fermentations, which are expected to more closely resemble commercial scale production.

Formaldehyde levels have been tested in preparations of the notified substance, as follows. Dried KBM from the 3(b) (4) batches run was resuspended at a 20mg/mL concentration in 0.05X PBS and assayed for acetaldehyde according to the Nash Reagent Absorbance method (**See Appendix 3-4**). Based on the extrapolation of a standard curve established with known amounts of formaldehyde, the method should detect concentrations as low as 0.01 mg/mL formaldehyde. The KBM samples were concentrated 20X more than the standard, and no signal was found to be more fluorescent than the blank control. This suggests that formaldehyde up to 0.0005 mg/mL could not be detected. Therefore, on a percentage basis there is no more than 0.0025% or 25 ppm formaldehyde in the notified substance. Formaldehyde is an approved animal feed additive, in accordance with 21 CFR 573.460, under which up to 2.5 gm/kg of feed is allowed. KnipBio therefore expects that formaldehyde levels in the notified substance, which are undetectable in the assay described above, will be below the maximum allowed concentration under 21 CFR 573.460.

Figure 3-2. Tunable UV Spectra of Strains KB200 and KB203

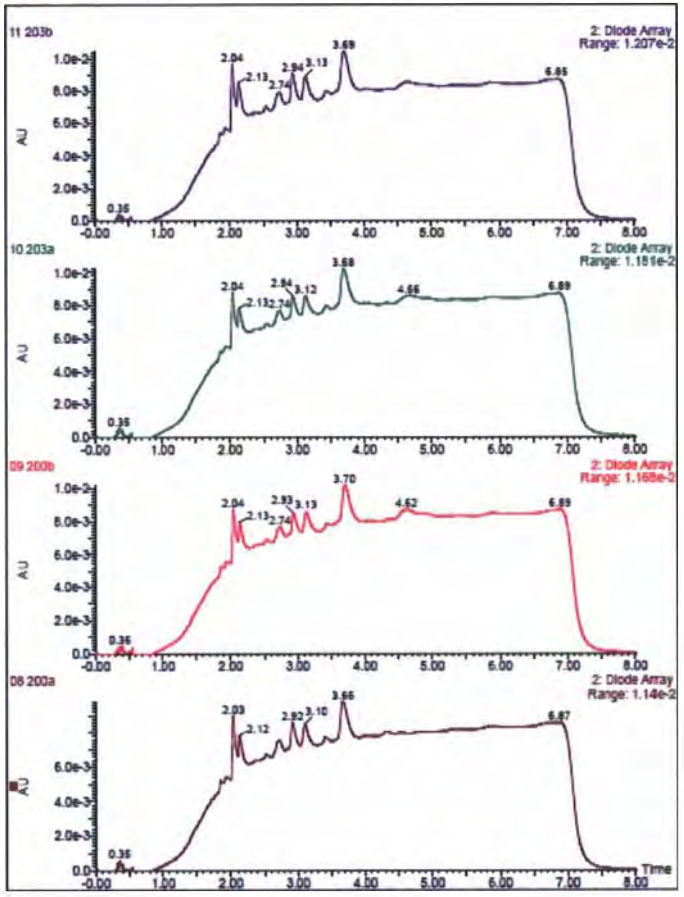
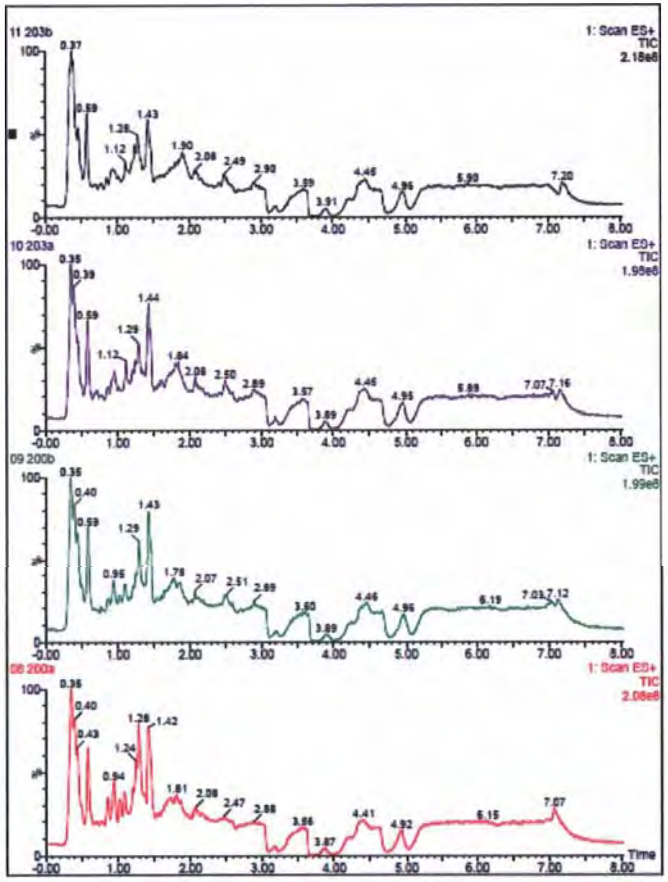


Figure 3-3. Mass Spectrometry Analysis of Strains KB200 and KB203



Concentration of methanol in the notified substance. Although methanol is used as a feedstock in the fermentation of the microorganism comprising the notified substance, concentrations of methanol in the notified substance are expected to be extremely low, if detectable at all. As fermentation progresses, methanol in the growth media is converted to microbial biomass and is therefore expected to be depleted in the natural course of the fermentation. Further, when collecting cells by centrifugation at the end of the run, it would be expected that any remaining methanol would primarily be retained in the supernatant rather than the cell pellet.

Methanol levels have been tested in preparations of the notified substance. Dried KBM from the 3 (b) (4) batches run was resuspended at a 20mg/mL in 0.05X PBS and assayed for methanol according to the Nash Reagent Absorbance and Alcohol oxidase method (See **Appendix 3-4**). Based on the extrapolation of a standard curve established with known amounts of Methanol, MeOH could be detected in the samples at concentrations as low as 0.005% (0.04 mg/mL). Since the samples were ran at a 4X concentration compared to the standards, and no signal was found to be more fluorescent than the blank control, KnipBio is confident there is less than 0.00125% MeOH (0.01mg/mL) in the notified substance. As a percentage basis there was no more than 0.05% MeOH or 500 ppm in the notified substance. Since KBM will be no more than 10% of the total feed, KnipBio does not expect the methanol concentration to be more than 0.005% (50 ppm) of the diet.
According to 21 CFR Part 573.637, free methyl alcohol is allowed in animal feed as long as it does not exceed 0.015%. Furthermore, according to a 2011 Position Paper from the UK Advisory Committee on Animal Feedingstuffs (UK Advisory Committee on Animal Feedingstuffs, 2011), citing (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2010):

> In December 2010, EFSA published an Opinion on the use of glycerine as a co-product from biodiesel production from Category 1 animal by-products (ABP) and vegetable oils. EFSA stated that inclusion rates of glycerol are usually up to 15% of the diet of ruminants and up to 10% in non-ruminant diets, with no adverse effects on animal health. It also found that residual amounts of methanol (up to 0.5%) and sodium (up to 1%) had no adverse effects on animal health. However, a maximum level of 0.2% methanol is proposed for the EU Catalogue of Feed Materials required under Regulation 767/2009, but we envisage that any MPLS should only apply to category 3 tallow.

KnipBio therefore expects that methanol levels in the notified substance, which are undetectable in the assay described above, will be below the maximum allowed concentration.

(b) When the intended use is in food for food-producing animals, you must provide:

(b)(1) The potential quantities of any residues that humans may be exposed to in edible animal tissues, including:
(i) Residues of the notified substance;

(ii) Residues of any other substance that is expected to be formed in or on the animal food because of the use of the notified substance; and
(iii) Residues from any other substance that is present with the notified substance whether naturally, due to its manufacture (e.g., contaminants or by-products), or produced as a metabolite in edible animal tissues when the notified substance is consumed by a food producing animal

The protein content of the notified substance that is being provided to the target species will be incorporated in the flesh of the fish, as is the case for all ingested proteins, so that there would not be any safety concerns regarding human consumption of the product, nor is there the need for any specific exposure assessment for humans.

With regard to the potential presence of PHBs in the notified substance, as discussed above it is expected that any such concentrations in the substance will not exceed 25%, and that such concentrations will be diluted at least 10-fold because the notified substance will be incorporated into aquafeed at no greater than 10% of the total diet. It is further expected that the PHBs will be metabolized or digested in the GI tract of the fish to some extent (Jendrossek and Handrick, 2002; Defoirdt *et al.*, 2009; Liu *et al.*, 2010) and further that, as a high molecular weight substance, PHBs are unlikely to be deposited in fish flesh to any appreciable degree. It is thus expected that human exposure to any potential PHB presence will be extremely low.

(b)(2) The data and information you rely on to establish, in accordance with paragraph (b)(1) of this section, the potential quantities of any residues that humans may be exposed to in edible animal tissues.

As noted above, at the level of inclusion in aquaculture feed that KnipBio intends (up to 10% w/w in the total diet) the maximum level of PHB to which the target species would be exposed would be approximately 1-2.5%. The literature discussed below in Part 6 provides evidence that PHBs would be degraded to fatty acids in fish intestinal tract, such that human consumers of fish that have been fed the notified substance would not be expected to be exposed to significant levels of PHBs, and so there would be no adverse effects on human consumers of such aquatic products.

Part 4. Self-limiting levels of use.

In circumstances where the amount of the notified substance that can be added to animal food is limited because animal food containing levels of the notified substance above a particular level would become unpalatable or technologically impractical, in Part 4 of your GRAS notice you must include data and information on such self-limiting levels of use.

There are no self-limiting levels of use for the notified substance.

Part 5. Experience based on common use in food before 1958.

If the statutory basis for your conclusion of GRAS status is through experience based on common use in animal food, in Part 5 of your GRAS notice you must include evidence of a substantial history of consumption of the notified substance for food use by a significant number of animals of the species to which the substance is intended to be fed prior to January 1, 1958, and evidence of a substantial history of consumption by humans consuming human foods derived from food-producing animals prior to January 1, 1958.

This GRAS Notice is not based on common use in food prior to 1958.

Part 6. Narrative.

Summary

The data and literature described throughout this dossier unequivocally support KnipBio's conclusion that use of Single Cell Protein preparation derived from *Methylobacterium extorquens,* when incorporated at 10% or less of aquaculture feed is safe. This conclusion is corroborated by a number of studies, described above, in which aquatic animals of several species were fed this biomass preparation, with no adverse effects seen, and no effect on one of the most sensitive parameters: growth of the animal. This conclusion is also corroborated by ample evidence from the literature and other experimental data derived by KnipBio and others.

The following sections summarize the basis for this determination.

(a)(1) You must explain why the data and information in your notice provide a basis for your view that the notified substance is safe under the conditions of its intended use for both the target animal and for humans consuming human food derived from food producing animals.

The data and information included in this Notice provide a basis for KnipBio's view that the notified substance is safe under the conditions of its intended use for both the target animal and for humans consuming human food derived from food producing animals. The following is a summary of this information. Except as explicitly noted, all the data and information described below is available to the public.

1) Target Species; Suitability of the Notified Substance for Aquaculture Feed.

According to the United Nations Food and Agriculture Organization (UN-FAO), there are as many as 580 aquatic species currently farmed all over the world, representing the protein sector with the greatest genetic diversity (Ababouch *et al.*, 2016). Cattle for comparison, contains many different breeds used for beef, dairy or draft animal applications, are found on every continent humans occupy, but is typically collectively characterized by the single taxonomic classification of *Bos taurus.* In contrast, the breakdown for aquaculture species having economic value is 362 finfishes, 104 mollusks, 62 crustaceans, 6 amphibians, 9 invertebrates and 37 aquatic plants. In all, the world market for aquaculture products for the decade between 2005-2014 in aquaculture grew by ~6%. [(http://www.nmfs.noaa.gov/aquaculture/aquaculture_in_us.html). With demands from the industry from rapid growth, coupled with the dramatic genetic diversity, it is impractical to conduct efficacy and safety tests in hundreds of different varieties in a time-efficient manner to best serve the aquaculture industry.

One approach to solve this paradox is to determine highly qualified aquatic candidates to serve as a representative model for all other aquatic species. Among the important considerations for a proposed demonstration species would be life cycle of the animal, diet consumed in nature, physiology and metabolism, degree of background information available, and commercial relevance. An ideal demonstration species would be one that

might be seen as a "canary" (i.e., "canary in a coal mine") that is sensitive to the parameters being tested, while also having an extensive academic history, would be ideal to determine if novel feed ingredients or supplements can be shown to have a significant effect in terms of growth, survival and overall nutrition. In a field with such diversity, it is likely that a single model cannot be inclusive enough to draw a complete conclusion for the entire field.

Here, KnipBio proposes that the data from investigations of the species reported in this GRAS Notice, the common salmonid *Oncorhynchus mykiss* (rainbow trout) and *Penaeus vannamei* (Pacific white shrimp), and *Salmo salar* (Atlantic salmon) is sufficient to support the broader use of the notified substance for all aquaculture feed, with these species fulfilling all of the required criteria of covering a broad diversity of species that are well-studied, sensitive to testing, and commercially relevant. Recently, a precedent was established for this approach with the approval of the ingredient taurine for broad adoption across all of aquaculture with data provided from a subsect of species. (NOAA Fisheries, 2017; Northwest Fisheries, 2017).

Taxonomically, the proposed model species diverge at levels below the kingdom Animalia, with *O. mykiss* belonging to the phylum Chordata and *P. vannamei* belonging to the Phylum Arthropoda. A Google Scholar search of *"(Lipo)Penaeus vannamei"* reveals approximately 33,600 results and *"Oncorhynchus mykiss"* returned ~118,000 citations demonstrating a wealth of accumulated knowledge of these two species. As a comparison, *"Bos taurus"* which was domesticated approximately 10,500 years ago, yielded ~145,000 hits. Salmonids are good indicators for nutrition as they are known to be sensitive to allergenic materials, as stomach inflammation (gastroenteritis) is also well characterized as an effect of eating diets containing terrestrial proteins like soy. Shrimp are particularly sensitive to diseases, including bacterial infections due to the low auto-immune memory generally afflicting crustaceans. While there are hundreds of species raised for economical gain in aquaculture, salmonids (i.e. salmon and trout) and crustaceans (i.e. Pacific white shrimp) represent approximately 40% of the total commercial value for the industry (Tacon, 2002; Mente *et al.*, 2006; Glencross *et al.*, 2007; Gjedrem *et al.*, 2012; Ababouch *et al.*, 2016).

Additionally, rainbow trout are an acceptable model for finfish for the following reasons:

- Production is conducted responsibly, in highly monitored laboratory settings, in compliance with the Institutional Animal Care and Use Committee (IACUC).
- The rainbow trout is a hardy fish that readily spawns, is fast growing, and is tolerant to a range of environments.
    - Trout can withstand temperature variation (0-27 °C), but spawning and growth occurs in a narrower range (9-14 °C), with the optimum below 21 °C.
    - The animal's sensitivity to temperature and food availability has been shown to influence growth and maturation, making it a good indicator species.
- The versatility of the trout's lifestyle reflects its metabolic flexibility. It is capable of occupying habitats ranging from an anadromous life history (living in the ocean but spawning in gravel-bottomed, fast-flowing, well-oxygenated rivers and streams) to permanently inhabiting lakes.
- Large fry can be easily weaned on to an artificial diet, making this a practical system for nutrition studies.

- Females are able to produce up to 2,000 eggs/kg of body weight, and through the use of selective breeding and photoperiod adjustment hatchery strains have developed that can mature earlier and spawn all year round.

Pacific white shrimp are an important model for crustaceans:

- Production is conducted responsibly, in highly monitored laboratory settings, in compliance with the Institutional Animal Care and Use Committee (IACUC).
- Broodstock of *P. vannamei* can be collected naturally, harvested from ponds, or purchased from tank-reared SPF/SPR broodstock from the United States of America representing a diverse genetic pool.
- The life cycle and rearing of Pacific white shrimp is well understood. Nauplii, the juvenile stage of can be harvested by light attraction, disinfected and transferred directly to larval rearing tanks.
- *P. vannamei* is very efficient at utilizing Biofloc in ponds or consuming formulated feeds representing a diverse, omnivorous diet. Under intensive culture conditions, lower protein content feeds are used representing a greater range of diet formulations. For example, lower cost, less-processed terrestrial proteins like soy can be incorporated.
- The major disease problems suffered by *P. vannamei* include viruses and bacteria entering the ponds and other systems. *P. vannamei* have little to no immune-memory so the effect of nutrition against invasive microorganisms or viruses is more pronounced than in other animal systems.
- Super-intensive cultivation strategies (i.e. indoor raceway systems enclosed in greenhouses) are biosecure, eco-friendly, have a small ecological footprint and can produce cost-efficient, quality shrimp.

For these reasons, KnipBio believes that the data and information presented in this dossier, while primarily limited to rainbow trout, Pacific white shrimp and Atlantic salmon is sufficient to support the finding that the notified substance is Generally Recognized as Safe for use in any aquaculture feed, when incorporated at 10% or less of the feed.

2) *Methylobacterium extorquens.*

a) Natural history, biology

The notified substance is a Single Cell Protein preparation derived from *Methylobacterium extorquens*. Bacteria of the *Methylobacterium* genus are facultative methylotrophs: they have the ability to grow by reducing carbon compounds with one or more carbon atoms but not by reducing carbon-carbon bonds (Chistoserdova *et al.*, 2003). These species are generally non-motile, rod-shaped, obligately aerobic, and often have a distinctive pink pigmentation. Bacteria of the *Methylobacterium* genus can utilize methanol from various natural sources, including that emitted by the stomata of plants (Abanda-Nkpwatt *et al.*, 2006). Some *Methylobacterium* strains are suggested to be part of the natural human food flora and others have even been suggested to add to the flavor of strawberries (MicrobeWiki, no date). This bacterium can be found mostly in soils, on leaves, and in other parts of plants and (Lidstrom and Chistoserdova, 2002)

The α-proteobacterium *M. extorquens* (previously identified as a *Pseudomonas* sp.) has been studied for over 50 years (Large *et al.*, 1961). It is a facultative aerobic Gram-negative naturally occurring bacterium found in nature as a leaf symbiont (Balachandar *et al.*, 2008; Dourado *et al.*, 2013; Gourion *et al.*, 2006; Kutschera, 2007). It has the ability to use C1 compounds such as methanol as a carbon source and therefore has been the model system of choice for uncovering the genetic basis of growth on these compounds (i.e., methylotrophy) (Chistoserdova *et al.*, 1991, 2003; Gourion *et al.*, 2006; Marx *et al.*, 2003; Muller *et al.*, 2011; Šmejkalová *et al.*, 2010; Andersen, 2014; Ward *et al.*, 2004).

The complete genome sequences are now available for 7 strains of *M. extorquens* as well as about 20 other species in the genus (NCBI) (Marx *et al.*, 2012; Ward *et al.*, 2004; Peyraud *et al.*, 2011; Vuilleumier *et al.*, 2009). Reference sequences can be found on the following websites:
https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=419610
http://hamap.expasy.org/proteomes/METEP.html
http://www.ebi.ac.uk/Tools/dbfetch/expasyfetch?CP000908
https://www.ncbi.nlm.nih.gov/genome/?term=Methylobacterium%20extorquens%20PA1
https://biocyc.org/

The ability of *M. extorquens* to use renewable carbon sources such as methanol makes it a promising candidate for new biotechnology development (Dourado *et al.*, 2015; Schrader *et al.*, 2009; Ochsner *et al.*, 2014; Fei *et al.*, 2014; Strong *et al.*, 2016).

Besides being naturally rich in antioxidants and pigments, *Methylobacterium* has been used for a few decades for the production of plastics polymers such as PHA/PHB* by fermentation (Bélanger *et al.*, 2004; Bourque *et al.*, 1995; Höfer *et al.*, 2010) and much effort has been invested in producing maximum levels of PHB through bioprocess development.

A great body of work has been published about improvement of bioprocess (e.g. media development) for increasing production of valuable molecules using C1 compounds as a principal carbon source (Bormann and Roth, 1999; Delaney *et al.*, 2013; Kim*et al.*, 2003; Kim *et al.*, 1996; Mokhtari-Hosseini *et al.*, 2009; Peyraud *et al.*, 2012). Scale-up in large fermenters has been published for production either of PHA/PHB or biomass for agriculture applications (Bogosian 2016; Miguez *et al.*, 2006; Groleau *et al.*, 1994).

b) Evidence of lack of pathogenicity or toxicity.

KnipBio believes that the production organism for the notified substance, *Methylobacterium extorquens*, is a safe organism that will not pose any health risks to the target species that will be fed the notified substance, or to human consumers who eat such aquatic animals. We base this belief on extensive literature searches which show that there is no evidence that this species is pathogenic, toxic or allergenic. The following is a summary of these literature searches and their findings.

* Polyhydroxybutyrate (PHB) is one species of the broader category of polymeric molecules known as polyhydroxyalkanoates (PHA). In citing literature references in this document, these acronyms may be used interchangeably.

Pathogenicity

A Google Scholar search "*Methylobacterium extorquens* pathogenicity" on September 12, 2017 yielded 1460 hits, most of which related to plant pathogenicity. When the word plant was removed from the search, ~ 393 hits are generated (See **Appendix 6-1**). Most of the papers are not directly relevant to *M. extorquens* pathogenicity, for several reasons. First, in many cases, it is clear from the titles of these papers that they are reports that organisms of entirely different genera and species are pathogens or have pathogenic properties, and when species of *Methylobacterium* were mentioned, it was only peripherally. Sometimes *Methylobacterium* species have been cited merely because certain genes in the alleged pathogenic species had homology to certain *Methylobacterium* genes. In some cases where the title points to a different microbial species, it is not at all clear what reference there may be for *Methylobacterium.* There are other papers where the term "pathogen" appears to have been used in a completely different context, such as discussions of factors affecting the growth of environmental pathogens. Further, as discussed below, there were papers which pertained to species of *Methylobacterium* other than *M. extorquens*, although some of these are discussed below. We discuss those articles deemed to be relevant as follows.

We first note that several of the papers uncovered in the literature search report that different *Methylobacterium* species may be opportunistic pathogens that can cause illness in immunocompromised individuals. *Methylobacterium* species have also been isolated from various medical-related sources such as catheters, endoscopes and hospital water supplies, but with little or no evidence that any of the isolates are pathogenic. (Truant *et al.*, 1998) said that, as of its 1998 publication date, "to our knowledge, *only* 21 cases of human methylobacterium infection have been reported in the world's literature (emphasis added)," a figure which was updated two years later to 29 cases in (Sanders *et al.*, 2000). A more recent review (Kovaleva *et al.*, 2014) reports that *Methylobacterium* species have been reported as sources of health care-associated infections, including infections in immunocompromised hosts (see references cited in this paper).

In many of the papers identified in the literature search, the authors identified the isolated microorganism simply as a species of *Methylobacterium* without assigning a definitive species identification (Sanders *et al.*, 2000; Furuhata *et al.*, 2006; Kelley *et al.*, 2004; Truant *et al.*, 1998))†. Other papers, particularly ones from the 1990s or earlier, assigned species identifications that are now known not to be correct, or which KnipBio believes are erroneous, based on more up-to-date taxonomic tools such as 16S rRNA sequencing.

We first summarize those papers that have explicitly identified *M. extorquens* as the strain implicated in opportunistic pathogenicity. (Hogue *et al.*, 2007) reports that *M. extorquens* has been implicated as an opportunistic pathogen (see Table 1 in this paper), but also says that "The clinical relevance of *Methylobacterium* species is not always clear due to the limited information available in many publications. The seemingly low pathogenicity of this organism, coupled with the fact that it primarily affects immunocompromised persons, makes the determination of clinical relevance difficult". Another paper (Kaye *et al.*, 1992) reports that *M. extorquens* was found to be the source of a catheter-borne infection, but we

† Note that the Truant et al. paper is identified as "Tmant" et al. on page 4 of the literature search in Appendix 6-1.

believe this strain classification is incorrect, because it was done based on morphological and physiological tests. For instance, the isolated strains grew on xylose and fructose, while typical *M. extorquens* strains identified by 16S RNA sequencing are not able to utilize these compounds. In any event, this paper characterized the putative *M. extorquens* strain as "low virulence" and that the infection it caused was only "low-grade or intermittent".

(Greub, *et al.*, 2004) isolated a microorganism resembling *M. extorquens* from amoebal cocultures from nasal swabs, although these authors did not report any direct evidence that this culture was pathogenic. (Patel and Gutierrez, 1992) reported an unusual case of pneumonia with full-blown septic shock syndrome which they attributed to an infection of *M. extorquens* infection in a non immunocompromised adult host (a 66-year old man). The microorganism was identified by CDC as *M. extorquens*, but KnipBio believes that this classification, from the early 1990s, may not be reliable. (Lambert *et al.*, 1983) reported that lesions obtained from a 48-year-old Puerto Rican woman with extensive ulcers on her buttocks, right arm, and thighs showed the presence bacilli which were identified as *Vibrio extorquens*, which they characterized as a partially acid-fast methanolophilic organism "not previously associated with disease in humans". (*V. extorquens* has since been reclassified as *Methylobacterium extorquens*). (Frank, *et al.*, 2007) tentatively identified a microbial strain isolated from toys in a pediatric waiting room as *M. extorquens*, although the paper expressed some doubt about the accuracy of this classification, and did not assert that the presence of this organism on the toys posed any risk to children.

Many of the papers identified in the search implicate *Methylobacterium mesophilicum* (previously known as *M. mesophilica*) as an opportunistic pathogen. These papers include (Sanders *et al.*, 2000; Imbert *et al.*, 2005; Engler and Norton, 2017; Fernandez *et al.*, 1997); see also (Flournoy *et al.*, 1992), not cited in the literature search. There are many references in the literature which indicate that, at one time, there was confusion in the field about whether *M. mesophilicum* and *M. extorquens* were the same or different species, but the consensus in the field is that these are separate and distinct species (see for example, (MicrobeWiki, no date), which lists both as distinct species). It is known that *M. mesophilicum* and *M. extorquens* utilize different carbon sources, among other differences between these species. Therefore, KnipBio will not comment further on references reporting that *M. mesophilicum* is an opportunistic pathogen, other than to note the presence of such references in the literature search in **Appendix 6-1**, and to specifically mention the several papers cited above.

It has been speculated that the ability of *Methylobacterium* species to form biofilms and to develop resistance to high temperatures, drying, and disinfecting agents may explain why *Methylobacterium* are often found in the hospital environment. Kovaleva *et al.* (2014) presents an overview of documented infections and cross-contaminations with *Methylobacterium* related to endoscopic procedures. With regard to *M. extorquens*, this paper says:

> Despite low virulence, *Methylobacterium* is able to cause colonization and infections in immunocompromised patients. *Methylobacterium mesophilicum*, *Methylobacterium zatmanii*, and *Methylobacterium extorquens* are the three most commonly reported species

> isolated from normally sterile body sites, i.e., blood, liquor cerebrospinalis, bone marrow, synovia, and ascitic and peritoneal fluids.

Kovaleva *et al.* (2014) also reported that *M. extorquens* has been found in contaminated endoscopes, which it attributed to its ability to form biofilms, described as follows:

> *M. extorquens* had a strong biofilm-producing ability, with the highest biofilm amount and the maximum metabolic activity after 7 days incubation in R2A broth. The use of 1% peracetic acid disinfectant caused a marked inhibition of *M. extorquens* growth in 2-, 5-, and 7-day biofilms directly after treatment.

Although *M. extorquens* is reported to have biofilm-producing ability, the biofilm-forming phenotype would only be expected to be expressed when the organisms are metabolically active; and since the organism will be nonviable in the final manufactured product, this is not likely to cause any problems (e.g. possible intestinal adhesion) when the notified substance is ingested by the target species. In addition, based on literature reports suggesting the importance of cellulose in the formation of biofilms or activation of the immune response in several microbial species (Romling and Galperin, 2015; Matthysse *et al.*, 2005; Spiers *et al.*, 2003; Danhorn and Fuqua, 2007), it is believed that the deletion of the *cel* locus in the production strain would diminish the ability of *M. extorquens* to form biofilms.

On November 7, 2017, KnipBio conducted a Google Scholar search using the old name of *M. extorquens*. A search using the keywords "*Pseudomonas extorquens* pathogenicity" uncovered only 43 hits (see **Appendix 6-3**). Most of these papers were quite old, dating from the 1970s, and none of the papers appeared relevant to the potential pathogenicity of this strain, other than a small number of references which also were found in the search described above.

In addition to the above literature searches, KnipBio conducted a search of the PATRIC database (Pathosystems Resource Integration Center, https://www.patricbrc.org/view/Genome/419610.10t) which showed that *M. extorquens* does not contain any virulence factors. Results of this search can be made available to FDA CVM upon request.

Toxicity

A Google Scholar search "*Methylobacterium extorquens* toxicity -plant" on September 12, 2017 yielded 558 hits, most of which were not relevant to any alleged toxicity of *Methylobacterium* species (for reasons discussed below), and none of which were relevant to *M. extorquens* (see **Appendix 6-2**), but note that several papers already identified in the "pathogenicity" search above also were found in this search. Among the reasons for ruling out most of the references in this search were that the search picked up references to the toxicity of methanol or formaldehyde, or irrelevant references to other toxic molecules not found in or produced by *Methylobacterium*. In fact, several papers reported that *Methylobacterium* species could reduce the toxicity of metallic waste streams. Previous Google searches using the same or similar keywords also provided no hits relevant to mammalian or human toxicity. The only reference found by KnipBio that is remotely relevant is (Balachandran *et al.*, 2012), who reported a *Methylobacterium* soil isolate which

they called *Methylobacterium* sp. (ERI-135), which showed promising antibacterial and cytotoxic activity *in vitro*.

Antibiotic Resistance

Methylobacterium extorquens is naturally resistant to trimethoprim, a pyrimidine inhibitor of dihydrofolate reductase, which is an enzyme of the central carbon metabolism during C1 compound (methanol) assimilation (see Šmejkalová *et al.*, 2010).

A Google search on June 20, 2017 for antibiotic resistance in *M. extorquens* yielded no useful hits, except (Hu and Lidstrom, 2012) which reports the presence of the tetracycline resistance gene tetR in *M. extorquens* strain AM1 (see also (Ma *et al.*, 2016)). This gene is not present in *M. extorquens* PA1, which is the starting strain for the strain used in the notified substance.

A search conducted on July 13, 2017 in the ARDB-Antibiotic Resistance Genes Database (Center for Bioinformatics and Computational Biology- University of Maryland- College Park, MD 20742) yielded only one putative resistance gene in *M. extorquens* PA1, identified by homology, as shown below. KnipBio has confirmed that its strain is resistant to bacitracin.

Gene ID	Gene	Profile	Description
Mext_2387	baca	Bacitracin	Undecaprenyl pyrophosphate phosphatase, which consists in the sequestration of Undecaprenyl pyrophosphate.

Symbiotic Relationships

As noted above, *M. extorquens* occurs in nature as a leaf symbiont (Balachandar *et al.*, 2008; Dourado *et al.*, 2013; Gourion *et al.*, 2006; Kutschera, 2007); see also (Knief *et al.*, 2010) and (Koskimäki *et al.*, 2015). It is not believed that this species maintains any other symbiotic relationships other than with the plant species described in these references.

Summary

KnipBio believes that, based on the literature identified and discussed above, the production organism for the notified substance, *Methylobacterium extorquens*, is a safe organism that will not pose any health risks to the target species that will be fed the notified substance, or to human consumers who eat such aquatic animals.

3) Production strain

a) Genetic manipulation to create production strain

(b) (4)

(b) (4)

Evidence was also presented in Part 2 of this Notice and its Appendices to verify that the deletions took place as planned, and to show that these genetic modifications to the strain are stable through at least 20 generations.

b) Effects of genetic manipulation.

The genetic manipulation has not introduced any new genes or biochemical functions into the wild type strain *M. extorquens* PA1 (Knief *et al.*, 2010). The manipulation has only removed two genetic pathways.

The (b) (4) to eliminate or substantially decrease the production of cellulose by the *M. extorquens* production organism, in order to reduce the amount of cell clumping that might occur during fermentation.

The (b) (4) to ensure that the production strain did not yield any of the carotenoid compound spirilloxanthin. *M. extorquens* is a natural producer of carotenoids, but in the specific parental strain employed by KnipBio, it has recently been found that the spirilloxanthin pathway is inactive. This was not learned until the genetic manipulation to remove the pathway was completed, and so there should be no appreciable impact of the manipulation on the characteristics of the organism.

c) Safety of KB203 and impact of genetic manipulation on safety.

M. extorquens has been classified as Biosafety Level 1 (ATCC, 2017). The genetic manipulations made to the starting strain consist only of gene deletions, and so it is extremely unlikely that such manipulations could affect traits such as pathogenicity, infectivity, etc. to any extent, much less to an extent that could change the biosafety level classification of the strain.

The most direct evidence for the safety of the production strain KB203 are the various feeding studies that are described in detail in Part 2 of this Notice and its Appendices, which are also summarized below.

4) Fermentation and product formulation.

a) Fermentation process.

As described in detail in Part 3 above, the notified substance is produced from biomass of *Methylobacterium extorquens*, which has been grown to sufficient volume via fermentation using conditions well-established for this microorganism. Specifically, KnipBio has used a standard minimum media composition, based on the published media formulation of Choi

et al. (1989). This media includes certain salts and minerals, includes an antifoaming agent, and utilizes methanol as the carbon source. At the conclusion of each production run, the biomass is separated from the spent media by centrifugation and is then spray-dried for preparation of the Single Cell Protein material, as described in **Appendix 2-4**. The resulting product has the composition shown in Part 2 and discussed below, and analysis of this material has shown no evidence that any toxic, infectious or any other hazardous component is present in these preparations.

KnipBio currently expects that commercial production of the notified substances may involve one or more toll manufacturers, operating under contract with KnipBio. All such manufacturing activities will be conducted in compliance with the Food Safety Modernization Act and will utilize Standard Operating Procedures in accordance with Good Manufacturing Practice.

b) Food-grade materials used in production: safety implications.

Throughout the manufacturing process, food-grade materials of suitable purity are used. These are summarized in Tables in **Appendix 2-4**. All such materials will either be AAFCO-approved or are the subject of FDA GRAS regulations, with the exceptions noted in the Appendix. Specifically, food-grade sources of potassium phosphate monobasic, methanol, and the antifoaming agent have been identified and will be used in commercial production. Although experiments and pilot fermentation runs to date have made use of boric acid in the growth media, boric acid will not be included in the growth media for commercial fermentations.

c) Safety implications of fermentation and the production process.

Production of the notified substance will take place using the most appropriate procedures in accordance with Good Manufacturing Practice, using only materials that are of known purity and are suitable for use in animal food. Fermentation workers will be appropriately trained, and all safety precautions consistent with Good Industrial Large Scale Practice for microorganisms designated as Biosafety Level 1 will be utilized. Furthermore, it is necessary for the efficiency and productivity of the fermentation for strict controls to be in place to prevent contamination of the production organism with other microorganisms, and so the resulting biomass from each production run should be free of any such contamination.

5) *Methylobacterium extorquens* protein product (the "notified substance")

a) Background

The notified substance is a Single Cell Protein (SCP) prepared, as described above, from cultures of *Methylobacterium extorquens* fermented using methanol as its carbon source. There is a long history of development and use of Single Cell Proteins in human and animal food, as has been extensively documented in the literature (Anupama, 2000; Øverland *et al.*, 2010; Nasseri *et al.*, 2011; Suman *et al.*, 2015; Matassa *et al.*, 2016). The term Single Cell Protein was coined in the 1960s, but products have been commercially manufactured since BP's production of Toprina® SCP as early as the 1950s. Pruteen,® produced by ICI, was the first commercial SCP used as animal feed additive, and it was produced using *Methylophilus methylotrophus* cultured on methanol and had 72 % protein content. The Tables below

summarize the microorganisms that have been evaluated and/or sold commercially for the production of SCP for animal feed.

Table 6-1. Microorganisms Investigated for Use in Single Cell Protein Products.

Raw Material	Organism	Scale	Market	Product	Manufacturer
Glucose	Fusarium venenatum	50-100 T/ year	Human food	Mycoprotein (known as Quorn)	Ranks Hovis McDougall, UK
Cheese whey	Penicillium cyclopium	300 T/ year	Animal feed		Heurty, France
Coffee waste	Trichoderma harzianum	40,000 L	Animal feed		ICAITI, Guatemala & El Salvador
Sulfite waste liquor	Paecilomyces variotii	10,000 T/year	Animal feed		Finland
Pulp mill wastes	Cheetomium cellulyticom	1T/ day	Animal feed		Envirocon Ltd, Canada
Methanol	Methylophilus methylotrophus	60,000T/year	Animal feed	PRUTEEN	ICI, US
Alkanes	Candidar lipolytica	20,000 T/year	Animal feed	TOPRINA	BP (France & England)
Ethanol	Yeast		Flavor enhancer	TORUTEIN	Amoco Company
Waste	Bacteria		Animal feed	ProFloc	Nutrinsic

Sources: (Goldberg, 1985); (Anupama, 2000); (Øverland *et al.*, 2010); (Nasseri *et al.*, 2011); (Suman *et al.*, 2015); (Matassa *et al.*, 2016).

Table 6-2. Microorganisms Currently Used in Single Cell Protein Products.

Organism	Substrate	Name	Production volume (T/y)	Application	Product Name	Manufacturer	Reference
Mycoprotein	Glucose	Fusarium venenatum	22,000	Human feed	Quorn	Monde Nissin Corporation (ICI)	Quorn.co.uk
Bacteria	Waste	mixture	5,000	Animal and fish	Profloc	Nutrinsinc	Nutrinsinc (2015)
Bacteria	Methane	Methylococcus capsulatus		Aquafeed	FeedKind	Calysta	Byrne (2016)
Bacteria	Methanol	Methylophilus methylotrophus	60,000	Animal feed	Pruteen	ICI	
Bacteria	Methanol	Methylomonas clara			Probion	Hoechst	Thacker & Kirkwood (1992)
Bacteria	Methanol	Methylomonas methanica			Norprotein	Norsk-Hydro	Thacker & Kirkwood (1992)

Sources: (Goldberg, 1985); (Anupama, 2000); (Øverland *et al.*, 2010); (Nasseri *et al.*, 2011); (Suman *et al.*, 2015); (Matassa *et al.*, 2016).

A single cell protein product for fish feed is currently marketed in the EU by Calysta under the trademark FeedKind. This product is produced by fermentation of the methanotroph *Methylococcus capsulatus* (i.e. different from the methylotroph used to produce the notified substance that is the subject of this Notice), using methane as the feedstock. It was originally developed by the Norwegian firm BioProtein Engineering AS under the name

BioProtein, and was approved for feed use in fish and certain terrestrial animals by the EU in 1995. It was later the subject of additional safety reviews when approvals were sought for expanded use in other terrestrial animal species. Analysis by the EU in 2005 expressed some concerns over the use of the product in terrestrial animals, some of which were attributed to the presence of microorganisms other than *M. capsulatus* (EFSA, 2005). Analysis by the Norwegian Scientific Committee for Food Safety in 2006 concluded that "an inclusion level of [BioProtein] of 6% in the diets to terrestrial target animals and a 10% maximum inclusion level in salmon feed (both for fresh- and seawater stages) would reduce the risk of potentially adverse effects in the animals. The risk associated with the human consumption of products from animals fed on [BioProtein] is considered negligible" (Norwegian Scientific Committee for Food Safety, 2006). The 1995 EU approval remains in effect and the product remains on the market under its new trademark.

b) Composition

The notified substance is a Single Cell Protein preparation that provides an excellent source of protein for the target fish species, and in particular includes concentrations of specific amino acids comparable to other sources of protein. Detailed information on the composition of the notified substance is provided above in Part 2 and in **Appendix 2-8** of this Notice. Also presented in Part 2 are comparisons of the composition of the notified substance with that of other sources of protein provided by fishmeal or soybean meal, showing that the notified substance is a comparable source of protein as compared to soybean meal and some fishmeal (i.e. tuna or local mixed species), although it is not as good a source of fat or fiber as those other meals.

As summarized in **Appendix 2-8**, the notified substance is also expected to contain certain low concentrations of metals. **Table 6-3** below shows the expected concentrations of these metals in the notified substance, derived from the analyses reported in **Appendix 2-8**. Because the notified substance will make up no greater than 10% of the overall diet of the targeted species, the maximum concentrations of these metals to which the target species will be exposed would be ten-fold lower than the figures shown in the first column of the **Table**. The **Table** also shows reported literature values for the daily requirements for shrimp, trout and salmon for certain of these metals (where such data are available in the literature). It can be seen that the expected exposure of these three species to these metals in feed containing the notified substance would fall within the species' dietary requirements for that metal, making it highly unlikely that the metals present in the notified substance would have any deleterious effect on the target species.

As discussed in Part 2 above, the notified substance, although produced from live microorganisms, is not expected to contain any appreciable levels of live cells. KnipBio has found that the initial step of spray-drying the cell biomass resulting from fermentation results in approximately a 4-log reduction of viable cell numbers, and the company expects that the final step of extruding the spray-dried material into feed pellets at high temperatures will result in significant additional reduction in viable cell counts.

Table 6-3. Metal Concentrations in the Notified Substance and Dietary Requirements for the Target Species.

	KBM-203	Requirement (mg/kg dry diet)			10% inclusion	Tolerance (NRC 2005)
Mineral	mg/kg	Shrimp (L. v.)	Trout	Salmon	mg/kg	mg/kg
Calcium	260-440	5,000-20,000	24,000		25-50	9,000
Phosphorus	9,400-10,500	300-7,000	7,000	6,000	900-1,100	10,000
Sodium	3,000-4,000		600	> 600	300-400	
Chloride	240-390				24-40	
Magnesium	820-900	2,600-3,000	500-700		80-90	3,000
Manganese	65.4-76.80		12-13	7-10,5	7-8	
Iron	167-263		30-170	33-100	17-26	
Zinc	40.7-65.8		15-30	37 – 67	4-7	250
Copper	11.9-19.7	16-32	3- 3.5	3	1-2	100
Potassium	4,960-6,200		15-150	8,000	500-620	
Cobalt	5.7-6.2		0.05-1	0.05-1	0.6-0.7	
Molybdenum	12.00-14.8				1-1.5	10
Sulfur	3,430-4,050				300-400	
Selenium	0.05< -0.11	0.2-0.4	0		<0.01	2

Sources: (Chanda *et al.*, 2015), (Watanabe, Kiron and Satoh, 1997), (National Research Council, 2011a), (Lall, 2000), (Tacon, 1992); (National Research Council, 2011b) (National Research Council, 2005)
http://www.fao.org/docrep/field/003/ab470e/ab470e06.htm
http://www.fao.org/fileadmin/user_upload/affris/docs/Trout/English/table_2.htmhttp://www.fao.org/fileadmin/user_upload/affris/docs/Trout/English/table_2.htm

c) Potential contaminants

i) Polyhydroxybutyrates (PHBs)

As described in detail in above, the microbial strain on which the notified substance is based, *Methylobacterium extorquens*, is a natural producer of polyhydroxybutyrates (PHBs)(Korotkova and Lidstrom, 2001). The notified substance is therefore expected to contain certain amounts of PHBs, which might range from 15-25% depending on the

fermentation conditions. KnipBio believes that the expected levels of PHBs in the notified substance will be well below the levels that might be expected to be harmful to the target species, and furthermore there is evidence in the literature that PHBs may have beneficial effects in animal diets.

Polyhydroxybutyrates are ubiquitous compounds, naturally produced by a broad range of microorganisms (Anderson and Dawes, 1990; Hempel *et al.*, 2011; Khanna and Srivastava, 2005; Lee, 1996; Lu *et al.*, 2009; Lundgren *et al.*, 1965) and used by such bacteria as an intracellular carbon and energy storage compound (Khanna and Srivastava, 2005). PHBs have been found by several investigators to be produced in *M. extorquens*, at concentrations ranging from 25-33%, depending on growth conditions (Bourque *et al.*, 1992), (Höfer *et al.*, 2011a,b), (Höfer *et al.*, 2010). PHBs have been found to be synthesized by numerous other bacterial species, including *Ralstonia eutropha, Alcaligenes eutrophus, Alcaligenes latus, Azotobacter vinelandii,* and *Bacillus megaterium* (Hempel *et al.*, 2011), (Lee, 1996). It has been hypothesized that microorganisms respond to feast-famine cycles by accumulating storage polymers such as PHBs when food sources are present, but then using them for growth when the external food source is depleted (van Loosdrecht *et al.*, 1997).

There is a considerable body of literature attesting to the presence of PHBs in animal diets, as summarized in **Table 6-4** below. No deleterious effects were seen from the presence of these compounds in animal feed, and in some cases positive effects were noted. For example, reports in the literature mention that PHA/PHB polymers can be degraded into β-hydroxy-short chain fatty acids (see references in the **Table 6-4** and discussion below). (Defoirdt *et al.*, 2007) and (Halet *et al.*, 2007) used that observation to show that PHB-containing bacteria could offer protection from pathogenic *Vibrio campbellii.* Numerous other publications in the literature report seeing positive effects of PHBs in animal diets (Boon *et al.*, 2013; Defoirdt *et al.*, 2009; De Schryver *et al.*, 2010; Ludevese-Pascual *et al.*, 2017; Situmorang *et al.*, 2016), with several noting antimicrobial effects of PHBs and one publication (Suguna *et al.*, 2014) suggesting that PHBs could stimulate both specific and nonspecific immune mechanisms. Poly-β-hydroxybutyrate is also produced by some lactic acid bacteria that are used as probiotics (Aslim *et al.*, 1998; Yüksekda *et al.*, 2003; Yilmaz *et al.*, 2005).

A 2013 review (Kunasundari *et al.*, 2013) summarized previous studies as follows:

> It is evident from previous studies that PHAs are not toxic and have been used in animal feed (Waslien and Calloway, 1969; Forni *et al.*, 1999a, b; Peoples *et al.*, 2001; Forni *et al.*, 1999). The *C. necator* H16 cells containing PHB was found to be tolerated by animals. These studies also reported the poor digestibility of PHAs by monogastric animals.

Table 6-4 summarizes numerous studies in the literature, including those cited above, in which aquatic animals were fed diets that included PHBs as one component, and sums up the findings and outcomes of these studies. No significant adverse effects were seen in any of those studies, and some showed beneficial effects on parameters such as growth rate.

There is significant reason to expect that PHB-producing bacteria are naturally found in fish guts, largely because production of PHBs by bacteria is so ubiquitous across many genera and species. A wide variety of bacteria and fungi have the ability to degrade extracellular PHB as they are able to secrete extracellular PHB depolymerase enzymes (Jendrossek and Handrick, 2002). (Defoirdt *et al.*, 2009) indicate that PHB particles are partially degraded in the gut of brine shrimps nauplii. (Liu *et al.*, 2010) isolated PHB-degrading bacteria from the gastrointestinal environment of a few aquatic animals (sturgeon, European sea bass, prawns). Any PHBs found in the feed would be expected to be degraded by such bacteria, therefore making it very unlikely that human consumers would be exposed to any significant levels of PHBs. Studies by De Schryver *et al.*, (2010) suggest that PHB (used at 5% w/w in a diet) is degraded during the gastrointestinal passage of juvenile sea bass. This group showed that, during a 6-week trial period, the dietary PHB acted as an energy source for the fish, likely indicating that PHB was degraded and used during gastrointestinal passage. In addition, the group observed that gut pH decreased from 7.7 to 7.2, suggesting that the presence of PHB in the gut led to the increased production of (short-chain fatty) acids. In addition, (Defoirdt *et al.*, 2007) provided evidence to show that PHB particles were at least partially degraded in the intestines of nauplii of the brine shrimp *Artemia fransiscana*. (Najdegerami *et al.*, 2012) reported results in Siberian sturgeon that were also consistent with PHB degradation in the fish gut. Apart from microbial degradation, PHB has also been shown to be degraded in animal tissues such as rats or rabbits (Freier *et al.*, 2002), (Qu *et al.*, 2006) .

In addition, as noted above, at the level of inclusion in aquaculture feed that KnipBio intends (up to 10% w/w in the total diet) the maximum level of PHB would be approximately 1-2.5%. The literature cited and discussed above indicates that such levels would not be expected to have an effect of the health of the fish, and since any PHBs in the notified substance would be expected to be degraded in the fish gut, there would be no adverse effects on the health of humans consuming fish which have fed on the notified substance.

Finally, we note that PHB produced by fermentation with *Ralstonia eutropha* has recently been approved for use in animal feed by the European Union. In the recently published Commission Regulation (EU) 2017/1017 of 15 June 2017 (European Union, 2017), the EU announced that it authorized the use of the following ingredient in animal feed:

> Product containing 3-hydroxybutyrate and 3-hydroxyvalerate, produced via fermentation with *Ralstonia eutropha*, and non-viable bacterial protein meal remaining from the producing bacteria and fermentation broth.

(*Table 6-4 begins on the next page. The rest of this page has been left blank*).

Table 6-4. Literature Reports of the Presence of Polyhydroxybutyrates in Fish Diets.

Species	Age/ size	% PHB	PHB origin	Trial length	Challenge	Outcome	Reference
Shrimp Penaeus monodon	PL1- PL30	3.4-4.1	Bacillus spp.	30 days	Vibrio campbellii	No effect on body weight or body length	Laranja *et al.* (2014) Veterinary Microbiol. 173: 310-317
Shrimp Penaeus monodon	PL5	55% in Bacillus sp.	Bacillus spp.	16 days	Vibrio campbellii	Improvement of immune response	Laranja *et al.* (2017) Fish & Shellfish Immunology 68:202-210
European sea bass Dicentrarchus labrax	1 month old juvenile	2-10	Chemical	6 weeks	none	Slight decrease in body weight at 10%	De Schryver et al (2010) Appl. Microbiol. Biotechnol. 86: 1535-1541
Siberian sturgeon Acipenser baerii	17 gm	2-5	Chemical	10 weeks	none	No significant effect on growth or FCR	Najdegerami *et al.* (2012) FEMS Microbiol Ecol 79:25–33
Rainbow trout Oncorhynchus mykiss	~110 mg	0.5-2	Chemical	6 weeks	none	No significant effect on body weight, protein content, or lipid content of the fish	Najdegerami *et al.* (2015) Aquaculture Nutrition
Rainbow trout Oncorhynchus mykiss	~13 gm	1	Chemical	12 weeks	none	No significant effect on body weight	Rodriguez-Estrada *et al.* (2009) Aquaculture Sci. 57 (4):609 - 617
Nile Tilapia Oreochromis niloticus	~ 27 mg	0.5-5	Chemical	28 days		No significant effect on body weight	Situmorang et al. (2016) Veterinary Microbiol. 182: 44-49
	larvae				Edwardsellia ictaluri	20% increase survival	Situmorang et al. (2016) Veterinary Microbiol. 182: 44-49
Chinese mitten crab Eriocheir sinensis	larval stage 1	0.01?	rotifers		Vibrio anguillarum	Increase in survival rate	Sui *et al.* (2012) Journal of Fish Diseases 35:359–364
Giant freshwater prawn Macrobrachium rosenbergii	larval stage 1	0.5	Artemia nauplii + PHB		none	increase in survival rate of larvae	Nhan *et al.* (2010) Aquaculture 302:76–81
Mossambique Tilapia Oreochromis mossambicus	25 gm	1-5	Bacillus thuringiensis	28 days	Aeromonas hydrophila	increase survival rate proportional to PHB%	Suguna *et al.* (2014) Fish & Shellfish Immunology 36: 90-97
Siberian sturgeon Acipenser baerii		5					Liu *et al.* (2010) FEMS Microbiol Ecol 74:196–204

European sea bass Dicentrarchus labrax		10					Liu *et al.* (2010) FEMS Microbiol Ecol 74:196–204
giant river prawn Macrobrachium rosenbergii		0.5					Liu *et al.* (2010) FEMS Microbiol Ecol 74:196–204
Brine shrimp Artemia franciscana	nauplii			48h	Vibrio campbellii		Liu *et al.* (2010) FEMS Microbiol Ecol 74:196–204
Brine shrimp Artemia franciscana	nauplii	0.01- 0.1		48h	Vibrio campbellii	increased survival rate	Baruah *et al.* (2015) SCIENTIFIC REPORTS | 5: 9427
Brine shrimp Artemia franciscana	nauplii	1-2 ?		48h	Vibrio harveyi	PHB decreases colonization by Vibrio	Van Cam (2009) Aquaculture 291:23–30
Brine shrimp Artemia franciscana		0.01- 0.1		48h	Vibrio campbellii	Increase survival	Defoirdt *et al.* (2007) Environm. Microbiol. 9(2): 445-452
Pacific white shrimp Litopenaeus vannamei	~4 gm	2		6 weeks		Slightly higher survival and productivity and lower total bacterial count in the intestine. Growth paramaters no different from the control	Corrêa da Silva *et al.* (2016) J. World Aquaculture Society
Brine shrimps Artemia franciscana	nauplii	0.001- 0.02	B. casei MSI04	48h	Vibrio		Kiran *et al.* (2016) npj Biofilms and Microbiomes 2
Pacific white shrimp Litopenaeus vannamei	~ 5gm	1-5		35 days		No effect on growth, FCR, weight gain	Duan *et al.* (2017) Fish & Shellfish Immunology 60: 520e528
Pacific white shrimp Litopenaeus vannamei		2				PHB could improve the digestive capacity of the shrimps	Correa da Silva *et al.* (2016) J. World Aquaculture Society
Blue mussel Mytilus edulis	larvae	0.1- 10 mg/L		14-18 days		Improved survival	Van Hung *et al.* (2015) Aquaculture 446: 318-324
Brine shrimp Artemia franciscana	nauplii	32% VSS		48h	Vibrio campbellii	Improved survival	Halet et al. (2007) FEMS Microbiol. Ecol. 60: 363-369
Giant Tiger prawn Penaeus monodon	post-larvae	artemia +PHB		15 days	Vibrio campbellii	Improved survival	Ludevese-Pascual (2017) Aquaculture Nutrition 23: 422-429

ii) Other Potential Contaminants

As described above, no other contaminants are expected to be found in the notified substance. Although it is possible that certain strains of *M. extorquens* are able to produce spirilloxanthin, the strain KB203 used by KnipBio does not produce this compound. Moreover, data are presented above to show that the genetic manipulation of the parental strain has not affected the spectrum of carotenoids KB203 produces. *M. extorquens* is a natural producer of the C30 class of carotenoids, but these compounds, when ingested by aquatic animals, do not impart color to the flesh (Takaichi, 2009; Torrissen *et al.*,1989; Konovalova *et al.*, 2007; Delgado-Vargas *et al.*, 2000). As shown above, the production of C30 carotenoids by the KB203 production strain is the same as production by the KB200 parent strain, and in both strains, total C30 carotenoid concentrations are no greater than 60 ppm.

As discussed above, the concentrations of methanol and formaldehyde, which might arise in the notified substance due to its method of manufacture, are expected to be no more than 0.05% (500 ppm) and 0.0025% (25 ppm) respectively. Both substances are therefore expected to be present at levels below the maximum allowed under applicable regulations: 21 CFR 573.460 for formaldehyde and 21 CFR Part 573.637 for methanol, as discussed in Section 3(a)(3) above.

d) Corroborative Target Animal studies

The notified substance has utility in the use as a protein supplement for aquaculture. KnipBio has carried out a number of studies in which the notified substance was fed to fish of several different species. These studies have shown the utility of the notified substance as a source for protein in the fish diet, and have also demonstrated that no adverse effects arose from the inclusion of the notified substance in the diet.

(Tlusty *et al.*, 2017) describes several animal feeding studies conducted by KnipBio using the notified substance, including feeding studies on Pacific white shrimp (*Litopenaeus vannamei*) growth and consumer taste preference, a study for which FDA has reviewed the protocol. KnipBio has also conducted a 12-week feeding study on trout (*Oncorhynchus mykiss*), for which FDA also reviewed the protocol. In each of these studies, animals performed equivalently when fed diets containing *M. extorquens* as when fed a standard aquaculture diet.

The results reported in (Tlusty *et al.*, 2017) demonstrate the potential broad applicability of *Methylobacterium extorquens* protein as a viable protein source for use in aquafeeds. When fed to fishes it resulted in equivalent performance in growth for grunts, and apparent digestibility coefficient for salmon as trials using traditionally formulated diets containing fishmeal. The salmon demonstrated higher digestibility for amino acids in the diets containing the notified substance. The Hardy *et al.* manuscript submitted to Aquaculture Research, included in **Appendix 2-11**, shows that the notified substance, when fed to trout,

resulted in equivalent performance for growth, feed utilization and nutrient retention, as compared to traditionally formulated diets containing fishmeal.

Table 2-8 in Part 2 and **Appendix 2-11** summarized the results of these studies as well as other unpublished studies that KnipBio has conducted or sponsored in which fish were fed the notified substance (in some cases, early formulations of the notified substance). These studies all show that the notified substance caused no harmful effects on the fish. Full study records for these studies, particularly including the studies published in Tlusty *et al.*, are available at the KnipBio offices should FDA be interested in reviewing them.

6) Summary of Safety Argument; Assertion of GRAS Status

KnipBio asserts that the generally available data and information that establish safety in accordance as discussed above provide a basis for our conclusion that the notified substance is generally recognized, among qualified experts, to be safe under the conditions of its intended use for both the target animal and for humans consuming human food derived from food producing animals.

a) Safety to target animals

The notified substance is based on a naturally-occurring microorganism, *Methylobacterium extorquens*, classified as Biosafety Level 1, that has never been reported to have pathogenic, toxic, or other hazardous properties (as confirmed in the company's literature searches), although strains of this species and other *Methylobacterium* species have been isolated from healthcare-associated infections in immunocompromised hosts. The starting wild type strain has been subjected to genetic manipulation only to remove two biosynthetic pathways, and since no heterologous, foreign or synthetic coding DNA has been introduced into the strain, the manipulation has not introduced any new biochemical functions into the strain.

The notified substance will be manufactured commercially using a well-understood, well-characterized growth medium, using standard fermentation procedures. AAFCO approved and/or food-grade materials of suitable purity will be used for all components of the growth media for all fermentations, and Good Manufacturing Practice and suitable Standard Operating Procedures will be used at all stages of manufacture.

The notified substance will have a well-characterized composition that will provide an excellent source of protein for the target species. The product will contain no impurities that might cause harm to the target species. The only impurity that is expected to be present in the notified substance would be certain levels of polyhydroxybutyrates, but since the notified substance will be added to aquafeed diets at a maximum of 10% by weight, the levels of PHBs to which the target species would be exposed would likely be no greater than 1-2.5% by weight in the total diet. Data from the literature summarized in this Notice indicate that the presence of PHBs in fish diets do not cause any adverse or negative effects and in fact may offer some benefits to the fish, such as enhanced growth rates.

Finally, the feeding studies conducted by KnipBio, including the published studies (Tlusty *et al.*, 2017), indicate that the product can be safely ingested by the target species with no adverse effects.

b) Safety to humans

KnipBio also believes that there will not be any adverse effects on the health of humans who consume aquaculture product that have been fed the notified substance, because there will be no exposure of humans to any deleterious substance. As described above, the notified substance itself is safe for ingestion by the target species and is unlikely to have any harmful component. The notified substance will largely consist of protein and amino acids, which when ingested by the target species will be metabolized and incorporated into proteins and other molecules within the fish gut. All components of the notified substance will be digested in the gut of the target species like normal feed ingredients. The entire product will therefore be metabolized by the aquatic animal like normal ingredients, and we do not anticipate any safety issue for human consumption. As discussed above, although the notified substance is expected to contain levels of PHBs no greater than 25%, such levels will be diluted at least tenfold in the fish diet. Further there is strong evidence, as discussed above, that microorganisms capable of degrading PHBs into short chain fatty acids can be found in the gut of many fish species., and so it is expected that there will be no residual concentrations of PHBs in the tissue of fish to which the notified substance has been fed, thus posing no health risk to humans who consume such fish.

Finally, KnipBio has conducted taste-testing with the Food Innovation Center (Oregon State) in which human subjects ingested small amounts of fish that had been fed preparations made from KnipBio's *M. extorquens* strain, and although those studies were not designed to test safety per se, there were no adverse effects noted in any individuals taking part in that study (Kampachi Sashimi Consumer Test, 2017).

For these reasons, KnipBio maintains that the notified substance is Generally Recognized as Safe for use in aquaculture feed, when used as an additive of up to 10% by weight in animal feed.

7) Discussion of (any) data inconsistent with GRAS determination

KnipBio has disclosed all safety data of which it is aware and have found none that is inconsistent with the GRAS determination.

8) Identification, justification for claims of confidentiality

All the data in this dossier are nonconfidential and are available to the public, except for specific information which constitutes trade secrets of KnipBio, Inc., and which the company claims as confidential in accordance with 21 CFR Part 171.1(h)(1). The two categories of information that are claimed as confidential trade secrets are (a) information regarding the manufacturing process for the notified substance; and (b) specific Standard Operating Procedures developed and maintained by KnipBio, which are used in laboratory and fermentation processes in the manufacture of the notified substance, or for quality control in manufacture of the notified substance. Public disclosure of information in each of these categories would result in substantial harm to KnipBio and its business, by providing

the company's competitors a significant advantage in allowing them to recreate the company's proprietary processes.

In addition, KnipBio has claimed as confidential the results of one fish feeding study described in **Appendix 2-11**, because this study has not been published in the scientific literature. KnipBio has not relied on this study in its substantiation of GRAS status but it has been cited herein as corroborative evidence. The claim of confidentiality arises from traditional courtesies towards KnipBio's academic collaborator maintaining confidentiality of studies before they are published in the scientific literature.

KnipBio, Inc. maintains internal procedures and practices to maintain the confidentiality of its trade secrets and other information. Documents containing confidential or trade secret information are marked as Confidential. Disclosure of such information within the Company is on a need-to-know basis and all Company employees have signed employment contracts that include strict confidentiality provisions, including a prohibition on unauthorized disclosure of information such as the confidential or trade secret information. Any individual or company outside of the Company who needs to know the confidential or trade secret information in the course of their business with the Company must, before receiving any such information, sign a written nondisclosure agreement to hold the information confidential and proprietary to the Company. KnipBio can provide further information to FDA CVM regarding its procedures and policies for maintaining the confidentiality of its trade secret information.

Part 7: List of supporting data and information in this GRAS notice.

This GRAS Notice relies on the following literature references in support of the finding of GRAS status for the notified substances. All these references are available in the public domain.

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Appendix 1

Spirilloxanthin production by PA1 and related strains

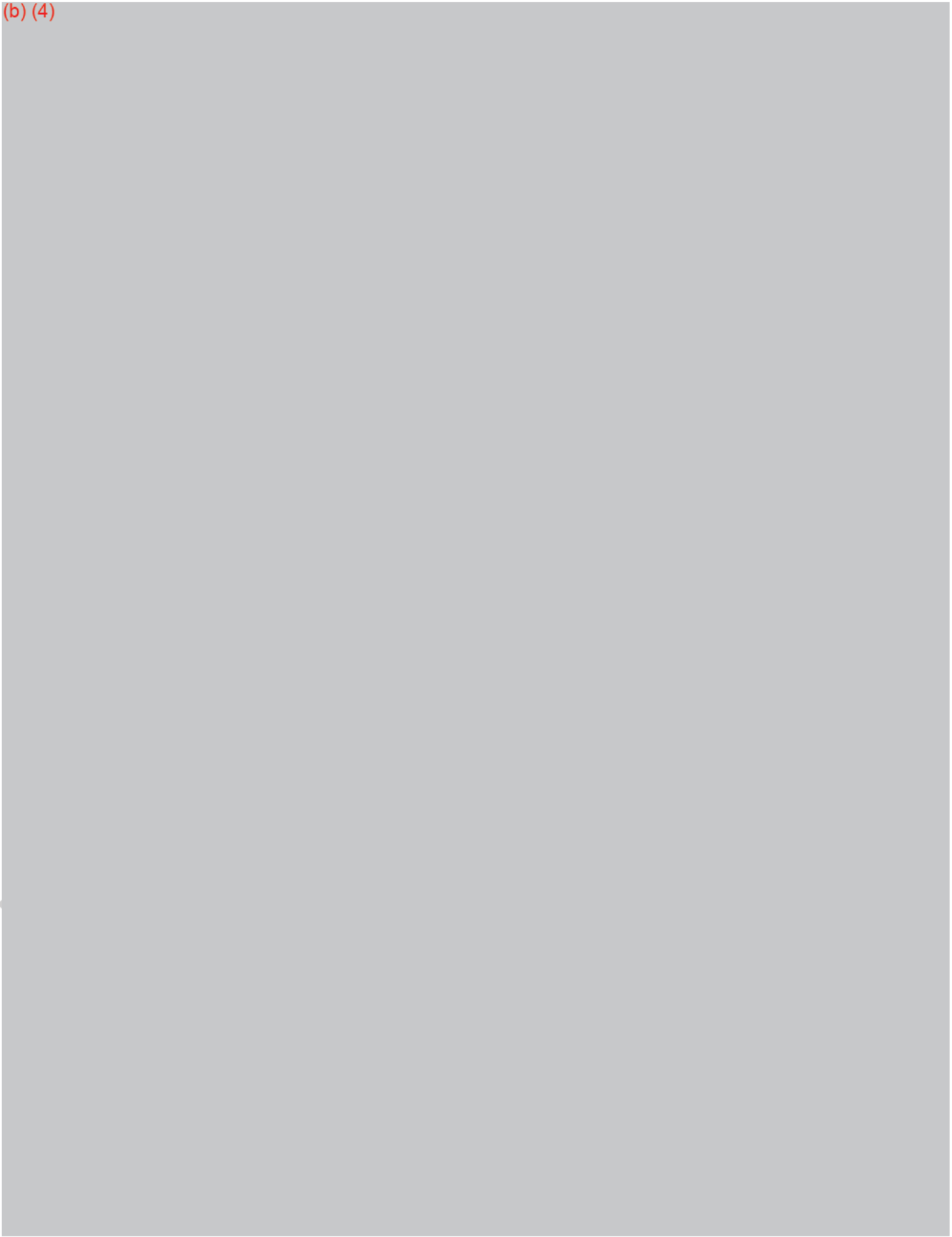
1. Purpose

Identify location of spirilloxanthin peak in tunable UV and MS spectra in strain extracts. Compare spirilloxanthin production of strains with and without intact crtCDF genes (for biosynthesis of spirilloxanthin from lycopene) and with and without the intact ppsR gene (which downregulates C40 biosynthetic genes in relevant growth conditions).

2. Strain Selection and Rationale

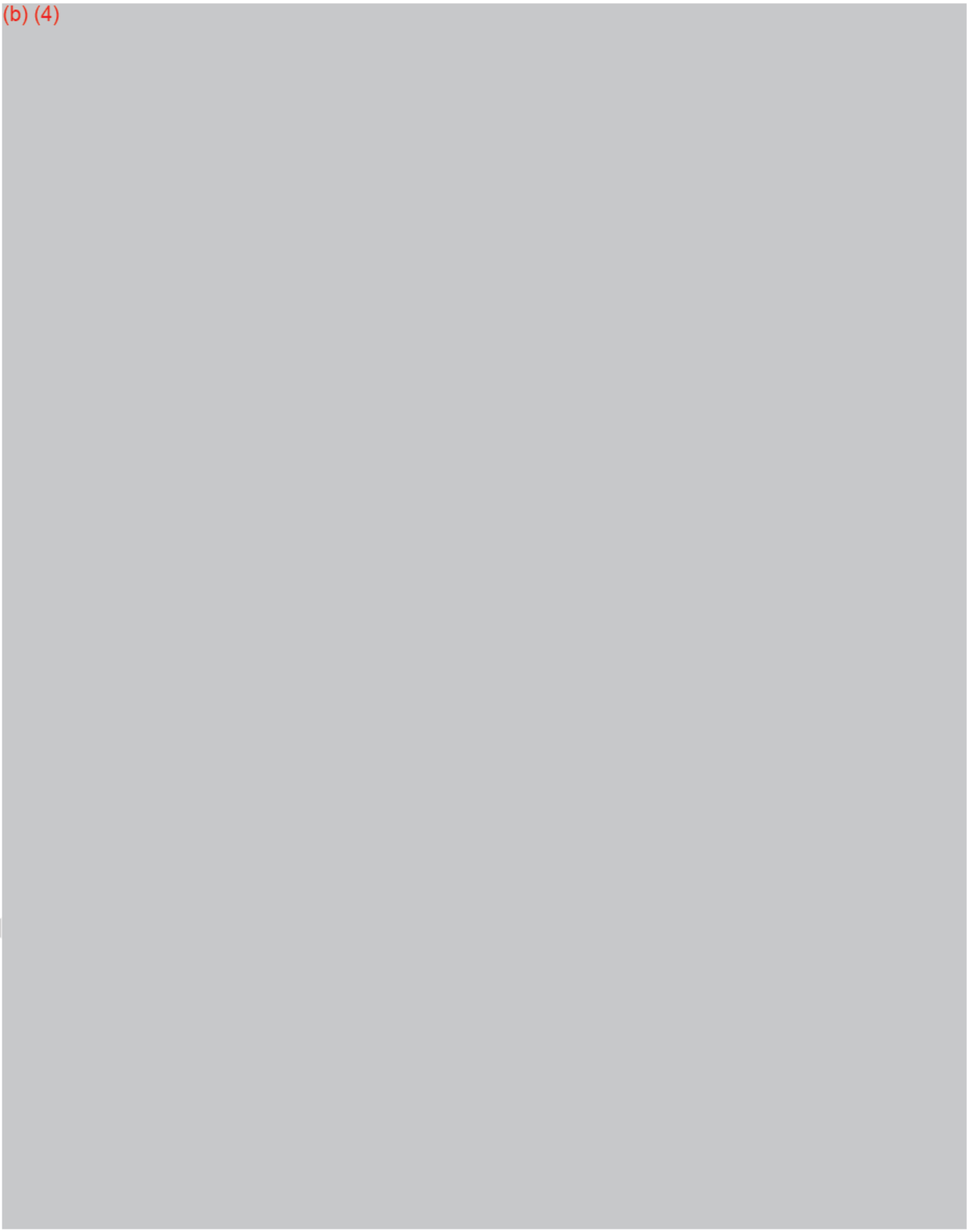
(b) (4)

(b) (4)



4. Procedure

(b) (4)

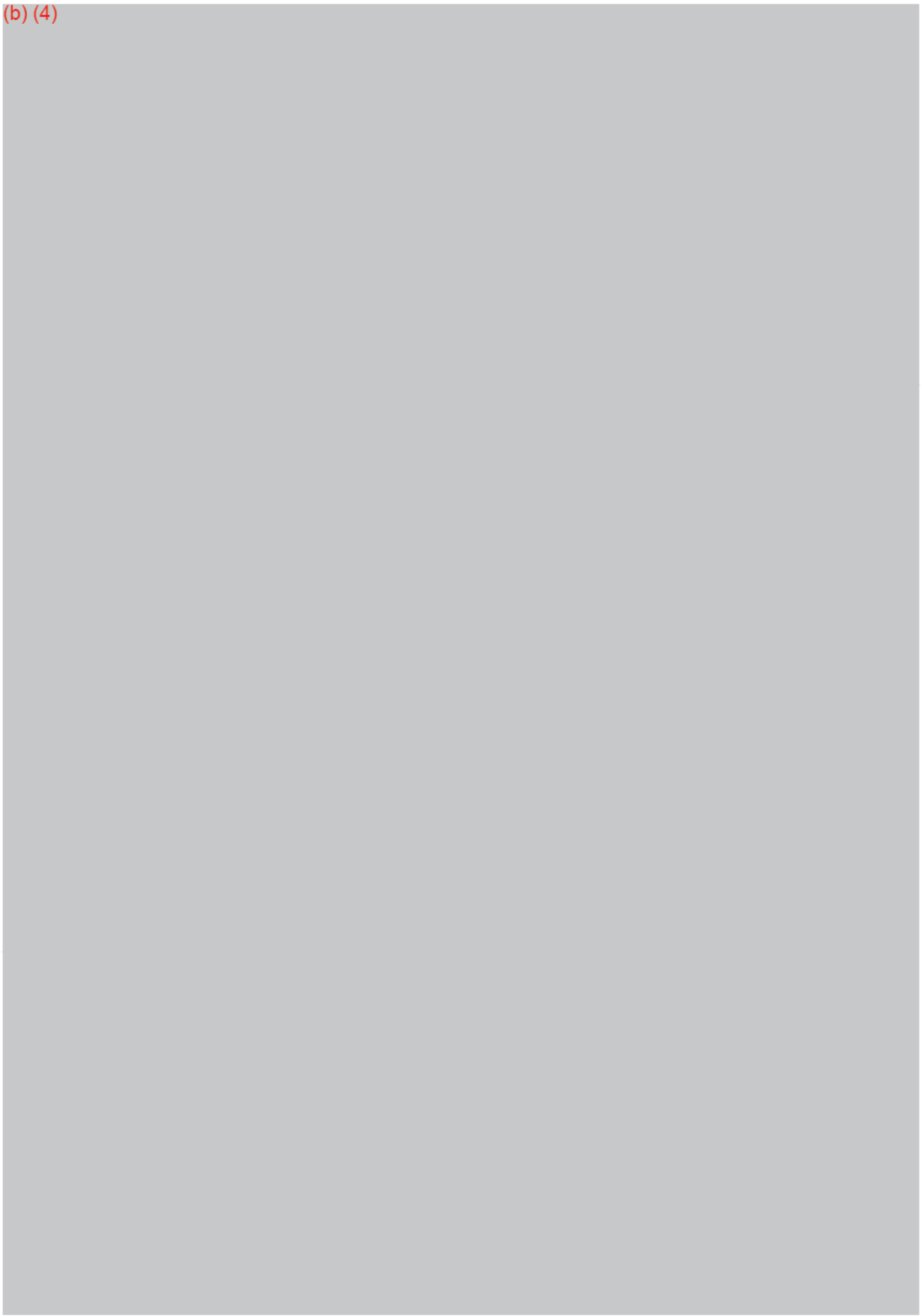


5. Analysis

(b) (4)

(b) (4)

(b) (4)



(b) (4)

6. References and notes

(b) (4)

Appendix 2

(b) (4)

ISO 9001
Certified Worldwide

July 13, 2018

(b) (4)

SUBJECT: FDA 21 CFR AND CVM COMPLIANCE – (b) (4)

To whom it may concern,

This product complies with the United State Food and Drug Administration's Code of Federal Regulations Title 21-Part 173.340, Secondary Direct Food Additives Permitted in Food for Human Consumption when used as a defoaming agent and its ingredients are listed under §173.340(a)(3). This product's use as a defoamer in wet milling of corn carries no usage limitation imposed by the regulation other than current good manufacturing practices (cGMPs) and usage is in an amount not in excess of that reasonably required to inhibit foaming.

The Center for Veterinary Medicine has used regulatory discretion and not taken action against the use of substances approved for use in human food as antifoaming or defoaming agents in animal feeds (21 CFR Part 173.340) when used according to the existing regulations.

We hope this information is useful to you. If you should have any further questions please feel free to contact us. Our corporate office number is (b) (4)

Sincerely,

(b) (4)

Regulatory Assistant

(b) (4)

(b) (4)

ISO 9001
Certified Worldwide

July 13, 2018

(b) (4)

Subject: Ingredient Composition – (b) (4)

To Whom It May Concern:

The composition of (b) (4) (b) (4) is as follows as noted on the label:

INGREDIENT LIST:

1. "Polyglycol"
[alpha]-Hydro-omega-hydroxy-poly
(oxyethylene)/poly(oxypropylene)/ poly(oxyethylene) block
copolymer

We hope this information is useful to you. If you should have any further questions please feel free to contact us. Our corporate office number is (b) (4).

Regards,

(b) (4)

Regulatory Assistant

(b) (4)

(b) (4)

Appendix 3

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

September 11, 2003

Mr. Gary Yingling
Kirkpatrick & Lockhart LLP
1800 Massachusetts Avenue, NW
Second Floor
Washington, DC 20036-1221

Dear Mr. Yingling:

You requested, on behalf of the Enzyme Technical Association, that OFAS review the use of certain defoaming and flocculating agents in the manufacture of enzyme preparations used in food. You provided information related to these compounds in your letters of December 20, 1996 (to Dr. Alan Rulis), 4-24-1998 (to Dr. Zofia Olempska-Beer), and 11-30-99 (to Dr. Zofia Olempska-Beer). You also arranged for a teleconference between ETA members and OFAS representatives, facilitated telephone contacts with technical experts from ETA member companies, and responded to numerous requests for clarification. We appreciate your and ETA's cooperation.

We reviewed the information on defoaming and flocculating agents that you submitted as well as the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. The enclosed attachment provides a brief overview of our evaluation and itemizes the evaluated defoamers (Table 1) and flocculants (Table 2). We conclude that these compounds are used by enzyme manufacturers in accordance with the principles of good manufacturing practice (GMP).

Sincerely yours,

Laura M. Tarantino, Ph.D.
Acting Director
Office of Food Additive Safety, HFS-200
Center for Food Safety and Applied Nutrition

Defoaming and Flocculating Agents Used in the Manufacture of Enzyme Preparations Used in Food

Enzyme Preparations

Most enzymes currently used in food are derived from microorganisms. The manufacturing process of such enzymes includes three major steps: fermentation, enzyme recovery, and enzyme formulation. The formulated products are generally referred to as enzyme preparations. In addition to the enzymes of interest, enzyme preparations contain added substances such as diluents, preservatives, and stabilizers. They may also contain metabolites derived from the production microorganism and the residues of substances used in the manufacturing process, such as components of the fermentation medium or defoaming and flocculating agents used during fermentation and recovery. When FDA reviews safety data on enzyme preparations, it considers all components of the preparation.

Defoaming Agents

Defoaming agents (defoamers) are used by enzyme manufacturers to reduce or prevent foaming during fermentation and recovery. They are formulated with ancillary ingredients such as surface-active agents or carriers. Defoamers currently used in the manufacture of food enzymes are listed in Table 1. The Table includes five major defoamers that are identified by a double asterisk and several compounds that are used either as secondary defoamers or ancillary ingredients in defoamer formulations.

The major defoamers are added to the fermentation broth at levels within the range of 0.05-1% on a weight basis. Some of these defoamers, for example, polyoxyethylene-polyoxypropylene block copolymer, may contain trace levels of ethylene oxide, propylene oxide, and 1,4-dioxane which are known to cause cancer in laboratory animals. The Office of Food Additive Safety (OFAS) has evaluated the use of defoamers listed in Table 1 and determined that human exposure to the residues of these defoamers in enzyme preparations does not present human safety concern.

Flocculating Agents

Flocculating agents (flocculants) are used in the enzyme recovery step to separate microbial cells and cell debris from the fermentation broth containing the dissolved enzyme. The flocculation typically consists of two steps - primary flocculation and secondary flocculation. In the primary flocculation, inorganic salts (such as calcium chloride or aluminum sulfate) or "low molecular weight" polymers (such as polyamines) are used to agglomerate the cellular debris. The primary flocculation is usually followed by the secondary flocculation in which "high molecular weight" polymers are used to aid the formation of larger agglomerates that are subsequently removed by centrifugation or filtration. The polymers used as flocculants can be either cationic or anionic. The cationic polymers are added to the fermentation broth at levels not higher than 1% on a

weight basis. The anionic polymers are used at levels at or below 0.025%.

The flocculants used in the manufacture of food enzymes are listed in Table 2. They include inorganic salts, polyamines, and polyacrylamides. Several of these compounds are regulated in 21 CFR either as food additives or GRAS substances. Certain polyamines may contain traces of epichlorohydrin and 1,3-dichloro-2-propanol. Polyacrylamides usually contain very low levels of acrylamide. These contaminants of polyamines and polyacrylamides are known to cause cancer in laboratory animals. OFAS has evaluated all polymers included in Table 2 and determined that human exposure to the residues of these flocculants in enzyme preparations does not present human safety concern.

Sources of Information on Defoamers and Flocculants

OFAS compiled data on defoamers and flocculants listed in Tables 1 and 2 using information voluntarily submitted by the Enzyme Technical Association. OFAS also relied on the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. Other sources of information included published articles, computer searches, and Material Safety Data Sheets issued by manufacturers of defoamers and flocculants.

Table 1. Defoamers Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Polypropylene glycol**	25322-69-4	Average MW: 2000
Polyglycerol polyethylene-polypropylene glycol ether oleate**	78041-14-2	
Polyoxyethylene-polyoxypropylene block copolymer**	9003-11-6	Average MW: 2000
Polypropylene glycol monobutyl ether**	9003-13-8	
Polydimethylsiloxane**	63148-62-9 68083-18-1	
Silica	7631-86-9 63231-67-4	
Stearic acid	57-11-4	
Sorbitan sesquioleate	8007-43-0	
Glycerol monostearate	123-94-4	
Polysorbates (polyoxyethylene sorbitan fatty acid esters)		Polysorbate 60 (CAS No. 9005-67-8), Polysorbate 65 (CAS No. 9005-71-4), and polysorbate 80 (CAS No. 9005-65-6) are regulated as food additives and components of defoamer formulations
Rape oil mono- and diglycerides	93763-31-6	
White mineral oil	64742-47-8	

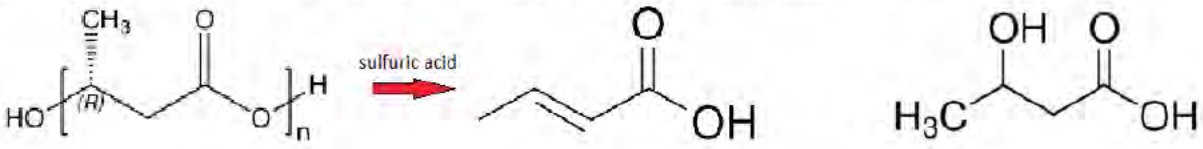
Table 2. Flocculants Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Dimethylamine-epichlorohydrin copolymer	25988-97-0	Cationic polyamine
Methylamine-epichlorohydrin copolymer	31568-35-1	Cationic polyamine
Dimethylamine-epichlorohydrin-ethylenediamine terpolymer	42751-79-1	Cationic polyamine
Polyacrylamide modified by condensation with formaldehyde and dimethylamine	67953-80-4	Cationic polyacrylamide
Acrylamide-acryloxyethyl-trimethyl-ammonium chloride copolymer	69418-26-4	Cationic polyacrylamide
Acrylamide-acrylic acid copolymer	25987-30-8 9003-06-9	Anionic polyacrylamide
Aluminum sulfate	10043-01-3	
Calcium chloride	10035-04-8 10043-52-4	

Appendix 4

1. *Background and Overall Summary*

The method adapted and verified here is based on a HPLC assay published by Karr *et al.* 1983 (2) to measure Polyhydroxybutyrate (PHB). PHB is converted by sulfuric acid into crotonic acid. Crotonic acid can be measured in crude reactions by absorbance at 210 nm or more precisely by separation and measurement on an HPLC or UPLC. Following conversion, there are no major peaks other than crotonic acid when measured by absorbance at 210nm.



PHB | Crotonic acid | 3-Hydroxybutryic acid

Our method showed excellent repeatability, linearity, low limits of detection and quantitation, and acceptable specificity. Our adaptation to using a UPLC reduced per sample times from 30 minutes in Karr (2) to 3 minutes per sample. The data presented herein supports the use of Method 1 for the analysis of PHB content in Knipbio produced single cell protein (SCP) containing 0.05 to 30% PHB (w/w). Collectively, the results presented here suggest the out largest contribution to error are in measuring dry cell weights and standard preparation.

Table 1: Summary of acceptance criteria and results.

Verification	Acceptance Criteria A	Acceptance Criteria B	Acceptance Criteria C	Passed Criteria	Failed Criteria
Repeatability Precision	RSD_r < 4 %.			A	
Linearity	r > 0.995	r^2 > 0.99	y in. < 2% area of target 500ug	A, B, C	
LOD†	S/N > 5			A	
LOQ‡	S/N > 10	RSD < 10%		A, B	
Specificity	Signal in matrix blank < twice the LOQ	2 Spike samples with 1mg PHB have < 10% Dev	Crotonic acid values within 5% on average	A, B, C	
Accuracy	4 Spike samples with 1mg PHB have <10% Dev on average			A	

*Linearity is tested using standards of amount 50, 100, 250, 500, 1000, 2000, 3000 ug.
†LOD is (b) (4) ug/mL or an equivalent amount of (b) PHB in Method 1.
‡LOQ is (b) (4) ug/mL or an equivalent amount of (b) ug PHB in Method 1.

2. Description of Method 1 and Method 2:

Sample processing:
Five to fifteen milligram of dried biomass is transferred to a glass tube to which 0.5 mL sulfuric acid is added. The sample is boiled for 30 minutes and allowed to cool. The sample is diluted to 3 mL total by three additions of water. To generate sample to separate to be injected and separated on a UPLC, the sample are further diluted in sample vials by adding 1, 5, 10, 20, 50, or 100uL to enough MilliQ water to equal 1 mL total sample volume. The sample are injected on a UPLC and a UV detector is used to measure the absorbance over time. Concentrations can be calculated by comparing the peak area to those of known standards PHB that have been subjected to the same process. By dividing the amount of PHB obtained by the dry cell weight, the percent PHB can be determined.

Method 1:

LC: Waters UPLC H-Class with CM-A, QSM, TUV, SM-FTN
MS: Waters 3100 Mass Detector
Column: Waters Acquity UPLC BEH C18, 1.7µm , 2.1x50mm, 130Å (186002350)
Column temperature: 32C
Sample temperature: 10C
Injection volume: 5µL typical. Dilutions down to 1 µL
Buffers:

- A: MilliQ water + 0.1% (v/v) Formic Acid
- B: MeOH + 0.1% (v/v) Formic Acid
- Seal/Purge Wash: 1:1 MeOH: H20
- Needle Wash: 100% MeOH

Run conditions:

- Isocratic separation using 90%A, 10%B at 0.45mL/min for 2.5 minutes.
- 0.5mL pre-equilibration

Method 2:

LC: Waters UPLC H-Class with CM-A, QSM, TUV, SM-FTN
MS: Waters 3100 Mass Detector
Column: Waters Acquity UPLC BEH C18, 1.7µm , 2.1x50mm, 130Å (186002350)
Column temperature: 32C
Sample temperature: 10C
Injection volume: 5µL typical. Dilutions down to 1uL
Buffers:

- A: MilliQ water + 0.1% (v/v) Formic Acid
- B: MeOH + 0.1% (v/v) Formic Acid
- Seal/Purge Wash: 1:1 MeOH: H20
- Needle Wash: 100% MeOH

Run conditions:

- Isocratic using 60%A, 40%B at 0.45mL/min for 2 minutes.
- 0.5mL pre-equilibration

Explanation for modifying original method:

Background:

The previous submission used the older UPLC Method 2, which separated the crotonic acid using an isocratic method with less Buffer A (MilliQ water + 0.1% formic Acid) and more Buffer B (Methanol + 0.1% FA). Method 2 used a 60:40 ratio of A:B and different peak fitting settings. Method 2 utilizes a 90:10 ratio of A:B and the Waters MassLynx peak fitting settings were changed to utilize Apex peak fitting. The 90:10 A:B method, Method 1, had increased peak areas, decreased background, increased signal to noise, and decreased use of organic solvents relative to the older 60:40 A:B ratio in Method 2. Additionally, increasing the aqueous component increase ESI+ mass spec signal of crotonic acid. We are including data supporting this change below.

Test procedure:

Standards comprising 100, 250, 500, or 1000µg PHB were prepared by two users (U1, U2) and analyzed using a UPLC in a manner similar to Methods 1 and 2 above. The ratio of Buffer A to Buffer B were tested at 50:50, 60:40, 70:30, 80:20, and 90:10 and compared. The peak area of the samples was determined using Waters' Masslynx. Relative Standard Deviation (RSD) was determine to compare the variability in repeat injections of the same sample. The signal to noise ratio was calculated by dividing the average peak area by the average peak area of multiple blanks to determine which method would have the lowest limit of detection. Lastly, the linearity of the two sets of standards was compared to ensure that which method would be better for quantitation.

Results from different ratios of Buffer A and B:

Increasing %A to 90% led to increased retention times and greater peak area (Table 2The increase in retention (Fig. 2) time moved the crotonate peak into an area with a better baseline, which increased signal to noise (Table 4), and allowed for better peak fitting which decreased the RSD of multiple injections (Table 2). All methods were found to have no major differences in linearity (Table 4). Consistent with the higher peak area in the 90:10 A:B Method 1, the mass spec signal of two crotonate ions was greater than other methods (Fig. 3)

Fig 2. Overlay of Diode array traces. From left to right – 50:50, 60:40, 70:30, 80:20, 90:10 shows an increase in retention time with increasing amounts of Buffer A.

Fig. 3. Overlay of 68.9 major mass spec ion. From left to right – 50:50, 60:40, 70:30, 80:20, 90:10.
Waters 3100 Mass Spec. ESI+ mode. Similar results seen with 86.9+ MS ion.

Table 2: Higher ratios of Buffer A increase peak areas.

Sample name	PHB assayed (µg_	Peak area 50:50	Peak area 60:40	Peak area 70:30	Peak area 80:30	Peak area 90:10	Peak area 90:10 repeat
U1 - blank	(b) (4)						
U1 - 100							
U1 - 250							
U1 - 500							
U1 - 1000							
U2 - blank							
U2 - 100							
U2 - 250							
U2 - 500							
U2 - 1000							

Table 3: Higher ratios of Buffer A decrease injection RSD, due to better peak fitting.

Sample	RSD (%) 3 inj. 50:50	RSD (%) 3 inj. 60:40	RSD (%) 3 inj. 70:30	RSD (%) 3 inj. 80:30	RSD (%) 3 inj. 90:10	RSD (%) 3 inj. 90:10 repeat
U1 - blank	(b) (4)					
U1 - 100						
U1 - 250						
U1 - 500						
U1 - 1000						
U2 - blank						
U2 - 100						
U2 - 250						
U2 - 500						
U2 - 1000						
Avg. RSD						

Table 4: Higher rations of Buffer A have higher signal to noise.

Sample	S/N 50:50	S/N 60:40	S/N 70:30	S/N 80:30	S/N 90:10	S/N 90:10 repeat
U1 - 100	(b) (4)					
U1 - 250						
U1 - 500						
U1 - 1000						
U2 - 100						
U2 - 250						
U2 - 500						
U2 - 1000						

Table 5: Increasing amounts of aqueous buffer A did not affect linearity.

	50:50	60:40	70:30	80:30	90:10	90:10 repeat
JA	(b) (4)					
DS						

3. Repeatability Precision.

Rationale:
Determine the repeatability of two aspects of our method so we can ascertain the precision of the measurement system by:
(1) Comparing the same sample injected three times will tell you the precision of the UPLC
(2) Comparing ten separate dilutions of the same analyte will tell you the precision of the pipettes and user.

Test procedure:
Following sulfuric acid derivatization and dilution to 3mL, the 500ug PHB standard was diluted ten fold in MilliQ water ten times by User 1. Each replicate was injected three times using Method 1. Repeatability was also retested with a new UPLC column with a 500ug PHB standard diluted 50 times in MilliQ water ten times by User 2. Retention time, peak area, and peak height were recorded for all injections.

Acceptance criteria:
According to (1), the Quantitative Method Acceptability Criteria RSD_r ranges from 22% and 2% depending on method levels. The effective concentration in the sample vial of 500 ug sample diluted ten fold is 16.6ug/mL or 16.6mg/kg. The acceptable RSD_r values for this concentration are between 4 and 6%. If the vial is diluted fifty fold, the effective concentration is 3.33 ug/mL or 3.33 mg/kg. The acceptable RSD_r values for this concentration are between 6 and 8%. Here, the peak height RSD of ten independent dilutions must be less than 4%.

Results:
RSD_r of 2.426 for peak height (Table 6) was below the 4-6% values for 100 to 10mg/kg method levels. RSD for repeat injections of the same replicate are much smaller and range from 0.08-

0.22% for peak area, and 0.01 to 0.43% for peak height, demonstrating the high precision of the UPLC. Similar results were seen with a different user and a new column. RSD_r of 0.541 for peak height (Table 7) is well within the acceptance criteria.

Table 6: Method 1 using 500ug PHB standard - User 1 on older column.

Replicate	Mean Ret. time	Mean peak area	Mean peak height	RSD Ret. time	RSD peak area	RSD peak height
1	(b) (4)					
2						
3						
4						
5						
6						
7						
8						
9						
10						
1st inj. Only 1-10						
All injections 1-10						

Table 7: Method 1 using 500ug PHB standard - User 2 on new column.

Replicate	Mean Ret. time	Mean peak area	Mean peak height	RSD Ret. time	RSD peak area	RSD peak height
1	(b) (4)					
2						
3						
4						
5						
6						
7						
8						
9						
10						
1st inj. 1-10						
All injections 1-10						

4. Linearity.

Rationale:
Determine the ability of a method to quantify an analyte based on its proportion to the response of a known set of standards within a certain range.

Test procedure:
Six standard solutions resulting from reacting at least six amounts of PHB (50, 100, 250, 500, 750, 1000, 1500, 2000, 3000 µg) with sulfuric acid were used to test Method 1 for linearity. PHB

amounts are typically 10-30% dry cell weight, but can be 1% or lower for certain strains or under certain growth conditions. For ~5mg of DCW, this equates to 500- 1500 µg or as little as 50 µg. For ~10mg of DCW, which is typically used if the %PHB is lower, this equates to 1000 to 2000 µg or as little as 100 µg. The current standards are linear from 10 to 600% of a 500 µg target concentration or 20% to 300% of a 1000 µg concentration.

Acceptance criteria:
The correlation coefficient for at least six standards generated from reacting purified PHB in amounts between 50 and 3000 µg must be greater than 0.995. The coefficient of determination must be greater than 0.99. The y-intercept must be less than 2% of the target response (peak area) of 500 µg. If a standard set fails either of these criteria, they are not used and new ones are made.

Results:
Method 1 results for four separately prepared standards from three users are below. The U1, U2-2, and U3 standards had acceptable correlation coefficients, coefficients of determination, and y intercept values relative to the target response of 500 µg (Table 8). The U2-1 standard's coefficient of determination and correlation coefficients were too low to meet the acceptance criteria.

Table 8: Linearity test of three standard sets

Curve	Stand. set	Fit Type	Equation used	R^2	r	r^2	%y interc. of targ. Resp.	Results
1	(b) (4)							
2								
3								
4								
5								
6								
7								

5. *Limits of Detection/ Quantification (LOD/LOQ)*

Rationale:
Establish the limits of detection and limits of quantitation for the method by determining:
(1) the minimum amount that is quantifiable
(2) the range over which the method can be accurately used.

Acceptance criteria:
LOD is lowest concentration where the average peak area of six dilutions is five times greater than the average peak area of at least 6 blanks (S/N peak area > 5:1). LOQ is the lowest concentration where the average peak area of six dilutions is 10 times greater than the average

peak area of at least 6 blanks (S/N peak area >10:1) and RSD is less than 10% for six replicates (RSD <10%).

Initial linearity test procedure:
The 50 µg PHB standard was diluted down to 10 or 25 µg equivalents. Instead of diluting the 50 µg 100µL with 900µL MilliQ water (1.66µg/mL), 20 µl and 50 µL of the 3 mL PHB H2SO4 solution were added to 980 µL and 950 µL MilliQ water, respectively. Each sample was injected six times at 5, 4, 3, 2, 1, 0.5 µL using Method 1. In each case, multiple blanks were used to determine the baseline area and height for signal to noise (S/N) ratios.

Initial Results:
The average peak height and area of blanks are given in Table 9. The average peak area and height of the diluted PHB standards are given in Table 10 and 11 respectively. The initial linearity test did not reach LOD or LOQ. The S/N for all peak areas tested is too high (>96) suggesting LOD is lower than 0.033 µg/mL. The samples were diluted more in the final linearity test procedure below.

We also looked at linearity for both peak height ($y = 26766x + 37.708 R^2 = 0.9995$) and peak area ($y = 1054.3x - 5.9221$; $R^2 = 0.9997$) for dilutions made by reducing injection volume suggesting the UPLC is more than adequate for reaching lower dilutions by decreasing injection volumes. However, the RSD of multiple 0.5 uL injections of the same analyte was much greater than the 5, 4, 3, 2, 1 ul injections (See Table 10). Therefore, injections below 1uL will not be used.

Table 9: Average peak area & height of 33 blanks using Initial linearity test procedure and Method 1.

	Average	STDEV	RSD	MAX
peak area	(b) (4)			
peak height				

Table 10: Peak area of initial linearity test procedure using Method 1

injection volume	µg/mL	ug equiv	Avg. peak area	STDEV	Mult. Inj. RSD (%)	S/N
0.5	(b) (4)					
1						
2						
3						
4						
5						
0.5						
1						
2						
3						
4						
5						

Table 11: Peak Height of initial linearity test procedure using Method 1

injection volume	µg/mL	ug equiv	Avg. peak height	STDEV	Mult. Inj. RSD (%)	S/N
0.5	(b) (4)					
1						
2						
3						
4						
5						
0.5						
1						
2						
3						
4						
5						

Final linearity test procedure (new column and standard):
A new column was installed for the results below. All the standards and samples were prepared by adding 20uL from the diluted H2SO4 (3mL) reaction to 980µL MilliQ-H_20. For the LOD/LOQ test the 50 and 100 µg PHB standard were diluted six times using 10, 5, 1 ul into a 990, 995, and 999 uL MilliQ-H_20.
Thus, the 50 µg PHB standard was diluted down to 25, 12.5, and 2.5 µg equivalents, and the100 µg PHB standard was diluted down to 50, 25, and 5µg equivalents. The thirty six resulting equivalents (six each of 50, 25, 25, 12.5, 5, 2.5 ug) were injected three times each at 5, 2.5, and 1 µL using Method 1. The 0.5uL injection volume was not used as the reinjection RSD increased considerably over 1uL injection (see initial test procedure results above). In each case, multiple blanks were used to determine the baseline area and height for signal to noise (S/N) ratios. The new standard had acceptable linearity (r > 0.99295, r2 > 0.998590, %y intercept to 500ug area response = 0.230%).

Final linearity test results:
The average peak height and area of blanks are given in Table 12. The average peak area and height of the diluted PHB standards are given in Table 13 and 14 respectively.

The LOD is (b) (4) ug/mL or an equivalent amount of (b) PHB. Lower dilutions had signal to noise levels below 5. The LOQ is (b) (4) ug/mL or an equivalent amount of (b) ug PHB. Lower dilutions had RSD greater than 10. Similar results were found when using peak height, except LOQ was higher at (b) (4) ug/mL and (b) ug PHB.

The low concentration samples also compared to their theoretical value to see if linearity extended down to the LOD or LOQ. For samples with greater than a 5 ug PHB equivalence (0.0333 µg/mL concentration), the % deviation was less than 10% (See Table 13). Linearity

between the theoretical and measured values was acceptable (r2 > 0.9992). Linearity could be extended to this level if necessary but would require additional standards.

Table 12: Average Peak area and height of 32 blanks using final test procedure and Method 1.

	Average	STDEV	RSD	MIN	MAX
peak area	(b) (4)				
peak height					

Table 13: Peak area of the final test procedure using Method 1

Standard (ug)	injection volume	µg equiv.	µg/mL	Avg. peak area	µg PHB measured	% DEV	STDEV	RSD	S/N	Failed peak fits
50	(b) (4)									
50										
50										
50										
50										
50										
50										
50										
50										
100										
100										
100										
100										
100										
100										
100										
100										
100										

Table 14: Peak height of the final test procedure using Method 1

Standard (ug)	injection volume	µg equiv.	µg/mL	Avg. peak height	STDEV	RSD	S/N	Failed peak fits
50	(b) (4)							
50								
50								
50								
50								
50								
50								
50								

50	(b) (4)
100	
100	
100	
100	
100	
100	
100	
100	
100	

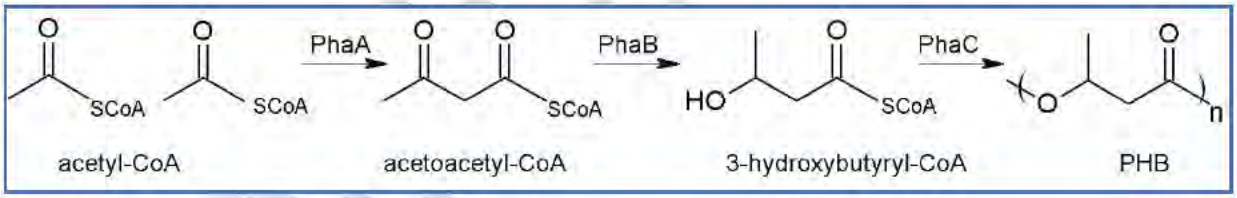
6. *Specificity and Accuracy*

Rational:

(1) Determine how specific the UPLC response is and whether there are other substances in matrix blanks lacking PHB that could interfere with determining PHB content.
(2) Determine the accuracy or closeness of the results to a theoretical value.

Test procedure:

Cultures of several strains were grown in flask with minimal media supplemented with either methanol or succinate as the sole carbon source. Strains with deletions in PHB synthesis genes *phaA, phaB, phaC,* or *phaAB* are not expected to make PHB and will serve as matrix blanks (see Table 13).

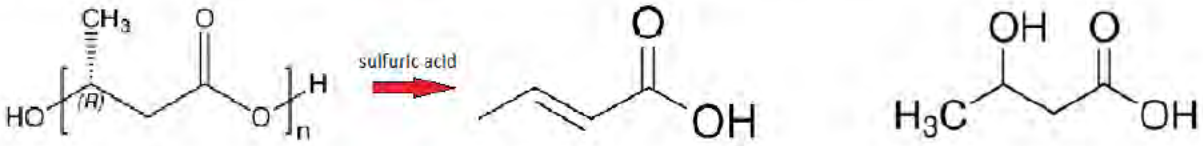


To harvest, 4 mL of culture was pelleted, washed with 4mL 0.05X PBS, pelleted again, and the resulting biomass was lyophilized in pre-weighed glass tubes. Some cultures were harvested multiple times to test the specificity and accuracy by spiking them with different amounts of PHB standards.

Spiking the samples occurred while making the U3 standard set (See Table 8). Briefly, A 5mg/mL resuspension of PHB in chloroform was prepared in a glass tube and weighed. After sonicating and heating at 55-58C until the PBH appears dissolved, the tube was allowed to cool and then reweighed. Based on density, additional chloroform was added back to match the original weight. The volume necessary from the amount of PHB needed for standards or as a spike was added to a clean glass tube or the lyophilized cell pellet, respectively. After drying off the chloroform in a Speed Vac, the tubes were reweighed to determine if the correct mass of PHB had been added.

The experiment was run on a newly installed column as above with new U3 standards, which had acceptable linearity (r > 0.99295, r2 > 0.998590, %y intercept to 500ug area response =

0.230%). A second standard was also prepared. A stock of crotonic acid was made at 2mg/mL and then diluted sequentially to generate several values that should match the amount of crotonic acid generated by the PHB standards used here. By comparing the calculated values, we can determine the conversion efficiency. PHB should convert to the same mass of crotonic acid; the repeating 3-hydroxybutryic acid in PHB has the same molecular formula and weight.



PHB | Crotonic acid | 3-Hydroxybutryic acid

Specificity acceptance criteria:
No signal in the matrix blanks should be greater than 2 times the LOQ (<2 .5ug). Frequently blanks will 'measure' 1.2 to 1.3ug (average 1.206, RSD 2.07% for 32 blanks. The values for 50, 25, 10, 5, 2.5, 1 ug/mL crotonic acid should be within 5% on average their expected value. Spiked samples of 2 matrix blanks with 1mg PHB should be within 10% of their theoretical values

Accuracy acceptance criteria:
Spiked samples of 4 matrixes with 1 mg PHB should be within 10% on average of their theoretical values.

Results:
The mutant strains *ΔphaA, phaB, phaAB* had no measurable PHB below 2.3ug (Table 15). The mutant *ΔphaC* had a detectable amount of PHB that was 10 times the LOQ (See Fig. 3). This may be due to accumulation of 3-hydroxybutryl-CoA or an alternative PhaC enzyme.

Table 15: Measured PHB in WT, *phaA, phaB, phaC, phaAB* mutant strains and their spiked amounts

Sample	Strain	ug PHB added theor.	Carbon	WT	Avg ug PHB	Std	Mult. Inj. RSD	ug PHB added measured	% Dev
D1	(b) (4)								
D2									
D3									
D4									
D5									
D5 ½ inj.									
D6									
D7									
D8									
D9									
D10									
D10 ½ inj.									
D11									

D12	(b) (4)
D13	
D14	
D15	
D16	
D17	
D18	
D19	
D20	
D21	
D22	
D23	
D24	
D25	
D26	

(b) (4)

Fig. 3. *ΔphaA, phaB, phaAB* mutants had no detectable PHB. The *ΔphaC* had PHB 10X higher the LOQ.

PHB conversion efficiency to crotonate:
The calculated concentration of crotonic acid dilutions matched well the theoretical value (Table 16). The mean difference was 3.9%. Higher amounts of crotonic acid overloaded the column when concentrations were greater than 50ug/mL. A similar upper limit was seen when determining the linearity results above. When making the 3mg standard by adding 100uL of the 3mL initial solution to 900uL MilliQ H_20 (100ug/mL) distorted peaks would occur and the linearity measures r and r^2 would decrease. This did not occur when diluting the 3mg standard

by using 50uL into 950uL MilliQ H_20 (50ug/mL). This suggest the maximum amount that can be loaded onto the column is ~0.25 ug.

Table 16: Conversion efficiency - comparison of crotonate standard to PHB standard.

Crotonate	ug/mL	Average	STDEV	RSD	Equivalent ug PHB	% Diff.	Notes
A	(b) (4)						
B							
C							
D							
E							
F							
G							
H							

Standard preparation accuracy:
The weight of the PHB added after drying was close when 1 mg or more PHB was added (Table 17). The average % deviation was 11.6. This improves when higher amounts of PHB were added (6.9% average for 2 and 3mg). Below 1mg, the % weight error was greater than 10%; however, the % deviation by UPLC was not as high (compare Table 15 and Table 17). This suggest weight measurement of samples may be a major contribution to overall error.

Table 17: Accuracy in measuring small amounts of PHB additions to preweighed tubes.

Type	mg PHB added	additional weight	% error	Type	mg PHB added	additional weight	% error
Standard	(b) (4)						
Standard							
Standard							
Standard							
Standard							
Standard							
Standard							
Standard							
Standard							
Standard							
Standard							
Standard							

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Appendix 5

1. *Background and Overall Summary*

The purpose of the method is to test the amounts of methanol and formaldehyde in spray dried batches of biomass by adapting a colorimetric method commonly used for quantifying methanol in aqueous solutions that was published by Anthon and Barret (1). To test for methanol, an alcohol oxidase from *Pichia pastoris* is first used to convert methanol to formaldehyde prior to adding the Nash reagent (2). The Nash reagent is a mixture of acetylacetone and ammonia which reacts specifically with formaldehyde to produce a chromophore that can be detected with absorbance or fluorescence. To test for formaldehyde, samples are simply mixed with the Nash reagent.

Formaldehyde and methanol are not expected to be in high concentrations at the end of methanol fermentation of *Methylobacterium extorquens* due to the residual enzyme activities from multiple methanol oxidases and formate activating enzyme (3-5). Furthermore, any residual formaldehyde is expected to react with proteins, lipids, and other compounds in the biomass. Methanol which is much more volatile than water (vapor pressure 13.02 kPA vs 2.34 kPa at 20 °C), would be expected to evaporate during spray drying, drum drying, or lyophilization.

Summary:
Below are several experiments in which we adapt and verify a published method (1) to measure methanol and formaldehyde in dried cellular biomass. Our modifications are:

(1) To extract any cellular methanol or formaldehyde, we resuspend the dried biomass in dilute phosphate buffered saline (PBS) and subject the cellular material to freeze thaw cycles that is frequently used to lyse bacterial cells. The mixture is pelleted, and the resulting supernatant is tested for methanol or formaldehyde as described above.
(2) To test for lower amounts of methanol or formaldehyde, we include higher amounts of the sample in the reaction relative to the standards. If we add 2ul of a standard and 16uL of a sample and correct for volume changes, we should be able to measure formaldehyde or methanol 8 folds lower.

However, based on the data herein, we can state that the levels of methanol and formaldehyde, if present are below 0.004% (w/v) and below the limit of detection (0.00004%), respectively.

2. *Methanol Summary*

In Experiments 1-3 below, we show our modification and verification of the method of Anthon and Barret (1) to attempt to measure methanol in spray dried biomass.

We established the linearity of the detection between (b) (4) and (b) (4) (w/v) and demonstrate that by increasing sample volumes relative to standards, it is possible to detect methanol as low as 0.002475% (w/v). We show that the methanol detected is specific in spray dried samples when spiked in at 0.05 and 0.005%.

Three powder biomass samples were subjected to freeze thaw lysis to ensure the methanol present is in solution and tested exhaustively to try and determine the amount of methanol present. Based on the data in this experiment, the safest estimate of methanol present is less than 0.004% (w/v).

3. *Experiment 1: Determining linearity and LOD*

Rationale:

(1) Determine the linearity of methanol method using standards of (b) (4) to (b) (4) MeOH (v/v) ((b) (4) mM).
(2) Determine the effects on linearity of increasing sample volume relative to standard volume to detect lower amounts of methanol.

Acceptance criteria:

Linearity is achieved if the standards from 0.05 to 0.25% (v/v) methanol have a $r^2 > 0.99$. For testing if increased samples give responses that are linear with their expected concentration a $r^2 > 0.95$ is considered acceptable.

Results:

Table 1 below shows at least three assays for three different users where linearity was achieved. When linearity is below our acceptance criteria of $r^2 < 0.99$ (**bolded** values), it is usually due to the highest standard of 0.25% MeOH. When this standard is removed r^2 increases to over 0.99. Typically, samples measuring outside the acceptable linear range, such as those giving values above 0.25% or below 0.05%, are discarded. They are retested following dilution, or a larger amount of the sample is compared to the standard (see below). As in (1), 0.25% ethanol, acetone, or 2-propanol did not have any signal above the alcohol free blank (data not shown).

Table 1: Linearity results from three users of methanol method standards.

User	r^2	% y intercept of 0.1% MeOH response	Standard range used in experiments	r^2 without 0.25% standard	Date
User 1	(b) (4)				170627
User 1					170628
User 1					170711
User 1					170712
User 2					180305
User 2					180419
User 2					180501
User 2					180523
User 3					180726
User 3					180726
User 3					180727

The standard reaction uses 2uL of the standard solution in a total volume of 202 ul, making the effective concentration of the standards between 0.0122 to 0.611mM. To measure lower concentrations, 4, 8, 16, or 32ul of sample can be used. After taking into account the increased volume, the percent methanol can be measured below 0.005%.

Table 2 below lists three experiments where the volume of the lower concentration standards was increased to 4, 8, 16, or 32uL. A linear fit between the expected value and the corrected response was generally good with $r^2 > 0.0975$. Thus, increasing the sample amount relative to the standard allows measuring methanol in the ug/mL range.

Table 2. Linearity results of samples at different volumes and lowest possible detectable limits.

Experiment	% Standard diluted	vol standards within range	r^2	Lowest Detectable % (v/v)	Lowest Detectable (mM)	Lowest detectable (mg/mL)
1A	(b) (4)					
1A						
1A						
1A						
1B						
1B						
1B						
1B						
1C						
1C						

Table 3. Linearity and LOD summary.

	% (v/v)	mM	% (w/v)
Linearity lower limit	0.005	1.23	0.0396
Linearity higher limit	0.25	61.74	0.198
Limit of detection	0.0003125	0.077	0.002475

MW Methanol:32.04 g/mol
Density: 786 g/L

4. *Experiment 2: Determination of the specificity and accuracy measurement.*

Rationale:
Test for specificity, accuracy, linearity, and limit of detection of methanol from cells grown in flask in absence of presence of methanol. Determine specificity using methanol spiked samples

Experimental Design:
We utilized the following samples:

1. KB203 grown with methanol as the sole carbon source.
    - Culture used for inoculation was grown in minimal medium with 0.5% methanol.

- Culture used for assay was inoculated with preculture of OD_{600} 0.05 into minimal media amended with 0.05% methanol.
- Cultures were fed an additional 0.05% methanol after 23 hours growth.
- Cell pellets from 4mL culture and culture supernatants were collected before feeding, where most of the initial methanol was depleted, and 3.5 hours after feeding.

2. KB203 grown with succinate as the sole carbon source.
   - Culture used for inoculation was grown in minimal medium with 30mM succinate.
   - Culture used for assay was inoculated with preculture of OD_{600} 0.05 into minimal media amended with 30mM succinate.
   - Cultures were fed an additional 30mM succinate after 23 hours growth.
   - Cell pellets from 4 mL culture and culture supernatants were collected before feeding, where most of the initial succinate was depleted, and 3.5 hours after feeding.

Sample processing:
All cell pellets were washed with 1mL 0.05X PBS. Following re-pelleting and removing the wash, the pellets were saved at -20°C until analysis. For analysis, cell pellets were resuspended by adding 200uL 0.05X PBS and mixing by pipetting.

To one tube of each cell pellet and culture supernatant, 1uL of 10% Methanol was added to spike in ~0.05% methanol. To one tube of each cell pellet after feeding, 10ul of 1mg/mL formaldehyde was added to spike in approximately 1.6mM formaldehyde (~0.048%) (see experiment 5). All cell pellet resuspensions were subjected to three freeze thaw cycles of at least ten minutes in a dry ice ethanol slurry and three minutes of bath sonication at room temperature. Following centrifugation at 15,000xG for 2 minutes, approximately 150uL of supernatant was transferred to a second tube and tested or methanol or formaldehyde levels. Samples were tested at 2, 4, 8, volumes relative to the 2uL standard volume.

Acceptance criteria:
Specificity is met if samples grown without methanol (succinate) have no detectable methanol in their supernatant and no detectable methanol in their cells. Spiked samples of methanol in succinate supernatants should be within 10% of the theoretical value. Accuracy is met if spiking of methanol results in methanol response that is within 10% of the theoretical value. Linearity is met if $r^2 > 0.95$ when comparing the measured methanol concentration of samples with increased volume relative to standards to their expected concentration.

Results:
None of the cells grown in methanol or succinate showed any detectable methanol (See Table 4). As expected the supernatant of the succinate grown cultures did not have detectable levels of methanol.

In the MeOH spiked samples, less MeOH than expected was seen for all cells, including succinate, suggesting possible residual methanol oxidase activity. Evidence that this activity is ablated in the spray drying process is given in Experiment 3 below. The succinate sample 2, did

have the expected amount of methanol in the spiked supernatant samples from before and after feeding, suggesting it did not have extracellular methanol oxidase activity.

In most cases, the linearity was acceptable when comparing the theoretical and measured amounts when samples were measure at 2, 4, and 8ul. Exceptions (**bold** in Table 4) were found in samples where the higher volume gave responses that were outside the linear range. Sample can thus be measured to at least 0.00125% methanol.

Table 4. Specificity and accuracy or addition of samples spiked with Methanol.

sample	Feed	sample	Spike	% MeOH	Linearity check r^2	Linearity check over .25%	expected	result	%Diff.
1	(b) (4)								
1									
1									
1									
1									
1									
1									
1									
2									
2									
2									
2									
2									
2									
2									
2									

Using the data above and the limit of detections tested, none of the cells from samples grown in flask had methanol > 0.0016% w/v (800ppm) (see Table 5 below). In subsequent experiments 3 and 4 below, we were able to determine methanol is even lower in dried powder because we use higher volumes of sample relative to standards.

Table 5. Maximum possible methanol possible in cell biomass from Experiment 2.

Sample	Feed	OD	DCW (mg)	(mg/mL) cell susp.	Maximum possible MeOH (% w/v)
1	(b) (4)				
1					
2					
2					

5. Experiment 3: Testing for methanol and formaldehyde in spray dried biomass

Rationale:

(1) Measure methanol and formaldehyde in spray dried biomass using the following samples:

1. Powder 1 – June 2015 run
2. Powder 2 – May 2016 run
3. Powder 3 – October 2016 run

(2) Determine the minimum amount of methanol detectable by testing linearity of increasing sample volumes relative to standard volumes.

Sample Processing:

Powder was weighed and resuspended with 0.05X PBS to reach a concentration of 20mg/mL. The resuspension was aliquoted into two tubes of 200uL. To one tube of each powder, 1uL of 10% Methanol was added to spike in 0.05% methanol. All six tubes were subjected to three freeze thaw cycles of at least ten minutes in a dry ice ethanol slurry and three minutes of bath sonication at room temperature. Following centrifugation at 15,000xG for 2 minutes, approximately 150uL of supernatant was transferred to a second tube and tested or methanol. Samples were tested at 2, 4, 8, 16, 32uL volumes relative to the 2uL standard volume.

Acceptance criteria:

Linearity is met if $r^2 > 0.95$ when comparing the measured methanol concentration of samples with increased volume relative to standards to their expected concentration. Accuracy is met if spiking of methanol results in methanol response that is within 10% of the theoretical value.

Results:

All of the powders had very low, but linear increases (r^2 >0.8 to 0.98) in absorbance with increasing sample volume consistent with them having low amounts of methanol (Table 6). However, this increase in absorbance may also be due to proteins and other components.

The spiked samples had measured values that were on average 5.6% of the expected values (Table 6). Unlike the cells in Experiment 2 above, the spray dried powder has very little or no residual methanol oxidase activity. This is likely due to denaturation caused by heating during the spray dried process. The spiked samples also had acceptable linearity with increasing sample volumes (r^2 >0.995).

Table 6: Specificity and accuracy or addition of 20mg dried powder samples spiked with Methanol

	% MeOH	Sample vol. used	Total vol.	Adjusted % MeOH	Spike	r^2	sample volumes in range	Expected from spike	Measured from spike	%Diff.
P1	(b) (4)									
P1										
P2										
P2										
P3										
P3										

The amount of Methanol in spray dried powders is presented in Table 7.

Table 7: Maximum possible methanol possible in cell biomass from Experiment 3.

Sample	mg/mL cell susp.	Methanol % (v/v)	Methanol % (w/v)
P1 (June 2015 run)	(b) (4)		
P2 (May 2016 run)			
P3 (Oct. 2016 run)			

6. *Experiment 4: Formaldehyde*

Rationale:

(1) Determine the linearity of formaldehyde method using standards.
(2) Determine the effects on linearity of increasing sample volume.
(3) Determine the lowest amount of formaldehyde possible.

Acceptance criteria:

Linearity is achieved if the standards from 0.01 mg/mL (0.33mM) to 0.5 mg/mL (16.7mM) have a $r^2 > 0.99$. For testing if increased samples give responses that are linear with their expected concentration a $r^2 > 0.95$ is considered acceptable.

Results:

We generally have acceptable linearity when using formaldehyde standards of 0.01 mg/mL (0.001%) to 0.5 mg/mL (0.05%). Table 8 below shows several assays from different users. When linearity is below our acceptance criteria of $r^2 < 0.99$, it is usually due to a pipetting error. Samples outside the acceptable linear range, such as those giving values above 0.5 mg/mL or below 0.01 mg/mL are discarded. They are retested following dilution, or a larger amount of the sample is compared to the standard (see below). As expected (1), acetaldehyde did not increase response above background (not shown).

As in the Methanol assay, the amount of formaldehyde measured can be lowered by using more sample than standard. When tested between 0.00004% and 0.001% (w/v) linearity was an acceptable $r^2>0.9918$. Linearity of the samples describe in experiment 2 using 2, 10, 20uL sample

that were not outside the linear range were consistent with being able to measure lower concentrations by increasing sample volume (r^2 >.9551, 0.9532).
None of the extractions from the powders had any response above the LOD (0.00004%) even when sample were 25 times greater than the formaldehyde standard (50uL sample volume).

Table 8: Linearity results from two users of formaldehyde method standards.

	r^2	Background Corrected Response of 10mM	b	Percent y intercept of response	Standard range used	Date
User 1	(b) (4)					170718
User 1						170725
User 1						170801
User 1						170809
User 2						171020
User 3						180727

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Appendix 6

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ORIGINAL ARTICLE

Partial replacement of soybean meal with *Methylobacterium extorquens* single-cell protein in feeds for rainbow trout (*Oncorhynchus mykiss* Walbaum)

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Abstract

A feeding trial was conducted with juvenile rainbow trout (15 16 g initial weight) to assess the effects of including single-cell protein (SCP) produced from *Methylobacterium extorquens* in trout feeds. Three isonitrogenous and isoenergetic diets were produced: a control diet and two experimental diets containing 5% or 10% bacterial protein meal replacing soybean meal. Triplicate tanks, each containing 35 fish, were fed each diet to apparent satiation in a constant-temperature (15°C), flow-through tank system for 12 weeks. No statistically significant differences in final fish weight or other fish growth parameters were observed. Similarly, feed efficiency parameters showed no significant differences among groups. Nutrient retention indices (protein, fat, energy) were relatively high and similar among fish in each dietary treatment group, as were whole body proximate compositions. Fish survival was high, with a small but statistically significant increase for the 10% SCP diet. Overall, results demonstrate that SCP from *M. extorquens* is a safe and effective alternative protein for rainbow trout diets at the low inclusion levels tested. Slightly lower weight gain in fish fed the 10% SCP diet was largely due to lower feed intake, suggesting that adding palatability-enhancing ingredients to feeds may allow higher levels of *M. extorquens* SCP to be used without compromising fish growth.

KEYWORDS
alternative proteins, rainbow trout, single-cell protein, sustainable feeds

Appendix 7

Corroborative Data

KnipBio Inc

Pacific white shrimp *Litopenaeus vannamei*

Note: The details described below are part of a larger study and only the corroborative data to support the notified substance is shown.

2. Material and Methods

Primary ingredients were analyzed at (b) (4) (b) (4)) for proximate and amino acid composition (Table 4). All experimental diets were produced at the (b) (4) at the (b) (4) under the direction of (b) (4) using the standard procedures for the shrimp feeds described by Qiu and Davis, (2016). Briefly, diets were prepared by mixing the pre-ground dry ingredients in a food mixer (b) (4)) for 10–15 minutes. Hot water was then blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 2.5-mm die. The wet pellets were then placed into a fan-ventilated oven (< 50 °C) overnight in order to attain a moisture content of less than 10%. Dry pellets were crumbled, packed in sealed bags, and stored in a freezer until use. The diets were analyzed at (b) (4) (b) (4)) for proximate and amino acid composition at (b) (4) for proximate and mineral composition (Table 3).

2.2. Growth trial

This trial was conducted at the (b) (4) (b) (4) Pacific white shrimp post larvae (PL) were obtained from (b) (4) (b) (4) and nursed in an indoor recirculating system. PLs were fed a commercial feed (b) (4) using an automatic

feeder for ~1 week, and then switched to crumbled commercial shrimp feed (b) (4) (b) (4) for ~1- 2 weeks.

For the diet trial, the recirculating system consisted of 24 aquaria (135 L) connected to a common reservoir, biological filter, bead filter, fluidized biological filter and recirculation pump. Four replicate groups of shrimps (0.15 g initial mean weight, 10 shrimp / tank) were offered diets using our standard feeding protocol over 6 weeks. Based on historic results, feed inputs were pre-programmed assuming the shrimp would double their weight weekly up to one gram then gain 0.8-1.3 g weekly with a feed conversion ratio (FCR) of 1.8. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality. For each tank, a fixed ration of 0.39 g day^{-1} for the first week, 0.83 g day^{-1} for the second week, 1.66 g day^{-1} for the third week, 2.24 g day^{-1} for the fourth week, 2.53 g day^{-1} for the fifth week, and 3.05 g day^{-1} for the last week was also allotted in 4 portions per day.

At the conclusion of each growth trial, shrimp were counted and group-weighted. Mean final weight, FCR, WG, biomass, and survival were determined (Table 2).

2.3. Water quality monitoring

For all trials, dissolved oxygen (DO), water temperature and salinity were measured twice daily by using a (b) (4) 650 multi-parameter instrument ((b) (4)). Hydrogen potential (pH) was measured twice weekly by using a waterproof (b) (4) ((b) (4) (b) (4) Total ammonia-nitrogen (TAN) and nitrite were evaluated every week by using the methods described by Sororzano (1969) and Spotte (1979).

2.4. Statistical analysis

All the data were analyzed using (b) (4) ((b) (4)). Data from three growth trials were analyzed using one-way ANOVA to determine significant differences

(P<0.05) among treatments followed by the Tukey's multiple comparison test to determine difference between treatments in each trial. The pooled standard errors were used across growth trials, as the variance of each treatment is the same. Arcsine square root transformation was used prior to analysis for the proportion data. False discover rate (FDR) controlling procedures were used to adjust the P-value to control the FDR for data from nutrient contents of whole body and amino acid retention. Data from digestibility trial were analyzed using non-parametric (kruskal-wallis) one-way ANOVA to determine significant differences (P<0.05) among treatments followed by the Tukey's multiple comparison test to determine differences between treatments.

3. Results

3.1. Water quality

In this study, temperature, salinity, pH, TAN, and nitrite were maintained at 6.96 ± 0.31 mg L^{-1}, 28.1 ± 0.3 °C, 8.2 ± 0.6 ppt, 7.0 ± 0.3, 0.05 ± 0.04 mg L^{-1}, and 0.12 ± 0.12 mg L^{-1}, respectively. Water quality conditions was considered suitable for normal growth and survival of this species.

3.2. Growth trials

The final biomass of the shrimp was significantly reduced when 26.6% KBM was added in the diet compared to the diet without KBM supplementation. KBM compared to the treatment supplemented with 0, 6% and 13.3% saw statistically equivalent growth, FCR and no significant difference was detected in survival (90 to 100%) across treatments.

4. Discussion & Conclusion

The findings indicated that no significant differences were observed in terms of growth performance and FCR when the diets supplemented with KBM up to 13.3% under the conditions

of the present study. However, negative effects on growth response, FCR, and protein as well as amino acids retention efficiency were observed for shrimp grown on diets containing 26.6% KBM.

Table 1 Composition (% as is) of test diets utilized in diet trial.

Ingredients	Diet code			
	D_1	D_2	D_3	D_4
Fish meal[1]	6.00	6.00	6.00	6.00
Soybean meal[2]	53.00	46.50	46.50	40.10
Corn protein concentrate[3]	8.00	8.00	8.00	8.00
Bacterial biomass[4]	0.00	6.00	13.30	26.60
Fish oil[2]	5.92	5.97	5.81	5.70
Trace mineral premix[6]	0.50	0.50	0.50	0.50
Vitamin premix[7]	1.80	1.80	1.80	1.80
Choline chloride[5]	0.20	0.20	0.20	0.20
Stay C[8]	0.10	0.10	0.10	0.10
Mono-dicalcium phosphate[9]	2.50	2.80	2.80	2.90
Lecithin[10]	1.00	1.00	1.00	1.00
Cholesterol[5]	0.08	0.08	0.08	0.08
Methionine[11]	0.05	0.04	0.03	0.02
Lysine[11]	0.00	0.04	0.05	0.09
Corn starch[5]	20.85	20.97	13.83	6.91

1 (b) (4)

2 De-hulled solvent extract soybean meal, (b) (4)

3 (b) (4)

4 (b) (4)

5 (b) (4)

6 (b) (4)

7 (b) (4)

8 (b) (4)

9 (b) (4)

10 (b) (4)

11 (b) (4)

Table 2 Performance of juvenile shrimp *L. vannamei* (Initial weight 0.15g) offered diets formulated to partially replace soybean meal on a digestible protein basis for six weeks.

Diet	KBM levels (%)	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
D_1	0	42.68^{a}	4.74^{a}	3160.39^{a}	1.72^{c}	90.0
D_2	6	43.15^{ab}	4.30^{ab}	2813.38^{ab}	1.90^{bc}	100.0
D_3	13.3	45.38^{ab}	4.54^{a}	2732.16^{abc}	1.73^{c}	100.0
D_4	26.6	35.05^{b}	3.60^{c}	2304.94^{c}	2.26^{a}	97.5
PSE[1]		1.1420	0.0710	57.1783	0.0338	1.9084
P-value		0.0406	0.0002	0.0008	0.0001	0.3194

[1] PSE: Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 3 Proximate composition[1] (% as is) and mineral composition[1] (g kg^{-1}: phosphorus, sulfur, potassium, magnesium, calcium, sodium; mg kg^{-1}: iron, manganese, copper, zinc) of the test diets used.

Composition[1]	D_1	D_2	D_3	D_4
Crude protein	35.7	33.7	38.4	41.1
Moisture	8.7	11.71	8.39	10.46
Crude fat	6.71	7.57	8.2	7.26
Crude fiber	3.1	2.47	7.1	8.3
Ash	7.08	6.67	7.08	6.56
Sulfur	0.40	0.36	0.44	0.43
Phosphorus	1.36	1.36	1.29	1.37
Potassium	1.33	1.16	1.23	1.11
Magnesium	0.18	0.16	0.19	0.17
Calcium	1.31	1.27	1.37	1.37
Sodium	0.10	0.11	0.14	0.16
Iron (ppm)	149	127	166	161
Manganese (ppm)	40.1	38.1	67.8	69.4
Copper (ppm)	16.8	15.9	16.9	15.8
Zinc (ppm)	183	168	213	177

[1] Diets were analyzed at (b) (4)

Table 4 Proximate composition (% as is), phosphorus content (% as is), and amino acid profile (% as is) of the ingredients used in the growth and digestibility trials.

Composition[1]	KBM	Fish meal	Soybean meal
Crude protein	52.42	62.78	44.89
Moisture	3.50	7.99	10.97
Crude fat	1.29	10.56	3.78
Crude fiber	0.00	0.00	3.20
Ash	4.25	18.75	6.67
Phosphorus	1.00	3.15	0.66
Alanine	3.81	3.91	2.04
Arginine	3.23	3.68	3.35
Aspartic acid	3.72	5.34	5.10
Cysteine	0.30	0.47	0.62
Glutamic acid	6.13	7.47	8.24
Glycine	2.73	4.88	2.04
Histidine	0.80	1.63	1.20
Isoleucine	1.80	2.42	2.17
Leucine	3.24	4.21	3.57
Lysine	2.79	4.67	3.06
Methionine	0.86	1.61	0.66
Phenylalanine	2.04	2.39	2.35
Proline	2.25	3.08	2.39
Serine	1.21	2.11	1.90
Taurine	0.08	0.73	0.13
Threonine	2.00	2.41	1.75
Tryptophan	0.10	0.62	0.62
Tyrosine	1.34	1.67	1.64
Valine	2.84	2.99	2.34

[1] Ingredients were analyzed at (b) (4)

Appendix: Raw Data

Exp1634											
System: d	STOCK:	9/14/2016									
						6	weeks				
			Initial	9/14/2016	Terminate	10/26/2016					
TK	Diet code	# shrimp	Biomass	Mean wt	# shrimp	Biomass	Mean wt	survival	WG	WG%	FCR
1	D1	10	1.5	0.15	9	39.3	4.4	90.0	4.2	2811.1	1.87
3	D4	10	1.6	0.16	10	34.7	3.5	100.0	3.3	2068.8	2.34
10	D3	10	1.65	0.17	10	46	4.6	100.0	4.4	2687.9	1.71
12	D2	10	1.48	0.15	8	32.2	4.0	80.0	3.9	2619.6	2.10
13	D2	10	1.45	0.15	11	49.7	4.5	110.0	4.4	3016.0	1.77
15	D1	10	1.43	0.14	9	44.6	5.0	90.0	4.8	3365.4	1.63
16	D4	10	1.53	0.15	9	34.9	3.9	90.0	3.7	2434.5	2.13
19	D4	10	1.38	0.14	10	36.2	3.6	100.0	3.5	2523.2	2.23
23	D3	10	1.5	0.15	10	46.1	4.6	100.0	4.5	2973.3	1.70
24	D1	10	1.4	0.14	9	40.1	4.5	90.0	4.3	3082.5	1.82
25	D1	10	1.49	0.15	9	46.7	5.2	90.0	5.0	3382.5	1.57
27	D4	10	1.5	0.15	10	34.4	3.4	100.0	3.3	2193.3	2.35
30	D2	10	1.58	0.16	10	43.6	4.4	100.0	4.2	2659.5	1.87
32	D3	10	1.63	0.16	10	43.3	4.3	100.0	4.2	2556.4	1.82
34	D3	10	1.64	0.16	10	46.1	4.6	100.0	4.4	2711.0	1.71
35	D2	10	1.4	0.14	11	47.1	4.3	110.0	4.1	2958.4	1.87
Summary											
Diet code	# shrimp	Biomass	Mean wt	# shrimp	Biomass	Mean wt	survival	WG	WG%	FCR	
D1	10	1.46	0.15	9	42.68	4.74	90.0	4.60	3160.39	1.72	
D2	10	1.48	0.15	10	43.15	4.30	100.00	4.15	2813.38	1.90	
D6	10	1.61	0.16	10	45.38	4.54	100.00	4.38	2732.16	1.73	
D9	10	1.50	0.15	9.75	35.05	3.60	97.50	3.45	2304.94	2.26	

Feed consumption

				Tank	1	3	10	12	13	15	16	18	19	23	24	25	27	30	32	34	35
Week	FCR	Date	Predict Growth	# shrimp	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Week 1	1.8	9/14	0.15		0.193	0.193	0.193	0.193	0.193	0.193	0.193	0.193	0.193	0.193	0.193	0.193	0.193	0.193	0.193	0.193	0.193
		9/15		2	0.386	0.386	0.386	0.386	0.386	0.386	0.386	0.386	0.386	0.386	0.386	0.386	0.386	0.386	0.386	0.386	0.386
		9/16		3	0.415	0.421	0.411	0.426	0.426	0.415	0.421	0.416	0.421	0.411	0.415	0.415	0.421	0.426	0.411	0.411	0.426
		9/17		4	0.415	0.421	0.411	0.426	0.426	0.415	0.421	0.416	0.421	0.411	0.415	0.415	0.421	0.426	0.411	0.411	0.426
		9/18		5	0.415	0.421	0.411	0.426	0.426	0.415	0.421	0.416	0.421	0.411	0.415	0.415	0.421	0.426	0.411	0.411	0.426
		9/19		6	0.415	0.421	0.411	0.426	0.426	0.415	0.421	0.416	0.421	0.411	0.415	0.415	0.421	0.426	0.411	0.411	0.426
		9/20		7	0.415	0.421	0.411	0.426	0.426	0.415	0.421	0.416	0.421	0.411	0.415	0.415	0.421	0.426	0.411	0.411	0.426
Week 2	1.8	9/21	0.3	1	0.831	0.842	0.822	0.852	0.852	0.831	0.842	0.832	0.842	0.822	0.831	0.831	0.842	0.852	0.822	0.822	0.852
		9/22		2	0.831	0.842	0.822	0.852	0.852	0.831	0.842	0.832	0.842	0.822	0.831	0.831	0.842	0.852	0.822	0.822	0.852
		9/23		3	0.831	0.842	0.822	0.852	0.852	0.831	0.842	0.832	0.842	0.822	0.831	0.831	0.842	0.852	0.822	0.822	0.852
		9/24	0.4	4	1.108	1.122	1.096	1.136	1.136	1.108	1.122	1.109	1.122	1.096	1.108	1.108	1.122	1.136	1.096	1.096	1.136
		9/25		5	1.108	1.122	1.096	1.136	1.136	1.108	1.122	1.109	1.122	1.096	1.108	1.108	1.122	1.136	1.096	1.096	1.136
		9/26		6	1.108	1.122	1.096	1.136	1.136	1.108	1.122	1.109	1.122	1.096	1.108	1.108	1.122	1.136	1.096	1.096	1.136
		9/27		7	1.108	1.122	1.096	1.136	1.136	1.108	1.122	1.109	1.122	1.096	1.108	1.108	1.122	1.136	1.096	1.096	1.136
Week 3	1.8	9/28	0.6	1	1.662	1.684	1.645	1.703	1.703	1.662	1.684	1.663	1.684	1.645	1.662	1.662	1.684	1.703	1.645	1.645	1.703
		9/29		2	1.662	1.684	1.645	1.703	1.703	1.662	1.684	1.663	1.684	1.645	1.662	1.662	1.684	1.703	1.645	1.645	1.703
		9/30		3	1.662	1.684	1.645	1.703	1.703	1.662	1.684	1.663	1.684	1.645	1.662	1.662	1.684	1.703	1.645	1.645	1.703
		10/1		4	1.662	1.684	1.645	1.703	1.703	1.662	1.684	1.663	1.684	1.645	1.662	1.662	1.684	1.703	1.645	1.645	1.703
		10/2		5	1.662	1.684	1.645	1.703	1.703	1.662	1.684	1.663	1.684	1.645	1.662	1.662	1.684	1.703	1.645	1.645	1.703
		10/3		6	1.662	1.684	1.645	1.703	1.703	1.662	1.684	1.663	1.684	1.645	1.662	1.662	1.684	1.703	1.645	1.645	1.703
		10/4		7	1.662	1.684	1.645	1.703	1.703	1.662	1.684	1.663	1.684	1.645	1.662	1.662	1.684	1.703	1.645	1.645	1.703
Week 4	1.8	10/5	0.8	1	1.994	2.245	2.193	1.817	2.498	2.216	2.245	2.217	2.245	2.193	2.216	2.216	2.245	2.271	2.193	2.193	2.498
		10/6		2	1.994	2.245	2.193	1.817	2.498	2.216	2.245	2.217	2.245	2.193	2.216	2.216	2.245	2.271	2.193	2.193	2.498
		10/7		3	1.994	2.245	2.193	1.817	2.498	2.216	2.245	2.217	2.245	2.193	2.216	2.216	2.245	2.271	2.193	2.193	2.498
		10/8		4	1.994	2.245	2.193	1.817	2.498	2.216	2.245	2.217	2.245	2.193	2.216	2.216	2.245	2.271	2.193	2.193	2.498
		10/9		5	1.994	2.245	2.193	1.817	2.498	2.216	2.245	2.217	2.245	2.193	2.216	2.216	2.245	2.271	2.193	2.193	2.498
		10/10		6	1.994	2.245	2.193	1.817	2.498	2.216	2.245	2.217	2.245	2.193	2.216	2.216	2.245	2.271	2.193	2.193	2.498
		10/11		7	1.994	2.245	2.193	1.817	2.498	2.216	2.245	2.217	2.245	2.193	2.216	2.216	2.245	2.271	2.193	2.193	2.498
Week 5	1.8	10/12	0.9	1	2.492	2.525	2.467	2.044	2.810	2.492	2.273	2.495	2.525	2.467	2.492	2.492	2.273	2.555	2.467	2.467	2.810
		10/13		2	2.492	2.525	2.467	2.044	2.810	2.492	2.273	2.495	2.525	2.467	2.492	2.492	2.273	2.555	2.467	2.467	2.810
		10/14		3	2.492	2.525	2.467	2.044	2.810	2.492	2.273	2.495	2.525	2.467	2.492	2.492	2.273	2.555	2.467	2.467	2.810
		10/15	1	4	2.769	2.806	2.741	2.271	3.123	2.769	2.525	2.772	2.806	2.741	2.769	2.769	2.525	2.839	2.741	2.741	3.123
		10/16		5	2.769	2.806	2.741	2.271	3.123	2.769	2.525	2.772	2.806	2.741	2.769	2.769	2.525	2.839	2.741	2.741	3.123
		10/17		6	2.769	2.806	2.741	2.271	3.123	2.769	2.525	2.772	2.806	2.741	2.769	2.769	2.525	2.839	2.741	2.741	3.123
		10/18		7	2.769	2.806	2.741	2.271	3.123	2.769	2.525	2.772	2.806	2.741	2.769	2.769	2.525	2.839	2.741	2.741	3.123
Week 6	1.8	10/19	1.1	1	3.046	3.087	3.015	2.498	3.435	2.742	2.778	3.049	3.087	3.015	2.742	3.046	3.087	3.123	3.015	3.015	3.435
		10/20		2	3.046	3.087	3.015	2.498	3.435	2.742	2.778	3.049	3.087	3.015	2.742	3.046	3.087	3.123	3.015	3.015	3.435
		10/21		3	3.046	3.087	3.015	2.498	3.435	2.742	2.778	3.049	3.087	3.015	2.742	3.046	3.087	3.123	3.015	3.015	3.435
		10/22		4	3.046	3.087	3.015	2.498	3.435	2.742	2.778	3.049	3.087	3.015	2.742	3.046	3.087	3.123	3.015	3.015	3.435
		10/23		5	3.046	3.087	3.015	2.498	3.435	2.742	2.778	3.049	3.087	3.015	2.742	3.046	3.087	3.123	3.015	3.015	3.435
		10/24		6	3.046	3.087	3.015	2.498	3.435	2.742	2.778	3.049	3.087	3.015	2.742	3.046	3.087	3.123	3.015	3.015	3.435
		10/25		7	3.046	3.087	3.015	2.498	3.435	2.742	2.778	3.049	3.087	3.015	2.742	3.046	3.087	3.123	3.015	3.015	3.435
				total feed	75.049	77.606	75.820	67.150	84.183	74.467	73.565	76.666	77.606	75.820	74.467	76.600	75.726	78.505	75.820	75.820	84.183

Appendix 8

US 20150044327A1

(19) **United States**

(12) **Patent Application Publication**

Feinberg et al.

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(43) **Pub. Date: Feb. 12, 2015**

(54) **METHYLOTROPHS FOR AQUACULTURE AND ANIMAL FEED**

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(72) Inventors: **Larry F. Feinberg**, Harvard, MA (US); **Christopher J. Marx**, Moscow, ID (US)

(21) Appl. No.: **14/454,816**

(22) Filed: **Aug. 8, 2014**

Related U.S. Application Data

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Publication Classification

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A23K 1/18	(2006.01)
C12N 15/74	(2006.01)

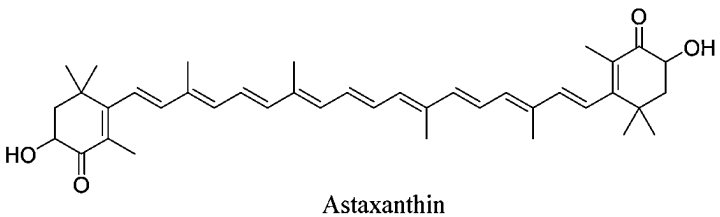
(52) **U.S. Cl.**

CPC ***A23K 1/1606*** (2013.01); ***C12N 15/74*** (2013.01); ***A23K 1/188*** (2013.01); ***A23K 1/16*** (2013.01)

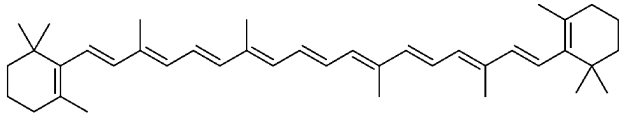
USPC **426/2**; 435/252.3; 426/61; 426/643

(57) **ABSTRACT**

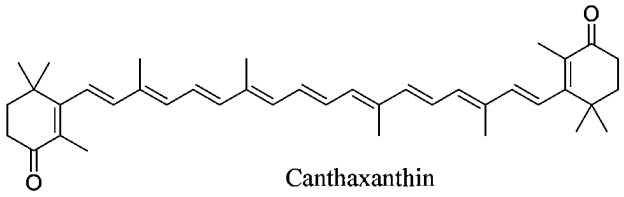
Disclosed are methods of producing carotenoid compounds in a methylotrophic bacterial host cell. Such a host cell may be an unmodified *Methylobacterium*, spontaneous mutant, or transformed cell, any of which exhibit favorable properties, such as overproduction of carotenoid compounds, increased carbon flux, improved growth, or the production of additional nutrients, such as protein, vitamins, antioxidants, or fatty acids. Also disclosed are feed compositions for use in aquaculture, or as animal feed, or as human nutritional supplements containing processed or unprocessed biomass from such cells, as are methods of preparation of the feed compositions.



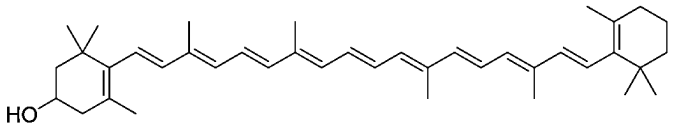
Astaxanthin



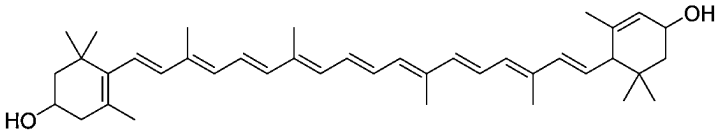
β-Carotene



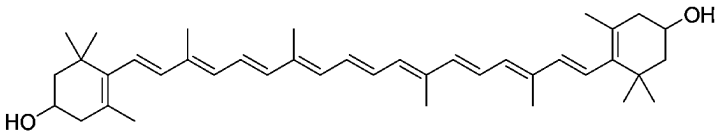
Canthaxanthin



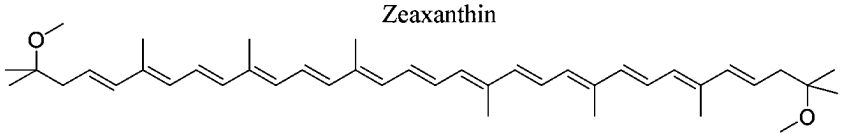
β-Cryptoxanthin



Lutein



Zeaxanthin



Spirilloxanthin

FIG. 1

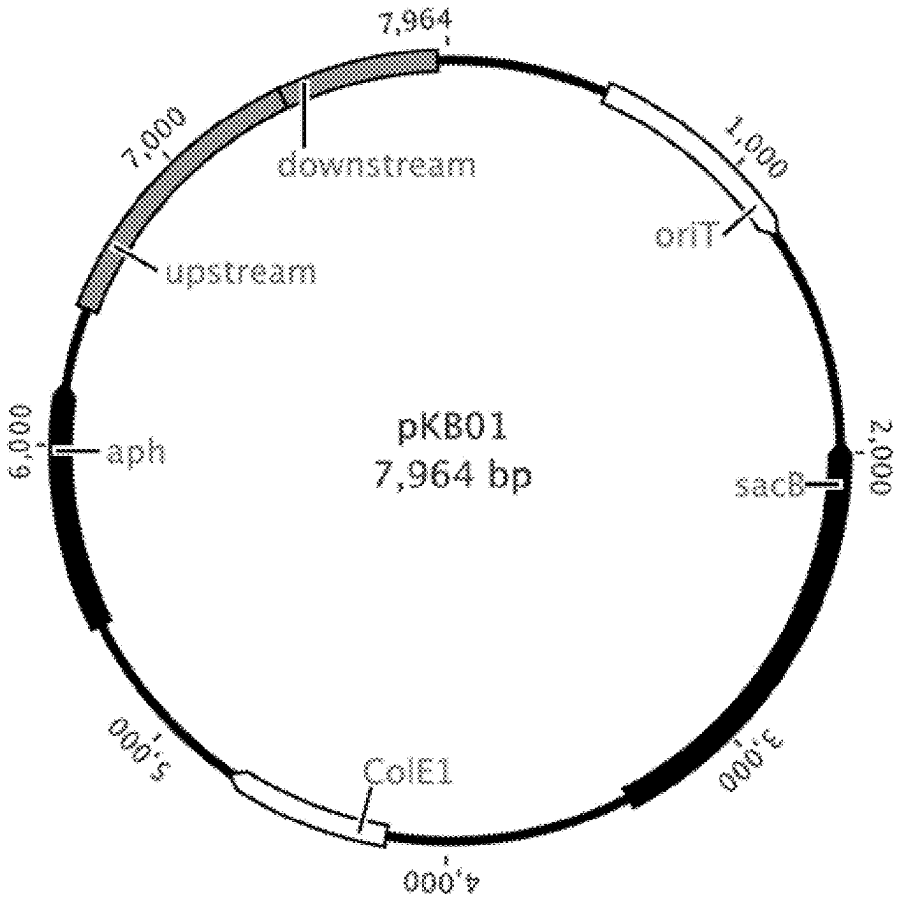

7,964
1,000
2,000
3,000
4,000
5,000
6,000
7,000
downstream
upstream
oriT
sacB
ColE1
aph
pKB01
7,964 bp

FIG. 2

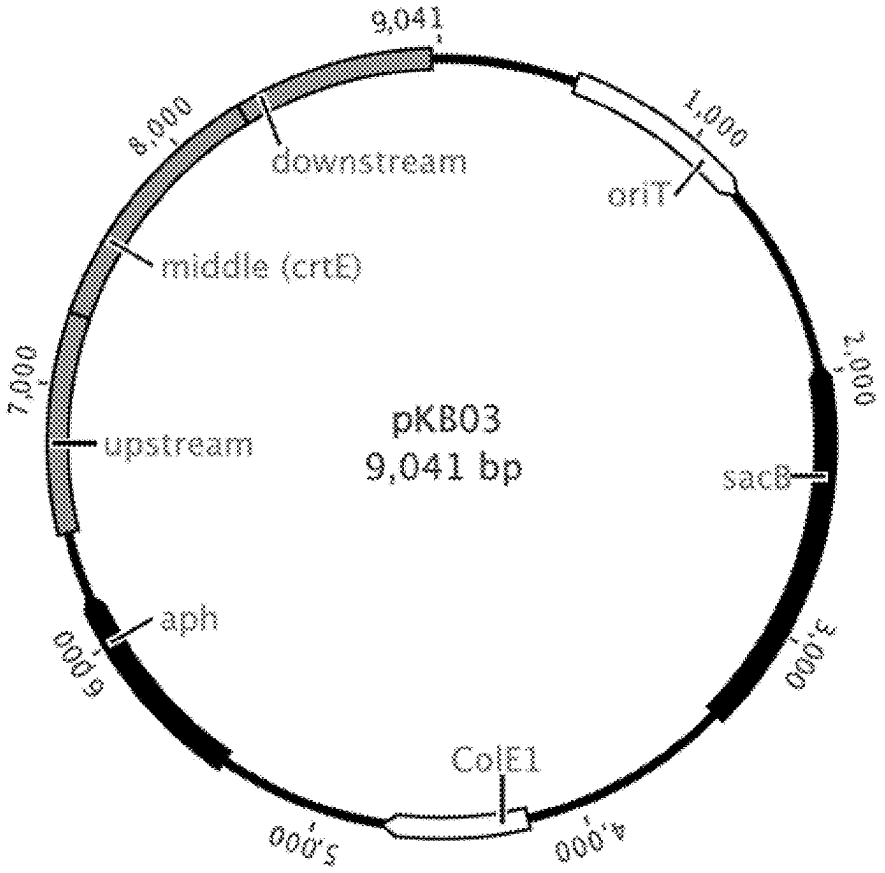

9,041
1,000
2,000
3,000
4,000
5,000
6,000
7,000
8,000
downstream
oriT
middle (crtE)
upstream
pKB03
9,041 bp
sacB
aph
ColE1

FIG. 3

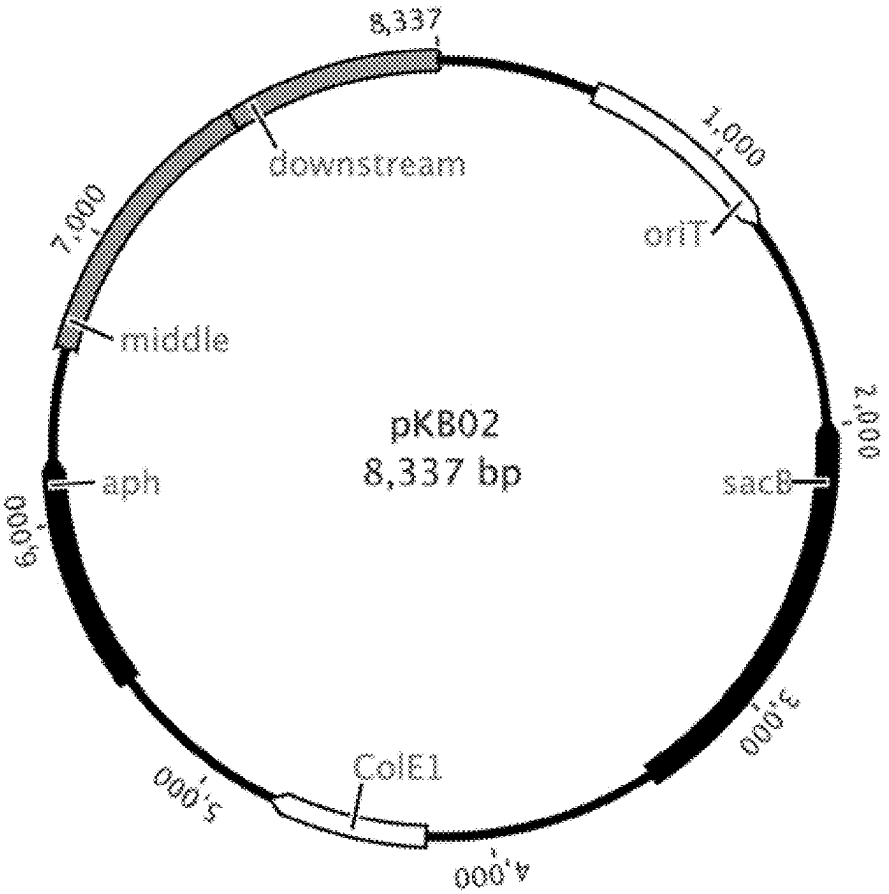

8,337
1,000
oriT
2,000
sacB
3,000
4,000
ColE1
5,000
6,000
aph
7,000
middle
downstream
pKB02
8,337 bp

FIG. 4

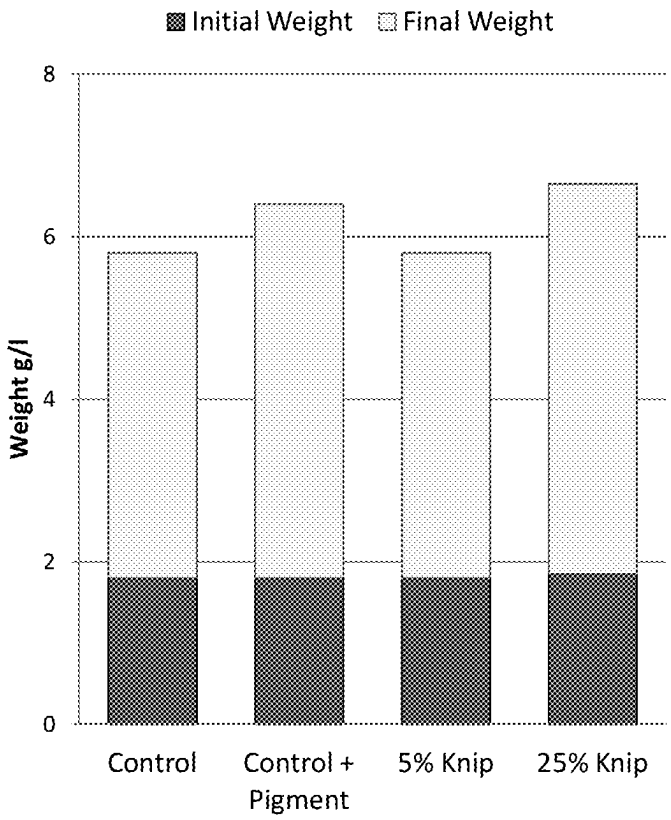

Initial Weight
Final Weight
Weight g/l
8
6
4
2
0
Control
Control + Pigment
5% Knip
25% Knip

FIG. 5

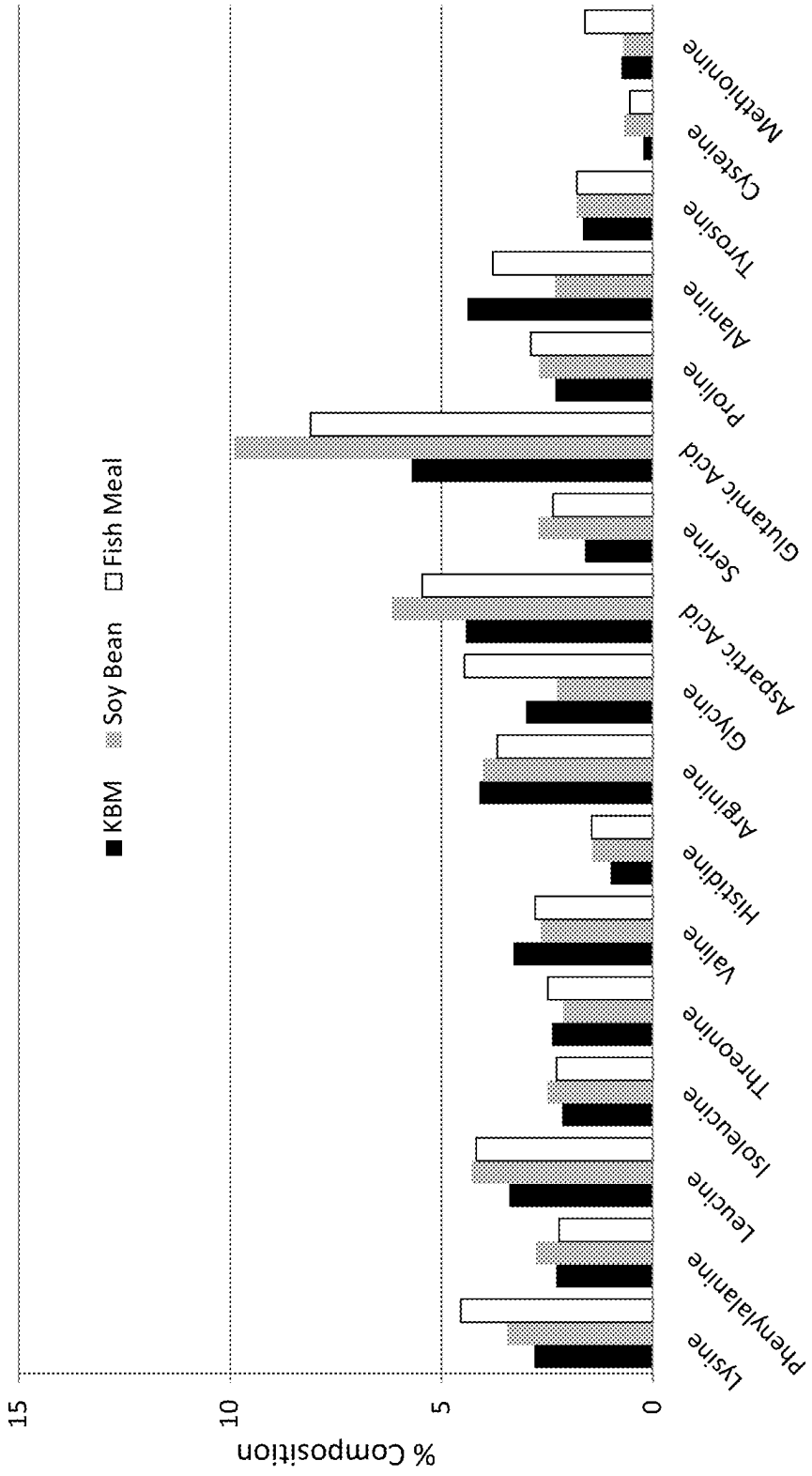

KBM
Soy Bean
Fish Meal
% Composition
15
10
5
0
Lysine
Phenylalanine
Leucine
Isoleucine
Threonine
Valine
Histidine
Arginine
Glycine
Aspartic Acid
Serine
Glutamic Acid
Proline
Alanine
Tyrosine
Cysteine
Methionine

FIG. 6

METHYLOTROPHS FOR AQUACULTURE AND ANIMAL FEED

PRIORITY

[0001] This application claims priority to U.S. provisional patent application No. 61/863,701, filed on Aug. 8, 2013, which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Aug. 7, 2014, is named 0114922-00002 SL.txt and is 11,507 bytes in size.

BACKGROUND

[0003] Carotenoids are a class of ubiquitous and structurally diverse natural pigments ranging in color from light yellow to orange to red. Carotenoids are responsible for the coloring of carrots, tomatoes, red peppers, and the petals of daffodils and marigolds, as well as lobsters, salmon, and other marine life. Carotenoids are produced by all photosynthetic organisms, as well as by some bacteria and fungi. Carotenoids have roles in photosynthesis, nutrition, and protecting against photooxidative damage. Animals cannot produce carotenoids themselves, but must obtain these nutritionally important compounds through their diet. Carotenoids are 40-carbon (C_{40}) terpenoids ultimately derived from the isoprene biosynthetic pathway, specifically from isopentenyl pyrophosphate (IPP), a five-carbon building block. This biosynthetic pathway can be divided into two portions: the upper isoprene pathway, which leads to the formation of IPP, and the lower carotenoid biosynthetic pathway, responsible for converting IPP into long chain (e.g., C_{30} and C_{40}) carotenogenic compounds.

[0004] Carotenoid compounds, such as β-carotene, astaxanthin, and spirilloxanthin, are used industrially as ingredients for food and feed stocks, both serving a nutritional role and often increasing desirability of the product to consumers. Carotenoids, such as astaxanthin and canthaxanthin, are often added to aquaculture feeds for the purpose of adding color to the flesh of aquacultured organisms; their wild counterparts have colored flesh resulting from consumption of carotenoids that occur naturally in Crustacea or algae, or in other fish that have consumed algae. For example, astaxanthin is widely used in salmon aquaculture to produce the orange coloration found in wild salmon. Some carotenoids are also precursors of vitamin A. Moreover, some carotenoids have antioxidant properties, and may have health benefits (see, for example, Jyonouchi et al., *Nutr. Cancer* 16:93, 1991; Giovannucci et al., *J. Natl. Cancer Inst.* 87:1767, 1995; Miki, *Pure Appl. Chem.* 63:141, 1991; Chew et al., *Anticancer Res.* 19:1849, 1999; Wang et al., *Antimicrob. Agents Chemother.* 44:2452, 2000). Several carotenoids (e.g., β-carotene, lycopene, and lutein) are currently sold as nutritional supplements.

[0005] A number of carotenoids have been produced in microbial organisms. For example, Intl Pat. Appl.Pub. No. WO 02/18617 describes a method of production of carotenoid compounds using microorganisms that metabolize single carbon substrates. Genes encoding elements of the carotenoid biosynthetic pathway have been cloned and expressed in fungi, yeast, and microbes. For example, lycopene has been produced from genetically engineered *E. coli* and *Candida utilis* (Farmer, W. R. et al. (2001) *Biotechnol. Prog.* 17: 57-61; Wang, C. et al., (2000) *Biotechnol Prog.* 16: 922-926; Misawa, N. and H. Shimada (1998) *J. Biotechnol.* 59: 169-181; Shimada, H. et al. (1998) *Appl. Environm. Microbiol.* 64: 2676-2680). Zeaxanthin has been produced from recombinant *E. coli* and *Candida utilis* (Albrecht, M. et al., (1999). *Biotechnol. Lett.* 21: 791-795; Miura, Y. et al. (1998) *Appl. Environm. Microbiol.* 64: 1226-1229). Astaxanthin has been produced from *E. coli* and *Pfaffia rhodozyma* (see, for example, U.S. Pat. No. 5,466,599 (incorporated by reference)). The nutrient β-carotene has been produced from *E. coli, Candida utilis* and *Pfaffia rhodozyma* (Albrecht, M. et al. (1999) *Biotechnol. Lett.* 21: 791-795; Miura, Y. et al. (1998) *Appl. Environm. Microbiol.* 64: 1226-1229; U.S. Pat. No. 5,691,190 (incorporated by reference)).

[0006] Genes encoding geranylgeranyl pyrophosphate synthase, lycopene cyclase, and phytoene dehydrogenase from *Erwinia herbicola* have been expressed in *E. coli* (see, for example, U.S. Pat. No. 5,545,816; U.S. Pat. No. 5,656,472; U.S. Pat. No. 5,530,189; and U.S. Pat. No. 5,530,188, all of which are incorporated by reference). Genes encoding such carotenoid products as geranylgeranyl pyrophosphate, phytoene, lycopene, β-carotene, and zeaxanthin-diglucoside, from *Erwinia uredovora* have been expressed in *E. coli, Zymomonas mobilis*, and *Saccharomyces cerevisiae* (U.S. Pat. No. 5,429,939). Carotenoid biosynthetic genes including crtE, crtB, crtI, crtY, and crtZ taken from *Flavobacterium* have been recombinantly expressed (see U.S. Pat. No. 6,124, 113).

[0007] Although the above methods can produce useful amounts of carotenoids, a need exists for improved methods. A particular long-appreciated need is for a process that produces useful yields of carotenoids from an inexpensive feedstock and also produces one or more nutrients (e.g., lipids or protein). The resulting carotenoid- and nutrient-rich microbial or plant biomass could then be processed into feed for aquaculture or agriculture, or used as a nutrient source for humans.

[0008] There are a number of microorganisms that utilize single-carbon substrates as their sole energy sources. Examples of single-carbon substrates include methane, methanol, formate, thiols, and methylated amines. These organisms are referred to as methylotrophs and also herein as "C1 metabolizers". Few methylotrophs have been successfully utilized to produce nutrients on an industrial scale. Despite the fact that single-carbon substrates are cost-effective energy sources, the lack of information about methylotroph genetics and the resulting difficulty in manipulation has limited their use primarily to the synthesis of native products.

[0009] A need also exists for low-cost, complete nutrition for use in aquaculture. Aquaculture is the propagation, cultivation and marketing of aquatic animals and plants in a controlled environment. The aquaculture industry is currently the fastest growing food production sector in the world. World aquaculture produces approximately 60 million tons of seafood at an annual value of more than $70 billion (USD). Presently, fish farming produces about half of all fish consumed globally and this percentage is growing as a result of declining yields from wild-caught fish in both marine and freshwater environments. Species groups produced in aquaculture include: carps and other cyprinids; oysters; clams,

cockles and arkshells; scallops; shrimps and prawns; salmons, trouts and smelts; mussels; and tilapias and other cichlids.

[0010] While certain species (e.g., tilapia) can be fed an exclusively vegetarian diet, others require a carnivorous diet. Feed for carnivorous fish typically comprises fishmeal and fish oil derived from wild caught species of small pelagic fish (predominantly anchovy, jack mackerel, blue whiting, capelin, sandeel and menhaden). The fishmeal and/or fish oil are processed into a pelleted or flaked feed, depending on the size of the fish to which it will be fed (e.g., fry, juveniles, adults). Other components of the aquaculture feed composition may include pigments, vegetable protein, vitamins, and minerals.

[0011] Fish oils from ocean-caught fish have traditionally been used as the sole dietary lipid source in commercial fish feed because of abundant supply, low cost, and high percentage of essential fatty acids. These "essential fatty acids" are required for normal growth, health, reproduction, and other functions. In fact, all vertebrate species, including fish, have a dietary requirement for both omega-6 and omega-3 polyunsaturated fatty acids ("PUFAs"). Eicosapentaenoic acid, or "EPA" (cis-5,8,11,14,17-eicosapentaenoic acid) is an omega-3 and docosahexaenoic acid, or "DHA" (cis-4,7,10,13,16,19-docosahexaenoic acid, a 22:6 omega-3) are two essential PUFAs.

[0012] About 87% of the global supply of fish oil is consumed for fish feed as a lipid source. Given that fish oil production has peaked at 1.5 million tons per year, the rapidly growing aquaculture industry will soon outpace the finite stocks of marine pelagic fish as a supply of fish oil. Therefore, it is essential to find and implement sustainable alternatives to fish oil that can keep pace with the ever growing global demand for fish products.

[0013] Many organizations recognize the limitations noted above with respect to fish oil availability and aquaculture sustainability. The National Oceanic and Atmospheric Administration and the Department of Agriculture (United States) have collaborated in an Alternative Feeds Initiative to " . . . identify alternative dietary ingredients that will reduce the amount of fishmeal and fish oil contained in aquaculture feeds while maintaining the important human health benefits of farmed seafood."

[0014] U.S. Pat. Appl. Pub. No. 2007/0226814 (incorporated by reference) discloses fish food containing at least one biomass obtained from fermenting microorganisms wherein the biomass contains at least 20% DHA relative to the total fatty acid content. Microorganisms from the genus *Stramenopiles* are mentioned as sources of DHA.

[0015] U.S. Pat. Appl. Pub. No. 2009/0202672 (incorporated by reference) discloses that stearidonic acid ("SDA"; 18:4 omega-3) can be added to aquaculture feed. This fatty acid can be obtained from a transgenic plant. Unfortunately, SDA is not converted efficiently to DHA in fish.

[0016] U.S. Pat. No. 7,932,077 (incorporated by reference) discloses that recombinantly engineered *Yarrowia lipolytica* may be a useful addition to most animal feeds, including aquaculture feeds, because it provides necessary omega-3 and/or omega-6 PUFAs, and based on its unique protein:lipid:carbohydrate composition, as well as unique complex carbohydrate profile (comprising an approximate 1:4:4.6 ratio of mannan:beta-glucans:chitin).

[0017] If the growing aquaculture industry is to sustain and even increase its contribution to world fish supplies, there is a need for alternative aquaculture feed compositions that: (i) reduce wild fish inputs by replacing fish oil and fish meal with non-fish derived sources; and (ii) use pigments that are not chemically synthesized, or otherwise derived from petroleum-based feedstocks, to provide pigmentation.

SUMMARY OF THE INVENTION

[0018] In certain embodiments, the present invention provides a biomass containing substantially one or more isolated methylotrophic bacterial cultures that are genetically modified or artificially pre-selected to produce elevated levels of a carotenoid compound relative to the corresponding unmodified or unselected bacterium. The carotenoid compound is, for example, β-carotene, lycopene, rhodopin, astaxanthin or spirilloxanthin. In certain embodiments, the bacterium is genetically modified so that one or more genes producing enzymes that divert isoprenoid compounds from the carotenoid biosynthetic pathway are blocked or deleted. In certain embodiments, the invention provides a bacterium that contains a non-lethal knock-out of shc, for example, *M. extorquens* comprising a non-lethal knock-out of shc. In other embodiments, the bacterium is selected by directed evolution as a spontaneous mutant that expresses a "dark pink" or "reddish" pigment.

[0019] In certain embodiments, the biomass can be in a dry, or substantially dry, form, e.g., containing less than 20%, 10%, 5%, 2% of moisture. In certain embodiments, the cultures are isolated by removing substantially all supernatant, such as by filtering, sedimentation, or centrifugation. In certain embodiments, the collection of cultures into the biomass and further processing of biomass excludes bacterial lysis step, e.g., by use of detergents or ultrasound. In certain embodiments, the processed bacterial cells maintain substantially whole cell membranes. In some embodiments, a substantial portion (e.g., more than 80%, 50%, 30%, 20%, 10% or 5%) of bacterial cells may maintain viability in the processed biomass.

[0020] The biomass of the invention may contain bacterial cultures selected from the group consisting of *Methylomonas, Methylobacter, Methylococcus, Methylosinus, Methylocystis, Methylomicrobium, Methanomonas, Methylophilus, Methylobacillus, Methylobacterium, Hyphomicrobium, Xanthobacter, Bacillus, Paracoccus, Nocardia, Arthrobacter, Rhodopseudomonas, Pseudomonas, Candida, Hansenula, Pichia, Torulopsis*, and *Rhodotorula*. In certain preferred embodiments, the bacterium is *M. extorquens*. In further embodiments, the strain of *M. extorquens* is selected from the group consisting of *M. extorquens* AM1, *M. extorquens* DM4, *M. extorquens* CM4, *M. extorquens* PA1, *M. extorquens* BJ001 (formerly *M. populi*), *M. radiotolerans, M. nodulans*, and *Methylobacterium* spp. 4-46.

[0021] In a further aspect, the invention provides a feed composition, comprising the biomass. The feed composition may contain at least 1% of the biomass by weight. In certain embodiments, the feed composition is optimized for consumption by fish, seafood, humans, or other animals. For example, the feed may comprise one or more of EPA, DHA, taurine, and one or more essential amino acids.

[0022] In yet another aspect, the invention provides a method of producing fish or seafood, comprising: farming fish or seafood, and providing a diet, which includes the feed of the invention, to the fish or seafood. With respect to aquaculture, the feed may be particularly useful for species (farmed for human consumption) that has pink-, reddish-, yellow- or orange-colored flesh. One advantage is that the farming of

fish may then fully exclude, or reduce the amount of, purified caratenoids used for supplementing the fish/seafood diet for esthetic purposes, thus substantially reducing the costs. Accordingly, the invention also provides a fish or seafood product exhibiting an elevated level of a carotenoid pigment in the flesh, wherein such elevated level is attributable to the diet comprising the feed composition of the invention. In certain embodiments, the fish meat contains at a higher level of at least one carotenoid compound than substantially same fish on a regular diet. Such a level may be higher by at least 10%, 15%, 20%, 25%, 50%, 80%, 100%, 200%, 300%, 400%, 500%, 1000% or more. In appearance, such a product would have a visibly darker, more appealing pigmentation. In related further embodiments, such food product is also characterized in that it does not contain, or contains less of, artificially introduced antibiotics or anti-inflammatory compounds, due to a healthier diet consumed by fish or seafood.

BRIEF DESCRIPTION OF THE FIGURES

[0023] FIG. **1** shows several exemplary carotenoid compounds.

[0024] FIG. **2** shows a map of pKB01: deletion construct for crtl-like gene, Mext_3011, described in Example 7.

[0025] FIG. **3** shows a map of pKB03: deletion construct for cluster of crtCDF (Mext_2725-26, -28), while preserving crtE (Mext_2727), described in Example 7.

[0026] FIG. **4** shows a map of pKB02: deletion construct for crtF (Mext_2728), described in Example 7.

[0027] FIG. **5** shows growth of the smallmouth grunt using 4 experimental diets, as described in Example 9, which included: (1) a standard commercially available grunt diet, (2) the standard diet plus astaxanthin pigment (~80PPM), (3) a diet containing 5% of the total feed pellet replaced by KnipBio single cell protein (KBM), and (4) a diet with 25% of the fish meal replaced by KBM (~60 PPM carotenoids).

[0028] FIG. **6** shows results of an amino acid profile analysis for the KBM feed described in Example 9.

DETAILED DESCRIPTION

Introduction

[0029] This invention provides, in one aspect, pigmented methylotrophic organisms (e.g., *Methylobacterium*) capable of producing one or more carotenoids. In certain embodiments, such organisms use methanol, methane, or another C1 energy source. In certain embodiments, such C1 energy source is the sole energy source for the organism. In certain embodiments, the methylotroph is *M. extorquens*. In certain embodiments, the *M. extorquens* or other methylotroph exhibits improved properties, such as improved yield of one or more carotenoids, production of a desired carotenoid spectrum, improved carotenoid levels per unit of biomass or as measured by a percentage of dry cell weight. In certain embodiments, the *M. extorquens* or other methylotroph is capable of producing specific desired nutrients, such as one or more proteins, one or more lipids, carbohydrates, and one or more vitamins. In certain embodiments, the protein produced is a complete nutrient source for aquaculture, agriculture, or humans.

[0030] The present invention also provides methods of engineering and culturing such methylotrophs, methods of using such methylotrophs to produce carotenoids, and methods of preparing carotenoid-containing compositions, such as food or feed additives, or nutritional supplements, using carotenoids produced in such methylotrophs. In particular, the present invention provides systems and methods for generating methylotrophs containing one or more oleaginic, proteinogenic and/or carotenogenic modifications that increase or alter their lipid-, protein-, or carotenoid-producing capabilities as compared with otherwise identical organisms that lack the modification(s). One preferred embodiment relates to an organism that produces one or more or all of the essential amino acids, for example lysine, valine, threonine, methionine, arginine, and taurine.

[0031] One aspect of this invention pertains to the field of aquaculture. More specifically, this invention pertains to aquaculture feed compositions comprising carotenoid-containing microbial biomass and a complete protein nutrition, that is, containing most or all amino acids necessary for healthy growth of the animal to which it is administered. The feed compositions may optionally contain omega-3 polyunsaturated fatty acid ratios of eicosapentaenoic acid to docosahexaenoic acid that are higher than currently available using fish oil, as well as further vitamins or other nutrients.

DETAILED DESCRIPTION

[0032] One common class of single carbon metabolizers is the methanotrophs, which are characterized by their ability to use methane as a sole source of carbon and energy. Methane monooxygenase is the enzyme required for the key step of methane metabolism. Its product is methanol (see Murrell et al., *Arch. Microbiol.* (2000), 173(5-6), 325-332). This reaction occurs at ambient temperature and pressures in sharp contrast to the industrial transformation of methane to methanol, which requires high temperatures (several hundred degrees Celsius) and high pressure (see WO 2000/007718 (incorporated by reference) and U.S. Pat. No. 5,750,821 (incorporated by reference)). This remarkable ability to transform methane under ambient conditions, along with the abundance of methane, makes the biotransformation of methane a potentially valuable process. No less desirable are methylotrophs capable of metabolizing methanol, which is itself an abundant and cheap feedstock. Being a liquid at room temperature, methanol is more easily utilized than methane for many applications.

[0033] The ketocarotenoid astaxanthin (3,3-dihydroxy-β, β-carotene-4,4'-dione) was first conceptualized as an oxidized form of β-carotene. Astaxanthin was subsequently found to be ubiquitous across many types of marine animals and algae. Few animals have the biosynthetic machinery to produce astaxanthin; most of them obtain it from their food. Astaxanthin is found in the plant kingdom principally in some species of cyanobacteria, algae and lichens.

[0034] Astaxanthin is a powerful antioxidant, being an inhibitor of lipid peroxidation (see, for example, Kurashige, M. et al. (1990) *Physiol. Chem. Phys. Med. NMR* 22:27). Also attributed to astaxanthin are chemopreventive effects such as significantly reducing the incidence of induced murine urinary bladder cancer (see Tanaka, T. et al. (1994) *Carcinogenesis* 15:15). Astaxanthin also exerts immunomodulating effects, inter alia enhancing antibody production (see Jyonouchi, H. (1993) *Nutr. Cancer* 19:269). The current, albeit incomplete, picture is that it appears to play an important role in cancer and tumor inhibition, as well as eliciting a positive response from the immune system.

[0035] Many methylotrophs contain an inherent isoprenoid pathway that enables them to synthesize other non-endog-

enous isoprenoid compounds. Some organisms are known to possess carotenogenic biosynthetic genes and the upper isoprene pathway which produces carotenogenic precursor molecules. Certain aspects of the isoprenoid biosynthesis pathway are conserved throughout the fungal, bacterial, plant and animal kingdoms. These include proteins or homologs corresponding to acetoacetyl-CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate pyrophosphate decarboxylase, IPP isomerase, FPP synthase, and GGPP synthase. An alternative isoprenoid biosynthesis pathway, sometimes called the "mevalonate-independent pathway", is utilized by some organisms (particularly bacteria). This pathway is initiated by the synthesis of DOXP (1-deoxy-D-xyloglucose-5-phosphate) from pyruvate and glyceraldehyde-3-phosphate. DOXP is then converted, via a series of biosynthetic steps, into IPP, which isomerizes into DMAPP and is then converted, via GPP and FPP, into GGPP.

[0036] Despite this knowledge, there is little precedent for genetically engineered C1 metabolizers producing specific, commercially valuable carotenoids. It is likely that the usefulness of these organisms for production of a larger range of chemicals is constrained by limitations including the relatively slow growth rates of methanotrophs, limited ability to tolerate methanol as an alternative substrate to methane, difficulty in genetic engineering, poor understanding of the roles of multiple carbon assimilation pathways present in methanotrophs, and potentially high costs due to the oxygen demand of fully saturated substrates such as methane. The problem to be solved is how to provide a cost effective method for the microbial production of carotenoid compounds, using organisms which utilize C1 compounds as their carbon and energy source.

[0037] Salmon and shrimp aquaculture would benefit from application of the present invention because of the importance of carotenoid pigmentation to the value of these organisms. (see Shahidi, F. et al. *Science* (1998) 38(1): 1-67). Lastly, carotenoids find applications in the synthesis of steroids, fragranaces, flavors, and compounds with electronics applications. Astaxanthin is the most expensive commercially used carotenoid compound, priced at thousands of dollars per kilogram. The disclosure herein provides a detailed description of the selection, modification, and use of appropriate C1 metabolizing microorganisms for the high-yielding production of various carotenoid compounds.

[0038] According to the present invention, carotenoid production in a host organism may also be accomplished through modifying the expression or regulating the activity of one or more proteins involved in isoprenoid biosynthesis. In certain embodiments the modification comprises removing alternative pathways that draw off intermediates at various stages. Genes encoding these enzymes can be cleanly removed using a marker-free allelic exchange system such as one based upon cre-lox (Marx, C. J. and Lidstrom, M. E. *BioTechniques* (2002) 33: 1062-1067), or a two-step, "in-out" system such as one based upon negative selection of sacB-containing strains (Marx, C. J. *BMC Research Notes* (2008) 1:1). Many of these genes are commonly clustered on the chromosome, thereby facilitating their removal. For example, one may remove one or more genes for enzymes or the enzymes themselves that make squalene and hopene on the route to hopanoid biosynthesis. Such genes and enzymes include squalene synthase, encoded by hpnC, dehydrosqualene synthase, encoded by hpnD, dehydrosqualene reductase, encoded by hpnE, or squalene-hopene synthase, encoded by she (also known as hpnF) (Bradley, A. S. et al. *Organic Geochemistry* (2010) 41: 1075-1081). Another offshoot that can be removed is the addition of a reduced geranylgeranyl group as an ester to bacteriochlorophyll (Addlesee, H. A. and Hunter, C. N. *Journal of Bacteriology* (1999) 181: 7248-7255). These reactions are accomplished by geranylgeranyl bacteriochlorophyll synthase, encoded by bchG, and geranylgeranyl-bacteriochlorophyll reductase, encoded by bchP. Finally, rather than synthesizing spirilloxanthin, for another product like astaxanthin it will be advantageous to remove enzymes downstream of where these pathways diverge. In this case, enzymes downstream of lycopene should be removed. These consist of hydroxyneurosporene dehydrogenase, encoded by crtC, methoxyneurosporene dehydrogenase, encoded by crtD, and hydroxyneurosporene methyltransferase, encoded by crtF. In certain embodiments, it will be advantageous to increase expression of endogenous genes upstream of lycopene. These include 1-deoxy-D-xylulose-5-phosphate synthase, encoded by dxs, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, encoded by dxr, isopentyl diphosphate isomerase, encoded by idi, farnesyl diphosphate synthase, encoded by ispA, geranylgeranyl diphosphate synthase, encoded by crtE, phytoene synthase, encoded by crtB, and phytoene desaturase, encoded by crtI. In certain embodiments, such modification comprises heterologous expression of isoprenoid biosynthesis polypeptides in the host organism and/or modifications of the expression or modifying the activity of one or more endogenous or heterologous isoprenoid biosynthesis polypeptides. Preferred carotenoids include astaxanthin and spirilloxanthin. In view of the considerable conservation of components of the isoprenoid biosynthesis polypeptides, one would expect that heterologous isoprenoid biosynthesis proteins would function even in significantly divergent organisms. In order to optimize expression in the methylotrophic host, such as *M. extorquens*, the sequence may be codon-optimized to match the most frequently used codons in the host organism. Indeed, in many cases proteins from different source organisms will function together (i.e., at the same time). In certain embodiments of the invention, a plurality of different heterologous isoprenoid biosynthesis polypeptides is introduced into the host cell. In certain embodiments, this plurality contains only proteins from the same source organism (e.g., two or more sequences of, or sequences derived from, the same organism); in other embodiments the plurality includes polypeptides independently selected from different source organisms (e.g., two or more sequences of, or sequences derived from, at least two different organisms). In certain embodiments, astaxanthin production will be accomplished by introducing lycopene cyclase, encoded by crtY, β-carotene ketolase, encoded by crtW, and β-carotene hydroxylase, encoded by crtZ. It is anticipated that the desired production will be supplied by the introduction of CrtY (lycopene cyclase) from *Bradyrhizobium* sp. ORS 278 [GenBank sequence ID: YP_001208335.1] or the like; CrtW (beta-carotene ketolase) from *Bradyrhizobium* sp. ORS 278 [GenBank sequence ID: YP_001208332.1] or the like; and CrtZ (β-carotene hydroxylase) from *Brevundimonas* sp. SD212 [GenBank sequence ID: AB181388] or the like.

[0039] In certain embodiments, it may be useful to change the levels of macromolecules within cellular material in order to provide beneficial properties to the feed. This may include changing or altering components, such as exopolysaccharides, poly-β-hydroxybutyrate storage polymer, or cellulose.

These modifications may divert more carbon flux toward other products, such as carotenoids, lipids, total protein, or engineered production of amino acids or vitamins.

[0040] In certain embodiments, genetic modifications will take advantage of freely replicating plasmid vectors for cloning. These may include small IncP vectors developed for use in *Methylobacterium*. These vectors may include pCM62, pCM66, or pHC41 for cloning (Marx, C. J. and M. E. Lidstrom *Microbiology* (2001) 147: 2065-2075; Chou, H.-H. et al. *PLoS Genetics* (2009) 5: e1000652).

[0041] In certain embodiments, genetic modifications will take advantage of freely replicating expression plasmids such as pCM80, pCM160, pHC90, or pHC91 (Marx, C. J. and M. E. *Lidstrom Microbiology* (2001) 147: 2065-2075; Chou, H.-H. et al. *PLoS Genetics* (2009) 5: e1000652).

[0042] In certain embodiments, genetic modifications will utilize freely replicating expression plasmids that have the ability to respond to levels of inducing molecules such as cumate or anhydrotetracycline. These include pHC115, pLC 290, pLC291 (Chou, H.-H. et al. *PLoS Genetics* (2009) 5: e1000652; Chubiz, L. M. et al. *BMC Research Notes* (2013) 6: 183).

[0043] In certain embodiments, genetic modifications will utilize recyclable antibiotic marker systems such as the cre-lox system. This may include use of the pCM157, pCM158, pCM184, pCM351 series of plasmids developed for use in *M. extorquens* (Marx, C. J. and M. E. Lidstrom *BioTechniques* (2002) 33: 1062-1067).

[0044] In certain embodiments, genetic modifications will utilize transposon mutagenesis. This may include mini-Tn5delivery systems such as pCM639 (D'Argenio, D. A. et al. *Journal of Bacteriology* (2001) 183: 1466-1471) demonstrated in *M. extorquens* (Marx, C. J. et al. *Journal of Bacteriology* (2003) 185: 669-673).

[0045] In certain embodiments, genetic modifications will utilize expression systems introduced directly into a chromosomal locus. This may include pCM168, pCM172, and pHC01 plasmids developed for *M. extorquens* AM1 (Marx, C. J. and M. E. Lidstrom *Microbiology* (2001) 147: 2065-2075; Lee, M.-C. et al. *Evolution* (2009) 63: 2813-2830).

[0046] In certain embodiments, genetic modifications will utilize a sacB-based system for unmarked exchange of alleles due to the sucrose sensitivity provided by sacB expression. This may include the pCM433 vector originally tested with *M. extorquens* (Marx, C. J. et al. *BMC Research Notes* (2008) 1:1).

[0047] In certain embodiments of the present invention that utilize heterologous isoprenoid biosynthesis polypeptides, the source organisms include as non-limiting examples fungi of the genera *Blakeslea, Candida, Cryptococcus, Cunninghamella, Lipomyces, Mortierella, Mucor, Phycomyces, Pythium, Rhodosporidium, Rhodotorula, Trichosporon, Yarrowia, Aspergillus, Botrytis, Cercospora, Fusarium* (*Gibberella*), *Kluyveromyces, Neurospora, Penicillium, Pichia* (*Hansenula*), *Puccinia, Saccharomyces, Schizosaccharomyces, Sclerotium, Trichoderms, Ustilago*, and *Xanthophyllomyces* (*Phaffia*). In certain embodiments, the source organisms are of a species including, but not limited to, *Cryptococcus neoformans, Fusarium fujikuroi, Kluyverimyces lactis, Neurospora crassa, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Ustilago maydis*, and *Yarrowia lipolytica*. In certain embodiments the source organism includes bacteria of the *Methylobacterium* genus or preferably species such as *M. extorquens*.

[0048] *Methylobacterium* strains are a diverse genus of largely plant-associated microbes. As of the past half-decade, genome sequences for several strains have been published, including *M. extorquens* AM1, *M. extorquens* DM4, *M. extorquens* CM4, *M. extorquens* PA1, *M. extorquens* BJ001 (formerly *M. populi*), *M. radiotolerans, M. nodulans*, and *Methylobacterium* spp. 4-46 (Vuileumier et al., 2009. *PLoS One*; Marx et al., 2012. *J. Bacteriology*). These strains offer various advantages and disadvantages, ranging from distinct growth rates on various substrates, to stark differences in genome size and mobile genetic element content. *M. extorquens* strains—of which there are five sequenced—pose the particular advantage of being able to draw from the tremendous knowledge about *M. extorquens* AM1, which has served as a workhorse for all of methylotrophy. Given recent discovery of a series of issues with the modern AM1 strain (Carroll et al., 2014. *BMC Microbiology*), however, some efforts have now focused on the genome streamlined, more robustly growing PA1 strain. These strains all share the majority of their genome content, and these genes are mainly 98% amino acid identical, or above. There are differences in gene content, however, which can be of critical importance to certain traits (Vuilleumier et al., 2009. *PLoS One*). As such, while a given genetic manipulation is likely to behave similarly across strains, there is also precedent for the occasional major differences.

[0049] Thus, in some embodiments, modified bacterium is a strain of *Methylobacterium*, e.g., *M. extorquens* AM1, *M. extorquens* DM4, *M. extorquens* CM4, *M. extorquens* PA1, *M. extorquens* BJ001 (formerly *M. populi*), *M. radiotolerans, M. nodulans*, and *Methylobacterium* spp. 4-46.

[0050] To date, there are three ways to generate carotenoid variants of *Methylobacterium*. First, key genes such as crtI can be knocked out that eliminate all coloration (Van Dien et al., 2003. *Applied & Environmental Microbiology*). Second, genes can be removed from branches that divert biosynthesis away from carotenoids, thereby enhancing coloration. An example of this is deleting shc, and thus the production of hopanoids. Finally, evolved variants from selection for growth in other conditions, such as rapid growth on 15 mM methanol (Lee et al., 2009. *Evolution*), can fortuitously lead to strains with increased or varied coloration.

[0051] In certain embodiments, methylotrophic bacteria of the invention are characterized in that they are genetically modified or artificially pre-selected to produce elevated levels of a carotenoid compound relative to the corresponding unmodified or unselected bacterium. Improved carotenoid production can be assayed in terms of mg carotenoid per gram of dry cell weight, such as using the methods described in Lemuth et al., 2011 (*Microbial Cell Factories*. 10:29). In some embodiments, the bacterial production of at least one carotenoid compound is elevated by at least 10%, 15%, 20%, 25%, 50%, 80%, 100%, 200%, 300%, 400%, 500%, 1000% or more.

[0052] The isoprenoid biosynthesis pathway is also used by organisms to produce non-carotenoid compounds, such as sterols, steroids, and vitamins, including vitamin E or vitamin K. Proteins that have isoprenoid biosynthesis pathway intermediates as their substrates, and divert them into biosynthesis of non-carotenoid compounds, are indirect inhibitors of carotenoid biosynthesis because they compete for the same intermediates as the desired carotenoid pathway. The present invention addresses this issue by enabling reductions of the

level or activity of such competing proteins, allowing for increased production of carotenoid compounds.

[0053] Beyond carotenoids and vitamins, a number of amino acids and other small metabolites are at limiting levels in feed sources. These may be amino acids, and in particular the set of arginine, threonine, valine, lysine, and methionine. Another molecule of interest is taurine (2-aminoethanosulfonic acid). In certain embodiments, directed genetic modifications of the relevant amino acid and taurine biosynthetic pathways augments the expression of key genes or removes side pathways and recycling pathways. In other embodiments selection may involve use of toxic analogues of the relevant compounds, such as ethionine to achieve methionine overproduction (see Lawrence et al. *Genetics* (1968) 58: 473-492). In yet other embodiments, experimental evolution of overproduction may occur through selection in the context of metabolic cross-feeding (Harcombe, W. R. *Evolution* (2010), 64(7), 2166-2172). In other embodiments, manipulations obtained by directed engineering, selection with analogues, and selection in consortia will be combined.

[0054] Carotenoids produced according to the present invention can be utilized in any of the applications mentioned herein, among which are their multifaceted biological or nutritional properties (antioxidant, antiproliferative, etc.) and their usefulness as pigments ranging in color from yellow to red. For example, according to the present invention, carotenoids may be used in pharmaceuticals (see, for example, Bertram et al., *Nutr. Rev.* 1999, 57:182; Singh et al., *Oncology* 1998, 12:1643; Rock, *Pharmacol. Ther.* 1997, 75:185; Edge et al., *J. Photochem Photobiol* 1997, 41:189; U.S. Patent Application 2004/0116514 (incorporated by reference); U.S. Patent Application 2004/0259959 (incorporated by reference)), food supplements (see, for example, Koyama et al., *J. Photochem Photobiol* 1991, 9:265; Bauemfeind, *Carotenoids as colorants and vitamin A precursors*, Academic Press, NY, 1981; U.S. Patent Application 2004/0115309 (incorporated by reference); U.S. Patent Application 2004/0234579 (incorporated by reference)), electro-optic applications, animal feed additives (see for example Krinski, *Pure Appl. Chem.* 1994, 66:1003; Polazza et al., *Meth. Enzymol.* 1992, 213:403), cosmetics (as anti-oxidants and/or as cosmetics, including fragrances; see, for example, U.S. Patent Application 2004/0127554 (incorporated by reference)), etc. Carotenoids produced in accordance with the present invention may also be used as intermediates in the production of other compounds (e.g., steroids).

[0055] For example, astaxanthin and/or esters thereof may be useful in a variety of pharmaceutical applications and health foods including treatment of inflammatory diseases, asthma, atopic dermatitis, allergies, multiple myeloma, arteriosclerosis, cardiovascular disease, liver disease, cerebrovascular disease, thrombosis, neoangiogenesis-related diseases, including cancer, rheumatism, diabetic retinopathy; macular degeneration and brain disorder, hyperlipidemia, kidney ischemia, diabetes, hypertension, tumor proliferation and metastasis; and metabolic disorders. Additionally, carotenoids and astaxanthin may be useful in the prevention and treatment of fatigue, for improving kidney function in nephropathy from inflammatory diseases, as well as prevention and treatment of other life habit-related diseases. Still further, astaxanthin has been found to play a role as inhibitors of various biological processes, including interleukin inhibitors, phosphodiesterase inhibitors, phospholipase A2 inhibitors, cyclooxygenase-2 inhibitors, matrix metalloproteinase inhibitors, capillary endothelium cell proliferation inhibitors, lipoxygenase inhibitors. See, for example, Japanese Publication No. 2006022121 (JP Appl No. 2005-301156); Japanese Publication No. 2006016408 (JP Appl No. 2005-301155); Japanese Publication No. 2006016409 (JP Appl No. 2005-301157); Japanese Publication No. 2006016407 (JP Appl No. 2005-301153); Japanese Publication No. 2006008717 (JP Appl No. 2005-301151); Japanese Publication No. 2006008716 (JP Appl No. 2005-301150); Japanese Publication No. 2006008720 (JP Appl No. 2005-301158); Japanese Publication No. 2006008719 (JP Appl No. 2005-301154); Japanese Publication No. 2006008718 (JP Appl No. 2005-301152); Japanese Publication No. 2006008713 (JP Appl No. 2005-301147); Japanese Publication No. 2006008715 (JP Appl No. 2005-301149); Japanese Publication No. 2006008714 (JP Appl No. 2005-301148); and Japanese Publication No. 2006008712 (JP Appl No. 2005-301146).

[0056] It will be appreciated that, in some embodiments of the invention, carotenoids produced by manipulated host cells as described herein are incorporated into a final product (e.g., food or feed supplement, pharmaceutical, cosmetic, dye-containing item, fragrance, nutraceutical, etc.) in the context of the host cell. For example, host cells may be lyophilized, freeze dried, frozen or otherwise inactivated, and then whole cells may be incorporated into or used as the final product. The host cell may also be processed prior to incorporation in the product to increase bioavailability (e.g., via lysis). This may include methods such as homogenization, with or without subsequent addition of ethoxyquin or other appropriate reductants to protect carotenoids or other nutritional components from subsequent oxidation. The host cell may be processed in the presence of a hydrophobic substance that may or may not be incorporated into the final formulation in order to aid in partial extraction and bioavailability of carotenoids. This may involve combining bacterial material with the fish oils, or other dietary oils prior to their joint addition to the eventual feed. Cell material may be provided as thawed "wet" cell material, or as dried bacterial "cake". Alternatively or additionally, a final product may incorporate only a portion of the host cell (e.g., fractionated by size, solubility), separated from the whole. For example, in some embodiments of the invention, lipid droplets are isolated from the host cells and are incorporated into or used as the final product; or a protein isolate may be incorporated into or used as the final product. In other embodiments, the carotenoids themselves, or individual carotenoid compounds are isolated and reformulated into the final product.

[0057] As stated above, fatty acid and glucoside esters are the predominant carotenoid esters found in nature, whereas additional esters (e.g., with organic acids or inorganic phosphate) can be synthesized to generate useful product forms. For delivery, carotenoid esters can also be formulated as salts of the ester form. See, e.g., US Publication No. 2005/0096477 (incorporated by reference).

[0058] The amount of carotenoid incorporated into a given product may vary dramatically depending on the product, and the particular carotenoid(s) involved. Amounts may range, for example, from less than 0.01% by weight of the product, to more than 1%, 10%, 20%, 30% or more; in some cases the carotenoid may comprise 100% of the product. Similarly, the addition of cell material in feed can range from small doses, such as 0.01%, up to 100% of the feed. In some embodiment, the feed contains at least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 50% or more of biomass of the invention.

[0059] In some embodiments of the invention, one or more produced carotenoids is incorporated into a component of food or feed (e.g., a food supplement). Types of food products into which carotenoids can be incorporated according to the present invention are not particularly limited, and include beverages, such as teas, juices, and liquors; confections, such as jellies and biscuits; fat-containing foods and beverages, such as dairy products; processed food products, such as rice and soft rice (or porridge); infant formulas; or the like. In some embodiments, it may be useful to incorporate the carotenoids within bodies of edible lipids as it may facilitate incorporation into certain fat-containing food products.

[0060] Examples of feedstuffs into which carotenoids produced in accordance with the present invention may be incorporated include, for instance, pet foods, such as cat foods, dog foods and the like, feeds for aquarium fish, cultured fish or crustaceans, etc., feed for farm-raised animals (including livestock and further including fish or crustaceans raised in aquaculture). The carotenoids and/or other caloric or nutritional supplements produced in accordance with the present invention can also be incorporated into food or vitamin supplements for human consumption. Food or feed material into which the carotenoid(s) produced in accordance with the present invention is incorporated is preferably palatable to the organism which is the intended recipient. This food or feed material may have any physical properties currently known for a food material (e.g., solid, liquid, soft).

[0061] In some embodiments of the invention, one or more produced carotenoids is incorporated into a cosmetic product. Examples of such cosmetics include, for instance, skin cosmetics (e.g., lotions, emulsions, creams and the like), lipsticks, anti-sunburn cosmetics, makeup cosmetics, fragrances, and other products for daily use (e.g., toothpastes, mouthwashes, bad breath preventive agents, solid soaps, liquid soaps, shampoos, conditioners).

[0062] In some embodiments, one or more produced carotenoids are incorporated into a pharmaceutical. Examples of such pharmaceuticals include, for instance, various types of tablets, capsules, drinkable agents, troches, gargles, etc. In some embodiments, the pharmaceutical is suitable for topical application. Dosage forms are not particularly limited, and include capsules, oils, granula, granula subtilae, pulveres, tabellae, pilulae, trochisci, or the like. Oils and oil-filled capsules may provide additional advantages both because of their lack of ingredient decomposition during manufacturing, and because inventive carotenoid-containing lipid droplets may be readily incorporated into oil-based formulations.

[0063] Pharmaceuticals according to the present invention may be prepared according to techniques established in the art including, for example, the common procedure as described in the United States Pharmacopoeia.

[0064] Carotenoids produced according to the present invention may be incorporated into any pigment-containing product including, for example, fabric, and paint. They may also be incorporated into a product which is an environmental indicator, or an instrument, such as a biosensor, for use as a detection agent.

[0065] Accordingly, the present invention further provides a process for production of carotenoids, such as, but not limited to, β-carotene, echinenone, β-cryptoxanthin, canthaxanthin, adonirubin, cis-adonixanthin, adonixanthin, astaxanthin, zeaxanthin, spirilloxanthin, and intermediates leading to spirilloxanthin, such as lycopene and rhodopin, the process comprising culturing a bacterial species in a nutrient medium including sources of carbon, nitrogen and inorganic substances; and recovering an individual carotenoid pigment or a mixture of carotenoid pigments from the bacterial cells, vesicles secreted therefrom and/or the growth medium.

[0066] Medium for production of carotenoids using the present microorganisms is, for example, as follows. It contains a carbon source, a nitrogen source and inorganic salts necessary for the growth of producer microorganisms, as well as, if necessary, any special required substances for the growth or thriving of the organism (for example, vitamins, amino acids, nucleic acids).

[0067] The carbon source may comprise sugars, such as glucose, sucrose, lactose, fructose, trehalose, mannose, mannitol, and maltose; organic acids, such as acetic acid, fumaric acid, citric acid, propionic acid, malic acid, pyruvic acid, malonic acid, and ascorbic acid; alcohols, such as ethanol, propanol, butanol, pentanol, hexanol, isobutanol, and glycerol; oil or fat, such as soybean oil, rice bran oil, olive oil, corn oil, sesame oil, linseed oil, and the like. The amount of the carbon source added varies according to the kind of the carbon source, and usually 1 to 100 g, or 2 to 50 g per liter of medium.

[0068] The nitrogen source may comprise potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulfate, ammonium phosphate, ammonia, urea, and the like, alone or in combination. Amount of the nitrogen source added varies according to the kind of the nitrogen source, and is usually 0.1 to 30 g, and preferably 1 to 10 g per liter of medium.

[0069] The inorganic salt may comprise potassium dihydrogen phosphate, dipotassium hydrogen phosphate, disodium hydrogen phosphate, magnesium sulfate, magnesium chloride, ferric sulfate, ferrous sulfate, ferric chloride, ferrous chloride, manganous sulfate, manganous chloride, zinc sulfate, zinc, chloride, cupric sulfate, calcium chloride, calcium carbonate, sodium carbonate, and the like, alone or in combination. Amount of inorganic salt varies according to the kind of the inorganic salt, and usually 0.001 to 10 g per liter of medium.

[0070] As special required substances, vitamins, nucleic acids, yeast extract, peptone, meat extract, malt extract, corn steep liquor, soybean meal, dried yeast etc., may be used alone or in combination. Amount of the special required substance used varies according to the kind of the substance, and usually ranges between 0.2 g to 200 g, and preferably 3 to 100 g per liter of medium.

[0071] The pH value of a medium is typically adjusted to pH 2 to 12, preferably 6 to 9. The medium may further comprise one or more buffers to maintain the culture at the desired pH. Typical buffers are known in the art and include phosphate, carbonate, acetate, PIPES, HEPES, and Tris buffers; the optimal buffer for a given organism can easily be determined by one of ordinary skill in the art. For *Methylobacterium*, a common medium (Lee, M.-C. et al. *Evolution* (2009) 63: 2813-2830) is a phosphate buffered medium that consists of 1 mL of trace metal solution (to 1 liter of deionized water the following were added in this order: 12.738 g of EDTA disodium salt dihydrate, 4.4 g of $ZnSO_4.7H_2O$, 1.466 g of $CaCl_2.2H_2O$, 1.012 g of $MnCl_2.4H_2O$, 0.22 g of $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.314 g of $CuSO_4.5H_2O$, 0.322 g of $CoCl_2.6H_2O$, and 0.998 g of $FeSO_4.7H_2O$; pH 5.0 was maintained after every addition), 100 mL of phosphate buffer (25.3 g of K_2HPO_4 and 22.5 g of NaH_2PO_4 in 1 liter of deionized water), 100 mL of sulfate solution (5 g of $(NH_4)_2SO_4$ and 0.98 g of

$MgSO_4$ in 1 liter of deionized water), and 799 mL of deionized water. All components were heat sterilized separately and then pooled together. An alternative medium recently developed for use with *Methylobacterium extorquens* takes advantage of an organic buffer and has a citrate-chelated trace metal mix. Culturing is carried out at temperature of 15 to 40° C., and preferably 20 to 35° C., usually for 1 to 20 days, and preferably 1 to 4 days, under aerobic condition provided by shaking or aeration/agitation. Common practice with *Methylobacterium* is at 30° C. As a membrane component, carotenoids may be produced to higher titer at temperatures that vary from optimal, in medium that becomes limiting for a nutrient such as N or P, by exposure to light (visible or ultraviolet), or by the addition of a stressful agent such as NaCl. Finally the carotenoid(s) and other product nutrients may be isolated and purified from the culture.

[0072] The protocol for making M-PIPES medium is described in Table S1 of Delaney et al., 2013. PLoS One (8:e62957). FIG. 2 in U.S. Ser. No. 61/863,701 shows an exemplary recipe for medium optimized for use with *M. extorquens.*

[0073] In order to generate dense cultures of strains such as *Methylobacterium*, it may be advantageous to use a fed-batch method. Methanol can be tolerated well at 0.5-1% v/v (~120-240 mM), and thus this step size of addition can be used repeatedly. Critically, pH levels drop during culturing on methanol, such that the use of a base such as KOH or NaOH would be important to maintain the pH around 6.5. Aeration can be achieved via physical agitation, such as an impeller, via bubbling of filtered air or pure oxygen, or in combination. In order to reduce production costs, the buffer can be replaced from phosphates or PIPES to a carbonate-buffered medium.

[0074] Typically, microbial cells are separated from the culture by a conventional means such as centrifugation or filtration. The cells may be isolated whole, or may be lysed to release their contents for extraction or further processing. The cells or the medium may be subjected to an extraction with a suitable solvent. As an optional step prior to extraction carotenoid loaded vesicles may be recovered from the medium, by for example, ultracentrifugation or filtration.

[0075] As a solvent for the extraction, any substance in which the carotenoids are soluble can be used. For example, organic solvents, such as acetone, chloroform, dichloromethane, hexane, cyclohexane, methanol, ethanol, isopropanol, benzene, carbon disulfide, and diethyl ether, are used, and preferably chloroform, dichloromethane, acetone, methanol, ethanol or isopropanol is used. The purification can be carried out by conventional procedures, such as absorption, elution, dissolving and the like, alone or preferably in combination.

[0076] According to the present invention, one or more of β-carotene, echinenone, β-cryptoxanthin, canthaxanthin, adonirubin, cis-adonixanthin, adonixanthin, astaxanthin, zeaxanthin, spirilloxanthin, and intermediates leading to spirilloxanthin such as lycopene and rhodopin are simultaneously produced and present in the cultured cells and/or medium.

[0077] One aspect of the invention is related to a method for the production of a carotenoid compound, the method comprising

[0078] (a) providing a pigmented methylotrophic bacterial host cell comprising:

[0079] (i) suitable levels of isopentenyl pyrophosphate for the production of the carotenoid compound; and (ii) at least one isolated nucleic acid molecule encoding an enzyme in the carotenoid biosynthetic pathway under the control of suitable regulatory sequences;

[0080] (b) contacting the host cell of step (a) under suitable growth conditions with an effective amount of a C1 carbon substrate whereby the carotenoid compound is produced.

[0081] In certain embodiments, the carotenoid compound is selected from the group consisting of non-natural carotenoids, antheraxanthin, adonixanthin, astaxanthin, canthaxanthin, capsorubrin, β-cryptoxanthin, α-carotene, β-carotene, ϵ-carotene, echinenone, γ-carotene, ƒ-carotene, α-cryptoxanthin, diatoxanthin, 7,8-didehydroastaxanthin, fucoxanthin, fucoxanthinol, isorenieratene, lactucaxanthin, lutein, lycopene, neoxanthin, neurosporene, hydroxyneurosporene, peridinin, phytoene, rhodopin, rhodopin glucoside, siphonaxanthin, spheroidene, spheroidenone, spirilloxanthin, uriolide, uriolide acetate, violaxanthin, zeaxanthin-β-diglucoside, zeaxanthin, and intermediates in the biosynthetic production of any of the foregoing carotenoid compounds.

[0082] In certain embodiments, the carotenoid compound is selected from the group consisting of β-carotene, lycopene, rhodopin, echinenone, β-cryptoxanthin, canthaxanthin, adonirubin, cis-adonixanthin, adonixanthin, astaxanthin, zeaxanthin, spirilloxanthin, and intermediates in the biosynthetic production of any of the foregoing carotenoid compounds.

[0083] In certain embodiments, the carotenoid compound is selected from the group consisting of β-carotene, lycopene, rhodopin, astaxanthin and spirilloxanthin.

[0084] In certain embodiments, the carotenoid compound is spirilloxanthin.

[0085] In certain embodiments, the C1 carbon substrate is selected from the group consisting of methane, methanol, formaldehyde, formic acid, methylated amines, methylated thiols, and carbon dioxide.

[0086] In certain embodiments, the C1 carbon substrate is selected from the group consisting of methanol, formaldehyde, and methylated amines.

[0087] In certain embodiments, the C1 carbon substrate is methanol.

[0088] In certain embodiments, the host cell is selected from the group consisting of *Methylomonas, Methylobacter, Methylococcus, Methylosinus, Methylocyctis, Methylomicrobium, Methanomonas, Methylophilus, Methylobacillus, Methylobacterium, Hyphomicrobium, Xanthobacter, Bacillus, Paracoccus, Nocardia, Arthrobacter, Rhodopseudomonas, Pseudomonas, Candida, Hansenula, Pichia, Torulopsis*, and *Rhodotorula.*

[0089] In certain embodiments, the host cell is a *Methylobacterium.*

[0090] In certain embodiments, the host cell is *Methylobacterium extorquens.*

[0091] In certain embodiments, the host cell comprises a functional Embden-Meyerhof carbon pathway, said pathway comprising a gene encoding a pyrophosphate dependent phosphofructokinase enzyme.

[0092] In certain embodiments, the host cell contains at least one gene encoding a fructose bisphosphate aldolase enzyme.

[0093] In certain embodiments, the host cell contains a functional Entner-Douderoff carbon pathway.

[0094] In certain embodiments, the suitable levels of isopentenyl pyrophosphate are provided by the expression of heterologous upper isoprenoid pathway genes.

[0095] In certain embodiments, the upper isoprenoid pathway genes are selected from the group consisting of D-1-deoxyxylulose-5-phosphate synthase (Dxs), D-1-deoxyxylulose-5-phosphate reductoisomerase (Dxr), 2C-methyl-D-erythritol cytidylyltransferase (IspD), 4-diphosphocytidyl-2-C-methylerythritol kinase (IspE), 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), CTP synthase (PyrG), LytB, and GcpE.

[0096] In certain embodiments, the host cell produces a non-natural spectrum of carotenoid compounds.

[0097] In certain embodiments, the host cell produces a spectrum of amino acids suitable for use as a nutritional supplement.

[0098] In certain embodiments, the spectrum of amino acids comprises all essential amino acids.

[0099] In certain embodiments, the host cell produces taurine.

[0100] In certain embodiments, the host cell produces one or more vitamins or antioxidants.

[0101] In certain embodiments, the host cell produces one or more fatty acids.

[0102] In certain embodiments, the one or more fatty acids comprises monounsaturated fatty acids, polyunsaturated fatty acids, or one or more essential omega-3 fatty acids.

[0103] In certain embodiments, the one or more essential omega-3 fatty acids is EPA, DHA, or both.

[0104] In certain embodiments, the host cell is a spontaneous mutant which overexpresses one or more carotenoid compounds relative to the non-mutant cell.

[0105] In certain embodiments, the isolated nucleic acid molecule encodes a carotenoid biosynthetic enzyme selected from the group consisting of geranylgeranyl pyrophosphate (GGPP) synthase, phytoene synthase, phytoene desaturase, lycopene cyclase, β-carotene hydroxylase, zeaxanthin glucosyl transferase, β-carotene ketolase, β-carotene C-4 oxygenase, β-carotene desaturase, spheroidene monooxygenase, carotene hydratase, carotenoid 3,4-desaturase, 1-OH-carotenoid methylase, farnesyl diphosphate synthetase, and diapophytoene dehydrogenase.

[0106] In certain embodiments, the host cell is a transformed cell comprising multiple copies of at least one gene encoding an enzyme selected from the group consisting of D-1-deoxyxylulose-5-phosphate synthase (Dxs), D-1-deoxyxylulose-5-phosphate reductoisomerase (Dxr), 2C-methyl-D-erythritol cytidylyltransferase (IspD), 4-diphosphocytidyl-2-C-methylerythritol kinase (IspE), 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), CTP synthase (PyrG), LytB, GcpE, isopentyl diphosphate isomerase, farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, phytoene synthase, phytoene desaturase, lycopene cyclase (CrtY), β-carotene ketolase (CrtW), and β-carotene hydroxylase (CrtZ).

[0107] In certain embodiments, the host cell is a transformed cell comprising at least one gene encoding an enzyme selected from the group consisting of D-1-deoxyxylulose-5-phosphate synthase (Dxs), D-1-deoxyxylulose-5-phosphate reductoisomerase (Dxr), 2C-methyl-D-erythritol cytidylyltransferase (IspD), 4-diphosphocytidyl-2-C-methylerythritol kinase (IspE), 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), CTP synthase (PyrG), LytB, GcpE, isopentyl diphosphate isomerase, farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, phytoene synthase, phytoene desaturase, lycopene cyclase (CrtY), β-carotene ketolase (CrtW), and β-carotene hydroxylase (CrtZ), operably linked to a strong promoter.

[0108] In certain embodiments, the host cell comprises at least one gene encoding an enzyme selected from the group consisting of lycopene cyclase (CrtY), β-carotene ketolase (CrtW), and β-carotene hydroxylase (CrtZ).

[0109] In certain embodiments, the host cell comprises one or more of the genes CrtY (lycopene cyclase) from *Bradyrhizobium* sp. ORS 278 [GenBank sequence ID: YP_001208335.1], CrtW (beta-carotene ketolase) from *Bradyrhizobium* sp. ORS 278 [GenBank sequence ID: YP_001208332.1], and CrtZ (β-carotene hydroxylase) from *Brevundimonas* sp. SD212 [GenBank sequence ID: AB181388].

[0110] In certain embodiments, the host cell is modified so that one or more genes producing enzymes that divert isoprenoid compounds from the carotenoid biosynthetic pathway are blocked or deleted.

[0111] In certain embodiments, the one or more blocked or deleted genes are selected from the group consisting of genes involved in hopanoid biosynthesis, genes involved in producing carotenoids other than astaxanthin, and genes involved in producing carotenoids other than spirilloxanthin.

[0112] In certain embodiments, the one or more blocked or deleted genes are selected from the group consisting of hpnC, hpnD, hpnE, she (hpnF), bchG, bchP, crtC, crtD, and crtF.

[0113] In certain embodiments, the host cell is a spontaneous mutant whose rate of growth is increased relative to a corresponding non-mutant.

[0114] In certain embodiments, the host cell is cultured under stress conditions selected from light depletion, nutrient depletion, nitrogen depletion, high salt, or a chemical that inhibits growth of the host cell, wherein the stress conditions induce changes in gene expression leading to increased carotenoid production.

[0115] One aspect of the present invention is a pigmented methylotrophic host cell that produces a carotenoid compound, comprising:

[0116] (i) suitable levels of isopentenyl pyrophosphate for the production of the carotenoid compound; and (ii) at least one isolated nucleic acid molecule encoding an enzyme in the carotenoid biosynthetic pathway under the control of suitable regulatory sequences; wherein the host cell produces a carotenoid compound.

[0117] In certain embodiments, the carotenoid compound is selected from the group consisting of non-natural carotenoids, antheraxanthin, adonixanthin, astaxanthin, canthaxanthin, capsorubrin, β-cryptoxanthin, α-carotene, β-carotene, ϵ-carotene, echinenone, γ-carotene, ƒ-carotene, α-cryptoxanthin, diatoxanthin, 7,8-didehydroastaxanthin, fucoxanthin, fucoxanthinol, isorenieratene, lactucaxanthin, lutein, lycopene, neoxanthin, neurosporene, hydroxyneurosporene, peridinin, phytoene, rhodopin, rhodopin glucoside, siphonaxanthin, spheroidene, spheroidenone, spirilloxanthin, uriolide, uriolide acetate, violaxanthin, zeaxanthin-β-diglucoside, zeaxanthin, and intermediates in the biosynthetic production of any of the foregoing carotenoid compounds.

[0118] In certain embodiments, the carotenoid compound is selected from the group consisting of β-carotene, lycopene, rhodopin, echinenone, β-cryptoxanthin, canthaxanthin, adonirubin, cis-adonixanthin, adonixanthin, astaxanthin,

zeaxanthin, spirilloxanthin, and intermediates in the biosynthetic production of any of the foregoing carotenoid compounds.

[0119] In certain embodiments, the carotenoid compound is selected from the group consisting of β-carotene, lycopene, rhodopin, astaxanthin and spirilloxanthin.

[0120] In certain embodiments, the carotenoid compound is spirilloxanthin.

[0121] In certain embodiments, the host cell is capable of using as an energy source a C1 carbon substrate selected from the group consisting of methane, methanol, formaldehyde, formic acid, methylated amines, methylated thiols, and carbon dioxide.

[0122] In certain embodiments, the C1 carbon substrate is selected from the group consisting of methanol, formaldehyde, and methylated amines.

[0123] In certain embodiments, the C1 carbon substrate is methanol.

[0124] In certain embodiments, the host cell is selected from the group consisting of *Methylomonas, Methylobacter, Methylococcus, Methylosinus, Methylocyctis, Methylomicrobium, Methanomonas, Methylophilus, Methylobacillus, Methylobacterium, Hyphomicrobium, Xanthobacter, Bacillus, Paracoccus, Nocardia, Arthrobacter, Rhodopseudomonas, Pseudomonas, Candida, Hansenula, Pichia, Torulopsis*, and *Rhodotorula.*

[0125] In certain embodiments, the host cell is a *Methylobacterium.*

[0126] In certain embodiments, the host cell is *Methylobacterium extorquens.*

[0127] In certain embodiments, the host cell comprises a functional Embden-Meyerhof carbon pathway, said pathway comprising a gene encoding a pyrophosphate dependent phosphofructokinase enzyme.

[0128] In certain embodiments, the host cell contains at least one gene encoding a fructose bisphosphate aldolase enzyme.

[0129] In certain embodiments, the host cell contains a functional Entner-Douderoff carbon pathway.

[0130] In certain embodiments, the suitable levels of isopentenyl pyrophosphate are provided by the expression of heterologous upper isoprenoid pathway genes.

[0131] In certain embodiments, the upper isoprenoid pathway genes are selected from the group consisting of D-1-deoxyxylulose-5-phosphate synthase (Dxs), D-1-deoxyxylulose-5-phosphate reductoisomerase (Dxr), 2C-methyl-D-erythritol cytidylyltransferase (IspD), 4-diphosphocytidyl-2-C-methylerythritol kinase (IspE), 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), CTP synthase (PyrG), LytB, and GcpE.

[0132] In certain embodiments, the host cell produces a non-natural spectrum of carotenoid compounds.

[0133] In certain embodiments, the host cell produces a spectrum of amino acids suitable for use as a nutritional supplement.

[0134] In certain embodiments, the spectrum of amino acids comprises all essential amino acids.

[0135] In certain embodiments, the host cell produces taurine.

[0136] In certain embodiments, the host cell produces one or more vitamins or antioxidants.

[0137] In certain embodiments, the host cell produces one or more fatty acids.

[0138] In certain embodiments, the one or more fatty acids comprises monounsaturated fatty acids, polyunsaturated fatty acids, or one or more essential omega-3 fatty acids.

[0139] In certain embodiments, the one or more essential omega-3 fatty acids is EPA, DHA, or both.

[0140] In certain embodiments, the host cell is a spontaneous mutant which overexpresses one or more carotenoid compounds relative to the non-mutant cell.

[0141] In certain embodiments, the isolated nucleic acid molecule encodes a carotenoid biosynthetic enzyme selected from the group consisting of geranylgeranyl pyrophosphate (GGPP) synthase, phytoene synthase, phytoene desaturase, lycopene cyclase, β-carotene hydroxylase, zeaxanthin glucosyl transferase, β-carotene ketolase, β-carotene C-4 oxygenase, β-carotene desaturase, spheroidene monooxygenase, carotene hydratase, carotenoid 3,4-desaturase, 1-OH-carotenoid methylase, farnesyl diphosphate synthetase, and diapophytoene dehydrogenase.

[0142] In certain embodiments, the host cell is a transformed cell comprising multiple copies of at least one gene encoding an enzyme selected from the group consisting of D-1-deoxyxylulose-5-phosphate synthase (Dxs), D-1-deoxyxylulose-5-phosphate reductoisomerase (Dxr), 2C-methyl-D-erythritol cytidylyltransferase (IspD), 4-diphosphocytidyl-2-C-methylerythritol kinase (IspE), 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), CTP synthase (PyrG), LytB, GcpE, isopentyl diphosphate isomerase, farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, phytoene synthase, phytoene desaturase, lycopene cyclase (CrtY), β-carotene ketolase (CrtW), and β-carotene hydroxylase (CrtZ).

[0143] In certain embodiments, the host cell is a transformed cell comprising at least one gene encoding an enzyme selected from the group consisting of D-1-deoxyxylulose-5-phosphate synthase (Dxs), D-1-deoxyxylulose-5-phosphate reductoisomerase (Dxr), 2C-methyl-D-erythritol cytidylyltransferase (IspD), 4-diphosphocytidyl-2-C-methylerythritol kinase (IspE), 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), CTP synthase (PyrG), LytB, GcpE, isopentyl diphosphate isomerase, farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, phytoene synthase, phytoene desaturase, lycopene cyclase (CrtY), β-carotene ketolase (CrtW), and β-carotene hydroxylase (CrtZ), operably linked to a strong promoter.

[0144] In certain embodiments, the host cell comprises at least one gene encoding an enzyme selected from the group consisting of lycopene cyclase (CrtY), β-carotene ketolase (CrtW), and β-carotene hydroxylase (CrtZ).

[0145] In certain embodiments, the host cell comprises one or more of the genes CrtY (lycopene cyclase) from *Bradyrhizobium* sp. ORS 278 [GenBank sequence ID: YP_001208335.1], CrtW (beta-carotene ketolase) from *Bradyrhizobium* sp. ORS 278 [GenBank sequence ID: YP_001208332.1], and CrtZ (β-carotene hydroxylase) from *Brevundimonas* sp. SD212 [GenBank sequence ID: AB181388].

[0146] In certain embodiments, the host cell is modified so that one or more genes producing enzymes that divert isoprenoid compounds from the carotenoid biosynthetic pathway are blocked or deleted.

[0147] In certain embodiments, the one or more blocked or deleted genes are selected from the group consisting of genes involved in hopanoid biosynthesis, genes involved in produc-

ing carotenoids other than astaxanthin, and genes involved in producing carotenoids other than spirilloxanthin.

[0148] In certain embodiments, the one or more blocked or deleted genes are selected from the group consisting of hpnC, hpnD, hpnE, she (hpnF), bchG, bchP, crtC, crtD, and crtF.

[0149] In certain embodiments, the host cell is a spontaneous mutant whose rate of growth is increased relative to a corresponding non-mutant.

[0150] In certain embodiments, the host cell is cultured under stress conditions selected from light depletion, nutrient depletion, nitrogen depletion, high salt, or a chemical that inhibits growth of the host cell, wherein the stress conditions induce changes in gene expression leading to increased carotenoid production.

[0151] In one aspect, the invention relates to a feed composition, comprising biomass from a host cell as described above.

[0152] In certain embodiments, the composition further comprises a source of protein comprising all of the essential amino acids.

[0153] In certain embodiments, the composition further comprises one or more vitamins or antioxidants.

[0154] In certain embodiments, the composition further comprises one or more fatty acids.

[0155] In certain embodiments, the one or more fatty acids comprises monounsaturated fatty acids, polyunsaturated fatty acids, or one or more essential omega-3 fatty acids.

[0156] In certain embodiments, the one or more essential omega-3 fatty acids is EPA, DHA, or both.

[0157] In certain embodiments, the biomass comprises whole cells.

[0158] In certain embodiments, the biomass comprises lysed cells.

[0159] In certain embodiments, the biomass is processed or partially processed.

[0160] In certain embodiments, the composition is for aquaculture, including aquaculture feed organisms such as krill, rotifers, or the like.

[0161] In certain embodiments, the composition is for use in agriculture as an animal feed.

[0162] In certain embodiments, the composition is for use with ornamental fish, shrimp, corals, or other hobbyist aquaculture.

[0163] In certain embodiments, the composition is for human use.

[0164] In certain embodiments, the human use is as a nutritional supplement.

[0165] In one aspect, the invention relates to a method of preparing a feed composition as described above, the method comprising

[0166] (a) culturing in an appropriate medium at least one host cell as described above;

[0167] (b) concentrating the medium to provide a biomass,

[0168] (c) optionally providing additional feed components, and

[0169] (d) producing the feed composition from the biomass.

[0170] In certain embodiments, step (b) comprises centrifugation.

[0171] In certain embodiments, step (b) comprises allowing the biomass to settle.

[0172] In certain embodiments, step (b) comprises filtration.

[0173] In certain embodiments, the method further comprises a pre-treatment of the biomass after step (a) with a chemical agent to disrupt the cell membranes of the biomass.

[0174] In certain embodiments, the chemical agent is a surfactant or solvent.

[0175] In certain embodiments, the method further comprises mechanical disruption of the cell membranes of the biomass after step (a).

[0176] In this disclosure, a number of terms and abbreviations are used. The following definitions are provided.

[0177] Carotenogenic modification: The term "carotenogenic modification", as used herein, refers to a modification of a host organism that adjusts production of one or more carotenoids, as described herein. For example, a carotenogenic modification may increase the production level of one or more carotenoids, and/or may alter relative production levels of different carotenoids. In principle, an inventive carotenogenic modification may be any chemical, physiological, genetic, or other modification that appropriately alters production of one or more carotenoids in a host organism produced by that organism as compared with the level produced in an otherwise identical organism not subject to the same modification. In most embodiments, however, the carotenogenic modification will comprise a genetic modification, typically resulting in increased production of one or more selected carotenoids. In some embodiments, the selected carotenoid is one or more of astaxanthin, β-carotene, canthaxanthin, lutein, lycopene, phytoene, zeaxanthin, modified zeaxanthin or astaxanthin (e.g., glycoside, esterified zeaxanthin or astaxanthin), spirilloxanthin, and intermediates leading to spirilloxanthin such as lycopene and rhodopin. In certain embodiments, the carotenoid is one or more xanthophylls, and/or a modification thereof (e.g., glycoside, esterified xanthophylls). In certain embodiments, the xanthophyll is selected from the group consisting of astaxanthin, lutein, zeaxanthin, lycopene, spirilloxanthin, and intermediates leading to spirilloxanthin such as rhodopin, and modifications thereof. In certain embodiments, the carotenoid is one or more of astaxanthin, β-carotene, canthaxanthin, lutein, lycopene, and zeaxanthin and/or modifications of zeaxanthin or astaxanthin. In certain embodiments, the carotenoid is β-carotene. In certain embodiments, the selected carotenoid is astaxanthin. In some embodiments, the selected carotenoid is spirilloxanthin. In certain embodiments, the selected carotenoid is astaxanthin. In some embodiments, the selected carotenoid is one or more intermediates that are precursors of spirilloxanthin such as, for example, lycopene or rhodopin.

[0178] Carotenoid: The term "carotenoid" is understood in the art to refer to a structurally diverse class of pigments derived from isoprenoid pathway intermediates. The commitment step in carotenoid biosynthesis is the formation of phytoene from geranylgeranyl pyrophosphate. Carotenoids can be acyclic or cyclic, and may or may not contain oxygen, so that the term carotenoids include both carotenes and xanthophylls. In general, carotenoids are hydrocarbon compounds having a conjugated polyene carbon skeleton formally derived from the five-carbon compound IPP, including triterpenes (C_{30} diapocarotenoids) and tetraterpenes (C_{40} carotenoids) as well as their oxygenated derivatives and other compounds that are, for example, C_{35}, C_{50}, O_{60}, C_{70}, C_{80} in length or other lengths. Many carotenoids have strong light absorbing properties and may range in length in excess of C_{200}. C_{30} diapocarotenoids typically consist of six isoprenoid units joined in such a manner that the arrangement of iso-

prenoid units is reversed at the center of the molecule so that the two central methyl groups are in a 1,6-positional relationship and the remaining non-terminal methyl groups are in a 1,5-positional relationship. Such C_{30} carotenoids may be formally derived from the acyclic $C_{30}H_{42}$ structure, having a long central chain of conjugated double bonds, by: (i) hydrogenation (ii) dehydrogenation, (iii) cyclization, (iv) oxidation, (v) esterification/glycosylation, or any combination of these processes. C_{40} carotenoids typically consist of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule so that the two central methyl groups are in a 1,6-positional relationship and the remaining non-terminal methyl groups are in a 1,5-positional relationship. Such C_{40} carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure, having a long central chain of conjugated double bonds, by (i) hydrogenation, (ii) dehydrogenation, (iii) cyclization, (iv) oxidation, (v) esterification/glycosylation, or any combination of these processes. The class of C_{40} carotenoids also includes certain compounds that arise from rearrangements of the carbon skeleton, or by the (formal) removal of part of this structure. More than 600 different carotenoids have been identified in nature. Carotenoids include but are not limited to: antheraxanthin, adonirubin, adonixanthin, astaxanthin, canthaxanthin, capsorubrin, β-cryptoxanthin, α-carotene, β-carotene, β,ψ-carotene, δ-carotene, ϵ-carotene, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, γ-carotene, ψ-carotene, 4-keto-γ-carotene, ƒ-carotene, α-cryptoxanthin, deoxyflexixanthin, diatoxanthin, 7,8-didehydroastaxanthin, didehydrolycopene, fucoxanthin, fucoxanthinol, isorenieratene, β-isorenieratene, lactucaxanthin, lutein, lycopene, myxobactone, neoxanthin, neurosporene, hydroxyneurosporene, peridinin, phytoene, rhodopin, rhodopin glucoside, 4-keto-rubixanthin, siphonaxanthin, spheroidene, spheroidenone, spirilloxanthin, torulene, 4-keto-torulene, 3-hydroxy-4-keto-torulene, uriolide, uriolide acetate, violaxanthin, zeaxanthin-β-diglucoside, zeaxanthin, and C30 carotenoids. Additionally, carotenoid compounds include derivatives of these molecules, which may include hydroxy-, methoxy-, oxo-, epoxy-, carboxy-, or aldehydic functional groups. Further, included carotenoid compounds include ester (e.g., glycoside ester, fatty acid ester) and sulfate derivatives (e.g., esterified xanthophylls).

[0179] Isoprenoid pathway: The "isoprenoid pathway" is understood in the art to refer to a metabolic pathway that either produces or utilizes the five-carbon metabolite isopentyl pyrophosphate (IPP). As discussed herein, two different pathways can produce the common isoprenoid precursor IPP—the "mevalonate pathway" and the "non-mevalonate pathway". The term "isoprenoid pathway" is sufficiently general to encompass both of these types of pathway. Biosynthesis of isoprenoids from IPP occurs by polymerization of several five-carbon isoprene subunits. Isoprenoid metabolites derived from IPP vary greatly in chemical structure, including both cyclic and acyclic molecules. Isoprenoid metabolites include, but are not limited to, monoterpenes, sesquiterpenes, diterpenes, sterols, and polyprenols such as carotenoids.

[0180] Oleaginic modification: The term "oleaginic modification", as used herein, refers to a modification of a host organism that adjusts the desirable oleaginy of that host organism, as described herein. In some cases, the host organism will already be oleaginous in that it will have the ability to accumulate lipid to at least about 20% of its dry cell weight. It may nonetheless be desirable to apply an oleaginic modification to such an organism, in accordance with the present invention, for example to increase (or, in some cases, possibly to decrease) its total lipid accumulation, or to adjust the types or amounts of one or more particular lipids it accumulates (e.g., to increase relative accumulation of triacylglycerol). In other cases, the host organism may be non-oleaginous (though may contain some enzymatic and regulatory components used in other organisms to accumulate lipid), and may require oleaginic modification in order to become oleaginous in accordance with the present invention. The present invention also contemplates application of oleaginic modification to non-oleaginous host strains such that their oleaginicity is increased even though, even after being modified, they may not be oleaginous as defined herein. In principle, the oleaginic modification may be any chemical, physiological, genetic, or other modification that appropriately alters oleaginy of a host organism as compared with an otherwise identical organism not subjected to the oleaginic modification. In most embodiments, however, the oleaginic modification will comprise a genetic modification, typically resulting in increased production and/or activity of one or more oleaginic polypeptides. In certain embodiments, the oleaginic modification comprises at least one chemical, physiological, genetic, or other modification; in other embodiments, the oleaginic modification comprises more than one chemical, physiological, genetic, or other modification. In certain aspects where more than one modification is utilized, such modifications can comprise any combination of chemical, physiological, genetic, or other modification (e.g., one or more genetic modification and chemical or physiological modification).

[0181] The term "feed premix" refers to the crude mixture of aquaculture feed components prior to processing, optionally at high temperature, into an aquaculture feed composition that is in the form of pellets or flakes.

[0182] An aquaculture feed composition is used in the production of an "aquaculture product", wherein the product is a harvestable aquacultured species (e.g., finfish, crustaceans), which is often sold for human consumption. For example, salmon are intensively produced in aquaculture and thus are aquaculture products.

[0183] Aquaculture compositions may also be used as feed for aquaculture feed organisms such as small fish like krill, rotifers, and the like, that are food sources for larger aquaculture organisms such as carnivorous fish. In addition, aquaculture compositions described herein can be used as feed for ornamental fish, shrimp, hobbyist aquaculture, and the like, that are not intended as food for other organisms.

[0184] The term "aquaculture meat product" refers to food products intended for human consumption comprising at least a portion of meat from an aquaculture product as defined above. An aquaculture meat product may be, for example, a whole fish or a filet cut from a fish, each of which may be consumed as food. In some embodiments, such a product can be referred to as a fish or seafood product.

[0185] "Eicosapentaenoic acid" ("EPA") is the common name for cis-5,8,11,14,17-eicosapentaenoic acid. This fatty acid is a 20:5 omega-3 fatty acid. The term EPA as used in the present disclosure will refer to the acid or derivatives of the acid (e.g., glycerides, esters, phospholipids, amides, lactones, salts or the like) unless specifically mentioned otherwise.

[0186] "Docosahexaenoic acid" ("DHA") is the common name for cis-4,7,10,13,16,19-docosahexaenoic acid. It is a 22:6 omega-3 fatty acid. The term DHA as used in the present disclosure will refer to the acid or derivatives of the acid (e.g.,

glycerides, esters, phospholipids, amides, lactones, salts or the like) unless specifically mentioned otherwise.

[0187] As used herein the term "biomass" refers to microbial cellular material. Biomass may be produced naturally, or may be produced from the fermentation of a native host or a recombinant production host. The biomass may be in the form of whole cells, whole cell lysates, homogenized cells, partially hydrolyzed cellular material, and/or partially purified cellular material (e.g., microbially produced oil).

[0188] The term "processed biomass" refers to biomass that has been subjected to additional processing such as drying, pasteurization, disruption, etc., each of which is discussed in greater detail below.

[0189] The term "C-1 carbon substrate" refers to any carbon-containing molecule that lacks a carbon-carbon bond. Examples are methane, methanol, formaldehyde, formic acid, formate, methylated amines (e.g., mono-, di-, and trimethyl amine), methylated thiols, and carbon dioxide. The term "C1 metabolizer" refers to a microorganism that has the ability to use a single carbon substrate as a sole source of energy and biomass. C1 metabolizers will typically be methylotrophs and/or methanotrophs capable of growth.

[0190] The term "methylotroph" means an organism capable of oxidizing organic compounds which do not contain carbon-carbon bonds. Where the methylotroph is able to oxidize CH_4, the methylotroph is also a methanotroph.

[0191] The term "methanotroph" means a prokaryote capable of utilizing methane as a substrate. Complete oxidation of methane to carbon dioxide occurs by aerobic degradation pathways. Typical examples of methanotrophs useful in the present invention include but are not limited to the genera *Methylomonas, Methylobacter, Methylococcus*, and *Methylosinus*.

[0192] The term "high growth methanotrophic bacterial strain" refers to a bacterium capable of growth using methane as its sole carbon and energy source.

[0193] The term "isoprenoid compound" refers to any compound which is derived via the pathway beginning with isopentenyl pyrophosphate (IPP) and formed by the head-to-tail condensation of isoprene units which may be of 5, 10, 15, 20, 30 or 40 carbons in length. There term "isoprenoid pigment" refers to a class of isoprenoid compounds which typically have strong light absorbing properties.

[0194] The term "upper isoprene pathway" refers to any of the genes and gene products (including homologs and mutants thereof, whether naturally-occurring or genetically engineered) associated with the isoprenoid biosynthetic pathway including the dxs gene (encoding 1-deoxyxylulose-5-phosphate synthase), the dxr gene (encoding 1-deoxyxylulose-5-phosphate reductoisomerase), the "ispD" gene (encoding the 2C-methyl-D-erythritol cytidyltransferase enzyme; also known as ygbP), the "ispE" gene (encoding the 4-diphosphocytidyl-2-C-methylerythritol kinase; also known as ychB), the "ispF" gene (encoding a 2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase; also known as ygbB), the "pyrG" gene (encoding a CTP synthase); the "lytB" gene involved in the formation of dimethylallyl diphosphate; and the gcpE gene involved in the synthesis of 2-C-methyl-D-erythritol 4-phosphate in the isoprenoid pathway. The term "Dxs" refers to the 1-deoxyxylulose-5-phosphate synthase enzyme encoded by the dxs gene.

[0195] The term "Dxr" refers to the 1-deoxyxylulose-5-phosphate reductoisomerase enzyme encoded by the dxr gene.

[0196] The term "YgbP" or "IspD" refers to the 2C-methyl-D-erythritol cytidyltransferase enzyme encoded by the ygbP or ispD gene. The names of the gene, ygbP or ispD, are used interchangeably in this application. The names of gene product, YgbP or IspD are used interchangeably in this application.

[0197] The term "YchB" or "IspE" refers to the 4-diphosphocytidyl-2-C-methylerythritol kinase enzyme encoded by the ychB or ispE gene. The names of the gene, ychB or ispE, are used interchangeably in this application. The names of gene product, YchB or IspE are used interchangeably in this application.

[0198] The term "YgbB" or "IspF" refers to the 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase enzyme encoded by the ygbB or ispF gene. The names of the gene, ygbB or ispF, are used interchangeably in this application. The names of the gene product, YgbB or IspF, are used interchangeably in this application.

[0199] The term "PyrG" refers to a CTP synthase enzyme encoded by the pyrG gene.

[0200] The term "IspA" refers to Geranyltransferase or farnesyl diphosphate synthase enzyme as one of prenyl transferase family encoded by ispA gene. The term "LytB" refers to protein having a role in the formation of dimethylallyl-pyrophosphate in the isoprenoid pathway and which is encoded by lytB gene.

[0201] The term "GcpE" refers to a protein having a role in the formation of 2-C-methyl-D-erythritol 4-phosphate in the isoprenoid pathway (Altincicek et al., *J. Bacteriol.* (2001), 183(8), 2411-2416; Campos et al., *FEBS Lett.* (2001), 488(3), 170-173).

[0202] The term "lower carotenoid biosynthetic pathway" refers to any of the following genes and gene products (including homologs and mutants thereof, whether naturally-occurring or genetically engineered) associated with the isoprenoid biosynthetic pathway, which are involved in the immediate synthesis of phytoene (whose synthesis represents the first step unique to biosynthesis of carotenoids) or subsequent reactions. These genes and gene products include the "ispA" gene (encoding geranyltransferase or farnesyl diphosphate synthase), the "ctrN" and "ctrN1" genes (encoding diapophytoene dehydrogenases), the "crtE" gene (encoding geranylgeranyl pyrophosphate synthase), the "crtX" gene (encoding zeaxanthin glucosyl transferase), the "crtY" gene (encoding lycopene cyclase), the "crtI" gene (encoding phytoene desaturase), the "crtB" gene (encoding phytoene synthase), the "crtZ" gene (encoding β-carotene hydroxylase), and the "crtO" gene (encoding a β-carotene ketolase). Additionally, the term "carotenoid biosynthetic enzyme" is an inclusive term referring to any and all of the enzymes in the present pathway including CrtE, CrtX, CrtY, CrtI, CrtB, CrtZ, and CrtO.

[0203] The term "IspA" refers to the protein encoded by the ispA gene, and whose activity catalyzes a sequence of 3 prenyltransferase reactions in which geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) are formed.

[0204] The term "CrtN1" or "CrtN, copyl" refers to copy 1 of the diapophytoene dehydrogenase enzyme encoded by crtN1 gene. The term "CrtN2" or "CrtN copy2" refers to copy 2 of the diapophytoene dehydrogenase enzyme (Crt) encoded by crtN2 gene.

[0205] The term "CrtE" refers to geranylgeranyl pyrophosphate synthase enzyme encoded by crtE gene which converts

trans-trans-farnesyl diphosphate and isopentenyl diphosphate into pyrophosphate and geranylgeranyl diphosphate.

[0206] The term "CrtX" refers to the zeaxanthin glucosyl transferase enzyme encoded by the crtX gene, and which glycosolates zeaxanthin to produce zeaxanthin-β-diglucoside. The term "CrtY" refers to the lycopene cyclase enzyme encoded by the ctfYgene and which catalyzes conversion of lycopene to β-carotene.

[0207] The term "Crtl" refers to the phytoene desaturase enzyme encoded by the crtl gene and which converts phytoene into lycopene via the intermediaries of phytofluene, zeta-carotene, and neurosporene by the introduction of four double bonds.

[0208] The term "CrtB" refers to the phytoene synthase enzyme encoded by the crtB gene which catalyzes the reaction from prephytoene diphosphate to phytoene. The term "CrtZ" refers to the β-carotene hydroxylase enzyme encoded by crtZgene which catalyzes the hydroxylation reaction from β-carotene to zeaxanthin.

[0209] The term "CrtO" refers to the β-carotene ketolase enzyme encoded by crtO gene which catalyzes conversion of β-carotene into canthaxanthin (two ketone groups) via echinenone (one ketone group) as the intermediate.

[0210] The term "HpnD" refers to putative dehydrosqualene synthase, which is thought to combine a dehydrated and a standard farnesyl-PP group to generate the C_{30} molecule dehydrosqualene and is encoded by the gene hpnD.

[0211] The term "HpnE" refers to putative dehydrosqualene reductase, which is thought to reduce dehydrosqualene to generate the C_{30} molecule dehydrosqualene and is encoded by the gene hpnE.

[0212] The term "HpnC" refers to squalene synthase, which combines two farnesyl-PP groups to generate the C_{30} molecule squalene and is encoded by the gene hpnC.

[0213] The term "SHC" refers to squalene-hopene cyclase that converts the linear squalene molecule into the pentacyclic molecule hopene and is encoded by the gene shc (also known as hpnF). In some embodiments, the modified bacteria of the invention contains a knockout of shc, e.g., as *M. extorquens* having a shc knockout which results in elevated levels of carotenoid production (see, e.g., Example 7).

[0214] The term "carotenoid compound" is defined as a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) consisting of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule so that the two central methyl groups are in a 1,6-positional relationship and the remaining nonterminal methyl groups are in a 1,5-positional relationship. All carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure (Formula I below), having a long central chain of conjugated double bonds, by (i) hydrogenation. (ii) dehydrogenation, (iii) cyclization, or (iv) oxidation, or any combination of these processes.

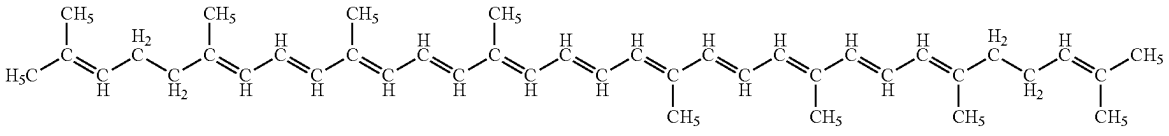
[0215] The present invention provides for the expression of genes involved in the biosynthesis of carotenoid compounds in microorganisms which are able to use single carbon substrates as a sole energy source. Such microorganisms are referred to herein as C1 metabolizers. The host microorganism may be any C1 metabolizer which has the ability to synthesize isopentenyl pyrophosphate (IPP) the precursor for many of the carotenoids. Many C1 metabolizing microorganisms are known in the art which are able to use a variety of single carbon substrates. Single carbon substrates useful in the present invention include but are not limited to methane, methanol, formaldehyde, formic acid, methylated amines (e.g., mono-, di- and tri-methyl amine), methylated thiols, and carbon dioxide. All C1 metabolizing microorganisms are generally classified as methylotrophs. Methylotrophs may be defined as any organism capable of oxidizing organic compounds which do not contain carbon-carbon bonds. A subset of methylotrophs is the methanotrophs, which have the distinctive ability to oxidize methane. Facultative methylotrophs have the ability to oxidize organic compounds which do not contain carbon-carbon bonds, but may also use other carbon substrates such as sugars and complex carbohydrates for energy and biomass. Obligate methylotrophs are those organisms which are limited to the use of organic compounds which do not contain carbon-carbon bonds for the generation of energy and obligate methanotrophs are those obligate methylotrophs that have the ability to oxidize methane.

[0216] Facultative methylotrophic bacteria are found in many environments, but are isolated most commonly from soil, landfill and waste treatment sites. Many facultative methylotrophs are members of the α, β, and γ subgroups of proteobacteria (Hanson et al., Microb. Growth 01 Compounds., [Int. Symp.], 7th (1993), 285-302. Editor(s): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK; Madigan et al., Brock Biology of Microorganisms, 8th edition, Prentice Hall, UpperSaddle River, N.J. (1997)). Facultative methylotrophic bacteria suitable in the present invention include but are not limited to, *Methylophilus, Methylobacillus, Methylobacterium, Hyphomicrobium, Xanthobacter, Bacillus, Paracoccus, Nocardia, Arthrobacter, Rhodopseudomonas*, and *Pseudomonas*. Preferred obligate methanotrophs are included in, but not limited to, the genera *Methylobacterium, Methylomonas, Methylobacter, Methylococcus, Methylosinus, Methylocyctis, Methylomicrobium*, and *Methanomonas*.

[0217] The ability to utilize single carbon substrates is not limited to bacteria but extends also to yeasts and fungi. A number of yeast genera are able to use single carbon substrates in addition to more complex materials as energy sources. Specific methylotrophic yeasts useful in the present invention include but are not limited to *Candida, Hansenula, Pichia, Torulopsis*, and *Rhodotorula*.

Formula I

(I)



[0218] Of particular interest in the present invention are high growth facultative methylotrophs having an energetically favorable carbon flux pathway. For example, the Applicants have discovered a specific strain of methylotroph having several pathway features which make it particularly useful for carbon flux manipulation and the production of carotenoids and additional nutrients. This type of strain has served as the host in the present application and is an α-proteobacterium known as *Methylobacterium extorquens*.

[0219] The C1 metabolizing microorganisms of the present invention are ubiquitous and many have been isolated and characterized. A general scheme for isolation of these strains includes addition of an inoculum into a sealed liquid mineral salts media, containing either methane or methanol. Care must be made of the volume:gas ratio and cultures are typically incubated between 25-55° C. Typically, a variety of different methylotrophic bacteria can be isolated from a first enrichment, if it is plated or streaked onto solid media when growth is first visible. Methods for the isolation of methanotrophs are common and well known in the art (see for example Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, Mass.; Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36: 227 (1992); or Hanson, R. S. et al. The Prokaryotes: a handbook on habitats, isolation, and identification of bacteria; Springer-Verlag: Berlin, New York, 1981; Volume 2, Chapter 118).

[0220] It is expected that the present teaching will enable the general identification and isolation of organisms exhibiting desired characteristics. One aspect of a C1 metabolizer is that it incorporates an active Embden-Meyerhof pathway as indicated by the presence of a pyrophosphate dependent phosphofructokinase. Another key characteristic of the present high growth strain is that it is a facultative methylotroph, able to use methanol (or other C1 substrates) as a sole carbon source; of course, for optimal growth, other carbon-containing nutrients may be included, or other C1 nutrients supplemented in addition to the methanol. Methods for the isolation of methanotrophs are common and well known in the art. Similarly, pyrophosphate dependent phosphofructokinase has been well characterized in mammalian systems and assay methods have been well developed (see for example Schliselfeld et al. *Clin. Biochem.* (1996), 29(1), 79-83; Clark et al., *J. Mol. Cell. Cardiol.* (1980), 12(10), 1053-64. The contemporary microbiologist will be able to use these techniques to identify the present high growth strain.

EXEMPLIFICATION

[0221] The invention having been described, it will be further understood by reference to the following non-limiting examples.

Example 1

Directed Evolution of Methylotrophic Bacteria

[0222] Directed evolution is capable of yielding enhancement of a desired trait, such as selection for highly pigmented organisms. The technique is adapted here for the selection/evolution of *M. extorquens* overproducing astaxanthin and a number of essential amino acids. According to the present invention, one route to carotenoid production is to simply evolve cultures under the desired industrial conditions in order to improve growth rates and/or survival under the relevant environmental parameters. As this is proceeding, the visible nature of carotenoids can be used as a screen for lineages that are either losing coloration while they adapted, or those that have fortuitously become more highly pigmented. An example selection regime would be serial transfers in minimal medium containing just methanol. Upon plating cultures occasional isolates may be noted for having a "dark pink" or "reddish" colony morphology. This approach has yielded various strains with increased or altered pigmentation, as noted in Table 1 of Lee et al. (2009. Evolution. 63:2816-2830). Upon genome resequencing, the basis of the pigmentation can be revealed and combined with other developments below. Application of selection/evolution will also lead to increased methanol tolerance by culturing under ever higher concentrations beyond the ~1% tolerated now, to 5%, 10%, or higher. Experimental evolution may be carried out by serial transfers performed every 48 hours (within 1 hour) by transferring 150 μL into 9.45 mL of fresh media (a 1/64 dilution, thus permitting six generations of growth before reaching stationary phase). This provides a population size at the end of each cycle of ~2×10^9 (9.6 mL). Populations can be maintained at 30° C. in 50 mL flasks with 225 rpm shaking. At regular intervals following the transfer of 1/64 of the population to fresh media, an appropriate dilution of the remaining culture can be plated to test for contamination, and then 750 μL of DMSO was added to the remaining liquid (~8% v/v DMSO final concentration) and duplicate vials of this mixture were preserved at −80° C. It is at the time of plating that colonies may be examined for variants with differing pigmentation. FIG. 11 in U.S. Ser. No. 61/863,701 is an image of "matchsticks" showing various levels of carotenoids in *Methylobacterium extorquens* strains: compared to the control (1), the next three (2-4) show evolved isolates, and (6) shows a hopanoid-deficient strain compared to its progenitor (5).

[0223] Directed evolution can also be utilized to select for increased production of diffusible molecules such as amino acids.

Example 2

Directed Genetic Engineering of Methylotrophic Bacteria Using Recyclable Antibiotic Marker System

[0224] By combining a "feeder" strain of *E. coli* that requires a given nutrient (such as methionine, or other amino acids) with the methylotroph utilized (such as *M. extorquens*) it is possible to select for strains whose amino acid production feed their partner and allow growth of the consortia. In order to correlate production with growth advantage of that new genotype it is essential to perform these experiments in a spatially structured manner, such as on agar or agarose-containing petri dishes that contain a food source only utilizable by *E. coli* (such as glucose or lactose) but omit the addition of the nutrient that that *E. coli* mutant requires. Selection conditions involve plating the two strains together for an extended period of time (multiple days or weeks), then washing the combined cell material, vortexing, and re-plating a dilution onto fresh medium as before. In some instances it may be beneficial to combine this approach with the addition of a toxic analogue that will create direct selection for increased production to overcome the inhibitory effects of the toxic analogue.

[0225] Directed genetic engineering can be used as a strategy to increase production of carotenoids, or other desirable

molecules. Two major approaches are envisioned. First, pathways which withdraw carbon to alternative products such as hopanoids, spirilloxanthin, and the addition of geranylgeranyl groups to bacteriochlorophyll. Allelic replacement constructs can be generated which contain the upstream and downstream flanks of the genes to be deleted in an allelic exchange vector such as pCM433 (Marx, C. J. *BMC Research Notes* (2008) 1:1). Through use of triparental matings such a construct can be introduced by selecting for tetracycline resistance, and then resolved by selecting for sucrose resistance (and counter-screening for tetracycline sensitivity; potential positives confirmed by PCR or sequencing). The next gene(s) to be removed can then occur in that background. The major targets—all described above—are: 1.) the genes that withdraw farnesyl diphosphate to generate hopanoids (collectively encoded by the hpnCDEF locus), 2.) those that withdraw lycopene to make spirilloxanthin (encoded by crtD, crtE and crtF), and 3.) the genes involved in decorating bacteriochlorophyll with a geranylgeranyl group (encoded by bchG and bchP). These removals can occur alone or together, and may be combined with other alterations.

[0226] One technique to be employed will utilize recyclable antibiotic marker systems such as the cre-lox system. This will include use of the pCM157, pCM158, pCM184, pCM351 series of plasmids developed for use in *M. extorquens* (Marx, C. J. and M. E. Lidstrom *BioTechniques* (2002) 33: 1062-1067). See FIG. 4 in U.S. Ser. No. 61/863,701, which shows a rationale for cre-lox marker recycling in *Methylobacterium* and other methylotrophs. The strategy for cre-lox recycling of antibiotic markers in *Methylobacterium* and other bacteria is illustrated in FIG. 3 of Marx and Lidstrom, 2002. BioTechniques (33:1062-1067). Cre recombinase is a site-specific recombinase from the P1 phage that catalyzes in vivo excision of DNA regions flanked by co-directional loxP recognition sites. The system used here consists of a mobilizable allelic exchange vector with a loxP-flanked antibiotic resistance cassette, pCM184 or pCM351, and an IncP plasmid that expresses the Cre recombinase, pCM157 or pCM158. We demonstrate the broad utility of this system by generating unmarked mutant strains of two phylogenetically distinct Gram-negative bacteria, *Methylobacterium extorquens* AM1 (an α-proteobacterium), and *Burkholderia fungorum* LB400 (a β-proteobacterium).

[0227] Materials and Methods

[0228] Media and Growth Conditions

[0229] *M. extorquens* AM1 strains were grown on a minimal salts medium containing carbon sources at the following levels, 0.2% citrate, 0.5% (v/v) methanol, 0.25% (wt/v) methylamine, and 0.4% (wt/v) succinate. *Escherichia coli* strains were grown on LB medium. Antibiotics were added at the following final concentrations, unless noted: 50 μg/mL ampicillin, 10 μg/mL chloramphenicol, 50 μg/mL (for *E. coli* and *M. extorquens* AM1) or 20 μg/mL (for *B. fungorum* LB400) kanamycin, 50 μg/mL rifamycin, 35 μg/mL streptomycin, and 10 μg/mL tetracycline. Chemicals were obtained from Sigma. Nutrient agar and Bacto-agar were obtained from Difco. Conjugation was performed using standard techniques.

[0230] Construction of a broad-host-range cre-lox system for antibiotic marker recycling

[0231] Two allelic exchange vectors, pCM184 and pCM351 (FIG. 5 in U.S. Ser. No. 61/863,701, which shows plasmids useful for cre-lox marker recycling in *Methylobacterium* and other methylotrophs. The plasmids used to enable cre-lox recycling of antibiotic markers in *Methylobacterium* and other bacteria are illustrated in FIGS. 1 and 2 of Marx and Lidstrom, 2002. BioTechniques (33:1062-1067).), were created by inserting a loxP-bounded antibiotic resistance cassettes into a variant of the mobilizable suicide plasmid, pAYC61. The 1.3 kb HindIII fragment bearing the kanamycin resistance cassette from pUC4K was inserted into pLox1 which had been cut with XbaI and blunted, to create pCM161. In order to introduce convenient multiple cloning sites, the loxP-bounded kanamycin cassette of pCM161 was amplified with following primer pair, CM-ufkMCS, 5'-TGACGTCTA-GATCTGAATTCAGCTGTACAATTGGTAC-CATGGATGCATATGGC GGCCGCA-3' (SEQ ID NO:1), and CM-dfkMCS, 5'-GACTAGTGAGCTCACCGGTTAA-CACGCGTACGTAGGGCCCGCGGTATCGATA AGCTG-GATCC-3' (SEQ ID NO:2). The resulting 1.4 kb PCR product was purified and cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.) to create pCM183. In order to preserve useful cloning sites, pAYC61 was cut with EcoRI and SmaI, blunted using T4 DNA polymerase, and self-ligated to produce pCM182. Finally, the 1.4 kb AatII-SpeI fragment from pCM183 containing the loxP-flanked kanamycin cassette was ligated between the AatII and XbaI sites of pCM182 to create pCM184 (GenBank accession number AY093429). A gentamycin-resistance conferring version, pCM351, was also generated. The loxP-flanked gentamycin-resistance cassette (encoded by aaaC1) was amplified from pLoxGen4 using CM-ufkMCS and CM-dfkMCS and cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.) to produce pCM350. The 1.0 kb AatII/SacI fragment from pCM350 was cloned between the AatII and SacI sites of pCM184 to generate pCM351 (GenBank accession number AY093430).

[0232] Two broad-host-range cre expression vectors, pCM157 and pCM158 (FIG. 5 in U.S. Ser. No. 61/863,701), were created based upon a pair of small, mobilizable IncP plasmids. The 1.1 kb XbaI-EcoRI fragment from pJW168 was cloned between the XbaI and EcoRI sites of pCM62 to generate the tetracycline-resistance conferring cre expression plasmid pCM157. A kanamycin-resistant version, pCM158, was generated by cloning the same XbaI-EcoRI fragment from pJW168 between the XbaI and EcoRI sites of pCM66. These plasmids contain cre behind the *E. coli* lac promoter. In *M. extorquens* AM1, this promoter provides only low constitutive activity. Despite this low expression, the majority of cells obtained from the first passage onto plates lacking kanamycin are already kanamycin sensitive (data not shown).

[0233] Generation of a Afae mutant of *M. extorquens* AM1

[0234] *M. extorquens* AM1 mutants defective for fae (encodes formaldehyde-activating enzyme) were generated using pCM184. The regions immediately flanking fae were amplified by PCR using the following primer pairs: CM-Dfae-1,5"-CGGGTTTCGTGACCTGTTC-3" (SEQ ID NO:3), and CM-Dfae-2,5"-GTTATGCGGCCGCCATCTG-CATGGAAGCCATCCTTGTTTGC-3" (SEQ ID NO:4); and CM-Dfae-3,5"-GCTTATCGATACCGTCGACCTCGAG-GCAGTCCTGGGCAGA-3" (SEQ ID NO:5), and CM-Dfae-4,5"-CGGGCATCGAGCGTTTCAC-3" (SEQ ID NO:6). The purified PCR products for fae-upstream and fae-downstream were cloned into pCR2.1 to produce pCM195, and pCM196, respectively. The 0.6 kb EcoRI-NotI fragment from pCM195 was introduced between the EcoRI and NotI sites of pCM184 to produce pCM197. Subsequently, the 0.6 kb ApaI-SacI fragment from pCM196 was ligated between the ApaI and SacI sites of pCM197 to produce pCM198.

[0235] A Δfae::kan mutant of *M. extorquens* AM1 was generated by introducing pCM198 by conjugation from *E. coli* S17-1. Kanamycin-resistant transconjugants obtained on succinate medium containing rifamycin were screened for tetracycline sensitivity to identify potential null mutants. To date, we have generated over thirty different null mutant strains utilizing this system, and the frequency of double-crossover events has varied from 5% to 80% (C. J. Marx and M. E. Lidstrom, unpublished data). One such Δfae::kan mutant, CM198K.1, was chosen for further study. The plasmid pCM157 was introduced by conjugation into CM198K.1 using the helper plasmid pRK2073. Tetracycline-resistant strains were streaked for purity until the resulting strain produced only kanamycin-sensitive colonies (generally only two transfers). Subsequently, pCM157 was cured from the strain by two successive transfers on medium lacking tetracycline to produce the Δfae strain CM198.1. Analytical PCR was performed with wild-type *M. extorquens* AM1, CM198K.1, and CM198.1 for confirmation of allelic exchange, and subsequent deletion of the kanamycin cassette (data not shown). Where examined, the sequence of the analytical PCR product indicated faithful recombination between the loxP sites (data not shown).

[0236] Generation of a ΔflhA mutant of *B. fungorum* LB400

[0237] *B. fungorum* LB400 mutants defective for flhA (predicted to encode a NAD- and glutathione-dependent formaldehyde dehydrogenase) were generated using pCM184, as described above with *M. extorquens* AM1. The regions flanking flhA were amplified by PCR using the following primer pairs: CM-BfflhAuf, 5-GGTGACGGCATTGAAGCTG-3 (SEQ ID NO:7), and CM-BfflhAur, 5-CATGCATCTTTG-GTCTTCATCGTGAATG-3 (SEQ ID NO:8); and CM-BfflhAdf, 5-ACCGCGGTCGTGCTGTACTAATCC-3 (SEQ ID NO:9), and CM-BfflhAur, 5-AGAGCTCGATACCGAC-CGATAGATCTC-3 (SEQ ID NO:10). The flhA upstream and downstream PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.) to produce pCM360 and pCM361, respectively. The 0.6 kb SacII-SacI downstream fragment from pCM361 was introduced between the SacII and SacI sites of pCM184 to produce pCM362. Subsequently, the 0.5 kb EcoRI-NsiI upstream fragment from pCM360 was ligated between the EcoRI and NsiI sites of pCM362 to produce pCM363.

[0238] A ΔflhA::kan mutant of *B. fungorum* LB400 was generated by introducing pCM363 by conjugation. Kanamycin-resistant transconjugants were obtained on citrate medium containing chloramphenicol (wild-type *B. fungorum* LB400 was found to be naturally resistant below 10-20 μg/mL). One tetracycline-sensitive strain representing a ΔflhA::kan mutant, CM363K.1, was chosen for further study. The plasmid pCM157 was used as described above to produce the ΔflhA strain CM363.1. Analytical PCR was performed with wild-type *B. fungorum* LB400, CM363K.1, and CM363.1 for confirmation (data not shown).

[0239] The minimal inhibitory concentration (MIC) of formaldehyde was determined by comparing the rate and extent of colony formation of wild-type *B. fungorum* LB400 to that of the flhA mutants CM363K.1 and CM363.1 on solid medium containing succinate as a growth substrate with various concentrations of formaldehyde. Formaldehyde was added to the plates immediately prior to the addition of the molten agar. Because an undetermined fraction of the formaldehyde will volatilize, the reported MIC of formaldehyde is a maximum value.

[0240] Results and Discussion

[0241] In order to test the broad-host-range cre-lox antibiotic marker recycling system, unmarked mutants were generated in *M. extorquens* AM1 (an α-proteobacterium) and *B. fungorum* LB400 (a β-proteobacterium). Analytical PCR confirmed replacement of each deleted gene with kan, and the subsequent excision of kan to produce the unmarked deletion (data not shown). The Mae mutant of *M. extorquens* AM1 grew like wild-type on succinate, but failed to grow on methanol or medium containing succinate and methanol. This mutant phenotype is in agreement with previous observations with a fae::kan mutant. The CM198.1 Mae strain can serve as a convenient host for structure-function studies that require expression of variant Fae proteins.

[0242] As a second demonstration of this broad-host-range antibiotic marker recycling system, a ΔflhA mutant of *B. fungorum* LB400 was generated. In other bacteria, the flhA gene encodes a glutathione-dependent formaldehyde dehydrogenase. This enzyme is involved in formaldehyde detoxification in *E. coli* and *Paracoccus denitrificans*, and is required for methylotrophic growth by the latter. The ΔflhA strain CM363.1 was found to be somewhat more sensitive to the presence of formaldehyde during growth on citrate than wild-type *B. fungorum* LB400, with a MIC of 0.1 mM compared to 0.2 mM for the wild-type. This finding demonstrates that the glutathione-dependent pathway is involved in formaldehyde detoxification across multiple branches of the proteobacteria.

[0243] In conclusion, this new broad-host-range cre-lox antibiotic marker recycling system offers the possibility to create unmarked mutants in a wide variety of Gram-negative bacteria. Utilization of allelic exchange with counter-selection against integrants, and an inherently unstable minimal IncP Cre expression plasmid, obviates the need for successful negative selection in the target organism, a feature of some previously developed marker recycling systems. Use of PCR to generate flanks for gene replacement allows for the facile generation of precise deletion mutants, as well as truncations through the introduction of start or stop codons in the primers, as needed. Variants of this system can be readily developed to allow the construction of chromosomal transcriptional or translational fusions (T. Strovas, C. J. Marx, and M. E. Lidstrom, unpublished data). Marker recycling systems such as ours described here offer a substantial advantage over standard allelic exchange methods due to the fact that it can be used iteratively to enable generation of unmarked strains bearing multiple genetic modifications. Our laboratory has already utilized this system to generate a *M. extorquens* AM1 strain bearing four separate mutations (C. J. Marx, L. Chistoserdova, and M. E. Lidstrom, unpublished data). Finally, engineered strains generated with these tools are more acceptable for environmental release owing to the absence of introduced antibiotic resistance markers.

Example 3

Directed Genetic Engineering of Methylotrophic Bacteria Using Alleic Exchange Vectors

[0244] Another option for multiple genetic manipulations, which also avoids leaving behind undesired scars, is to use an "in-out" system (FIG. 7 in U.S. Ser. No. 61/863,701 shows

rationale and plasmid useful for clean allelic exchange in *Methylobacterium* and other methylotrophs via sucrose counter selection. The strategy and plasmid used to enable antibiotic-free allelic exchange in *Methylobacterium* and other bacteria are illustrated in FIG. 1 of Marx, 2008. BMC Research Notes (1:1)). The basic idea behind these techniques is to first employ positive selection to select for single crossover integration of the entire donor vector due to recombination between a cloned region spanning the desired mutation in the vector and the corresponding chromosomal site. In the second step, negative selection is used to select for isolates that have recombined out the vector sequence. If the second recombination event excising the vector occurs on the same side of the introduced mutation as the first recombination event that introduced it onto the chromosome, the original chromosomal locus will be restored unchanged. If the second recombination event occurs on the opposite side of the introduced mutation, however, this results in excision of the original allele and the new mutation remains. As such, negative selection results in colonies with both resulting final states, as well as some percentage of false-positives that are resistant but have not excised the vector. As long as the false positives do not dominate, and the recombination rates to each side of the introduced mutation are reasonably balanced, screening of a modest collection of resulting recombinants will generate the desired unmarked mutation.

[0245] An "in-out" allelic exchange vector for generating unmarked mutations therefore must be able to be introduced into the recipient organism, be incapable of vegetative replication, and bear appropriate markers for positive and negative selection. Positive selection is generally accomplished using any number of antibiotic resistance genes, whereas comparably fewer options for negative selection generally exist. The most commonly used techniques are to use streptomycin (Sm) sensitivity, which comes as a pleiotropic effect of expressing the tetracycline (Tc) efflux pump, or sucrose-sensitivity that results from expression of levansucrase, encoded by sacB. Levansucrase activity is lethal in the presence of sucrose for most gram-negative bacteria. This paper presents a facile, broad-host-range "in-out" system based on sacB and has been specifically designed to allow facile unmarked allelic exchange in a wide variety of bacterial taxa. In order to test this system, allelic exchange has been performed at three different loci in *M. extorquens* AM1.

[0246] Results and Discussion

[0247] Construction of the "in-out" allelic exchange vector pCM433

[0248] In order to generate a facile system for marker-free allelic exchange across a wide variety of bacterial species, the loxP-flanked kanamycin (Km) resistance cassette of the broad-host-range marker-recycling vector, pCM184 was first excised and replaced with a synthetic linker that introduced three new restriction sites to the extensive multiple-cloning sites. Subsequently, a fragment from pDS132 bearing sacB and cat (encoding levansucrase and chloramphenicol (Cm) acetyltransferase, respectively) was introduced, generating pCM433 (FIG. 7 in U.S. Ser. No. 61/863,701)). It may be noted that initial attempts to were made to take advantage of the potential negative selection (Sm sensitivity) afforded by expression of the Tc efflux pump present on pCM184. Sm sensitivity was found to be enhanced in tet bearing cells, but the sensitivity was too modest to be utilized effectively for negative selection (Marx, unpublished results).

[0249] Allelic exchange at three loci in *M. extorquens* AM1

[0250] Three loci of interest in *M. extorquens* AM1 were chosen to test the utility of pCM433 for allelic exchange. These loci were hprA (encodes hydroxypyruvate reductase, a key enzyme of the serine cycle for assimilation of formaldehyde into biomass), mptG (encodes β-ribofuranosylaminobenzene 5'-phosphate synthase, the first dedicated enzyme for the synthesis of tetrahydromethanopterin, the C_1-carrier molecule used for this organism's formaldehyde oxidation pathway), and crtI (encodes phytoene desaturase, a necessary enzyme for carotenoid biosynthesis).

[0251] In all cases, constructs were made to convert the allele from wild-type (wt) to mutant, and the reciprocal reversion of mutant to wt. To accomplish this, both the ancestral, wt allele and the deletion (ΔhprA, ΔmptG) or insertion ($crtI^{502}$, generated by insertion of ISphoA/hah-Tc into crtI, followed by Cre-mediated excision of all but 132 bp of the IS) alleles were amplified by PCR, cloned into pCR2.1, sequenced, and then introduced into pCM433. Each of these donor plasmids were then introduced into the appropriate target strain via triparental conjugations and plated onto Tc plates (also containing Rif for counter-selection against *E. coli*). Tc^R transconjugants were obtained at a frequency of 10^{-6} to 10^{-7}. In some cases, even these single-crossover recombinants that contained both the wild-type and mutant alleles exhibited a phenotype. For example, the pool of single-crossover intermediates from either pCM441 (wt crtI allele) inserted into the white CM502 strain or pCM440 (defective $crtI^{502}$ allele) inserted into the pink CM501 strain each contained Tc^R colonies of both colors. As such, one pink and one white isolate from the conjugation into each background were isolated (CM1263 (white) and CM1264 (pink) from CM502, and CM1265 (pink) and CM1266 (white) from CM501). A polar effect of pCM433 insertion into this site was clearly observed. Irrespective of whether the wt allele was being introduced into the mutant, or vise versa, strains with the wild-type allele upstream, proximal to the gene's promoter (as determined by PCR analysis for strains CM1264 and CM1265), were pink, carotenoid-containing colonies, whereas the other strains (CM1263 and CM1266) had the $crtI^{502}$ allele upstream of pCM433 and were white.

[0252] In order to select for recombinants that have excised the vector, suspensions of Tc^R isolates were diluted and plated onto plates containing various levels of sucrose (2.5, 5, and 10% w/v). At all sucrose levels sucrose-resistant colonies were obtained at a frequency of 10^{-4} to 10^{-5}. These colonies were then screened for Tc sensitivity (indicating the expected loss of the pCM433-based construct), as well as the expected mutant phenotype (inability to grow on methanol for ΔhprA and ΔmptG, white colonies (versus pink) for $crtI^{502}$). These were confirmed via PCR analysis using primers situated outside the region of the locus where recombination occurred. In the cases presented here, differences in the size of amplified products sufficed to distinguish the alleles used, but primers designed to distinguish single-nucleotide substitutions (or sequencing) have been used in subsequent studies (Chou and Marx, unpublished). Overall, a false positive rate of $sucrose^R$, Tc^R strains generated here in *M. extorquens* AM1 was 26% (105/402). It should be noted, however, that the range of frequencies varied from 0% to 78% for different construct/recipient pairs. This is likely related to the rate of recombination for the flanking regions of each locus as compared to the rate of generating sucrose-resistance from other mechanisms. For all three loci, wild-type alleles were replaced by mutant alleles, and vise versa. In subsequent work, dozens of

allelic exchanges including the introduction of single-nucleotide substitutions have been successfully performed utilizing this system (Chou and Marx, unpublished).

[0253] The broad-host-range vector for marker-free allelic exchange described here has several features that greatly facilitate its use in various systems. First, unlike a number of similar vectors, such as pDS132 from which much some of the construct derives, pCM433 relies upon a pUC-derived ColE1 replicon, such that it can be maintained and easily purified in high quantities (5-10 μg DNA from 1.5 mL liquid culture) in any desired *E. coli* strain. Second, pCM433 contains a polylinker containing a substantially larger number of restriction sites than comparable tools we are aware of, facilitating the introduction of cloned DNA fragments. Third, the presence of three antibiotic markers on pCM433 permits use in a wide range of organisms in which they are applicable. Finally, pCM433 maintains features typically found in other broad-host-range systems such as the presence of an IncP oriT that allows conjugation to be utilized for delivery into the recipient strain.

[0254] Materials and Methods

[0255] Media, Growth Conditions, and Genetic Techniques

[0256] *M. extorquens* AM1 strains were grown at 30° C. on agar plates with "Hypho" minimal salts medium; *E. coli* were grown at 37° C. on Luria-Bertani agar. Substrates and antibiotics were used at the following concentrations: methanol (125 mM), succinate (15 mM), sucrose (5% w/v unless otherwise stated), 50 μg/mL Ap (ampicillin), 20 μg/mL Cm, 50 μg/mL Km, 50 μg/mL Rif (rifamycin), 35 μg/mL Sm, and 10 μg/mL Tc.

[0257] Tri-parental conjugations were performed by mixing the *E. coli* strain with the donor plasmid, the *M. extorquens* AM1 recipient strain, and an *E. coli* strain with the helper plasmid pRK2073. This mixture was grown overnight on permissive Nutrient agar plates at 30° C. before introducing some of mix (either by streaking with a loop or by washing with Hypho and re-plating) onto selective medium containing an appropriate C source, Rif for counter-selection against *E. coli*, and the selective antibiotic (Tc for pCM433-based donors; neither Ap nor Cm works effectively in *M. extorquens* AM1, Marx, unpublished). Sucrose selection was accomplished by suspending a loop of a given strain in 100 μl Hypho (approximately 10^9 mL^{-1}) and plating 50 μl of a 10^{-2} dilution of this suspension onto Hypho plates containing an appropriate C source (generally succinate) and 5% sucrose. Resulting strains were tested for Tc sensitivity, additional expected phenotypes (depending on the locus and allele being exchanged), and additionally, the chromosomal organization of all strains constructed was confirmed through PCR analysis. DNA concentrations were determined using a ND-1000 spectrophotometer (NanoDrop).

[0258] Construction of Plasmids and Generation of Strains

[0259] In order to generate the allelic exchange vector pCM433, the Km resistance cassette of pCM184 was excised with NdeI and SacII, and the remaining 5.4 kb vector backbone was ligated together with a synthetic linker designed to introduce three additional, unique cloning sites into the final vector (PstI, XhoI, and NotI). The linker was formed by boiling, and then slowly re-annealing at room temperature, a mixture of two oligos, CM-link1f (tatgctgcagctcgagcggccgc (SEQ ID NO:47) and CM-link1r (ggccgctcgagctgcagca (SEQ ID NO:48)), which were designed to have complementary overhangs to NdeI and SacII. The resulting plasmid, pCM432, was then transformed into the dam dcm *E. coli* strain, C2925H (ara-14 leuB6 fhuA31 lacY1 tsx78 gInV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD 1 R(zgb210::Tn10) Tc^s endA 1 rspL 136 (Sm^R) dam 13::Tn9 (Cm^R) xyIA-5 mtl-1 thi-1 mcrB1 hsdR2, New England Biolabs), enabling digestion at an otherwise methylated, and therefore blocked, MscI site. The 2.7 kb XbaI-XmaI fragment of pDS132 containing sacB and cat was then purified, blunted with Klenow enzyme, and ligated with the MscI-digested pCM432 vector to generate pCM433 (see FIG. 7 in U.S. Ser. No. 61/863,701). A construct with the sacB-cat fragment in the opposite orientation, pCM433r, was also obtained.

[0260] A series of constructs and strains were generated in order to test the ability of pCM433 to enable unmarked allelic exchange at three distinct loci in the *M. extorquens* AM1 chromosome. Donor constructs for allelic exchange at the mptG locus were generated by first amplifying a region including mptG from CM501 (an isolate of wild-type, Rif^R *M. extorquens* AM1), or the corresponding region from the ΔmptG strain, CM508 (an isolate of CM253.1), each of which were ligated into pCR2.1 (Invitrogen) to generate pCM411 and pCM424, respectively. These PCR-amplified inserts (and all other alleles described below that were cloned into pCR2.1) were sequenced to confirm no PCR errors were introduced during amplification. The 2.1 kb ApaI-BamHI fragment of pCM411 containing the mptG region was then introduced into pCM433 that had been digested with ApaI and BglII, resulting in the donor vector pCM436. Similarly, the 1.3 kb SacI-XhoI fragment of pCM438 with the ΔhprA region was cloned into the same sites of pCM433 to generate the donor vector pCM439. This allowed the use of pCM436 (containing the wild-type mptG allele) to reverse the lesion found in CM508, while pCM437 (ΔmptG allele) was introduced into CM501 to do the opposite, generating the deletion in a single step.

[0261] Similarly, donor constructs for allelic exchange at the crtI locus were generated by first amplifying a region including crtI (encodes phytoene desaturase) from the pink CM501 strain, or the corresponding region from the white crtI::ISphoA/hah (i.e., $crtI^{502}$) strain, CM502 (an isolate of AM1-W). These fragments were ligated into pCR2.1 (Invitrogen) to generate pCM417 and pCM426, respectively. The 1.6 kb BamHI-NsiI fragment of pCM411 containing the crtI region was then introduced into pCM433 that had been digested with BglII and NsiI, resulting in the donor vector pCM440. Similarly, the 1.7 kb BamHI-NotI fragment of pCM426 with the $crtI^{502}$ region was cloned between the BglII and NotI sites of pCM433 to generate the donor vector pCM441. This allowed the use of pCM440 (containing the wild-type crtI allele) to reverse the lesion found in CM502, while pCM441 ($crtI^{502}$ allele) was introduced into CM501 to do the opposite, generating the insertion allele.

[0262] Finally, for the third locus, hprA, an antibiotic-resistance free deletion strain was generated initially using the previously developed cre-lox system. In contrast to the system described here using pCM433, the process to generate the ΔhprA strain was substantially more involved (and resulted in leaving behind a loxP scar). First, the regions upstream and downstream of hprA, were amplified separately and cloned into pCR2.1 (Invitrogen) to generate pCM428 and pCM429, respectively. The 0.5 kb upstream region was then excised from pCM428 using BglII and NotI and ligated into the same sites of pCM184 to generate pCM430. Into this plasmid, the 0.6 kb ApaI-SacI fragment from pCM429 was cloned into the same sites to generate the donor plasmid pCM431. As previ-

ously described, this plasmid was introduced into both the wild-type (pink) *M. extorquens* AM1 strain, CM501, as well as the otherwise isogenic white strain with a crtI^{502} allele, CM502, leading to the isolation of the hprA::kan strains CM1122 and CM1123, respectively. pCM157 (expressing Cre recombinase) was introduced into these two strains to catalyze the excision of the kan cassette, and was subsequently cured, ultimately resulting in the antibiotic-resistance free ΔhprA strains CM1203 and CM1204 used below.

[0263] Donor constructs for allelic exchange of the hprA locus were generated by first amplifying a region including hprA from CM501, or the corresponding region from the ΔhprA strain generated above, CM1203. Ligation of these fragments into pCR2.1 (Invitrogen) generated pCM434 and pCM438, respectively. The 2.2 kb ApaI-BamHI fragment of pCM434 containing the hprA region was introduced into pCM433 that had been digested with ApaI and BglII, resulting in the donor vector pCM434. Similarly, the 1.3 kb SpeI-NsiI fragment of pCM438 with the ΔhprA region was cloned between the XbaI and NsiI sites of pCM433 to generate the donor vector pCM439. This allowed the use of pCM434 (containing the wild-type hprA allele) to reverse the lesion found in CM1203, while pCM439 (AhprA allele) was introduced into CM501 to do the opposite, generating the deletion in a single step.

Example 4

Directed Genetic Engineering of Methylotrophic Bacteria Using Recyclable Antibiotic Marker System

[0264] Over the past few years, the genetic "toolkit" available for use with *Methylobacterium extorquens* AM1 has expanded significantly. The *Methylobacterium* organisms selected for genetic modification in the present invention can be engineered using, for example, small IncP vectors including pCM62 (FIG. 3 in U.S. Ser. No. 61/863,701 shows plasmids useful for cloning in *Methylobacterium* and other methylotrophs. The base plasmids for cloning and expression in *Methylobacterium* and other bacteria are shown in FIGS. 2 and 4 of Marx and Lidstrom, 2001. Microbiology (147:2065-2075)), pCM66, or pHC41 for cloning (Marx, C. J. and M. E. Lidstrom Microbiology (2001) 147: 2065-2075; Chou, H.-H. et al. *PLoS Genetics* (2009) 5: e1000652). Genetic modifications will also take advantage of freely replicating expression plasmids such as pCM80 (see FIG. 3 in U.S. Ser. No. 61/863, 701), pCM160, pHC90, or pHC91 (Marx, C. J. and M. E. Lidstrom *Microbiology* (2001) 147: 2065-2075; Chou, H.-H. et al. *PLoS Genetics* (2009) 5: e1000652). Other plasmids have the ability to respond to levels of inducing molecules such as cumate or anhydrotetracycline. These include pHC115, pLC 290, pLC291 (Chou, H.-H. et al. *PLoS Genetics* (2009) 5: el 000652; Chubiz, L. M. et al. *BMC Research Notes* (2013) 6: 183). In certain embodiments, genetic modifications will utilize expression systems introduced directly into a chromosomal locus. These may include pCM168, pCM172, and pHC01 plasmids developed for *M. extorquens* AM1 (Marx, C. J. and M. E. Lidstrom Microbiology (2001) 147: 2065-2075; Lee, M.-C. et al. *Evolution* (2009) 63: 2813-2830).

[0265] FIG. 6 in U.S. Ser. No. 61/863,701 shows plasmids useful for insertional expression from a chromosomal locus in *M. extorquens* AM1. The plasmids used for chromosomal cloning and expression in *Methylobacterium extorquens* are illustrated in FIG. 2 of Marx and Lidstrom, 2004. Microbiology (150:9-19). As described in Marx, C. J. et al. *Microbiology* (2004), 150: 9-19, an insertional expression system has been developed that allows expression of genes from a stable, unmarked chromosomal locus. This system has been used to better understand the role of the tetrahydrofolate (H_4F) pathway in methylotrophy. Previously, it has not been possible to generate null mutants lacking either mtdA (encoding an NADP-dependent methylene-H_4F/methylene-tetrahydromethanopterin dehydrogenase) or fch (encoding methenyl-H_4F cyclohydrolase). An unmarked strain was generated that expressed the analogous folD gene (encoding a bifunctional NADP-dependent methylene-H_4F dehydrogenase/methenyl-H_4F cyclohydrolase) from *Methylobacterium chloromethanicum* CM4^T. In this strain, null mutants could be obtained that grew normally on multicarbon substrates but were defective for growth on C_1 substrates. Additionally, null mutants of mtdA and/or fch could also be generated in the wild-type by supplementing the succinate medium with formate. These strains were unable to grow on C_1 compounds but were not methanol-sensitive. These approaches have demonstrated that the apparent essentiality of mtdA and fch is due to the need for formyl-H_4F for biosynthesis of purines and other compounds, and have provided clear genetic evidence that the H_4F pathway is required for methylotrophy.

[0266] Directed genetic engineering can also be used to increase expression of biosynthetic pathways needed to generate lycopene. This can be accomplished by cloning the region flanking the native promoter upstream of such a gene (or operon) and replacing the promoter with one of moderate to high strength. These include the strong promoter driving expression of the methanol dehydrogenase operon (P_{mxaF}) or the rhizobiaphage promoter (P_R) (Chubiz, L. M. et al. *BMC Research Notes* (2013) 6: 183). As described above, these genes included dxs, dxr, and ispDEF that lead to isopentyl diphosphate, and idi, ispA, crtE, crtB, and ctrI to generate lycopene. These manipulations can occur alone or together, and may be combined with other alterations.

[0267] Directed genetic engineering can be used to introduce novel enzymatic capacities needed to synthesize novel biomolecules, such as astaxanthin. This can be accomplished by cloning the necessary genes in their native, codon optimized, or otherwise manipulated version. These enzymes can be introduced into the desired host with a replicating plasmid-based system, such as pCM80, pCM160, pHC90, pHC91, pHC115, pLC290, or pLC291. Alternatively, for stable maintenance in the absence of selection they can be introduced onto the chromosome using systems described above, including pCM168, pCM172, and pHC01 developed for *M. extorquens*. Just three enzymes are required to extend from lycopene to astaxanthin: lycopene cyclase, encoded by crtY, β-carotene ketolase, encoded by crtW, and β-carotene hydroxylase, encoded by crtZ. These can be expressed from individual loci or fused into a synthetic operon. In some embodiments crtY and crtW will originate from the closely related *Bradyrhizobium* sp. ORS 278. In some embodiments crtZ will originate from the fellow α-proteobacterium *Brevundimonas* sp. SD212.

[0268] Wild-type *M. extorquens* or an available high pigment strain may be grown on methanol in order to serve as a feedstock for fish. Methanol levels added in fed-batch method can be maximized, within the constraints of the other nutrients present in the medium. Total additions of methanol to 5-10% v/v are desirable. To enable this, additional nitrogen may need to be added in the form of ammonium sulfate or

ammonium chloride. Given the tendency for methanol growth to lower the pH of the medium, bases such as sodium bicarbonate or sodium hydroxide can be added to maintain pH close to initial levels (generally pH 6.2 to 7). The final optical density of the culture can be determined via dilutions analyzed spectrophotometrically.

Example 5

Inducible Expression Vectors for use in Methylotrophic Bacteria

[0269] To date, only one regulated expression system has been demonstrated to be functional in *M. extorquens*. Choi and coworkers constructed an inducible expression system utilizing the cumate responsive transcriptional repressor, CymR, from *Pseudomonas putida* F1 and the strong P_{mxaF} promoter that drives the expression of methanol dehydrogenase in *M. extorquens*. This hybrid system has been modified and utilized to test the fitness consequences of gene expression levels of different formaldehyde oxidation enzymes in *Methylobacterium*. While functional, this promoter-operator pairs are extremely "leaky", wherein the basal level of expression in non-inducing conditions is quite high. This limitation makes heterologous gene expression exceedingly difficult, and hampers the exploration of conditionally null phenotypes.

[0270] Building on these previous findings, we have employed an additional transcriptional repressor, TetR, from the transposon Tn 10. As the foundational member of the TetR-family of DNA binding proteins, of which CymR is also a member, TetR has been extensively studied yielding much data on ligand binding, DNA binding kinetics, and operator site specificity. In the absence of inducer, TetR and CymR bind tightly to their respective operator sites (see FIG. 8 in U.S. Ser. No. 61/863,701 shows cumate- and anhydrotetracycline-regulated promoter systems for use in *Methylobacterium*. The plasmids used to for regulated expression in *Methylobacterium* are illustrated in FIG. 1 of Chubiz et al., 2013. BMC Research Notes (6:183).), thereby inhibiting transcriptional initiation by RNA polymerase. Upon binding of ligands such as tetracycline or anhydrotetracycline (a high-affinity ligand) in the case of TetR, or cumate (p-isopropyl benzoate) with CymR, the affinity of TetR and CymR for their respective operator sites is nearly abolished, allowing for transcription initiation to proceed. Exploiting these characteristics, numerous studies have modified existing expression systems to behave in a dose-dependent manner. In fact, TetR and related transcriptional repressors have found use in numerous synthetic biology applications in bacteria, archaea, and eukaryotes.

[0271] Here we describe the construction of two McP-based, inducible expression vectors for use in *M. extorquens*, and possibly numerous other proteobacteria with minor modification. The novelty of these vectors lies in their use of two separate transcriptional repressors, TetR and CymR, along with a strong promoter from the rhizobial phage 16-3. We demonstrate the utility of these vectors by showing that i) induction is dose-dependent, ii) induction is continuous through time, and iii) the regulatory range of both systems exceeds those currently available for *M. extorquens*. Collectively, these results supply researchers investigating *M. extorquens*, and likely numerous other proteobacteria, with two alternative systems to express genes in traditional and synthetic biology applications.

[0272] Findings

[0273] Promoter Design and Rationale

[0274] During the process of selecting an appropriate promoter, we desired that the promoter i) be sufficiently active in *M. extorquens* and ii) not be subject to regulation by native transcription factors. Based on these two criteria, a natural source for such a promoter was from bacteriophage. Many bacteriophage promoters have a wide host range and often have strong, constitutive activity in the absence of their transcriptional control mechanisms. However, numerous well characterized coliphage-derived promoters such as λP_L, λP_R, T5 P_N 25, T7 P_{A1} are weakly active or inactive in *M. extorquens*. To this end, we looked to other bacteriophage promoters that have been shown to be active in α-proteobacteria. Based on this metric, we explored the use of promoters from the control region of the rhizobial phage 16-3 (P_L and P_R). Phage 16-3 has been extensively examined with physiological and biochemical studies in both its host, the α-proteobacterium *Sinorhizobium meliloti*, and *Escherichia coli*, suggesting that P_L and P_R may be functional in a variety of hosts. Additionally, the only transcriptional regulator known to interact with P_L and P_R is the 16-3 C repressor.

[0275] In a set of exploratory experiments, we found that P_R was active in *M. extorquens* (data not shown). As we desired to construct inducible systems, we focused attention to engineering P_R derivatives containing operator sites for the CymR and TetR regulators (FIG. 8 in U.S. Ser. No. 61/863,701 shows cumate- and anhydrotetracycline-regulated promoter systems for use in *Methylobacterium*). The plasmids used to for regulated expression in *Methylobacterium* are illustrated in FIG. 1 of Chubiz et al., 2013. BMC Research Notes (6:183).). The resulting hybrid promoters, $P_{R/cmtO}$ and $P_{R/tetO}$, were found to produce the widest regulatory range with-out interfering with PR promoter activity. Interestingly, we found that placing the operators, specifically tetO, throughout other regions of the promoter resulted in either loss of promoter repression or activity (data not shown). This was a somewhat surprising result given the flexibility of many other phage-derived systems to be manipulated with multiple repressor and activator operator sites. Collectively, these findings allowed us to engineer two inducible promoters with similar maximal activity (FIG. 9 in U.S. Ser. No. 61/863,701 shows titratability of the regulated promoter systems shown in FIG. 7). The response of expression from pLC290 and pLC291 with addition of inducer in *Methylobacterium* are shown in FIG. 2 of Chubiz et al., 2013. BMC Research Notes (6:183).).

[0276] Activation of $P_{R/cmtO}$ and $P_{R/tetO}$ is dose-Dependent

[0277] A desirable property for regulated expression systems is for levels of gene expression from the promoter to be proportional to the concentration of inducer. In order to explore the range of induction of $P_{R/cmtO}$ and $P_{R/tetO}$, the promoters along with their respective regulatory proteins were introduced onto broad-host-range plasmids (IncP compatibility group) to create the expression vectors pLC290 and pLC291 (FIG. 8 in U.S. Ser. No. 61/863,701). Since previous studies have demonstrated mCherry to be a sensitive measure of gene expression in *M. extorquens*, we decided to use mCherry fluorescence as a metric of promoter activity. We placed the red-fluorescent protein variant mCherry under the control of each promoter in pLC290 and pLC291 and introduced the resulting vectors (pJP18T and pJP22T) into *M. extorquens*. To induce expression from $P_{R/cmtO}$ and $P_{R/tetO}$, we supplied varied concentrations of cumate (Q) and anhydrotetracycline (aTc), respectively, to *M. extorquens* cultures.

[0278] In general, both promoters were found to be responsive to concentrations of Q and aTc that were in agreement with previous studies in *M. extorquens* or other organisms. The $P_{R/cmtO}$ promoter was observed to respond to a range of 0.1 to 5 μg/mL (0.6 to 30 μM) of Q and the $P_{R/tetO}$ promoter from 0.1 to 25 ng/mL (0.2 nM to 50 nM) aTc. Interestingly, the induction profile of $P_{R/cmtO}$ increased in a log-linear fashion over the entire concentration range, whereas $P_{R/tetO}$ was observed to have a much more concave profile. In terms of regulatory range, $P_{R/cmtO}$ and $P_{R/tetO}$ were observed to have 10-fold and 30-fold induction, respectively, with both promoters having the same maximum absolute levels of expression (FIG. 9 in U.S. Ser. No. 61/863,701). Importantly, the basal level of expression from $P_{R/cmtO}$ was found to be approximately 3-fold higher than that of $P_{R/tetO}$. Taken together, these data suggest that while $P_{R/cmtO}$ may be more tunable, $P_{R/tetO}$ serves as a superior expression system for genes requiring tight repression, such as cytotoxic proteins. Also, we found that there was minimal crosstalk between the CymR and TetR ligand specificity or promoter binding indicating these systems would work independent of one another (pJP18T: 4.6 Uninduced/4.2 with aTc; pJP22T: 1.0 Uninduced/1.1 with Q; Grown in succinate).

[0279] Comparing the levels of gene expression and regulatory range of $P_{R/cmtO}$ and $P_{R/tetO}$ to the cumate inducible P_{mxaF} promoter previously reported, we found that in *M. extorquens* these promoters achieve 33% of the maximal activity of P_{mxaF} (the strongest known *Methylobacterium* promoter) and provide a greater degree of repression. Specifically, a cumate-inducible P_{mxaF} mCherry expression vector, pHC115m, yielded relative fluorescence values of 15.6±1.5 (uninduced) to 157.1±3.7 (induced). While this 10-fold regulatory range was similar to $P_{R/cmtO}$, the minimal and maximal expression from $P_{R/cmtO}$ were both 3-fold lower. By comparison, $P_{R/tetO}$, with a 30-fold regulatory range, was able to repress expression 8-fold lower than the P_{mxaF} system with only a 3-fold difference in maximum expression. Collectively, these results demonstrate that both $P_{R/cmtO}$ and $P_{R/tetO}$ provide improvement over previously explored systems. However, we do note that P_{mxaF} may remain a superior promoter in cases when high-level protein over-expression is desired. Importantly, these hybrid promoters allow for more relevant exploration of cellular physiology as their expression levels and ranges fall well within or above native promoters in *M. extorquens*.

[0280] Maximal Activation of $P_{R/cmtO}$ and $P_{P/tetO}$ is Substrate Dependent

[0281] An issue with many expression systems designed with host-derived promoters is the possibility of interactions with native transcription factors. Specifically, the P mxaF promoter is known to be more highly active in cells grown on methanol as opposed to succinate. To explore this possibility, with respect to $P_{R/cmtO}$ and $P_{R/tetO}$, we cultured *M. extorquens* harboring pJP18T and pJP22T in media with either methanol or succinate as the sole carbon source. We found that succinate grown cells possessed a nearly 2-fold increase in maximal gene expression, compared to methanol grown cells; effectively, the opposite behavior seen with P_{mxaF}. We suspect that this disparity in maximal expression may be due to an external factor, such as different plasmid copy numbers, between methanol and succinate growth. Previously reported XylE and β-galactosidase promoter probe vectors used in *M. extorquens*, such as pCM130 and pCM132 (plasmids with the same back-bone as pLC290 and pLC291), exhibit between 2- and 3-fold increases in background activity during succinate versus methanol growth. As pCM130 and pCM132 possess no promoter sequences upstream of their reporter genes, the only likely variation that might exist is in plasmid copy number. Comparing these findings to our own, where $P_{R/cmtO}$ and $P_{R/tetO}$ contain no host-related transcription factor binding sites, we see similar fold changes in maximal expression suggesting that a similar mechanism may be affecting these expression systems. Taken together, these data indicate that single-copy or chromosomally integrated systems be used in situations where uniform expression is desired across substrates.

[0282] Induction of $P_{R/cmtO}$ and $P_{R/tetO}$ is Continuous

[0283] A problematic feature of many expression systems, particularly those associated with metabolic pathways, is that gene expression can exhibit phenotypic heterogeneity throughout the population of cells, such as an on-off, switch-like behavior. To explore this possibility, we grew *M. extorquens* strains bearing the mCherry expression vectors pJP18T and pJP22T to mid-log phase, induced cultures with either Q or aTc, and measured the time course of individual-cell fluorescence by flow cytometry. We found that over 8 hours of induction the induced populations activated transcription in a uniform, continuous manner (FIG. 10 in U.S. Ser. No. 61/863,701 shows unimodal expression during an induction time-course for each of the regulated promoter systems shown in FIG. 7 of U.S. Ser. No. 61/863,701). The time-course of smooth, non-bimodal regulation of expression from pLC290 and pLC291 in *Methylobacterium* are shown in FIG. 3 of Chubiz et al., 2013. BMC Research Notes (6:183).). Though we did observe residual uninduced cells, we suspect this may be due to debris introduced by our cell fixing method or possibly cells losing mCherry due to costly over-expression. These data demonstrate the utility of the $P_{R/cmtO}$ and $P_{R/tetO}$ expression systems in studying aspects of cellular physiology requiring uniform gene expression.

[0284] Complementation and Conditional Null Phenotypes Using $P_{R/tetO}$ Constructs

[0285] To examine the utility of these vectors for studying *M. extorquens* physiology, we complemented a gene encoding a key enzyme in methanol metabolism using the $P_{R/tetO}$-based plasmid pLC291. We chose to use utilize $P_{R/tetO}$ due to the tight induction properties we have observed using an mCherry reporter (FIG. 8 in U.S. Ser. No. 61/863,701). The product of ftfL (formate-tetrahydrofolate ligase) is required for the assimilation of formate into biomass during one-carbon metabolism. A disruption in ftfL results in a methanol minus growth phenotype. By complementing a ftfL knockouts using ftfL-expressing vectors under the control of $P_{R/tetO}$, in the presence of aTc, we found that we could fully restore growth on methanol. Importantly, in the absence of aTc, we observed that we were able to produce a complete null phenotype for ftfL. To date, no expression system for *M. extorquens* has been capable of producing conditional null phenotypes. These results demonstrate the utility of $P_{R/tetO}$ to study *M. extorquens* physiology and generate conditional null mutants regulated by aTc.

[0286] To date, only a handful of expression systems exist for bacterial models outside *E. coli* and other closely related γ-proteobacteria. In an effort to expand the genetic toolkit available to researchers working with *M. extorquens*, and presumably other proteobacteria, we have constructed a set of two inducible expression vectors that utilize the CymR and TetR (cumate and tetracycline repressors) in conjunction with

the strong PR promoter from phage 16-3. The pLC290 and pLC291 vectors were found to provide uniform, high-level expression in *M. extorquens* over a wide range of inducer concentrations. Importantly, compared to the only existing inducible system for *M. extorquens*, we found that $P_{R/cmtO}$ and $P_{R/tetO}$ have 3 and 8-fold increases in repression, respectively. This provides a significant improvement in the ability to explore *M. extorquens* cellular physiology. Further, as these promoters operate orthogonally to one another, we believe these expression systems will easily work in concert within a single strain to allow complex genetic engineering in a wider range of bacteria. For these reasons, we believe these vectors and promoter systems will be of great use to the bacteriological community in many research and industrial settings.

[0287] Materials and Methods

[0288] Bacterial Strains, Medium, and Growth Conditions

[0289] All bacterial strains used in this work are derivatives of *Escherichia coli* NEB10β (New England Biolabs), *E. coli* LC100 (F⁻rph-1 ilvG attλ::[spcR lacI^{Q} tetR]), *Methylobacterium extorquens* PA1 strain CM2730 (Δ ceIABCD) or *M. extorquens* AM1. Growth of all strains, except *E. coli*, was performed in modified 'Hypho' minimal medium as described by Chou and coworkers, with succinate at 5 mM or methanol at 20 mM. *E. coli* strains were cultured in Luria-Bertani broth as described by Miller or nutrient broth. Media was supplemented with kanamycin at 50 μg/mL or ampicillin at 100 μg/mL to select for the presence of all plasmids. Inducers anhydrotetracycline (aTc) and cumate-KOH (Q) were supplied at 25 ng/mL or 5 μg/mL from aqueous stocks, respectively, unless otherwise indicated. Growth and gene expression experiments were performed at 30° C. using an automated growth system described by Delaney and coworkers.

[0290] Plasmid and Strain Construction

[0291] Promoter designs were initially constructed and subsequently mutated in a pBluescript(SK−) (Stratagene) backbone. Synthetic oligonucleotides CAACAACTTATACCATGGCCTACAAAAAGGCAAACAATGGTACTTGACGACTC ATCACAA (SEQ ID NO:11) and GTCCGTTCGTTACAATCTACAACTACAATTGTTGTGATGAGTCGTCAAGTACC ATTG (SEQ ID NO:12) containing the sequence for a 91 nt region encoding the PR promoter from the rhizobial phage 16-3. The oligonucleotides were annealed to form a 91 bp dsDNA fragment, followed by PCR amplification with primers ATAGGGCCCCAACAACTTATACCATGGCCTAC (SEQ ID NO:13) and ATAGGTACCGTCCGTTCGTTACAATCTACAAC (SEQ ID NO:14) to introduce PspOMI and KpnI restriction sites. The resulting fragment was digested with PspOMI and KpnI and cloned into the respective sites in pBluescript(SK−) to form pLC265. TetR and CymR operator sites (tetO and cmtO), were introduced at the distal end of PR in pLC265 using enzymatic inverse PCR (EI-PCR) using primers ATACGTCTCATCCCTATCAGTGATAGAGAGTTGTAGATTGTAACGAACGGAC (SEQ ID NO:15), ATACGTCTCAGGGACGTCAAGTACCATTGTTTGCC (SEQ ID NO:16), ATACGTCTCAACAAACAGACAATCTGGTCTGTTTGTGGTACCCAATTCGCCCT AG (SEQ ID NO:17), and ATACGTCTCATTGTTTACAATCTACAACTACAATTGTTGTG (SEQ ID NO:18) followed by BsmBI digestion and ligation to generate plasmids pLC271 (PR/tetO containing) and pLC277 (PR/cmtO containing).

[0292] The subsequent broad-host-range vectors were constructed using the expression vector pHC115 as a template. A DNA region encoding Tn10 tetR was PCR amplified from LC100 using primers ATAGCTAGCAGGGAGAGACCCCGAATGATGTCTAGATTAGATAAAAGTAAAGT G (SEQ ID NO:19) and ATAGGGCCCTTAAGACCCACTTTCACATTTAAG (SEQ ID NO:20) containing NheI and PspOMI restriction sites. The resulting product was digested and ligated into the NheI and PspOMI sites of pHC115, thereby replacing the cymR coding region with tetR to form pLC261. From pHC115 and pLC261, the PmxaF region was excised with PspOMI and KpnI and replaced with subcloned PR/cmtO and PR/tetO fragments from pLC277 and pLC271. To the resulting plasmids, a trrnB terminator was PCR amplified from pHC01 using primers ACGCGAAATTCAAGCGCTAGGGCCAAGTTGGGTAACGCCAGGGTTTTCCC (SEQ ID NO:21) or ATGTGAAAGTGGGTCTTAAGGGCCAAGTTGG (SEQ ID NO:22) (Chubiz et al. *BMC Research Notes* (2013), 6:183) GTAACGCCAGGGTTTTCCC (SEQ ID NO:23) and TGTAGGCCATGGTATAAGTTGTTGGGATGCAAAAACGAGGCTAGTTTACC (SEQ ID NO:24) and cloned into the PspOMI site, using the method of Gibson and coworkers, to reduce transcriptional read-through into the PR/cmtO and PR/tetO promoter regions. Likewise a more comprehensive multiple cloning site was introduced into the KpnI and EcoRI sites using annealed synthetic oligonucleotides GATAGGTACCTCTAGAAGATCTACGCGTACTAGTGCATGCGAGCTCACCGGT GATTCATAG (SEQ ID NO:25) and CTATGAATTCACCGGTGAGCTCGCATGCACTAGTACGCGTAGATCTTCTAGAG GACCTATC (SEQ ID NO:26) to produce the final expression vectors pLC290 and pLC291. The mCherry expression vectors pJP18T and pJP22T were created by subcloning a KpnI and EcoRI digestion product containing mCherry from pHC115m into the corresponding sites in pLC290 and pLC291, respectively. The vectors pLC290 (GenBank Accession KC296704) and pLC291 (Gen Bank Accession KC296705) are publically available.

[0293] Unmarked ftfL knockouts were generated by transforming the Cre-recombinase expression plasmid pCM157 into *M. extorquens* AM1 derivatives CM216K.1 generating strain CM2336 (ΔftfL::loxP). The ftfL complementation vector was generated by subcloning a KpnI and EcoRI digestion product of a pHC115-based ftfL plasmid (SMC unpublished) into the corresponding sites of pLC291, creating plasmids pSC54. The vector, pSC54, was introduced into CM2336 via triparental mating using the helper plasmid pRK2073, to produce strains CM4103 (ΔftfL::loxP/pSC54). Complementation was performed by inoculation of succinate grown CM4103 into methanol minimal medium containing 0 μg/mL or 20 μg/mL aTc.

[0294] Fluorescence-Based Expression Assays

[0295] Assays to measure levels of mCherry protein expression were performed as follows. For dose-dependent response curves, *M. extorquens* strains harboring pJP18T or pJP22T were grown to saturation in 10 mL of Hypho-succinate medium. These cultures were then diluted 1:200 in fresh medium, followed by 630 μL aliquots being dispensed to clear, flat-bottom, 48-well microtiter plates (Costar). Cultures were grown for 4 hours on a plate shaking tower (Caliper) at 150 rpm in a 30° C. humidified room. After 4 hours of growth, 10 μl of fresh medium containing Q or aTc was added to supply Q and aTc at desired concentrations. Cultures were

allowed to continue growth for an additional 24 hours prior to fluorescence (excitation 587 nm/emission 610 nm) and optical density (600 nm) measurements made using a Tecan Safire2 plate reader. Relative fluorescence values reported are: Relative fluorescence (A.U.)=RFU/OD_{600}*10^{-3}.

[0296] Dynamic expression assays were conducted under similar conditions as above with the following exceptions. Cells (200 μL of culture) were harvested after induction at 0, 2, 4, 6, 8, and 24 hrs. Culture samples were pelleted by centrifugation (6,000×g) and resuspended in an equal volume of cold Hypho medium without succinate and supplemented with 100 mg/mL streptomycin to inhibit mCherry translation. Fixed cells were kept on ice prior to fluorescence measurements made using a BD LSR II Flow Cytometer. Flow cytometry data were then analyzed using the BioConductor flowCore package in R. Reported fluorescence values for flow cytometry are raw values from the BD LSR II and were not correlated to those of the Tecan Safire2.

Example 6

Harvesting of Biomass; Processing into Feed

[0297] Nutrient-rich biomass can be harvested via 1.) filtration, perhaps using a series of filters of decreasing pore size or tangential flow filtration 2.) continuous centrifugation, 3.) settling to the bottom of the fermentation vessel, or 4.) any combination of the above, or other approaches. Settling may be enhanced through the addition of a fining agent such as egg whites, gelatin, isinglass, the sequential addition of kieselsol and chitosan, carboxymethylcellulose, or other agents alone or in combination. Wet and dry cell mass can be determined before and after drying material in an oven. Total protein can be estimated via colorimetric assays (Bradford, M. M. *Analytical Biochemistry* (1976) 72: 248-254; Lowry, O. H. et al. *J. Biological Chemistry* (1951) 193: 265-275). Carotenoid content can be assessed spectrophotometrically following organic extraction (Takaichi and Shimada *Methods Enzymol.* (1992) 213: 374-385). Further characterization can occur via nuclear magnetic resonance or liquid chromatography-mass spectrometry (Holtin, K. et al. *Anal Bioanal Chem* (2009) 395: 1613-1622). Through comparison to standards, this can establish the identity and weight percentage of carotenoids present. Vitamins such as B-12 can be determined via bioassay (Berg, T. M. et al. *Appl. Environ. Microbiol.* (1976) 31: 459-464). Cellulose content can be determined enzymatically (Zhang, Y. H. et al. *Methods Mol. Biol.* (2009) 581: 213-231). Poly-β-hydroxybutyrate content can be determined by flow cytometry or spectorfluorometry (Degelau, A. et al. *Appl. Microbiol. Biotechnol.* (1995) 42: 653-657). Free amino acids can be quantified via derivativization and analysis via gas chromatography-mass spectrometry (Krömer, J. O. et al. *J. Bacteriol* (2004) 186: 1769-1784; Marx, C. J. et al. *PLoS Biology* (2005) 3: e16).

[0298] One method of preparing cell mass is via freeze-drying in a lyophilizer, and then readdition of dried cell powder into gel, pellet, or flake forms of fish food. Alternatively, fresh (wet) cell material may be added to other ingredients prior to preparation via drying or heating. In other methods, cell material may be disrupted via homogenization, sonication, enzymatic treatment, or other treatments alone or together in order to alter the bioavailability of pigments, other nutrients, and protein. This will likely be accompanied by addition of an antioxidant. The optimal method of preparation can be found by trial and error or by prediction based on the animal for which the feed is intended.

[0299] Trials to test the utility of pigmented methylotrophs as a carotenoid-rich protein source for aquaculture feed can proceed in various stages. As a simple first test of palatability, *Methylobacterium* can be added to a gel fish food at a smaller volume. Contingent upon interest in feeding, flavor additives such as fish hydrolysate can be adjusted, accordingly. As a second stage, the nutritional value of *Methylobacterium* cell material and the ability to deposit pigments can be assessed in a small, rapidly growing fish such as Amphiprion (i.e., clown fish). Using a combinatorial design, we can consider six initial treatments. Traditional fish food can be prepared with and without the addition of commercial astaxanthin. Pigment-free *Methylobacterium* and a high pigment strain (such as in Lee, M.-C. et al. *Evolution* (2009) 63: 2813-2830) can be added to varying levels, such as 5% and 25% total dry weight of feed, into 95% or 75% traditional feed. Further tests could compare additions to alternative technologies such as treated or untreated soy protein. From this we will be able to assess fish vigor, survival, weight gain and body dimensions, externally visible coloration in the scales, and pigment deposition in the flesh. Follow-up trials could assess the rate and specificity of deposition using isotopically-labelled biomass using ^{13}C-methanol or ^{15}N-ammonium. There are two grounds for determining the success of these trials. First, are the fish at least as healthy as the standard feed, or perhaps more healthy than a similar replacement with soy-based protein instead of *Methylobacterium*? Second, is there detectable pigmentation in the flesh and scales relative to the negative control, and how far toward (or above) the positive control is this coloration? Positive results in model organisms as indicators for larger, commercially relevant species will already indicate utility as a pigment-laden feed for ornamental fish, and may point to specific utility of sprilloxanthin if the coloration is distinct from that seen with astaxanthin. The ultimate effectiveness in aquaculture applications can be assessed with similar feeding trials performed with the commercial species to be utilized, such as salmon or shrimp. As above, among the important criteria are fish vigor, survival, weight gain, prevention of disease (e.g. enteritis), and body dimensions, externally visible coloration in the scales, and pigment deposition in the flesh.

Example 7

General Plasmid Construction

[0300] Deletion mutants were generated in *M. extorquens* PA1 using pPS004 (Michener et al, 2014. *J. Bacteriology.* 196:2101-2107), a kanamycin-resistance allelic exchange vector derived from pCM433 (Marx 2008). Briefly, 500+ bp regions flanking the target locus were PCR amplified and assembled into pPS04 using Gibson isothermal assembly (Gibson 2009). All plasmids relevant to this study are listed in Table 1.

TABLE 1

List of relevant plasmids

Plasmid	Description	Reference
pKB01	deletion construct for ctrI-like locus (Mext_3011)	This work

TABLE 1-continued

List of relevant plasmids

Plasmid	Description	Reference
pKB02	deletion construct for crtF (Mext_2528)	This work
pKB03	deletion construct for cluster of crtCDF (Mext_2725-26, -28)	This work
pCM433	Allelic exchange vector	Marx 2008
pPS04	kanR derivative of pCM433	Michener et al., 2014
pRK2073	helper plasmid for triparental matings	Figurski 1979

[0301] Construction of pKB01 to delete crtI-like locus (Mext_3011)

[0302] To delete Mext_3011 (a crtI-like gene), two flanking regions were amplified using the following oligonucleotide pairs: upstream, ATGGATGCATATGCTGCAGCTCGAGCGGCCGCGGCCCCCTTTGCCCTT (SEQ ID NO:27) plus ATCCGGCACGGTTGACACTATGGCTGGGA (SEQ ID NO:28); and downstream, GCGCTGACGAAAATCCCAGCCATAGTGTCAACCGTGCCGGATGCCCGT (SEQ ID NO:29) plus GGTTAACACGCGTACGTAGGGCCCGCGGCCGCGGGCGATGTTGGTGAA (SEQ ID NO:30). Underlined sequences denote overlapping regions designed to facilitate Gibson isothermal assembly. A map of the resulting plasmid—pKB01—is listed in FIG. **2**.

[0303] Construction of pKB03 to delete crtCDF (Mext_2725-26, -28)

[0304] The construct to delete crtCDF (Mext_2725-26, -28) while maintaining crtE (Mext_2727) was slightly more complex, requiring 3 PCR products with the following primer pairs: upstream flank of crtCD,

(SEQ ID NO: 31)
ATGGATGCATATGCTGCAGCTCGAGCGGCCGCCCGATTGCCTGCCCCTAG

plus

(SEQ ID NO: 32)
GGATCAACGGTGATGCGAGGCGGAGCGCATTTTCGGTGGCAGGCGCCTGA

GCGAAGTCC;

middle region encoding crtE

(SEQ ID NO: 33)
CTGCCACCGAAAATG

plus

(SEQ ID NO: 34)
TTAGCGCCGCGGCAAGGCCGGTTCT;

and downstream flank of crtF,

(SEQ ID NO: 35)
CGAGCGATGGCGTGAGAACCGGCCTTGCCGCGGCGCTAAGAGTGT

plus

(SEQ ID NO: 36)
GGTTAACACGCGTACGTAGGGCCCGCGGCCGCCGAATCGCCGCTGACA.

A map of the resulting plasmid—pKB03—is listed in FIG. **4**.

[0305] Construction of pKB02 for ΔcrtF (Mext_2728)

[0306] A construct to delete crtF (Mext_2728) was inadvertently created during the Gibson assembly of pKB03 fragments. In this construct, approximately 129 bp of spurious PCR product (from Mext_1932) were assembled upstream of the middle and downstream fragments of pKB03 described above. Given that this upstream fragment bears no homology to the target locus, this region behaved as "vector" and was lost in the double-crossover recombinant, resulting in a clean deletion as verified by PCR analysis and Sanger sequencing.

[0307] Strain Construction

[0308] Deletion constructs were introduced into *M. extorquens* PA1 using triparental matings with the helper plasmid pRK2073 (Figurski 1979). Mutants were engineered in several *M. extorquens* PA1 genetic backgrounds: "wild-type" *M. extorquens* PA1 (Knief 2010); a Δcel mutant deficient in cellulose biosynthesis (Delaney 2013); and a double Δcel Δshc strain lacking both cellulose biosynthesis and squalene-hopane cyclase. Clean genomic deletions were confirmed by PCR analysis and Sanger sequencing using a combination of primers from the constructs, as well as the following oligonucleotides designed outside the region of recombination: pKB01, CTCCCCATCCTCGTGATC (SEQ ID NO:37) and GAGGAAGGCGTCCGGGTC (SEQ ID NO:38); pKB02, GTGCCGGATGCCCG (SEQ ID NO:39) T and CGCCGAAACCCGGATG (SEQ ID NO:40); pKB03, GCTCGCCACCAAGTTCG (SEQ ID NO:41) and CGCCGAAACCCGGATG (SEQ ID NO:42).

REFERENCES CITED IN THIS EXAMPLE

[0309] Delaney N F, Kaczmarek M E, Ward L M, Swanson P K, Lee M-C, et al. (2013) Development of an optimized medium, strain and high-throughput culturing methods for *Methylobacterium extorquens*. PLoS ONE 8: e62957. doi: 10.1371/journal.pone.0062957.

[0310] Figurski D H, Helinski D R (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. P Natl Acad Sci Usa 76: 1648-1652.

[0311] Gibson D G, Young L, Chuang R-Y, Venter J C, Hutchison C A, et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Meth 6: 343-345. doi:10.1038/nmeth.1318.

[0312] Knief C, Frances L, Vorholt J A (2010) Competitiveness of diverse *Methylobacterium* strains in the phyllosphere of *Arabidopsis thaliana* and identification of representative models, including *M. extorquens* PA1. Microb Ecol 60: 440-452. doi:10.1007/s00248-010-9725-3.

[0313] Marx C J (2008) Development of a broad-host-range sacB-based vector for unmarked allelic exchange. BMC Research notes 1:1. doi:10.1186/1756-0500-1-1.

Example 8

Construction of CM3945

[0314] An allelic exchange plasmid was constructed from pCM433, a sacB-based suicide plasmid. The genomic region annotated as squalene hopene cyclase (shc) is numerically annotated in the reference *M. extorquens* PA1 genome as

Mext_1944. To knockout the gene, PCR products of sequences upstream and downstream shc were ligated into pCM433 to create cloning vector pAB194.

[0315] The primer pair taccatggatgcatatgctgcagctcgagcCCG CGC CGC AGG AAT TC (SEQ ID NO:43) (forward) and CGC ATC GTT CTC GCC TCG TTC (SEQ ID NO:44) (reverse) was used to amplify the region upstream of the shc locus. The primer pair gag aca gtc gaa cga ggc gag aac gat gcg GCA ACC TGA AGC GGG GCA AC (SEQ ID NO:45) (forward) and ggttaacacgcgtacgtagggcccgcggccGAT TGA GAC CCG CGG GTC ATC (SEQ ID NO:46) (reverse) was used to amplify the region downstream of the she locus. These primers were designed to add homology to the pCM433 backbone.

[0316] Following digestion of pCM433 with NotI, the upstream and downstream PCR products were ligated into the vector backbone via Gibson assembly, generating cloning vector pAB194.

[0317] CM3945 was generated by mating pAB194 into the recipient strain *M. extorquens* PA1 cel deletion strain (CM2730; Delaney et al., 2013). The allelic exchange was performed as described in Marx et al 2008. The deletion was confirmed by Sanger sequencing.

Example 9

Grunt Trial

[0318] An experiment was designed for growing the smallmouth grunt (*Haemulon chrysargyreum*) on four different experimental diets to determine if KnipBio's SCP, or KBM, was a suitable feed ingredient for a model fish. The four diets were composed of (1) a standard commercially available grunt diet, (2) the standard diet plus astaxanthin pigment (~80PPM), (3) a diet containing 5% of the total feed pellet replaced by KnipBio single cell protein (KBM), and (4) a diet with 25% of the fish meal replaced by KBM (~60 PPM carotenoids). CM3945 strain was used to produce KBM. Fish length, weight, feed conversion ratio, and gut microbiota were all assessed. Each condition was tested in triplicate (12 aquarium tanks) with approximately 15 fish in each tank, for a total of 180 fish.

[0319] FIG. **5** shows growth of the smallmouth grunt using 4 experimental diets including a 25% inclusion of KBM

[0320] In this pilot trial, smallmouth grunts were fed to satiation over the course of 34 days using the four experimental diets. The data suggests that the two diets with carotenoids (at roughly similar PPM levels), support the highest growth rates relative to the control diets without pigments over the same time (see FIG. **12**). Growth of the grunt was observed to be 370% and 391% for the Control+Pigment and the 25% KBM inclusion respectively. The averaged-out feed conversion ratio (FCR) ranged from 1.09-1.24.

[0321] An interesting indication from this data is that the pigments in KBM appear to be bio-accessible to the fish tested which implies the intense processing for pigment extraction typical with algae and yeast today is unnecessary in this system. Advantages to this include lower processing costs as well as longer viability of the anti-oxidant pigments as exposure to O2-damage is considerably lower while remaining intact.

[0322] In part, KnipBio's single cell protein (KBM) serves as a viable protein alternative for animal feeds given its natural composition and potential for enhanced expression. In aquaculture and agriculture, vegetable proteins (e.g., soy) are commonly used. However, these vegetable protein sources lack essential amino acids like lysine, methionine and others which require formulated feeds to add these essential nutrients exogenously. As seen in FIG. **6**, KBM as a raw ingredient is largely comparable to commercially available final feeds based on soy or fish meal. The genetic tractability of *M. extorquens* lends itself to the further fine tuning of specific or groups of amino acids. Another consideration for the use of vegetable proteins are the carbohydrates that are often associated as high as 10%. Certain animals (e.g., salmon) react unfavorably to excess sugar and result in stomach inflammation (enteritis). KBM carbohydrate composition can be an order of magnitude lower minimizing or avoiding these effects considerably. Blood meal and poultry byproducts are often included as part or in combination with our proteins for animal feeds. One of the significant drawbacks to this material is the undigested phosphorous content from bone that then subsequently enters the environment. The composition of KBM is 5-10× lower in phosphorous which means more of the feed is usable to the animal simultaneously resulting in a lower environmental footprint.

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<400> SEQUENCE: 30

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<400> SEQUENCE: 33

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<211> LENGTH: 25
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<211> LENGTH: 45
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<400> SEQUENCE: 38

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<400> SEQUENCE: 41

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<400> SEQUENCE: 46

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<210> SEQ ID NO 48
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 48

ggccgctcga gctgcagca 19

1. A biomass comprising substantially one or more isolated methylotrophic bacterial cultures, wherein the bacteria are genetically modified or artificially pre-selected to produce elevated levels of a carotenoid compound relative to the corresponding unmodified or unselected bacterium.

2. The biomass of claim **1**, wherein the biomass is in a dry or substantially dry form.

3. The biomass of claim **1**, wherein the bacterium of the cultures is selected from the group consisting of *Methylomonas, Methylobacter, Methylococcus, Methylosinus, Methylocyctis, Methylomicrobium, Methanomonas, Methylophilus, Methylobacillus, Methylobacterium, Hyphomicrobium, Xanthobacter, Bacillus, Paracoccus, Nocardia, Arthrobacter, Rhodopseudomonas, Pseudomonas, Candida, Hansenula, Pichia, Torulopsis*, and *Rhodotorula*.

4. The biomass of claim **3**, wherein the bacterium is a *Methylobacterium*.

5. The biomass of claim **4**, wherein the bacterium is *M. extorquens*.

6. The biomass of claim **5**, wherein the strain of *M. extorquens* is selected from the group consisting of *M. extorquens* AM1, *M. extorquens* DM4, *M. extorquens* CM4, *M. extorquens* PA1, *M. extorquens* BJ001 (formerly *M. populi*), *M. radiotolerans, M. nodulans*, and *Methylobacterium* spp. 4-46.

7. The biomass of claim **1**, wherein the carotenoid compound is selected from the group consisting of β-carotene, lycopene, rhodopin, astaxanthin and spirilloxanthin.

8. The biomass of claim **1**, wherein the genetically modified bacterium is modified so that one or more genes producing enzymes that divert isoprenoid compounds from the carotenoid biosynthetic pathway are blocked or deleted.

9. The biomass of claim **1**, wherein the bacterium comprises a non-lethal knock-out of shc.

10. The biomass of claim **1**, wherein the pre-selected bacterium is a spontaneous mutant that is selected to express a "dark pink" or "reddish" pigment.

11. A feed composition, comprising the biomass of claim **1**.

12. The feed composition of claim **11**, wherein the biomass is obtained without bacterial lysis.

13. The feed composition of claim **12**, that wherein the biomass is collected by filtering, sedimentation, or centrifugation.

14. The feed composition of claim **11**, wherein composition contains at least 1% of the biomass by weight.

15. The feed composition of claim **11**, wherein the composition is optimized for consumption by fish.

16. The method of claim **15**, wherein the fish is of species farmed for human consumption that has pink-, reddish-, yellow-, or orange-colored flesh.

17. The feed composition of claim **15**, wherein the composition comprises one or more of: EPA, DHA, taurine, and an essential amino acid.

18. A method of preparing a biomass, the method comprising:

(a) culturing in an appropriate medium methylotrophic bacteria, and

(b) collecting the biomass of claim **1**.

19. The method of claim **16**, wherein the biomass is collected by filtering, sedimentation, or centrifugation.

20. An *M. extorquens* comprising a non-lethal knock-out of shc.

21. A method of producing fish or seafood, the method comprising:

(a) farming fish or seafood, and

(b) providing the feed composition of claim **11** to the fish or seafood.

22. A fish or seafood product exhibiting an elevated level of a carotenoid pigment in the flesh, said level attributable to the diet comprising the feed of claim **11**.

* * * * *

Appendix 2-1. 16S Sequencing.

Methylobacterium extorquens strain CM2730 was acquired from (b) (4) laboratory. This strain was described in the following paper: Development of an Optimized Medium, Strain and High-Throughput Culturing Methods for *Methylobacterium extorquens* (Delaney *et al.,* 2013). Deletion of the spirilloxanthin pathway was performed as described in that paper, leading to strain KB203. During KnipBio's fermentation experiments, the culture was streaked out to determine if there was any contamination during in-house lab fermentation. We selected one pink colony from the culture and outsourced it for 16S sequencing to (b) (4) who used standard in-house 16S bacterial forward and reverse primers. The sequence obtained was then compared to the bacterial database using the BLAST program on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The results showed below confirm that strain KB203 is a *Methylobacterium extorquens* strain.

1. 203-16S-rRNA-F-ab1

TGCAGTCGAACGGGCACCTTCGGGTGTCAGTGGCAGACGGGTGAGTAACACGTGGGAACGTACCCTTCGGTTCGGAATAACT
CAGGGAAACTTGAGCTAATACCGGATACGCCCTTTTGGGGAAAGGTTTACTGCCGAAGGATCGGCCCGCGTCTGATTAGCTTG
TTGGTGGGGTAACGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGA
AGGCCTTAGGGTTGTAAAGCTCTTTTGTCCGGGACGATAATGACGGTACCGGAAGAATAAGCCCCGGCTAACTTCGTGCCAGC
AGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGGCGCGTAGGCGGCCGATTAAGTCGGGG
GTGAAAGCCTGTGGCTCAACCACAGAATTGCCTTCGATACTGGTTGGCTTGAGACCGGAAGAGGACAGCGGAACTGCGAGTG
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AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCCAGCCGTTGGCCTGCTTGCAGGT
CAGTGGCGCCGCTAACGCATTAAGCATTCCGCCTGGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCATCCCTTGACATGG

Description	Max score	Total score	Query cover	E value	Ident	Accession
Methylobacterium extorquens 16S ribosomal RNA gene, partial sequence	1633	1633	100%	0	99%	KF192614.1
Uncultured Methylobacterium sp. clone SB6-38 16S ribosomal RNA gene, partial sequence	1633	1633	100%	0	99%	KJ197806.1
Uncultured Methylobacterium sp. clone SB6-21 16S ribosomal RNA gene, partial sequence	1633	1633	100%	0	99%	KJ197793.1
Methylobacterium zatmanii strain M0505 16S ribosomal RNA gene, partial sequence	1633	1633	100%	0	99%	KF924223.1
Methylobacterium sp. CBMB43 16S ribosomal RNA gene, partial sequence	1633	1633	100%	0	99%	EF126748.1
Methylobacterium pseudosasae strain BL44 16S ribosomal RNA, partial sequence	1631	1631	100%	0	99%	NR_108240.1
Methylobacterium pseudosasae strain BL44 16S ribosomal RNA gene, partial sequence	1631	1631	100%	0	99%	EU912442.1
Methylobacterium zatmanii strain PSBB041, complete genome	1629	8149	100%	0	99%	CP021054.1
Methylobacterium extorquens strain PSBB040, complete genome	1629	8149	100%	0	99%	CP019322.1
Methylobacterium extorquens partial 16S rRNA gene, isolate 5-3-1.1(1)	1629	1629	100%	0	99%	LT601404.1
Uncultured bacterium clone BJ201307-22 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	KX508034.1
Methylobacterium sp. EP_L_58 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	KJ642436.1
Methylobacterium extorquens strain IARI-IIWP-43 16S ribosomal RNA gene, partial	1629	1629	100%	0	99%	KF572999.1

sequence						
Methylobacterium sp. IARI-HHS2-69 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	KF572997.1
Methylobacterium zatmanii gene for 16S ribosomal RNA, partial sequence, strain: z15a	1629	1629	100%	0	99%	AB698688.1
Methylobacterium zatmanii gene for 16S ribosomal RNA, partial sequence, strain: 37d	1629	1629	100%	0	99%	AB698687.1
Uncultured bacterium clone 16slp87-03h04.p1ka 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	FJ512822.1
Methylobacterium sp. SuP10 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	EU912450.1
Methylobacterium sp. SuP6 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	EU912449.1
Methylobacterium sp. 602 partial 16S rRNA gene, strain 602	1629	1629	100%	0	99%	FN868956.1
Uncultured bacterium clone TF76 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	GU272244.1
Methylobacterium sp. HS_8 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	GQ365189.1
Methylobacterium sp. 5b.2.1 collection-date 15-Sep-2003 from Germany 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	FJ157971.1
Methylobacterium sp. 5b.1.5 collection-date 15-Sep-2003 from Germany 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	FJ157970.1
Methylobacterium sp. 5b.1.4 collection-date 15-Sep-2003 from Germany 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	FJ157969.1
Methylobacterium sp. 5a.1.8 collection-date 15-Sep-2003 from Germany 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	FJ157966.1
Methylobacterium sp. 1c.1 collection-date 15-Sep-2003 from Germany 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	FJ157962.1
Methylobacterium extorquens DM4 str. DM4 chromosome, complete genome	1629	8149	100%	0	99%	FP103042.2
Methylobacterium extorquens AM1, complete genome	1629	8149	100%	0	99%	CP001510.1
Uncultured bacterium clone nbw391h07c1 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	GQ096960.1
Uncultured bacterium clone nbw830c11c1 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	GQ009608.1
Uncultured bacterium clone nbw830a11c1 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	GQ009592.1
Uncultured bacterium clone nbw829g05c1 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	GQ009304.1
Methylobacterium sp. M417 gene for 16S rRNA, partial sequence, strain: M417	1629	1629	100%	0	99%	AB461759.1
Methylobacterium extorquens PA1, complete genome	1629	8149	100%	0	99%	CP000908.1
Methylobacterium extorquens gene for 16S rRNA, partial sequence, strain: TA5 (=NEU 48)	1629	1629	100%	0	99%	AB298399.1
Methylobacterium sp. CBMB38 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	EF165044.1
Methylobacterium sp. TMAH-R0422 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	EF062507.1
Methylobacterium sp. GW2 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	DQ400509.1
Methylobacterium extorquens 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	AF531770.1
Methylobacterium sp. P1 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	AF148859.2
Methylobacterium sp. iRIII1 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	AY358000.2
Methylobacterium extorquens strain IAM 12631 16S ribosomal RNA gene, partial	1629	1629	100%	0	99%	NR_112230.1

sequence						
Methylobacterium extorquens 16S ribosomal RNA gene, partial sequence; tRNA-Ile and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	AF293375.1
Uncultured alpha proteobacterium partial 16S rRNA gene, clone MTAA10	1629	1629	100%	0	99%	AJ964950.1
Methylobacterium sp. 16S rRNA gene	1629	1629	100%	0	99%	D25305.1
Methylobacterium sp. 15R46 16S ribosomal RNA gene, partial sequence	1628	1628	100%	0	99%	KR094821.1
Uncultured Methylobacterium sp. clone SB6-7 16S ribosomal RNA gene, partial sequence	1628	1628	100%	0	99%	KJ197814.1
Uncultured Methylobacterium sp. clone SB6-39 16S ribosomal RNA gene, partial sequence	1628	1628	100%	0	99%	KJ197807.1
Uncultured Methylobacterium sp. clone SB6-34 16S ribosomal RNA gene, partial sequence	1628	1628	100%	0	99%	KJ197803.1
Uncultured Methylobacterium sp. clone SB6-32 16S ribosomal RNA gene, partial sequence	1628	1628	100%	0	99%	KJ197802.1
Uncultured Methylobacterium sp. clone SB6-2 16S ribosomal RNA gene, partial sequence	1628	1628	100%	0	99%	KJ197791.1
Methylobacterium extorquens strain Fab1 16S ribosomal RNA gene, partial sequence	1628	1628	99%	0	99%	GU992345.1
Methylobacterium sp. JW66.2a partial 16S rRNA gene, strain JW66.2a	1628	1628	100%	0	99%	FN556577.1
Uncultured Methylobacterium sp. clone SB6-8 16S ribosomal RNA gene, partial sequence	1626	1626	99%	0	99%	KJ197815.1
Uncultured Methylobacterium sp. clone SB6-16 16S ribosomal RNA gene, partial sequence	1626	1626	99%	0	99%	KJ197787.1
Uncultured bacterium clone JPL1_46 16S ribosomal RNA gene, partial sequence	1626	1626	100%	0	99%	DQ532136.1
Uncultured bacterium clone BJ201307-47 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	KX508056.1
Methylobacterium extorquens strain QTYC47b 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	KM974666.1
Methylobacterium sp. EP_L_93 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	KJ642434.1
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Uncultured Methylobacterium sp. clone SB6-37 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	KJ197805.1
Uncultured Methylobacterium sp. clone SB6-29 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	KJ197800.1
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Uncultured Methylobacterium sp. clone SB6-19 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	KJ197790.1
Uncultured Methylobacterium sp. clone SB6-15 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	KJ197786.1
Uncultured Methylobacterium sp. clone SB6-1 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	KJ197782.1
Methylobacterium extorquens strain CM4 16S ribosomal RNA gene, complete sequence	1624	1624	100%	0	99%	NR_074215.1
Methylobacterium sp. Bg2z partial 16S rRNA gene, isolate Bg2z	1624	1624	100%	0	99%	HF548411.1
Uncultured bacterium clone A45 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	JN882050.1
Uncultured organism clone ELU0028-T175-S-NIPCRAMgANa_000113 small subunit ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	HQ749723.1
Uncultured organism clone ELU0028-T175-S-NIPCRAMgANa_000035 small subunit ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	HQ749645.1
Uncultured organism clone ELU0021-T97-S-NI_000060 small subunit ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	HQ746582.1
Uncultured bacterium clone ncd2777f09c1 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	JF238902.1

Uncultured bacterium clone ncd2763a01c1 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	JF223114.1
Uncultured bacterium clone ncd2761c08c1 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	JF223072.1
Uncultured bacterium clone ncd29b03c1 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	HM252364.1
Methylobacterium zatmanii strain 6012 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	GU294335.1
Methylobacterium zatmanii strain 7211 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	GU294329.1
Uncultured bacterium clone TF78 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	GU272245.1
Uncultured bacterium clone 208 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	GU225982.1
Uncultured bacterium partial 16S rRNA gene, clone 13_F01	1624	1624	100%	0	99%	FN421635.1
Methylobacterium zatmanii strain NBCS25 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	GQ281065.1
Uncultured bacterium clone nbw829f02c1 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	GQ009291.1
Methylobacterium extorquens CM4, complete genome	1624	8121	100%	0	99%	CP001298.1
Methylobacterium extorquens partial 16S rRNA gene, strain F46	1624	1624	100%	0	99%	AM910538.1
Methylobacterium extorquens strain JSCtot103 16S ribosomal RNA (rrn) gene, partial sequence	1624	1624	99%	0	99%	DQ870723.1
Uncultured bacterium clone JSC9-I 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	DQ532265.1
Methylobacterium sp. iRIV1 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	AY358005.2
Methylobacterium extorquens gene for 16S ribosomal RNA, partial sequence, strain:NCIMB 13688	1624	1624	100%	0	99%	AB175630.1
Uncultured bacterium clone 16slp108-1f08.w2k 16S ribosomal RNA gene, partial sequence	1622	1622	100%	0	99%	GQ157975.1
Methylobacterium extorquens strain Hab1 16S ribosomal RNA gene, partial sequence	1622	1622	99%	0	99%	GU992354.1
Methylobacterium extorquens strain Fab2 16S ribosomal RNA gene, partial sequence	1622	1622	99%	0	99%	GU992346.1
Methylobacterium sp. D1B20 partial 16S rRNA gene, isolate D1B20	1620	1620	100%	0	99%	LN614691.1
Uncultured Methylobacterium sp. clone SB6-41 16S ribosomal RNA gene, partial sequence	1620	1620	99%	0	99%	KJ197809.1
Methylobacterium extorquens strain VRI2-7 16S ribosomal RNA gene, partial sequence	1618	1618	100%	0	99%	KY882061.1
Methylobacterium extorquens strain VRI2-2 16S ribosomal RNA gene, partial sequence	1618	1618	100%	0	99%	KY882058.1
Methylobacterium thiocyanatum strain TMV2-1 16S ribosomal RNA gene, partial sequence	1618	1618	100%	0	99%	KY882043.1
Uncultured bacterium clone DWTP1.3B.H04 16S ribosomal RNA gene, partial sequence	1618	1618	100%	0	99%	KU713506.1
Uncultured bacterium clone DWTP1.3A.F10 16S ribosomal RNA gene, partial sequence	1618	1618	100%	0	99%	KU713404.1

203-16S-rRNA-R-ab1

GGTCGCCTGCCTCCTTGCNGGTTGGCGCAGCGCCGTCGGGTAAGACCAACTCCCATGGTGTGACGGGCGGTGTGT
ACAAGGCCCGGGAACGTATTCACCGTGGCGTGCTGATCCACGATTACTAGCGATTCCGCCTTCATGCACCCGAGTT
GCAGAGTGCAATCCGAACTGAGACGGTTTTTGGGGATTTGCTCCACCTCGCGGCTTCGCGTCCCACTGTCACCGCC
ATTGTAGCACGTGTGTAGCCCATCCCGTAAGGGCCATGAGGACTTGACGTCATCCACACCTTCCTCGCGGCTTATC
ACCGGCAGTCTCCCTAGAGTGCCCAACTGAATGATGGCAACTAAGGACGTGGGTTGCGCTCGTTGCGGGACTTAA
CCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTGCACGCCTCCGAAGAGGATCCCCGATCT

CTCGAGGTAACATGCCATGTCAAGGGATGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCT
TGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGAATGCTTAATGCGTTAGC
GGCGCCACTGACCTGCAAGCAGGCCAACGGCTGGCATTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCC
TGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGAACCGGACCAGACAGCCGCCTTCGCCACTGGTGTTCTTGCG
AATATCTACGAATTTCACCTCTACACTCGCAGTTCCGCTGTCCTCTTCCGGTCTCAAGCCAACCAGTATCGAAGGCA
ATTCTGTGGTTGAGCCACAGGCTTTCACCCCCGACTTAATCGGCCGCCTACGCGCCCTTT

	Max score	Total score	Query cover	E value	Ident	Accession
Methylobacterium zatmanii strain PSBB041, complete genome	1646	8232	100%	0	99%	CP021054.1
Methylobacterium extorquens strain PSBB040, complete genome	1646	8232	100%	0	99%	CP019322.1
Methylobacterium extorquens partial 16S rRNA gene, isolate 5-3-1.1(2)	1646	1646	100%	0	99%	LT601405.1
Uncultured bacterium clone BJ201307-22 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	KX508034.1
Methylobacterium sp. AMS5, complete genome	1646	8232	100%	0	99%	CP006992.1
Methylobacterium extorquens strain QTYC47b 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	KM974666.1
Methylobacterium extorquens strain 11.2 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	KF681062.1
Methylobacterium zatmanii strain M0505 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	KF924223.1
Methylobacterium extorquens strain IARI-IIWP-43 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	KF572999.1
Methylobacterium sp. IARI-HHS2-69 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	KF572997.1
Methylobacterium extorquens strain MS1 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	KC625555.1
Methylobacterium extorquens strain CM4 16S ribosomal RNA gene, complete sequence	1646	1646	100%	0	99%	NR_074215.1
Methylobacterium zatmanii gene for 16S ribosomal RNA, partial sequence, strain: z15a	1646	1646	100%	0	99%	AB698688.1
Methylobacterium zatmanii gene for 16S ribosomal RNA, partial sequence, strain: 37d	1646	1646	100%	0	99%	AB698687.1
Uncultured bacterium clone A45 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	JN882050.1
Methylobacterium sp. AMS5 gene for 16S rRNA, partial sequence	1646	1646	100%	0	99%	AB600003.1
Methylobacterium sp. SuP10 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	EU912450.1
Methylobacterium sp. SuP6 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	EU912449.1
Uncultured bacterium clone 16slp120-1d12.p1k 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	GQ157319.1
Uncultured bacterium clone 208 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	GU225982.1
Methylobacterium sp. 5b.2.1 collection-date 15-Sep-2003 from Germany 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	FJ157971.1
Methylobacterium sp. 5b.1.5 collection-date 15-Sep-2003 from Germany 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	FJ157970.1
Methylobacterium sp. 5b.1.4 collection-date 15-Sep-2003 from Germany 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	FJ157969.1
Methylobacterium sp. 5a.1.8 collection-date 15-Sep-2003 from Germany 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	FJ157966.1
Methylobacterium extorquens DM4 str. DM4 chromosome, complete genome	1646	8232	100%	0	99%	FP103042.2
Methylobacterium extorquens AM1, complete genome	1646	8232	100%	0	99%	CP001510.1
Methylobacterium extorquens CM4, complete genome	1646	8232	100%	0	99%	CP001298.1
Methylobacterium extorquens PA1, complete genome	1646	8232	100%	0	99%	CP000908.1

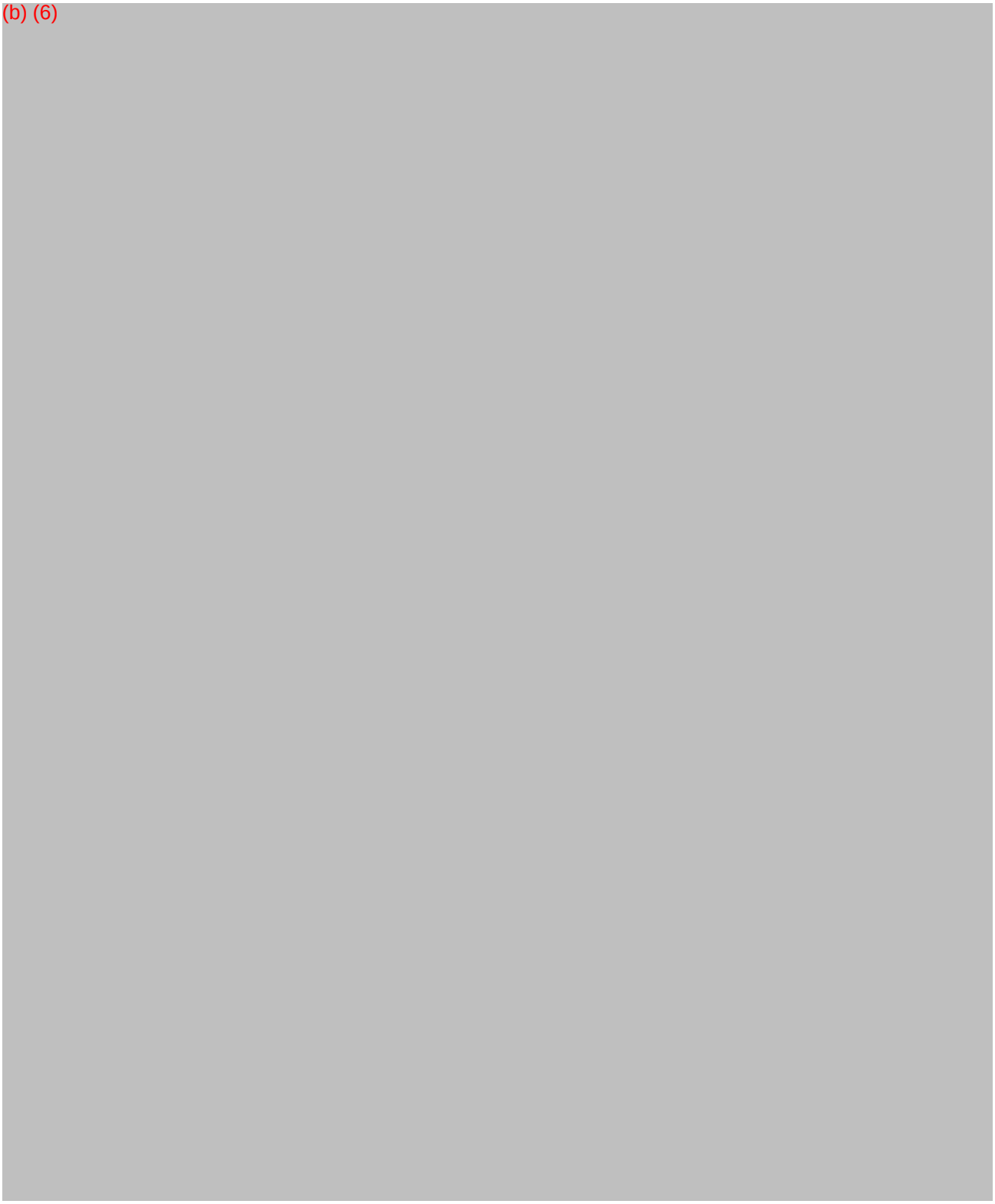
Methylobacterium sp. CBMB38 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	EF165044.1
Methylobacterium sp. CBMB43 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	EF126748.1
Methylobacterium sp. GW2 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	DQ400509.1
Uncultured bacterium clone JSC9-I 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	DQ532265.1
Methylobacterium extorquens 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	AF531770.1
Methylobacterium sp. P1 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	AF148859.2
Methylobacterium sp. iRIII1 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	AY358000.2
Methylobacterium extorquens strain IAM 12631 16S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	NR_112230.1
Methylobacterium extorquens gene for 16S ribosomal RNA, partial sequence, strain:NCIMB 13688	1646	1646	100%	0	99%	AB175630.1
Methylobacterium extorquens 16S ribosomal RNA gene, partial sequence; tRNA-Ile and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	1646	1646	100%	0	99%	AF293375.1
Uncultured alpha proteobacterium partial 16S rRNA gene, clone MTAA10	1646	1646	100%	0	99%	AJ964950.1
Uncultured bacterium partial 16S rRNA gene, clone 13_F01	1642	1642	100%	0	99%	FN421635.1
Uncultured bacterium clone JPL1_46 16S ribosomal RNA gene, partial sequence	1642	1642	100%	0	99%	DQ532136.1
Methylobacterium sp. iRIV1 16S ribosomal RNA gene, partial sequence	1642	1642	100%	0	99%	AY358005.2
Methylobacterium sp. iRII2 16S ribosomal RNA gene, partial sequence	1642	1642	100%	0	99%	AY357997.2
Methylobacterium sp. Bg2z partial 16S rRNA gene, isolate Bg2z	1640	1640	100%	0	99%	HF548411.1
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 0502TCLN014	1640	1640	100%	0	99%	AB695746.1
Methylobacterium sp. 602 partial 16S rRNA gene, strain 602	1640	1640	100%	0	99%	FN868956.1
Methylobacterium sp. DC2c-19 gene for 16S rRNA, partial sequence	1640	1640	100%	0	99%	AB552870.1
Uncultured bacterium clone 16slp108-1f08.w2k 16S ribosomal RNA gene, partial sequence	1640	1640	100%	0	99%	GQ157975.1
Methylobacterium chloromethanicum strain PVAS3 16S ribosomal RNA gene, partial sequence	1640	1640	100%	0	99%	GU130527.1
Methylobacterium chloromethanicum strain PVAS2 16S ribosomal RNA gene, partial sequence	1640	1640	100%	0	99%	GU130526.1
Methylobacterium sp. HS_8 16S ribosomal RNA gene, partial sequence	1640	1640	100%	0	99%	GQ365189.1
Uncultured bacterium partial 16S rRNA gene, clone 4_G01	1640	1640	100%	0	99%	FN421767.1
Uncultured bacterium partial 16S rRNA gene, clone 4_C03	1640	1640	100%	0	99%	FN421734.1
Uncultured bacterium partial 16S rRNA gene, clone 13_E04	1640	1640	100%	0	99%	FN421629.1
Methylobacterium sp. 1c.1 collection-date 15-Sep-2003 from Germany 16S ribosomal RNA gene, partial sequence	1640	1640	100%	0	99%	FJ157962.1
Methylobacterium extorquens partial 16S rRNA gene, strain F46	1640	1640	100%	0	99%	AM910538.1
Methylobacterium extorquens gene for 16S rRNA, partial sequence, strain: TA5 (=NEU 48)	1640	1640	100%	0	99%	AB298399.1
Methylobacterium sp. sh_20 16S ribosomal RNA gene, partial sequence	1639	1639	100%	0	99%	EU878886.1
Methylobacterium pseudosasae strain BL44 16S ribosomal RNA, partial sequence	1637	1637	100%	0	99%	NR_108240.1
Alpha proteobacterium F3H1_a8 16S ribosomal RNA gene, partial sequence	1637	1637	100%	0	99%	KF641676.1
Methylobacterium pseudosasae strain BL44 16S ribosomal RNA gene, partial sequence	1637	1637	100%	0	99%	EU912442.1
Methylobacterium extorquens strain VRI2-7 16S ribosomal RNA gene, partial sequence	1635	1635	100%	0	99%	KY882061.1
Methylobacterium extorquens strain VRI2-2 16S ribosomal RNA gene, partial sequence	1635	1635	100%	0	99%	KY882058.1
Uncultured bacterium clone BJ201307-47 16S ribosomal RNA gene, partial sequence	1635	1635	100%	0	99%	KX508056.1

Methylobacterium zatmanii partial 16S rRNA gene, isolate Bts 17	1635	1635	100%	0	99%	HG975596.1
Uncultured bacterium clone 16slp120-1e11.w2k 16S ribosomal RNA gene, partial sequence	1635	1635	100%	0	99%	GQ157320.1
Methylobacterium zatmanii strain NBCS25 16S ribosomal RNA gene, partial sequence	1635	1635	100%	0	99%	GQ281065.1
Uncultured bacterium clone S2-5-CL33 16S ribosomal RNA gene, partial sequence	1635	1635	100%	0	99%	EU769150.1
Methylobacterium sp. 16S rRNA gene	1635	1635	100%	0	99%	D25305.1
Methylobacterium extorquens strain 11.6 16S ribosomal RNA gene, partial sequence	1633	1633	99%	0	99%	KF681061.1
Methylobacterium sp. sh_28 16S ribosomal RNA gene, partial sequence	1631	1631	100%	0	99%	EU878893.1
Methylobacterium populi strain ICGV-1 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	KY882108.1
Methylobacterium populi strain N3-3 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	KY882097.1
Methylobacterium populi strain VRI2-A3 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	KY882063.1
Methylobacterium populi strain VRI2-3 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	KY882059.1
Methylobacterium extorquens partial 16S rRNA gene, strain MMB1	1629	1629	100%	0	99%	LT594351.1
Uncultured Methylobacterium sp. clone KWLB236 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	KU519610.1
Methylobacterium populi DNA, complete genome	1629	8149	100%	0	99%	AP014809.1
Methylobacterium populi strain VP2 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	KF955558.1
Methylobacterium thiocyanatum gene for 16S ribosomal RNA, partial sequence, strain: z4h	1629	1629	100%	0	99%	AB698683.1
Methylobacterium thiocyanatum gene for 16S ribosomal RNA, partial sequence, strain: 87a	1629	1629	100%	0	99%	AB698682.1
Methylobacterium populi strain L9-464 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	JQ659498.1
Methylobacterium populi strain L6-306 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	JQ659381.1
Methylobacterium sp. PX15_S1 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	JF274929.1
Methylobacterium sp. D24(2010) 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	GU566357.1
Methylobacterium sp. AL1(2010) 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	GU566336.1
Methylobacterium zatmanii strain 6012 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	GU294335.1
Methylobacterium zatmanii strain 7211 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	GU294329.1
Methylobacterium sp. CSL-1 gene for 16S rRNA, partial sequence	1629	1629	100%	0	99%	AB500944.1
Methylobacterium sp. 13635J 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	EU741082.1
Methylobacterium sp. 13632G 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	EU741076.1
Uncultured alpha proteobacterium clone S1-10-CL6 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	AY728074.1
Methylobacterium extorquens 16S ribosomal RNA gene, partial sequence	1629	1629	100%	0	99%	AF267912.1
Uncultured bacterium clone 16slp117-2d12.p1k 16S ribosomal RNA gene, partial sequence	1628	1628	100%	0	99%	GQ157867.1
Uncultured bacterium clone 16slp124-4a10.p1k 16S ribosomal RNA gene, partial sequence	1628	1628	100%	0	99%	GQ157434.1
Methylobacterium sp. sh_30 16S ribosomal RNA gene, partial sequence	1628	1628	99%	0	99%	EU878895.1
Methylobacterium populi strain L9-478 16S ribosomal RNA gene, partial sequence	1626	1626	100%	0	99%	JQ659500.1
Methylobacterium populi strain M2-1 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	KY882116.1
Methylobacterium populi strain M1-2 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	KY882115.1
Methylobacterium populi strain CO6-3 16S ribosomal RNA gene, partial sequence	1624	1624	100%	0	99%	KY882101.1

Appendix 2-2. Genetic constructions to delete (b) (4) and (b) (4)

Section A. Methodology used in the Deletions

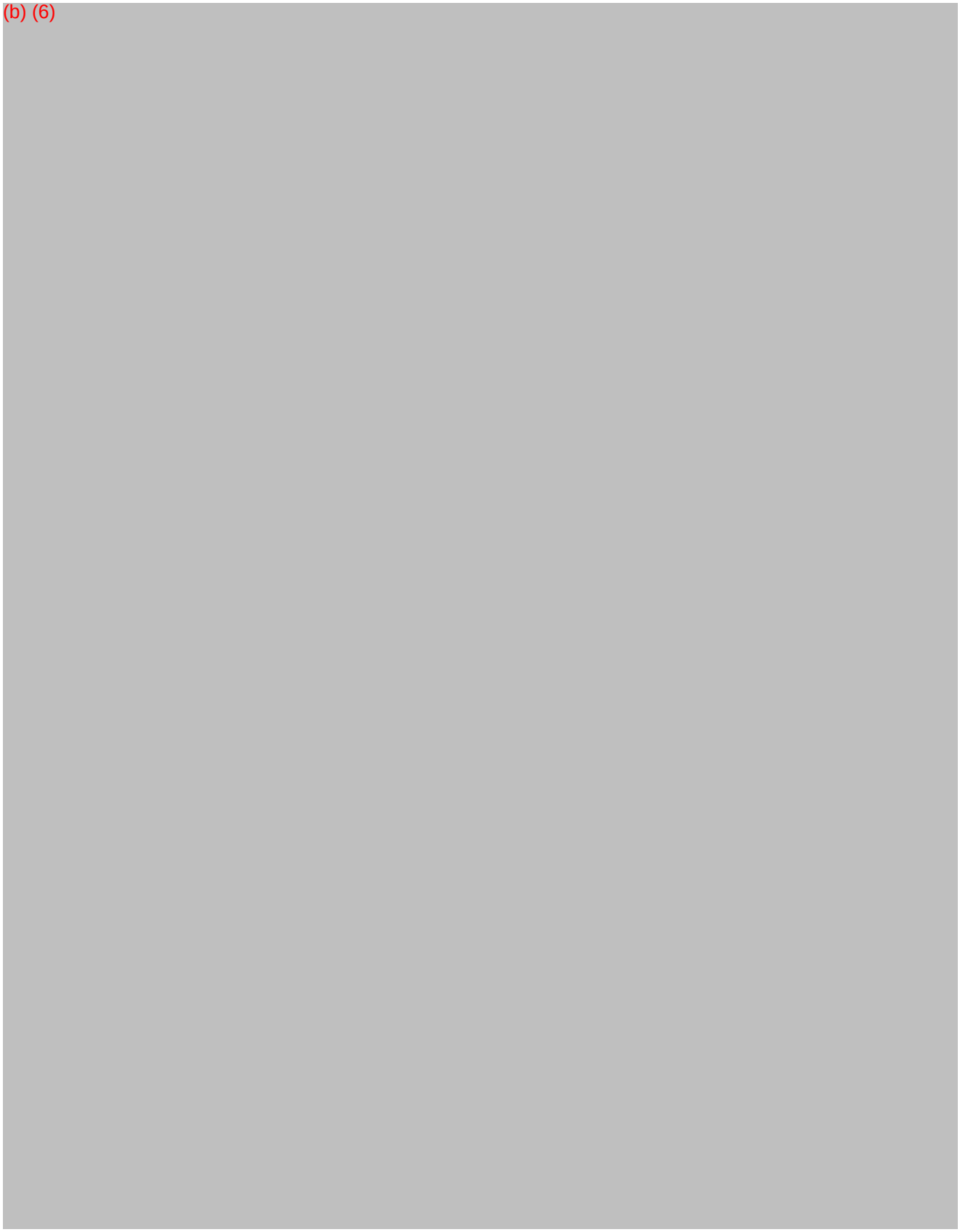
(b) (6)



(b) (6)

Section B: Verification of Deletion of Cellulase Pathway

(b) (6)



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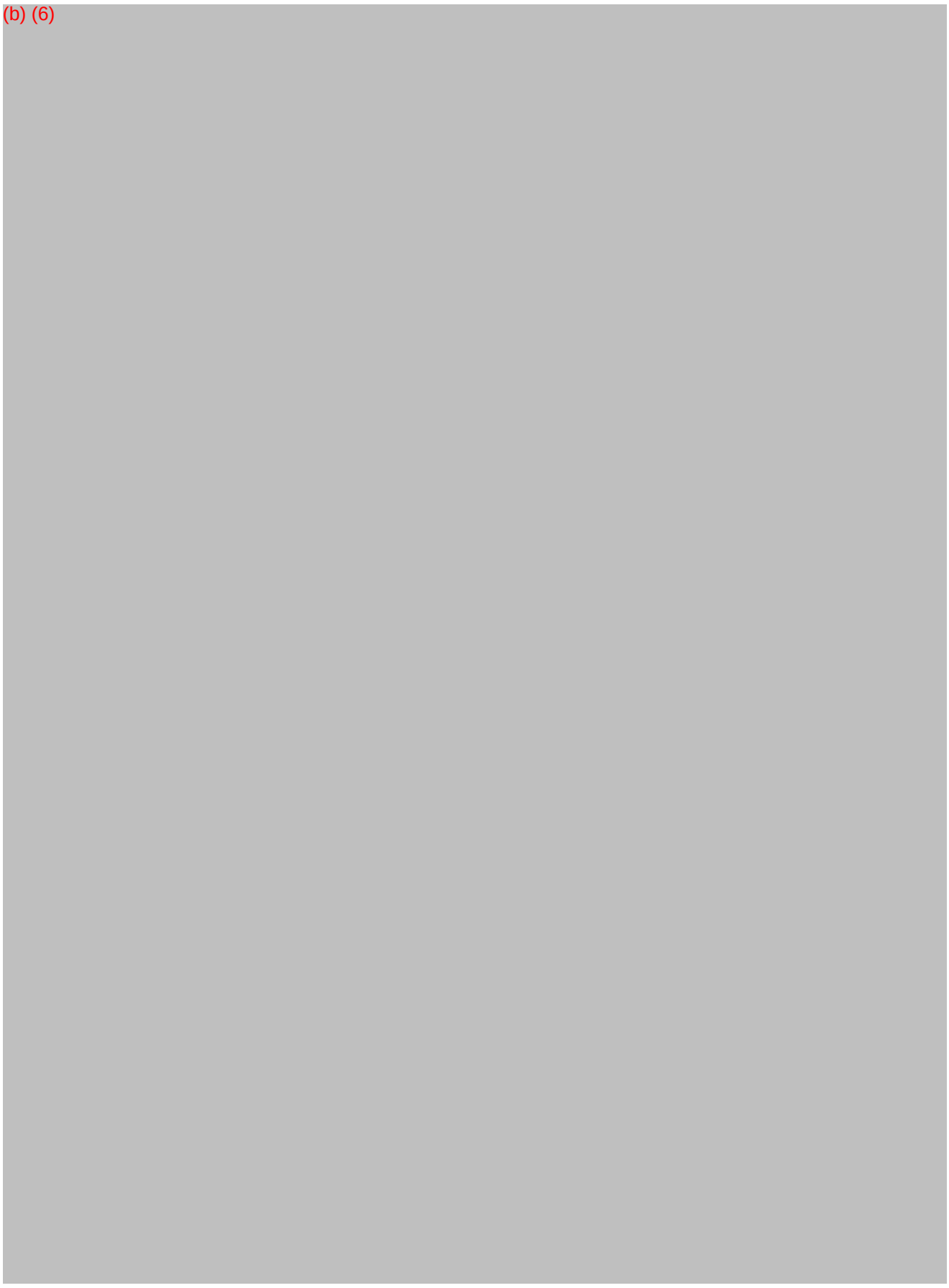

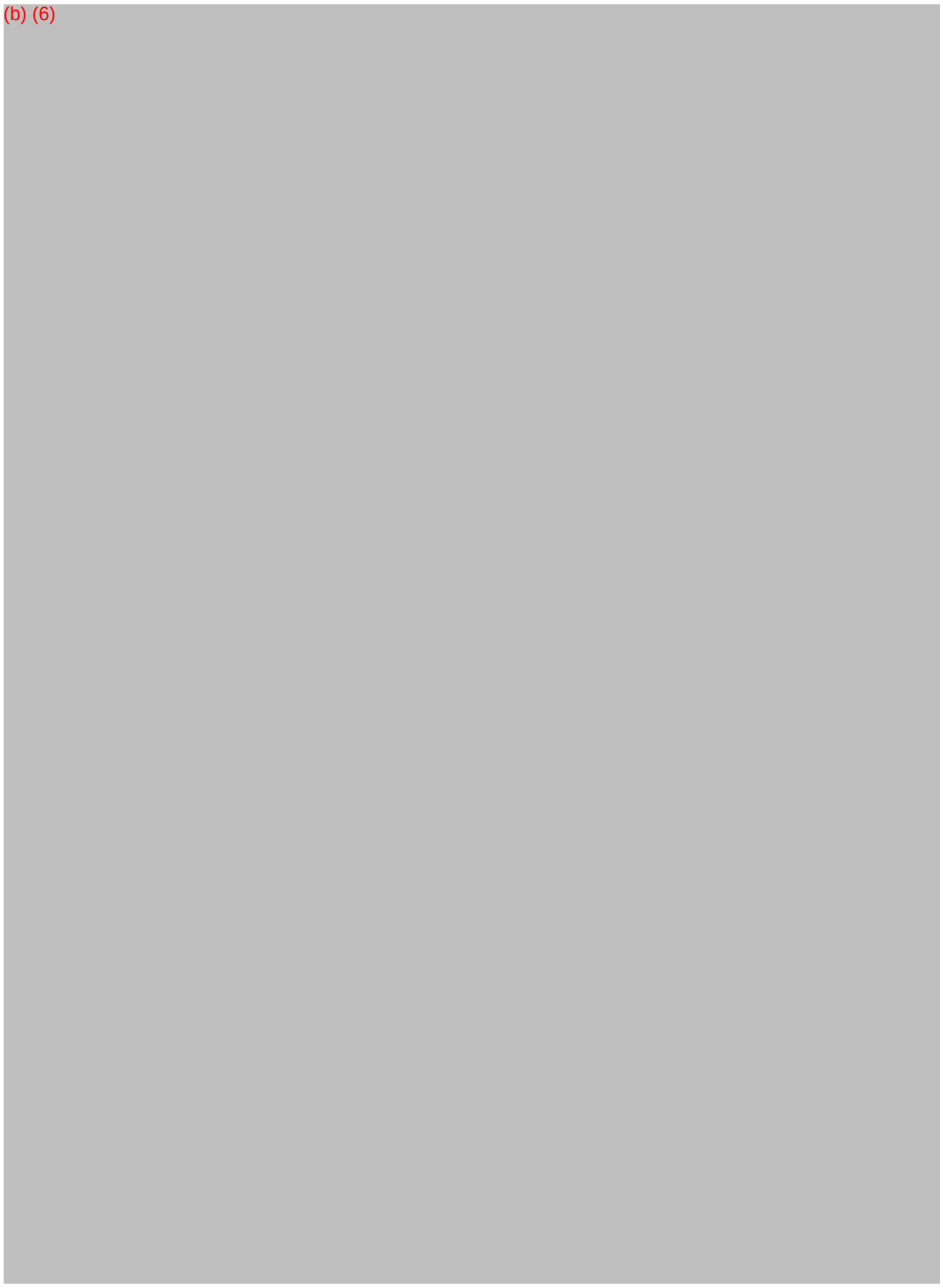
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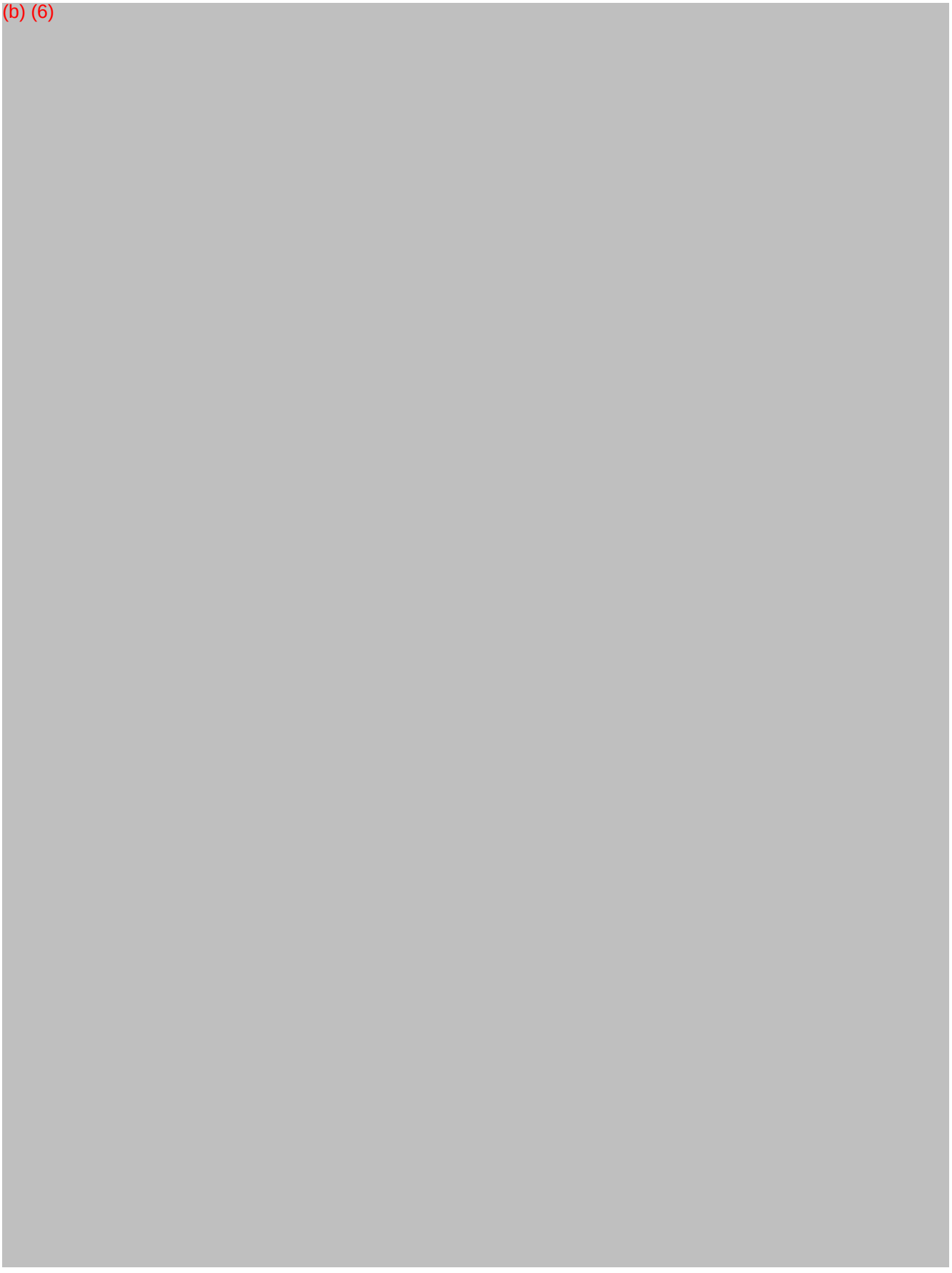
(b) (6)

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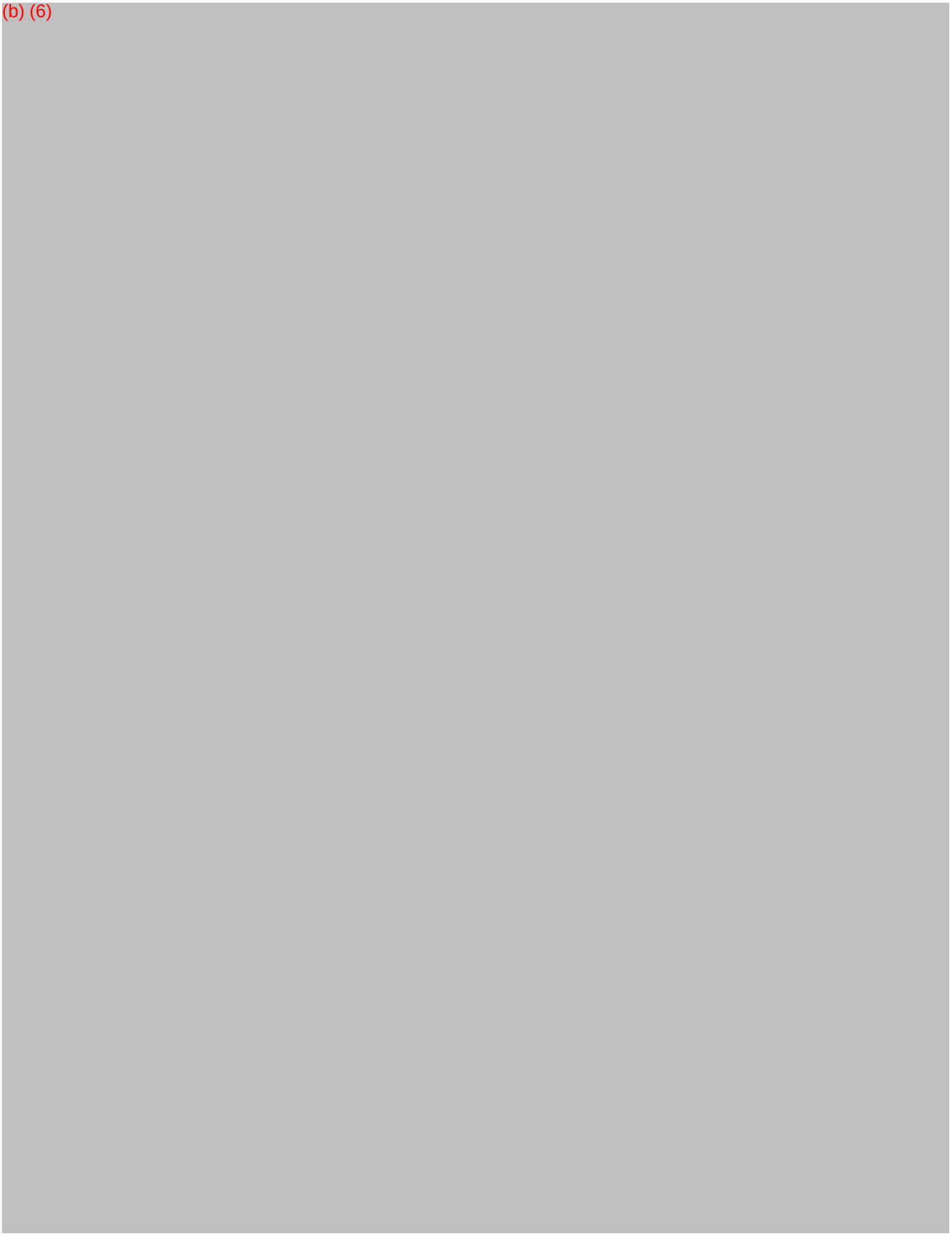
(b) (6)



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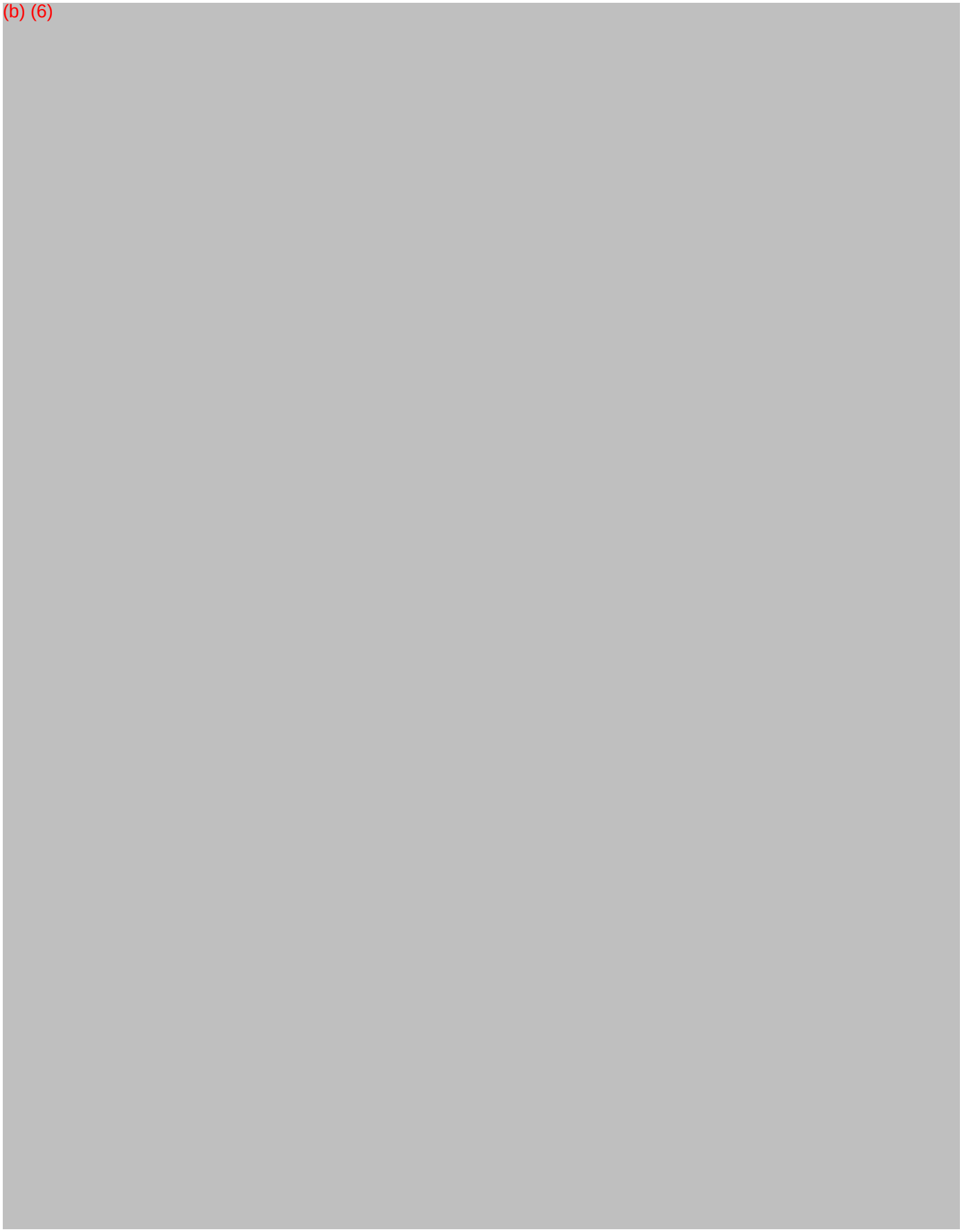

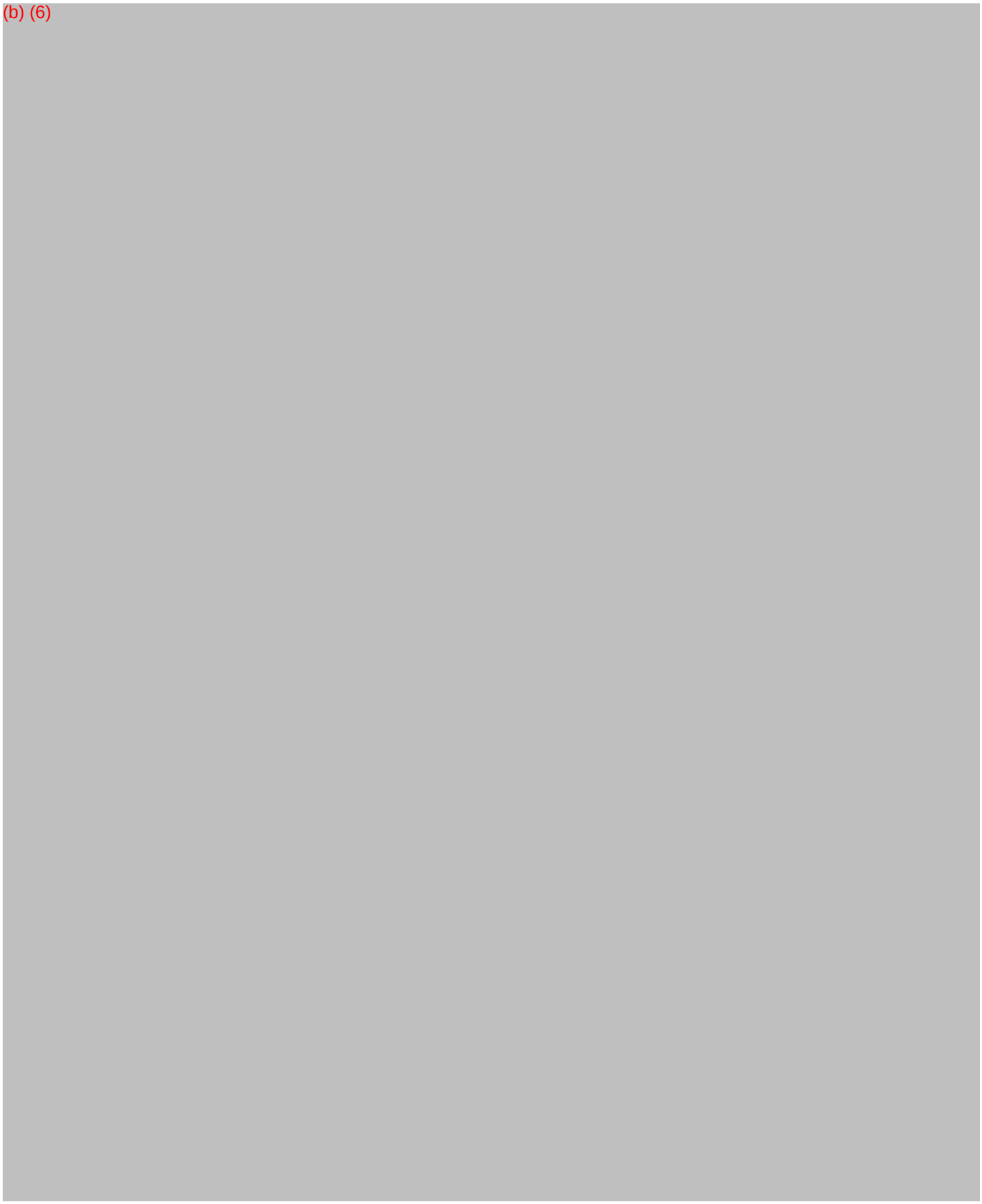
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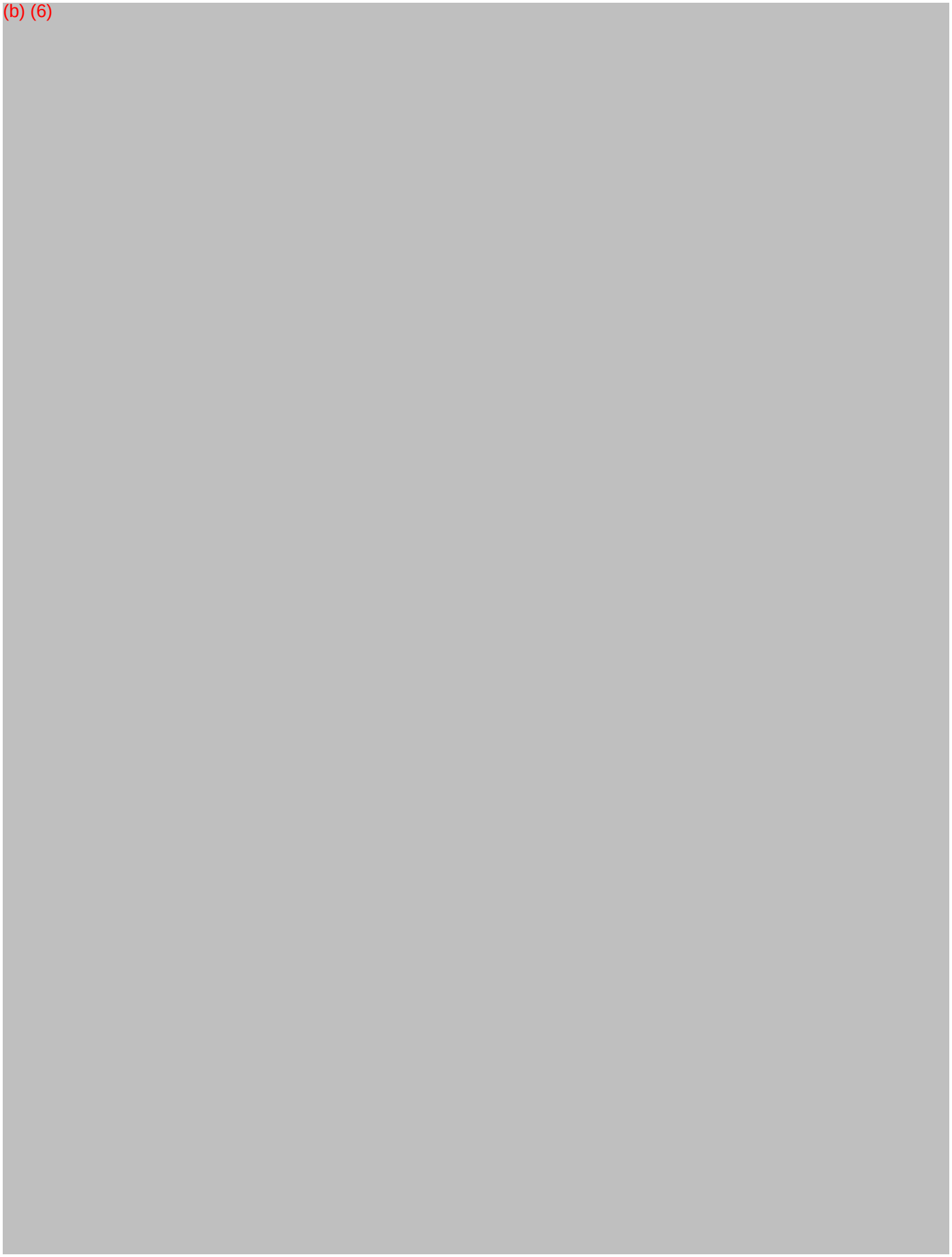
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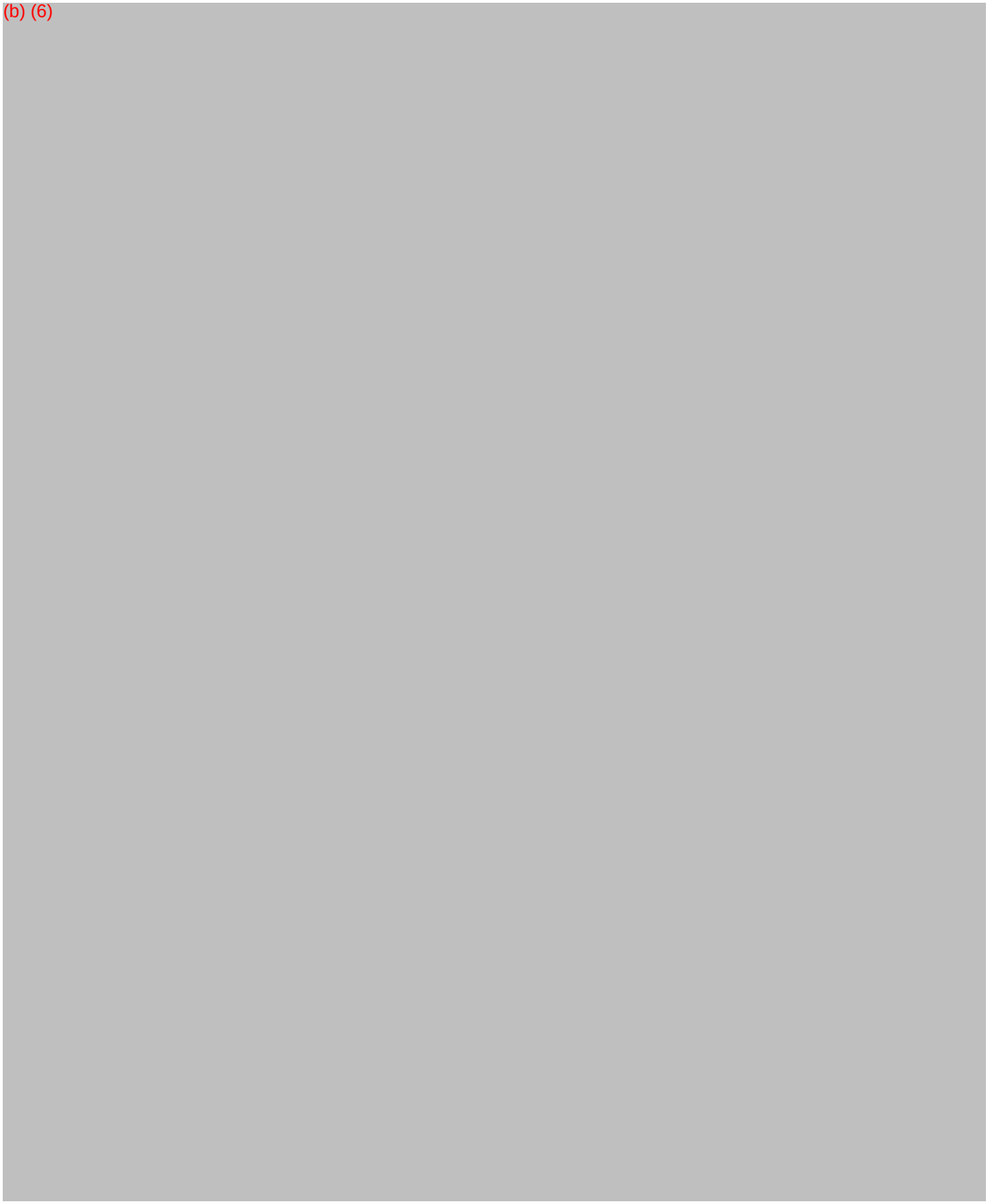
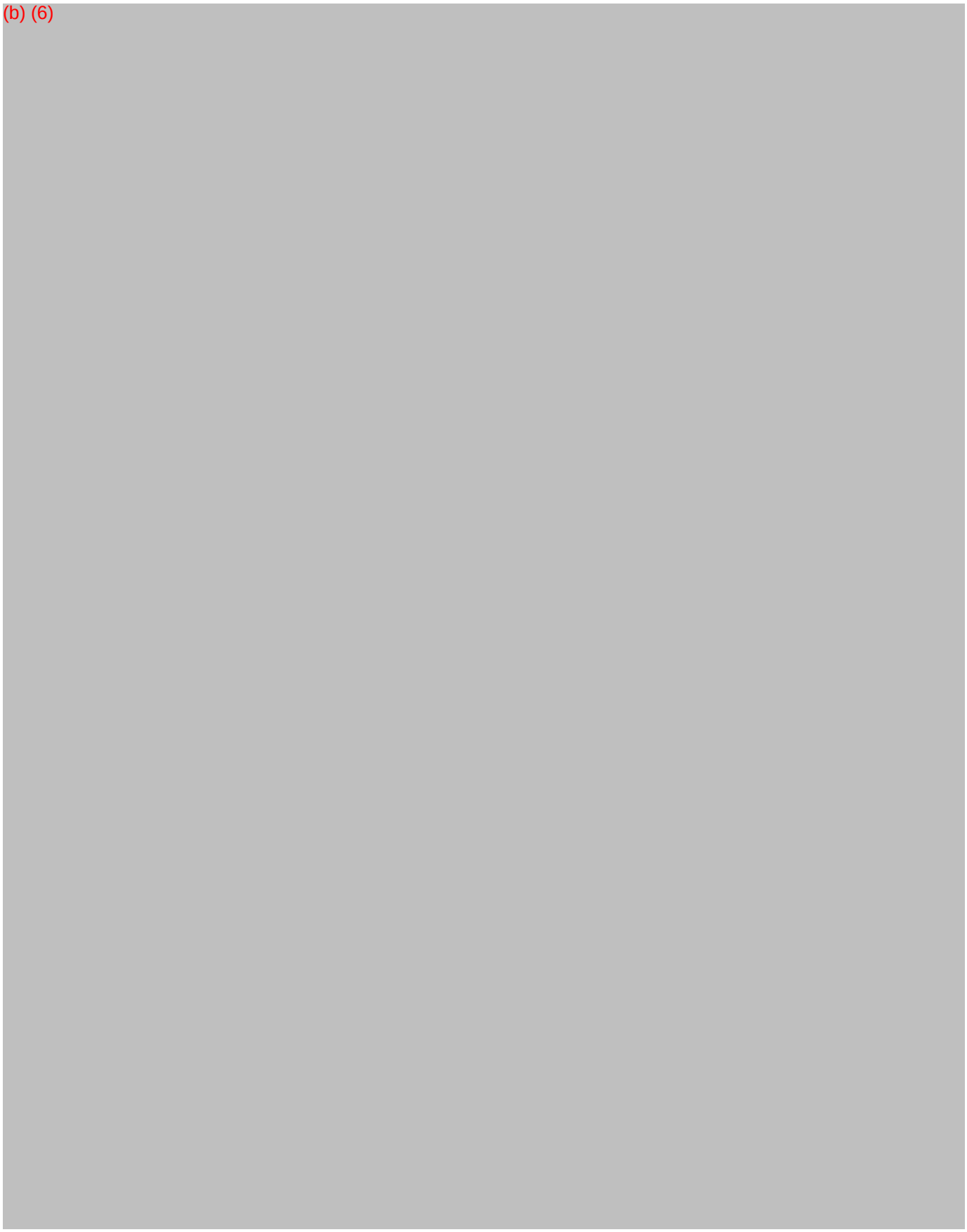

(b) (6)


(b) (6)

Section C: Verification of Deletion of Spirilloxanthin Pathway

(b) (6)



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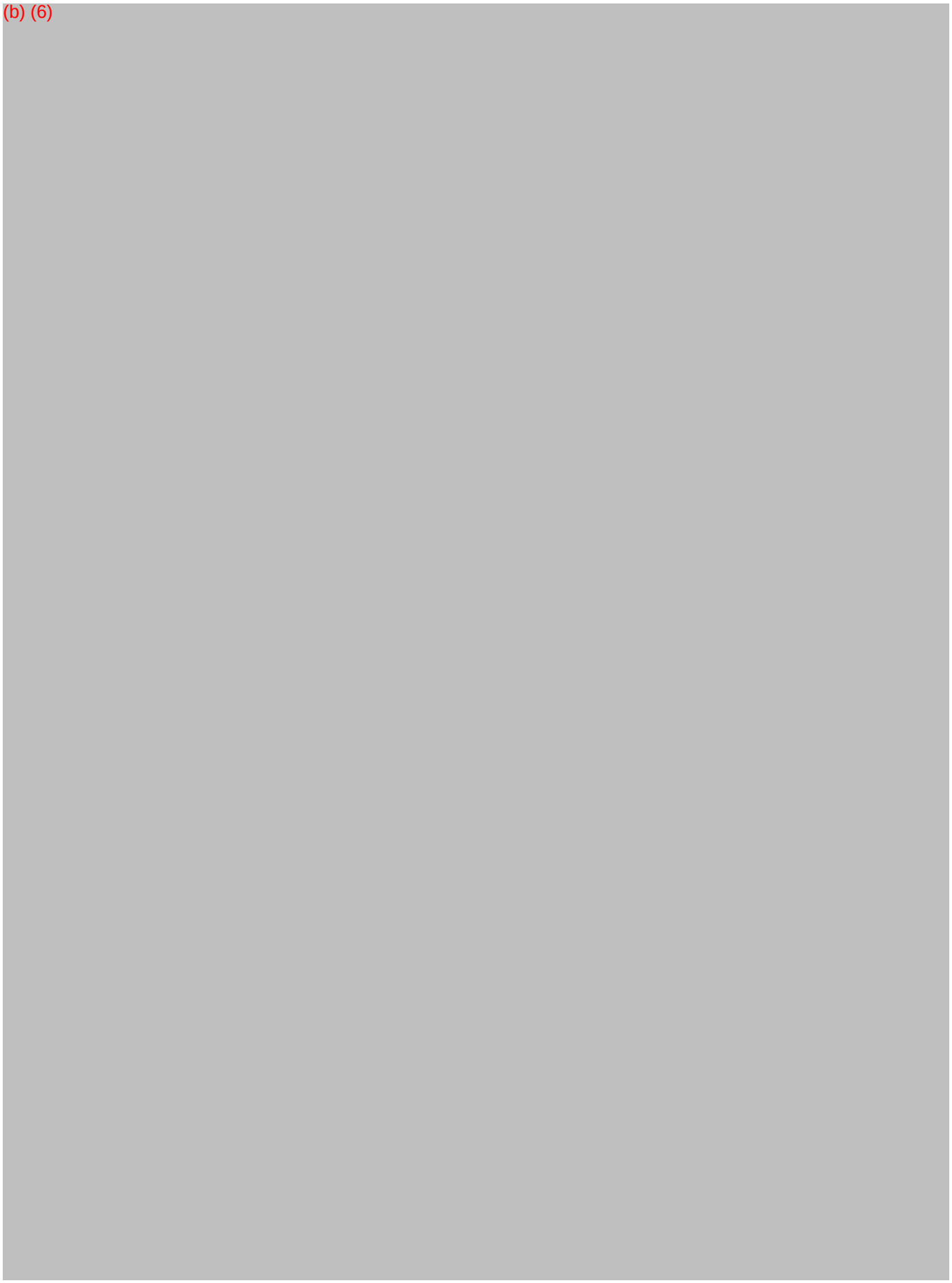

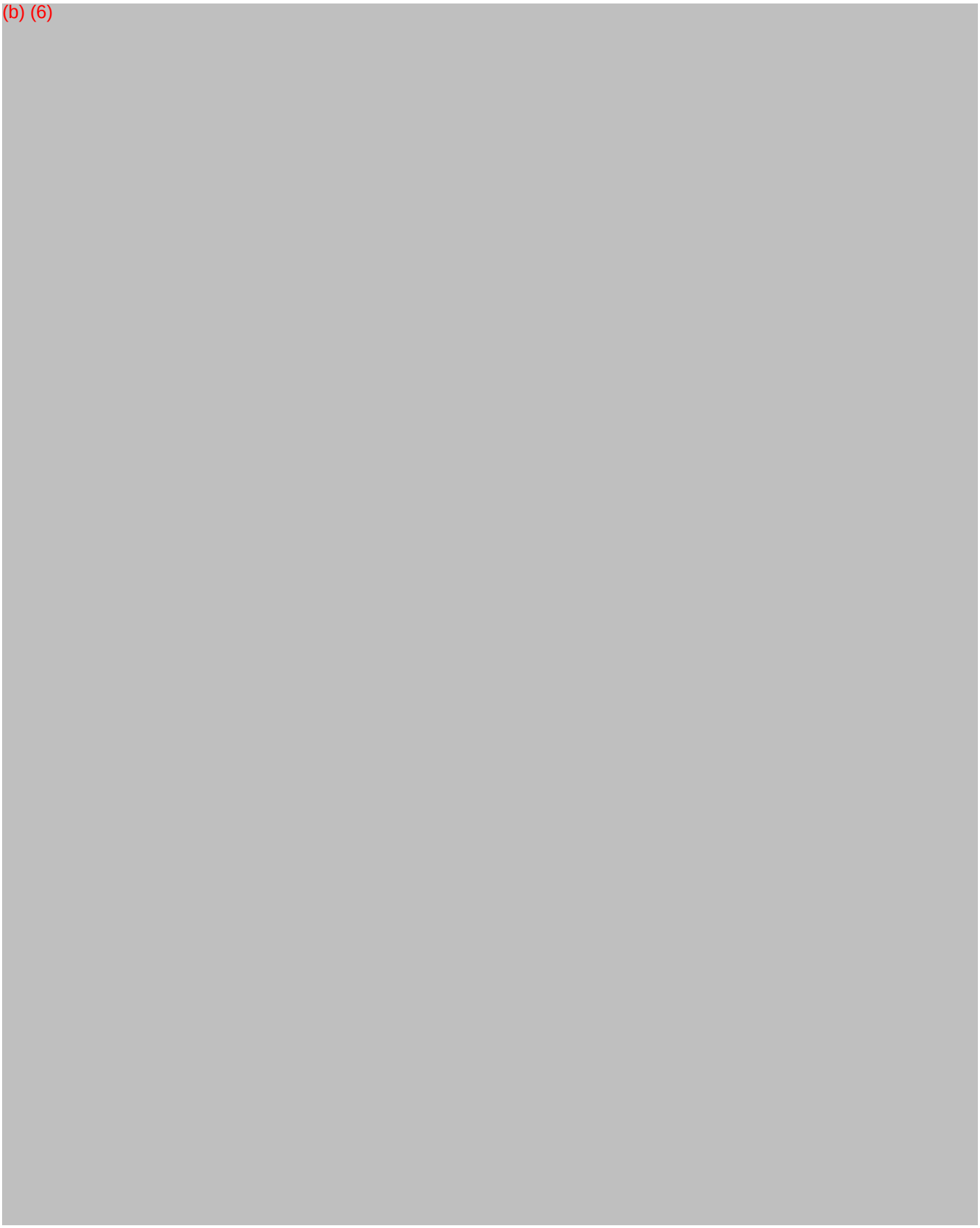
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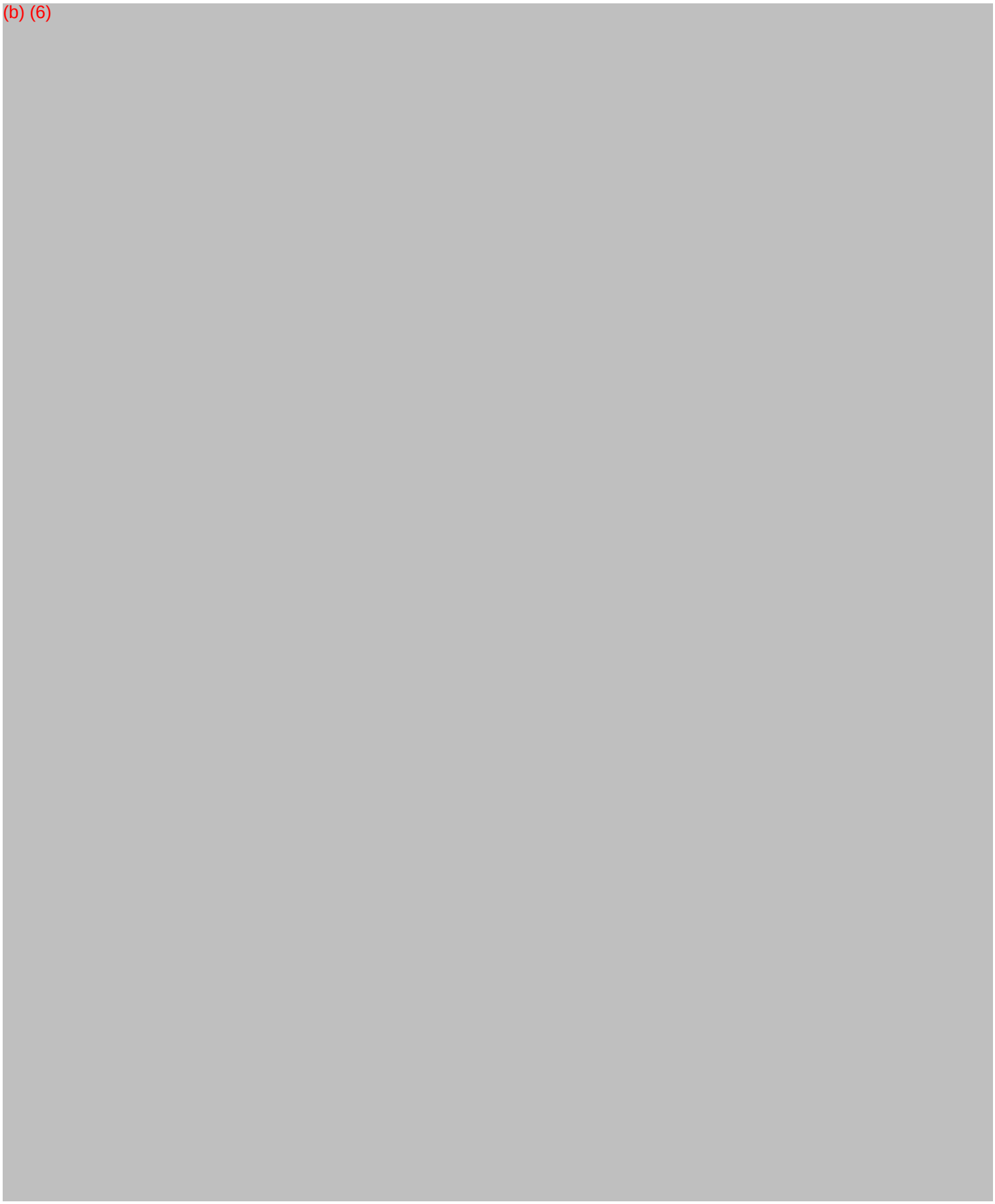

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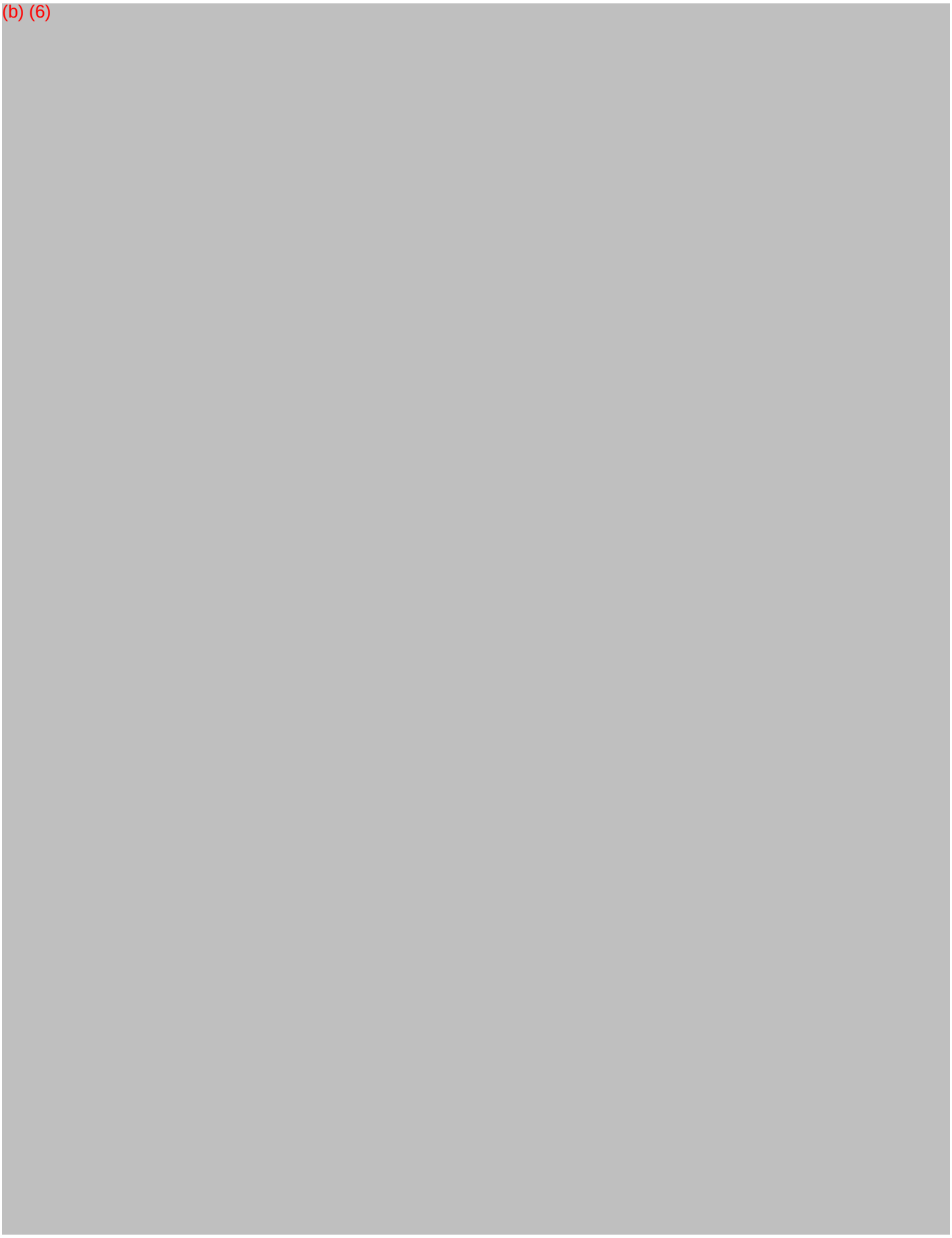
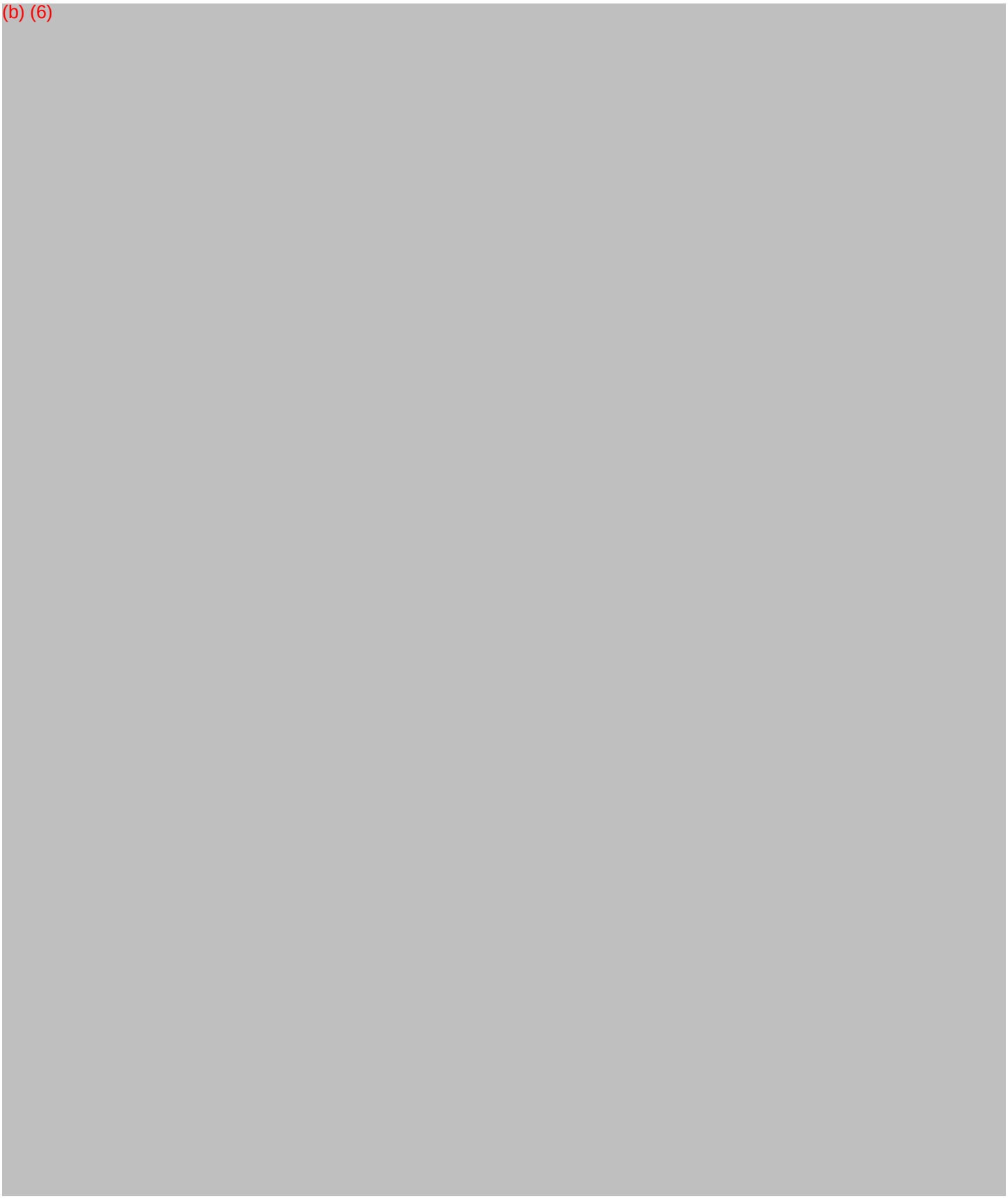



(b) (6)

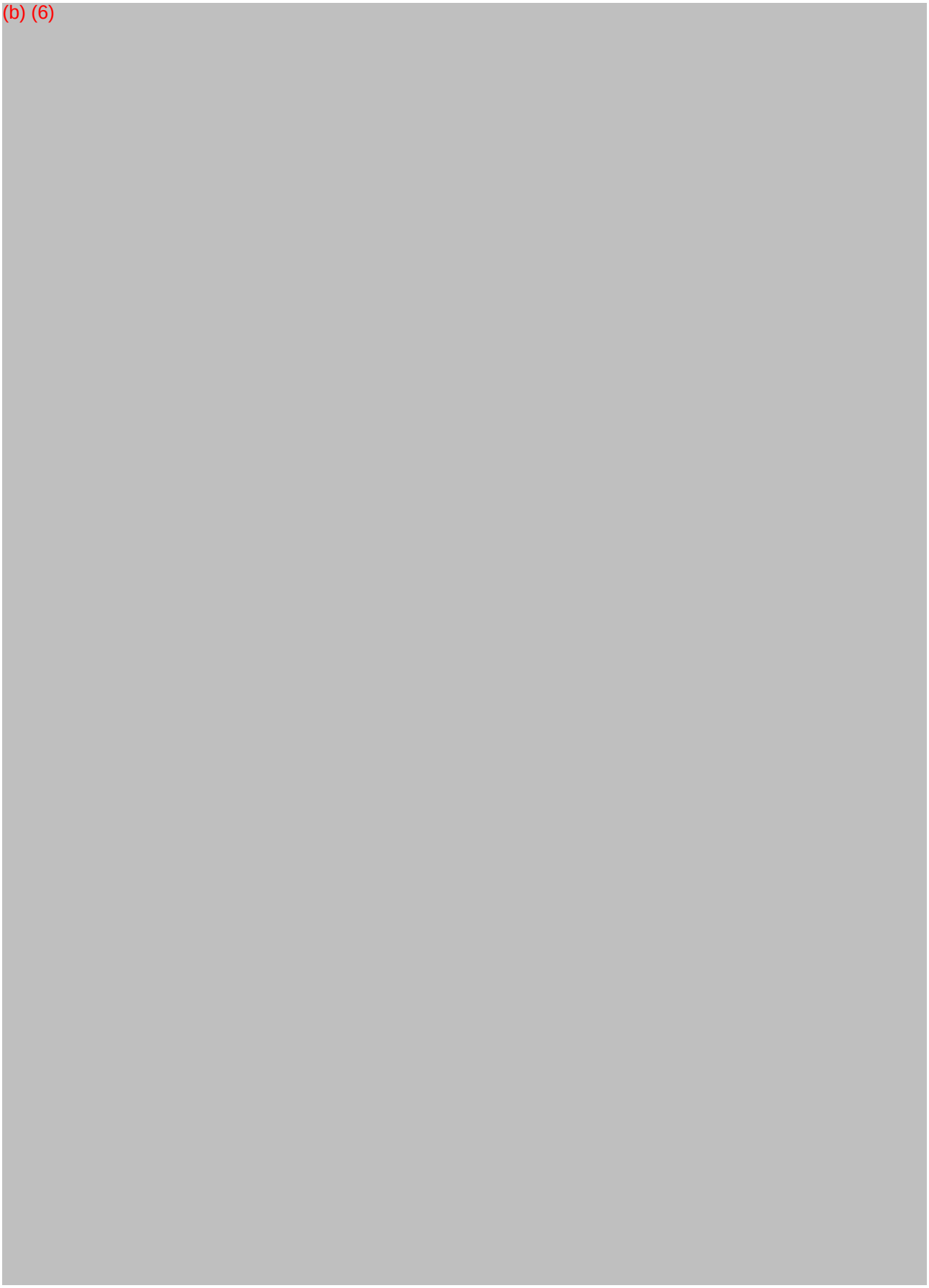
Load Example

BLAST Parameters

Appendix 2-3. Sequence analysis for strain stability

(b) (6)

(*shown on following page*)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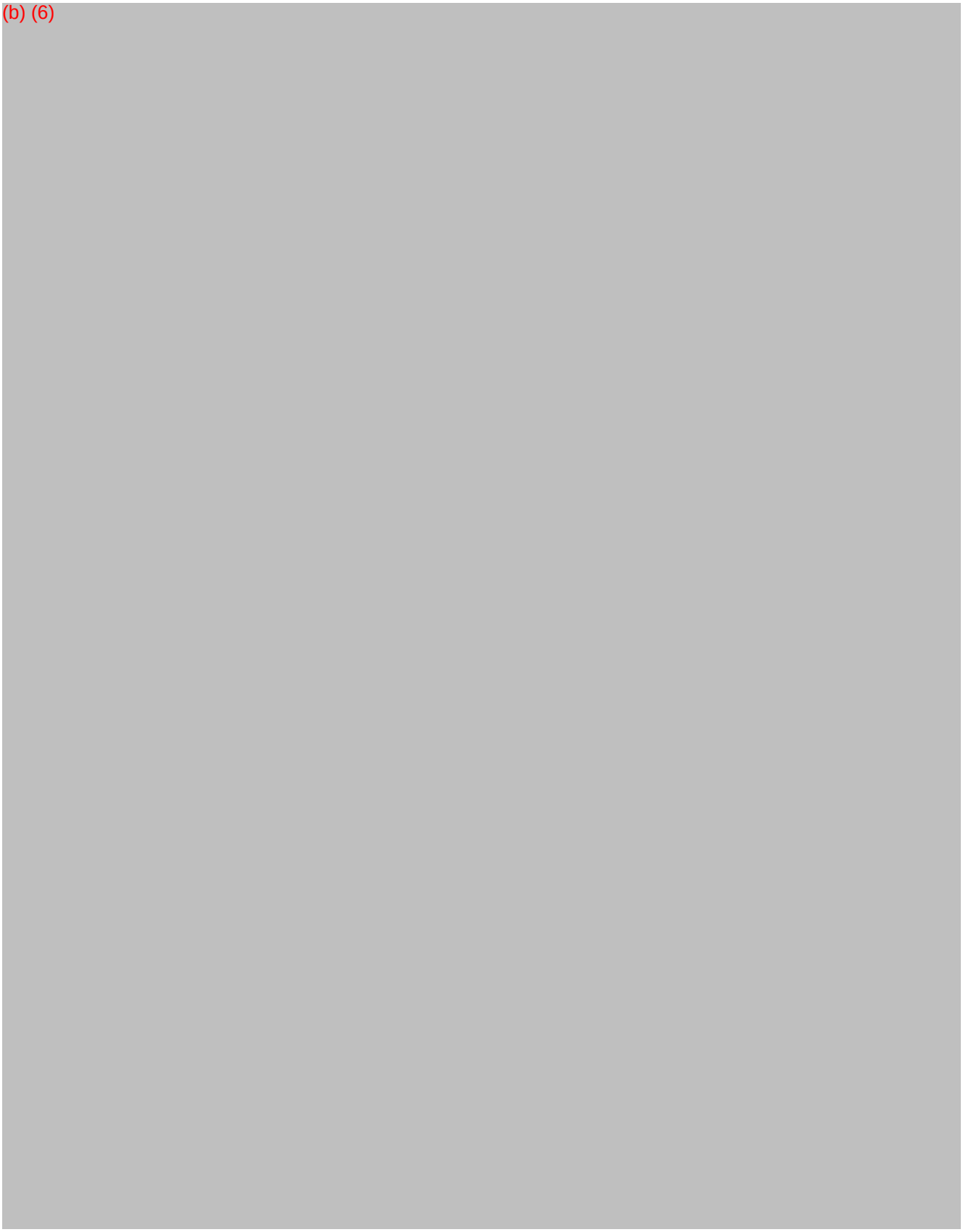
(b) (6)



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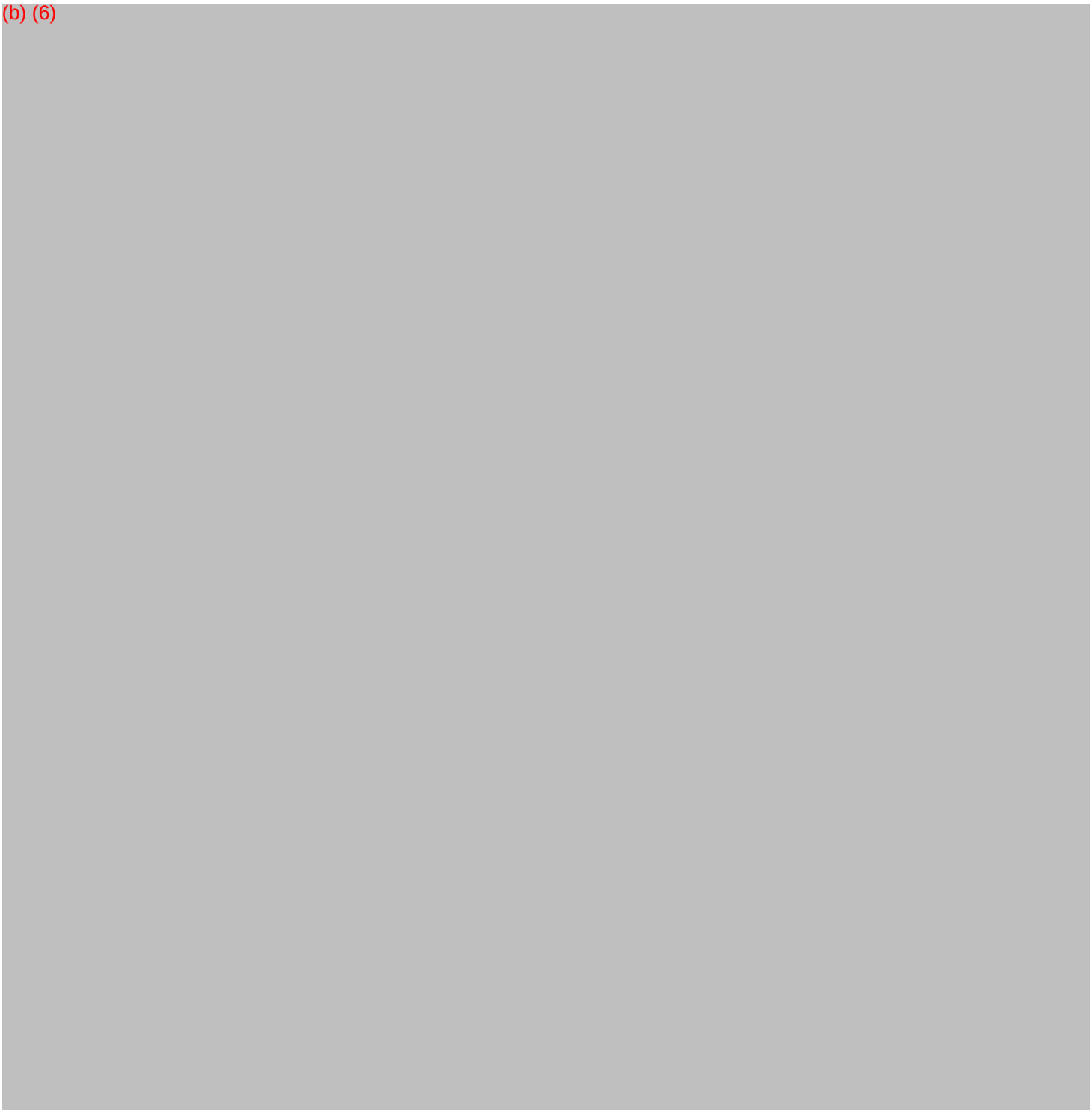

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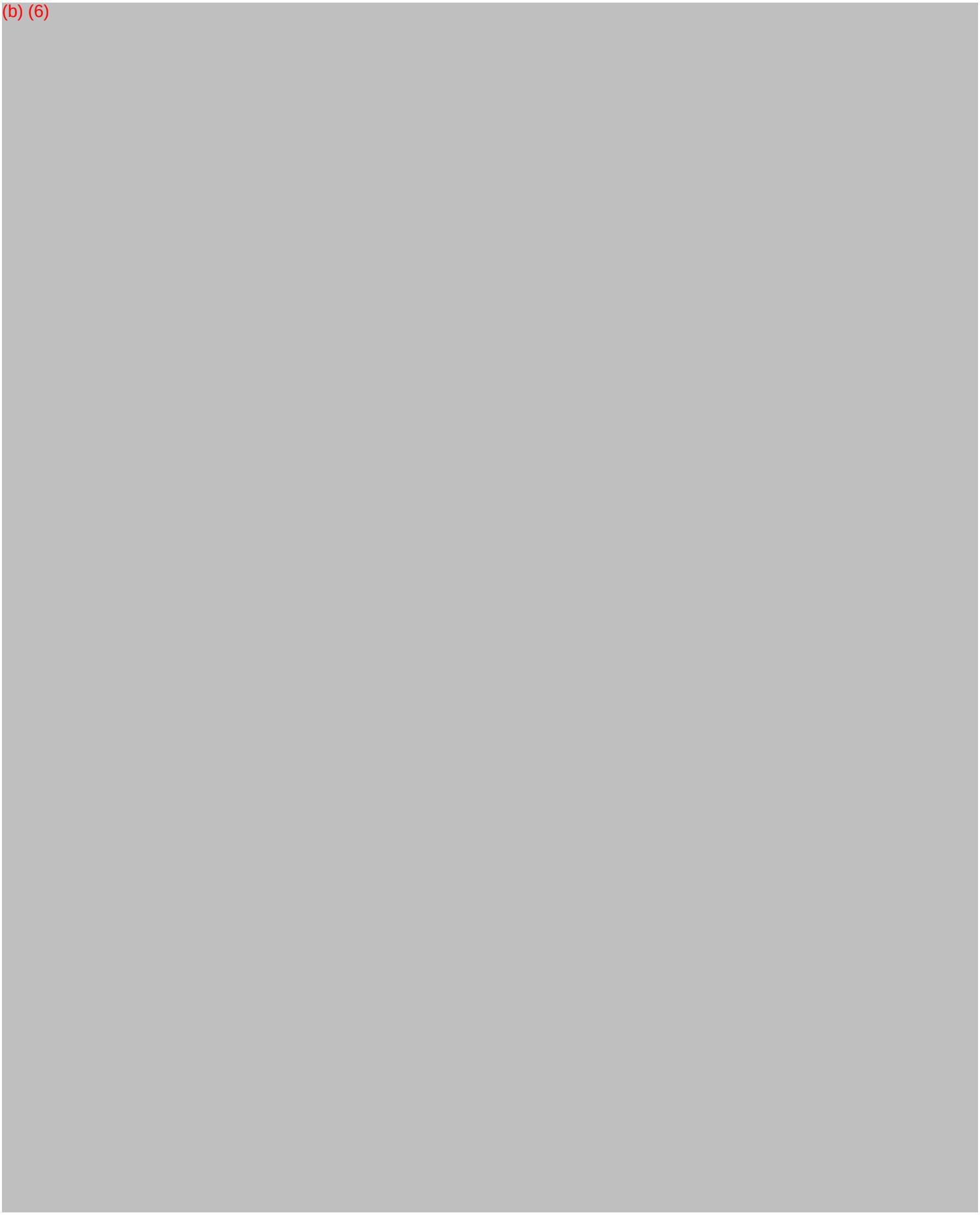
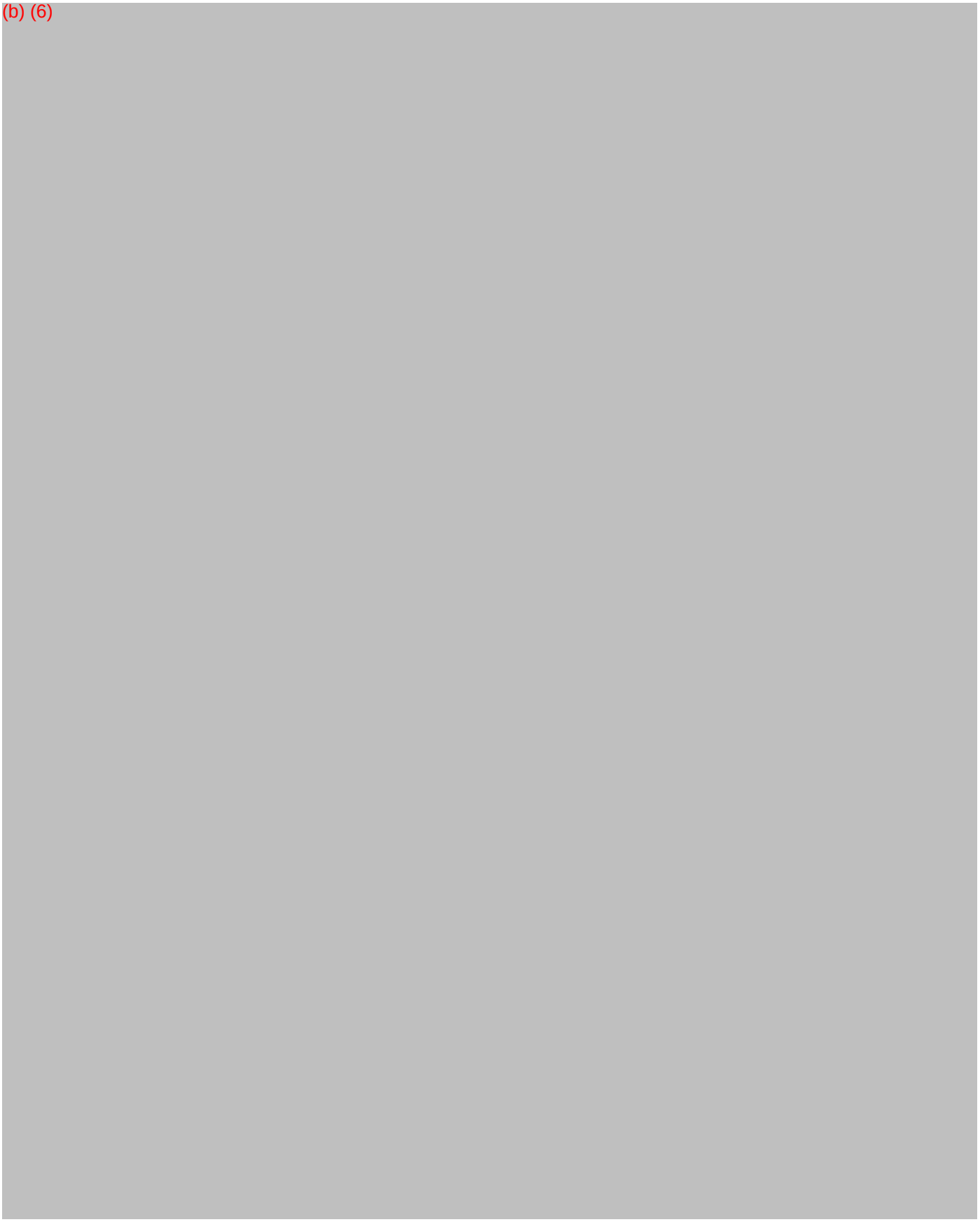
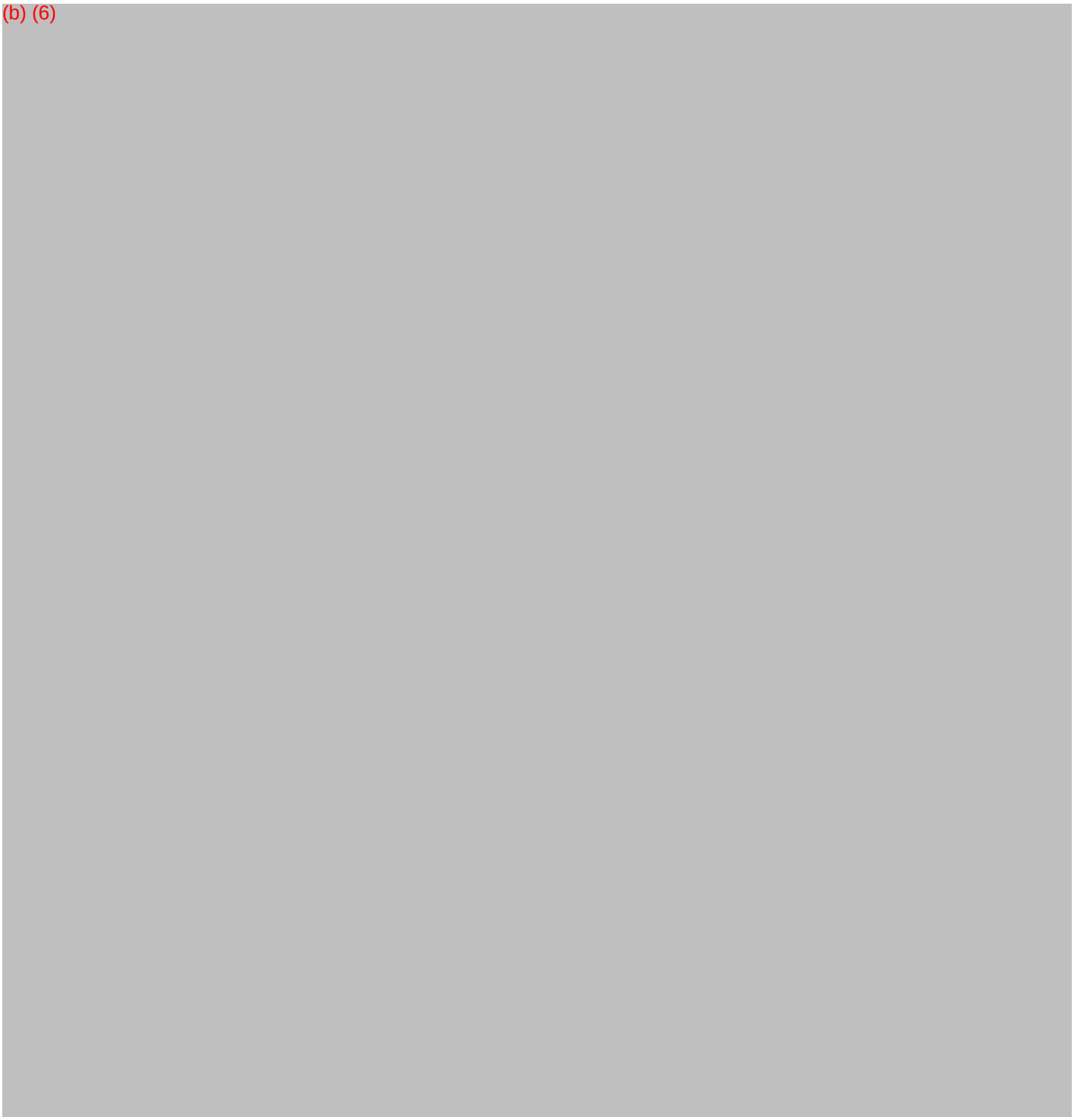


(b) (6)

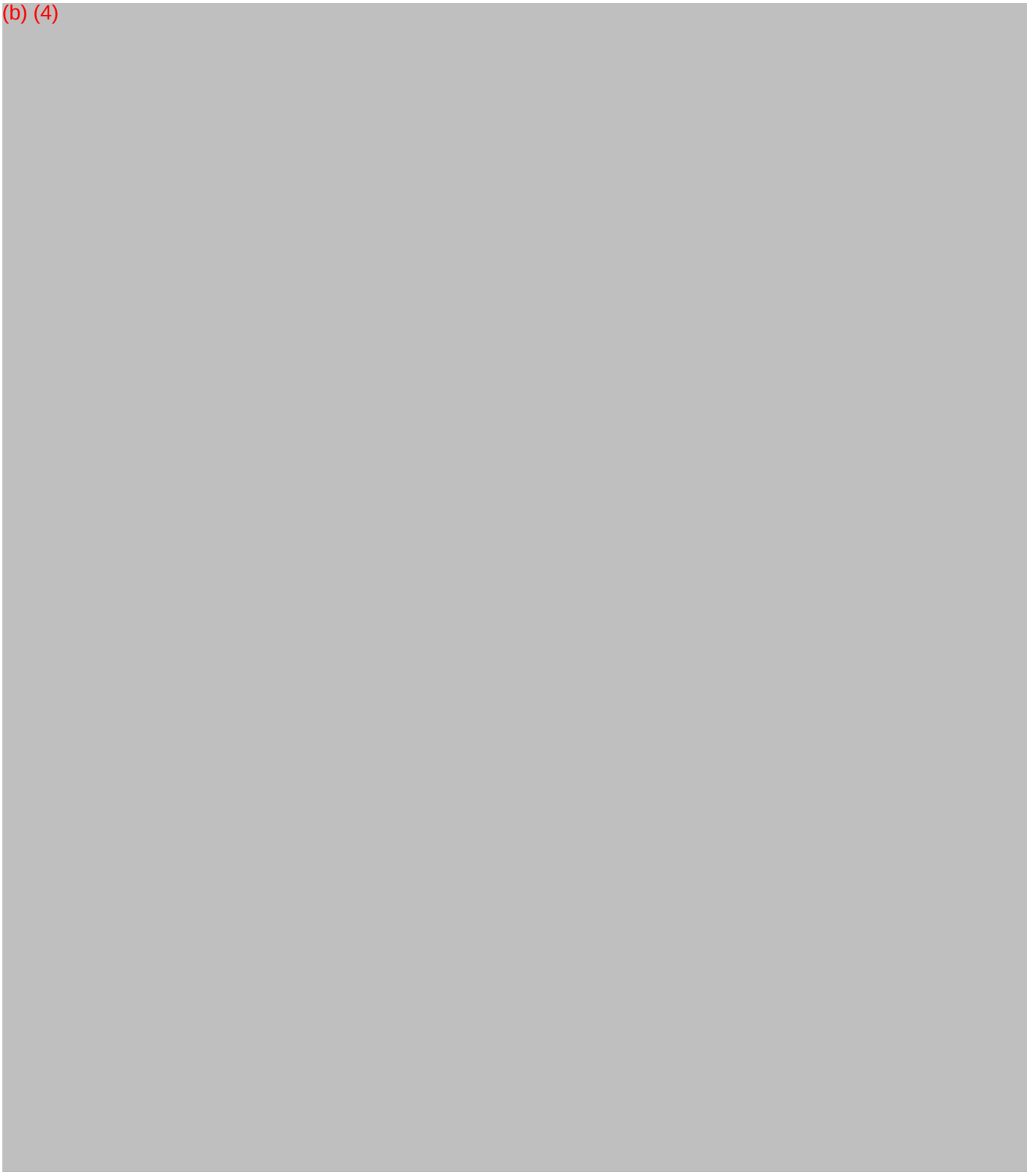



(b) (6)

(b) (6)

Appendix 2-4. Fermentation Conditions and Results.

(b) (4)



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Appendix 2-5

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Appendix 2-6

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Appendix 2-7

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(b) (4)

Appendix 2-8

KnipBio

KnipBio Meal Aka "FishKnip™"

Origin: United States

Data Sheet

Description: Single cell protein derived from a pure culture fermentation

Contents: Whole cell microbial single protein inclusive of protein content, amino acids, and carotenoids dried to lower moisture content to below 10%.

Physical Specifications Appearance: Light pink colored, very fine powder protein flour

Guaranteed Content

Protein min.:	50%	Total coliforms (cfu/g):	Negative (<5
Fat min.:	0.1%	Yeast (cfu/g):	Negative (<5)
Ash min.:	4.9%	Mold (cfu/ g):	Negative (<5)
Moisture min.:	4.2%	Salmonella:	Negative (<5)

Amino Acid Profile

Amino Acid	% (typical)	Amino Acid	% (typical)
Methionine	0.7-0.9	Glycine	2.5-2.8
Cysteine	0.3-0.4	Histidine	1.0-1.2
Lysine	2.5-3.3	Threonine	2.0-2.5
Tryptophan	~0.3	Valine	2.8-3.1
Arginine	3.3-3.8	Leucine	3.3-3.7
Isoleucine	1.7-2.1	Phenylalanine	2.0-2.2

Biosecurity controls

Preventative treatments:

- Pure culture fermentation and biotechnology techniques
- Traceability of lots
- Proper storage and handling

Packaging: Polypropylene plastic liners, polypropylene buckets (weight ~40kg); super sacks for bulk

Shelf life: > 1 year in optimal storage conditions (cool, dry place, at a temperature of max. 20 º C)

KnipBio Inc
110 Canal Street 4th Floor Lowell, MA 01852

(b) (4)

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 9/14/2015
Reported: 9/25/2015
(b) (4) ID : (b) (4)

KnipBio
[redacted]

Single Cell Protein KB17 8/11/15

Amino Acid	% of sample
Methionine	(b) (4)
Cystine	
Lysine	
Phenylalanine	
Leucine	
Isoleucine	
Threonine	
Valine	
Histidine	
Arginine	
Glycine	
Aspartic Acid	
Serine	
Glutamic Acid	
Proline	
Hydroxyproline	
Alanine	
Tyrosine	
TOTAL:	

Respectfully Submitted
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 9/14/2015
Reported: 9/25/2015

KnipBio
[redacted]

(b) (4) ID :
(b) (4)

Single Cell Protein KB17 8/11/15

	(b) (4)
Moisture	
Protein (crude)	
Fat (crude)	
Fiber (crude)	
Ash	

[redacted]

(b) (4)

[redacted] Submitted.
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 6/15/2016
Reported: 6/24/2016
(b) (4) **ID :** (b) (4)

KnipBio
[redacted]

Alternative Fishmeal Single cell protein Spray Dried - (b) (4) -KB203-(b) (4) 6/14/16

Amino Acid	% of sample
Methionine	(b) (4)
Cystine	
Lysine	
Phenylalanine	
Leucine	
Isoleucine	
Threonine	
Valine	
Histidine	
Arginine	
Glycine	
Aspartic Acid	
Serine	
Glutamic Acid	
Proline	
Hydroxyproline	
Alanine	
Tyrosine	
TOTAL:	

Respectfully Submitted,
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 6/15/2016
Reported: 6/20/2016
(b) (4) **ID :** (b) (4)

KnipBio
[redacted]

Alternative Fishmeal Single cell protein Spray Dried - (b) (4) KB203-(b) (4) 6/14/16

	(b) (4)
Moisture	
Protein (crude)	
.002% Pepsin Digestibility	
Fat (crude)	
Fiber (crude)	
Ash	

Respectfully Submitted,
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

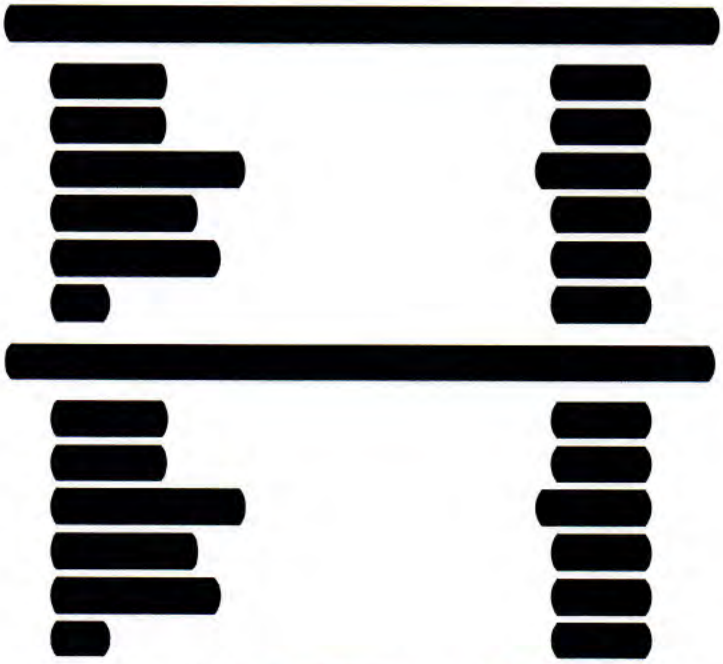
CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 8/17/2016
Reported: 8/22/2016

KnipBio

[redacted]

(b) (4) ID :
(b) (4)



(b) (4)

Alternative Fish Meal - (b) (4) *KB203-*(b) (4) *8/16/16*

(b) (4)

	(b) (4)
Moisture	
Nitrogen	
Protein (crude)	
Fat (crude)	
Fiber (crude)	
Ash	

Respectfully Submitted
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

KnipBio
[redacted]

Order ID: (b) (4)
Received: 9/9/2016
Reported: 9/14/2016

(b) (4) ID :
[redacted]

KB0203-(b) (4) 9/6/16

(b) (4) ID :
(b) (4)

	(b) (4)
Moisture	
Protein (crude)	
Fat (crude)	
Fiber (crude)	
Ash	
Calcium	
Phosphorus	
Sodium	
Chloride	
Magnesium	
Manganese	
Iron	
Zinc	
Copper	
Potassium	
Boron	
Cobalt	
Molybdenum	

Respectfully Submitted
(b) (4)

Results are reported on as-received basis unless specified otherwise.

** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

KnipBio
[redacted]

Order ID: (b) (4)
Received: 11/9/2016
Reported: (b) (4)

(b) (4) ID :

[redacted]

(b) (4) - *KnipBio A8.9 11/8/16* (b) (4)

Moisture	(b) (4)
Protein (crude)	
Fat (crude)	
Fiber (crude)	
Ash	

(b) (4) - *KnipBio B7.8 11/8/16* (b) (4)

Moisture	(b) (4)
Protein (crude)	
Fat (crude)	
Fiber (crude)	
Ash	

(b) (4) - *KnipBio C10.0 11/8/16* (b) (4)

Moisture	(b) (4)
Protein (crude)	
Fat (crude)	
Fiber (crude)	
Ash	

(b) (4) ID :

(b) (4) - *KnipBio D9.4 11/8/16* (b) (4)

Moisture	(b) (4)
Protein (crude)	
Fat (crude)	
Fiber (crude)	
Ash	

Respectfully Submitted
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 11/16/2016
Reported: 11/21/2016
(b) (4) **ID :** (b) (4)

KnipBio
[redacted]

(b) (4) - KnipBio KB203B-(b) (4) 11/15/16

	(b) (4)
Moisture	
Protein (crude)	
Non-Protein Nitrogen	
Fat (crude)	
Fiber (crude)	
Ash	
Sulfur	

Respectfully Submitted
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

NJFL INC

New Jersey Feed Laboratory, Inc.

Mailing Address:
PO Box 06650
Trenton, NJ 08650

Shipping Address:
1686 Fifth Street
Ewing, NJ 08638

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 2/10/2017
Reported: 2/20/2017
(b) (4) **ID :** (b) (4)

KnipBio
[redacted]

(b) (4) - KnipBio KB203-(b) (4) 2/8/17

Amino Acid	% of sample
Methionine	(b) (4)
Cystine	
Lysine	
Phenylalanine	
Leucine	
Isoleucine	
Threonine	
Valine	
Histidine	
Arginine	
Glycine	
Aspartic Acid	
Serine	
Glutamic Acid	
Proline	
Hydroxyproline	
Alanine	
Tyrosine	
Tryptophan	
TOTAL:	

Respectfully Submitted
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 2/10/2017
Reported: 2/20/2017
(b) (4) **D :** (b) (4)

KnipBio
[redacted]

(b) (4) - KnipBio KB203-(b) 2/8/17

Moisture	(b) (4)
Nitrogen	
Protein (crude)	
Non-Protein Nitrogen	
Urea and Ammoniacal Nitrogen	
Fat (crude)	
Fiber (crude)	
Ash	
Carbohydrates	
Calories	
Calcium	
Phosphorus	
Sodium	
Chloride	
Magnesium	
Manganese	
Iron	
Zinc	
Copper	
Potassium	
Selenium	(b) (4)
Sulfur	

Respectfully Submitted,
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 2/10/2017
Reported: 2/20/2017
(b) (4) ID : (b) (4)

KnipBio
[redacted]

Arsenic
Lead
Mercury
Boron
Cobalt
Molybdenum
Vitamin C

(b) (4)

Respectfully Submitted
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 2/10/2017
Reported: 2/20/2017
(b) (4) **ID :** (b) (4)

KnipBio
[redacted]

(b) (4) - KnipBio KB203-(b) (4) 2/8/17

	(b) (4)
Moisture	
Nitrogen	
Protein (crude)	
Non-Protein Nitrogen	
Urea and Ammoniacal Nitrogen	
Fat (crude)	
Fiber (crude)	
Ash	
Carbohydrates	
Calories	
Calcium	
Phosphorus	
Sodium	
Chloride	
Magnesium	
Manganese	
Iron	
Zinc	
Copper	
Potassium	
Selenium	(b) (4)
Sulfur	

Respectfully Submitted,
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 2/10/2017
Reported: 2/20/2017
(b) (4) **ID :** (b) (4)

KnipBio
[redacted]

Arsenic	(b) (4)
Lead	
Mercury	
Boron	
Cobalt	
Molybdenum	
Vitamin C	

Respectfully Submitted
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 5/17/2017
Reported: 5/26/2017
(b) (4) **ID** (b) (4)

KnipBio
[redacted]

(b) (4) - KnipBio KB203 (b) (4) 5/16/17

	(b) (4)
Moisture	
Protein (crude)	
Fat (crude)	
Fiber (crude)	
Ash	
Calcium	
Phosphorus	
Sodium	
Chloride	
Magnesium	
Manganese	
Iron	
Zinc	
Copper	
Potassium	
Selenium	
Sulfur	
Arsenic	
Lead	
Mercury	
Boron	
Cobalt	

Respectfully Submitted
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

Order ID: (b) (4)
Received: 5/17/2017
Reported: 5/26/2017
(b) (4) **ID** (b) (4)

KnipBio
[redacted]

Molybdenum
Vitamin C

(b) (4)

Respectfully Submitted
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

KnipBio
[redacted]

Order ID: (b) (4)
Received: 5/17/2017
Reported: 5/23/2017

(b) (4) ID :

(b) (4) - *KnipBio KB203-*(b) (4) *5/16/17* (b) (4)

	(b) (4)
Moisture	
Protein (crude)	
Fat (crude)	
Fiber (crude)	
Ash	

(b) (4) - *KnipBio KB203-*(b) (4) *5/16/17* (b) (4)

	(b) (4)
Moisture	
Protein (crude)	
Fat (crude)	
Fiber (crude)	
Ash	

[redacted]

Respectfully Submitted,
(b) (4)

Results are reported on as-received basis unless specified otherwise.
** indicates the marked result was carefully rechecked.*

(b) (4)

Appendix 2-9

Standard Operating Procedure

(b) (4)

(b) (4)

Appendix 2-10

(b) (4)

ANALYSIS REPORT

Customer No.
Customer KnipBio
110 Canal Street
M2D2-4th Floor
Lowell, MA
01854

Job ID No. (b) (4)
Report No. (b)
Time 0
Date Received December 5/16
Date Sampled December 5/16
Date Reported December 15/16
Page 1 of 1

Product ID and Condition	Protein Flour (b) (4) Good, Intact		DL	Date Tested	Method Used
Lab ID Number	(b)				
Analysis					
Total Coliforms (cfu/g) Presumptive	(b) (4)		(b) (4)	12/5/16	MFHPB-34*
Total Coliforms (cfu/g) Confirmed					
E. Coli (cfu/g) Presumptive				12/5/16	MFHPB-34*
E. Coli (cfu/g) Confirmed					
Standard Plate Count (cfu/g)				12/5/16	MFHPB-18*
Staphylococcus aureus (cfu/g) Presumptive				12/5/16	MFLP-21*
Staphylococcus aureus (cfu/g) Confirmed					
Salmonella (+ve/-ve) Presumptive				12/5/16	MFHPB-20*
Salmonella (+ve/-ve) Confirmed					
Yeast (cfu/g)				12/5/16	MFHPB-22*
Mold (cfu/g)				12/5/16	MFHPB-22*
Protein (%)				12/5/16	AOAC 2001.11* (PROT-01)
Fat Acidiy (mg/KOH/100g)				12/8/16	AACC 02 01A (FFA-03)
Appearance Hedonic Scale Rating					In-House
Appearance Comments					In-House
Odour Hedonic Scale Rating					In-House
Odour Comments					In-House
Overall Hedonic Scale Rating					In-House
Notes:	E-mail results to cpb@knipbio.com ; lfeinberg@knipbio.com				
Regular TAT	Microbial counts may be estimated as per specific method requirements.				

(b) (4)

Signature of Approval
Name: (b) (4)
Title: Laboratory Supervisor

(b) (4)

ANALYSIS REPORT

Customer No.	299745	**Job ID No.**	(b) (4)
Customer	KnipBio	**Report No.**	(b)
	110 Canal Street	**Week**	3
	M2D2-4th Floor	**Date Received**	December 28 /16
	Lowell, MA	**Date Sampled**	December 28/16
	01854	**Date Reported**	January 18/17
		Page	1 of 1

Product ID and Condition	Protein Flour (b) (4) Good, Intact		**DL**	**Date Tested**	**Method Used**
Lab ID Number	(b)				
Analysis					
Standard Plate Count (cfu/g)	(b) (4)		(b) (4)	12/28/16	MFHPB-19*
Yeast (cfu/g)				12/28/16	MFHPB-22*
Mold (cfu/g)				12/28/16	MFHPB-22*
Fat Acidiy (mg/KOH/100g)				12/30/16	AACC 02 01A (FFA-03)
Appearance Hedonic Scale Rating				12/30/16	In-House
Appearance Comments				12/30/16	In-House
Odour Hedonic Scale Rating				12/30/16	In-House
Odour Comments				12/30/16	In-House
Overall Hedonic Scale Rating				12/30/16	In-House
Notes:	E-mail results to cpb@knipbio.com ; lfeinberg@knipbio.com				
Regular TAT					

(b) (4)

Signature of Approval
Name: (b) (4)
Title: Laboratory Supervisor

(b) (4)

ANALYSIS REPORT

Customer No.	299745	**Job ID No.**	(b) (4)
Customer	KnipBio	**Report No.**	(b)
	110 Canal Street	**Week**	6
	M2D2-4th Floor	**Date Received**	January 16/17
	Lowell, MA	**Date Sampled**	January 16/17
	01854	**Date Reported**	January 24/17
		Page	1 of 1

Product ID and Condition	Protein Flour (b) (4) Good, Intact		**DL**	**Date Tested**	**Method Used**
Lab ID Number	(b)				
Analysis					
Standard Plate Count (cfu/g)	(b) (4)		(b) (4)	1/16/17	MFHPB-18*
Yeast (cfu/g)				1/16/17	MFHPB-22*
Mold (cfu/g)				1/16/17	MFHPB-22*
Fat Acidiy (mg/KOH/100g)				1/20/17	AACC 02 01A
Appearance Hedonic Scale Rating				1/20/17	In-House
Appearance Comments				1/20/17	In-House
Odour Hedonic Scale Rating				1/20/17	In-House
Odour Comments				1/20/17	In-House
Overall Hedonic Scale Rating				1/20/17	In-House
Notes:	E-mail results to cpb@knipbio.com ; lfeinberg@knipbio.com				
Regular TAT					

(b) (4)

Signature of Approval
Name: (b) (4)
Title: Laboratory Supervisor

(b) (4)

ANALYSIS REPORT

Customer No.	299745	**Job ID No.**	(b) (4)
Customer	KnipBio	**Report No.**	(b)
	110 Canal Street	**Week**	9
	M2D2-4th Floor	**Date Received**	February 6/17
	Lowell, MA	**Date Sampled**	February 6/17
	01854	**Date Reported**	February 17/17
		Page	1 of 1

Product ID and Condition	Protein Flour (b) (4) Good, Intact		**DL**	**Date Tested**	**Method Used**
Lab ID Number	(b)				
Analysis					
Standard Plate Count (cfu/g)	(b) (4)		(b) (4)	2/6/17	MFHPB-18*
Yeast (cfu/g)				2/6/17	MFHPB-22*
Mold (cfu/g)				2/6/17	MFHPB-22*
Fat Acidiy (mg/KOH/100g)				2/16/17	AACC 02 01A
Appearance Hedonic Scale Rating				2/16/17	In-House
Appearance Comments				2/16/17	In-House
Odour Hedonic Scale Rating				2/16/17	In-House
Odour Comments				2/16/17	In-House
Overall Hedonic Scale Rating				2/16/17	In-House
Notes:	E-mail results to cpb@knipbio.com ; lfeinberg@knipbio.com				
Regular TAT					

(b) (4)

Signature of Approval
Name: (b) (4)
Title: Laboratory Supervisor

(b) (4)

ANALYSIS REPORT

Customer No.	299745	**Job ID No.**	(b) (4)
Customer	KnipBio	**Report No.**	(b)
	110 Canal Street	**Week**	11
	M2D2-4th Floor	**Date Received**	February 20/17
	Lowell, MA	**Date Sampled**	February 21/17
	01854	**Date Reported**	February 27/17
		Page	1 of 1

Product ID and Condition	Protein Flour (b) (4) Good, Intact		DL	Date Tested	Method Used
Lab ID Number	(b)				
Analysis					
Standard Plate Count (cfu/g)	(b) (4)		(b) (4)	2/21/17	MFHPB-18*
Yeast (cfu/g)				2/21/17	MFHPB-22*
Mold (cfu/g)				2/21/17	MFHPB-22*
Fat Acidiy (mg/KOH/100g)				2/24/17	AACC 02 01A
Appearance Hedonic Scale Rating				2/27/17	In-House
Appearance Comments				2/27/17	In-House
Odour Hedonic Scale Rating				2/27/17	In-House
Odour Comments				2/27/17	In-House
Overall Hedonic Scale Rating				2/27/17	In-House
Notes:	E-mail results to cpb@knipbio.com ; lfeinberg@knipbio.com				
Regular TAT					

(b) (4)

Signature of Approval
Name: (b) (4)
Title: Laboratory Supervisor

(b) (4)

ANALYSIS REPORT

Customer No.	299745	**Job ID No.**	(b) (4)
Customer	KnipBio	**Report No.**	(b)
	110 Canal Street	**Week**	12
	M2D2-4th Floor	**Date Received**	February 27/17
	Lowell, MA	**Date Sampled**	February 27/17
	01854	**Date Reported**	March 6/17
		Page	1 of 1

Product ID and Condition	Protein Flour (b) (4) Good, Intact		**DL**	**Date Tested**	**Method Used**
Lab ID Number	(b)				
Analysis					
Standard Plate Count (cfu/g)	(b) (4)		(b) (4)	2/27/17	MFHPB-18*
Yeast (cfu/g)				2/27/17	MFHPB-22*
Mold (cfu/g)				2/27/17	MFHPB-22*
Fat Acidiy (mg/KOH/100g)				3/3/17	AACC 02 01A
Appearance Hedonic Scale Rating				3/6/17	In-House
Appearance Comments				3/6/17	In-House
Odour Hedonic Scale Rating				3/6/17	In-House
Odour Comments				3/6/17	In-House
Overall Hedonic Scale Rating				3/6/17	In-House
Notes:	E-mail results to cpb@knipbio.com ; lfeinberg@knipbio.com				
Regular TAT					

(b) (4)

Signature of Approval
Name: (b) (4)
Title: Laboratory Supervisor

(b) (4)

OOS SUMMARY REPORT

Date of OOS Result Mar 1/17. OOS Due Date Mar 3/17 Client: KnipBio

Intertek ID Number: (b) (4) Analysis: Fat Acidity

Dept: Chemical Technician(s): (b)

Quality Report Checklist:

Notified Management:	YES	X	NO		Initial:	A H	Date:	Mar 1/17.
Verified Sample	YES	X	NO		Initial:	A H	Date:	Mar 1/17.
Verified Test Procedure:	YES	X	NO		Initial:	A H	Date:	Mar 1/17.
Verified Test Equip.:	YES	X	NO		Initial:	A H	Date:	Mar 1/17.
Reviewed Standard Data	YES	X	NO		Initial:	A H	Date:	Mar 1/17.
Reviewed QC Data	YES	X	NO		Initial:	A H	Date:	Mar 1/17.
Reviewed Sample Data	YES	X	NO		Initial:	A H	Date:	Mar 1/17.
Reviewed Calculations	YES	X	NO		Initial:	A H	Date:	Mar 1/17.

Cause Identified and Corrected? YES [ ] NO [x] Describe in detail all subsequent findings:

Technician error: no errors found

Equipment: in good working order, no errors found

Test materials: freshly made and within spec, no errors found

Documentation: no errors found

Conclusion: no errors found

Was the sample re-analyzed? YES [ ] NO [x]

If YES, did repeat testing meet specifications?? YES [ ] NO [ ] NOT DONE [x]

Describe in detail repeat testing results vs. specifications:

QA/QC Manager Signature: (b) (4) Date: Mar 3/17

(b) (4)

CERTIFICATE OF ANALYSIS

Customer No.	299745	**Job ID No.**	(b) (4)
Customer:	KnipBio	**Report No.**	(b) (4)
	110 Canal Street	**Date Received**	May 5/17
	Lowell, MA	**Date Reported**	May 16/17
	01850		

Product ID and Condition	Protein Flour (b) (4) Good, Intact	Protein Flour (b) (4) Good, Intact	Protein Flour (b) (4) Good, Intact	**DL**	**Date Tested**	**Method Used**
Lab ID Number	(b) (4)	(b) (4)	(b) (4)			
Analysis						
Total Coliforms (cfu/g) Presumptive	(b) (4)				5/8/17	MFHPB-34*
Total Coliforms (cfu/g) Confirmation						
E.coli (cfu/g) Presumptive					5/8/17	MFHPB-34*
E.coli (cfu/g) Confirmation						
Standard Plate Count (cfu/g)					5/8/17	MFHPB-33*
Staphylococcus aureus (cfu/g) Presumptive					5/8/17	MFLP-21*
Staphylococcus aureus (cfu/g) Confirmation						
Salmonella (Positive/ Negative) Presumptive					5/8/17	MFHPB-20*
Salmonella (Positive/ Negative) Confirmation						
Yeast & Mold (cfu/g)					5/8/17	MFHPB-22*
Crude Protein (%)					5/9/17	AOAC 2001.11 (PROT-01*)

Notes: E-mail results to cpb@knipbio.com
Microbial counts may be estimated as per specific method requirements.
Regular TAT PO# (b) (4) DL: Detection Limit

(b) (4)

val
Name: (b) (4)
Title: Laboratory Supervisor

Date Effective: April /17
Date Replaced: Jan5/16

O:/QM/formwksh/
2ar-master.doc Ver 06.doc

"*" These tests encompass our ISO 17025 SCC Scope of Accreditation
SCC Lab Accreditation (b)

Authorized By: (b) (4)

Appendix 2-11. Summary of KnipBio Animal Trials

This appendix summarizes the results of studies reported in KnipBio's published paper Tlusty *et al*. (2017) as well as other unpublished studies that KnipBio has conducted or sponsored in which aquatic animals were fed the notified substance (in some cases, early formulations of the notified substance). These studies all show that the notified substance caused no harmful effects on the fish. Full study records for these studies, particularly including the studies published in Tlusty *et al*., are available at the KnipBio offices should FDA be interested in reviewing them.

This report includes the following studies, several of which have been published in Tlusty *et al.* (2017), as indicated:

A. Smallmouth grunt

B. Atlantic Salmon

C. Trout

D. Pacific White Shrimp

1. New England Aquarium
2. University of Alabama.

A.**Smallmouth grunt (*included in Tlusty et al. 2017*)**

1.Experimental design

Hatchery-raised smallmouth grunts (*Haemulon chrysargyreum*; N=120) were stocked at 10 fish/tank into twelve 110L glass aquaria. Each of the 12 experimental tanks was randomly assigned one of four experimental diets, totaling three replicates per treatment

Ingredient	Composition (g kg-1 as fed)			
	GRU-C1	GRU-C2	GRU-KL	GRU-KH
Menhaden fish meal	5000.0	5000.0	4700.0	3500.0
KnipBio meal	0.0	0.0	500.0	2500.0
Squid meal	1000.0	1000.0	1000.0	1000.0
Soy bean meal	1000.0	1000.0	1000.0	1000.0
Wheat starch	700.0	700.0	600.0	300.0
Wheat gluten	500.0	500.0	500.0	500.0
Menhaden fish oil	500.0	500.0	550.0	600.0
Soybean lecithin	100.0	100.0	100.0	100.0
Vitamin premix	200.0	200.0	200.0	200.0
Trace mineral premix	200.0	200.0	200.0	200.0
Alpha-cellulose	700.0	660.0	550.0	0.0
Astaxanthin	0.0	40.0	0.0	0.0
Methionine	50.0	50.0	50.0	50.0
Lysine	50.0	50.0	50.0	50.0

* Diet GRU-C2 was identical to GRU-C1 with an added 80 ppm carotenoid.

Composition of four experimental feeds used to test the efficacy of KnipBio single cell protein (KnipBio meal; KBM) as a fishmeal substitute using smallmouth grunt (*H. chrysargyreum*), where GRU-C1 = **GRU**nt **C**ontrol feed (modelled after Alam *et al*., 2012; Alam *et al*., 2008; Alam *et al*., 2009), GRU-C2 = GRU-C1 with 80 ppm carotenoid addition, and GRU-KL and GRU-KH are control feed with fishmeal replaced with KBM; KL = **K**nipBio meal **L**ow (10% replacement) and KH = **K**nipBio meal **H**igh (50% replacement).

o Each tank was fed until apparent satiation four times/day. Animal care and procedures used in this trial were approved by Roger Williams University Animal Care and Use Committee (IACUC protocol R-13-12-20).

o Wet weight (g) and standard length (mm) was determined for each fish on day 0 and 41 (N=120 and 112, respectively).

o On day 41, three fish were randomly selected from each treatment (N=12 total), freeze-dried for 48hr, homogenized using a mortar and pestle, and stored in borosilicate vials. Processed samples were then shipped to the (b) (4) for whole body proximate, amino acid, and fatty acid analyses (% composition).

oThe foregut of three additional fish from each treatment (N=12 total) was removed via dissection and immediately frozen on dry ice for gut microbial DNA analysis.

2.Results

o Diet had no effect on grunt mortality, length, weight, condition factor, or specific growth rate (SGR) (for all variables, one-way ANOVA $F_{3,8} > 2.85$). Only 12 of the 120 grunts died during the experiment, and no more than one fish was lost per tank. For all fish, the average weight increase was 353.2±45.9%, length increase was 48.4±6.2% and SGR was 3.8±0.3. Average feed conversion ratio (FCR) per treatment ranged from 1.09-1.24.

o Diet did not significantly affect grunt gut microbial community (adonis, $p > 0.05$). Regardless of diet, ~60% of the fish gut microbiome was composed of three types of bacteria: *Halomonas, Oxalobacteracaea*, and *Shewanella*.

3. Conclusion.

The results of this study show that, in smallmouth grunts during a 41-day growth period, the notified substance can be included up to 10% by replacing Menhaden fish meal in the grunt diet without significantly affecting grunt mortality, length, weight, condition factor, or specific growth rate.

B.**Atlantic Salmon (*included in Tlusty et al. 2017*)**

1.Experimental design

a.Tanks

The purpose of this trial was to establish the digestibility of KBM by Atlantic salmon (*Salmo salar*). Fish N=96; 635±97 g wet weight were stocked at 16 animal/tank into six 417L fiberglass tanks. Water quality was monitored weekly to ensure that a healthy environment was maintained during the trial. Dissolved oxygen and temperature were monitored daily. Animal care and procedures used in this trial were approved per USDA-ARS Animal Care and Use Committee (IACUC FY2014-001).

b.Feed

Yttrium oxide was added to the reference diet at 0.1% of dry weight to serve as an inert, indigestible marker. The

chromic oxide was diluted to 0.07% when the test ingredients were added. Diets were stored in polypropylene plastic bags at room temperature until fed. All diets were fed within 4 months of manufacture.

c.Digestibility

To determine the digestibility of KBM, a reference diet was formulated and then KBM was added in a standard 70%/30% (that is, with KBM substituted at 30% of the diet). Each of the six experimental tanks was randomly assigned to one experimental treatment, totaling three replicates per treatment. All tanks were fed the control diet (C) for 7-days prior to initiating the experimental treatments. The basal diet contained 40% protein and 25% lipid with an estimated digestible energy of 19.6 kJ g^{-1}. Fish were fed three times/day. The feeding strategy was developed from experimental growth models validated from commercial data and different genetic stocks (Ruohonen & Mäkinen, 1992; Ursin, 1967).

Ingredient	Composition (g kg^{-1} as fed)
	Sal-C
Squid meal	260.0
Soy protein concetrate	171.4
Corn gluten meal	83.4
Soybean meal	43.0
Wheat flour	283.3
Taurine	5.0
Menhaden fish oil	133.9
Vitamin premis, ARS 702	10.0
Choine chlorine	6.0
Vitamin C	2.0
Ytrimum oxide	1.0
Trace mineral premix	1.0

Composition of two experimental feeds used to test the digestibility of KnipBio single cell protein (KnipBio meal; KBM) as a fishmeal substitute using Atlantic salmon (*S. salmar*), where **SAL-C** = **SAL**mon **C**ontrol diet (modelled after Gaylord *et al*., 2009). And **SAL-K** = **SAL**mon control diet + 297.0 g kg^{-1} **K**BM (not shown).

Fecal material was collected from each tank 18 hr post feeding on day 2 and 4 (Austreng, 1978; Hajen *et al*., 1993), dried at 60℃ for 24 hr, placed into plastic bags and stored at -20℃ until analysis.

The methods of Cho *et al*. (1982) and Bureau *et al*. (1999) were used to estimate apparent digestibility coefficients.

Apparent digestibility coefficients of each nutrient in the experimental diets were calculated according to the equations from Kleiber (1961) and Forster (1999).

2.Results

Protein and amino acid digestibility:

(%) of Atlantic salmon (*S. salmar*) exposed to two experimental feeds used to determine the viability of KnipBio single cell protein (KnipBio meal; KBM) as a fishmeal substitute, where **SAL-C** = **SAL**mon **C**ontrol diet (modelled after Gaylord *et al*., 2009) and **SAL-K** = **SAL**mon control diet + 297.0 g kg^{-1} **K**BM.

KBM inclusion had no measurable effect on the Apparent Digestibility Coefficient (ADC) value. The ADC for protein was slightly greater for the

Nutrient	Digestibility (%)	
	SAL-C	SAL-K
Crude protein	72.32	69.12
Essential amino acids		
Lysine	74.76	79.37
Methionine	75.46	82.01
Histidine	78.14	80.95
Isoleucine	70.72	77.72
Leucine	70.51	81.63
Phenylalanine	75.83	75.26
Tryptophan	77.46	77.71
Valine	74.65	78.82
Nonessential amino acids		
Aspartic acid	69.13	70.24
Threonine	68.52	71.37
Serine	79.19	79.89
Glutamine	80.53	79.54
Proline	74.62	80.25
Glycine	70.26	69.73
Alanine	70.35	75.54
Cysteine	60.99	69.56

animals fed the control diet compared to the 30% inclusion KBM diet (67.8±2.8 and 63.0±3.1 (averages ±1 S.D.) respectively). Diet did have a positive although insignificant influence on amino acid digestibility, as the KBM diet (SAL-K) resulted in better digestibility for seven of the eight essential amino acids and six of the 11 non-essential amino acids than the control (SAL-C) diet (for all amino acids, binomial probability, = 0.5p < 0.08).

3. Conclusion.

The results of this study show that inclusion of the notified substance of up to 30% in the diet of salmon had no measurable effect on the digestibility of the fish meal.

C.**Trout (*submitted for publication; attached manuscript*)**

1. Experimental procedure

Trials were done at the USDA- University of Idaho's Hagerman Fish Culture Experiment station. All analyses were done in duplicate. Proximate composition (moisture, protein, fat and ash) were determined using AOAC (1990) procedures. A draft manuscript describing this study and its results is attached to this Appendix as **Attachment A**.

a.*Diets*

All experimental diets were formulated to contain 46% crude protein and 20% lipid. All the diets met or exceeded the nutrient requirements of rainbow trout (NRC, 2011). Fishmeal was replaced with KnipBio product such that 13% and 26% of fishmeal protein was replaced with an equivalent amount of KnipBio product protein
Diet samples were analyzed for proximate composition and gross energy at the Hagerman lab using standard protocols. Diets were analyzed for protein and lipid content prior to the start of the feeding trial to ensure that expected values are achieved.

A control plus two experimental diets were produced as follows:

Diet 1: Control – standard upper level of fishmeal in the formulation (32%)
Diet 2:13% of fishmeal protein replaced by KnipBio product
Diet 3:26% of fishmeal protein replaced by KnipBio product

	Diet 1	**Diet 2**	**Diet 3**
Ingredient	**Control/ reference diet**	**5% KB protein**	**10% KB protein**
Sardine fishmeal	**32.34**	**27.00**	**22.50**
KnipBio protein	**0.00**	**5.00**	**10.00**
Soy protein concentrate	10.00	11.00	12.50
Soybean meal, dehulled and solvent extracted	10.00	**9.51**	**7.10**

Poultry by-product meal	14.00	16.00	16.00
Wheat gluten meal	3.00	3.00	3.00
Wheat flour	13.30	10.00	9.00
Fish oil	14.57	14.61	14.98
Dicalcium phosphate	0.39	1.08	1.70
L-Lysine HCl	0.50	0.60	0.90
DL-Methionine	0.00	0.30	0.42
Vitamin C (Stay-C)	0.20	0.20	0.20
Choline chloride	0.60	0.60	0.60
Trace mineral mix, Trouw Nutrition[1]	0.10	0.10	0.10
Vitamin premix, ARS 702[2]	1.00	1.00	1.00
Total	***100.0***	***100.0***	***100.0***
Calculated composition (%)			
Moisture	8.02	7.86	7.70
Crude protein (total)	45.49	46.25	45.98
Digestible protein	42.00	42.00	41.00
Fishmeal protein replacement by KB protein	**0.00**	**13.00**	**26.00**
Crude lipid	20.00	20.00	20.00
Ash	7.28	7.49	7.54
Lysine	3.64	3.40	3.55
Phosphorus	1.50	1.50	1.50

[1] Trace mineral premix supply the following to the diet (mg/kg diet): Zn (as $ZnSO_4$ $7H_2O$), 50; Mn (as $MnSO_4$), 7.5; Cu (as $CuSO_4$ $5H_2O$), 2.5; I (as KIO_3), 1; selenium, 0.05.

[2] Vitamin premix supply the following to the diet (mg/kg diet): D calcium pantothenate, 46.47; pyridoxine (pyridoxine HCl), 13.68; riboflavin, 9.58; niacinamide, 21.78; folic acid, 2.49; thiamine (thiamine mononitrate), 9.1; inositol, 599; biotin, 0.33; vitamin B_{12}, 0.03; menadione sodium bisulfite complex, 1.1; vitamin E (DL α-tocopherol acetate), 131.9 IU; vitamin D_3 (stabilized), 6594 IU; vitamin A (vitamin A palmitate, stabilized), 9641 IU; ethoxyquin, 198.

Experimental feeds were produced by extrusion pelleting similar to commercial fish feed production technology at the (b) (4) were stored in plastic lined paper bags, shipped to the Hagerman Fish Culture Experiment Station and stored at room temperature until fed.

b. Fish and Feeding schedule

Rainbow trout fry, hatched from eggs purchased from a commercial source ((b) (4)) were used in the study. Fifty fish (5-10g) were stocked into each of nine 145L tanks. Fish was weighed and counted every three weeks for the duration of the study (12 weeks). An initial sample of 20 fish from the population used in the study was sacrificed and frozen for later analysis. At the end of the study, fish was sacrificed (5 per tank) for proximate analysis on pooled samples (pooled by replicate tank). All fish handling and sampling, plus the experimental protocols used in the growth trial and subsequent digestibility trial was approved in advance by the University of Idaho's Institutional Animal Care and Use Committee.

c.Statistical Analysis of Data

Tank means was used as units of observation for statistical analysis. Fish growth, feed performance and body composition data will be tested for normality and homogeneity of variance prior to one-way Analysis of Variance (ANOVA). If required, data were transformed to achieve normal distribution. If significant differences were found, data were subjected to Tukey's HSD test to separate the means at a significance level of $P<0.05$.

d.Results after 12 weeks

					Total	Total	Average	Average	Average	Feed	% Body				% individual			Growth conversion
Tank	Diet	Start #	Morts	End #	Start wt	End wt	Start wt	End wt	Wt gain	Per Fish	Wt fed/d	F.C.R.	SGR	% Survival	DGI	wt gain	TGC	efficiency
129	control-12w	35	1	34	542	8057	15.5	237	221	183	1.73	0.83	3.25	97.1	4.4	1430	0.2857	188
133		35	2	33	537	7717	15.3	234	219	191	1.82	0.87	3.24	94.3	4.38	1424	0.2842	178
135		35	1	34	555	8062	15.9	237	221	187	1.76	0.85	3.22	97.1	4.38	1395	0.2843	183
Mean	1	35	1.3	34	545	7945	15.6	236	220	187	1.77	0.85	3.24	96.2	4.38	1417	0.2847	183
131	5%-12w	35	1	34	556	8335	15.9	245	229	186	1.7	0.81	3.26	97.1	4.46	1443	0.2895	192
134		35	0	35	554	8182	15.8	234	218	183	1.74	0.84	3.21	100	4.34	1377	0.2821	184
137		35	1	34	545	7529	15.6	221	206	169	1.7	0.82	3.16	97.1	4.23	1322	0.2746	186
Mean	2	35	0.7	34	552	8015	15.8	233	218	180	1.71	0.82	3.21	98.1	4.34	1381	0.2821	187
130	10%-12w	35	0	35	538	7411	15.4	212	196	166	1.74	0.84	3.12	100	4.14	1278	0.2685	180
132		35	0	35	551	7976	15.7	228	212	180	1.75	0.85	3.18	100	4.29	1348	0.2784	181
136		35	0	35	538	7695	15.4	220	204	172	1.74	0.84	3.17	100	4.23	1330	0.2744	182
Mean	3	35	0	35	542	7694	15.5	220	204	172	1.74	0.84	3.16	100	4.22	1318	0.2738	181

2. Conclusion.

The results of this study show that, in juvenile rainbow trout during a 12-week growth period, the notified substance can be included up to 10% by replacing soybean meal in rainbow trout diet without significantly affecting growth performance, feed utilization and nutrient retention.

D. Pacific white Shrimps

1.Study 1 New England aquarium (*included in Tlusty et al. 2017*)

a. Experimental design

Experimental diets used for all animal trials were produced using commercial manufacturing methods. Diets were stored in polypropylene plastic bags at room temperature until fed. All diets were fed within 4 months of manufacture.

Hatchery-raised Pacific white shrimp (*L.s vannamei*) were acquired from SKy8 Shrimp Farm, LLC (Stoughton, MA, USA) and stocked at 60 shrimp/tank into twelve 110L glass aquaria. Animal care and procedures used in this trial were approved by Roger Williams University Animal Care and Use Committee (IACUC protocol R-13-12-20).

To determine the effect of KBM on shrimp growth and survival, three diets of varying KBM inclusion were formulated (Table). Each of the 12 experimental tanks was randomly assigned one of the three diets, totaling four replicates per

Ingredient	Composition (g kg^{-1} as fed)		
	SHR-C	SHR-KL	SHR-KH
Menhaden fish meal	1200.0	600.0	0.0
KnipBio meal	0.0	630.0	1260.0
Soybean meal	3800.0	3800.0	3800.0
Menhaden fish oil	307.0	371.0	435.0
Corn starch	348.0	174.0	0.0
Whole wheat	3400.0	3400.0	3400.0
Trace mineral premix	50.0	50.0	50.0
Vitamin premix	180.0	180.0	180.0
Choline clorine	20.0	20.0	20.0
Vitamin C	10.0	10.0	10.0
CaP-diebasic	200.0	280.0	360.0

treatment. Each tank was fed to apparent satiation four times/day.

Composition of three experimental feeds used to test the efficacy of KnipBio single cell protein (KnipBio meal; KBM) as a fishmeal substitute using Pacific white shrimp (*L. vannamei*), where SHR-C = **SHR**imp **C**ontrol feed (modelled after Jobling, 2012) and SHR-KL and SHR-KH are control feed with fishmeal replaced with KBM; KL = **K**nipBio meal **L**ow (50% replacement) and KH = **K**nipBio meal **H**igh (100% replacement).

The gross wet weight (g) of all shrimp per tank was measured at day 0 (N=60), day 60 (N=45-55, depending on tank), and day 105 (N=19-20, depending on tank). At day 60, 20 shrimps from each tank (N=80 per treatment) were randomly selected and returned to their original tank and maintained according to the above experimental design for an additional 90 days. The remaining shrimps not used for the second 90-day trial (N=25-35, depending on tank) were euthanized, placed on ice, and wet weight (g), and carapace length (mm) were measured for each individual. On day 150, the remaining shrimps in each tank were enumerated and wet weight (g) and carapace length (mm) were measured for each individual.

b. Results

Diet had no effect on shrimp survival (one-way ANOVA, $F_{2,9} = 2.4$, $p > 0.1$, combined average = 84.7±5.6%); however, diet did influence shrimp growth (one-way ANOVA, % weight gain, $F_{2,9} = 5.4$, $p < 0.5$; SGR, $F_{2,9} = 8.6$, $p < 0.01$). Shrimp fed diet with 100% FM replacement (SHR-KH) grew less than those fed the control diet (SHR-C), and shrimp fed diet with 50% FM replacement (SHR-KL) showed growth intermediate to, and not statistically different from either SHR-C or SHR-KH. Diet influenced shrimp feed efficiency (one-way ANOVA, $F_{2,9} = 5.27$, $p < 0.5$). The food conversion ratio (FCR) of shrimp fed diets containing KBM were not statistically different than those fed the control diet.

c. Conclusion.

The results of this study show that, in Pacific White shrimp during a 15-week growth period, the notified substance even when constituting 50% or 100% of the fish diet had no effect on shrimp survival with only minor differences in growth rate.

2.Study 2 (b) (4)

This is an unpublished study, which KnipBio considers to be ***CONFIDENTIAL INFORMATION***.

a.Experimental design

In this Study, three trials were conducted to evaluate the biological response of shrimp to BB (Bacterial Biomass) in soy-based diets in terms of growth. In the trial 1 and 2, test diets were formulated to be isonitrogenous and isolipidic (35% protein and 8% lipid). All experimental diets were produced at the (b) (4) (b) (4) using the standard procedures for the shrimp feeds described by (Qiu and Davis, 2016)). Dry pellets were crumbled, packed in sealed bags, and stored in a freezer until use.

The growth trials were conducted at the (b) (4) (b) Pacific white shrimp post larvae (PL) were obtained from (b) (4) and nursed in an indoor recirculating system. PLs were fed a commercial feed (b) (4) (b) (4) using an automatic feeder for ~1 week, and then switched to crumbled commercial shrimp feed ((b) (4)) for ~1- 2 weeks.

In trial 1, three experimental diets (T_1D_1 - T_1D_3) were formulated to contain increasing levels (0, 6, and 12%) of BB as a replacement of SBM.

Composition (% as is) of test diets utilized in trial 1.

Ingredient	Diet code		
	T_1D_1	T_1D_2	T_1D_3
Soybean meal[1]	54.10	47.40	40.50
Corn protein concentrate[2]	8.00	8.00	8.00
Whole wheat[3]	25.00	25.00	25.00
Bacterial biomass[4]	0.00	6.00	12.00
Fish oil[2]	6.05	6.14	6.24
Trace mineral premix[5]	0.50	0.50	0.50
Vitamin premix[6]	1.80	1.80	1.80
Choline chloride[3]	0.20	0.20	0.20
Stay C[7]	0.10	0.10	0.10
Mono-dicalcium phosphate[8]	2.50	2.50	2.50
Lecithin[9]	1.00	1.00	1.00
Cholesterol[3]	0.05	0.05	0.05
Corn starch[3]	0.70	1.31	2.11

[1] De-hulled solvent extract soybean meal, (b) (4)

[2] (b) (4)

[3] (b) (4)

[4] KnipBio Inc., Lowell, MA, USA.

[5] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate,

13.193; Alpha-cellulose, 69.664.

[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.

[7] (b) (4)

[8] (b) (4)

[9] (b) (4)

Proximate composition (% as is) and amino acid profile (% as is) of the test diets used in trial 1.

Composition[1]	T_1D_1	T_1D_2	T_1D_3
Crude Protein	37.67	36.37	36.77
Moisture	5.41	8.34	6.66
Crude Fat	9.54	8.71	8.49
Crude Fiber	4.05	3.48	3.05
Ash	6.06	5.65	5.51

[1] Diets were analyzed at (b) (4)
(b) (4)

The recirculating system consisted of 12 aquaria (160 L) with four replicate groups of shrimps (1.51 g initial mean weight; 8 shrimps/ tank) that were offered diets using a standard feeding protocol over 6 weeks. Based on historic results, feed inputs were pre-programmed and daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality.

Results for trial 1

Performance of juvenile shrimp *L. vannamei* (Initial weight 1.51g) offered diets with different bacterial biomass levels (0, 6, and 12 %) for six weeks in trial 1.

Diet	BB levels (%)	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_1D_1	0	49.25	8.26[a]	440.04[a]	1.65[a]	75.0[b]
I_1D_2	6	53.85	6.96[ab]	370.55[ab]	1.99[b]	96.9[a]
T_1D_3	12	45.70	5.72[b]	280.94[b]	2.61[b]	100.0[a]
PSE[1]		1.1211	0.1646	1.7274	0.0603	12.4145
P-value		0.0831	0.0014	0.0012	0.0010	0.0047

[1] PSE: Pooled standard error.

[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).

[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.

Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

b. In trial 2, to confirm the results in trial 1 and investigate the effects of low inclusion levels of BB, six experimental diets (T_2D_1 - T_2D_6) were formulated to supplement with increasing levels (0, 1, 2, 4, 6, and 12%) of BB as a replacement of SBM.

Composition (% as is) of test diets utilized in trial 2.

Ingredient	Diet code

	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6
Fish meal[1]	6.00	6.00	6.00	6.00	6.00	6.00
Soybean meal[2]	53.00	51.90	50.80	48.60	46.50	40.10
Corn protein concentrate[3]	8.00	8.00	8.00	8.00	8.00	8.00
Bacteria biomass[4]	0.00	1.00	2.00	4.00	6.00	12.00
Fish oil[2]	5.92	5.93	5.94	5.95	5.97	6.01
Trace mineral premix[6]	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[7]	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride[5]	0.20	0.20	0.20	0.20	0.20	0.20
Stay C[8]	0.10	0.10	0.10	0.10	0.10	0.10
Mono-dicalcium phosphate[9]	2.50	2.50	2.60	2.60	2.80	2.90
Lecithin[10]	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol[5]	0.08	0.08	0.08	0.08	0.08	0.08
Methionine[11]	0.05	0.05	0.04	0.04	0.04	0.02
Lysine[11]	0.00	0.01	0.01	0.03	0.04	0.07
Corn starch[5]	20.85	20.93	20.93	21.10	20.97	21.22

[1] (b) (4)
[11] (b) (4)

Proximate composition (% as is) of the test diets used in trial 2.

Composition[1]	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6
Crude protein	36.33	35.52	36.42	34.29	34.48	36.10
Moisture	7.15	8.57	7.34	9.47	9.42	8.08
Crude fat	9.39	9.44	8.94	9.36	9.83	8.15
Crude fiber	3.21	3.28	3.01	2.99	2.73	2.69
Ash	6.86	6.75	6.70	6.62	6.60	6.55

[1] Diets were analyzed at (b) (4)
(b) (4)

The recirculating system consisted of 24 aquaria (135 L) with four replicate groups of shrimps (10 shrimp / tank). Shrimps were offered diets using standard feeding protocol over 6 weeks. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality.

Results for trial 2

Growth Performance of juvenile shrimp *L. vannamei* (Initial weight 0.98g) offered diets with different bacteria biomass levels (0, 1, 2, 4, 6, and 12 %) for six weeks in trial 2.

Diet	BB levels (%)	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_2D_1	0	79.3^{a}	8.4^{ab}	766.6^{ab}	1.64^{bc}	95.0
T_2D_2	1	84.9^{a}	9.2^{a}	836.8^{a}	1.50^{c}	92.5
T_2D_3	2	84.0^{a}	8.6^{ab}	811.1^{ab}	1.56^{bc}	97.5

T_2D_4	4	85.3[a]	8.5[ab]	765.3[ab]	1.63[bc]	100.0
T_2D_5	6	75.3[a]	7.7[b]	697.5[b]	1.83[b]	97.5
T_2D_6	12	58.1[b]	5.8[c]	493.70[c]	2.50[a]	100.0
PSE[1]		1.2191	0.1031	13.7258	0.0303	1.0623
P-value		<0.0001	<0.0001	<0.0001	<0.0001	0.1458

[1] PSE: Pooled standard error

[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).

Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Proximate composition (moisture: % as is; protein and lipid: % dry weight) and amino acid profile[2] (% dry weight) of whole shrimp body.

Diet	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6	PSE[1]	*P*-value	Adjust *P*-value
BB levels (%)	0	1	2	4	6	12			
Moisture	75.65[ab]	75.48[ab]	75.79[ab]	75.17[b]	76.93[ab]	77.23[a]	0.2091	0.0117	0.0351
Protein	75.08[b]	74.97[b]	74.39[b]	74.80[b]	75.28[b]	77.77[a]	0.1807	<0.0001	0.0003
Lipid	6.37[b]	6.54[ab]	7.92[a]	7.26[ab]	5.76[b]	3.62[c]	0.1706	<0.0001	<0.0001
Alanine	4.27	4.33	4.44	4.28	4.40	4.31	0.0365	0.5092	0.5555
Arginine	5.45[bc]	5.23[c]	5.43[c]	5.47[bc]	5.72[b]	6.17[a]	0.0325	<0.0001	<0.0001
Aspartic Acid	6.76	6.94	6.79	6.71	6.84	6.86	0.0322	0.2140	0.3425
Cysteine	0.60	0.61	0.61	0.60	0.62	0.63	0.0039	0.0673	0.1614
Glutamic Acid	10.18	10.41	10.16	10.06	10.22	10.31	0.0559	0.3439	0.4855
Glycine	5.01[cd]	4.83[d]	5.01[cd]	5.19[bc]	5.49[b]	6.08[a]	0.0350	<0.0001	<0.0001
Histidine	1.49	1.52	1.49	1.48	1.50	1.52	0.0115	0.6861	0.7159
Hydroxylysine	0.14	0.15	0.17	0.18	0.17	0.17	0.0070	0.5032	0.5555
Hydroxyproline	0.20	0.22	0.23	0.21	0.21	0.20	0.0051	0.4981	0.5555
Isoleucine	2.95	3.00	2.95	2.93	2.97	2.95	0.0111	0.3829	0.5105
Leucine	4.95	5.03	4.94	4.92	4.97	5.01	0.0162	0.1896	0.3250
Lysine	4.92	5.04	4.98	4.93	5.04	5.11	0.0239	0.0874	0.1907
Methionine	1.46[c]	1.49[c]	1.50[bc]	1.51[abc]	1.55[ab]	1.57[a]	0.0065	0.0002	0.0010
Phenylalanine	3.16	3.23	3.20	3.18	3.20	3.27	0.0169	0.3398	0.4855
Proline	4.09[a]	4.15[a]	4.18[a]	4.00[ab]	3.88[ab]	3.67[b]	0.0415	0.0036	0.0143
Serine	2.35	2.38	2.35	2.34	2.36	2.36	0.0195	0.9934	0.9934
Threonine	2.62[b]	2.76[a]	2.62[b]	2.60[b]	2.64[b]	2.61[b]	0.0135	0.0049	0.0169
Tryptophan	0.87	0.85	0.85	0.85	0.85	0.88	0.0048	0.1132	0.2263
Tyrosine	2.51	2.33	2.49	2.52	2.45	2.57	0.0405	0.4536	0.5555
Valine	4.10	4.19	4.12	4.16	4.17	4.00	0.0257	0.1632	0.3014
Total	68.05	68.66	68.49	68.08	69.23	70.23	0.2581	0.0629	0.1614

[1] PSE: Pool standard error.

[redacted]

[2] Proximate composition and amino acid profile of whole body samples were analyzed at (b) (4)
(b) (4)

Values within a row with different superscripts are significantly different based on Tukey's multiple range test.

iii. In trial 3, five experimental diets (T_3D_1 - T_3D_5) were formulated (Table 3). Additionally, a reference diet was utilized to determine digestibility coefficients in conjunction with 1% chromic oxide as an inert marker and 70:30 replacement strategy.

Composition (% as is) of test diets utilized in trial 3.

Ingredients	Diet code				
	T_3D_1	T_3D_2	T_3D_3	T_3D_4	T_3D_5
Fish meal[1]	6.00	6.00	6.00	6.00	6.00
Soybean meal[2]	53.00	46.50	46.50	40.10	40.10
Corn protein concentrate[3]	8.00	8.00	8.00	8.00	8.00
Bacteria biomass[4]	0.00	6.00	13.30	12.00	26.60
Fish oil[2]	5.92	5.97	5.81	6.01	5.70
Trace mineral premix[6]	0.50	0.50	0.50	0.50	0.50
Vitamin premix[7]	1.80	1.80	1.80	1.80	1.80
Choline chloride[5]	0.20	0.20	0.20	0.20	0.20
Stay C[8]	0.10	0.10	0.10	0.10	0.10
Mono-dicalcium phosphate[9]	2.50	2.80	2.80	2.90	2.90
Lecithin[10]	1.00	1.00	1.00	1.00	1.00
Cholesterol[5]	0.08	0.08	0.08	0.08	0.08
Methionine[11]	0.05	0.04	0.03	0.02	0.02
Lysine[11]	0.00	0.04	0.05	0.07	0.09
Corn starch[5]	20.85	20.97	13.83	21.22	6.91

Proximate composition (% as is) of the test diets used in trial 3.

Composition[1]	T_3D_1	T_3D_2	T_3D_3	T_3D_4	T_3D_5
Crude protein	35.7	33.7	38.4	34.7	41.1
Moisture	8.7	11.71	8.39	9.7	10.46
Crude fat	6.71	7.57	8.2	7.64	7.26
Crude fiber	3.1	2.47	2.64[2]	2.62	2.39[2]
Ash	7.08	6.67	7.08	6.61	6.56

[1] Diets were analyzed at (b) (4)

[2] Diets were analyzed at (b)

The recirculating system consisted of 24 aquaria (135L) with four replicate groups of shrimps (10 shrimps/ tank) that were fed as described for trial 2 for 6 weeks

Results for trial 3

Performance of juvenile shrimp *L. vannamei* (Initial weight 0.15g) offered diets formulated to partially replace soybean meal on a digestible protein basis for six weeks in trial 3.

Diet	BB levels (%)	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_3D_1	0	42.68[a]	4.74[a]	3160.39[a]	1.72[c]	90.0
T_3D_2	6	43.15[ab]	4.30[ab]	2813.38[ab]	1.90[bc]	100.0
T_3D_3	13.3	45.38[ab]	4.54[a]	2732.16[abc]	1.73[c]	100.0
T_3D_4	12	38.48[ab]	3.84[bc]	2438.14[bc]	2.11[ab]	100.0
T_3D_5	26.6	35.05[b]	3.60[c]	2304.94[c]	2.26[a]	97.5
PSE[1]		1.1420	0.0710	57.1783	0.0338	1.9084
P-value		0.0406	0.0002	0.0008	0.0001	0.3194

[1] PSE: Pooled standard error

[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).

[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.

Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Proximate composition of whole shrimp body and protein retention efficiency (PRE) offered diets formulated to utilize bacterial biomass (BB) partially replace soybean meal on a digestible protein basis for six weeks in trial 3.

Diet	BB levels (%)	Protein[2] (%)	Moisture (%)	Lipid[2] (%)	Fiber[2] (%)	Ash[2] (%)	PRE[3] (%)
T_3D_1	0	70.83[b]	76.1	8.40[a]	5.25	11.50[c]	30.50[a]
T_3D_2	6	70.68[b]	76.7	7.89[a]	5.26	11.80[c]	29.37[a]
T_3D_3	13.3	72.52[ab]	76.6	5.07[b]	5.30	12.56[bc]	27.97[a]
T_3D_4	12	72.59[ab]	77.0	6.00[ab]	5.48	13.35[ab]	25.43[ab]
T_3D_5	26.6	73.55[a]	77.4	4.07[b]	5.68	14.01[a]	20.11[b]
P-value		0.0107	0.2379	0.0003	0.3623	<0.0001	0.0002
PSE[1]		0.2819	0.1932	0.2803	0.0849	0.1399	0.6234

[1] PSE: Pool standard error.

[2] Dry weight basis.

[3] Protein retention (%) = (Final weight × Final protein content) - (Initial weight × Initial protein content) × 100 / Protein offered.

Conclusion.

The results of this study show that, during a 12-week growth period, the notified substance can be included up to 10% by replacing soybean meal in shrimps without significantly affecting growth performance, FCR, and protein as well as amino acids retention efficiency. Moreover, an increase in survivability was observed in the first trial, suggesting a beneficial effect of the notified substance in the conditions tested.

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Partial replacement of soybean meal protein with bacterial protein meal in feeds for rainbow trout (*Oncorhynchus mykiss* Walbaum)

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Abstract

A feeding trial was conducted with juvenile rainbow trout (15-16g initial weight) to assess the effects of replacement of a portion of soybean meal in the diet with bacterial protein meal produced from *Methylobacterium extorquens*. Three isonitrogenous and isoenergetic diets were produced: a control diet and two experimental diets containing 5% or 10% bacterial protein meal replacing soybean meal. Triplicate tanks, each containing 35 fish, were fed each diet to apparent satiation in constant-temperature (15 °C), flow-through spring water for twelve weeks. No statistically significant differences in final fish weight or other fish growth parameters were observed, although the average weight and total feed consumption of fish fed the 10% bacterial protein meal were numerically lower. Feed efficiency parameter showed no significant differences among groups. Nutrient retention indices (protein, fat, energy) were relatively high and similar among fish in each dietary treatment group, as were whole body proximate compositions. Nitrogen retention, expressed as g^{-1} kg body weight gain, was approximately 27 g whereas nitrogen loss was 37-39 g, with both being similar among treatment groups. Fish survival was high, with a small but statistically significant increase for the 10% bacterial protein meal diet. Overall, results demonstrate that bacterial protein meal from *Methylobacterium extorquens* is a suitable alternative protein for rainbow trout diets at all low inclusion levels tested. Slightly lower weight gain in fish fed the 10% bacterial protein meal diet was largely due to lower feed intake, suggesting that adding palatability-enhancing ingredients to feeds may allow higher levels of bacterial protein to be used without compromising fish growth.

Introduction

Single-cell proteins (SCPs) have been constituents of fish feeds for decades. Research on the nutrition of trout conducted at the Cortland Hatchery, a research facility operated under a cooperative agreement between the New York State Conservation Department, the U.S. Fish and Wildlife Service and Cornell University, demonstrated that adding 8% brewer's yeast (*Saccharomyces cerevisiae*), a by-product of beer production, to hatchery-made feeds improved fish growth compared to standard trout feed formulations (Phillips, Brockway, Kolb, & Maxwell, 1950). Thereafter, brewer's yeast was routinely included in trout feeds, albeit at relatively low levels. Fish feed formulations of the day were developed empirically through trial-and-error substitution of ingredients because the dietary requirements of fish for the 40 or so essential nutrients were not yet known. Over the next two decades, dietary requirements of many essential nutrients for salmonids were established, making it possible to formulate fish feeds based on nutrient contents of a given feed mixture (NRC, 1972). This led to improved fish performance and enabled the use of feed formulation programs to formulate feeds that met the nutritional requirements of the species being fed at the lowest cost per kg. The responsibility of the feed formulator was to place restrictions or limits on feed ingredient levels to ensure that formulations were palatable, able to be pelleted, durable and met other such practical considerations not accounted for by computer-run feed formulation programs.

For many years, fishmeal was the primary source of protein in formulated feeds for salmonids due to its competitive price relative to other protein ingredients as well as its contribution of a range of nutrients to feeds. However, a major disruption in global supply associated with an El Niño event in Peru and Chile in 1972-73 caused fishmeal prices to triple (Kolhonen, 1974). This increased fish feed prices and stimulated efforts to identify and evaluate alternative protein ingredients for their suitability as fishmeal replacements. Among the alternate proteins evaluated at the time were SCPs from yeast and bacteria (Nose, 1974; Matty & Smith, 1978; Beck, Gropp, Koops, & Tiews, 1979; Spinelli, Mahnken, & Steinberg, 1979; Kaushik & Luquet, 1980). Collectively, studies showed that SCPs were suitable ingredients to supply a portion of dietary protein in trout feeds, although suitable levels in feeds depended on the source of the SCP (yeast or bacteria), the substrate upon which it was grown, which fish species and life history stage was tested, and how the experimental feed was formulated. SCP use in livestock and poultry feeds was limited by the nucleic acid content of SCPs, but trout have high levels of uricase that allows them to metabolize nucleic acids (Rumsey, Hughes, Smith, Kinsella, & Shetty, 1991a).

Bacterial SCP produced using methane as a feedstock was first evaluated in rainbow trout feeds by Kaushik and Luquet (1980), who reported that up to 80% of fishmeal could be replaced without compromising fish performance. Skrede, Berge, Storebakken, Herstad, Aarstad, & Sundstø (1998) and Storebakken, Kvien, Shearer, Grisdale-Helland, Helland, & Berge (1998) examined digestibility of meal produced from methanotrophic bacteria and reported values slightly lower than those for high-quality fishmeal. Perera, Carter, & Houlihan (1995) found that bacterial SCP could be included in rainbow trout diets at 17.4%, replacing 25% of the fishmeal, without adverse effects on feed consumption (based on radiography), feed efficiency or growth rate. However, 17.4% dietary bacterial SCP reduced protein efficiency ratio and increased nitrogen excretion. Even though not significant, there was also a reduction in protein digestibility (85% vs. 79.9%), which reduced protein retention. These effects were attributed, in part, to the nucleic acid content of bacterial SCP. Nucleic acids contribute to dietary nitrogen content but are non-protein nitrogen compounds.

Methylobacterium extorquens is a non-pathogenic, plant epiphyte that is notable for the ability to consume methanol, in addition to a variety of multi-carbon substrates such as ethanol, glycerol, or many organic acids (Ochsner, Sonntag, Buchhaupt, Schrader & Vorholt, 2014). Methanol is a particularly attractive feedstock due to its abundant availability from either geological or biological sources of natural gas. Because of this, methanol-based biotechnology has seen considerable development in the past decade (for review Schrader et al., 2009) and the vast majority of this has occurred in *M. extorquens* (Bélanger, Figueira, Bourque, Morel, Béland, Laramée, Groleau, & Míguez, 2004; Tlusty, Rhyne, Szczebak, Bourque, Bowen, Burr, Marx, & Feinberg, 2017) because of its genetic tractability (Marx and Lidstrom, 2001; Marx, 2008; Schada von Borzyskowski, Remus-Emsermann, Weishaup, Vorholt & Erb, 2014) and proven track record for high-density fermentation (Bélanger et al., 2001, Bourque, Pomerleau, & Groleau, 1995; Tlusty et al., 2017). As a model system, the nutritional value of the new protein products (bacterial protein) can be manipulated to enhance levels of targeted essential amino acids, alter levels of major biomass constituents, or to produce other high value molecules.

KnipBio is a US Corporation that has very recently demonstrated that *M. extorquens* (KnipBio meal, or KBM) can serve as a suitable feed ingredient for a variety of organisms (Tlusty et al., 2017). Initial studies with KBM in fish and shrimp feeds reported positive growth results with Pacific white shrimp (*Litopenaeus vannamei*) and smallmouth grunts (*Haemulon chrysargyreum*). Feeds with KBM had no effect on sensory attributes of Pacific white shrimp.

Interestingly, addition of KBM to feeds did not lead to significant changes in the shrimp gut microbiome, as is sometimes the case with alternative feeds (Zhou, Ringø, Olsen, & Song, 2016). KBM was also found to be highly digestible to Atlantic salmon (*Salmo salar*) (Tlusty et al., 2017).

The objective of the current study was to evaluate growth and safety of KBM as a feed component for rainbow trout (*Oncorhynchus mykiss*) in a laboratory scale feeding trial. The length of the trial was of sufficient duration to assess the apparent nutritional quality of feeds in which soybean meal (SBM) was partially replaced with KBM when other protein sources were kept constant. Evaluation criteria included nutrient retention, an important consideration from the perspective of regulatory compliance for freshwater aquaculture, as well as survival.

Materials and methods

Experimental Feeds*:* KBM supplied by Knipbio was analyzed for proximate composition as described below prior to feed formulation (Table 1). *M. extorquens* (strain KB203) was grown by aerobic fermentation at 30 °C with standard procedures for assuring purity of the seed cultures, as described previously (Bélanger et al., 2004; Tlusty et al., 2017). The defined CHOI4 medium and trace metals stock solution were used throughout the process, ultimately culminating in growth in a 1500 L fermenter as before (Bélanger et al., 2004; Tlusty et al., 2017). The collected biomass was spray-dried (Tlusty et al., 2017) to generate a flour that was used in the feeds (i.e., KBM).

Three experimental diets, a control diet plus two experimental diets, were developed with formulation software (WinFeed 2.8, Cambridge, UK) to contain 45% crude protein, 18% lipid, 3.1% lysine and 1% methionine (as-is basis, Table 2). Amino acid composition data provided by KnipBio was used for feed formulation. The diets were as follows:

Diet 1: Control – standard level of fishmeal in commercial trout feeds
Diet 2: 5% KBM replaced SBM on a crude protein basis
Diet 3: 10% KBM replaced SBM on a crude protein basis

All diets met or exceeded the minimum nutrient requirements of rainbow trout (NRC, 2011). Experimental feeds were produced by extrusion pelleting at the Bozeman Fish Technology Center, Bozeman, MT. All ingredients were ground to a particle size of <200 µm using an air-swept pulverizer (Model 18H, Jacobsen, Minneapolis, MN) and processed into pellets using a

twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with a ~25 sec exposure to 127 °C in the extruder barrel (average across 5 sections). Pellets were dried in a pulse bed drier (Buhler AG, Uzwil, Switzerland) for 20 minutes at 102 °C with a 10 minute cooling period, resulting in final moisture levels of less than 10%. Added oil was top-coated after the pellets were cooled using a vacuum-coater (AJ Mixing, Ontario, CA). Diets were placed in plastic lined paper bags, shipped to the University of Idaho's Hagerman Fish Culture Experiment Station and stored at room temperature until fed. Feed samples were analyzed for proximate composition and gross energy prior to the start of the feeding trial to ensure that expected values were achieved.

Fish and Feeding: The feeding trial was conducted at the Hagerman Fish Culture Experiment Station. Rainbow trout fingerlings, hatched from eggs purchased from a commercial source (TroutLodge, Sumner, WA,) were used in the study. Thirty-five fish (initial average weight: 15.6 g) were stocked into each of nine 145-L tanks. Each tank received 10-12 L/min of constant temperature (15 °C) spring water supplied by gravity. Using a completely randomized design, three triplicate tanks were assigned to each of the three experimental diets. Each diet was fed by hand by trained staff three times per day to apparent satiation, six days per week, for 12 weeks. Photoperiod was held constant at 14 h light: 10 h dark with fluorescent lights on electric timers. Tanks were cleaned daily and any mortality was removed and recorded when first noticed. Fish were bulk-weighed and counted every three weeks for the duration of the study. Twenty fish from the initial population used in the study were sacrificed and frozen at -20 °C for later whole-body proximate analysis. At the end of 12 weeks, five fish per tank were removed and euthanized with MS-222 (250 mg/L). Length and weight of fish were measured to calculate condition factor. Fish were pooled by tank and frozen at -20 °C for proximate analysis. Samples of proximal and distal intestine from three fish per tank were also collected for histological assessment. Experimental protocols used in the growth trial were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee.

Chemical analysis: Proximate composition (percent moisture, protein, fat and ash) of bacterial meal, experimental feeds and whole-body fish samples were determined using AOAC (2002) procedures. Fish samples pooled by tank were pureed using an industrial food processer. Briefly, samples were dried in a convection oven at 105 °C for 12 h to determine percent moisture. Dried samples were finely ground by mortar and pestle and analyzed for crude protein (total nitrogen × 6.25) using the combustion method with a nitrogen determinator (TruSpec N, LECO Corporation, St. Joseph, MI). Crude fat in the extruded feeds was measured after acid hydrolysis with an ANKOM HCL hydrolysis system (ANKOM Technology, Macedon, NY)

by extraction with petroleum ether using an ANKOM XT15 extractor. For other samples, crude fat was analyzed without the acid hydrolysis step. Ash was analyzed by incineration at 550 °C in a muffle furnace for 5 hr. Energy contents of samples were determined using an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Company Inc., Moline, IL). All analyses were conducted in duplicate.

Calculations: Using the live-weight and feed consumption data, the following indices were calculated as per Hardy and Barrows (2002):

Weight gain (g/fish) = (g mean final weight – g mean initial weight);

Mean weight gain (%) = [(g mean final weight – g mean initial weight)/g mean initial weight] × 100;

Specific growth rate (SGR, %/d) = [(ln mean final weight – ln mean initial weight)/number of days] × 100;

Daily growth index (DGI, $g^{1/3}$/d) = {[g mean final weight$^{(1/3)}$ – g mean initial weight$^{(1/3)}$]/number of days} × 100;

Feed consumed (g/fish) = g total feed consumed/number of surviving fish;

Daily feed consumption (DFC, % bw/d) = [(g feed consumed/g mean body weight)/number of days] × 100,

where mean body weight = (g mean initial weight + g mean final weight)/2;

Feed conversion ratio (FCR) = g feed consumed per fish/ g wet weight gain per fish;

Survival (%) = (number of fish at the end of the trial/number of fish at the beginning) × 100;

Protein efficiency ratio (PER) = g weight gain/g protein consumed;

Condition factor (K) = {g fish weight/(cm fish length)^3} × 100;

Nutrient retention (%) = (g nutrient gain/g nutrient consumed) × 100; and

Energy retention (ER, %) = (MJ energy gain/MJ energy consumed) × 100.

Statistical Analysis of Data: Tank mean values were used as units of observation for statistical analysis. Fish growth performance, feed utilization, body composition, and nutrient retention data were tested for normality and homogeneity of variance prior to one-way Analysis of Variance (ANOVA). When significant differences were found, data were subjected to Tukey's HSD test to separate the means at a significance level of $P<0.05$. All statistical tests were performed with SAS 9.4 software for personal computers (SAS Institute Inc. Cary, NC).

Results

The proximate and amino acid composition of KBM is presented in Table 1. Crude protein content was 51%; fat and ash levels were low in the product. The gross energy was 21.2 MJ/kg. The sum of amino acids in the product indicated that approximately 9% of the product was non-protein nitrogen. The proximate composition and energy content of diets used in the growth trial are presented in Table 2. Moisture was low in the diets (2.07%-2.53%). Crude protein content was similar across the diets (~48.6%). Measured crude fat level ranged from 14.8% (Diet 1, Control) to 15.62% (Diet 3, 10KBM). Dietary ash levels were similar (10.12% to 10.35%), as were gross energy levels, 21.5 MJ/kg in the control diet to 21.9 MJ/kg in diet 3.

After 12 weeks of feeding, mean final weight of fish varied from 220 g (diet 3) to 236 g (control diet), with no statistically significant differences among treatment groups (Table 3), although all growth indices decreased numerically with increasing percentages of KBM protein in the diet. Mean feed intake (g/fish) also decreased numerically with increasing percentages of KBM in the diet, whereas feed conversion ratios were low and similar (0.82-0.85) among the dietary treatments. Protein efficiency ratio varied from 2.42 (control diet) to 2.49 (diet 2). There were no significant differences among the dietary groups with respect to the feed utilization indices.

Whole-body proximate composition and energy content of fish did not vary significantly among the dietary treatments (Table 4). Whole-body dry matter (~31%), crude protein (16.6-17%), crude fat (11.6-12.2%) and ash levels (1.91-2.0%) were similar among the dietary groups. Energy levels varied from 8.56 MJ/kg (5KBM) to 8.72 MJ/kg (10KBM). There was no trend in the whole body proximate categories with respect to the dietary inclusion level of KBM.

There was no significant difference among the dietary groups in nutrient retention (Table 4). Lipid retention ranged from 94.3% (diet 2) to 97.3% (control). Protein retention in rainbow trout ranged from 40.7% (control) to 43.0% (diet 2). Energy retention varied from 48.2% (control) to 49% (diet 2). Similarly, nitrogen intake, retention or loss values were not significantly different among the dietary treatments (Table 5). For each kg body weight gain, N intake varied slightly from 64.2 g (diet 2) to 66.1 g (control), N retained from 26.9 g (control) to 27.6 g (diet 2) and N loss from 36.6 g (diet 2) to 39.2 g (control). There was no clear trend for N balance with regard to the graded levels of KBM in the diet.

Survival was high across the treatments (96.2 to 100%). Fish fed the 10KBM diet had a small, but statistically significant increase in survival compared to those fed the control diet. The fish

fed the 5KBM diet had intermediate survival not statistically separable from either of the other two treatments. Condition factor values of fish varied from 1.51 (diet 3) to 1.56 (diet 2) and were not significantly different among the dietary treatments.

Discussion

The results of this study demonstrate that KBM produced from *Methylobacterium extorquens* is a suitable dietary ingredient for use in trout feeds at relatively low levels of inclusion. Although growth indices numerically decreased with increased inclusion of KBM, this was mostly due to a reduction in feed intake as dietary KBM levels increased. Kaushik and Luquet (1980) also recorded a decrease in feed intake in trout fed diets with increasing levels of meal produced from methanotrophic bacteria. Similarly, Kiessling and Askbrandt (1993) noted a decrease in feed intake and trout weight gain when fish were fed a diet containing bacterial protein meal produced from *Corynebacterium glutamicum* at only 4% of the feed, but not when fish were fed a diet with up to 16% bacterial protein meal produced from *Brevibacterium lactofermentum*. In contrast, other studies with trout or Atlantic salmon have not reported a reduction in feed intake associated with dietary level of meal produced from methanotrophic bacteria (Aas, Grisdale-Helland, Terjesen, & Helland, 2006a; Aas, Hatlen, Grisdale-Helland, Terjesen, Bakke-McKellep, & Helland, 2006b). While the source of bacterial protein meal and the process by which it is dried likely affect feed intake, the primary factor accounting for these differences are the feed formulations used in various trials. For example, Aas et al. (2006b) did not observe reductions in weight gain of trout fed diets containing up to 27% methanotrophic bacteria meal over the course of a 76-day feeding trial. However, fishmeal levels in feeds were high, 63.5% in the control diet and 40.7% in the diet containing the highest level of bacterial meal increased. This contrasts with the study of Kaushik and Luquet (1980) in which the fishmeal level was 35% in the control diet and other protein sources were used in experimental feeds. The statement by Kaushik and Luquet that 80% of fishmeal could be replaced with methanotrophic bacteria meal must be tempered by the fact that the highest level of bacterial meal in their study was 35%, and that feed intake and fish performance were reduced at levels above 21%. In a study in which feeding rate was held constant, three levels of bacterial protein were used to replace fishmeal in diets for rainbow trout (Perera et al., 1995). At each level of bacterial protein in diets (17.4%, 43.5% and 69.5%), fish weight gain was significantly reduced. In this context, it is notable that increasing levels of KBM did not compromise either the feed conversion ratio or the protein efficiency ratio.

In the present study, the nitrogen budget was similar to those found in Atlantic salmon (Aas et al., 2006a) and rainbow trout (Aas et al., 2006b). Inclusion of KBM did not affect nitrogen loss (fecal and non-fecal) in rainbow trout. Research with methanotrophic bacterial SCP in feed for Atlantic salmon has produced results similar to those found with rainbow trout. Aas et al. (2006a) fed diets containing 0, 4.5, 9, 18 and 36% methanotrophic bacteria SCP that replaced fishmeal in diets for salmon. The highest and lowest levels of fishmeal in diets were 65% and 35.4%. Over the course of the 52 days study, fish approximately doubled their initial weight (170g). In contrast to the results of Storebakken et al. (2004), who had found decreased growth for bacterial SCP above 20% inclusion, fish weight gain increased with increasing levels of methanotrophic bacteria meal in the diet. Nitrogen retention also increased with increasing bacterial SCP in the diet, despite the fact that digestibility values for nitrogen, sum of amino acids, lipid and energy decreased as the bacterial SCP level in the diet increased. Increased retention suggests that diets containing higher levels of bacterial SCP resulted in more efficient metabolism of these nutrients rather than more efficient digestion, resulting in lower catabolic losses. A challenge for interpretation of these studies, however, was the very high and variable percentages of fishmeal used. Importantly, fishmeal levels in the current study were held constant at 30% across the three experimental diets, with KBM replacing soybean meal. This formulation thus provides a more realistic assessment of KBM as an alternative feed ingredient for salmonids than many previous studies that used extremely high and impractical fishmeal levels.

Although the total mortality was low throughout the feeding trial, 10% KBM inclusion led to a small but statistically significant increase in survival compared to the control diet (with the 5% inclusion intermediate). Additional work will be required to understand this effect, but it should be noted that there is precedent for SCP sources to increase survival and reduce gastroenteritis (Dabrowski, 1984; Laranja, Ludevese-Pascual, Amar, Sorgeloos, Bossier & De Schryver, 2014; Banerjee, Azad, Vikineswary, Selvaraj, & Mukherjee, 2000; Romarheim, Øverland, Mydland Skrede, & Landsverk, 2011) and thus this may represent a significant value added even at a modest inclusion rate.

These initial findings that KBM represents a suitable protein replacement for trout opens the door to a variety of future innovations. As it has been found that bacterial protein products' quality and nutrient profile vary widely due to substrate and conditions of fermentation, type of bacteria and processing after fermentation (Øverland, Romarheim, Hovin, Storebakken, & Skrede., 2010), there exists a wide space of design choices that can be exploited to produce KBM that might further enhance the growth or survival traits conferred.

Conclusion

The present study evaluated KBM as an alternative protein source in feeds for juvenile rainbow trout during a 12-week growth trial. Results showed that KBM can be included up to 10% replacing soybean meal in a rainbow trout diet without significantly affecting growth performance, feed utilization, nutrient retention or fish health. A small but statistically significant increase in survival under benign testing conditions warrants further investigation to assess if KBM increases fish survival under production conditions.

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Table 1. Proximate and amino acid composition of bacterial protein meal (% as-is basis unless mentioned otherwise)

Nutrient	Percent
Dry matter	96.33
Crude protein (N x 6.25)	50.88
Crude fat	0.84
Ash	4.12
Organic matter	92.21
Gross energy (MJ/kg)	21.2
Essential and semi-essential amino acids	
Arginine	3.28
Histidine	1.13
Isoleucine	1.72
Leucine	3.32
Lysine	2.5
Methionine	0.88
Cysteine	0.34
Phenylalanine	2.03
Tyrosine	1.44
Threonine	1.97
Valine	2.75
Non-essential amino acids	
Alanine	3.75
Aspartic acid	4.39
Glutamic acid	6.32
Glycine	2.52
Hydroxyproline	0.02
Proline	1.87
Serine	1.88

Table 2. Ingredient and proximate composition of experimental diets with graded levels of bacterial protein meal fed to juvenile rainbow in the growth trial (g/kg, as-fed basis unless mentioned otherwise).

	Diet 1	Diet 2	Diet 3
Ingredients	Control	5% KBM	10% KBM
Fishmeal, sardine	300.00	300.00	300.0
Poultry by-product meal, feed grade	120.5	120.5	120.5
Soybean meal, dehulled & solvent-extracted	**160.0**	**108.0**	**56.0**
Bacterial protein meal	**0.0**	**50.0**	**100.0**
Soy protein concentrate, Profine VF	90.0	90.0	90.0
Wheat gluten meal	20.0	20.0	20.0
Wheat flour	161.1	160.7	160.7
Dicalcium phosphate	4.2	6.5	8.4
Trace mineral mix, Trouw Nutrition[1]	1.0	1.0	1.0
Vitamin premix, ARS 702[2]	10.0	10.0	10.0
Choline chloride (60%)	6.0	6.0	6.0
Vitamin C (Stay-C, 35%)	2.0	2.0	2.0
Fish oil	125.2	125.3	125.4
Proximate composition (analyzed)			
Moisture	25.3	24.9	20.7
Crude protein (N × 6.25)	486.3	486.2	485.8
Crude fat	148.0	153.6	156.2
Ash	101.9	103.5	101.2
Gross energy (MJ/kg)	21.5	21.6	21.9

[1] Trace mineral premix supply the following to the diet (mg/kg diet): Zn (as $ZnSO_4$ $7H_2O$), 50; Mn (as $MnSO_4$), 7.5; Cu (as $CuSO_4$ $5H_2O$), 2.5; I (as KIO_3), 1; selenium, 0.05.

[2] Vitamin premix supply the following to the diet (mg/kg diet): D calcium pantothenate, 46.47; pyridoxine (pyridoxine HCl), 13.68; riboflavin, 9.58; niacinamide, 21.78; folic acid, 2.49; thiamine (thiamine mononitrate), 9.1; inositol, 599; biotin, 0.33; vitamin B_{12}, 0.03; menadione sodium bisulfite complex, 1.1; vitamin E (DL α-tocopherol acetate), 131.9 IU; vitamin D_3 (stabilized), 6594 IU; vitamin A (vitamin A palmitate, stabilized), 9641 IU; ethoxyquin, 198.

Table 3. Growth performance and feed utilization of juvenile rainbow trout fed diets with graded levels of bacterial protein meal for 12 weeks[1,2].

	Diet 1	Diet 2	Diet 3
	Control	5% KBM	10% KBM
Initial weight (g/fish)	15.6±0.2	15.8±0.1	15.5±0.1
Final weight (g/fish)	236±1	233±7	220±5
Weight gain (g/fish)	220±1	218±7	204±5
Mean weight gain (%)	1417±11	1381±35	1318±21
Specific growth rate (SGR, %/d)	3.24±0.01	3.21±0.03	3.16±0.02
Daily growth index (DGI, $g^{1/3}$/d)	4.38±0.01	4.34±0.07	4.22±0.04
Feed consumed (g/fish)	187±2	180±5	172±4
Daily feed consumption (% Body weight/day)	1.77±0.03	1.71±0.01	1.74±0.01
Feed conversion ratio	0.85±0.01	0.82±0.01	0.84±0.00
Survival (%)[3]	96.2±0.9[b]	98.9±0.9[ab]	100[a]
Protein efficiency ratio (PER)	2.42±0.04	2.49±0.02	2.44±0.01
Condition factor[4]	1.55±0.05	1.56±0.04	1.51±0.01

[1]Mean±SE (n=3) in the same row that share the same superscript or do not have superscripts are not statistically different (P>0.05; Completely Randomized Design, One-factor ANOVA; Tukey's HSD Test).

[2]All calculations were performed on an average fish weight basis.

[3]Each treatment group consisted of 105 fish (3 tanks, 35 fish per tank).

[4]Five fish per tank were measured for weight and length to calculate condition factor.

Table 4. Whole-body proximate composition and nutrient retention of juvenile rainbow trout (average initial weight, 15.6 g) fed experimental diets containing graded levels of bacterial protein meal for 12 weeks (%, wet basis)[1,2].

		Diet 1	Diet 2	Diet 3
	Initial fish[3]	Control	5% KBM	10% KBM
Proximate composition				
Dry matter	23.5	31.0±0.4	31.0±0.3	31.2±0.4
Crude protein	13.8	16.6±0.1	17.0±0.1	16.7±0.1
Crude fat	7.07	11.9±0.5	11.6±0.3	12.2±0.5
Ash	2.13	2.00±0.01	1.91±0.07	2.00±0.11
Gross energy (MJ/kg)	6.03	8.61±0.16	8.56±0.14	8.72±0.19
Nutrient retention				
Fat		97.3±3.4	94.3±2.0	95.3±4.3
Protein		40.7±0.8	43.0±0.7	41.3±0.4
Energy		48.2±0.6	49.0±0.6	48.4±1.1

[1]Mean±SE (n=3) in the same row that share the same superscript or do not have superscript are not statistically different (P>0.05; Completely Randomized Design, One-factor ANOVA).
[2]Five fish from each tank were used for analysis.
[3]Initial fish composition are for reference only and were not included for statistical analysis.

Table 5. Nitrogen budget in juvenile rainbow trout (average initial weight, 15.6 g) fed experimental diets containing graded levels of bacterial protein meal for 12 weeks (g/kg body weight gain)[1,2].

	Diet 1	Diet 2	Diet 3
	Control	5% KBM	10% KBM
N intake	66.1±1.0	64.2±0.6	65.6±0.1
N retained	26.9±0.2	27.6±0.2	27.1±0.3
N lost	39.2±1.2	36.6±0.8	38.5±0.4

[1]Mean±SE (n=3) in the same row that share the same superscript or do not have superscript are not statistically different (*P*>0.05; Completely Randomized Design, One-factor ANOVA).
[2]Five fish from each tank were used for analysis.

Appendix 3-1

(b) (4)

(b) (4)

(b) (4)

Appendix 3-2

CONTAINS CONFIDENTIAL INFORMATION OF KNIPBIO, INC.			
[redacted]	**Research and Development**	**SOP #**	13
		Revision #	2
		Implementation Date	2017-07-27
Page #	1 of 3	**Last Reviewed/Update Date**	
SOP Owner	Daniel Smith	**Approval**	

(b) (4)

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Appendix 3-3

Carotenoid analysis PA1 and related strains

1. Purpose

Compare carotenoid profile of *Methylobacterium extorquens* PA1 (internally designated KB200) to related strains KB203 (KB200 ΔcelABC ΔcrtCDF), KB208 (KB203 ΔMext_4818-4824), KB440 (KB203 ΔMext_3434-3441).

2. Materials

(b) (4)

3. Procedure

(b) (4)

(b) (4)

4. References and notes

(b) (4)

Appendix 3-4

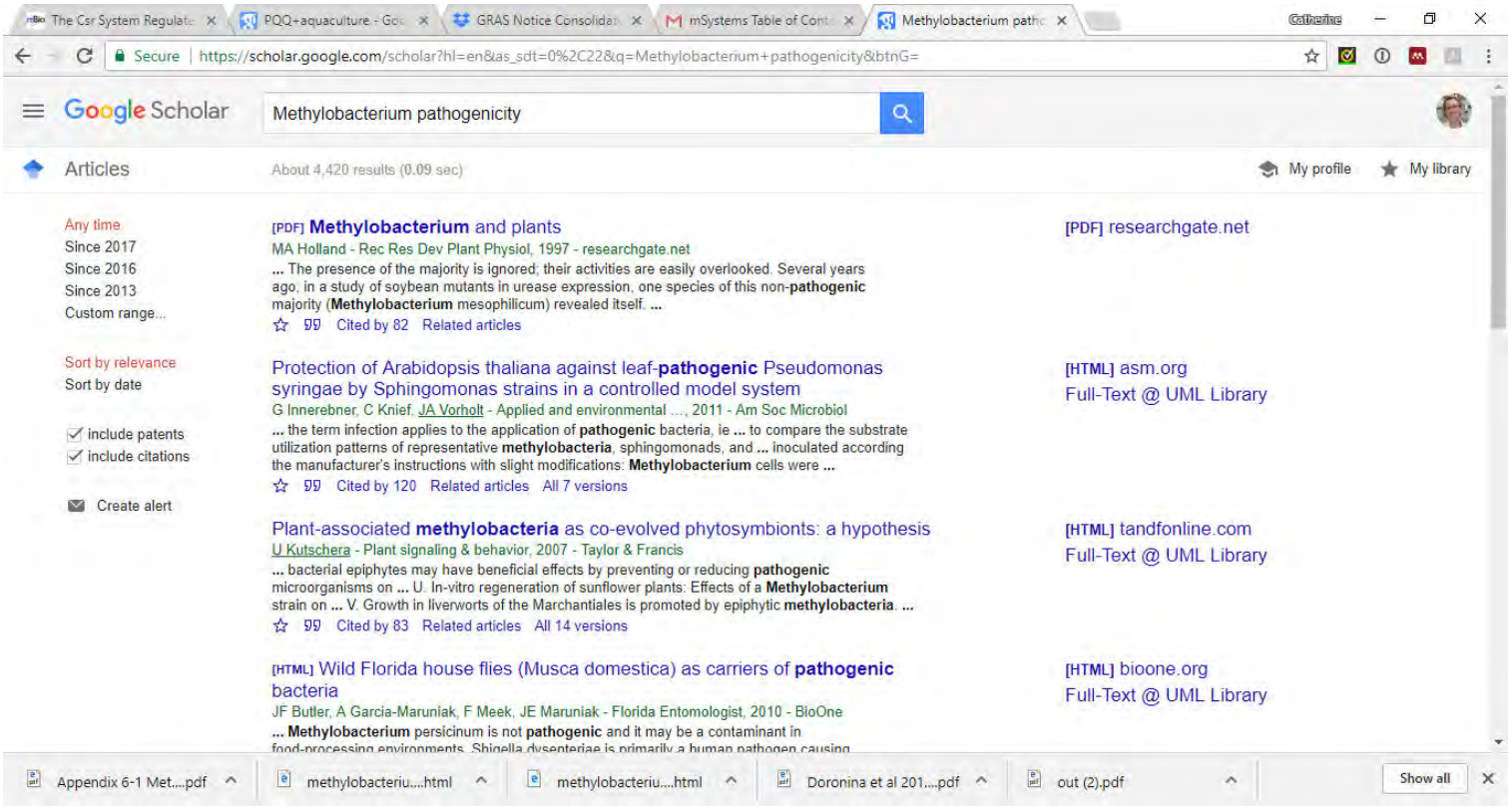
(b) (4)

(b) (4)

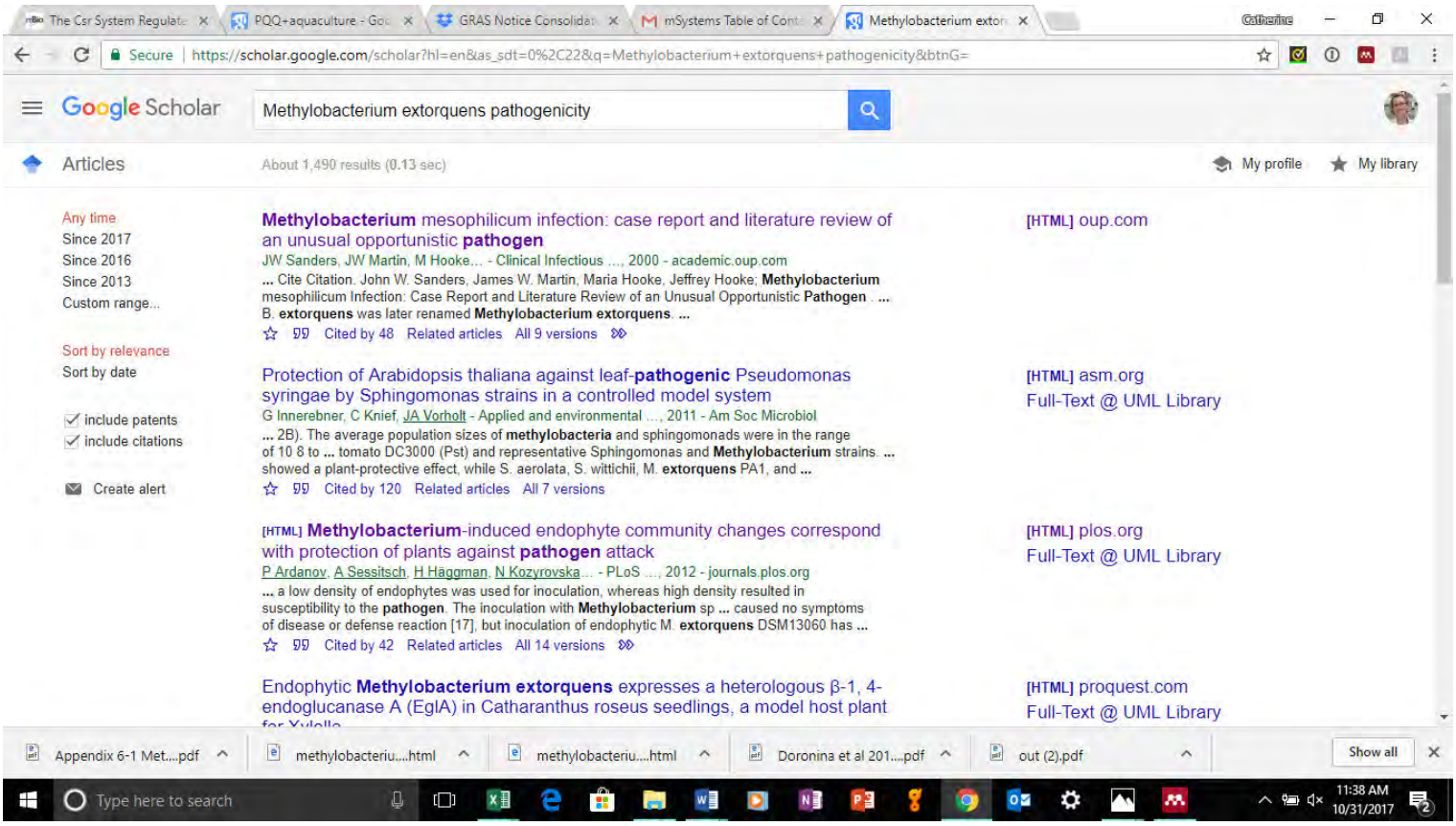
(b) (4)

Appendix 6-1

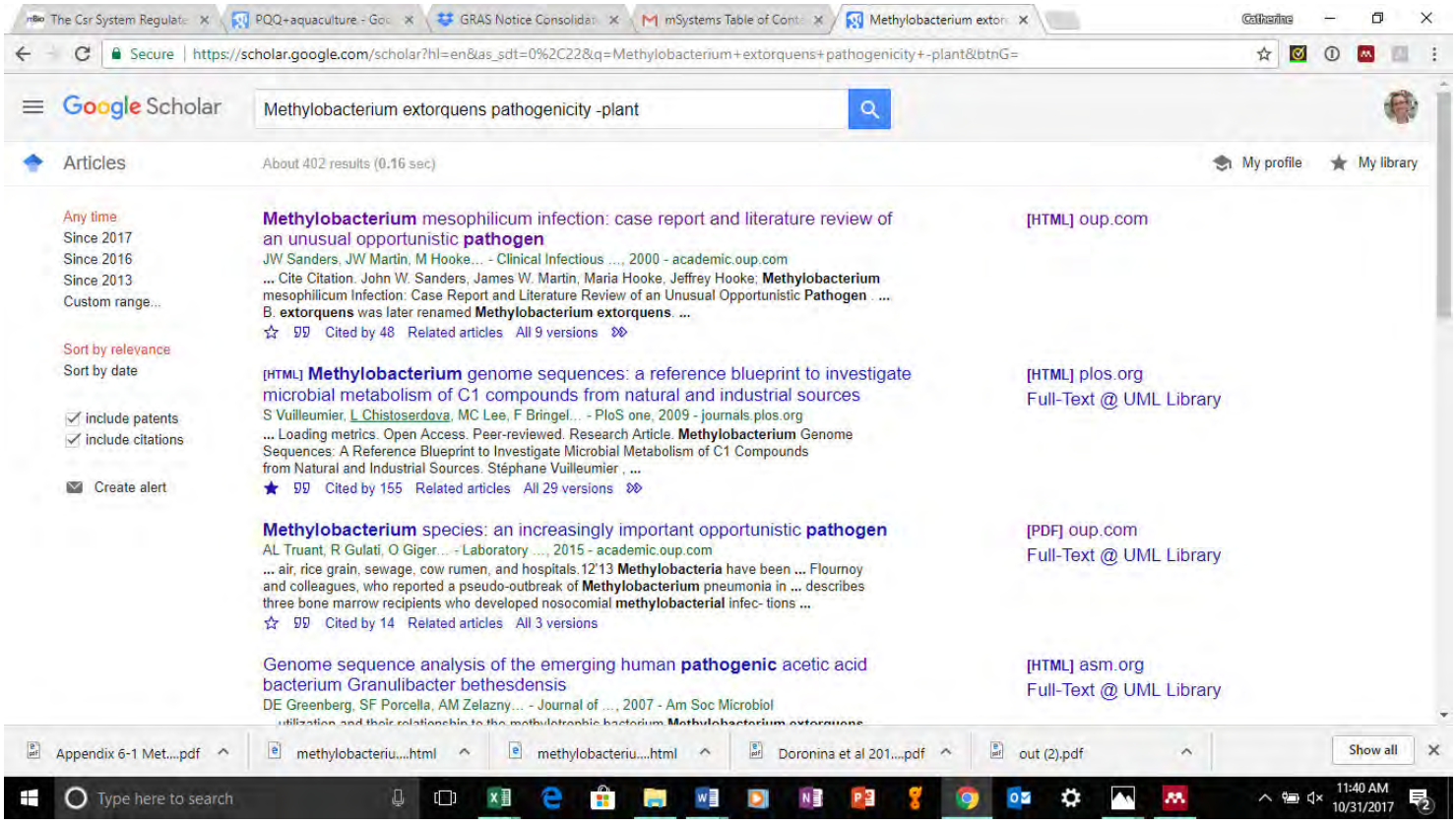
A Google Scholar search "*Methylobacterium* pathogenicity" on October 31, 2017 yielded 4420 hits.



A Google Scholar search adding the species extorquens "*Methylobacterium extorquens* pathogenicity" on September 12, 2017 yielded 1490 hits, most of which related to plant pathogenicity.



When the word plant was removed from the search, ~ 400 hits are generated. The list of all the papers are in attached as part of this Appendix.



Most of the papers identified in this search are not directly relevant to *M. extorquens* pathogenicity, for several reasons. First, in many cases, it is clear from the titles of these papers that they are reports that organisms of entirely different genera and species are pathogens or have pathogenic properties, and when species of *Methylobacterium* were mentioned, it was only peripherally. Sometimes *Methylobacterium* species have been cited merely because certain genes in the alleged pathogenic species had homology to certain *Methylobacterium* genes. In some cases where the title points to a different microbial species, it is not at all clear what reference there may be for *Methylobacterium.* There are other papers where the term "pathogen" appears to have been used in a completely different context, such as discussions of factors affecting the growth of environmental pathogens. Further, as discussed below, there were papers which pertained to species of *Methylobacterium* other than *M. extorquens*, although some of these are discussed below. We discuss those articles deemed to be relevant as follows.

We first note that several of the papers uncovered in the literature search report that different *Methylobacterium* species may be opportunistic pathogens that can cause illness in immunocompromised individuals. *Methylobacterium* species have also been isolated from various medical-related sources such as catheters, endoscopes and hospital water supplies, but with little or no evidence that any of the isolates are pathogenic. (Truant *et al.*, 2015) said that, as of its 1998 publication date, "to our knowledge, *only* 21 cases of human

methylobacterium infection have been reported in the world's literature (emphasis added)":

> **Truant, A.L., Gulati, R., Giger, O., Satishchandran, V. and Caya, J.G., 2015. Methylobacterium species: an increasingly important opportunistic pathogen. *Laboratory Medicine*, *29*(11), pp.704-710.**
>
> Methylobacterium species rarely cause human disease. Those isolated from humans are usually found as opportunistic pathogens in patients weakened by an underlying disease process. This report describes a case of Methylobacterium bacteremia in a 35-year-old woman with AIDS, compares the clinical presentation of this case with that of other previously reported cases of Methylobacterium infection, and provides a history of the Methylobacterium genus and its relevant taxonomy. Recommendations for presumptive identification include pink to orange colony growth on blood or Sabouraud agar but not on MacConkey agar; gram-negative, vacuolated bacillus or coccobacillus; growth at 25 to 30 degrees C and not at 42 degrees C; and positive results on tests for oxidase and urease. Antibiotics with demonstrated efficacy against Methylobacterium species include amikacin, gentamicin, ciprofloxacin, trimethoprim-sulfamethoxazole, ceftizoxime, and ceftriaxone.

In 2000, Sanders *et al.* reported 29 cases of infection putatively linked to Methylobacterium mesophilicum.

> **Sanders, J.W., Martin, J.W., Hooke, M. and Hooke, J., 2000. Methylobacterium mesophilicum infection: case report and literature review of an unusual opportunistic pathogen. *Clinical Infectious Diseases*, *30*(6), pp.936-938.**
>
> Methylobacterium mesophilicum is a methylotrophic, pink pigmented, gram-negative rod that was initially isolated from environmental sources that is being increasingly reported as a cause of opportunistic infections in immunocompromised hosts. We present the case of an immunocompromised woman who developed a central catheter infection with M. mesophilicum and review the other 29 cases reported in the literature, noting that it is frequently resistant to beta-lactam agents but is generally susceptible to aminoglycosides and quinolones.

A more recent review (Kovaleva *et al.*, 2014) reports that *Methylobacterium* species have been reported as sources of health care-associated infections, including infections in immunocompromised hosts (see references cited in this paper).

> **Kovaleva, J., Degener, J.E. and van der Mei, H.C., 2014. Methylobacterium and its role in health care-associated infection. *Journal of clinical microbiology*, *52*(5), pp.1317-1321.**
>
> *Methylobacterium* species are a cause of health care-associated infection, including infections in immunocompromised hosts. The ability of*Methylobacterium* species to form biofilms and to develop resistance to high temperatures, drying, and disinfecting agents may explain the colonization of *Methylobacterium* in the hospital environment in, e.g., endoscopes. Due to its slow growth, it can be easily missed during microbiological surveillance of endoscope reprocessing. The purpose of this minireview is to present an overview of documented infections and cross-contaminations with *Methylobacterium* related to endoscopic procedures and to illustrate the health care-associated relevance of this slow-growing bacterium.

In many of the papers identified in the literature search, the authors identified the isolated microorganism simply as a species of *Methylobacterium* without assigning a definitive species identification (see above (Sanders *et al.*, 2000); (Truant *et al.*, 1998)) as well as (Furuhata *et al.*, 2006); (Kelley *et al.*, 2004).

Furuhata, K., Kato, Y., Goto, K., Hara, M., Yoshida, S.I. and Fukuyama, M., 2006. Isolation and identification of Methylobacterium species from the tap water in hospitals in Japan and their antibiotic susceptibility. *Microbiology and immunology*, *50*(1), pp.11-17.

Contamination of tap water by *Methylobacterium* species has become a serious concern in hospitals. This study was planned to examine the distribution of *Methylobacterium* species inhabiting tap water used in Japanese hospitals and antibiotic sensitivity of the isolates in 2004. Species identification of 58 isolates was performed based on the homology of a partial sequence of 16S rDNA. The dominant *Methylobacterium* species in hospital water were *M. aquaticum* and *M. fujisawaense*. To examine the biochemical properties of these isolates, a carbon source utilization was tested using an API50CH kit. The phenotypic character varied widely, and was not necessarily consistent with the results of phylogenic analysis based on the partial 16S rDNA sequence, suggesting that the biochemical properties are not suitable for identification of *Methylobacterium* species. The isolates were also subjected to antibiotic sensitivity tests. They were resistant to 8 antibiotics, but highly sensitive to imipenem (MIC_{90}=1 μg/ml) and tetracycline (MIC_{90}=8 μg/ml). These findings concerning the isolates revealed the presence of *Methylobacterium* species with resistance to multiple antibiotics in hospital tap water.

Kelley, S.T., Theisen, U., Angenent, L.T., Amand, A.S. and Pace, N.R., 2004. Molecular analysis of shower curtain biofilm microbes. *Applied and environmental microbiology*, *70*(7), pp.4187-4192.

Households provide environments that encourage the formation of microbial communities, often as biofilms. Such biofilms constitute potential reservoirs for pathogens, particularly for immune-compromised individuals. One household environment that potentially accumulates microbial biofilms is that provided by vinyl shower curtains. Over time, vinyl shower curtains accumulate films, commonly referred to as “soap scum,” which microscopy reveals are constituted of lush microbial biofilms. To determine the kinds of microbes that constitute shower curtain biofilms and thereby to identify potential opportunistic pathogens, we conducted an analysis of rRNA genes obtained by PCR from four vinyl shower curtains from different households. Each of the shower curtain communities was highly complex. No sequence was identical to one in the databases, and no identical sequences were encountered in the different communities. However, the sequences generally represented similar phylogenetic kinds of organisms. Particularly abundant sequences represented members of the α-group of proteobacteria, mainly *Sphingomonas* spp. and *Methylobacterium* spp. Both of these genera are known to include opportunistic pathogens, and several of the sequences obtained from the environmental DNA samples were closely related to known pathogens. Such organisms have also been linked to biofilm formation associated with water reservoirs and conduits. In addition, the study detected many other kinds of organisms at lower abundances. These results show that shower curtains are a potential source of opportunistic pathogens

associated with biofilms. Frequent cleaning or disposal of shower curtains is indicated, particularly in households with immune-compromised individuals.

Other papers, particularly ones from the 1990s or earlier, assigned species identifications that are now known not to be correct, or which KnipBio believes are erroneous, based on more up-to-date taxonomic tools such as 16S rRNA sequencing.

We summarize those papers that have explicitly identified *M. extorquens* as the strain implicated in opportunistic pathogenicity. (Hogue *et al.*, 2007) reports that *M. extorquens* has been implicated as an opportunistic pathogen (see Table 1 in this paper), but also says that "The clinical relevance of *Methylobacterium* species is not always clear due to the limited information available in many publications.".

Hogue, R., Graves, M., Moler, S. and Janda, J.M., 2007. Pink-pigmented non-fermentative gram-negative rods associated with human infections: a clinical and diagnostic challenge. *Infection*, *35*(3), p.126.

Over the past several decades, the appearance of pink-pigmented bacteria in clinical specimens has gone from being a microbiologic curiosity in the clinical laboratory to the recognition of these aerobic microorganisms as etiologic agents of human disease, most notably bloodstream infections. Advances in the fields of molecular taxonomy and phylogenetics indicate that at least four distinct genera and eight different species are associated with clinical infections in susceptible patient populations. However, these bacteria are slow growing and present multiple diagnostic challenges to the microbiology laboratory including culture, isolation, and identification to species rank. This article provides a current review of these unusual non-fermentative chromogenic bacteria including their disease spectrum, taxonomy, and laboratory identification. The review also highlights the pitfalls or shortcomings we currently have in our knowledge of these microbes and their disease-producing capabilities.

Another paper (Kaye *et al.*, 1992) reports that *M. extorquens* was found to be the source of a catheter-borne infection, but we believe this strain classification is incorrect, because it was done based on morphological and physiological tests. For instance, the isolated strains grew on xylose and fructose, while typical *M. extorquens* strains identified by 16S RNA sequencing are not able to utilize these compounds. In any event, this paper characterized the putative *M. extorquens* strain as "low virulence" and that the infection it caused was only "low-grade or intermittent".

Kaye, K.M., Macone, A. and Kazanjian, P.H., 1992. Catheter infection caused by Methylobacterium in immunocompromised hosts: report of three cases and review of the literature. *Clinical infectious diseases*, *14*(5), pp.1010-1014.

Three cases of catheter infection due to *Methylobacterium extorquens* are reported. Each patient had a history of acute leukemia and was immunocompromised; two had undergone bone marrow transplantation, and the third was receiving consolidation chemotherapy. All three patients survived after removal of the central venous catheter and antibiotic treatment. The clinical features of these cases are compared with those of the 12 previously reported cases of infection due to *Methylobacterium* species.

(Greub, *et al.*, 2004) isolated a microorganism resembling *M. extorquens* from amoebal cocultures from nasal swabs, although these authors did not report any direct evidence that this culture was pathogenic.

> **Greub, G., 2004. Amoebae-resisting bacteria isolated from human nasal swabs by amoebal coculture.**
>
> Amoebae feed on bacteria, and few bacteria can resist their microbicidal ability. Amoebal coculture could therefore be used to selectively grow these amoebae-resisting bacteria (ARB), which may be human pathogens. To isolate new ARB, we performed amoebal coculture from 444 nasal samples. We recovered 7 (1.6%) ARB from 444 nasal swabs, including 4 new species provisionally named Candidatus Roseomonas massiliae, C. Rhizobium massiliae, C. Chryseobacterium massiliae, and C. Amoebinatus massiliae. The remaining isolates were closely related to Methylobacterium extorquens, Bosea vestrii, and Achromobacter xylosoxidans. Thus, amoebal coculture allows the recovery of new bacterial species from heavily contaminated samples and might be a valuable approach for the recovery of as-yet unrecognized emerging pathogens from clinical specimens.

(Patel and Gutierrez, 1992) reported an unusual case of pneumonia with full-blown septic shock syndrome which they attributed to an infection of *M. extorquens* infection in a non immunocompromised adult host (a 66-year old man). The microorganism was identified by CDC as *M. extorquens*, but KnipBio believes that this classification, from the early 1990s, may not be reliable.

> **Patel, Y. and Gutierrez, C., 1992. Severe Pneumonia With Sepsis Due To Methylobacterium Extorquens. *Southern Medical Journal*, *85*(9), pp.3S-42.**
>
> *Methylobacterium extorquens* is a pink facultative methylotrophic organism previously known as *Pseudomonoas mesophilica*. There are a few reports in the microbiology literature (none in the medical literature) describing occasional isolation of similar bacteria from clinical specimens, but the specific role of this organism in human pathogenesis is virtually unknown. We describe an unusual case of pneumonia with full-blown septic shock syndrome due to *Methylobacterium extorquens* infection in a nonimmunocompromised adult host.

(Lambert *et al.*, 1983) reported that lesions obtained from a 48-year-old Puerto Rican woman with extensive ulcers on her buttocks, right arm, and thighs showed the presence bacilli which were identified as *Vibrio extorquens*, which they characterized as a partially acid-fast methanolophilic organism.

> **Lambert, W.C., Pathan, A.K., Irnaeda, T., Kaminski, Z.C. and Reichman, L.B., 1983. Culture of Vibrio extorquens from severe, chronic skin ulcers in a Puerto Rican woman. *Journal of the American Academy of Dermatology*, *9*(2), pp.262-268.**
>
> A 48-year-old Puerto Rican woman developed extensive ulcers on her buttocks, right arm, and thighs over a 3 1/2-year period, The lesions began as small, subcutaneous nodules which subsequently ulcerated and expanded up to 19 cm in diameter. Biopsy of both ulcerated and nonulcerated lesions showed acid-fast bacilli. Culture of both types of lesions grew *Vibrio extorquens*, a partially acid-fast methanolophilic organism not previously associated with disease in humans. The patient developed agglutinating antibody, titer 1:80,

to this organism. The disease responded to treatment with antibiotics to which the organism was sensitive in vitro.

(Frank, *et al.*, 2007) tentatively identified a microbial strain isolated from toys in a pediatric waiting room as *M. extorquens*, although the paper expressed some doubt about the accuracy of this classification, and did not assert that the presence of this organism on the toys posed any risk to children.

> **Frank, J., Rodriguez, D. and Guzman, J., Methylobacterium extorquens which is found in the soil and is not harmful. We feel that we just scratched the surface on the amount of research that needs to be done to ensure that these waiting rooms are clean and safe for our kids. Sierra College Journal of Microbiology, p.16.**
>
> We obtained organisms from pediatric waiting room toys to be observed and identified. We swabbed toys from both Sutter and Kaiser facilities and did numerous tests on them to decipher what kinds of organisms were living on the toys. We got mixed results being that Kaiser had less bacteria growing than Sutter, but the problem that we encountered was that we did not know whether the organisms growing were harmful or not. The one organism that we did identify was Methylobacterium extorquens which is found in the soil and is not harmful. We feel that we just scratched the surface on the amount of research that needs to be done to ensure that these waiting rooms are clean and safe for our kids.

Many of the papers identified in the search implicate *Methylobacterium mesophilicum* (previously known as *M. mesophilica*) as an opportunistic pathogen. These papers include (Sanders *et al.*, 2000) see above; and the following:

> **Imbert, G., Seccia, Y. and La Scola, B., 2005. Methylobacterium sp. bacteraemia due to a contaminated endoscope. *Journal of Hospital Infection*, *61*(3), pp.268-270.**
>
> *Methylobacterium mesophilicum* is a fastidious Gram-negative bacterium that belongs to the alpha subgroup of Proteobacteria. It forms pink colonies on agar plates and is a common contaminant in water.[1] Although an opportunistic pathogen in immunocompromised hosts, it does cause pseudo-infections. When retrieved, the most common source of contamination is environmental contamination or exposure.[1,2] We present a patient who developed bacteraemia due to *M. mesophilicum* following endoscopic retrograde cholangiopancreatography (ERCP), and report the investigation of the source of contamination.
>
> **Norton, C.E.R., 2001. Recurrent Methylobacterium mesophilicum sepsis associated with haemodialysis. *Pathology*, *33*(4), pp.536-537.**
>
> Methylobacterium mesophilicum is an environmental organism that has infrequently been implicated as a human pathogen. Most reported cases are in immunocompromised hosts. The natural ecology of this organism is related to vegetation or soil. We report the case of a 51-year-old man who developed a recurrent bacteraemia with this organism while receiving haemodialysis through an indwelling intravascular catheter. The literature on the clinical significance of this organism is reviewed together with specific microbiological identifying features which could be of use in the routine diagnostic laboratory.

Fernandez, M., Dreyer, Z., Hockenberry-Eaton, M. and Baker, C.J., 1997. Methylobacterium mesophilica as a cause of persistent bacteremia in a child with lymphoma. *The Pediatric infectious disease journal*, *16*(10), pp.1007-1008.

Methylobacterium mesophilica, a Gram-negative, pink-pigmented rod bacterium, was first isolated from leaf surfaces.[1] It is an opportunistic pathogen that causes disease primarily in immunocompromised patients. Its low virulence probably accounts for the low attendant mortality, but this organism can cause morbidity in the immunosuppressed. Among the infectious manifestations reported in such patients, those associated with indwelling intravascular devices are common.[2, 3] These infections have been indolent and non-life-threatening, leading Kaye et al.[2] to recommend monotherapy with an aminoglycoside, without removal of the intravascular device.

We present a pediatric patient with protracted central venous catheter-associated infection caused by *M. mesophilica.*Our patient's bacteremia and prolonged febrile episodes did not resolve until the catheter was removed.

Flournoy, D.J., Petrone, R.L. and Voth, D.W., 1992. A pseudo-outbreak ofMethylobacterium mesophilica isolated from patients undergoing bronchoscopy. *European Journal of Clinical Microbiology & Infectious Diseases*, *11*(3), pp.240-243.

An unusual, slow growing, pink-pigmented gramnegative bacillus was isolated from bronchoscopy specimens of seven patients over a three-month period. The organism was identified as*Methylobacter mesophilica*. None of the patients were believed to be infected with*Methylobacter mesophilica*. The results of environmental cultures showed that the organism was present in tap water from the bronchoscopy room.

There are many references in the literature which indicate that, at one time, there was confusion in the field about whether *M. mesophilicum* and *M. extorquens* were the same or different species, but the consensus in the field is that these are separate and distinct species (see for example, (MicrobeWiki, no date), which lists both as distinct species). It is known that *M. mesophilicum* and *M. extorquens* utilize different carbon sources, among other differences between these species. KnipBio will not comment further on references reporting that *M. mesophilicum* is an opportunistic pathogen, other than to note the presence of such references in the literature search, and to specifically mention the several papers cited above.

The following pages list all the papers identified in this search.

Methylobacterium mesophilicum infection: case report and literature review of an unusual opportunistic **pathogen**
JW Sanders, JW Martin, M Hooke... - Clinical Infectious ..., 2000 - academic.oup.com
... Cite Citation. John W. Sanders, James W. Martin, Maria Hooke, Jeffrey Hooke; **Methylobacterium** mesophilicum Infection: Case Report and Literature Review of an Unusual Opportunistic **Pathogen** . ... B. **extorquens** was later renamed **Methylobacterium extorquens**. ...
Cited by 45 Related articles All 9 versions Cite Save More

Methylobacterium genome sequences: a reference blueprint to investigate microbial metabolism of C1 compounds from natural and industrial sources
S Vuilleumier, L Chistoserdova, MC Lee, F Bringel... - PloS one, 2009 - journals.plos.org
... Loading metrics. Open Access. Peer-reviewed. Research Article. **Methylobacterium** Genome Sequences: A Reference Blueprint to Investigate Microbial Metabolism of C1 Compounds from Natural and Industrial Sources. Stéphane Vuilleumier , ...
Cited by 154 Related articles All 29 versions Cite Save More

Methylobacterium species: an increasingly important opportunistic **pathogen**
AL Truant, R Gulati, O Giger... - Laboratory ..., 2015 - academic.oup.com
... air, rice grain, sewage, cow rumen, and hospitals.12'13 **Methylobacteria** have been ... Flournoy and colleagues, who reported a pseudo-outbreak of **Methylobacterium** pneumonia in ... describes three bone marrow recipients who developed nosocomial **methylobacterial** infec- tions ...
Cited by 14 Related articles All 3 versions Cite Save

Genome sequence analysis of the emerging human **pathogenic** acetic acid bacterium Granulibacter bethesdensis
DE Greenberg, SF Porcella, AM Zelazny... - Journal of ..., 2007 - Am Soc Microbiol
... utilization and their relationship to the methylotrophic bacterium **Methylobacterium extorquens** AM1 ... help to determine the roles of these pathways in G. bethesdensis **pathogenesis**. ... the former also found in the virtually pathognomonic CGD **pathogen** Chromobacterium violaceum ...
Cited by 48 Related articles All 12 versions Cite Save

Catheter infection caused by **Methylobacterium** in immunocompromised hosts: report of three cases and review of the literature
KM Kaye, A Macone... - Clinical infectious diseases, 1992 - academic.oup.com
... Since each patient had symp- toms, we believe that M. **extorquens** was acting as a **pathogen**, not simply ... York City Public Health Laborato- ries [7] and the Laboratory Centre for Disease Control in Ottawa [8] have reported the isolation of **Methylobacterium** from multiple ...
Cited by 42 Related articles All 9 versions Cite Save More

Methylobacterium sp. bacteraemia due to a contaminated endoscope
G Imbert, Y Seccia, B La Scola - Journal of Hospital ..., 2005 - journalofhospitalinfection.com
... using BLAST software, and showed over 99% similarity with that of **Methylobacterium extorquens** (access number ... M. **extorquens** is considered to be synonymous with M. mesophilicum. ... 1 Fortunately, due to the low **pathogenicity** of this micro-organism, death occurs rarely. ...
Cited by 14 Related articles All 7 versions Cite Save

Protein kinase G from **pathogenic** mycobacteria promotes survival within macrophages
A Walburger, A Koul, G Ferrari, L Nguyen... - ..., 2004 - science.sciencemag.org
... **Pathogenicity** of mycobacteria is tightly linked to their survival in host macrophages. Normally, phagocytosed microorganisms are rapidly transferred from phagosomes to lysosomes and are then degraded. However, **pathogenic** mycobacteria resist lysosomal delivery and ...
Cited by 460 Related articles All 20 versions Cite Save

Detection of **Methylobacterium** species by 16S rRNA gene-targeted PCR.
T Nishio, T Yoshikura, H Itoh - Applied and environmental ..., 1997 - Am Soc Microbiol
... As some clinical isolates of pink- pigmented bacteria have been reported as M. mesophilicum (15, 16, 29), studies on the **pathogenicity** of M ... 1990. Characteristics and antibiotic suscep- tibility of **Methylobacterium extorquens** isolated from drinking water and air in the hospital. ...
Cited by 27 Related articles All 9 versions Cite Save

Repeated, selection-driven genome reduction of accessory genes in experimental populations

MC Lee, CJ Marx - PLoS Genetics, 2012 - journals.plos.org

... as large chromosomal islands that are adaptive for specialized traits such as **pathogenicity**. ... We uncovered that most replicate populations of **Methylobacterium extorquens** AM1 evolved in the ... has been shown for Shigella flexneri, a facultative intracellular **pathogen** of primates ...

Cited by 81 Related articles All 16 versions Cite Save More

Isolation and identification of **Methylobacterium** species from the tap water in hospitals in Japan and their antibiotic susceptibility

K Furuhata, Y Kato, K Goto, M Hara... - Microbiology and ..., 2006 - Wiley Online Library

... 6) Furuhata, K., and Koike, KA 1990. Characteristics and antibiotics susceptibility of **Methylobacterium extorquens** isolated from drinking water and air in the hospital. ... **Methylobacterium** species: an increasingly important opportunistic **pathogen**. Lab. Med. 29: 704–710. ...

Cited by 25 Related articles All 7 versions Cite Save

Amoebae-resisting bacteria isolated from human nasal swabs by amoebal coculture

G Greub - 2004 - wwwnc.cdc.gov

... as it shared 99.3% 16S rDNA sequence homology with M. **extorquens** strain JCM ... study, we recovered additional alphaproteobacteria including a strain of **Methylobacterium** extorquans, a ... Preliminary report on the **pathogenicity** of Legionella pneumophila for freshwater and soil ...

Cited by 90 Related articles All 16 versions Cite Save More

Cytoplasmic ATPase involved in ferrous ion uptake from magnetotactic bacterium Magnetospirillum magneticum AMB-1

T Suzuki, Y Okamura, A Arakaki, H Takeyama... - FEBS ..., 2007 - Wiley Online Library

... the quantities of iron necessary to cause infection [5,6]. In these **pathogenic** microorganisms, ferrous ... MS-1 homologous protein (ZP00055824); Caulobacter, Caulobacter crescentus homologous protein (AAK24454); **Methylobacterium**, **Methylobacterium extorquens** strain AM1 ...

Cited by 15 Related articles All 9 versions Cite Save More

Combined sacB-based negative selection and cre-lox antibiotic marker recycling for efficient gene deletion in Pseudomonas aeruginosa

L Quénée, D Lamotte, B Polack - Biotechniques, 2005 - researchgate.net

... has been sequenced (1) substantial progress has been made in the study of this Gram- negative opportunistic **pathogen**, which is ... Marx and Lidstrom (6) recently described a method for marker recycling in Gram-negative bacteria, namely **Methylobacterium extorquens** ...

Cited by 81 Related articles All 9 versions Cite Save More

Isolation of an unidentified pink-pigmented bacterium in a clinical specimen.

T Odugbemi, C Nwofor, KT Joiner - Journal of clinical ..., 1988 - Am Soc Microbiol

... No **pathogen** was recovered from the throat and nose swabs of the patient. ... The exact **pathogenic** role of the organism is unclear because only one blood culture was available ... constituted their cluster 2; the 14 strains in their cluster 1 were similar to **Methylobacterium extorquens**. ...

Cited by 21 Related articles All 10 versions Cite Save

The structure and computational analysis of Mycobacterium tuberculosis protein CitE suggest a novel enzymatic function

CW Goulding, PM Bowers, B Segelke, T Lekin... - Journal of molecular ..., 2007 - Elsevier

... and that M. tuberculosis CitE may be critical for **pathogenesis**, encompassing part of a ... The bacterial **pathogen** M. tuberculosis causes the world's deadliest infectious disease ... K_pne ; Klebsiella pneumoniae, E_col; Escherichia coli, M_ext, **Methylobacterium extorquens**; and the ...

Cited by 26 Related articles All 15 versions Cite Save

Methylobacterium zatmanii, A PINK PIGMENTED FACULTATIVE METHYLOTROPHIC (PPFM) BACTERIUM ISOLATED FROM THE HUMAN ORAL CAVITY

TM CARVAJAL, RL TAN, AC LEE - Philippine Journal of ..., 2011 - researchgate.net

... **Methylobacterium** dichloromethanicum as later subjective synonyms of **Methylobacterium extorquens** and of **Methylobacterium** lusitanum as a later subjective synonym of ... 2000. Letter to the Editor: Monitoring **methylobacteria** in water systems ... and **Methylobacterium** aerolatum sp. ...

Cited by 2 Related articles All 6 versions Cite Save More

The oxidation of methanol in gram-negative bacteria

C Anthony - FEMS Microbiology Letters, 1990 - Elsevier

... 1989) The second subunit of methanol dehydrogenase of **Methylobacterium extorquen~"** AM1. ... A rapid method for the purification of methanol dehydrogenase from **Methylobacterium extorquens**. ... and Energy Generation in the Cystic Fibrosis **Pathogen** Pseudomonas aeruginosa. ...

Cited by 17 Related articles All 6 versions Cite Save More

The 2.3-Å crystal structure of the shikimate 5-dehydrogenase orthologue YdiB from Escherichia coli suggests a novel catalytic environment for an NAD-dependent …

J Benach, I Lee, W Edstrom, AP Kuzin, Y Chiang... - Journal of Biological …, 2003 - ASBMB

... alignment of YdiB with members of the same cluster of orthologous genes (50) and with MtdA (**Methylobacterium extorquens** AM1 methylene ... Interestingly, the genome of the **pathogenic** E. coli strain O157 (15) contains one additional orthologue of shikimate 5-dehydrogenase ...

Cited by 55 Related articles All 9 versions Cite Save

Molecular analysis of shower curtain biofilm microbes

ST Kelley, U Theisen, LT Angenent... - Applied and …, 2004 - Am Soc Microbiol

... setting that may provide a persistent reservoir for **pathogenic** microorganisms is ... perhaps with the same ecological roles as the **Methylobacteria**, were encountered. ... several clones representing organisms closely related to **Methylobacterium extorquens** and **Methylobacterium** ...

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Development of Recombinant Escherichia coli Strain for the Production of Coenzyme B 12

KO Yeounjoo, S ASHOK, SK AINALA... - 한국생물공학회 학술 …, 2013 - dbpia.co.kr

... Forkhead box O1 (FoxO1) transcription factors regulate the activity of important target genes involved in the **pathogenesis** of acne. ... OP62 NAD-dependent FDH1 Beta Subunit from **Methylobacterium extorquens** AM1 as NAD(H)-regenerating Enzyme HyunJun CHOE, SuMi LEE ...

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Genome Sequence Analysis of the

EHPA Acid - J. Bacteriol, 2007 - Citeseer
... help to determine the roles of these pathways in G. bethesdensis **pathogenesis**. ... the former also found in the virtually pathognomonic CGD **pathogen** Chromobacterium violaceum ... utilization and their relationship to the methylotrophic bacterium **Methylobacterium extorquens** AM1 ...
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NAD-dependent FDH1 B eta Subunit from **Methylobacterium extorquens** AM1 as NAD (H)-regenerating Enzyme

C HyunJun, LEE SuMi, HJ HWANG, JC JOO... - 한국생물공학회 학술 ..., 2013 - dbpia.co.kr
... Forkhead box O1 (FoxO1) transcription factors regulate the activity of important target genes involved in the **pathogenesis** of acne. ... OP62 NAD-dependent FDH1 Beta Subunit from **Methylobacterium extorquens** AM1 as NAD(H)-regenerating Enzyme HyunJun CHOE, SuMi LEE ...
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Synthetic microbial ecology for biofuel production from lignocellulose

M Thommes, A Lubbe, J Lee - orau.gov
... chose each organism based on its metabolic capacity, oxygen requirement, **pathogenicity**, and genome ... All community members grow aerobically, are not **pathogenic**, and have had their ... putida is a soil-dwelling bacterium that degrades lignin, **Methylobacterium extorquens** is a ...
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Neoechinulin A isolated from marine-derived Microsporum sp. suppresses sebum accumulation in insulin-like growth factor (IGF)-1 differentiated human ...

AHN Byul-Nim, LI Yong-Xin, KIM Se-Kwon - 한국생물공학회 학술대회, 2013 - dbpia.co.kr
... Forkhead box O1 (FoxO1) transcription factors regulate the activity of important target genes involved in the **pathogenesis** of acne. ... OP62 NAD-dependent FDH1 Beta Subunit from **Methylobacterium extorquens** AM1 as NAD(H)-regenerating Enzyme HyunJun CHOE, SuMi LEE ...
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OP61 Neoechinulin A isolated from marine-derived Microsporumsp. suppresses sebum accumulation in insulin-like growth factor (IGF)-1 differentiated human ...

AHN Byul-Nim, LI Yong-Xin, KIM Se-Kwon - dev02.dbpia.co.kr
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Functional analysis of Xylella genome by identification of proteins and putative-virulence-relatade-low molecular weight compounds

SM Tsai - 1999
Cite Save

Polymer Nanoparticle-mediated Genetic Engineering of Cell Sheets to Enhance Therapeutic Angiogenesis

P Hyun-Ji, LEE Joan, JUN Indong, TJ KANG... - 한국생물공학회 학술 ..., 2013 - dbpia.co.kr
... Forkhead box O1 (FoxO1) transcription factors regulate the activity of important target genes involved in the **pathogenesis** of acne. ... OP62 NAD-dependent FDH1 Beta Subunit from **Methylobacterium extorquens** AM1 as NAD(H)-regenerating Enzyme HyunJun CHOE, SuMi LEE ...
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Wide distribution of genes for tetrahydromethanopterin/methanofuran-linked C1 transfer reactions argues for their presence in the common ancestor of ...

L Chistoserdova - Frontiers in microbiology, 2016 - ncbi.nlm.nih.gov
... and with the increasing representation of environmental versus **pathogenic** microbes, the ... Novel methylotrophy genes of **Methylobacterium extorquens** AM1 identified by using transposon mutagenesis ... lost and genes found: evolution of bacterial **pathogenesis** and symbiosis. ...
Cited by 1 Related articles All 8 versions Cite Save

Draft genomic DNA sequence of the facultatively methylotrophic bacterium Acidomonas methanolica type strain MB58

N Higashiura, H Hadano, H Hirakawa, M Matsutani... - 2014 - academic.oup.com

... Recently, an A. methanolica strain CGDAM1 was isolated from a patient with chronic granulomatous disease exacerbated with cervical lymphadenopathy indicating the emerging **pathogenicity** of these bacteria (Chase et al., 2012). ...

Cited by 4 Related articles All 8 versions Cite Save More

Methylobacterium mesophilica as a cause of persistent bacteremia in a child with lymphoma

M Fernandez, ZA Dreyer... - The Pediatric ..., 1997 - journals.lww.com

... 1 It is an opportunistic **pathogen** that causes disease primarily in immunocompromised patients. ... The species M. mesophilica and **Methylobacterium extorquens** are considered synonymous, 5 although DNA analyses indicate that they actually are different species. 6. ...

Cited by 21 Related articles All 6 versions Cite Save

The three domains of a bacterial sialidase: a β-propeller, an immunoglobulin module and a galactose-binding jelly-roll

A Gaskell, S Crennell, G Taylor - Structure, 1995 - Elsevier

... Abstract. Background: Sialidases, or neuraminidases, have been implicated in the **pathogenesis** of many diseases, but are also produced by many non-**pathogenic** bacteria. Bacterial sialidases are very variable in size, often ...

Cited by 204 Related articles All 9 versions Cite Save

Genomes back-to-back: when sequencing race is a good thing

MY Galperin - Environmental microbiology, 2004 - Wiley Online Library

... (2004) The genomic sequence of the accidental **pathogen** Legionella pneumophila. ... **Methylobacterium extorquens** AM1 from a genomic point of view. ... konkukian (serotype H34) superinfection: case report and experimental evidence of **pathogenicity** in immunosuppressed ...

Cited by 5 Related articles All 7 versions Cite Save More

Session II. Regulation of gene expression in Prokaryotes

H Bogusz, R Płocińska, J Dziadek - actabp.pl

... Several bacterial species, for instance **Methylobacterium** ex- torquens AM1 and **Methylobacterium extorquens** DM4, also con ... to the environment seems to be crucial for the **pathogenicity** of tubercle bacilli considered as the most dangerous hu- man bacterial **pathogen**. ...

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Crystal structures of Mycobacterial MeaB and MMAA-like GTPases

TE Edwards, L Baugh, J Bullen, RO Baydo... - Journal of structural and ..., 2015 - Springer

... MysmA.00200.b, PDB ID 3NXS), M. thermoresistibile (MythA.00200.a, PDB ID 3TK1), **Methylobacterium extorquens** (MeaB, PDB ... M. **extorquen** (B). ... 2WWW, and 2QM7 were used for M. tuberculosis, M. smegmatis, M. thermoresistible, H. sapiens, and M. **extorquens**, respectively. ...

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Comparative analysis of prototype two-component systems with either bifunctional or monofunctional sensors: differences in molecular structure and ...

R Alves, MA Savageau - Molecular microbiology, 2003 - Wiley Online Library

... Next article in issue: A C-terminal EGF-like domain governs BAD1 localization to the yeast surface and fungal adherence to phagocytes, but is dispensable in immune modulation and **pathogenicity** of Blastomyces ... **Methylobacterium extorquens**, O30796, MXAB. ...

Cited by 102 Related articles All 11 versions Cite Save

Serologic reactivity to the emerging **pathogen** Granulibacter bethesdensis

DE Greenberg, AR Shoffner... - The Journal of ..., 2012 - academic.oup.com

... Article Navigation. Serologic Reactivity to the Emerging **Pathogen** Granulibacter bethesdensis. David E. Greenberg David E. Greenberg. 1. Laboratory of Clinical Infectious Diseases. Correspondence: David Greenberg, MD, University ...

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Recurrent **Methylobacterium** mesophilicum sepsis associated with haemodialysis

CER Norton - Pathology, 2001 - Taylor & Francis

... is an environmental organ- ism that has infrequently been implicated as a human **pathogen**. ... of earthworms by Bassalik in 1913.1 He named this Bacillus **extorquens**. ... synonymous with Pseudomonas mesophilica which has been reclassified as **Methylobacterium** mesophilicum. ...

Cited by 12 Related articles All 8 versions Cite Save

University of Medical Centre St. Radboud, Radboud University Nijmegen, The Netherlands Andreas Voss University of Medical Centre St. Radboud
T Schülin - Catheter-Related Infections, 2004 - CRC Press
Related articles Cite Save

Revisiting the glyoxylate cycle: alternate pathways for microbial acetate assimilation

SA Ensign - Molecular microbiology, 2006 - Wiley Online Library
... and Chistoserdova, L. (2005) Identification of genes involved in the glyoxylate regeneration cycle in **Methylobacterium extorquens** AM1, including ... Estrella, Proteomic Analysis Reveals That Iron Availability Alters the Metabolic Status of the **Pathogenic** Fungus Paracoccidioides ...
Cited by 40 Related articles All 5 versions Cite Save

Bioinformatics classification and functional analysis of PhoH homologs

AE Kazakov, O Vassieva, MS Gelfand... - In silico ..., 2003 - content.iospress.com
... **Methylobacterium extorquens*** + ... Moreover, fatty acids seem to be a crucial catabolic source during the second stage of the **pathogenic** growth of ... of these changes to the metal homeostasis and RNA modification can bring more light to understanding of **pathogenicity** of these ...
Cited by 33 Related articles All 6 versions Cite Save

Adenosyltransferase tailors and delivers coenzyme B12

D Padovani, T Labunska, BA Palfey, DP Ballou... - Nature chemical ..., 2008 - nature.com

... B 12 and convert it to the active coenzyme forms 5 . In **Methylobacterium extorquens**, a facultative ... 14)—and perhaps also by heme scavenging proteins in **pathogenic** organisms 15 . ... relevance of the direct transfer mechanism is supported by genetic studies on M. **extorquens**. ...

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Positional Scanning-Synthetic Peptide

PDP Cross-Reactivity - J Immunol, 2002 - pdfs.semanticscholar.org

... Page 4. Table I. Self- and **pathogen**-derived peptides selected on the basis of their potential crossreactivity with Melan-A ... 62 164.77 **Methylobacterium Extorquens** Hypothetical protein in PHAC 3 region 37–46 63 163.99 Salmonella typhimurium LTKB homolog 180–189 ...

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The eukaryotic-like Ser/Thr protein kinases of Mycobacterium tuberculosis

Y Av-Gay, M Everett - Trends in microbiology, 2000 - Elsevier

... bacteria and the host is provided by the human gastric epithelial **pathogen** Helicobacter pylori. ... that glutamine metabolism, and perhaps also PknG, are important in the **pathogenicity** of M ... have been shown to be involved in cell development, stress response and **pathogenesis**. ...

Cited by 334 Related articles All 10 versions Cite Save

Roseomonas keratitis after remote penetrating keratoplasty

S Goyal, DB Warner - Graefe's Archive for Clinical and Experimental ..., 2013 - Springer

... as unnamed, pink-pigmented, non-fermentatitive bacteria that phenotypically resembled **Methylobacterium extorquens** but were ... factors and its **pathogenic** potential in the eye are still ill-defined ... and imipenem [5]. In our experience once the sensitivities of the **pathogen** are known ...

Cited by 1 Related articles All 7 versions Cite Save

Methylobacterium bacteremia in AIDS

AL Tmant, R Gulati, O Giger... - Clinical microbiology ..., 1998 - Wiley Online Library

... individual [5]. The genus **Methylobacterium** includes eight species, **Methylobacterium extorquens**, M.fujisawaense ... examined for vacuoles, which are present in the **methylobacteria** but absent ... JW Sanders, JW Martin, M. Hooke, J. Hooke, **Methylobacterium** mesophilicum Infection ...

Cited by 8 Related articles All 7 versions Cite Save

Genomic analysis of carbon source metabolism of Shewanella oneidensis MR-1: predictions versus experiments

MH Serres, M Riley - Journal of bacteriology, 2006 - Am Soc Microbiol

... In this context, the proteins of the opportunistic **pathogen** Pseudomonas aeruginosa and the **pathogen** Pasteurella multocida have many similarities to those in S. oneidensis MR-1. Nevertheless, it ... Methylotrophy in **Methylobacterium extorquens** AM1 from a genomic point of view ...

Cited by 77 Related articles All 15 versions Cite Save

Methylotrophic bacteria in trimethylaminuria and bacterial vaginosis

AP Wood, FJ Warren, DP Kelly - Handbook of Hydrocarbon and Lipid ..., 2010 - Springer

... 1992; Sanders et al., 2000), but other species (including M. **extorquens**) are also ... Apart from **Methylobacterium**, Afipia, Klebsiella, Micrococcus, Achromobacter, Pseudomonas, Bacillus and Mycobacterium ... or overproduction was caused by a third-party **pathogen** or environmental ...

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Process for producing oxazopyrroloquinolines, novel oxazopyrroloquinolines, and use of oxazopyrroloquinolines

T Urakami, M Oda, C Itoh, H Kobayashi... - US Patent ..., 1995 - Google Patents

... Removal of **pathogenic** microorganisms and tumor cells is effected by the by immune response of T cells of lymphocytes and ... **Methylobacterium extorquens** DSM 1337 (=JCM 2802=NCIB 9399), JCM 2803 (=NCIB 10409), ATCC 8457 (=DSM 1340=IAM 1081=JCM 2804=NCIB ...

Cited by 1 Related articles All 2 versions Cite Save

Constraint-based analysis of metabolic capacity of Salmonella typhimurium during host-**pathogen** interaction

A Raghunathan, J Reed, S Shin... - BMC systems ..., 2009 - bmcsystbiol.biomedcentral.com

... a model system for studying the **pathogenesis** of intracellular bacterial infections. Genome-scale modeling of bacterial metabolic networks provides a powerful tool to identify and analyze pathways required for successful intracellular replication during host-**pathogen** interaction. ...

Cited by 134 Related articles All 16 versions Cite Save More

Culture of Vibrio **extorquens** from severe, chronic skin ulcers in a Puerto Rican woman

WC Lambert, AK Pathan, T Irnaeda... - Journal of the American ..., 1983 - Elsevier

... of the patient that played a role in the etiology or **pathogenesis** of her ... organisms were readily identified histologically but extensive attempts to culture the **pathogen** were unsuccessNl ... belong to a species, particularly a nonmycobacterial species such as V. **extorquens**, other than ...

Cited by 16 Related articles All 6 versions Cite Save More

INDeGenIUS, a new method for high-throughput identification of specialized functional islands in completely sequenced organisms

S Shrivastava, CV Siva Kumar Reddy, SS Mande - Journal of biosciences, 2010 - ias.ac.in

... GI-5 Flagellary system Motility island **Methylobacterium** GI-1 Type II secretion system Secretion island GI-2 Cobalamine biosynthesis Metabolic island ... GI-4 Flagellary system Motility island GI-5 Phage associated proteins **Pathogenicity** island ...

Cited by 19 Related articles All 13 versions Cite Save More

An initial appraisal of the clinical significance of Roseomonas species associated with human infections

M Struthers, J Wong, JM Janda - Clinical infectious diseases, 1996 - academic.oup.com

... new group of unnamed pink-pigmented nonfermentative bacteria that pheno- typically resembled **Methylobacterium extorquens** (Pseudomo- nas ... species with patient populations, disease presentations, risk factors, and **pathogenic** potential. ... of strains) Primary **pathogen** 7 2 3 13 ...

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Microflora on the skin of European eel (Anguilla anguilla L., 1758) sampled from Creek Yuvarlakcay, Turkey

A Ugur, F Yılmaz, N Sahin - 2002 - evols.library.manoa.hawaii.edu

... It can be contaminated at any time from the moment of capture until it is eaten. Contamination can occur because **pathogenic** microorganisms form part of the normal flora of the fish. ... Proteus penneri 5.14 - + - + **Methylobacterium extorquens** 3.44 - + + - Serratia sp. 1.72 - - ...

Cited by 9 Related articles All 4 versions Cite Save

Insight in to the phylogeny of polyhydroxyalkanoate biosynthesis: horizontal gene transfer

VC Kalia, S Lal, S Cheema - Gene, 2007 - Elsevier

... In addition, phaA and phaB genes are also involved in glyoxylate regeneration eg, in Proteobacteria (α): methylotroph **Methylobacterium extorquens**, Caulobacter crescentus CB15, Rhodobacter sphaeroides and Actinobacterium: S. coelicolor A3(2) (Korotkova et al., 2002). ...

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MICROBIOLOGY COMMENT

S TYPE IV - 2005 - pdfs.semanticscholar.org

... The papers included deal with systems involved in **pathogenesis** and conjugation and highlight the significant ... of an upstream regulatory sequence that mediates the transcription of mox genes in **Methylobacterium extorquens** AM1 M. Zhang ... PATHOGENS AND **PATHOGENICITY** ...

Cite Save More

Community acquired Roseomonas infection in a pre-existing Tubercular lung lesion

NM Kaore, Z Khan, AR Aher... - Lung India: official organ ..., 2014 - ncbi.nlm.nih.gov

... 9] described a new group of unnamed pink-pigmented non-fermentative bacteria that phenotypically resembled **Methylobacterium extorquens** (Pseudomonas mesophilica ... appears to have low **pathogenic** potential for humans, but some species may cause clinically significant ...

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Oxazopyrroloquinolines and use of oxazopyrroloquinolines

T Urakami, M Oda, C Itoh, H Kobayashi... - US Patent ..., 1993 - Google Patents

... Removal of **pathogenic** microorganisms and tumor cells is effected. ... **Methylobacterium extorquens** DSM 1337 (=JCM 2802=NCIB 9399), JCM 2803 (=NCIB 10409), ATCC 8457 (=DSM 1340=IAM 1081=JCM 2804=NCIB 2879), ATCC 14718 (=DSM 1338=JCM 2805=NCIB 9133 ...

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Sclerosing inflammatory pseudotumor of the eye

HS Uy, QD Nguyen, J Arbour, M Gravel... - Archives of ..., 2001 - jamanetwork.com

... yielded growth of coral pink negative rods interpreted as **Methylobacterium extorquens**, sensitive to ... of the infectious disease consultant that the finding of M **extorquens** in the ... The proposed **pathogenesis** of sclerosing pseudotumor is exaggerated proliferation of fibroblasts and ...

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Bacterial diversity associated with the rotifer Brachionus plicatilis sp. complex determined by culture-dependent and-independent methods

R Ishino, S Iehata, M Nakano, R Tanaka... - Biocontrol ..., 2012 - jstage.jst.go.jp

... WC33 + 1 clone a- Proteobacterla 99 **Methylobacterium extorquens** (GU992347) 13 1° WC15+80lones **Methylobacterium** radiotolerans (GQ895736) 1° W037 ... Austin, B., Austin, D., Sutherland, R, Thompson, F., and Swings, J. (2005) **Pathogenicity** of vibrios to rainbow trout ...

Cited by 6 Related articles All 9 versions Cite Save

Miscellaneous organisms

A Voss - INFECTIOUS DISEASE AND THERAPY SERIES, 1997 - books.google.com

... Various synonyms have been used for **Methylobacterium**, such as Pseudomonas mesophilica, Protaminobacter rubra ... M. **extorquens** is an opportunistic **pathogen** of low virulence, causing CRI, fever ... respiratory tract flora and has only rarely been implicated as a human **pathogen**. ...

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Fluorescence lifetime imaging to quantify sub-cellular oxygen measurements in live macrophage during bacterial invasion

J Dragavon, M Amiri, B Marteyn... - ... of SPIE Vol, 2011 - proceedings.spiedigitallibrary.org
... The recognition of oxygen's role in bacterial **pathogenesis** further demonstrates the need to ... a collective response from the macrophage to the invading **pathogen** likely in ... of the regulation of pyrroloquinoline quinone biosynthesis in **Methylobacterium extorquens** AM1," Journal of ...
Cited by 3 Related articles All 6 versions Cite Save

The (d) evolution of methanotrophy in the Beijerinckiaceae—a comparative genomics analysis

I Tamas, AV Smirnova, Z He, PF Dunfield - The ISME journal, 2014 - ncbi.nlm.nih.gov
... SC2, and more distantly with **Methylobacterium extorquens** (Supplementary Figure 8). Other Alphaproteobacteria methylotrophs have very different arrangements (for example, Hyphomicrobium sp. MC1; Vuilleumier et al., 2011), or lack this island entirely. ...
Cited by 27 Related articles All 9 versions Cite Save

Characterization and cloning of a 37.6-kb plasmid carried by Legionella pneumophila recovered from patients and hospital water over a 12-year period

SD Mansfield, GS Bezanson... - Canadian journal of ..., 1997 - NRC Research Press
... prevalent in both patients and the environment, and show the greatest **pathogenicity** in guinea ... electroporation of the methylotrophic bacteria Hyphornicrobiurn facilis, Hyphomicrobium denitrificans, Methylobacillus glycogenes, **Methylobacterium extorquens**, and Methylophilus ...
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Methylobacterium extorquens which is found in the soil and is not harmful. We feel that we just scratched the surface on the amount of research that needs to be ...

J FRANK, D RODRIGUEZ, J GUZMAN - Sierra College Journal of Microbiology - microsc.net
... is that we did not know all of the kinds of bacteria found whether they were just normal flora or if they were highly **pathogenic**. ... We came to a roadblock when our organism that came back from UC Davis **Methylobacterium extorquens** was gram negative and all of the research we ...
Related articles Cite Save More

Comprehensive analysis of bacterial flora in postoperative maxillary cyst fluid by 16S rRNA gene and culture methods

N Sano, Y Yamashita, K Fukuda... - ISRN ..., 2012 - downloads.hindawi.com
... Identification of **pathogenic** bacteria was also performed by culture methods. ... Clostridium Granulicatella Tannerella Gemella Syntrophococcus Leptotrichia Anaerococcus Capnocytophaga Enterococcus Kingella **Methylobacterium** Sporanaerobacter Peptostreptococcus ...
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Microbial metabolism of C1-nitrogen compounds other than cyanide

PJ Large - FEMS Microbiology Letters, 1990 - Elsevier
... J. Bacteriol., 170 (1988), pp. 2254–2262. [26]. DN Nunn, C. Anthony. The nucleotide sequence and deduced amino acid sequence of the cytochrome cL gene of **Methylobacterium extorquens** AM1, a novel class of c-type cytochrome. Biochem. J., 256 (1988), pp. 673–676. [27]. ...
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The microbiology of metal working fluids

IP Thompson, CJ van der Gast - Handbook of Hydrocarbon and Lipid ..., 2010 - Springer
... If left uncontrolled, such microbial contamination can lead to fouling, bio-deterioration of the MWF, and compromise of key metal working properties of the fluids, in addition to increasing the risk of bacterial **pathogen** growth. ... Alphaproteobacteria. **Methylobacterium extorquens**. 1. A ...
Cited by 6 Related articles All 5 versions Cite Save More

Crystal structure of MabA from Mycobacterium tuberculosis, a reductase involved in long-chain fatty acid biosynthesis

M Cohen-Gonsaud, S Ducasse, F Hoh, D Zerbib... - Journal of molecular ..., 2002 - Elsevier
... the fatty-acid elongation system called FAS-II. 6., 7. and 8. The FAS-II system has been isolated from a non-**pathogenic** mycobacterium, Mycobacterium smegmatis. 9 This complex of several monofunctional enzymes catalyses ...
Cited by 86 Related articles All 13 versions Cite Save

Whole-proteome analysis of twelve species of Alphaproteobacteria links four pathogens

Y Zhou, DR Call, SL Broschat - Pathogens, 2013 - mdpi.com

... Rhizobium leguminosarum WSM1325, NC_012850, 4,767,043, 4,565. **Methylobacterium extorquens** DM4, NC_012988, 5,943,768, 5,594. Rhodomicrobium vannielii ATCC 17100, NC_014664, 4,014,469, 3,565. 2. Results and Discussion. ...

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Moore, SA, see Ryan, KA (248) 47 Morita, M., see Nakamiya, K.(248) 17 Murakami, K., see Hirota, K.(248) 37

K Hirota - FEMS Microbiology Letters, 2005 - Wiley Online Library

... production of enterocin P from Enterococcus faecium P13 in the methylotrophic bacterium **Methylobacterium extorquens** (248) 125 ... with 2-methacryloyloxyethyl phos- phorylcholine (MPC) co-polymer significantly reduces retention of human **pathogenic** microorganisms (248) 37 ...

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Large-effect beneficial synonymous mutations mediate rapid and parallel adaptation in a bacterium

D Agashe, M Sane, K Phalnikar... - Molecular biology ..., 2016 - academic.oup.com

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Cloning and characterization of putative zinc protease genes of Ehrlichia canis

CH Teng, SC Barr, YF Chang - DNA Sequence, 2003 - Taylor & Francis

... pPROAB, which was used for complementation analysis in **Methylobacterium extorquens** AM1, was created in two steps. ... Serum from an uninfected dog or specific-**pathogen**-free (SPF) dog did not recognize rProA or rProB (Fig. 8). Subcellular Localization ... M. **extorquens** AM1. ...

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Phylogenetic placement and characterization of a new alpha-2 proteobacterium isolated from a patient with sepsis.

G Blomqvist, L Wesslen, C Påhlson... - Journal of clinical ..., 1997 - Am Soc Microbiol

... Few species of the alpha subdivision of the proteobacteria (30) have been confirmed to be **pathogenic** to humans ... was found to be 95.7 and 96.1% similar to Afipia clevelandensis and Afipia felis and 95.0 and 95.7% similar to **Methylobacterium extorquens** and **Methylobacterium** ...

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A KIKUCHI, KFHT YOSHIMORI - 2016 - pdfs.semanticscholar.org

... Novel working hypothesis for **pathogenesis** of hematological malignancies: combination of mutations-induced cellular phenotypes determines the disease ... PqqE from **Methylobacterium extorquens** AM1: a radical S-adenosyl-L-methionine enzyme with an unusual tolerance to ...

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Spacelab J air filter debris analysis

DC Obenhuber - 1993 - ntrs.nasa.gov

... HAEMOPHILUS APHROPHILUS KLEBSIELLA PNEUMONIAE A KLEBSIELLA PNEUMONIAE A **METHYLOBACTERIUM EXTORQUENS** PSEUDOMONAS FLUORESCENS B XANTHOMONAS CAMPESTRIS ... Analysis for potentially **pathogenic** organisms, including ...

Cite Save

Discordant carbapenem susceptibility in **Methylobacterium** species and its application as a method for phenotypic identification

GJ Zaharatos, A Dascal, MA Miller - Journal of clinical ..., 2001 - Am Soc Microbiol

... has previously placed these species within two major subclusters (subcluster I, M. **extorquens** and M ... Detection of **Methylobacterium** species by 16S rRNA gene-targeted PCR. ... rium mesophilicum infection: case report and literature review of an unusual opportunistic **pathogen**. ...

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Decreased in vivo virulence and altered gene expression by a Brucella melitensis light-sensing histidine kinase mutant

CR Gourley, E Petersen, J Harms... - Pathogens and ..., 2015 - ncbi.nlm.nih.gov

... an effect on gene expression in a facultative intracellular **pathogen** had been ... mice may be explained by impaired intracellular replication or **pathogenesis**, increased initial ... A proteomic study of **Methylobacterium extorquens** reveals a response regulator essential for epiphytic ...

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Why prokaryotes have pangenomes

JO McInerney, A McNally... - Nature Microbiology, 2017 - eprints.whiterose.ac.uk

... Lee and Marx38 have shown selection-driven genome reduction in **Methylobacterium extorquens** AM1 experimental ... **extorquens** AM1 outside the environment in which the deletions were selected. ... At one extreme the obligate intracellular **pathogen** C. trachomatis has a core ...

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The histidine kinase PdhS controls cell cycle progression of the **pathogenic** alphaproteobacterium Brucella abortus

C Van der Henst, F Beaufay, J Mignolet... - Journal of ..., 2012 - Am Soc Microbiol

... PdhS is an essential and polarly localized histidine kinase in the **pathogenic** alphaproteobacterium Brucella abortus. ... Three homologs of PleC and DivJ HKs are found in Brucella abortus (16, 18), a facultative intracellular **pathogen** responsible for a worldwide zoonosis called ...

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Draft genomes of Nautella italica strains CECT 7645 T and CECT 7321: Two roseobacters with potential **pathogenic** and biotechnological traits

L Rodrigo-Torres, MJ Pujalte, DR Arahal - Marine genomics, 2016 - Elsevier

... Cover image Cover image. Draft genomes of Nautella italica strains CECT 7645 T and CECT 7321: Two roseobacters with potential **pathogenic** and biotechnological traits. ... Biotic relationship, Free-living, Free-living. **Pathogenicity**, Not reported, Not reported. ...

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Foam adsorption

L Blank, B Kuepper, EMDA Villa... - US Patent App. 14/ ..., 2012 - Google Patents
... cell. In some embodiments the prokaryotic cell is a gram-negative bacterial cell. In some embodiments the host cell is non-**pathogenic** for humans. In ... cell. In some embodiments the host cell is non-**pathogenic** for humans. In ...
Cited by 1 Related articles All 2 versions Cite Save

Quorum sensing and bacterial biofilms

JS Dickschat - Natural product reports, 2010 - pubs.rsc.org
... the symptoms caused by planktonic cells. 17 In many cases the human **pathogen** P. aeruginosa permanently colonises the lungs of cystic fibrosis patients and forms a biofilm in the sputum. 13 These chronic infections result ...
Cited by 212 Related articles All 5 versions Cite Save

The c-type cytochromes of methylotrophic bacteria

C Anthony - Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1992 - Elsevier
... Although applicable to the cytochrome from **Methylobacterium**, Methylophihc~ and ilyphomicrobtum, because their isoelectric points differ by about 4 pH units, the ... Cytochrome c~. is the only cytochrome c able to act as an electron acceptor for MDH in M. **extorquens** [21-23]. ...
Cited by 91 Related articles All 4 versions Cite Save

Profiling of Intestinal Microbial Diversity by PCR-DGGE Genes Coding for 16S rDNA and Immunity Status of the Orange Spotted Grouper (Epinephelus coioides) ...

AR Purwandari - 2012 - etd.lib.nsysu.edu.tw
... knowing the types, numbers, and sources of bacteria commonly associated with different developmental stages, could be useful for manipulating microbiota as a strategy to prevent **pathogenic** infection or to improve nutrition. Furthermore, we able ...
Related articles All 2 versions Cite Save

YEAST SYSTEMS BIOLOGY

PCA PCA, SU Screens, EMA Profile - Yeast Systems Biology - Springer
... 5, 92, 181, 266, 348, 408, 431, 448, 467, 485 **Methylobacterium extorquens**..... 438 BioGRID..... ... YEAST SYSTEMS BIOLOGY Subject Index 531 P PASTA analysis..... 133–148 **Pathogen** Aspergillus..... 515 Candida..... ...
All 2 versions Cite Save

Peptide Metabolism in Cytoplasm of Brain Cells

NH THOMA, PF LEADLAY, RN PERHAM, PA RECHE... - pdfs.semanticscholar.org
... Molecular structure of an unusual cytochrome c2 determined at 2.0 A; the cytochrome cH from **Methylobacterium extorquens** Stopped-flow studies on dimethylsulphoxide reductase from Rhodobacter capsulatus: kinetic competence of the dimethylsulphide-reduced intermediate ...
Cite Save More

A cytochrome c peroxidase from Pseudomonas nautica 617 active at high ionic strength: expression, purification and characterization

T Alves, S Besson, LC Duarte, GW Pettigrew... - ... et Biophysica Acta (BBA ..., 1999 - Elsevier
... 6] and N. europaea [7]; HP-Ph: hypothetical protein from P. horikoshii [35]; PCP-Ec: putative CCP from E. coli [36]; MG-Me, MG-Mm, MG-Mf and MG-Pd: methylamine utilization protein MauG precursors from respectively **Methylobacterium extorquens** [37], Methylophilus ...
Cited by 46 Related articles All 8 versions Cite Save

The MoxR ATPase RavA and its cofactor ViaA interact with the NADH: ubiquinone oxidoreductase I in Escherichia coli

KS Wong, JD Snider, C Graham, JF Greenblatt, A Emili... - PloS one, 2014 - journals.plos.org
... For example, MoxR of the MRP subfamily in Paracoccus denitrificans and **Methylobacterium extorquens** is important for the activation of ... suggests diverse functions, including surface adhesion, fibrinogen binding, metal insertion into protoporphyrin IX, and **pathogenesis** [15]–[18 ...
Cited by 15 Related articles All 13 versions Cite Save More

Tokyo University of Agriculture and Technology Tokyo, Japan

S Igarashi, K Sode - Enzyme Functionality: Design: Engineering, ..., 2003 - books.google.com
... Using the sensor system, we aimed to detect the specific DNA sequence of a **pathogenic** bacteria, Salmonella virulence (invA) gene. ... Routine use of DNA-based analyses, such as SNP or **pathogen** detection, will require Page 281. ...
Related articles Cite Save

The mosaicism of plasmids revealed by atypical genes detection and analysis

E Bosi, R Fani, M Fondi - BMC genomics, 2011 - bmcgenomics.biomedcentral.com

... not evenly distributed (Figure 2), ranging from 59 (found in **Methylobacterium extorquens** AM1) to ... relevant since it has probably allowed these strains to acquire **pathogenic** adaptation [31 ... After these two biological processes, we found that **pathogenesis**- and antibiotic resistance ...

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Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism?

CG Friedrich, D Rother, F Bardischewsky... - Applied and ..., 2001 - Am Soc Microbiol

... For the facultative methylotroph **Methylobacterium extorquens**(1, 45) essential Sox proteins were deduced from the partial genome sequence (Table 1). Partial sox gene clusters with the same order of genes as P. pantotrophus were detected in S. novella and ...

Cited by 429 Related articles All 13 versions Cite Save

Metabolism and biology of tryptophan

RR Brown - Recent Advances in Tryptophan Research, 1996 - Springer

... that such binding may be important in the as yet unclear mechanism of prion **pathogenesis** (Stahl and ... Mutants of **Methylobacterium extorquens** and Paracoccus denitrificans deficient in c -type cytochrome biogenesis synthesize the methylamine-dehydrogenase polypeptides ...

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A new group of cosmopolitan bacteriophages induce a carrier state in the pandemic strain of Vibrio parahaemolyticus

R Bastías, G Higuera, W Sierralta... - Environmental ..., 2010 - Wiley Online Library

... Among the **pathogenic** strains, a particular group that is clonal in nature and ... the non-pandemic strains, including several genes related to **pathogenicity** in a ... 41 753–42 121, Hypothetical protein, Hypothetical protein Mext_2445 **Methylobacterium extorquens** PA1 (YP_001639911 ...

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Science, marketing and wishful thinking in quantitative proteomics

M Hackett - Proteomics, 2008 - Wiley Online Library

... C show spectral counting data acquired to measure protein relative abundance changes observed when the oral **pathogen** Porphyromonas gingivalis ... Bosch, G., Skovran, E., Xia, Q., Miller, JA et al., Compre- hensive proteomics of **Methylobacterium extorquens** AM1 metabolism ...

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Expression and Simple Purification Strategy for the Generation of Anti-microbial Active Enterocin P from Enterococcus faecium Expressed in Escherichia coli ...

TN Le, TH Do, TN Nguyen, NT Tran... - Iranian Journal of ..., 2014 - ijbiotech.com

... In addition, mixtures of EntP and the **pathogenic** bacteria that were kept at 4°C for different durations, before they were spread on ordinary LB ... EntP has been expressed in different host strains, such as E. coli, **Methylobacterium extorquens** ATCC 55366, L. lactis, P. pastoris (8, 26 ...

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Fluorescence in situ hybridization-flow cytometry-cell sorting-based method for separation and enrichment of type I and type II methanotroph populations

MG Kalyuzhnaya, R Zabinsky... - Applied and ..., 2006 - Am Soc Microbiol

... MATERIALS AND METHODS. Bacterial strains and culture conditions.**Methylobacterium extorquens** AM1, Methylobacillus flagellatus KT, Methylosinus trichosporium OB3b, Methylosarcina lacus LW14, Methylomonas sp. strain LW13, Methylosinus sp. ...

Cited by 105 Related articles All 19 versions Cite Save

Sample preparation procedures utilized in microbial metabolomics: An overview

M Patejko, J Jacyna, MJ Markuszewski - Journal of Chromatography B, 2017 - Elsevier

... Toxins are most often analyzed with the use of metabolomic, genomic and proteomic approaches. Foodomics studies allow also to get insight into the mechanism of toxicity, as well as to answer the question about potential **pathogenicity** [27]. ... Methylbacterium **extorquens**, [9]. ...

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Metabolism of dimethylsulphoniopropionate by Ruegeria pomeroyi DSS-3

CR Reisch, WM Crabb, SM Gifford, Q Teng... - Molecular ..., 2013 - Wiley Online Library

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From Phage lambda to human cancer: endogenous molecular-cellular network hypothesis

G Wang, X Zhu, L Hood, P Ao - Quantitative Biology, 2013 - journal.hep.com.cn

... Cell , 102, 33-42 . doi: 10.1016/S0092-8674(00)00008-8 pmid:.10929711. 73, Zhao, Z., Yang, P., Eckert, RL and Reece, EA (2009) Caspase-8: a key role in the **pathogenesis** of diabetic embryopathy. Birth Defects Res. B Dev. Reprod. Toxicol. , 86, 72-77 . ...

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Distribution of a Nocardia brasiliensisCatalase Gene Fragment in Members of the GeneraNocardia, Gordona, andRhodococcus

L Vera-Cabrera, WM Johnson, O Welsh... - Journal of clinical ..., 1999 - Am Soc Microbiol

... fragment also showed similarity, although to a lesser extent, to catalases fromDrosophila melanogaster, Streptomyces coelicolor,P. aeruginosa, and **Methylobacterium extorquens**. ... II. The growth requirements, catalase activities and **pathogenic** properties of INH-resistant mutants ...

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Genetic disorders of vitamin B 12 metabolism: eight complementation groups–eight genes

DS Froese, RA Gravel - Expert reviews in molecular medicine, 2010 - cambridge.org

... 15). More than 50 different disease-causing mutations have been identified and are summarised in Ref. 14 (Fig. 3). The most common is the c.271dupA mutation, which causes a frameshift truncation, accounting for 42% of **pathogenic** alleles (Ref. 14). ...

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The copper responding surfaceome of Methylococccus capsulatus Bath

OA Karlsen, Ø Larsen, HB Jensen - FEMS microbiology letters, 2011 - academic.oup.com

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Mycobacterium avium genes associated with the ability to form a biofilm

Y Yamazaki, L Danelishvili, M Wu... - Applied and ..., 2006 - Am Soc Microbiol

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Characterisation of two putative protein Ser/Thr kinases from actinomycete Streptomyces granaticolor both endowed with different properties

T Vomastek, R Nádvorník, J Janeček... - The FEBS ..., 1998 - Wiley Online Library

... were also found in the thermophilic acti- nomycete Thermomonospora curvata [9] and in the **pathogenic** Mycobacterium tuberculosis ... 11-amino-acid tryptophan- docking motif previously identified in methanol dehydrogenase (MDH) from **Methylobacterium extorquens** [29] and in ...

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Primer caso en España de bacteriemia por **Methylobacterium** thiocyanatum procedente de catéter de Hickmann en una paciente inmunodeprimida con ...

T García-Lozano, PL Alegre, JLJ Bañón... - Medicina ..., 2012 - Elsevier Doyma

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Proteomics of Porphyromonas gingivalis within a model oral microbial community

M Kuboniwa, EL Hendrickson... - BMC ..., 2009 - bmcmicrobiol.biomedcentral.com

... Porphyromonas gingivalis is a periodontal **pathogen** that resides in a complex multispecies microbial ... further maturation is characterized by the colonization of more **pathogenic** gram-negative ... are considered responsible for destruction of periodontal tissues, **pathogenicity** is only ...

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γ-Glutamylmethylamide is an essential intermediate in the metabolism of methylamine by Methylocella silvestris

Y Chen, J Scanlan, L Song, A Crombie... - Applied and ..., 2010 - Am Soc Microbiol

... 39). Gram-negative bacteria such as **Methylobacterium extorquens** and Paracoccus denitrificans utilize MMA dehydrogenase, a multisubunit enzyme that generates formaldehyde and ammonium from MMA aerobically (9, 16). ...

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Protein destabilization and loss of protein-protein interaction are fundamental mechanisms in cblA-type methylmalonic aciduria

T Plessl, C Bürer, S Lutz, WW Yue... - Human ..., 2017 - Wiley Online Library

... 2010). MMAA is highly conserved from bacteria to human, and studies in the **Methylobacterium extorquens** (M. ext.) ... The nonsense mutation c.433C>T (p.Arg145*), resulting in a premature stop-codon, accounts for the majority of **pathogenic** alleles (Lerner-Ellis et al. 2004). ...

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On the unfounded enthusiasm for soft selective sweeps

JD Jensen - Nature Communications, 2014 - search.proquest.com

... a wide range of antibiotic-resistance mutations in **pathogenic** microbial populations ... studying adaptation to the antibiotic rifampicin in the **pathogen** Pseudomonas, MacLean ... replicated populations of **Methylobacterium extorquens** identifying as many as 17 simultaneous benecial ...

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The effectors and sensory sites of formaldehyde-responsive regulator FrmR and metal-sensing variant

D Osman, C Piergentili, J Chen, LN Sayer... - Journal of Biological ..., 2016 - ASBMB
... In addition, several mechanisms for the generation of formaldehyde at the host-**pathogen** interface have recently been proposed (2). Inducible formaldehyde detoxification mechanisms have now been recognized in most bacteria (2, 3, 19). ...
Cited by 7 Related articles All 8 versions Cite Save

The Multiple Scientific Disciplines Served by EcoCyc

PD Karp - Systems Biology and Biotechnology of Escherichia coli, 2009 - Springer
... tool for understanding other nonpathogenic bacterial species; 27% use it as a tool for understanding **pathogenic** bacterial species ... SJ, Strovas T, Lidstrom ME (2003) Quantification of central metabolic fluxes in the facultative methylotroph **Methylobacterium extorquens** AM1 ...
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Expressed genome of Methylobacillus flagellatus as defined through comprehensive proteomics and new insights into methylotrophy

EL Hendrickson, DAC Beck, T Wang... - Journal of ..., 2010 - Am Soc Microbiol
... Mfla_0718 to Mfla_0720) and FDH4 (Mfla_0338), named after the homologous enzymes formerly characterized in **Methylobacterium extorquens** AM1 (8 ... proteins with homology to adhesins and hemagglutinins, proteins typically synthesized and excreted by **pathogenic** bacteria. ...
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Proteomics of Streptococcus gordonii within a model developing oral microbial community

EL Hendrickson, T Wang... - BMC ..., 2012 - bmcmicrobiol.biomedcentral.com
... disease, are among the most common instances of bacterial **pathogenesis** in humans. ... transfer within a community can play an important role in **pathogenicity** [7]. Co ... S. gordonii resulted in increased virulence of the periodontal **pathogen** Aggregatibacter actinomycetemcomitans. ...
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Relations and functions of dye-linked formaldehyde dehydrogenase from Hyphomicrobium zavarzinii revealed by sequence determination and analysis

AC Schwartz, G Gockel, J Groß, B Moritz... - Archives of ..., 2004 - Springer
... sequences resembling the special promoters observed upstream of enzymes of methanol oxidation in **Methylobacterium extorquens** (Laufer and ... REW, Lory S, Olson MV (2000) Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic **pathogen**. ...
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Metabolite profiling uncovers plasmid-induced cobalt limitation under methylotrophic growth conditions

P Kiefer, M Buchhaupt, P Christen, B Kaup... - PLoS ..., 2009 - journals.plos.org
... is of great interest for different research areas such as the spread of antibiotic resistance among **pathogenic** microorganisms, plasmid ... **Methylobacterium extorquens** AM1 is a model methylotrophic bacterium that is able to grow in the presence of methanol and methylamine. ...
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New aerobic ammonium-dependent obligately oxalotrophic bacteria: description of Ammoniphilus oxalaticus gen. nov., sp. nov. and Ammoniphilus oxalivorans gen. ...

GM Zaitsev, IV Tsitko, FA Rainey... - ... of Systematic and ..., 1998 - ijs.microbiologyresearch.org
... unclear (30, 31, 33, 36, 40, 42). Among aerobic bacteria, ' Pseudomonas oxalaticus' strain Ox 1 (4 l), **Methylobacterium extorquens** strain AM 1, previously classified as Pseudomonas sp. (5, 15, 49, and among the anaerobic ...
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Global genome comparative analysis reveals insights of resistome and life-style adaptation of Pseudomonas putida strain T2-2 in oral cavity

XY Chan, KO Chua, KY How, WF Yin... - The Scientific World ..., 2014 - hindawi.com
... As there is reason to believe that the species is an opportunistic **pathogen** with ability to cause life-threatening ... N. Korotkova, L. Chistoserdova, V. Kuksa, and ME Lidstrom, "Glyoxylate regeneration pathway in the methylotroph **Methylobacterium extorquens** AM1," Journal of ...
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Generating and navigating proteome maps using mass spectrometry

CH Ahrens, E Brunner, E Qeli, K Basler... - Nature reviews. ..., 2010 - search.proquest.com

... FB24 5.08 4,536 3,220 71% LC-MS/MS 139. **Methylobacterium extorquens** AM1. ~6.0 7,556 4,447 58% LC-MS/MS 140. Eukaryotes. ... 41. Malmstrom, J. et al. Proteome-wide cellular protein concentrations of the human **pathogen** Leptospira interrogans. Nature 460, 762765 (2009) ...

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Population Dynamics and Seasonal Variation of Bacterial System Utilizing Single Carbon from River Cooum and River Adyar, Chennai, Tamilnadu, India

J Shanmugam, V Ponnusamy, M Gopal... - Int. J. Curr. Microbiol. ..., 2016 - researchgate.net

... Chistoserdova, L., Chen, SW, Lapidus, A., Lidstrom, ME 2003. Methylotrophy in **Methylobacterium extorquens** AM1 from a Genomic Point of View. J. Bacteriol., 185(10): 2980–2987. ... Enumeration and identification of **pathogenic** pollution indicators in Cauvery River, South India. ...

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Methylotrophic bacterium for the production of recombinant proteins and other products

M Figueira, L Laramee, J Murrell... - US Patent App. 09/ ..., 2001 - Google Patents

... [0010]. Such methods would include a new non-**pathogenic** prokaryotic microbial system, as an alternative to E. coli, for recombinant ... [0017]. In another preferred embodiment of the present invention the methylotrophic bacterium is **Methylobacterium extorquens** ATCC 55366. ...

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Investigating the metabolic capabilities of Mycobacterium tuberculosis H37Rv using the in silico strain iNJ 661 and proposing alternative drug targets

N Jamshidi, BØ Palsson - BMC systems biology, 2007 - bmcsystbiol.biomedcentral.com

... Background: Mycobacterium tuberculosis continues to be a major **pathogen** in the third world, killing almost 2 million people a year by the most recent estimates. ... Tuberculosis continues to be a devastating **pathogen** throughout the world, particularly in developing nations. ...

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Molecular and biochemical characterization of urease and survival of Yersinia enterocolitica biovar 1A in acidic pH in vitro

N Bhagat, JS Virdi - BMC microbiology, 2009 - bmcmicrobiol.biomedcentral.com

... Yersinia enterocolitica, an important food- and water-borne enteric **pathogen** is represented ... clinical, epidemiological and experimental evidences indicate their potential **pathogenicity** [5]. Some ... However the exact mechanisms underlying the **pathogenesis** by biovar 1A strains ...

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Chapter Twelve-Reannotation of Genomes by Means of Proteomics Data

J Armengaud - Methods in enzymology, 2017 - Elsevier

... Most published examples are centered on the reannotation of the genome of a given organism and the most recent studies are relying on next-generation proteomics high-throughput approaches: reannotation of **Methylobacterium extorquens** (Kumar, Mondal, et al., 2014 ...

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Methylobacterium mesophilicum cellulitis and lymphangitis following a dog bite

NM Abdel-Haq, BI Asmar - Journal of Pediatric Infectious ..., 2008 - thieme-connect.com

... case report and litera- ture review of an unusual opportunistic **pathogen**, Clin Infect ... al., Protracted cervical lym- phadenitis with Mycobacterium avium and **Methylobacterium** mesophilicum, Pediatr ... Imaeda, ZC Kaminski and LB Reichman, Culture of Vibrio **extorquens** from severe ...

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The biochemistry, physiology and genetics of PQQ and PQQ-containing enzymes

PM Goodwin, C Anthony - Advances in microbial physiology, 1998 - Elsevier

Pyrrolo-quinoline quinone (PQQ) is the non-covalently bound prosthetic group of many quinoproteins catalysing reactions in the periplasm of Gram-negative bacter.

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D Kumar, G Bansal, A Narang, T Basak, T Abbas... - ..., 2016 - Wiley Online Library

... Infection biology has gained a lot from integrative omics. The detailed mechanisms of many host-**pathogen** interactions have been worked out. **Pathogen** surveillance and epidemiological data have specially provided new dimensions to public health. ...

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Heterologous extracellular production of enterocin P from Enterococcus faecium P13 in the methylotrophic bacterium **Methylobacterium extorquens**

J Gutiérrez, D Bourque, R Criado, YJ Choi... - FEMS microbiology ..., 2005 - academic.oup.com

... Article Navigation. Heterologous extracellular production of enterocin P from Enterococcus faecium P13 in the methylotrophic bacterium **Methylobacterium extorquens**. Jorge Gutiérrez Jorge Gutiérrez. Departamento de Nutrición ...

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Quantitative detection of methanotrophs in soil by novel pmoA-targeted real-time PCR assays

S Kolb, C Knief, S Stubner... - Applied and Environmental ..., 2003 - Am Soc Microbiol

... DNA was extracted from lyophilized cells of Methylopila helvetica (DSM 6342), **Methylobacterium extorquens** (DSM 1337), and the ammonia-oxidizing strain Nitrosomonas europaea (NCIMB 11850) by heating for 10 min at 100°C in 25 µl of TE buffer (pH 7.2), followed by ...

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Use of the yeast Pichia pastoris as an expression host for secretion of enterocin L50, a leaderless two-peptide (L50A and L50B) bacteriocin from Enterococcus ...

A Basanta, B Gómez-Sala, J Sánchez... - Applied and ..., 2010 - Am Soc Microbiol

... Enterococcus faecium L50 is a multiple-bacteriocin producer strain isolated from a Spanish dry-fermented sausage (7), which inhibits several spoilage and food-borne **pathogenic** bacteria as well as clinical human and animal pathogens (2, 8, 9, 10). ...

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Bacterial genome evolution within a clonal population: from in vitro investigations to in vivo observations

M Beaume, N Monina, J Schrenzel... - Future microbiology, 2013 - Future Medicine

... term laboratory evolution of E. coli [10], and one with an engineered strain of **Methylobacterium extorquens** [11]. ... One of the most abundant bacteria in CF is the opportunistic **pathogen** P. aeruginosa, which can ... Evidently, phages play an important role in bacterial **pathogenicity**. ...

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Purification and properties of the formate dehydrogenase and characterization of the fdhA gene of Sulfurospirillum multivorans

RPH Schmitz, G Diekert - Archives of microbiology, 2003 - Springer

... Laukel M, Chistoserdova L, Lidstrom ME, Vorholt JA (2003) The tungsten-containing formate dehydrogenase from **Methylobacterium extorquens** AM1: Purification ... Vliet AH, Whitehead S, Barrell BG (2000) The genome sequence of the food-borne **pathogen** Campylobacter jejuni ...

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RR Brown - ... Advances in Tryptophan Research: Tryptophan and ..., 2012 - books.google.com

... that such binding may be important in the as yet unclear mechanism of prion **pathogenesis** (Stahl and ... Mutants of **Methylobacterium extorquens** and Paracoccus denitrificans deficient in c-type cytochrome biogenesis synthesize the methylamine-dehydrogenase polypeptides but ...

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NRL Adler, EM Allwood, DD Lucas... - BMC ..., 2016 - bmcgenomics.biomedcentral.com

... Burkholderia pseudomallei is a highly **pathogenic** Gram-negative organism and the causative agent ... to 25 °C. This system may be involved in **pathogenesis**, but its ... 96 % coverage) to the 5, 10-methylene tetrahydromethanopterin reductase in **Methylobacterium extorquens** that is ...

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Structure, mechanism and physiological roles of bacterial cytochrome c peroxidases

JM Atack, DJ Kelly - Advances in microbial physiology, 2006 - Elsevier

... and archaeal groups. Among Gram-negative bacteria, there is no particular correlation with metabolic lifestyle or type of organism, and a wide variety of both free-living and **pathogenic** bacteria contain ccp genes. Figure 1 shows ...

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Metabolic pathways and engineering of polyhydroxyalkanoate biosynthesis

K Taguchi, S Taguchi, K Sudesh, A Maehara... - Biopolymers ..., 2005 - Wiley Online Library

... (1998). Ectothiorhodospira shaposhnikovii, NO, AF307334, phaCEAPB, (1), Zhang et al. (2000). **Methylobacterium extorquens** IBT6, S, L07893, phaC, (5), Valentin and Steinbüchel (1993). Nocardia corallina, S, M, AF019964, phaC, (5), Hall et al. (1998). ...

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System and method for monitoring an analyte

J Lambert, A Fisher, M Borchert - US Patent App. 11/463,834, 2006 - Google Patents

... Hydrogenophaga pseudoflava, Kingella denitrificans, Kingella kingae, Kingella species, Kluvera ascorbata, Flavobacterium indologenes, **Methylobacterium extorquens**, **Methylobacterium** species, Micrococcus ... to identify it as: (i) harmless; (ii) a strain of a known **pathogen**; or (iii ...

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Rhodopseudomonas palustris CGA010 Proteome Implicates Extracytoplasmic Function Sigma Factor in Stress Response

MS Allen, GB Hurst, TYS Lu, LM Perry, C Pan... - Journal of Proteome ..., 2015 - osti.gov

... 71 ECF σ factors are involved in the regulation of genes involved in a number of cellular 72 responses including periplasmic stress, iron transport, and **pathogenesis** (for further 73 ... **Methylobacterium extorquens** (13). In that organism, the response regulator PhyR was 98 ...

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The LovK-LovR two-component system is a regulator of the general stress pathway in Caulobacter crescentus

R Foreman, A Fiebig, S Crosson - Journal of bacteriology, 2012 - Am Soc Microbiol

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Engineering Pseudomonas putida S12 for efficient utilization of D-xylose and L-arabinose

JP Meijnen, JH de Winde... - Applied and ..., 2008 - Am Soc Microbiol

... Liu, Q., JR Kirchhoff, CR Faehnle, RE Viola, and RA Hudson. 2006. A rapid method for the purification of methanol dehydrogenase from **Methylobacterium extorquens**. Protein Expr. Purif. 46:316-320. ...

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Characterization and heterologous gene expression of a novel esterase from Lactobacillus casei CL96

YJ Choi, CB Miguez, BH Lee - Applied and environmental ..., 2004 - Am Soc Microbiol

... The ORF of estI was isolated by PCR and expressed in Escherichia coli, the methylotrophic bacterium **Methylobacterium extorquens**, and the methylotrophic yeast Pichia pastoris under the control of T7, methanol dehydrogenase (P mxaF), and alcohol oxidase (AOX1 ...

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The evolution of conflict resolution between plasmids and their bacterial hosts

SÉ Mc Ginty, DJ Rankin - Evolution, 2012 - Wiley Online Library
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A Bissell - 2013 - repository.library.northeastern.edu
... identify novel antimicrobials. Mycobacterium tuberculosis is an obligately-aerobic respiratory **pathogen** with several known Mycobacterial-selective compounds with ... to Mycoplasma sp. based on morphology, **pathogenic** properties, and adherence to host cells (Messick, 2008). ...
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New vectors for chromosomal integration enable high-level constitutive or inducible magnetosome expression of fusion proteins in Magnetospirillum gryphiswaldense

S Borg, J Hofmann, A Pollithy, C Lang... - Applied and ..., 2014 - Am Soc Microbiol
... increasing their copy number. In similar approaches, Choi and coworkers integrated double copies of the cym repressor into **Methylobacterium extorquens**, thereby achieving tight repression of an inducible promoter (45). In the same ...
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Protein Engineering of POO Glucose Dehydrogenase

S Igarashi, K Sode - Enzyme Functionality: Design: Engineering, ..., 2003 - books.google.com
... Using the sensor system, we aimed to detect the specific DNA sequence of a **pathogenic** bacteria, Salmonella virulence (inv4) gene. ... Routine use of DNA-based analyses, such as SNP or **pathogen** detec- tion, will require both simplicity and sensitivity in sensor design. ...
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Proteogenomic insights into salt tolerance by a halotolerant alpha-proteobacterium isolated from an Andean saline spring

C Rubiano-Labrador, C Bland, G Miotello, P Guérin... - Journal of ..., 2014 - Elsevier
Tistlia consotensis is a halotolerant Rhodospirillaceae that was isolated from a saline spring located in the Colombian Andes with a salt concentration close to.
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Knockout and overexpression of pyrroloquinoline quinone biosynthetic genes in Gluconobacter oxydans 621H

T Hölscher, H Görisch - Journal of bacteriology, 2006 - Am Soc Microbiol
... Genes involved in PQQ synthesis have been characterized for several bacteria, including Klebsiella pneumoniae, Acinetobacter calcoaceticus, **Methylobacterium extorquens** AM1, and Pseudomonas sp. (reviewed in reference 19). ...
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Corynebacterium glutamicum harbours a molybdenum cofactor-dependent formate dehydrogenase which alleviates growth inhibition in the presence of formate

S Witthoff, L Eggeling, M Bott, T Polen - Microbiology, 2012 - mic.microbiologyresearch.org
... eg 52% with FDH from Streptomyces coelicolor and S. lividans) and from different mycobacteria including the human **pathogen** M. tuberculosis (48 %). However, a high sequence identity of 46 % also exists with FDH4 from **Methylobacterium extorquens**, which has been ...
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Metabolomics in molecular microbiology

ML Reaves - 2013 - search.proquest.com
... Figure 1. Overview of metabolomics in systems microbiology.....Page 2Figure 2. Elucidation of the methanol assimilation pathway in **Methylobacterium extorquens**. ... Figure 2. Elucidation of the methanol assimilation pathway in **Methylobacterium extorquens**. ...
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Elucidation of an alternate isoleucine biosynthesis pathway in Geobacter sulfurreducens

C Risso, SJ Van Dien, A Orloff, DR Lovley... - Journal of ..., 2008 - Am Soc Microbiol
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Development of a novel prokaryotic two-hybrid system for the detection and analysis of protein-protein interactions in vivo

PA Clarke - 2000 - doras.dcu.ie

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Detection of bacteria

M Danzer, H Polin, K Hofer, B Fiedler, J Radler... - US Patent ..., 2013 - Google Patents

... 230 **pathogenic** bacteria ... lentum, Francisella tularensis, Fusobacterium necrophorum, Fusobacterium nucleatum, Gardnerella vaginalis, Haemophilus influenzae, Helicobacter pylori, Kingella kingae, **Methylobacterium extorquens**, **Methylobacterium** mesophilicun, Mycobacterium ...

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A switch III motif relays signaling between a B12 enzyme and its G-protein chaperone

M Lofgren, D Padovani, M Koutmos... - Nature chemical ..., 2013 - nature.com

Heterotrimeric G proteins contain a switch III motif that regulates enzyme function. Structural and biochemical studies now identify a similar switch III loop in a nonheterotrimeric G-protein chaperone that explains the debilitating effects of mutations linked to methylmalonic aciduria ...

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Distribution, Evolution, and Physiology of Oxidative Fermentation

K Matsushita, M Matsutani - Acetic Acid Bacteria, 2016 - Springer

... In addition to methylotrophic bacteria, **Methylobacterium extorquens** and Paracoccus denitrificans, and AAB ancestors ... context identical to that of gene clusters in both M. **extorquens** and P ... SM (2007) Genome sequence analysis of the emerging human **pathogenic** acetic acid ...

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Recombinant cytochromes c biogenesis systems I and II and analysis of haem delivery pathways in Escherichia coli

RE Feissner, CL Richard-Fogal... - Molecular ..., 2006 - Wiley Online Library

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Pentanol and benzyl alcohol attack bacterial surface structures differently

T Yano, Y Miyahara, N Morii, T Okano... - Applied and ..., 2016 - Am Soc Microbiol

... nodulation-cell division (RND) transporter which is responsible for efflux of many chemotherapeutic agents in **Methylobacterium extorquens** DM1, has ... **Methylobacterium** mesophilicum infection: case report and literature review of an unusual opportunistic **pathogen**. ...

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Polyhydroxyalkanoates in Nanobiotechnology: Application to Protein–Protein Interaction Studies

SY Lee, JP Park - ... Self-assembly Systems and Biopolymer-based ..., 2006 - books.google.com

... 75 Nanobio Applications of Microbial Polyester butyrate from methanol: characterization of a new isolate of **Methylobacterium extorquens**. Appl. ... microarray for the specific immo- bilization of recombinant proteins and its ap- plication in immunodiagnostics and **pathogen** direction ...

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Type I pyridoxal 5′-phosphate dependent enzymatic domains embedded within multimodular nonribosomal peptide synthetase and polyketide synthase ...

T Milano, A Paiardini, I Grgurina... - BMC structural ..., 2013 - bmcstructbiol.biomedcentral.com

... 2. C3AKA5. Bacillus mycoides. GSA. KS–CP–PLP1. ? Nrps-transatpks. Asn-Mal-Gly-Nrp-Nrp-Nrp-Thr-Gly. 3. C5B3B9. **Methylobacterium extorquens**. TPA. CP–ABHy–PLP1– FMO. ? ? ? 4. C6XWU5. Pedobacter heparinus. GSA. A–CP–KS–AT–CP–PLP1. Nrp. Nrps-t1pks. ...

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Molecular characterization of ltp3 and ltp4, essential for C24-branched chain sterol-side-chain degradation in Rhodococcus rhodochrous DSM 43269

MH Wilbrink, R van der Geize... - ..., 2012 - mic.microbiologyresearch.org

... 2). Possibly, their encoded proteins form (hetero) multimeric structures as in other aldol-lyase enzymes, such as malyl-CoA lyase of **Methylobacterium extorquens** (formerly Pseudomonas AM1) (Hacking & Quayle, 1974) and citrate lyase of Leuconostoc mesenteroides (Bekal et ...

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Draft genomic sequence of Nereida ignava CECT 5292 T, a marine bacterium of the family Rhodobacteraceae

DR Arahal, MJ Pujalte... - Standards in ..., 2016 - standardsingenomics.biomedcentral ...

... Salinity. 3,5 %. TAS [1]. MIGS-22. Oxygen requirement. Aerobic. TAS [1]. MIGS-15. Biotic relationship. Free-living. TAS [1]. MIGS-14. **Pathogenicity**. Not reported. NAS. MIGS-4. Geographic location. Vinaroz, Spain. TAS [1]. MIGS-5. Sample collection. 1990. TAS [1]. MIGS-4.1. Latitude ...

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Isolation of Methylophaga spp. from marine dimethylsulfide-degrading enrichment cultures and identification of polypeptides induced during growth on dimethylsulfide

H Schäfer - Applied and environmental microbiology, 2007 - Am Soc Microbiol

... Previously, mxaF' knockout mutants of **Methylobacterium extorquens** (similar to xoxF) were not affected in their ability to grow on methanol or methylamine, and a phenotype associated with this gene has not yet been identified (8). Induction of a thiol-specific alkyl ...

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Identification of Staphylococcus aureus proteins recognized by the antibody-mediated immune response to a biofilm infection

RA Brady, JG Leid, AK Camper... - Infection and ..., 2006 - Am Soc Microbiol

... Microarray development and construction.DNA microarrays were constructed and made available by The Institute for Genomic Research, **Pathogen** Functional Genomics Resource Center (PFGRC), through a grant by the National Institute of Allergy and Infectious Diseases of ...

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Knowns and unknowns of membrane lipid synthesis in streptomycetes

M Sandoval-Calderón, Z Guan, C Sohlenkamp - Biochimie, 2017 - Elsevier

Bacteria belonging to the genus Streptomyces are among the most prolific producers of antibiotics. Research on cellular membrane biosynthesis and turnover is la.

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Less is more: selective advantages can explain the prevalent loss of biosynthetic genes in bacteria

G D'Souza, S Waschina, S Pande, K Bohl, C Kaleta... - ..., 2014 - Wiley Online Library

... 2012; Lee and Marx 2012). Interestingly, in a study with the bacterium **Methylobacterium extorquens** AM1 (Lee and Marx 2012), the observed fitness advantage did not seem to result from a general shortening of the genome, but was rather due to the loss of specific genes. ...

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Methods for treating bacterial infections

S Page, S Garg - US Patent 9,539,223, 2017 - Google Patents

... enteropathogenic E. coli; ETEC=enterotoxigenic E. coli; ExPEC=extraintestinal **pathogenic** E. coli ... Leptospira yanagawae.—Leptotrichiaceae:—Leptotrichia buccalis; Streptobacillus moniliformis.—Methylobacteriaceae:—**Methylobacterium extorquens** group; **Methylobacterium** ...

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Proteomics of Fusobacterium nucleatum within a model developing oral microbial community

EL Hendrickson, T Wang, DAC Beck... - ..., 2014 - Wiley Online Library

... 1996), and the categorization of F. nucleatum as a traditional **pathogen** or commensal is debatable. However, in current models of oral **pathogenicity**, health or disease states are not viewed in the context of a single **pathogenic** organism but rather of dysbiotic microbial ...

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The cytochrome c maturation locus of Legionella pneumophila promotes iron assimilation and intracellular infection and contains a strain-specific insertion sequence ...

VK Viswanathan, S Kurtz, LL Pedersen... - Infection and ..., 2002 - Am Soc Microbiol

... could facilitate growth under low-iron conditions (83). Thus, the investigation of L. pneumophila is yielding new insights into bacterial iron acquisition and **pathogenesis**. In the present study, we further characterized a second ...

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Protein

P Lind, C Soderberg - US Patent App. 10/187,171, 2002 - Google Patents

... J Bacteriol, June 1993; 175(2): 3776-83) discloses the genetics of malyl coenzyme A lyase in **Methylobacterium extorquens** AM1 ... example, to screen for compounds that increase or decrease the effect or activity of citrate lyase, with the potential of alleviating **pathogenic** effects of ...

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Human Genome and Diseases:¶ WD-repeat proteins: structure characteristics, biological function, and their involvement in human diseases

D Li, R Roberts - Cellular and molecular life sciences, 2001 - Springer

Page 1. Human Genome and Diseases: Review WD-repeat proteins: structure characteristics, biological function, and their involvement in human diseases D. Li and R. Roberts* Department of Medicine, Section of Cardiology ...

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A complex regulatory network controls aerobic ethanol oxidation in Pseudomonas aeruginosa: indication of four levels of sensor kinases and response regulators

DS Mern, SW Ha, V Khodaverdi... - ..., 2010 - mic.microbiologyresearch.org

... has been solved at 2.6 A° resolution and reveals that the backbone folding and the active site of QEDH are very similar to those of the large subunits of the PQQ-dependent quinoprotein methanol dehydrogenases (QMDHs) of **Methylobacterium extorquens** and Methylophilus ...

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南海北部不同深度的菌相與多樣性研究

HC Lai - 臺灣大學海洋研究所學位論文, 2010 - airitilibrary.com

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Experimental evolution in fungi: An untapped resource

KJ Fisher, GI Lang - Fungal Genetics and Biology, 2016 - Elsevier

... Many other bacterial and viral models have since been used in "evolve and transfer" experiments including Pseudomonas fluorescens (Barrett et al., 2005), **Methylobacterium extorquens** (Chou et al., 2011), Myxococcus xanthus (Velicer et al., 1998), and bacteriophage (Bull et ...

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Stealth adaptation of viruses: Review and updated molecular analysis on a stealth adapted African green monkey simian cytomegalovirus (SCMV)

W John Martin - J Hum Virol Retrovirol, 2014 - researchgate.net

Page 1. J Hum Virol Retrovirol 2014, 1(4): 00020 Journal of Human Virology & Retrovirology Submit Manuscript | http://medcraveonline.com Abstract The available DNA sequence data on an African green monkey simian cytomegalovirus ...

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Cofactor Editing by the G-protein Metallochaperone Domain Regulates the Radical B12 Enzyme IcmF

Z Li, K Kitanishi, UT Twahir, V Cracan... - Journal of Biological ..., 2017 - ASBMB

... The **Methylobacterium extorquens** orthologs of MCM, ATR and the G-protein (known as MeaB), have been characterized extensively (8, 11–17), and our understanding of the mechanism of cofactor repair derives primarily from studies on this system. ...

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Propionate metabolism in Mycobacterium tuberculosis: characterization of the vitamin B12-dependent methylmalonyl pathway

SA Savvi - 2009 - wiredspace.wits.ac.za

... chemotherapy (Anon, 1981, 1982), and the parallel demographic evolution of expanding **pathogen** and patient populations (Wirth et al., 2008), has elevated the magnitude of concern ... Successful containment of the **pathogen** results in latent infection. Various models have ...

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Use of the usp45 lactococcal secretion signal sequence to drive the secretion and functional expression of enterococcal bacteriocins in Lactococcus lactis

J Borrero, JJ Jiménez, L Gútiez, C Herranz... - Applied microbiology ..., 2011 - Springer

... 2006; Morello et al. 2008). The potent antimicrobial activity of the bacteriocins EntP and HirJM79 against **pathogenic** bacteria such as Listeria monocytogenes has driven interest for their biotechnological production by heterologous hosts (Gutiérrez et al. 2006; Sánchez et al. ...

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Metabolic resource allocation in individual microbes determines ecosystem interactions and spatial dynamics

WR Harcombe, WJ Riehl, I Dukovski, BR Granger... - Cell reports, 2014 - Elsevier

... We apply COMETS to the study of a previously built E. coli/S. enterica synthetic consortium (Harcombe, 2010) and to a new three-member consortium that incorporates **Methylobacterium extorquens** AM1 into the E. coli/S. enterica system. Results. ...

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Development of bacteriocinogenic strains of Saccharomyces cerevisiae heterologously expressing and secreting the leaderless enterocin L50 peptides L50A and ...

A Basanta, C Herranz, J Gutiérrez... - Applied and ..., 2009 - Am Soc Microbiol

... variety of ribosomally synthesized proteins or peptides, referred to as bacteriocins, displaying antimicrobial activity against a broad range of gram-positive bacteria and, to a lesser extent, gram-negative bacteria, including spoilage and food-borne **pathogenic** microorganisms (11 ...

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Recombinant prokaryotes and use thereof for production of O-glycosylated proteins

TU Gerngross, GE Henderson, E Anderson - US Patent 9,051,593, 2015 - Google Patents

... Microbiol. 68:5925-5932), **Methylobacterium extorquens** (Belanger, et al. (2004) FEMS Microbiol Lett. ... J. 94:17-24). PAS labeling of sialic acid moieties can occur under milder conditions, but like UDP-GalNAc, CMP-NANA is not produced in non-**pathogenic** E. coli strains. ...

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Prevention Strategy of Urogenital Infections by Using Lactobacilli with Probiotic Properties

L Pascual, L Barberis - Urinary Tract Infections, 2011 - intechopen.com

... bacterial strains has been suggested to enable them to form a physical-chemical barrier that prevents colonization by **pathogenic** bacteria. ... co-aggregation could be an important factor in maintaining vaginal health because it produces an area around the **pathogen** where the ...

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Caenorhabditis elegans as a model for obesity research

J Zheng, FL Greenway - International journal of obesity, 2012 - search.proquest.com

... This rapid growth and quick turnover allows rapid screening protocols looking for bioactive compounds.C. elegans grows on agar plates and uses non-**pathogenic** Escherichia coli (E. coli ... Glyoxylate regeneration pathway in the methylotroph **Methylobacterium extorquens** AM1. ...

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