Identification of Clinically Significant Bacteria

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Laboratory Diagnosis of Infectious Diseases:

From Basics to Molecular Methods Workshop

Friday 18 March 2011
Westmead Education & Conference Centre

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The Royal College of Pathologists of Australasia has received
Australian Government funding under the Specialist Training Program

Difficult Gram Positive Cocci

- *Streptococcus mutans*
- *Alloiococcus otitidis*
- *Gemella haemolysans*
- *Helcococcus kunzii*

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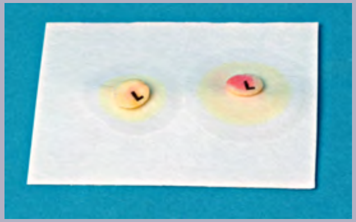
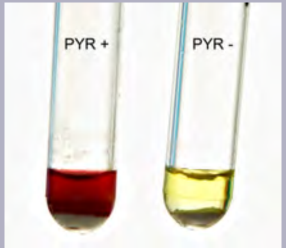
Tests that you need to perform

- Gram stain, haemolysis, motility, catalase
- **Morphology in BHI Broth**, 24hrs @ 37 °C ?clusters or chains
- Vancomycin susceptibility
- Bile esculin





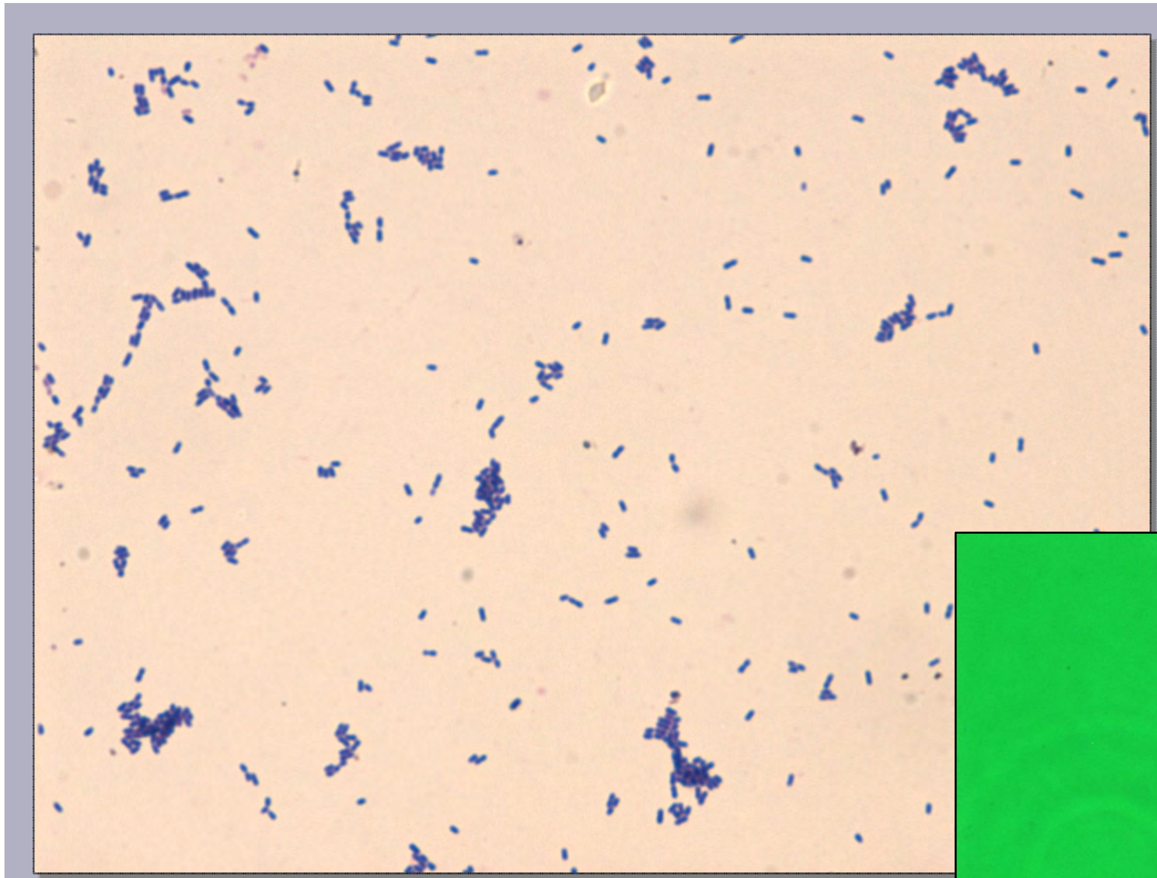
- PYR, LAP, ADH, Aesculin, hippurate – available as

  rapid disc tests (Remel, Rosco) or part of commercial kit
- Growth in 6.5% NaCl, Tween 80 broths and @ 45°C
- Gas from MRS broth

Streptococcus mutans

Key features

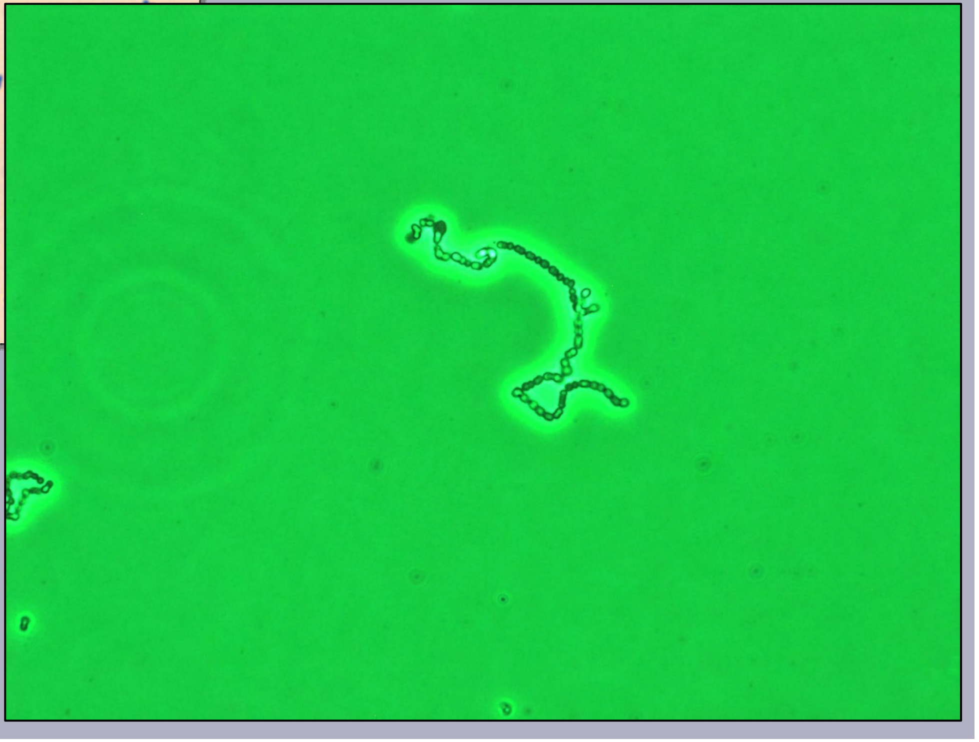
- The key is to recognise that this small GPR is actually a streptococcus
- White, dry colonies α-haemolytic or non-haemolytic on HBA
- Conversion to cocci in chains in BHI, PYG or Thioglycollate broth
- Identifies well on most ID systems



Streptococcus mutans

gs x1000

small GPR/coccobacilli



Streptococcus mutans

grown in BHI broth

x400 phase contrast

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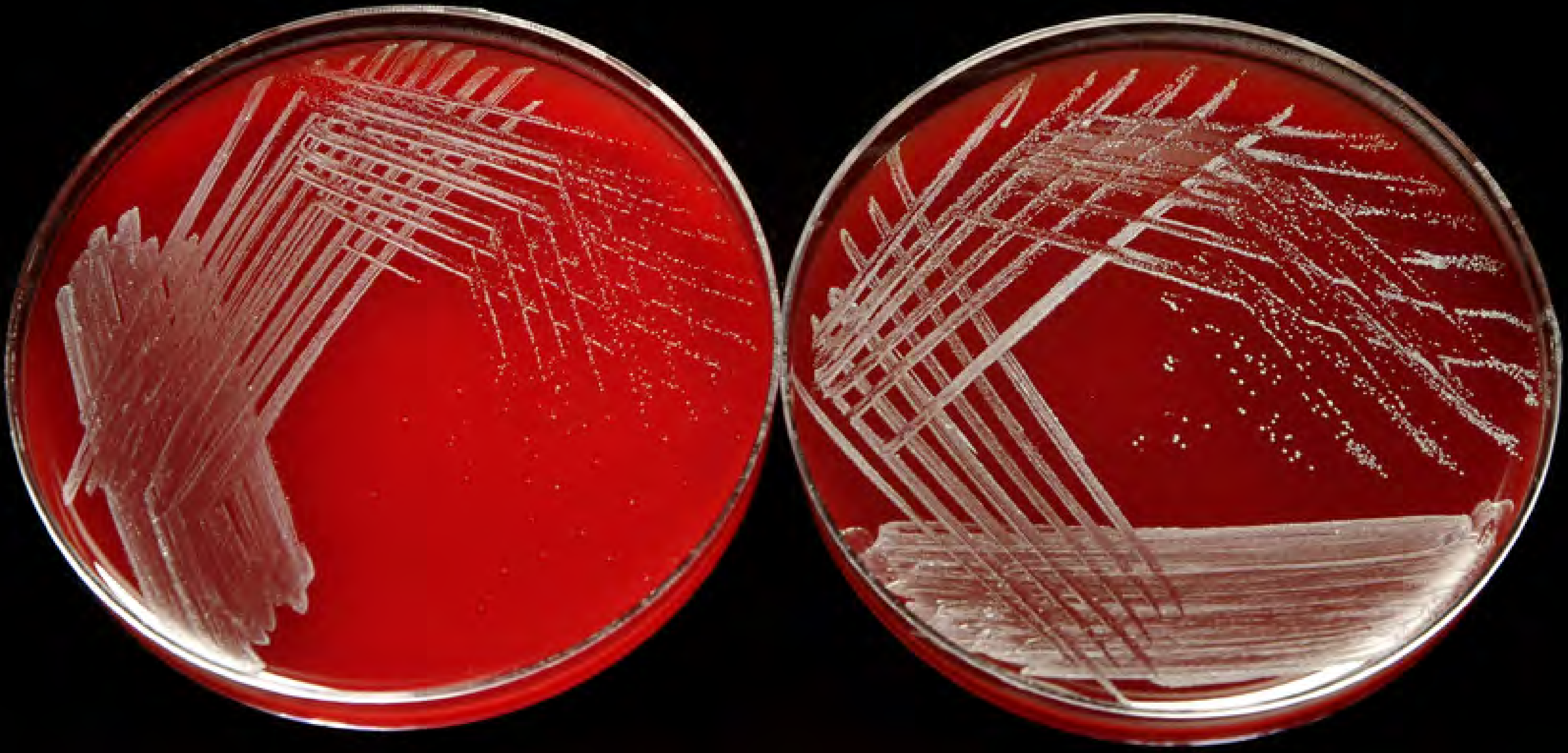

Streptococcus mutans - HBA 24 & 48 hours

Alloiococcus otitidis

Key features

- GPC regular size, in pairs, tetrads, clusters
- Slow-growing (48-72hrs) due to lipid requirement
- Strict aerobe and asaccharolytic (unusual for GPC)
- Non-haemolytic to α-haemolytic with age
- Identified by API 20 Strep & ID 32 Strep
- Key tests: Catalase +/+^{w}, PYR+, LAP+, 6.5% NaCl+ (slow), 45°C-, growth on BE but aesculin-, poor or no growth on CA

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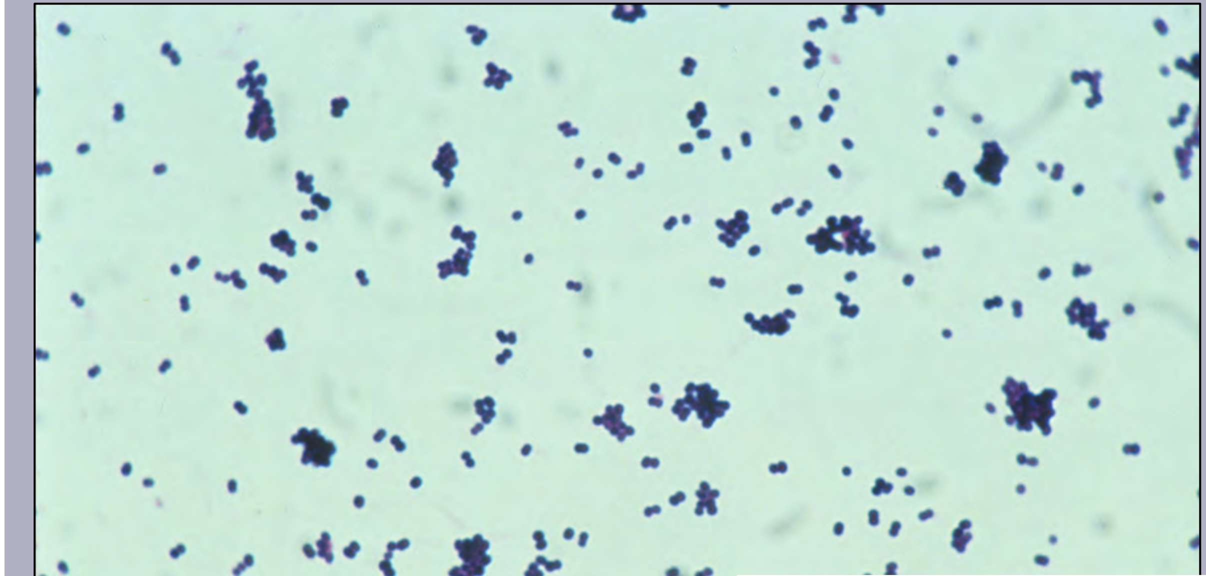
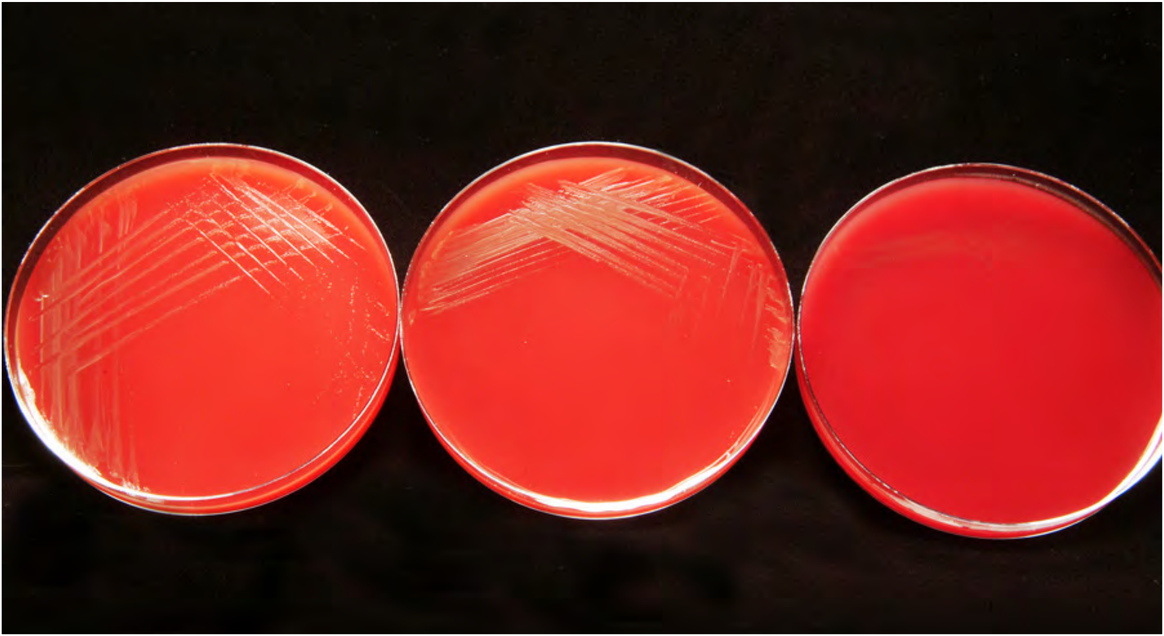


Alloiococcus otitidis

gs x1000

Always check morphology in BHI broth (I know I go on about this!!)



Alloiococcus otitidis

HBA @ 48hrs

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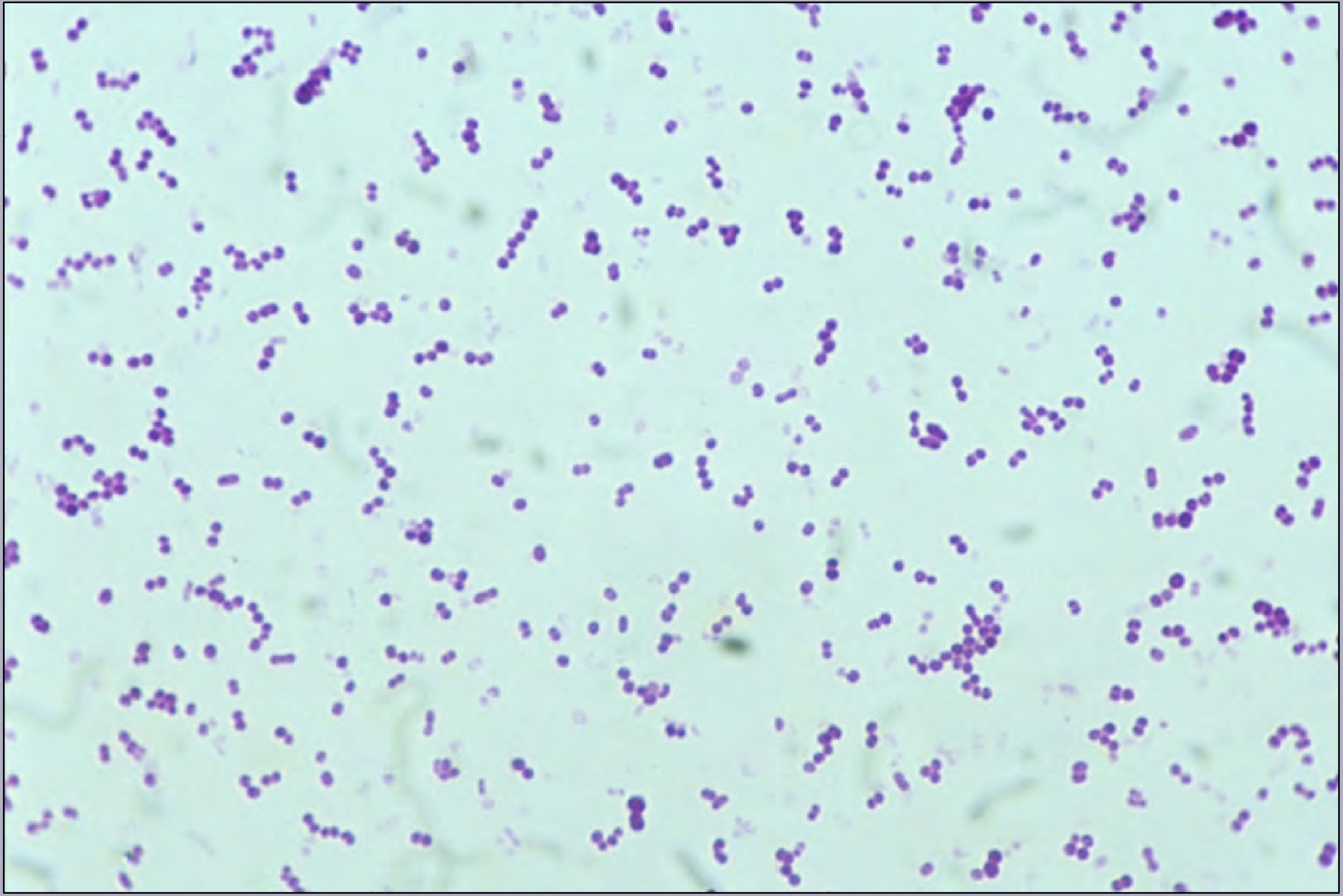
Gemella haemolysans

Key features

- Gram-variable cocci in pairs, clusters & small chains
- Slow-growing
- CO_2 enhances growth – *G. morbillorum* prefers to grow anaerobically, *G. haemolysans* prefers to grow aerobically
- Colonies are α-haemolytic or non-haemolytic
- Growth stimulated by Tween 80
- May be confused with NVS but not B6 dependent
- Key tests: PYR+/V (requires heavy inoculum), LAP (V), BE-, 45°C-, *Gemella haemolysans* NO_2+, *Gemella morbillorum* NO_2-



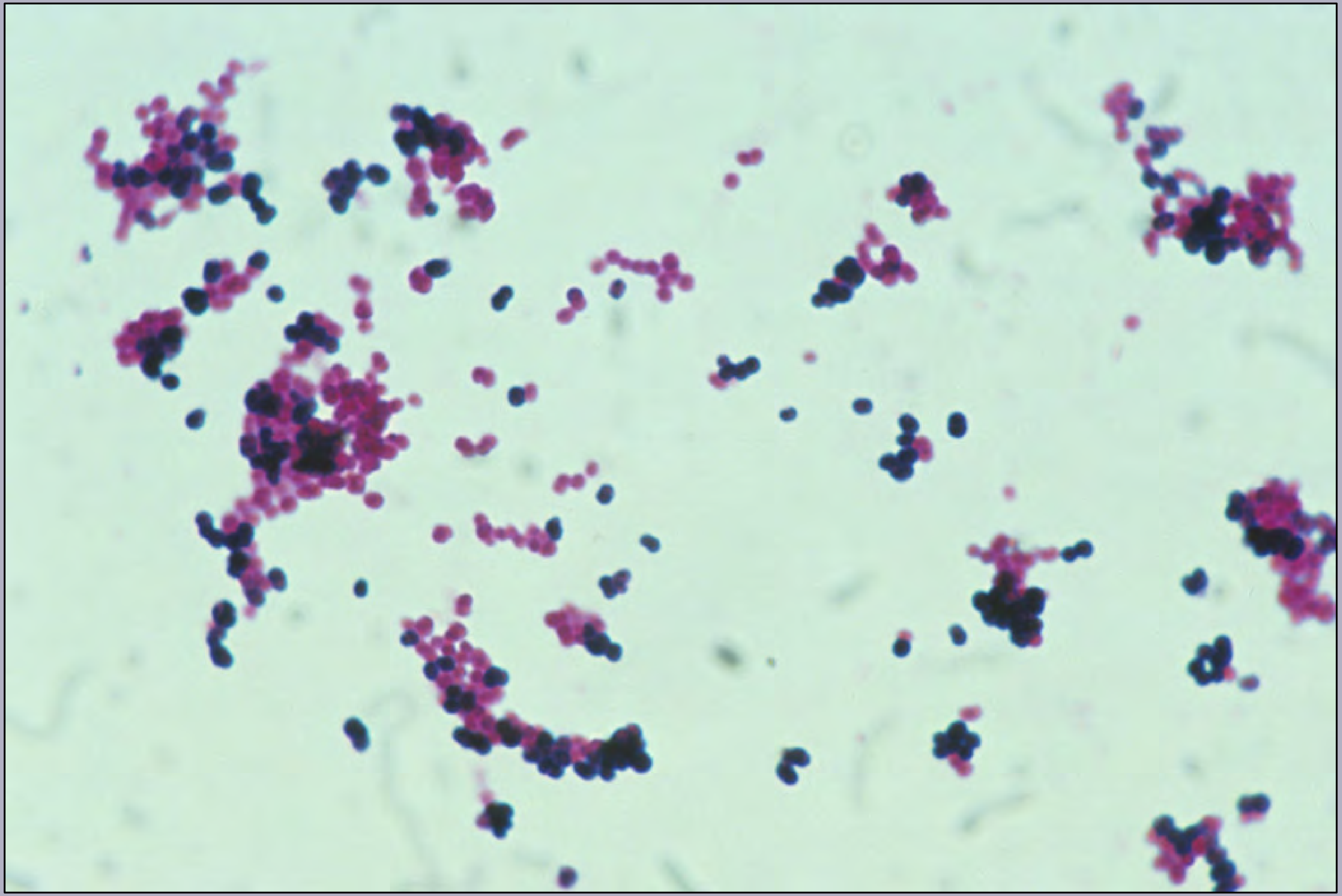
***Gemella haemolysans* – gs x100 cocci are often decolourised**

Helcococcus kunzii

Key features

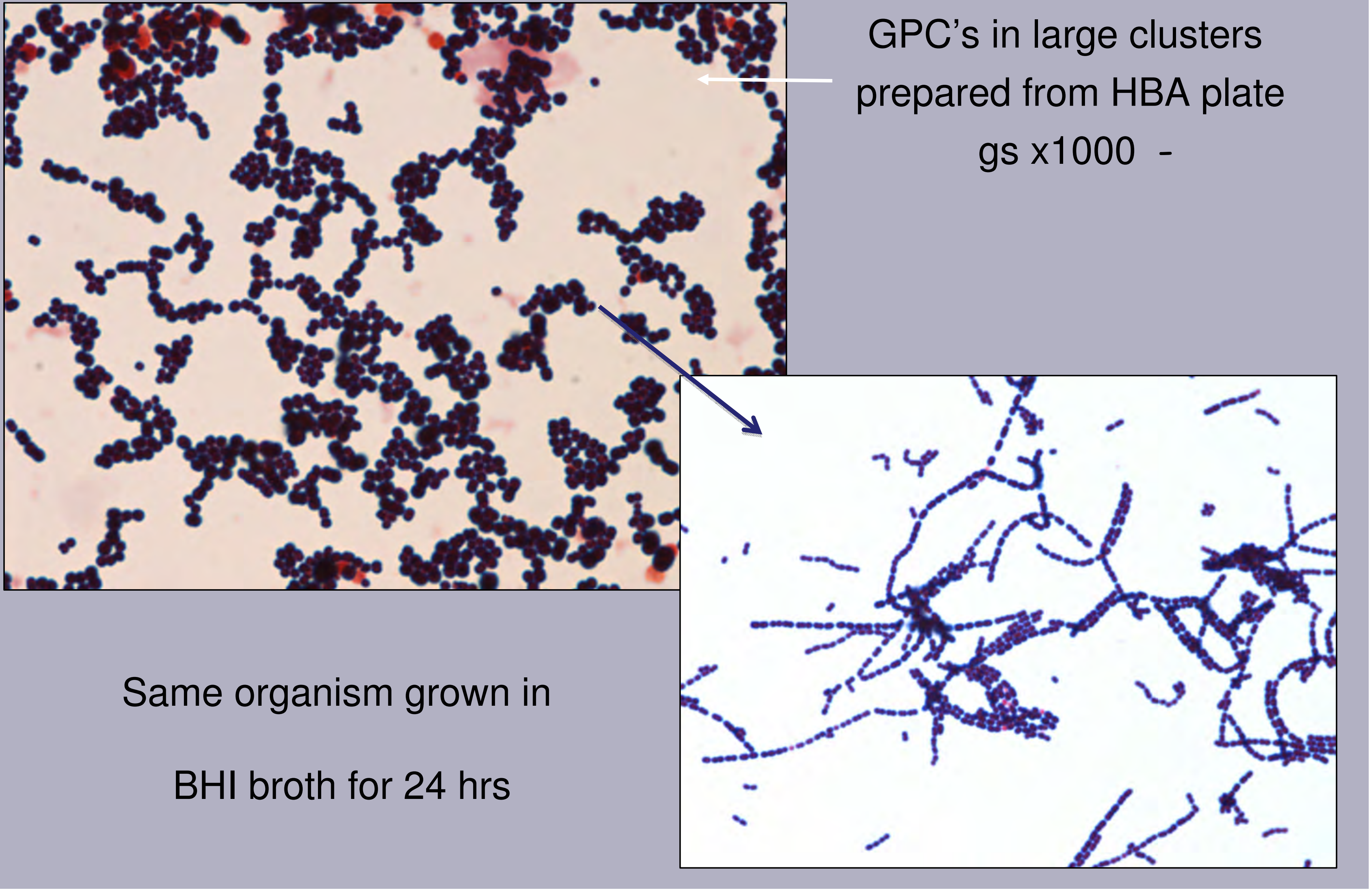
- Large irregular GPC in clusters – "*Aerococcus*-like"
- Non-haemolytic – some strains weakly α-haemolytic
- Lipophilic – growth stimulated by Tween 80
- Not included on all commercial kits/system databases, but

  ID 32 Strep gives a profile 4100413 – "doubtful" *A. viridans*
- Key tests to differentiate from *Aerococcus*: LAP-, PYR+, aesculin+, hippurate-, NG @ 45°C, Tween 80 stimulation
- Note: Follow manufacturer's instructions for rapid disc tests



Helcococcus kunzii – gs x1000 cocci are large and in clusters

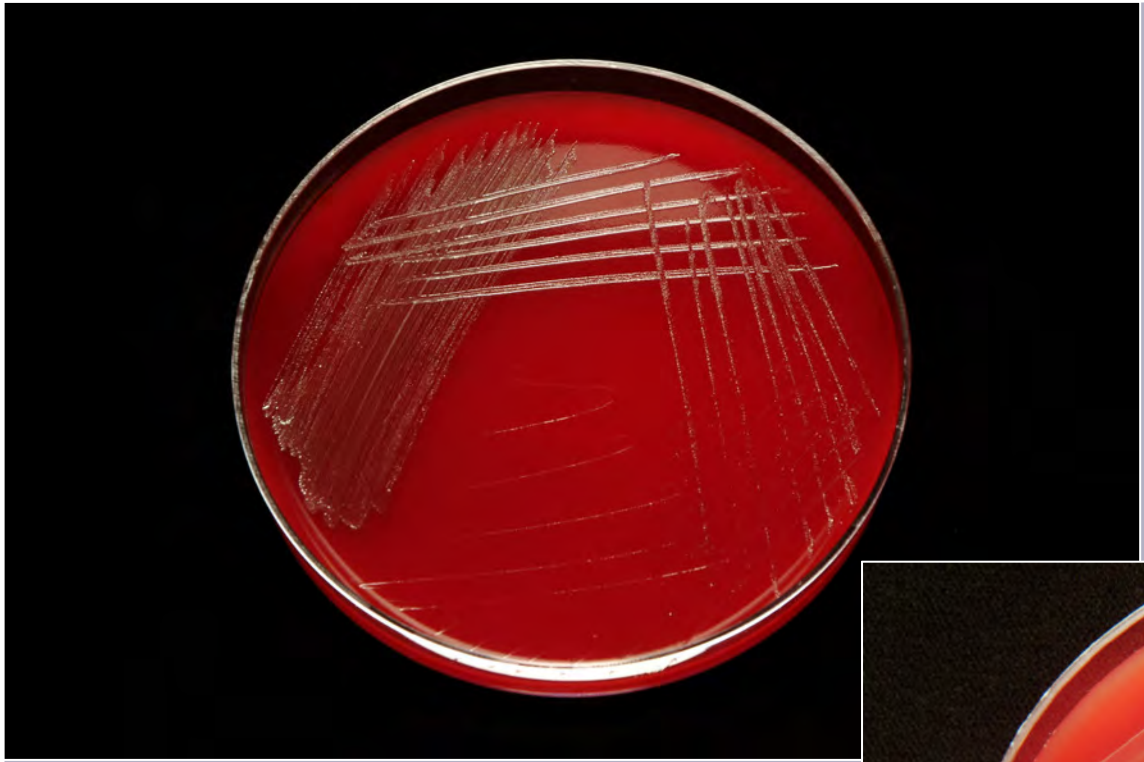
Always check morphology in broth (BHI) – DO NOT RELY ON GRAM STAIN FOR MORPHOLOGY – see next slide!



GPC’s in large clusters
prepared from HBA plate
gs x1000 -

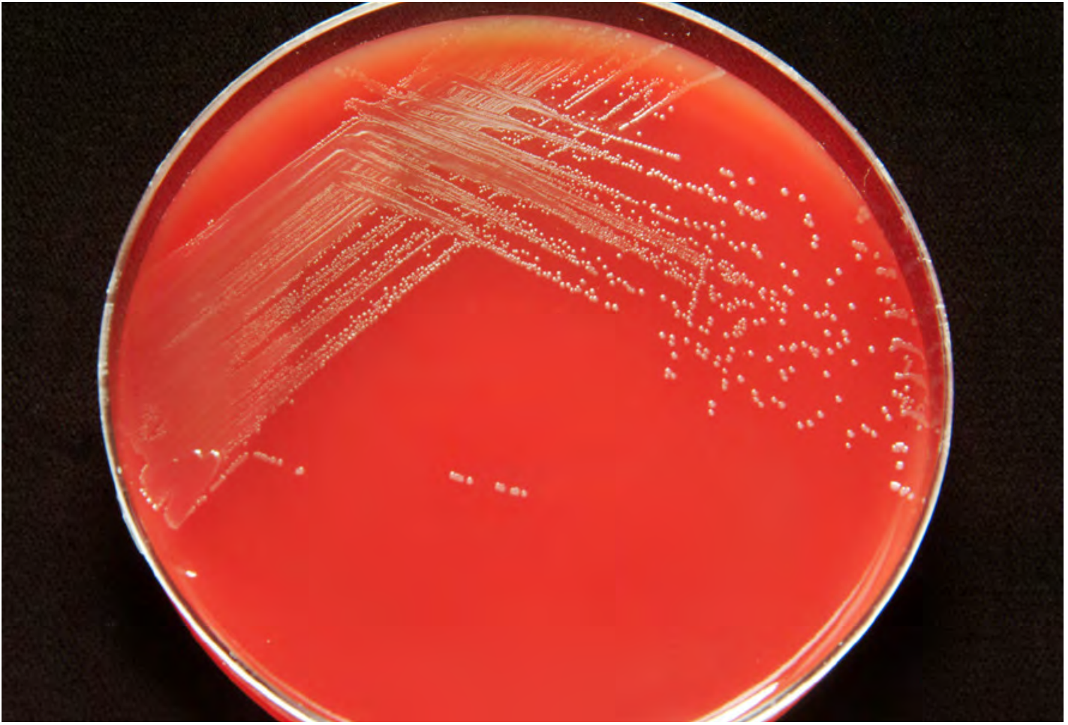
Same organism grown in

BHI broth for 24 hrs



Helcococcus kunzii

HBA @ 24hrs



Helcococcus kunzii

HBA @ 48hrs

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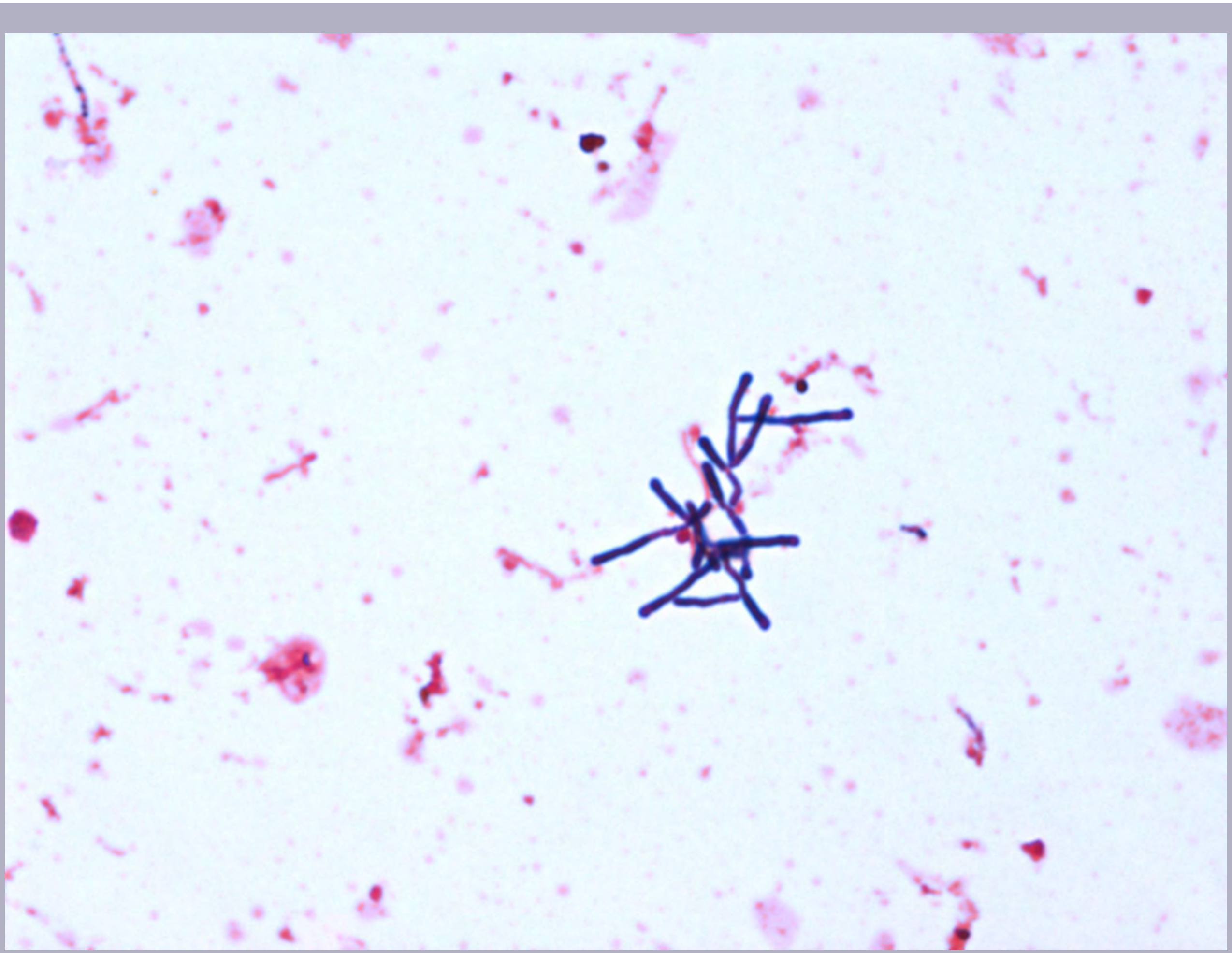
Difficult Gram Positive Rods



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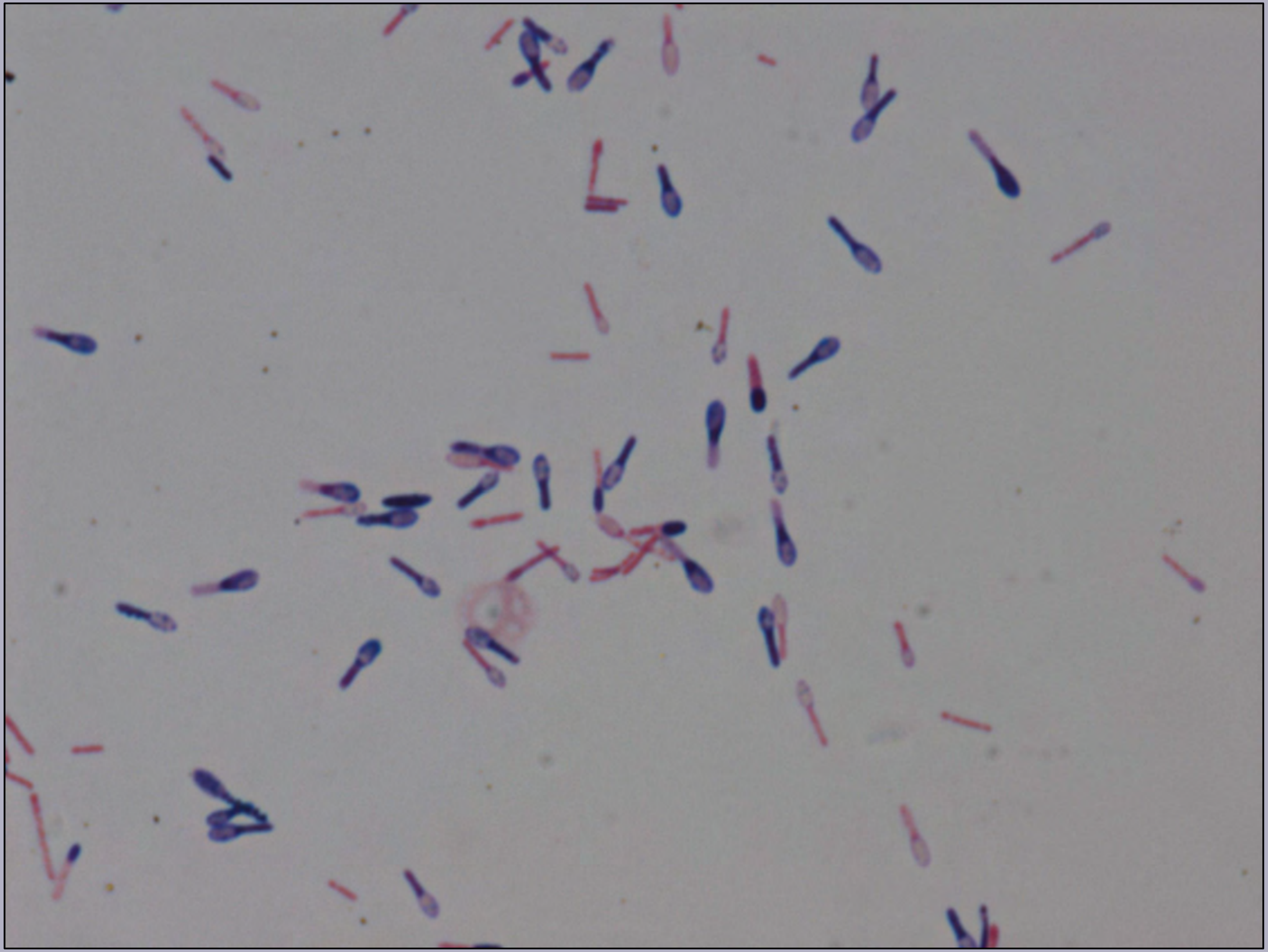
Anaerobes
Clostridium tertium

Features

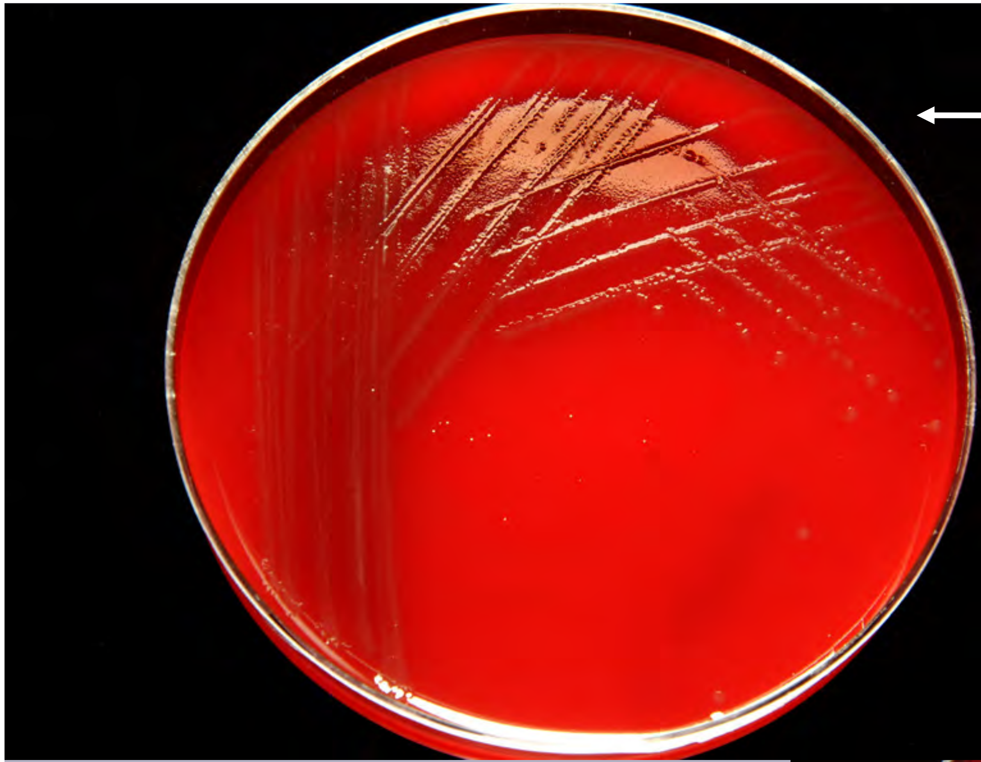
- Slender long Gram-positive rods with oval, terminal spores <u>anaerobically</u> but not aerobically

- Aerotolerant *Clostridia*: *C. tertium*, *C. histolyticum, C. Carnis*

- Mis-identified as *lactobacillus* if spores not detected or *Bacillus* species if growth conditions not examined.
  Key tests: *Bacillus* spp*:* cat +, sporulates aerobically
  C. tertium: cat -, sporulates anaerobically

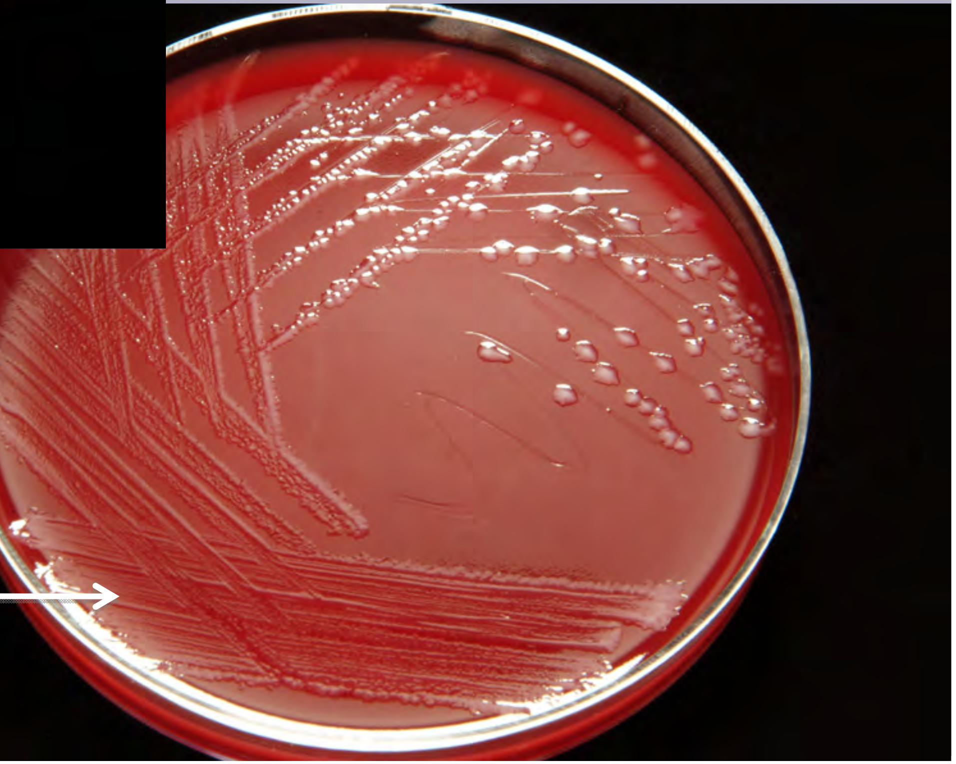


Clostridium tertium – gs x1000, terminal, oval spores



Clostridium tertium

HBA, CO_2



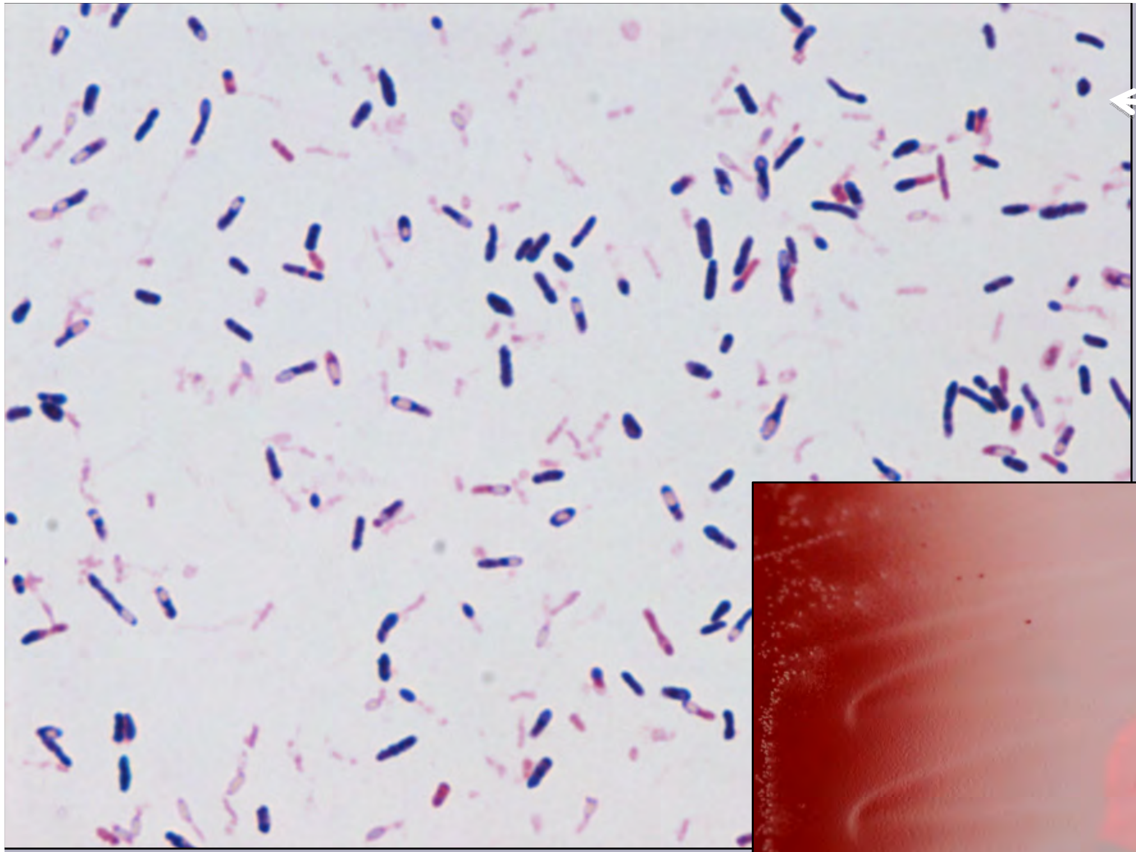
Clostridium tertium

HBA, ANO_2

Clostridium septicum

Key features

- Gram-positive rods – medium to large, some "lemon" shaped rod forms, staining often uneven
- Spores – oval, central to subterminal, distends cell
- Strict anaerobe, saccharolytic
- Metronidazole = S
- Catalase negative
- Highly motile – swarms over plate in 24hrs!
- Must distinguish from *C. sporogenes* – lipase, lactose, mannose, enzyme profile on Remel RapID ANA II or other commercial kit (PRO & PYR enzymes)

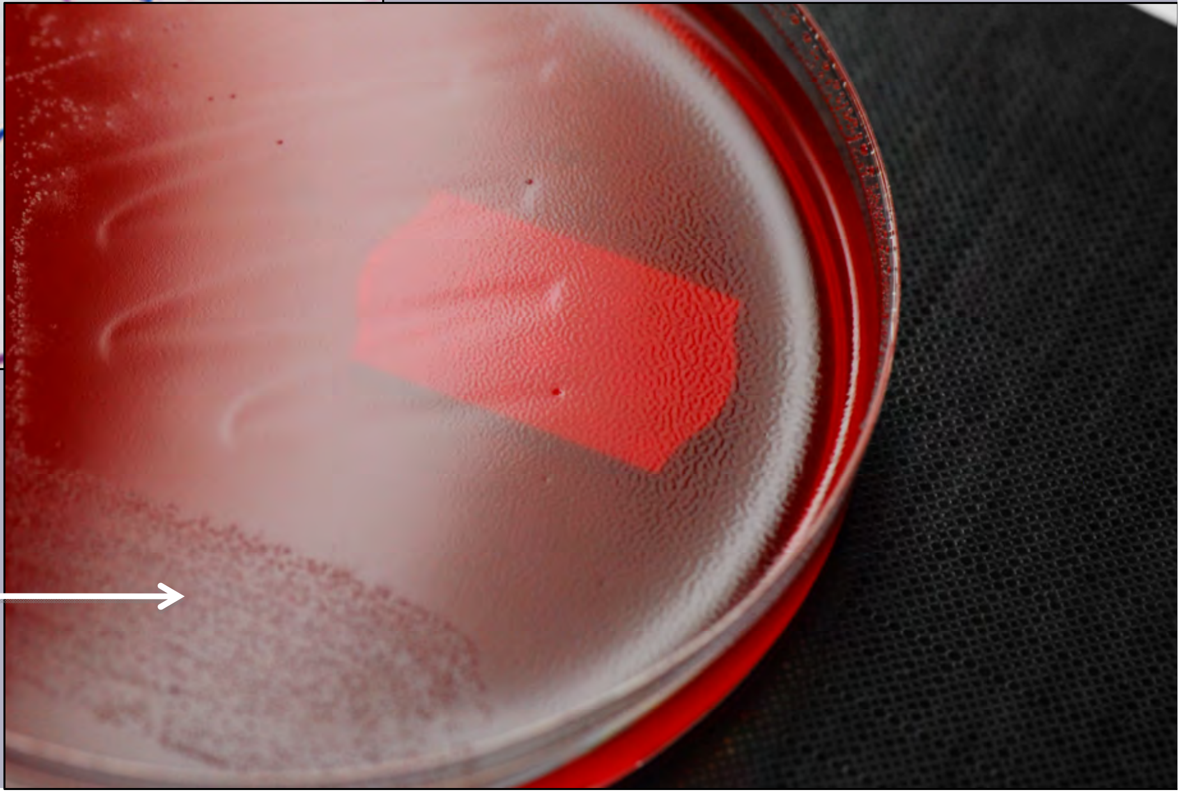


Clostridium septicum

gs x1000

central to subterminal spores,

lemon-shaped cells



Swarming over HBA

plate in 24 hrs

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Bifidobacterium longum

Special features

- Habitat intestinal tract of man and animals
- Anaerobic GPR - some species are aerotolerant
- Curved rods, rudimentary branching and "bifid" forms, dog bone, long club forms - Gram stain morphology is the key!
- Generally resistant to MTZ
- Fermentative
- >30 species – *B. dentium* (previously *B. eriksonii* only pathogen)

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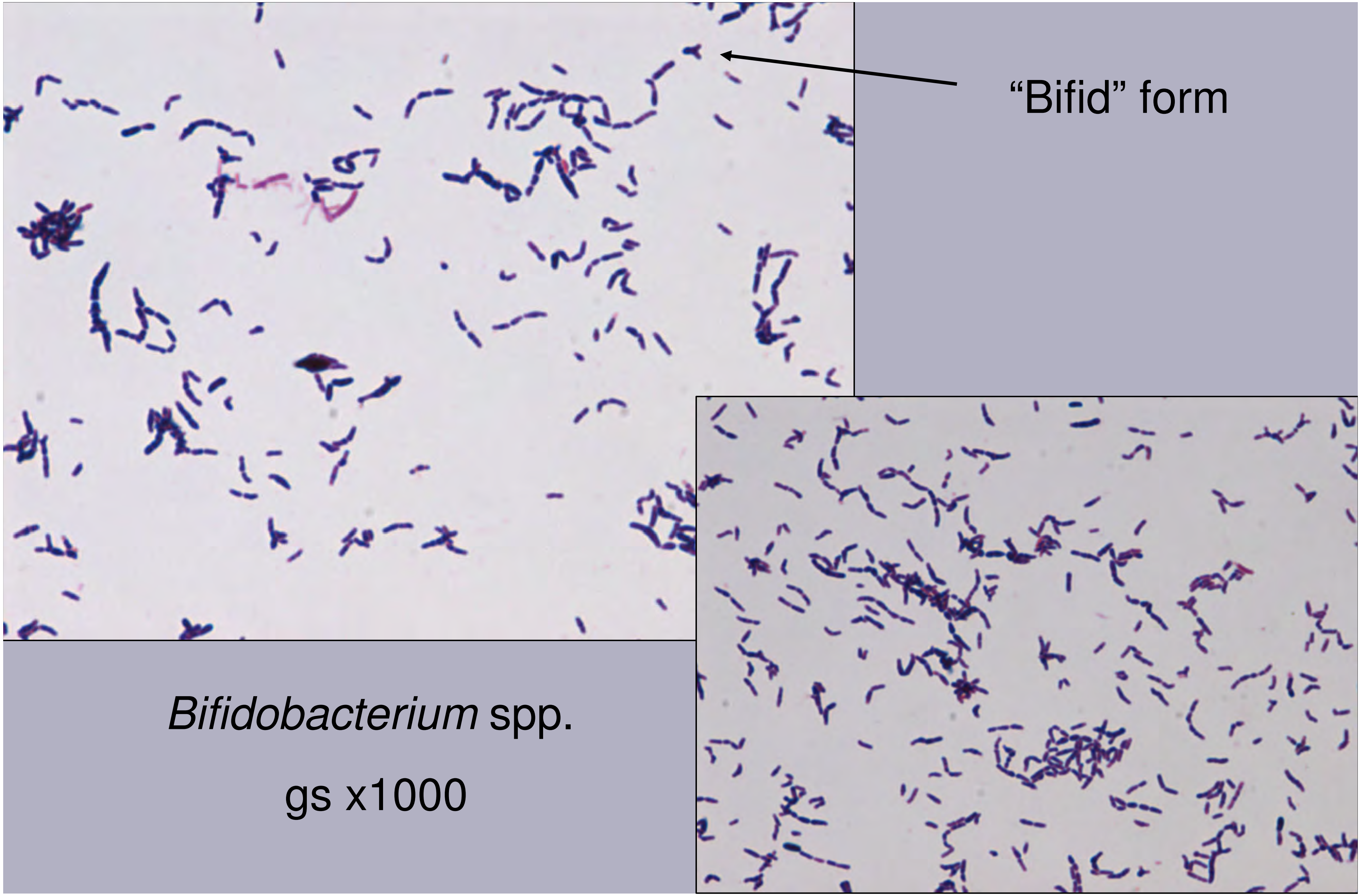
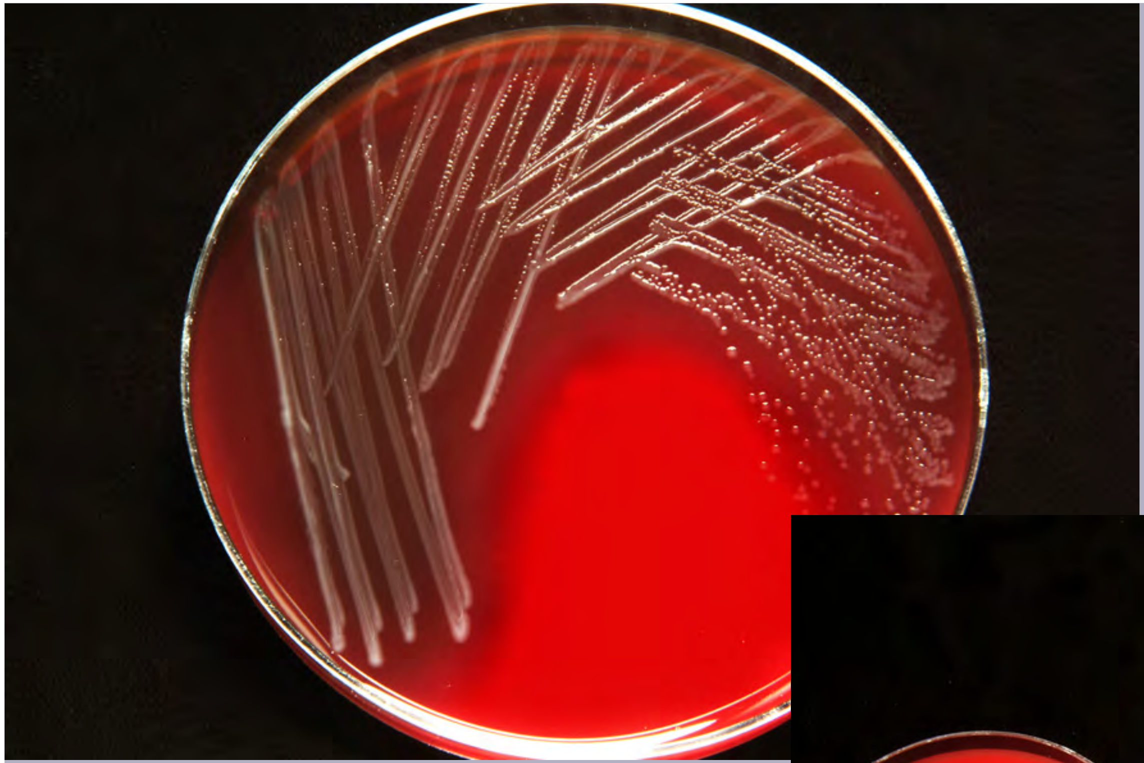


“Bifid” form

Bifidobacterium spp.

gs x1000



Bifidobacterium spp.

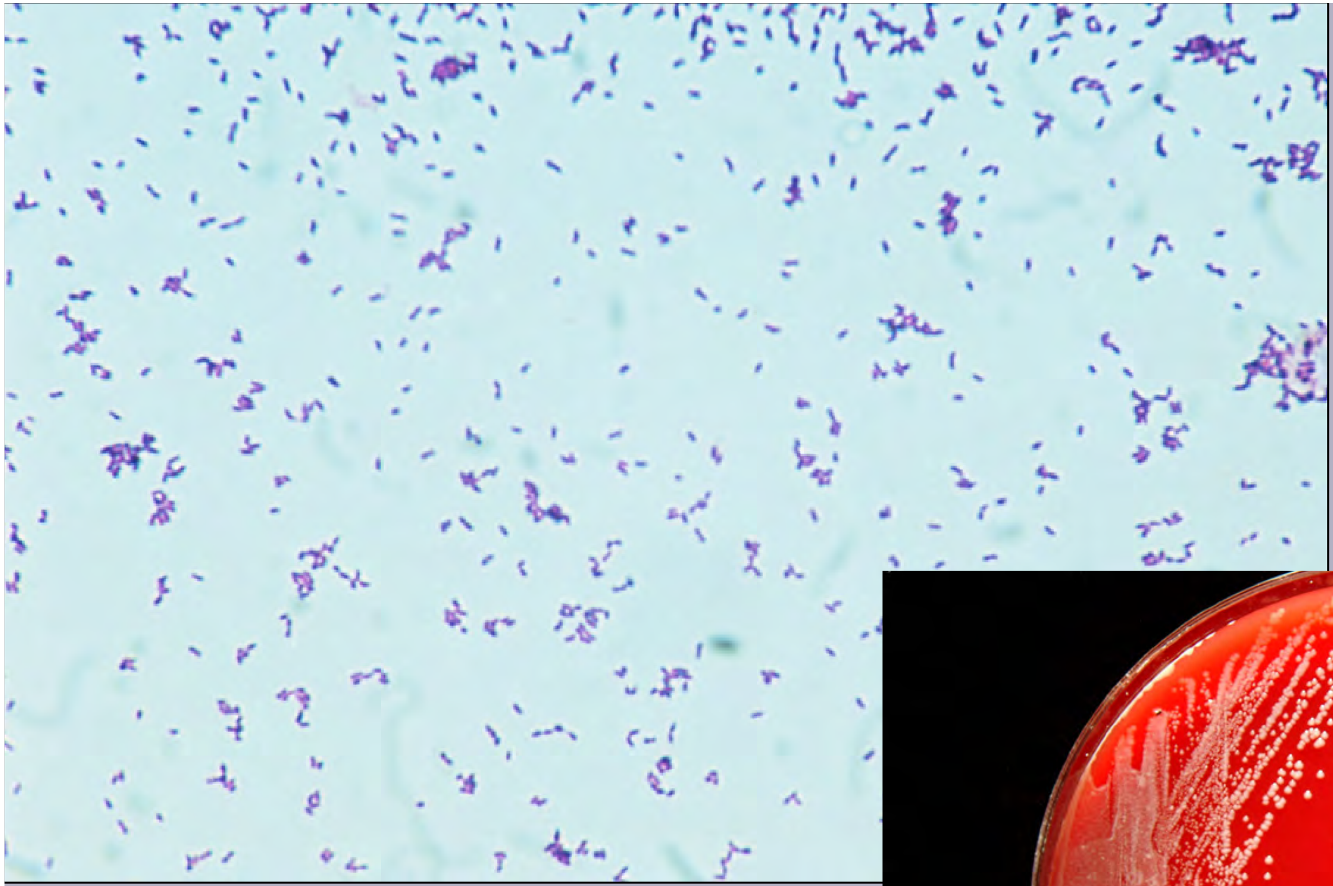
some species are
aerotolerant

Check growth conditions!

Dermabacter hominis

Key Features

- GPR – small coccoid to tear drop shaped coryneform rods
- Colonies white to grey, shiny, can be sticky
- Fermentative metabolism
- Identifies on API Coryne
- Unusual reactions: LDC +, ODC +, aesculin +



Dermabacter hominis

gs x1000

Dermabacter hominis

48hrs HBA

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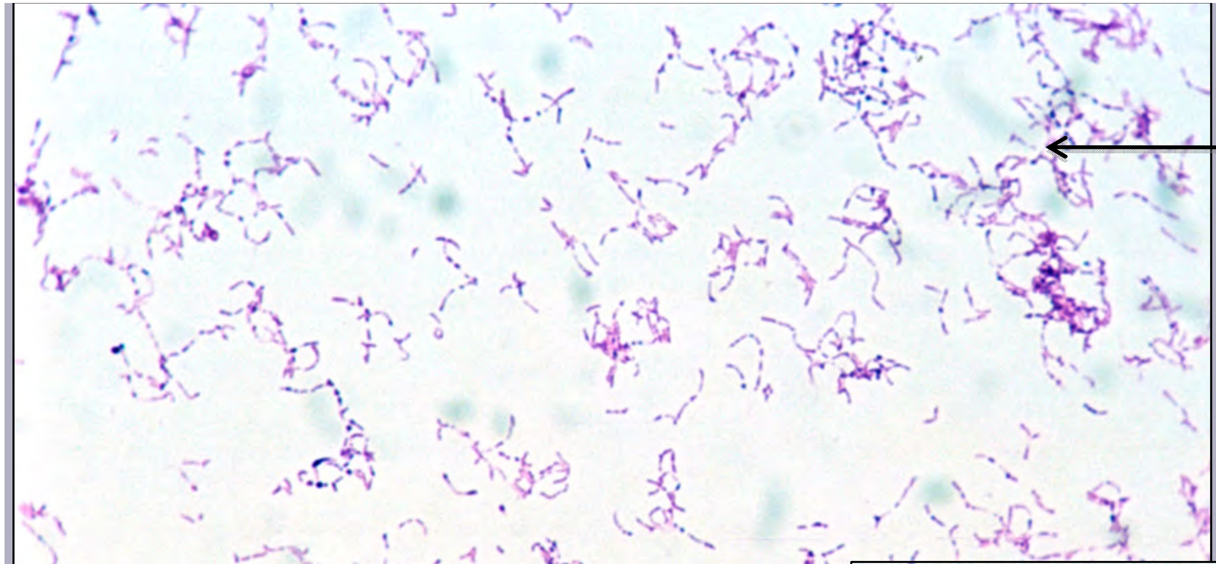
Actinomyces species

- GPR, coryneform, curved, irregular, some branching or extensive branching

  Note: Some newer *Actinomyces* spp. show very little branching and may appear coryneform
- Colony appearance varies with species
- Non-haemolytic, α-haemolytic or β-haemolytic
- Hints that an isolate may be an *Actinomyces* are:

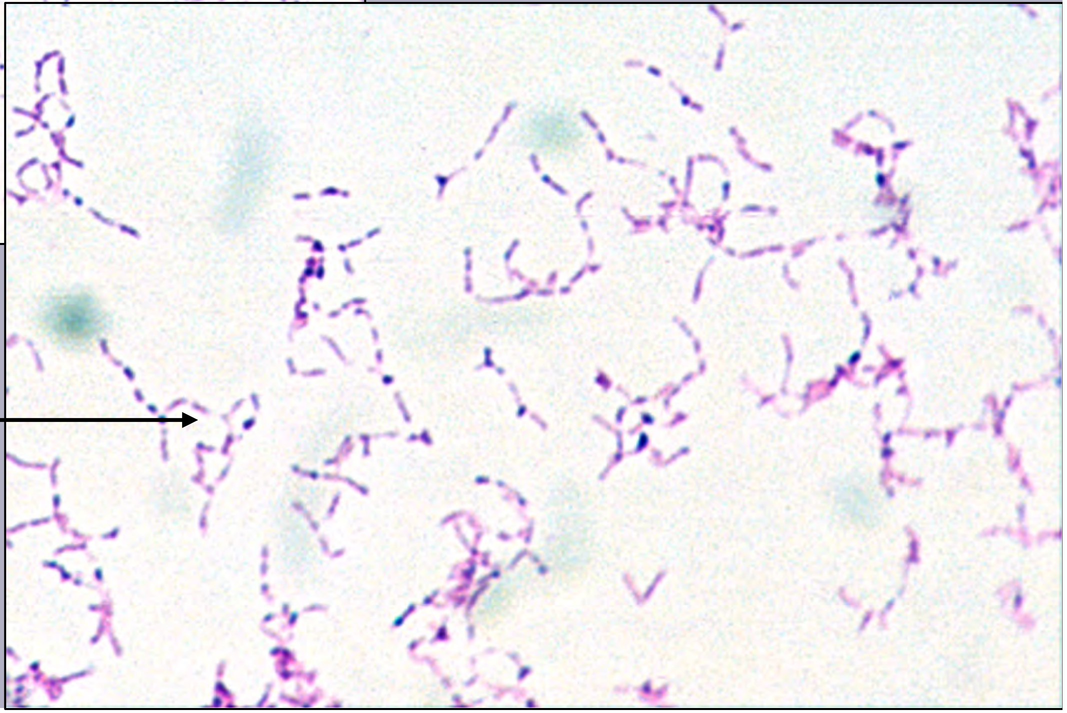
  -fermentation of xylose, lactose or aesculin hydrolysis

  -growth conditions
- API Coryne ID: *Microbacterium/Cellulomonas, G. vaginalis*



Actinomyces turicensis

gs x1000



Actinomyces turicensis

Close-up

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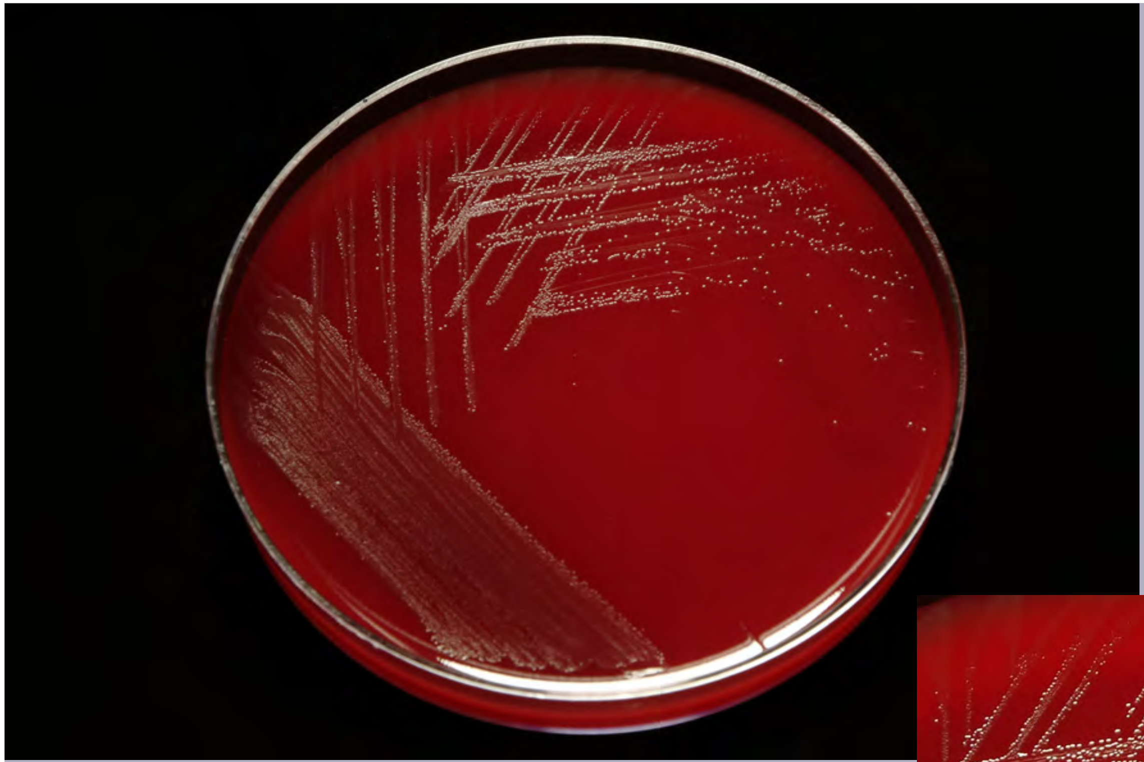
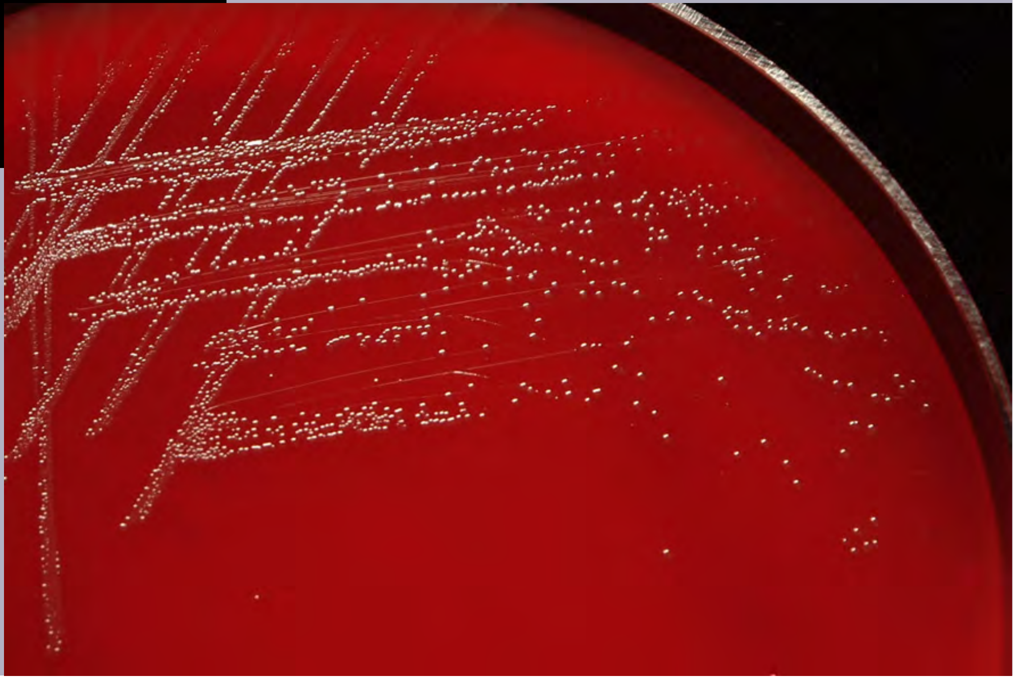
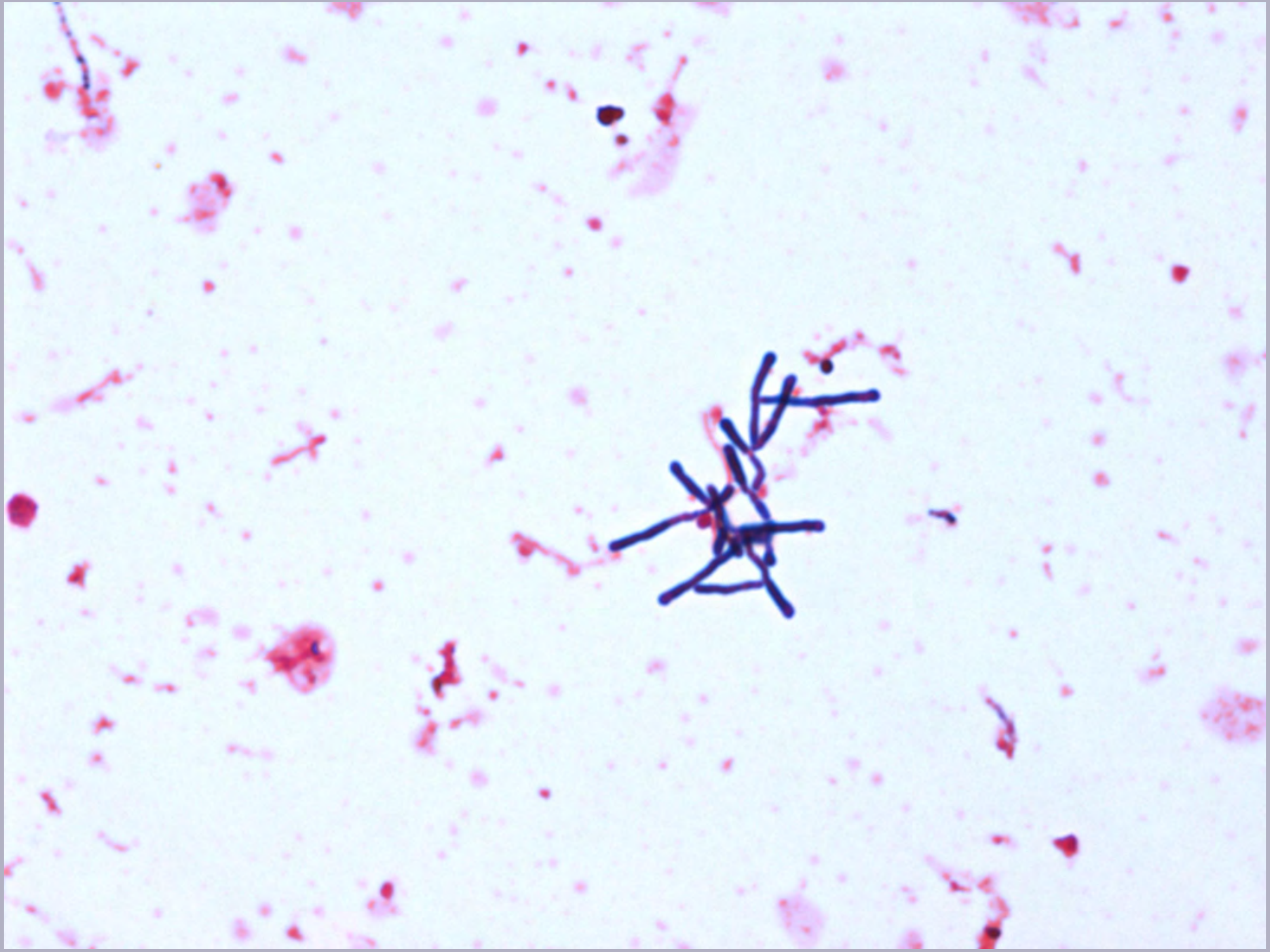
Actinomyces turicensis

24 & 48hr HBA







Actinomyces israelii – gs x1000 – microcolony grown in BHI broth

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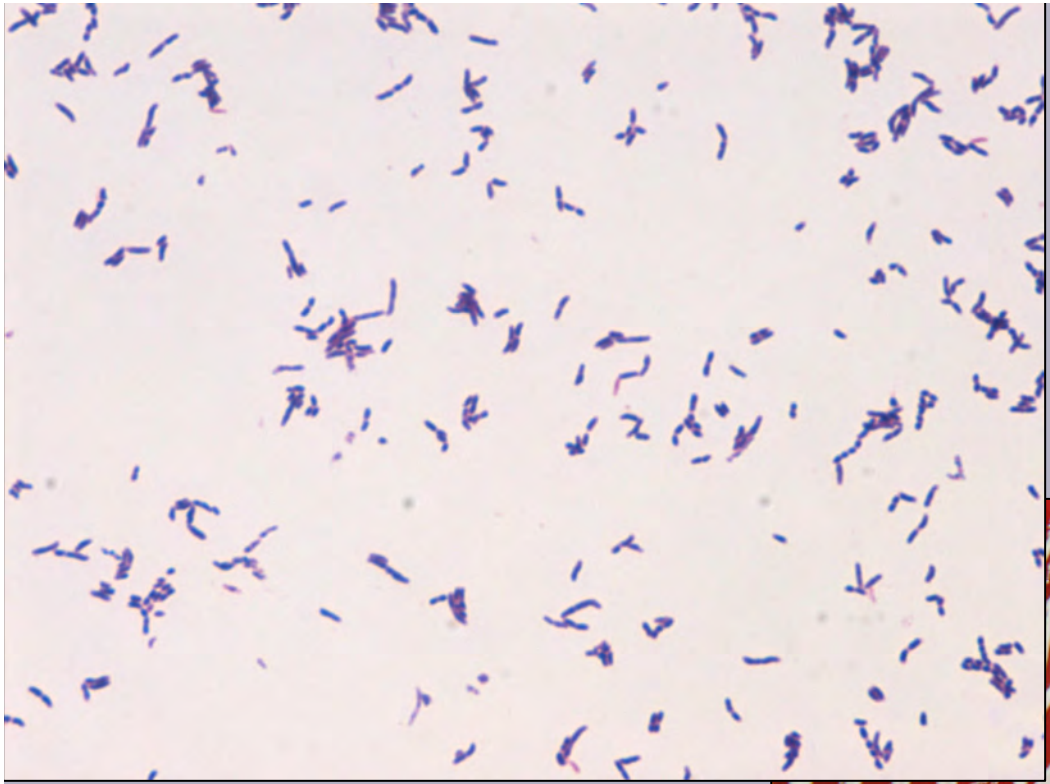
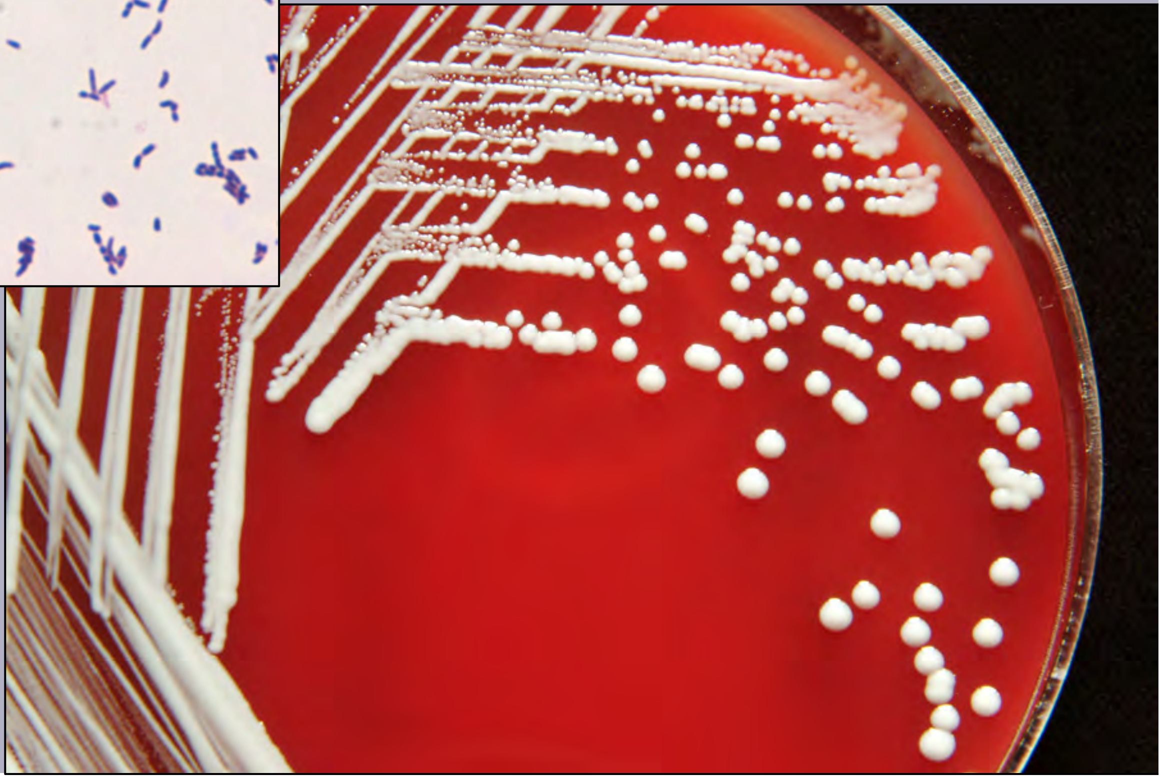


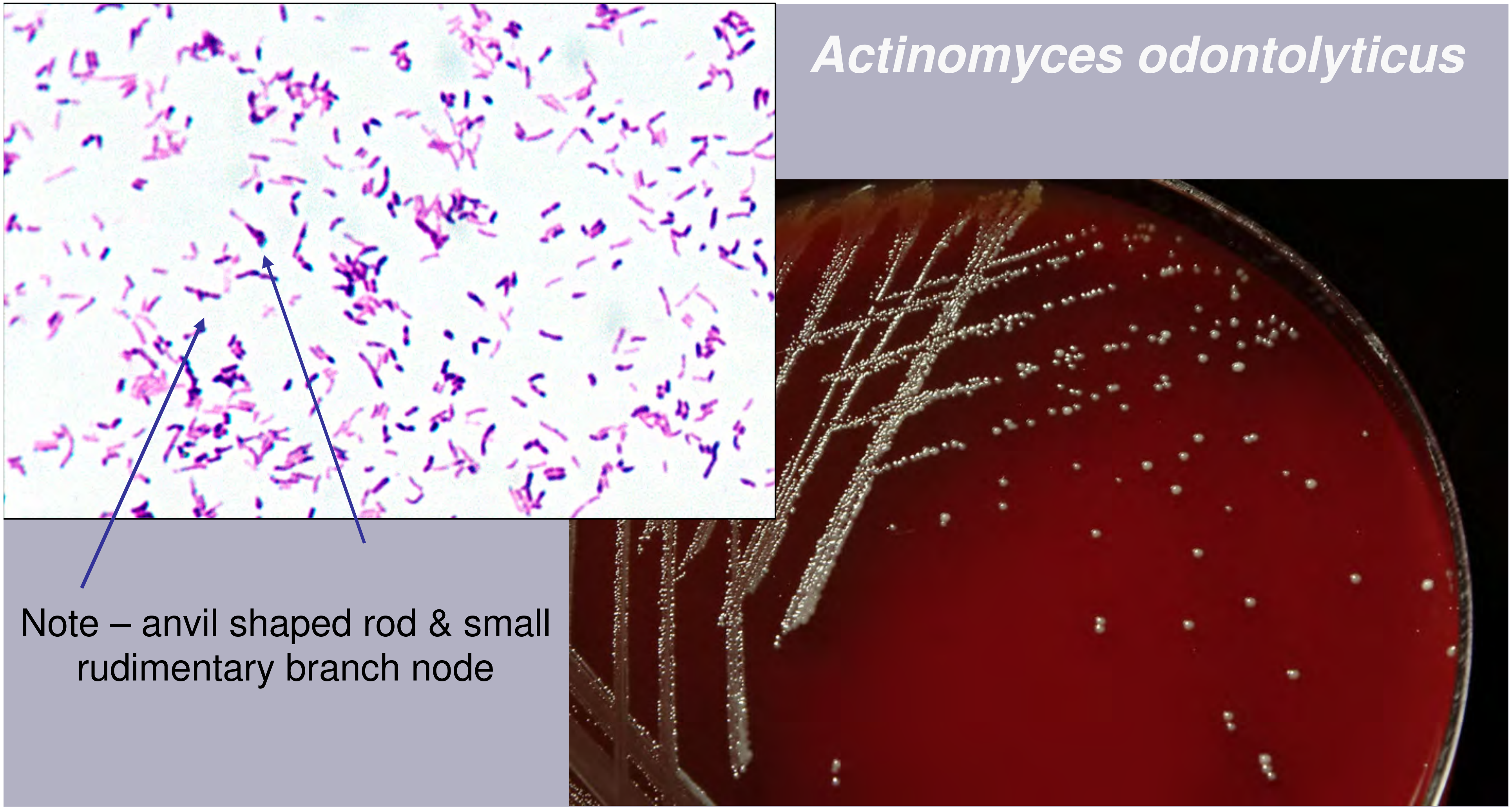
Actinomyces neuii spp. *neuii*

gs x1000



Note – irregular GPR, curved rods with tapered ends

Actinomyces spp. vary in their Gram stain appearance



Actinomyces odontolyticus

Note – anvil shaped rod & small rudimentary branch node

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Corynebacterium sundsvallense

Special features

- GPR - irregular pleomorphic rods
- Catalase +
- Fermentative
- Non-haemolytic
- Non-pigmented
- Non-motile
- No substrate or aerial mycelium
- Colonies resemble aerobic actinomycete but growth conditions are consistent with *Corynebacterium*

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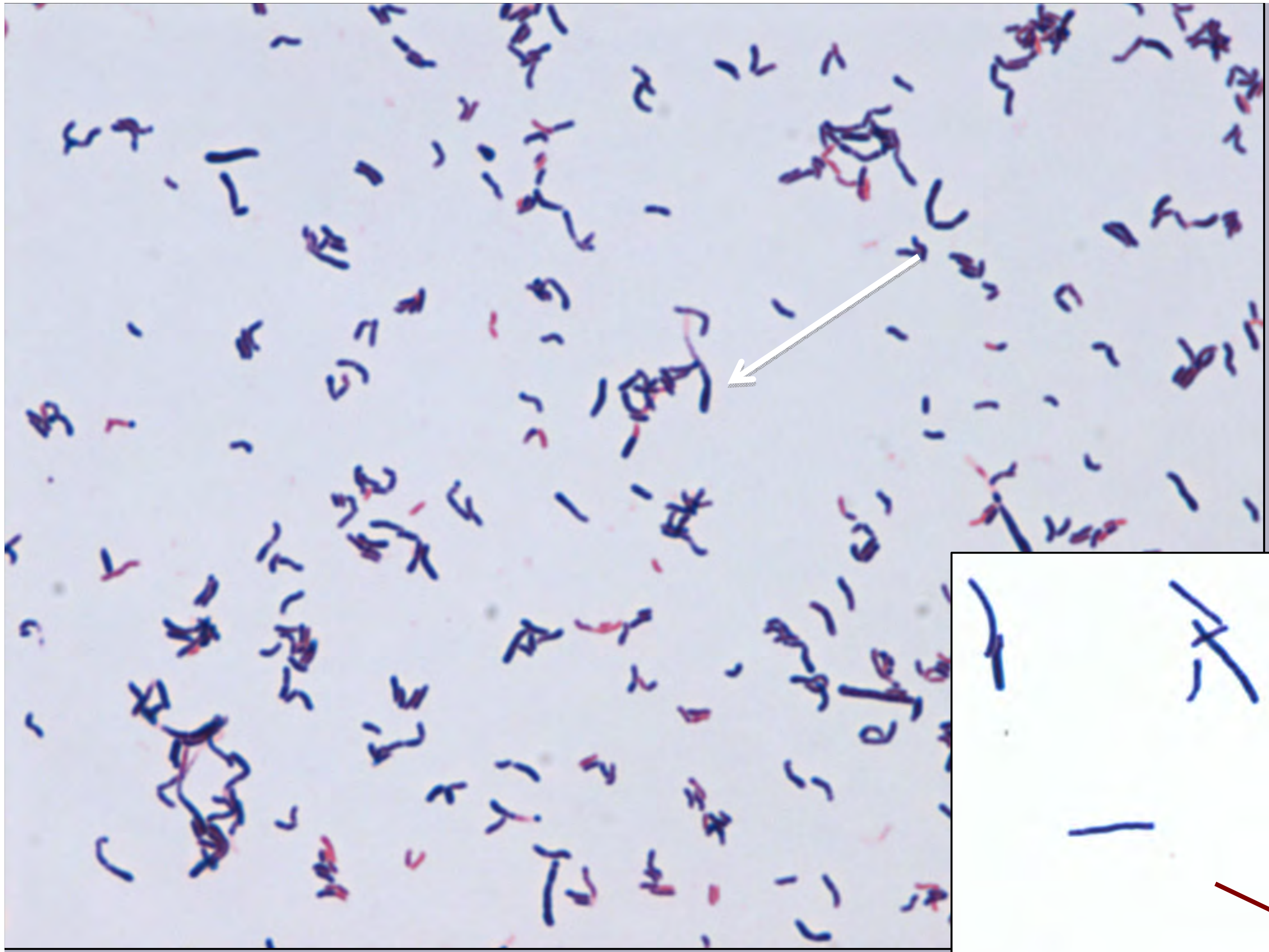

C. Sundsvallense
gs x1000
showing swellings
at end of rods

C. Matruchotii
gs x1000 - whip handles

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***Corynebacterium sundsvallense* – HBA, 72hrs**

C. durum, C. matruchotii & *Rothia dentocariosa* share similar characteristics

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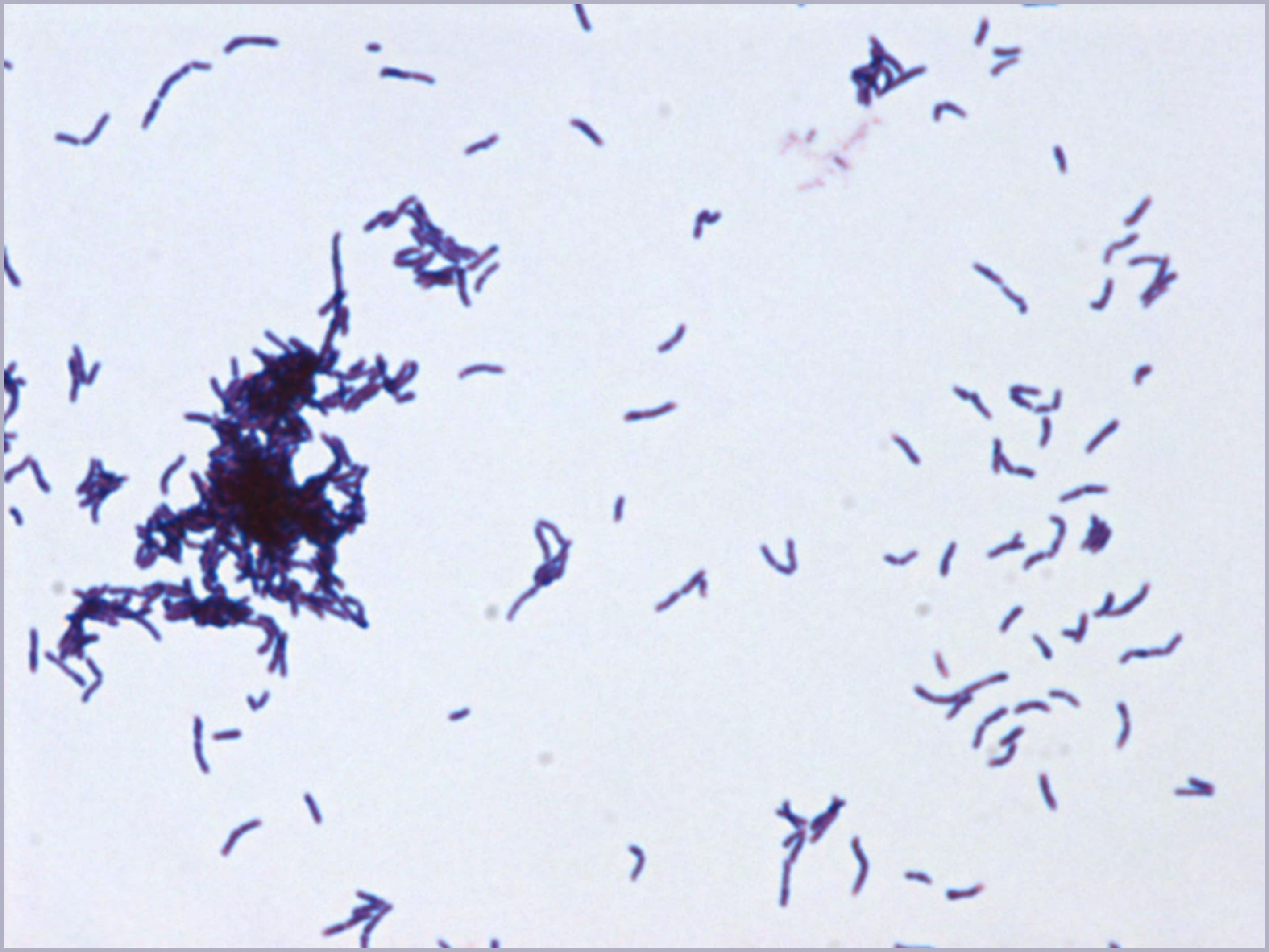
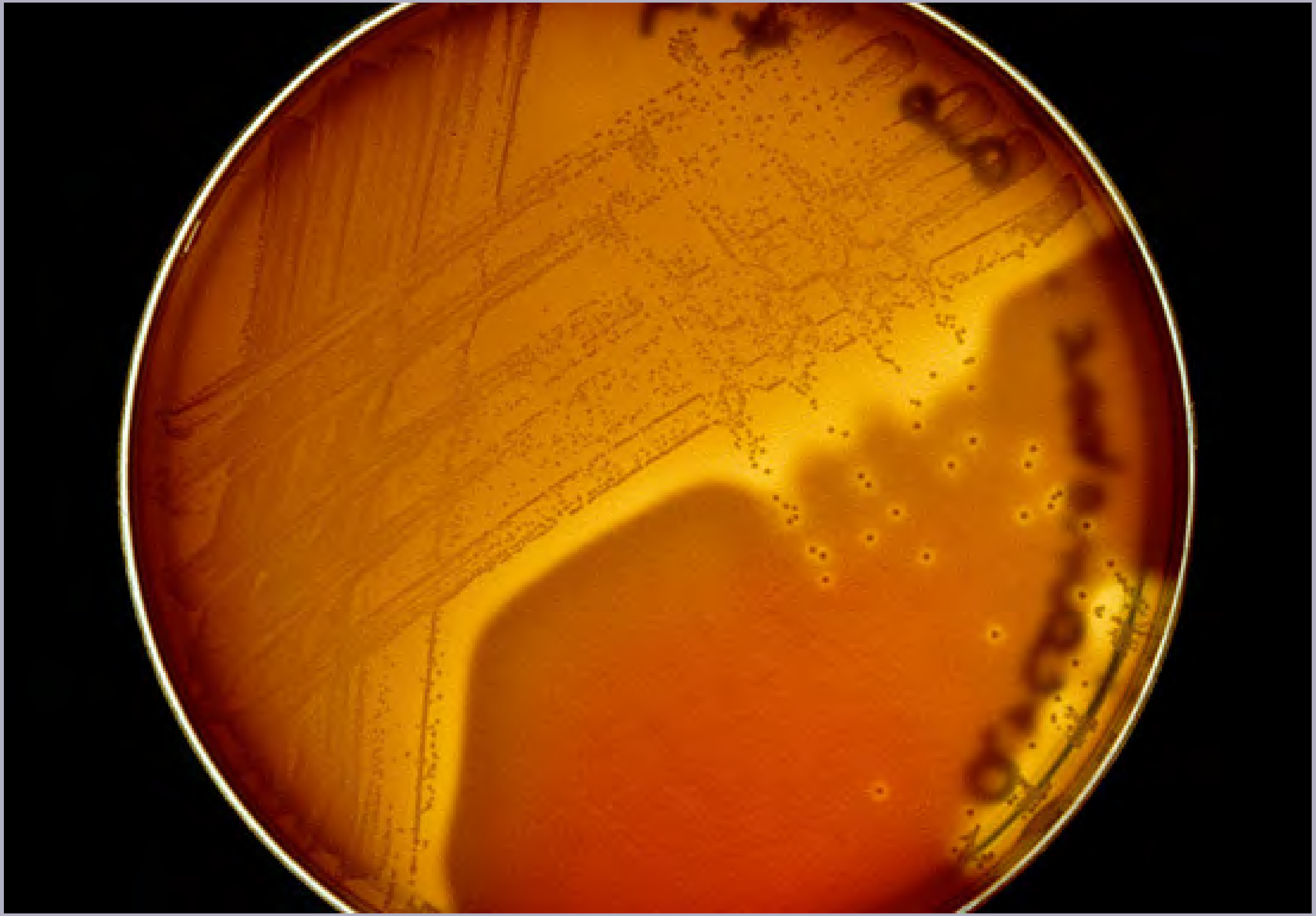
Arcanobacterium haemolyticum

Key features

- Gram-positive coryneform & irregular, curved rods
- Colonies 0.5mm smooth, dry, whitish colonies at 24hrs
- β-haemolytic colonies best observed @ 48hrs
- 3 medically relevant species: *A. haemolyticum, A. pyogenes, A. bernardiae*
- Identifies well on API Coryne
- key tests to differentiate species - gelatin, xylose, glycogen



A. haemolyticum – gs x1000 irregular gram positive rods



Arcanobacterium haemolyticum - showing β-haemolysis at 48hrs

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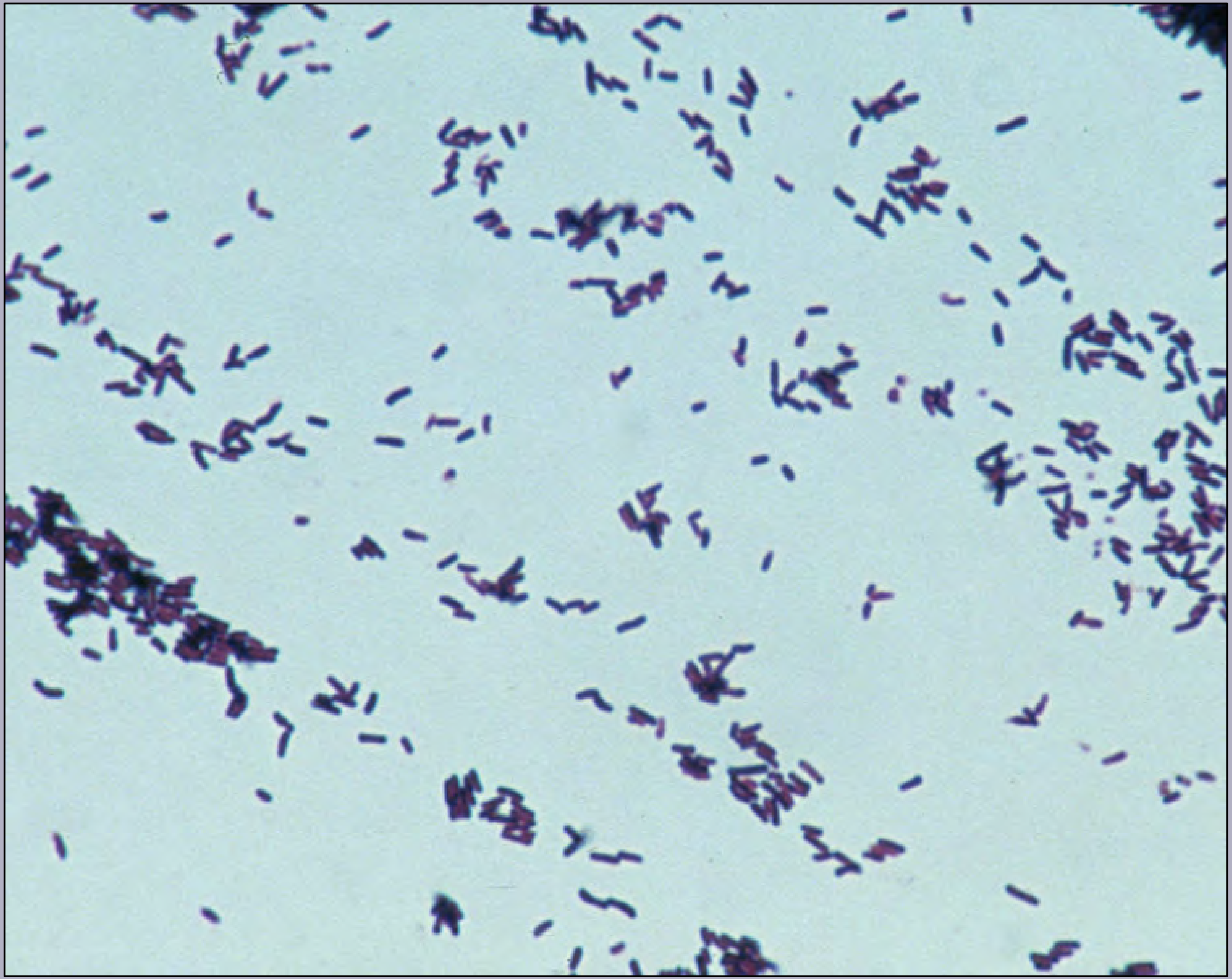
Microbacterium oxydans

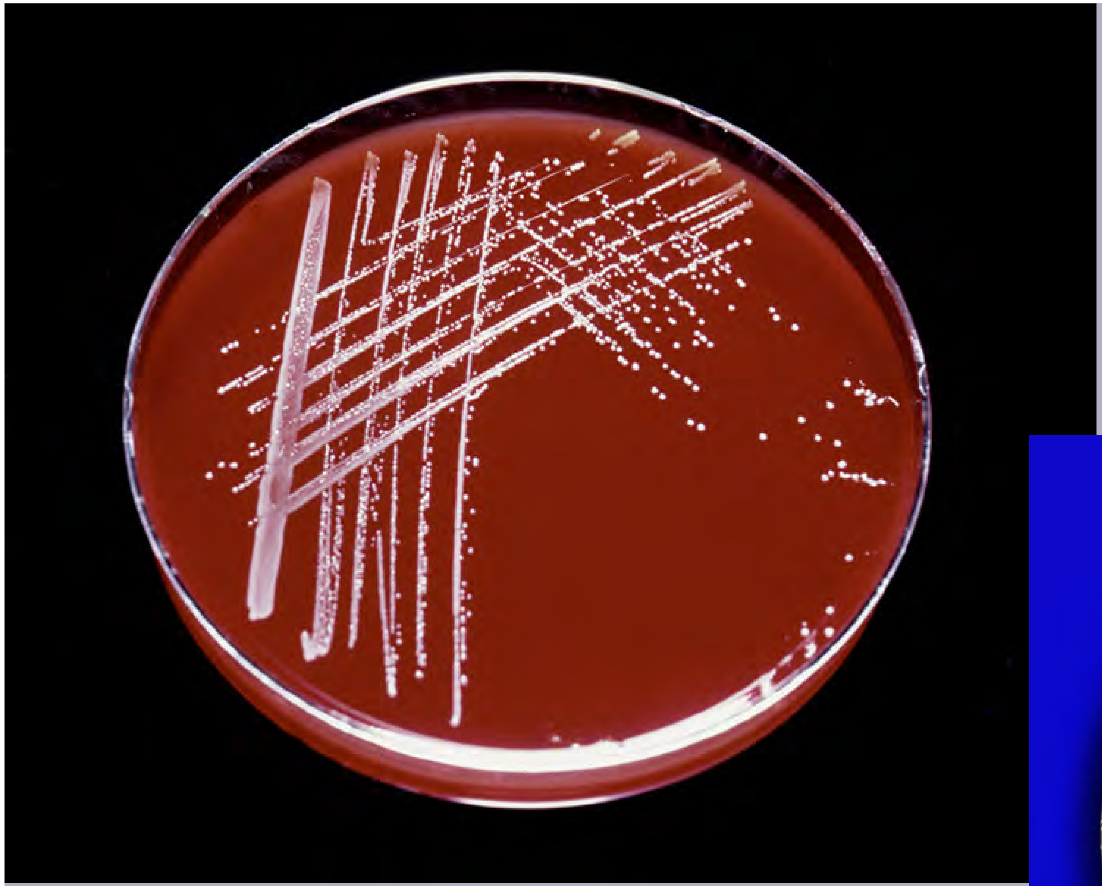
Key features

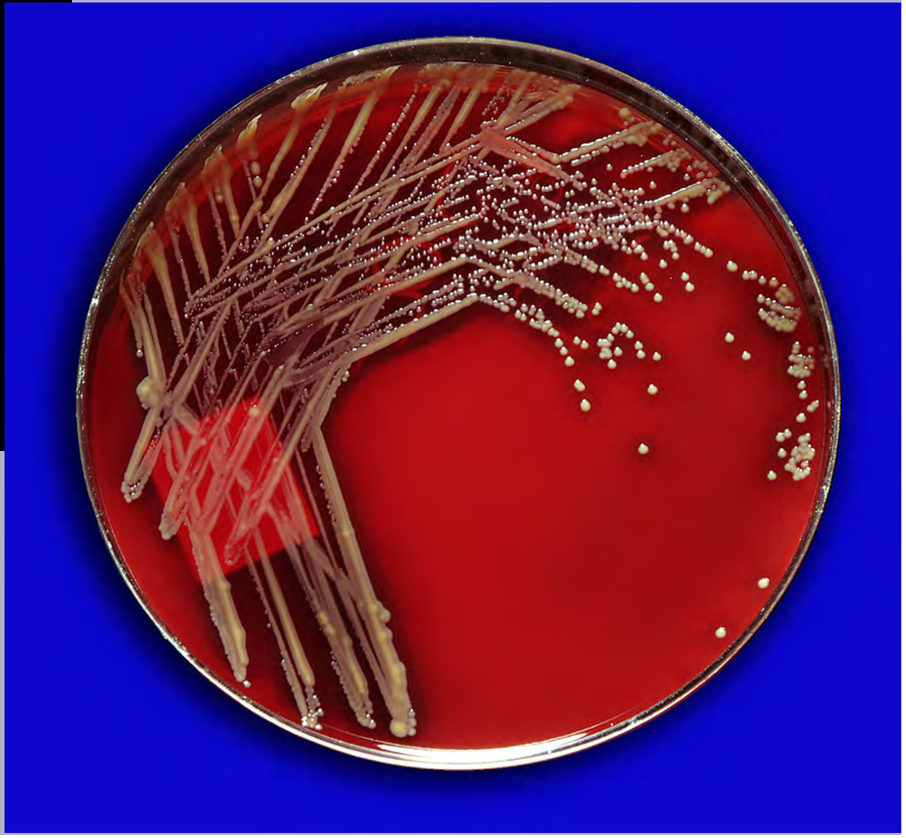
- GPR regular or pleomorphic, curved
- Colonies moist white-cream or yellow with age
- Motile or non-motile
- Oxidative metabolism but genus is a mixture of both oxidative & fermentative species – makes ID confusing
- API Coryne usually gives a clue (mannitol +, Aesc +) but check growth conditions



Microbacterium spp. - gs x1000

Microbacterium spp.
HBA @ 48hrs





pigment varies from white,
grey to yellow

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Aerobic Actinomycetes
Before we get started -
Questions you need to answer

- Is the isolate a strict aerobe (oxidative) or facultative anaerobe (fermentative)
- Is the organism a GP branching rod or irregular non-branching rods
- Do the rods stain poorly
- Is there a substrate mycelium <u>and</u> aerial hyphae
- Is there substrate mycelium <u>only</u>
- Is there <u>neither</u> substrate *nor* aerial hyphae
- Is the isolate acid fast by ZN or modified ZN

First - Why are some bugs acid fast?

- Cells that are acid fast contain mycolic acids (large group of long chain fatty acids of varying length)
- The amount of mycolic acids will confer varying degrees of resistance to chemicals, permeability & acid fastness (stain binds to cell wall mycolic acids)

Mycolic acid chain length (carbon number)

0 20 30 40 50 60 70 80 90

Coryne Dietzia Nocardia Tsukamurella Mycobacterium

Rhodococcus

Gordonia

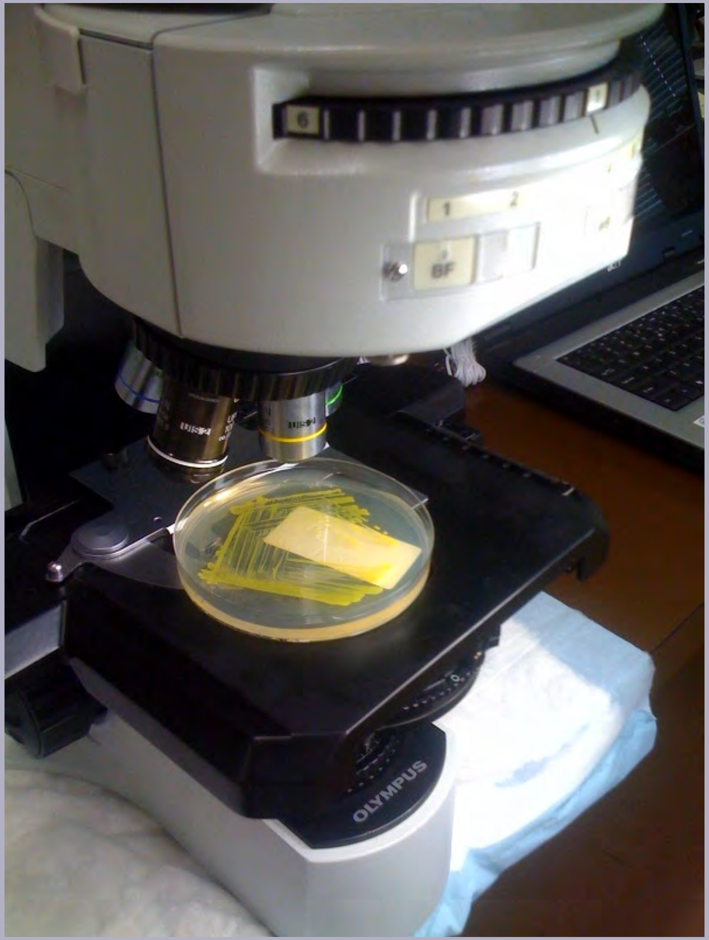
Secondly – Aerial hyphae & substrate mycelium

Are you sure you know what

you’re looking for and

how to go about it?

Looking for Substrate and Aerial Hyphae?

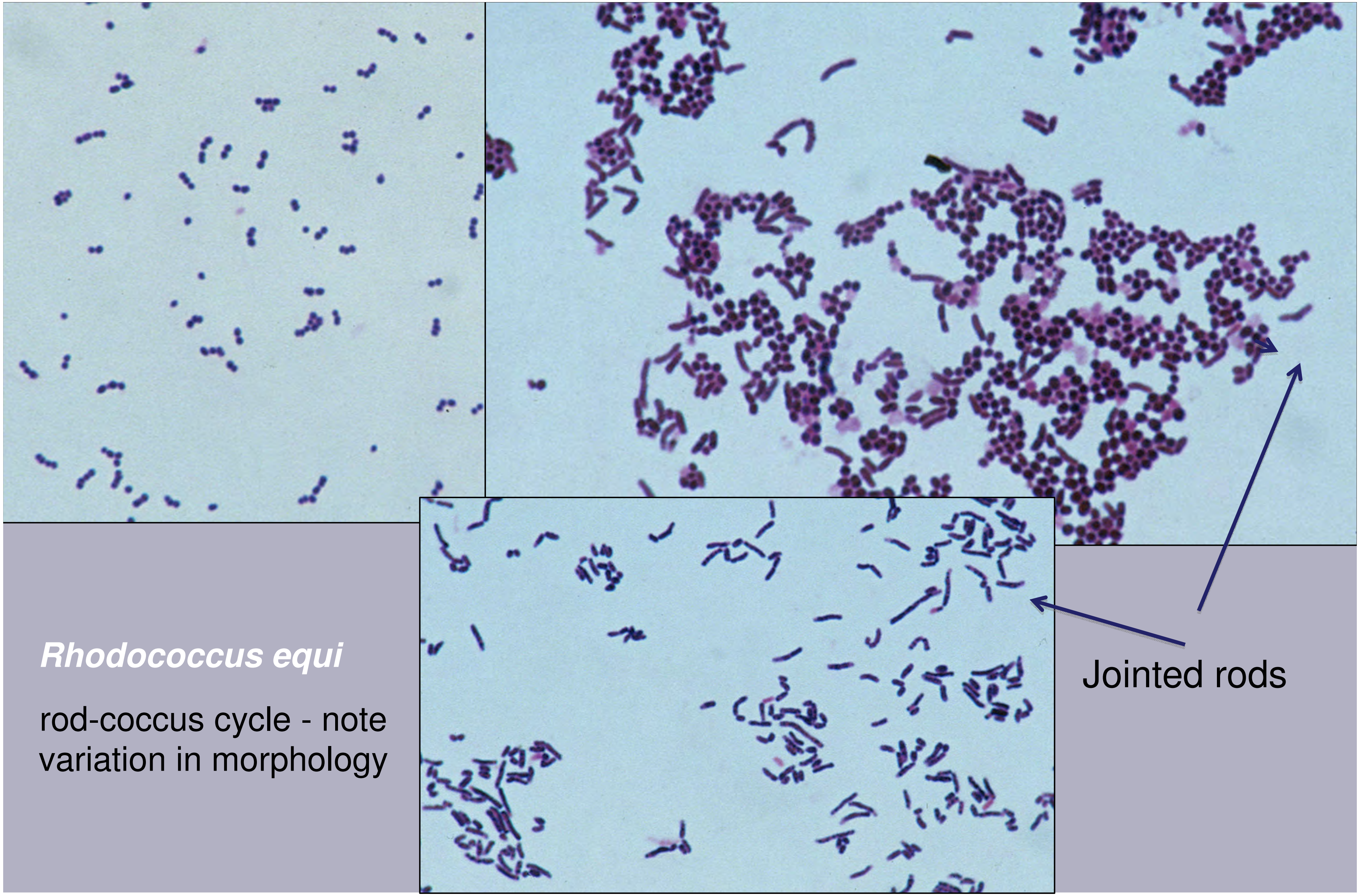


- Place culture plate on stage - must use clear medium e.g. NA, MHA, SAB
- Drop condenser to increase contrast
- Start with x10 objective to locate individual colonies
- Change to x20 objective to examine structures more closely, BUT don’t end up in the agar!

Rhodococcus equi

Special features

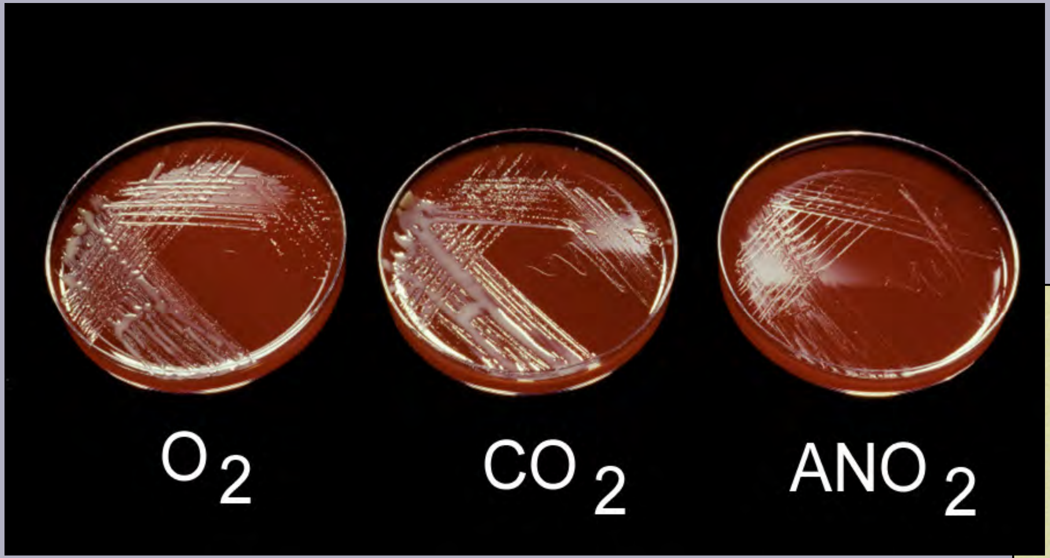
- GP rods – coryneform, jointed or rudimentary branching – morphology varies according to progression of the rod-coccus cycle
- Non-motile
- Oxidative metabolism
- Colonies – translucent mucoid, salmon pink with age
- Marked rod-coccus cycle but no substrate or aerial hyphae
- May be partially acid fast (Modified ZN)
- Identifies on API Coryne
- Note: mucoid pink colonies could be *Roseomonas* spp. or other pink oxidative GNR – check gram & do vancomycin or string test



Rhodococcus equi

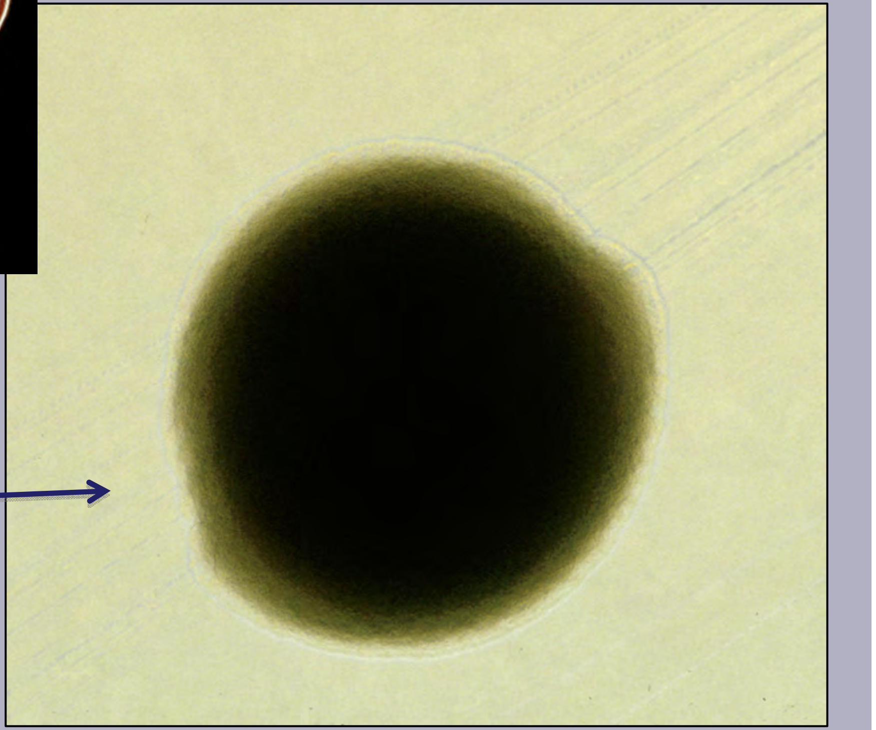
rod-coccus cycle - note variation in morphology

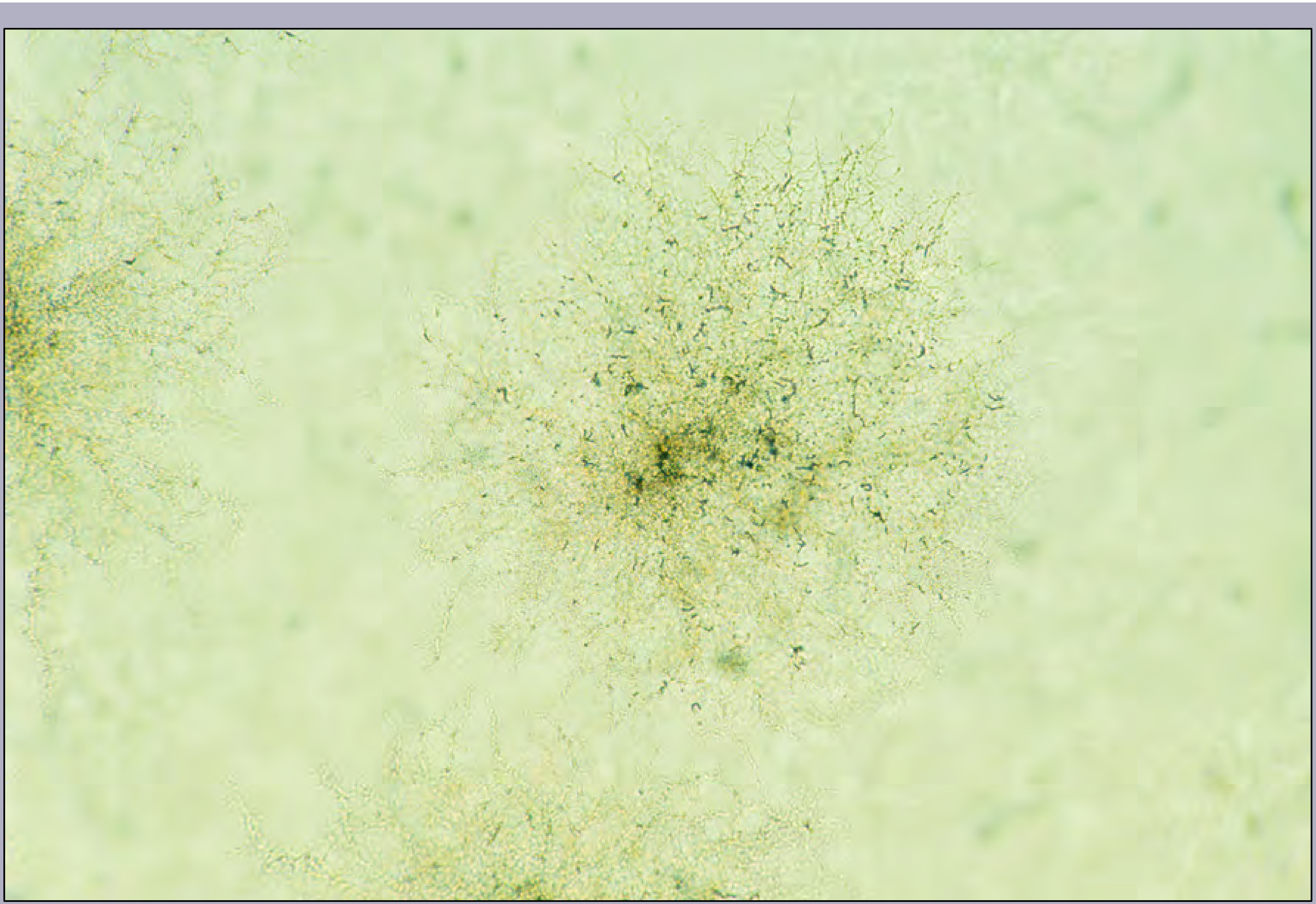
Rhococcus equi – showing oxidative growth pattern



No substrate or aerial hyphae

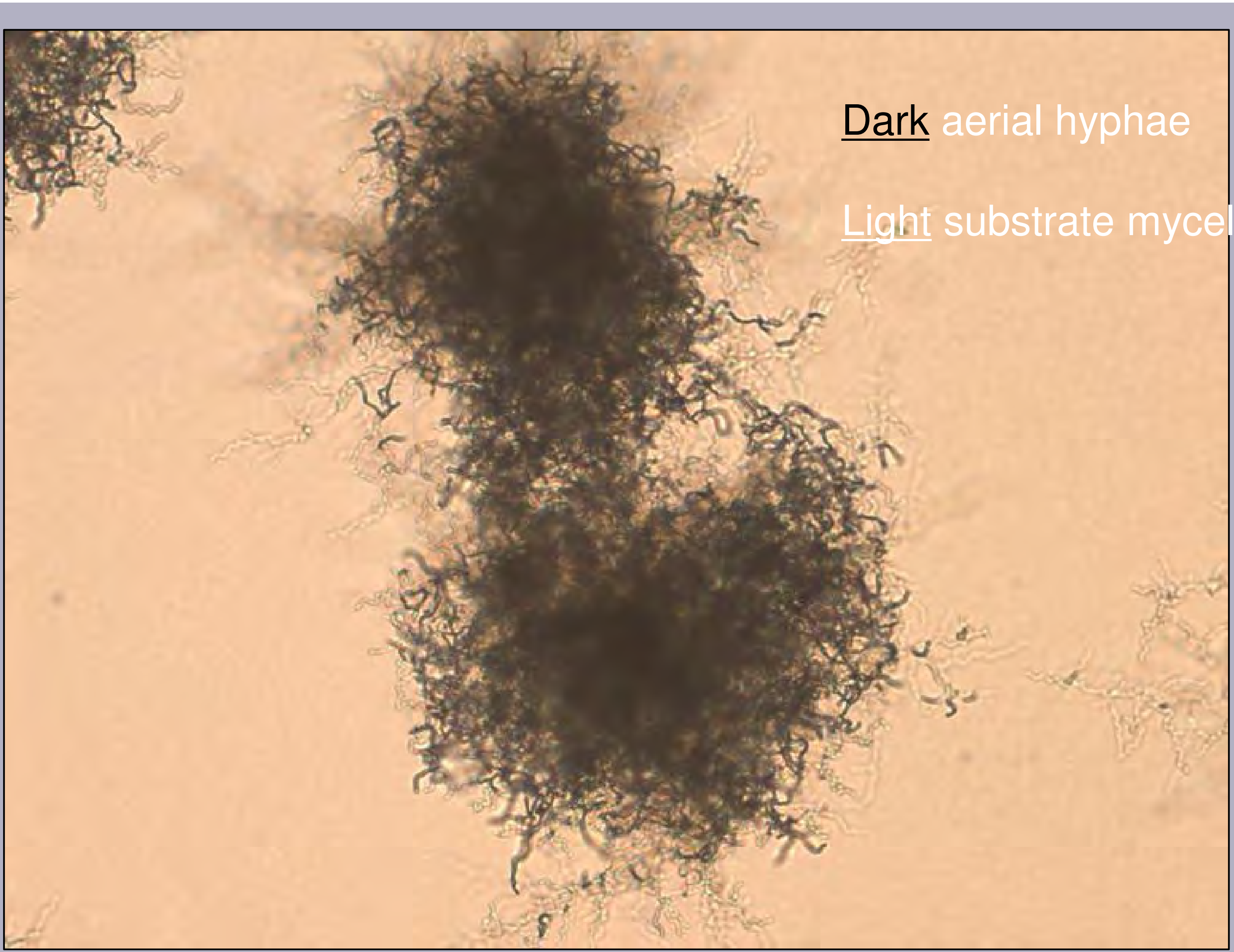
Compare this image to the next slide





***Nocardia* spp. – x400**

Early colony development showing substrate mycelium



Nocardia spp. NA x10 - note substrate & aerial hyphae

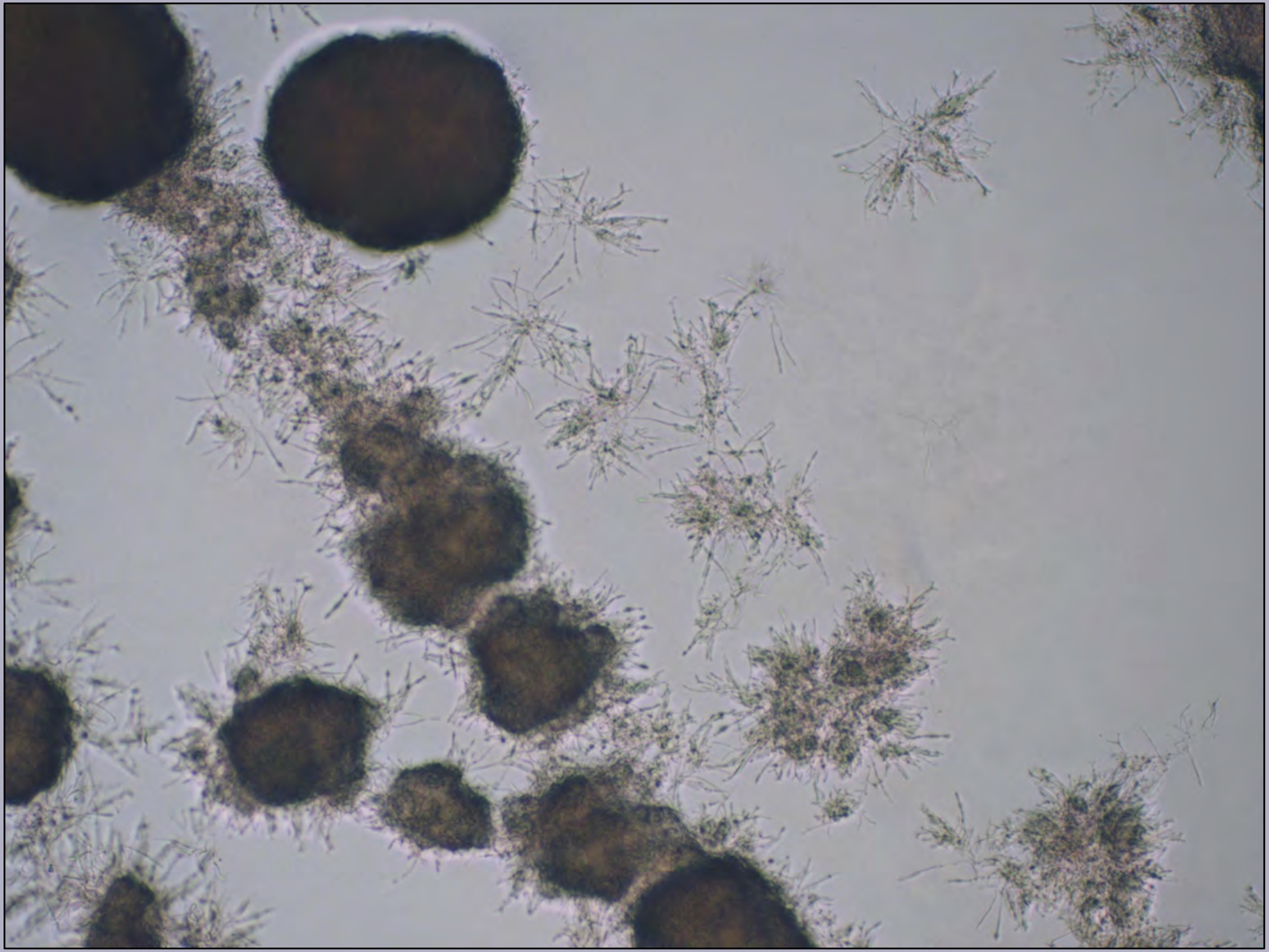
Important Note

Don’t be tempted to look for

substrate mycelium or

aerial hyphae too soon!

Check out the next slide …….

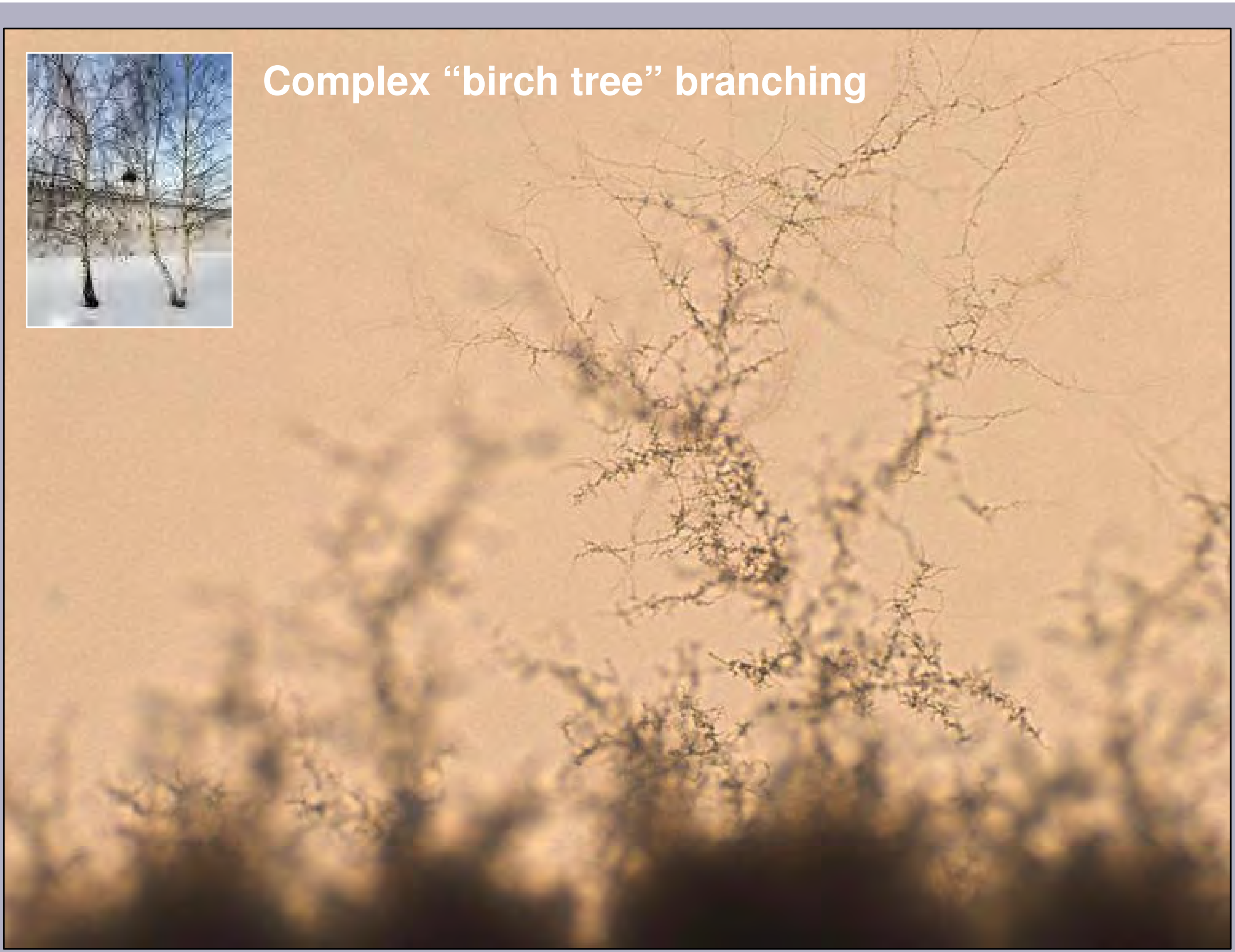


Corynebacterium sundsvallense – MHA, 4 days, x100

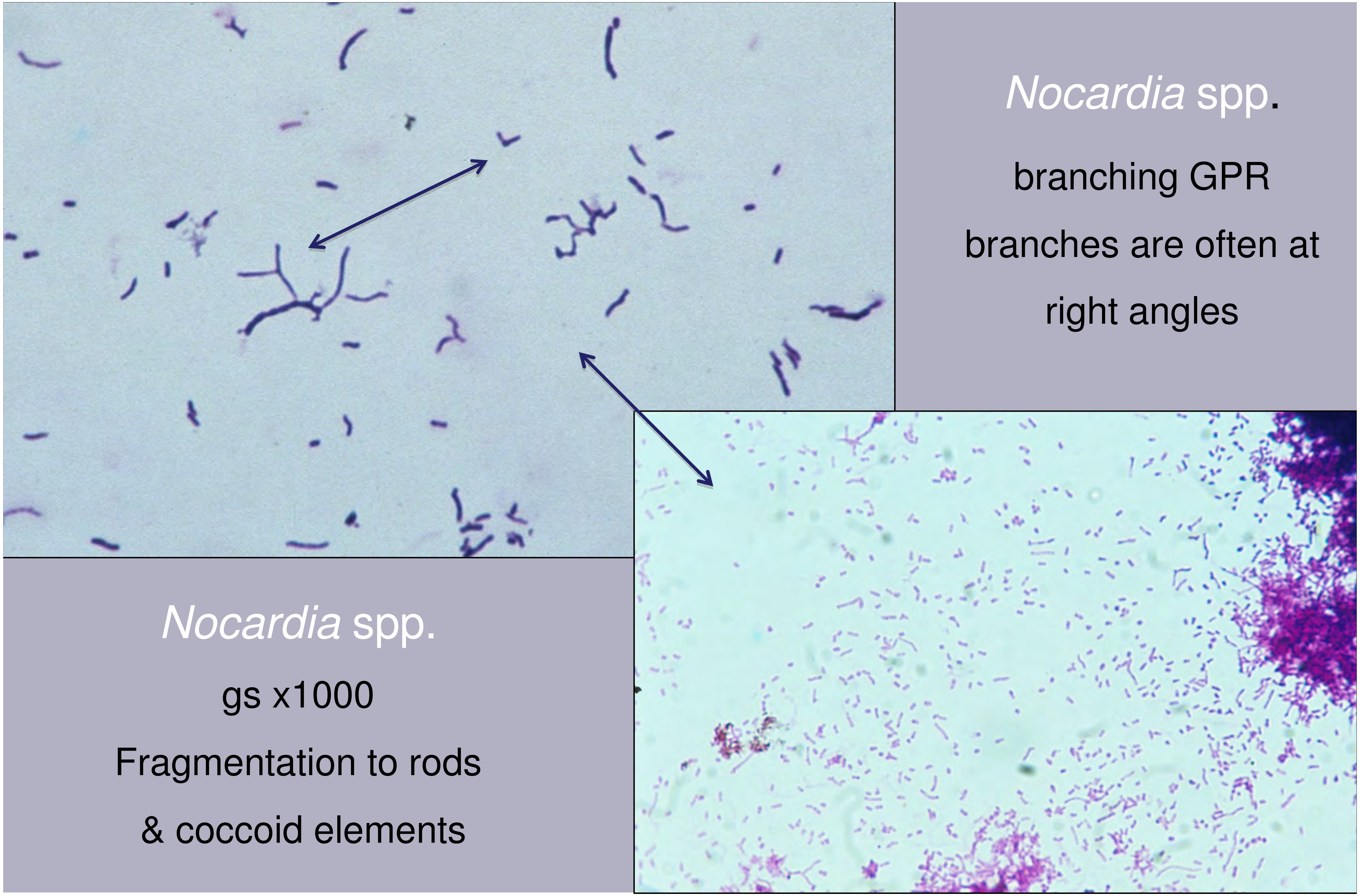
Nocardia species

Key features

- GP beaded branching rods, fragment to non-motile rods coccobacilli
- Colonies adherent, dry, chalky, heaped & folded with age
- Strict aerobe
- Acid-fast - modified ZN stain (Kinyoun)
- Substrate mycelium & aerial hyphae
- Key tests: Lysozyme = R, speciate by antibiotic susceptibility, assimilation reactions, amino acid hydrolysis reactions & Arylsulfatase test - (+ for *N. nova* only)



Nocardia spp. - NA x100



Nocardia spp.

branching GPR

branches are often at

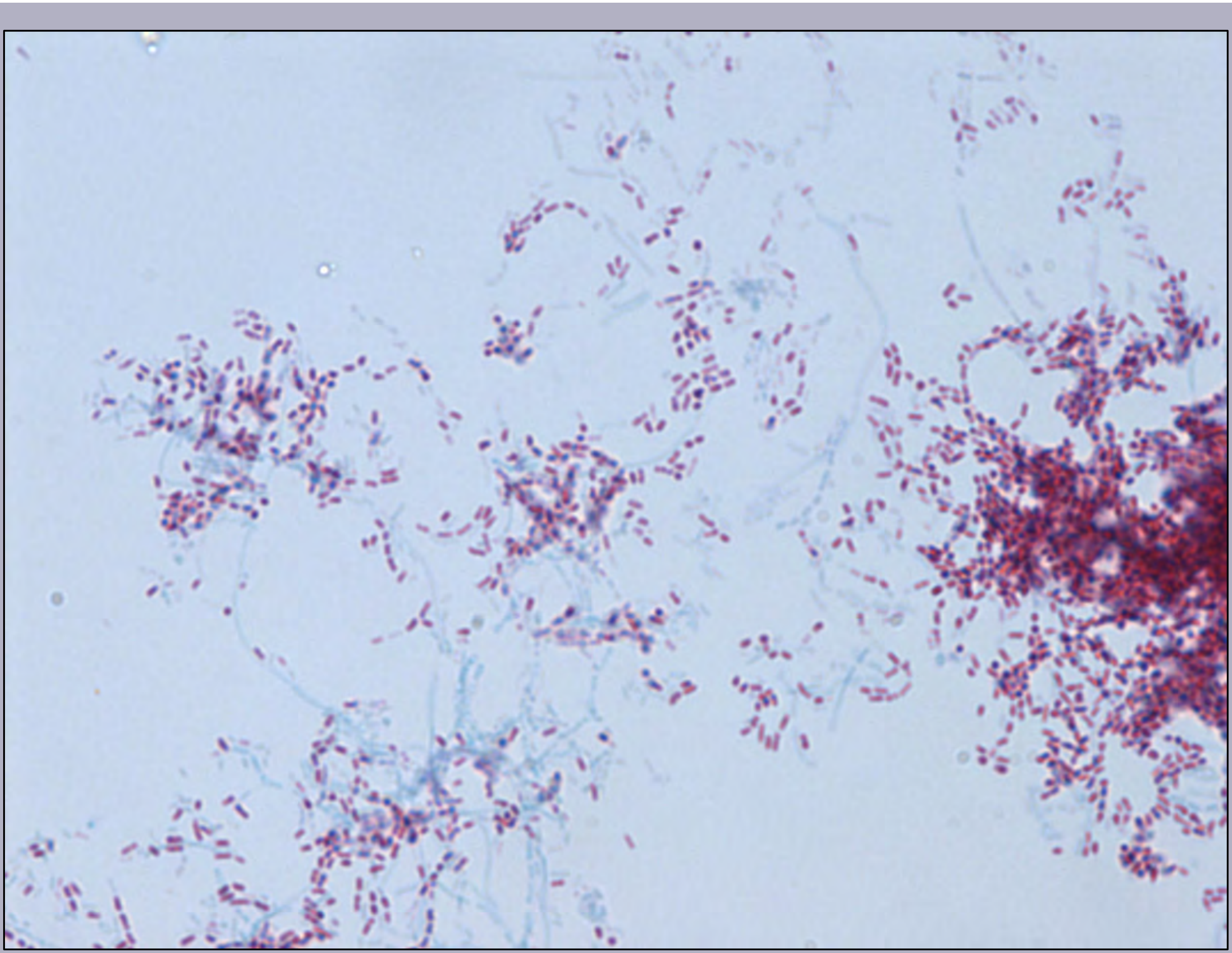
right angles

Nocardia spp.

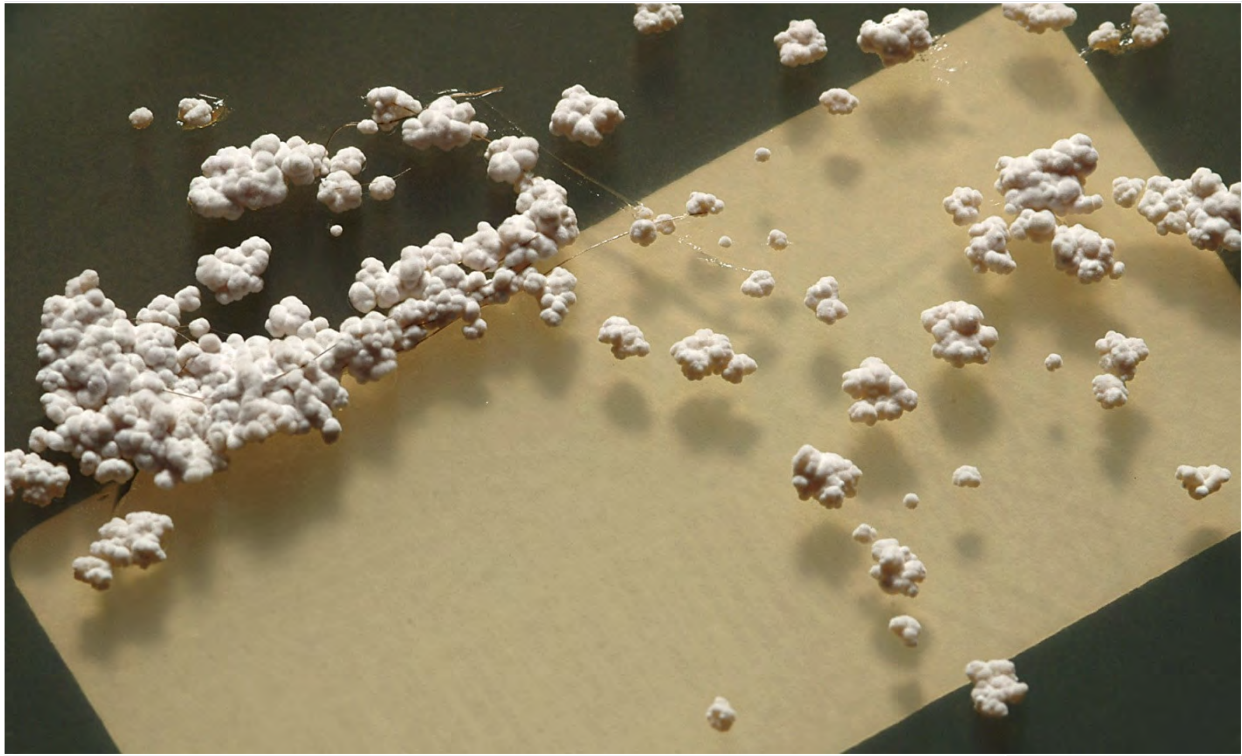
gs x1000

Fragmentation to rods

& coccoid elements



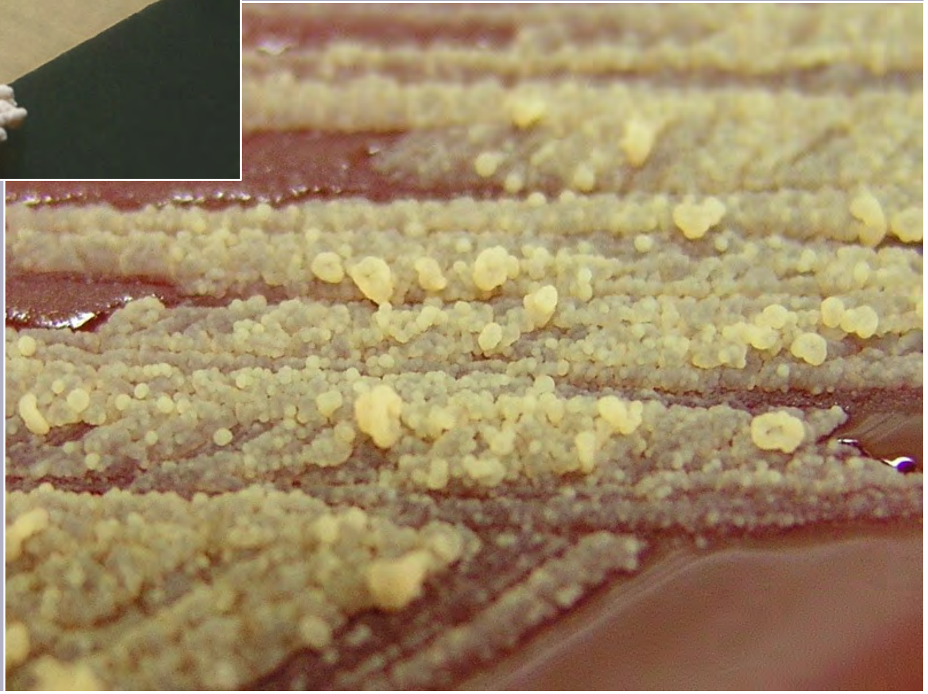
Nocardia spp. – Modified ZN stain
showing partial acid fast coccoid-rod elements



Nocardia spp.

NA slope @ 7 days

Pigment varies from chalk white, salmon pink, orange

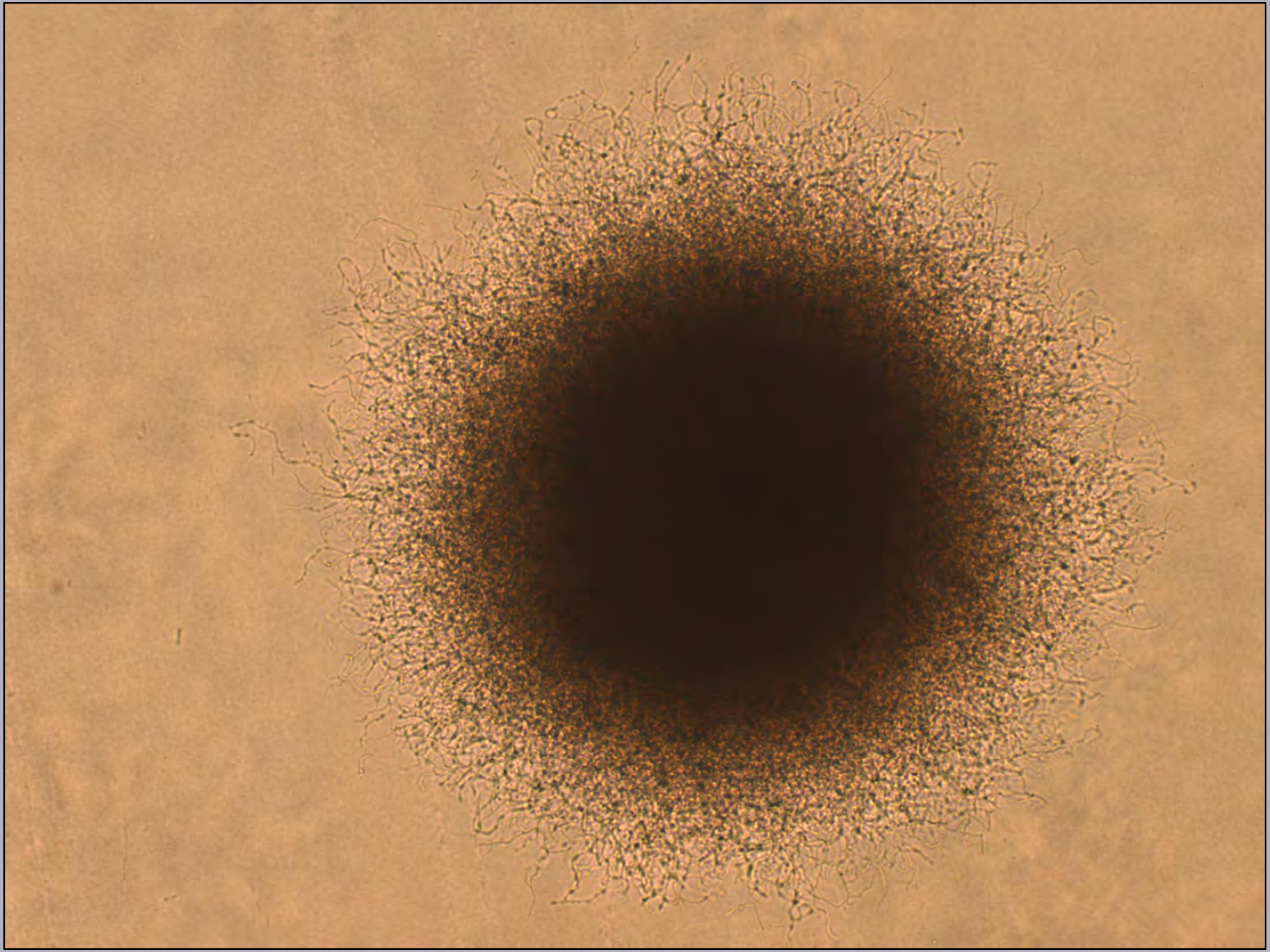


Colonies are adherent, dry, heaped and folded with earthy odour

Streptomyces species

Key features

- Long filamentous gram positive filaments with minimal branching
- Colonies khaki grey, heaped, folded, adherent, become chalky white with age, earthy odour
- Strict aerobe
- NOT acid-fast
- Substrate mycelium, <u>occ. aerial hyphae</u> that form chains of conidia
- No Fragmentation occur
- Lysozyme = S, biochemically difficult to speciate – perform16SrRNA gene sequencing



Streptomyces griseus, x100 - MHA at 48-72 hours

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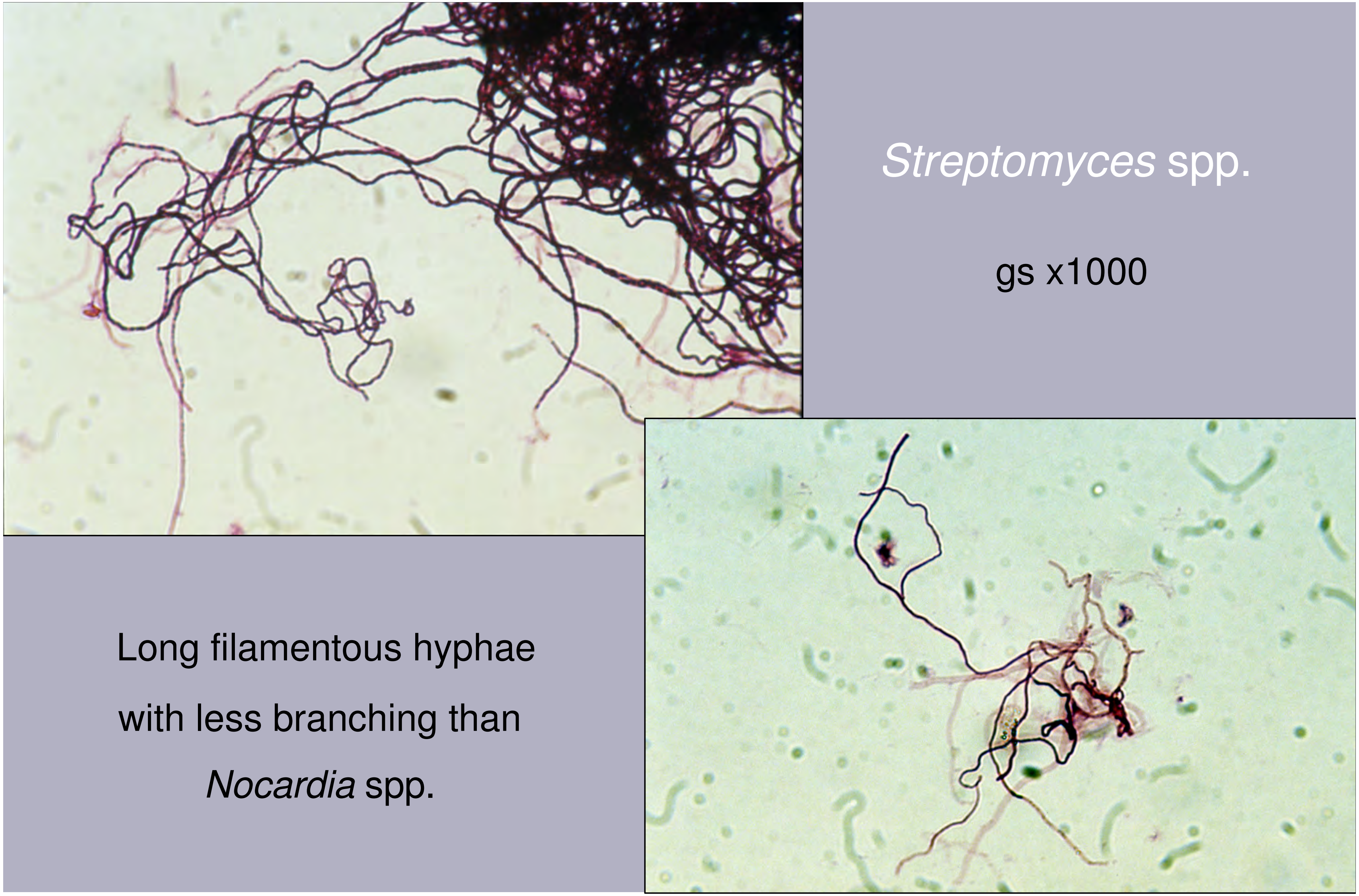


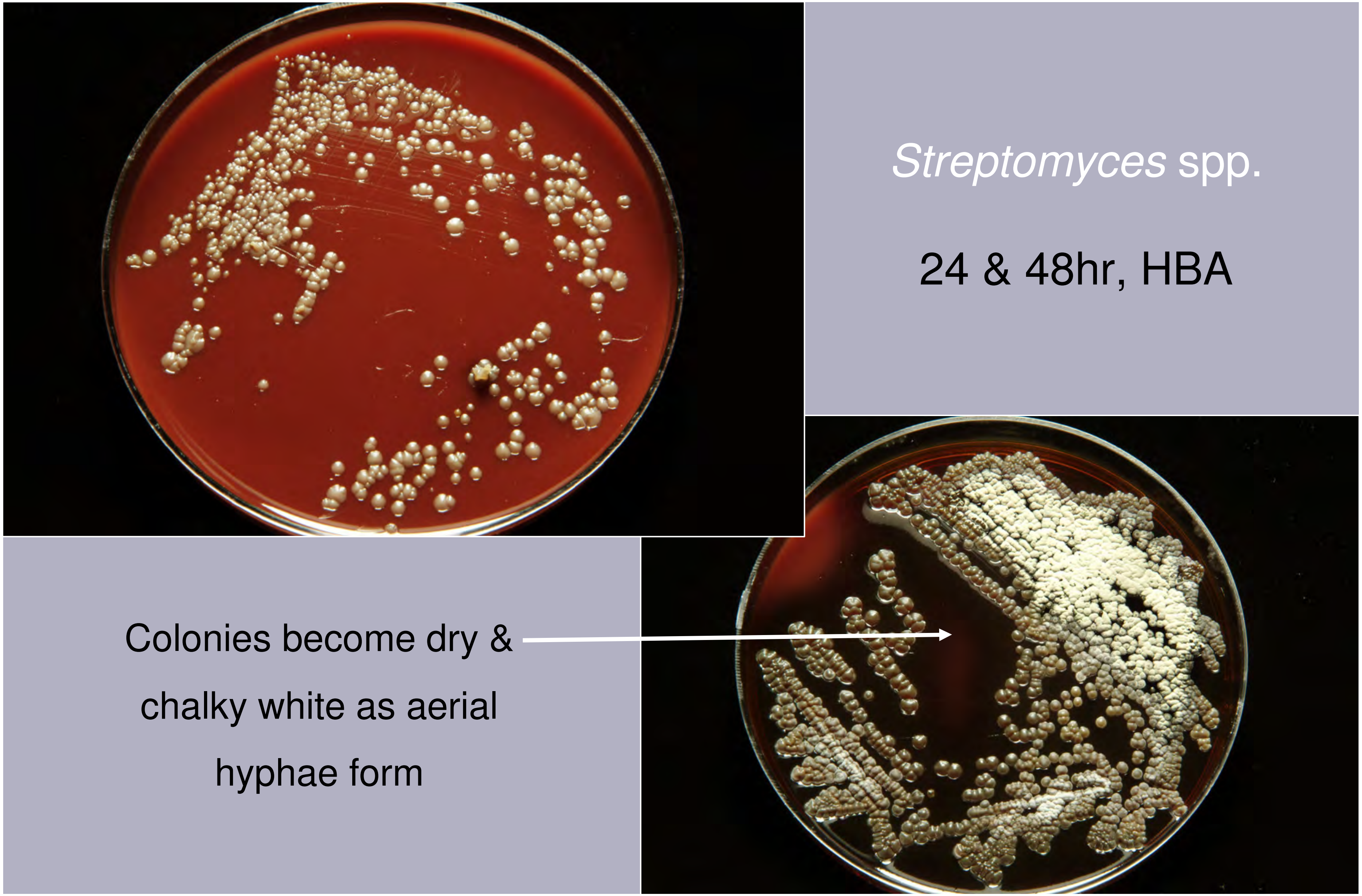
Streptomyces spp.

gs x1000

Long filamentous hyphae
with less branching than
Nocardia spp.

Streptomyces spp.

24 & 48hr, HBA

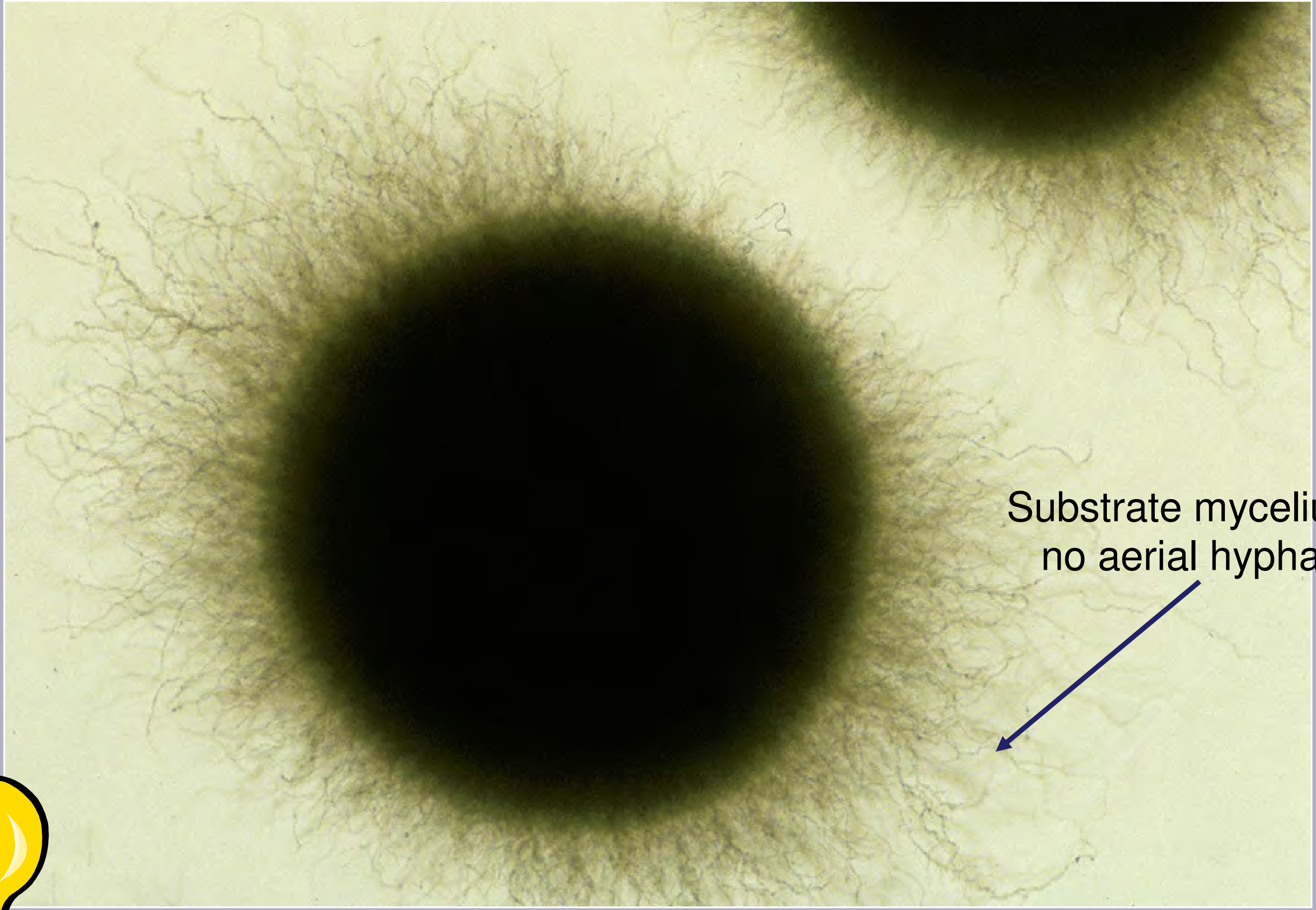


Colonies become dry & chalky white as aerial hyphae form

Oerskovia species

Key features

- Irregular GPR, branching filaments
- Colonies - smooth, glistening, bright yellow
- Facultative anaerobe, Fermentative
- NOT acid fast
- Substrate mycelium that fragments into motile elements but NO aerial hyphae.
- This differentiates *L. aquatica* and *Microbacterium* from *Oerskovia*
- API Coryne identifies *Oerskovia*
- Key tests: hydrolysis of casein, gelatin, xanthine, hypoxanthine



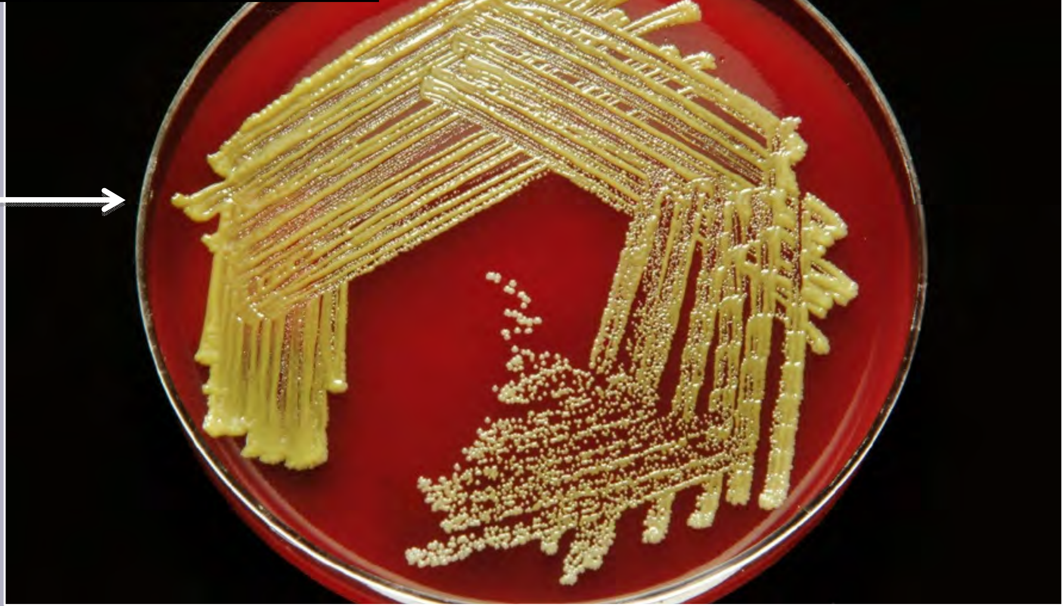
Substrate mycelium,
no aerial hyphae

Oerskovia spp. - NA, x1000, Substrate mycelium only

Oerskovia spp.

Facultative anaerobe

Excludes *Leifsonia aquatica*



Bright yellow pigment

2 species –

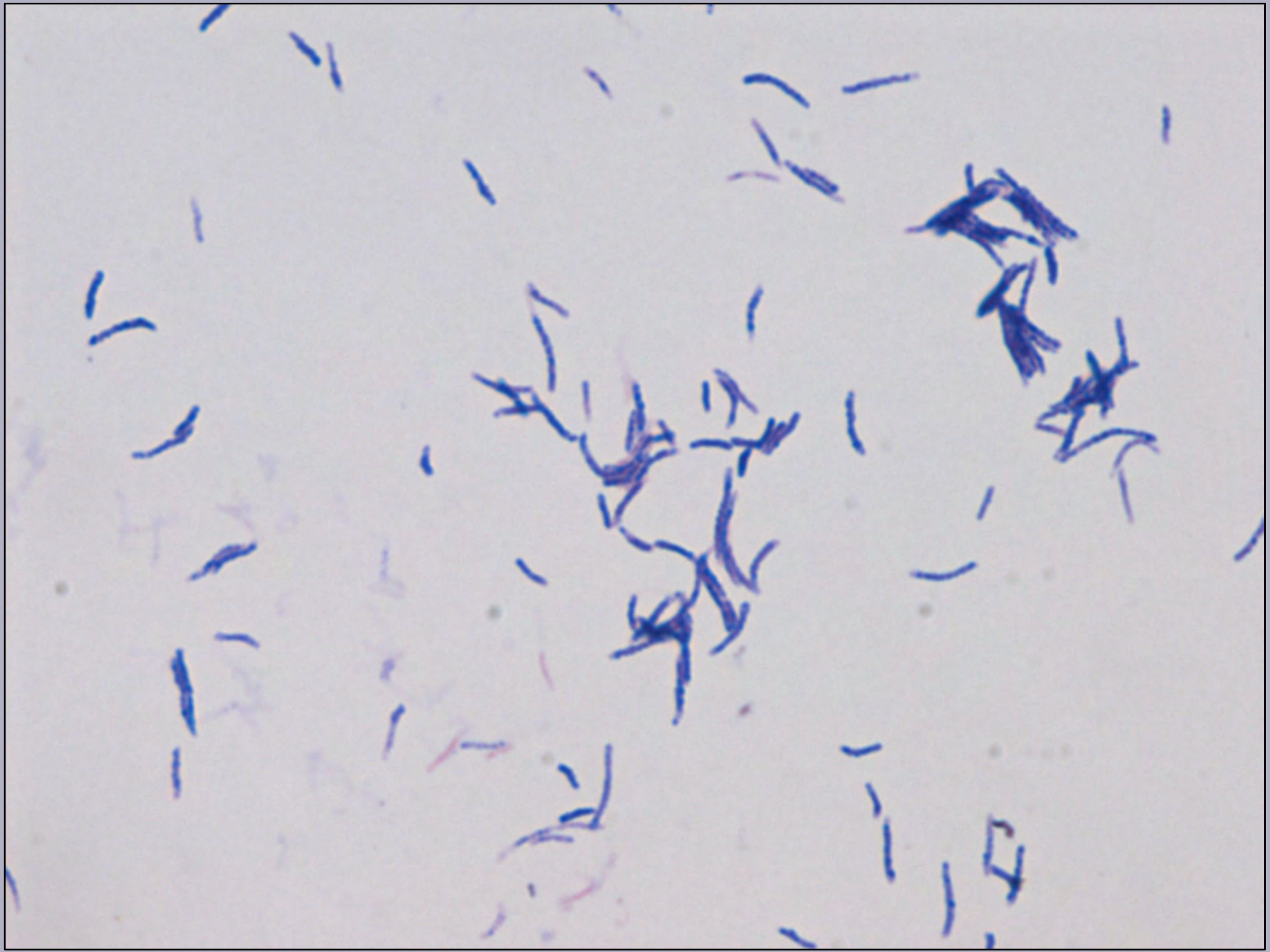
Oerskovia turbata

Cellulosimicrobium xanthinolytica

Tsukamurella species

Key features

- Irregular GPR – no obvious branching, may stain gram variable
- Rough & highly wrinkled colonies in 1-2 days
- Pigment varies with species – wh, cr, yell, orange
- Aerobic
- Partially & weakly acid fast by ZN & Modified ZN
- No substrate or aerial hyphae
- Lysozyme = R, 3 Day Arylsulfatase test –, Urea +



Tsukamurella spp. – gs x1000

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Tsukamurella inchonensis - HBA @ 72hrs

Oxidative metabolism – no growth ANO_2

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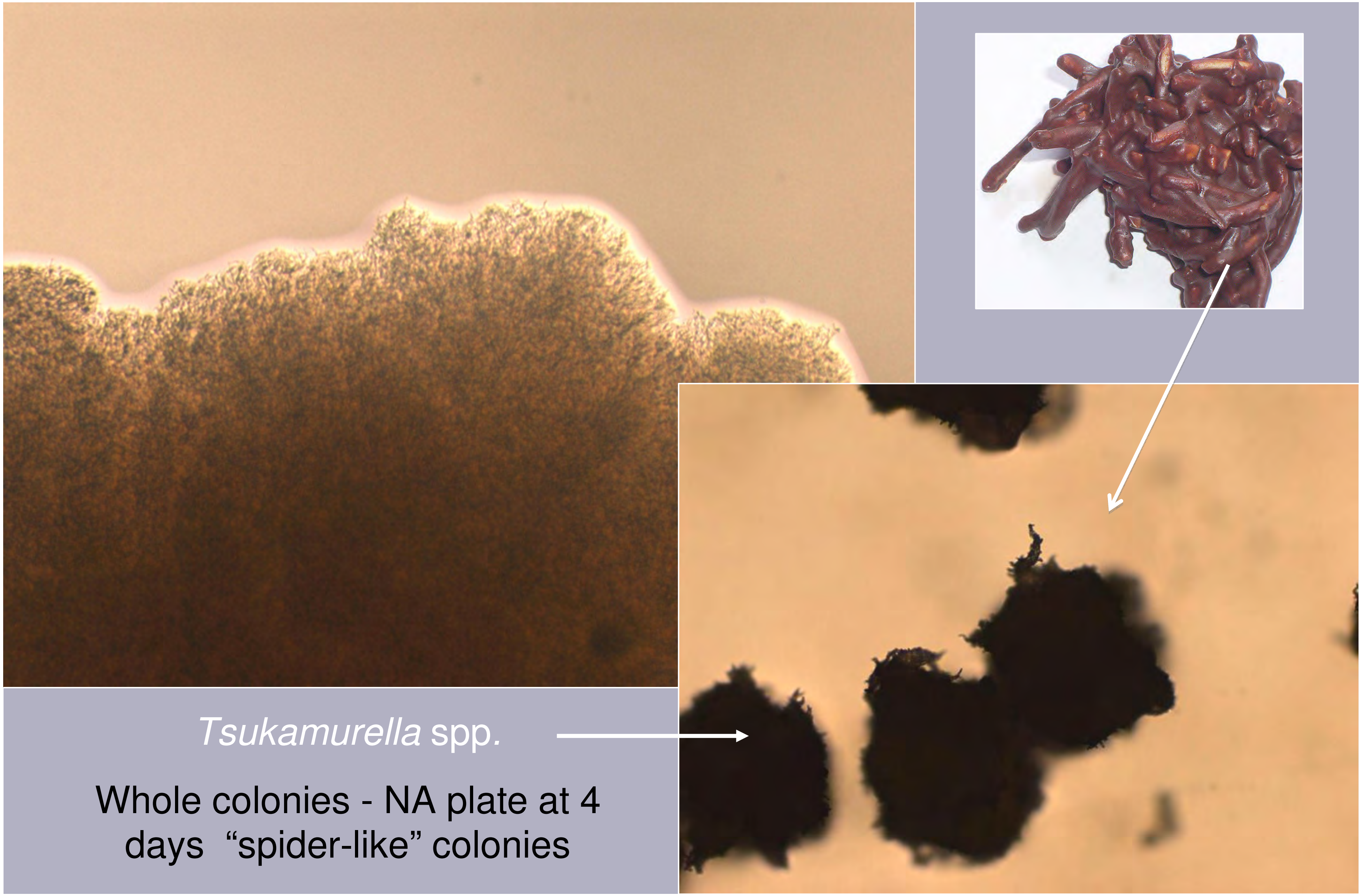
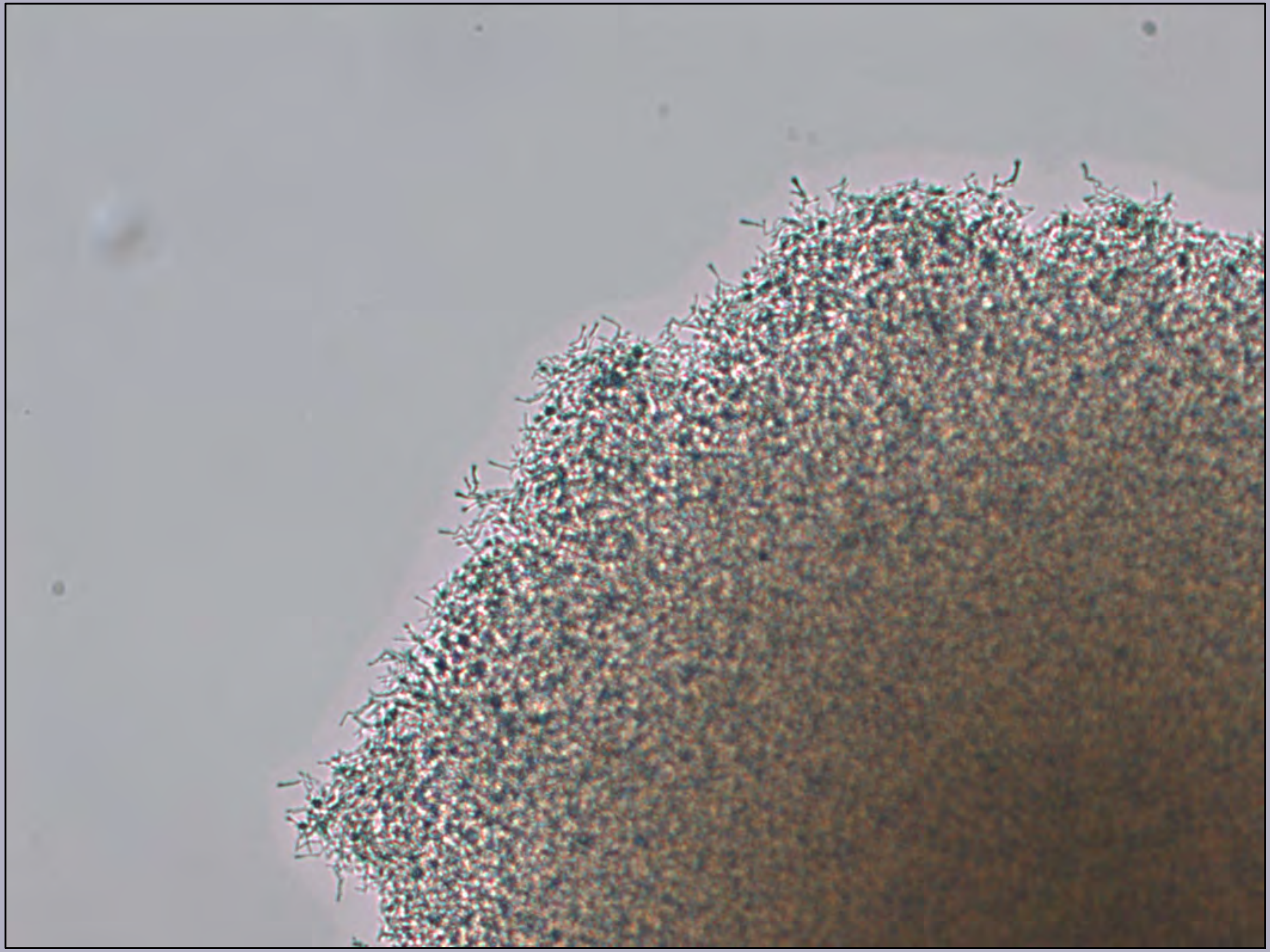


Tsukamurella spp.

Whole colonies - NA plate at 4 days “spider-like” colonies



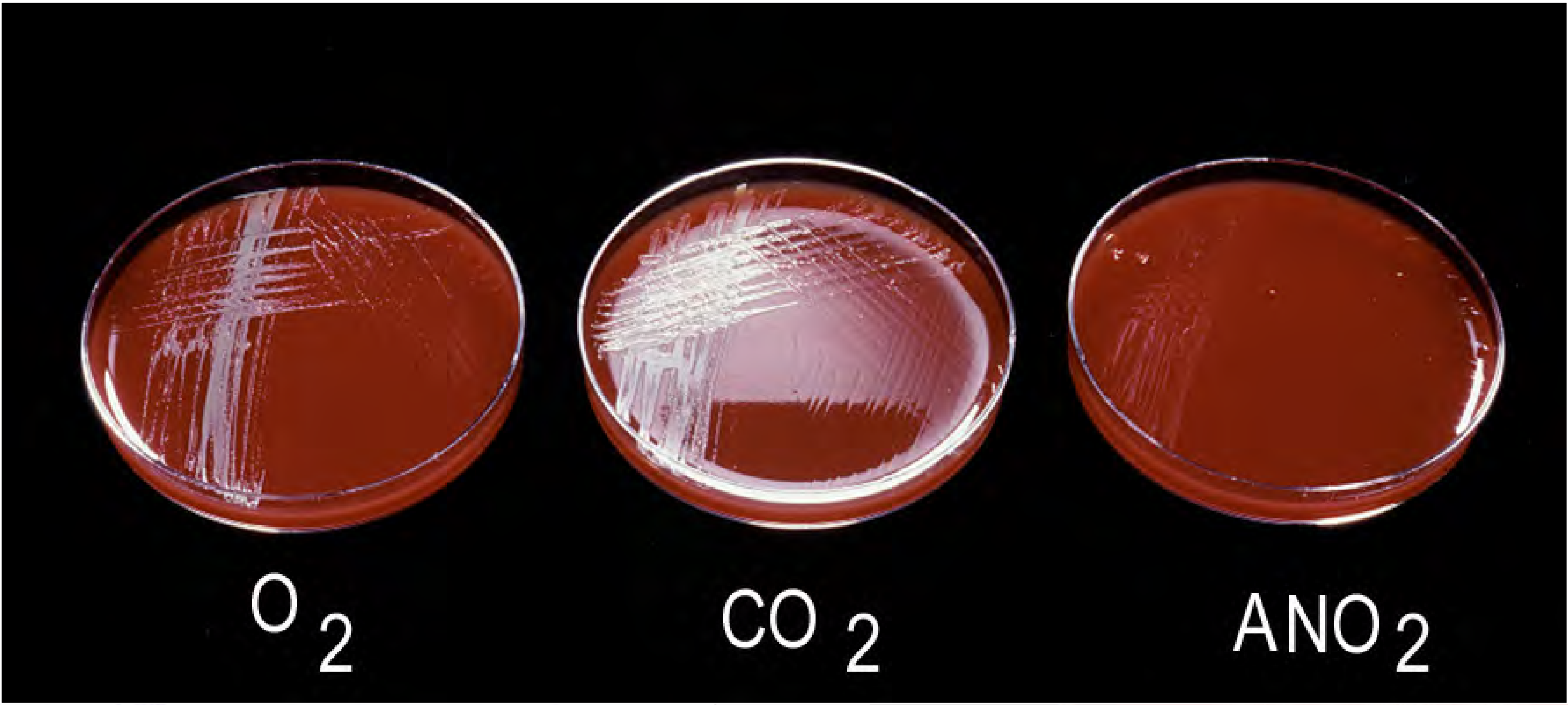
Tsukamurella colonies, NA plate at 7 days – closer view
This is not substrate or aerial hyphae! – only sticky rods

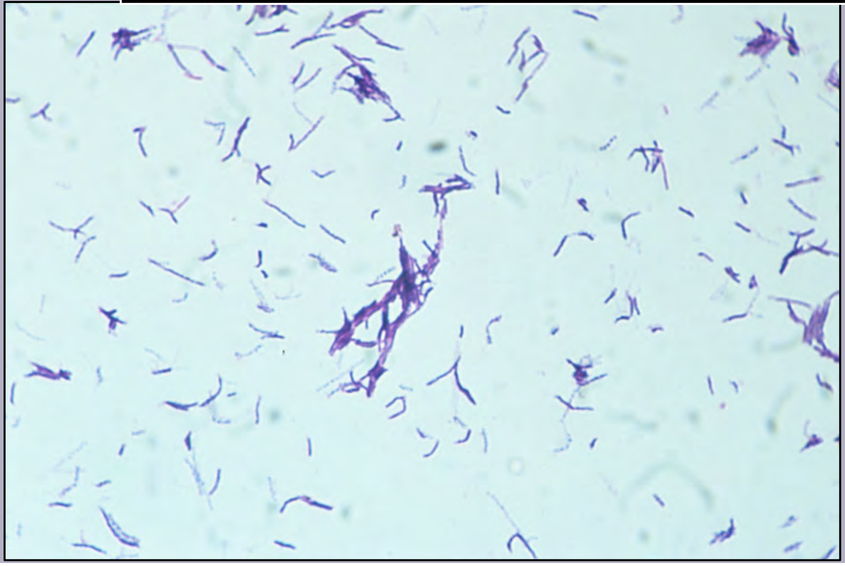
Mycobacterium fortuitum

Mycobacterium abscessus

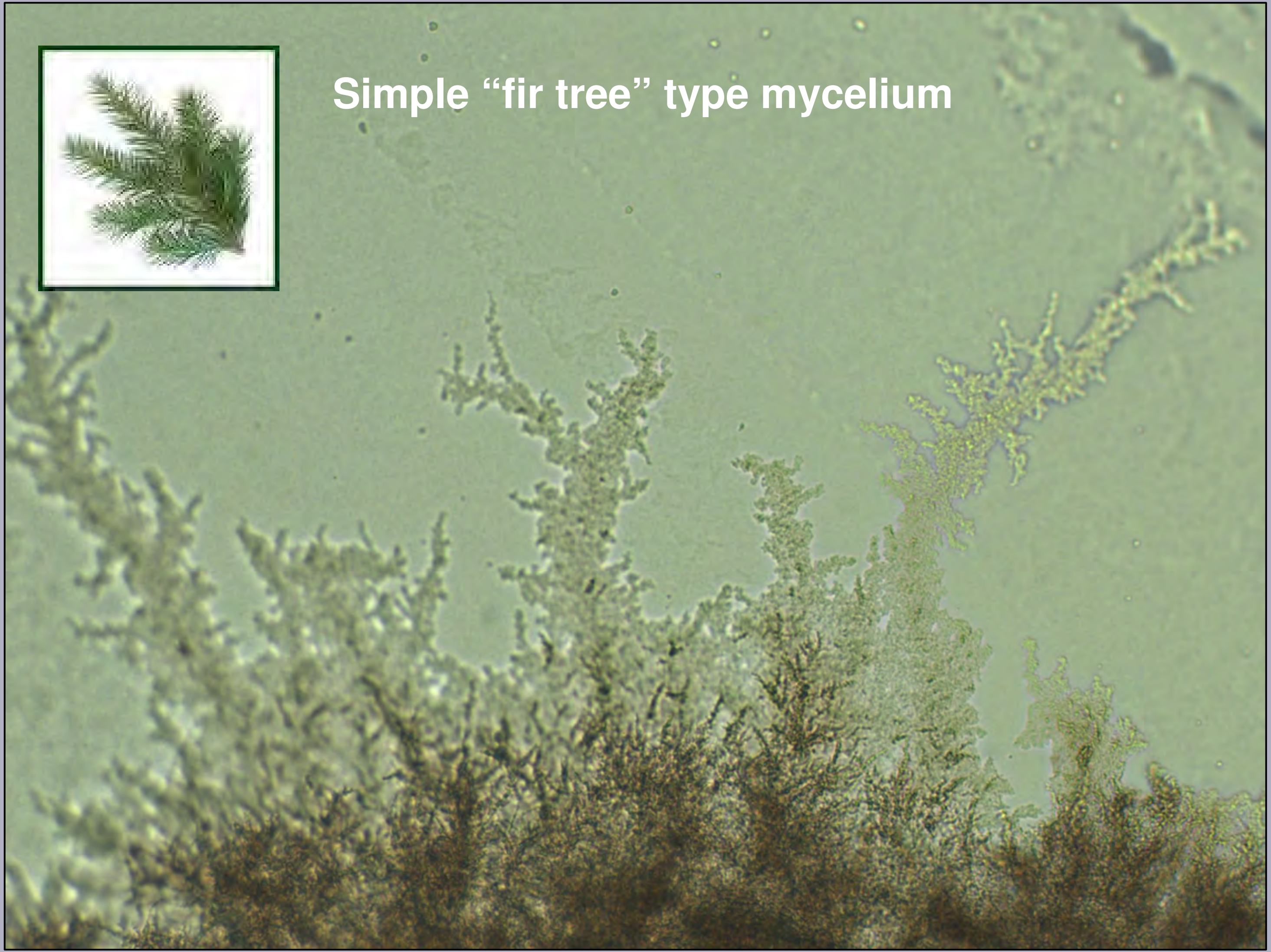
Key features

- Faint staining, gram-variable filaments & curved rods, “ghost” cells (branching filaments can occur with *M. fortuitum)*
- Slow-growing (3-5 days) aerobe
- Colonies non-pigmented, buff to yellow, smooth or dry
- Acid-fast but may be weak or partial
- Members of *M. chelonae* complex – highly resistant to antimicrobial therapy





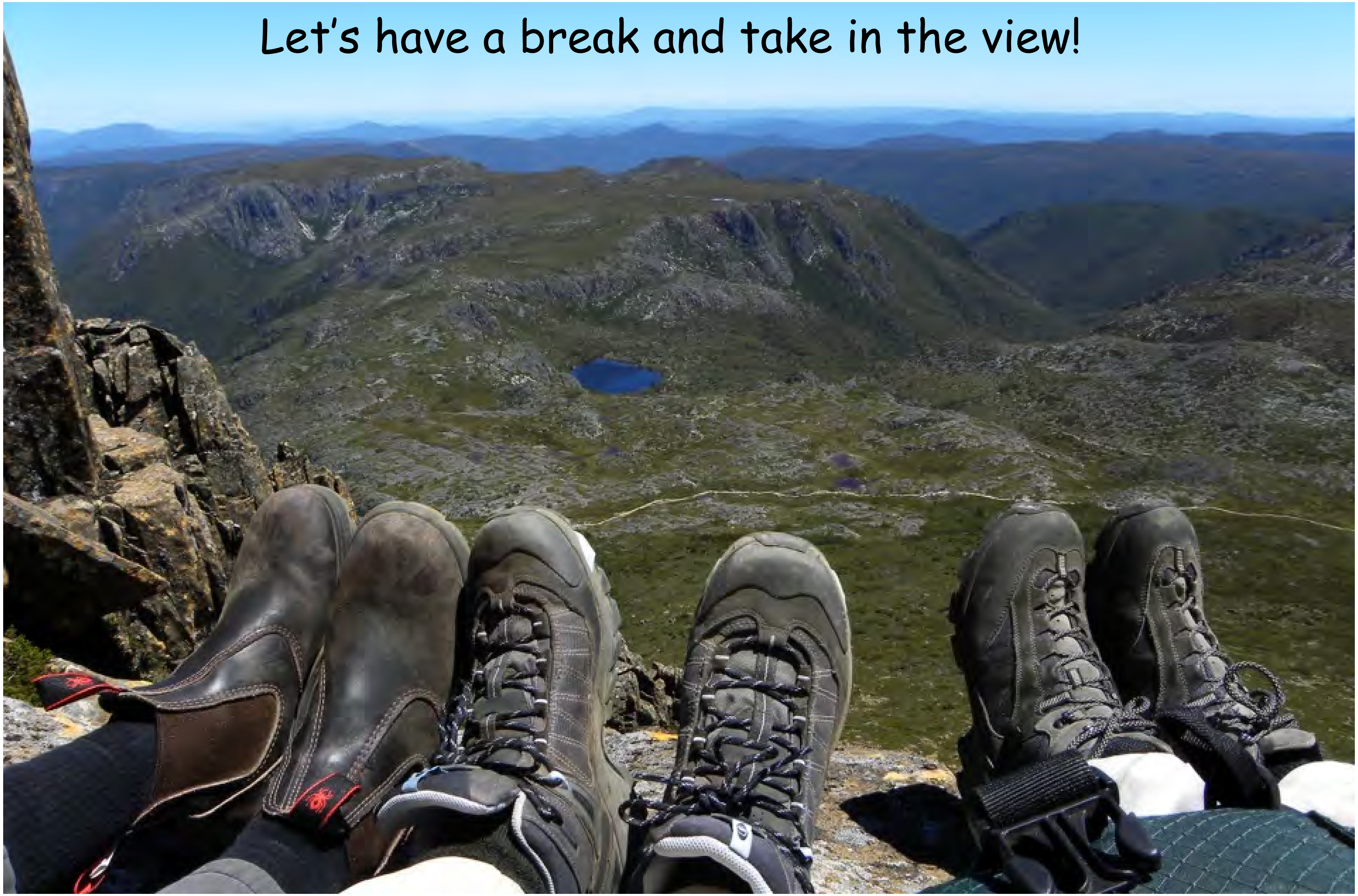
Close up – colonies can be dry or moist



Simple “fir tree” type mycelium

Mycobacterium fortuitum – 3 months on NA

Let’s have a break and take in the view!



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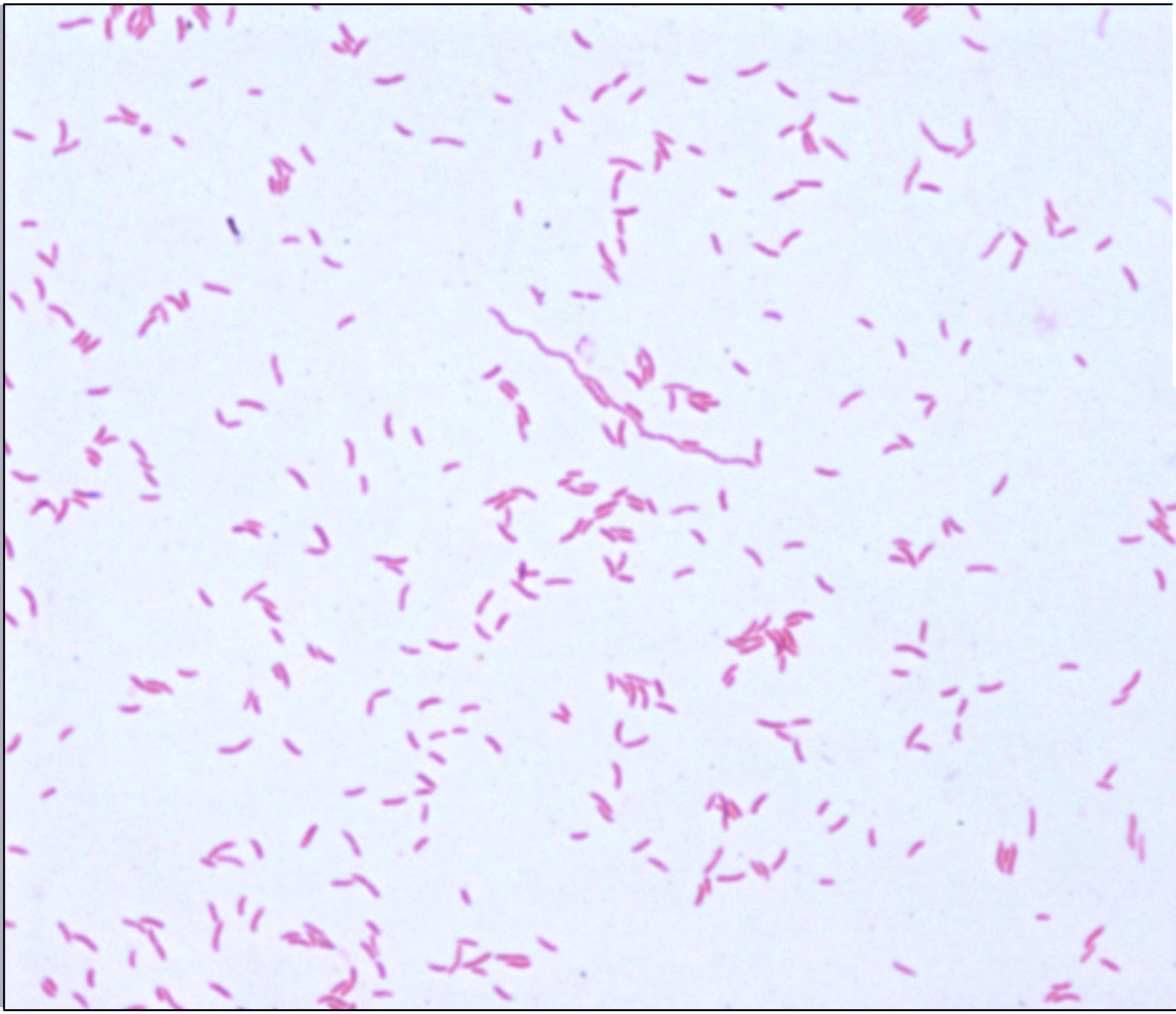
Gram Negative Rods



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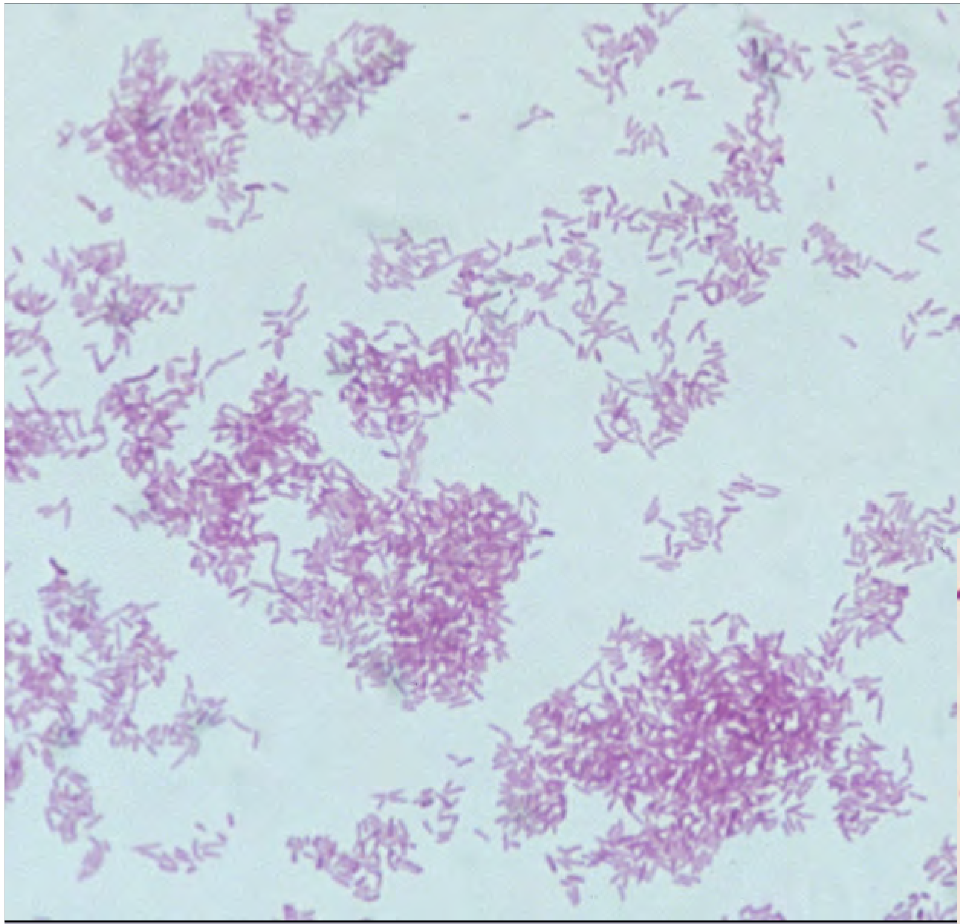
Bordetella holmesii

Key features

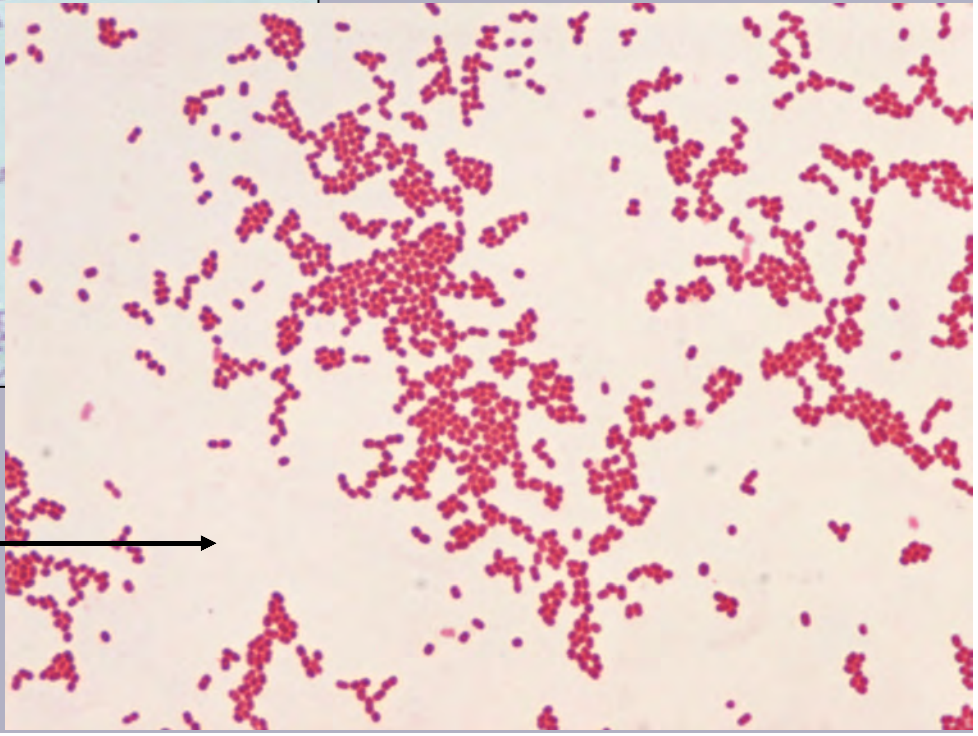
- Small to medium slender GNR, some curved rods
- Non-haemolytic, slow growing aerobe
- Oxidase -, Catalase V, MAC + but growth is slow
- Oxidative
- Non-motile
- Non-reactive - browning on tyrosine agar
- Not on database of commercial ID kits/systems

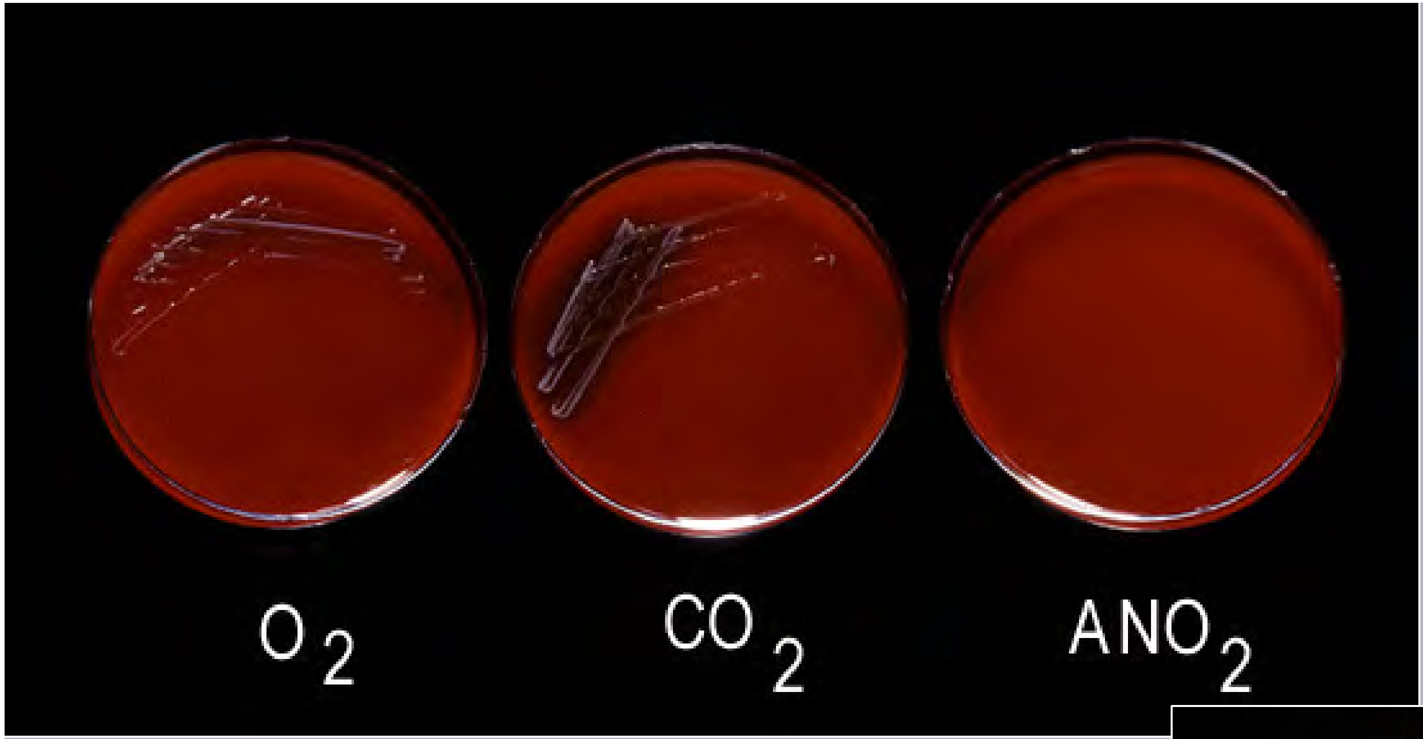


Bordetella holmesii
gs x1000
small-medium slender GNR
some curved

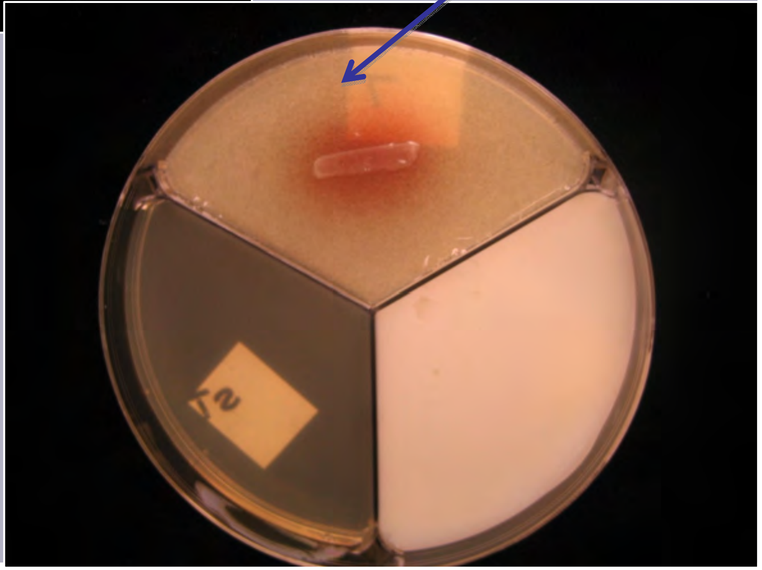
mis-identifies as
Acinetobacter spp.

BUT Gram stain morphology is not plump GNCB!





Browning on Tyrosine agar



Bordetella holmesii

growth O_2 & CO_2 but not anaerobic

typical of oxidative organisms

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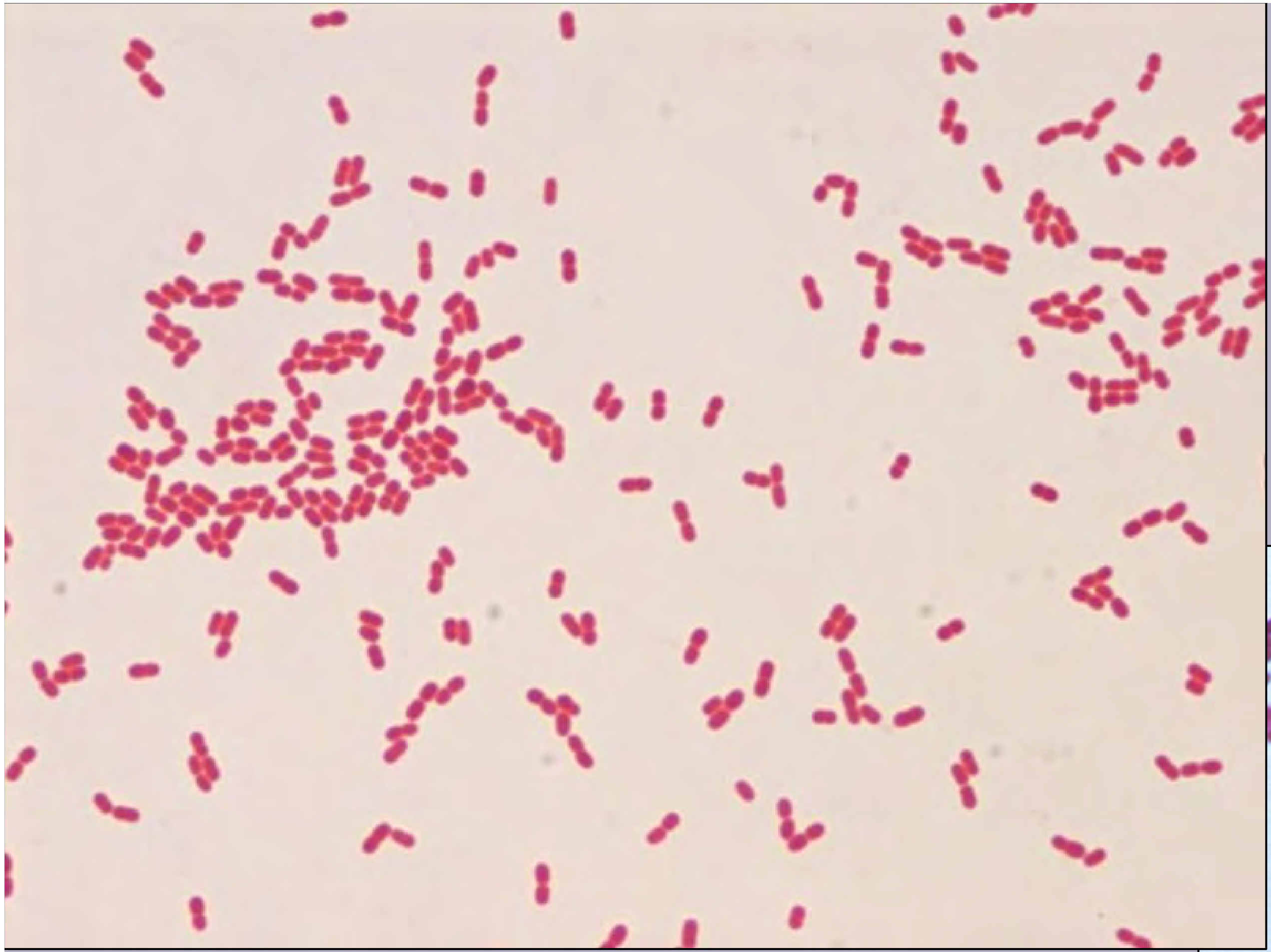
Moraxella atlantae

Key features

- Plump GNCB, often stains gram variable
- Strict aerobe - Oxidative
- Growth stimulated by bile salts (MAC growth is better or equal to HBA)
- Non-motile buy twitching has been observed (pili)
- OX+ (excludes *Acinetobacter* spp.), CAT+, asaccharolytic
- *M. osloensis* – some strains tributyrin +, ß-Lactamase +, vancomycin, R

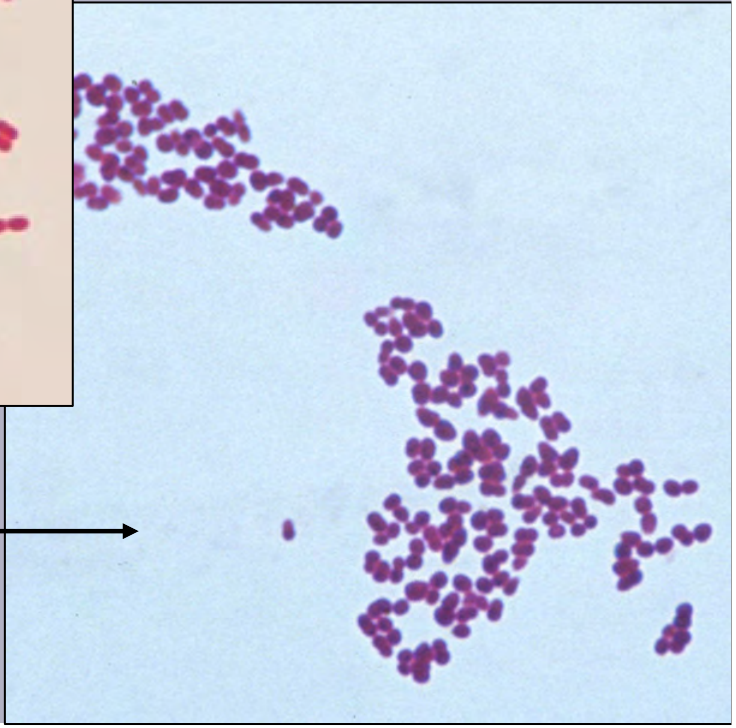


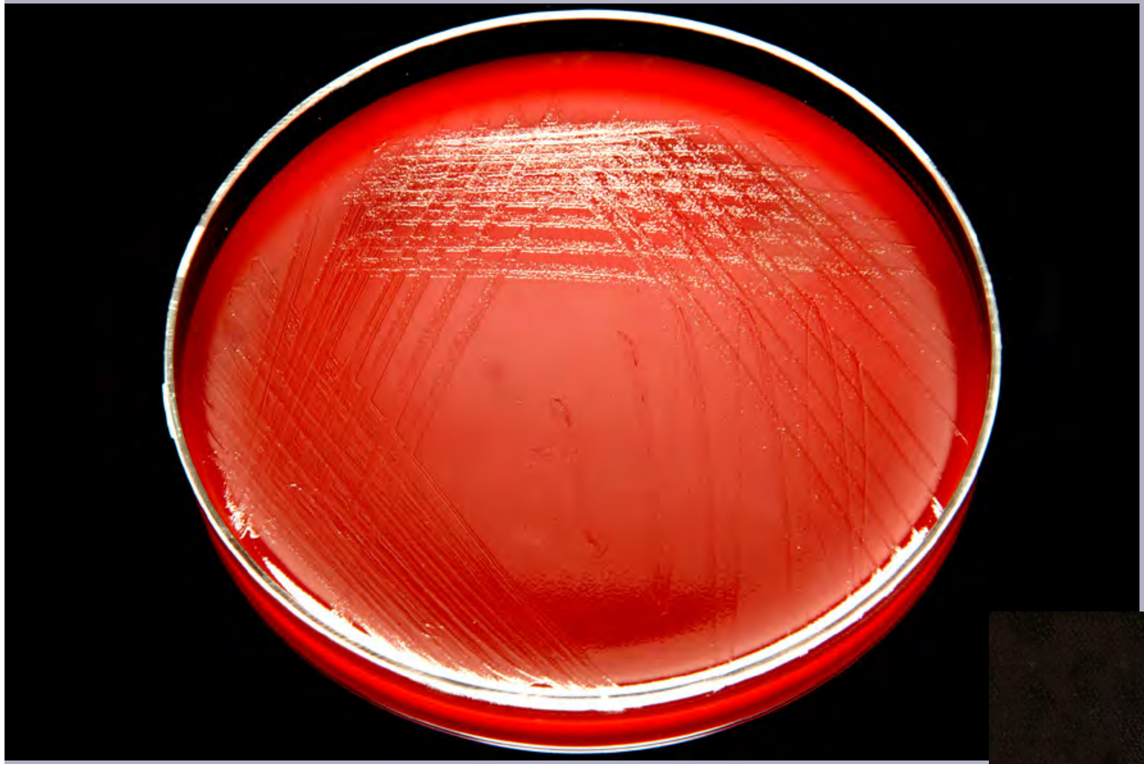
Moraxella atlantae

gs x1000

Plump GNCB

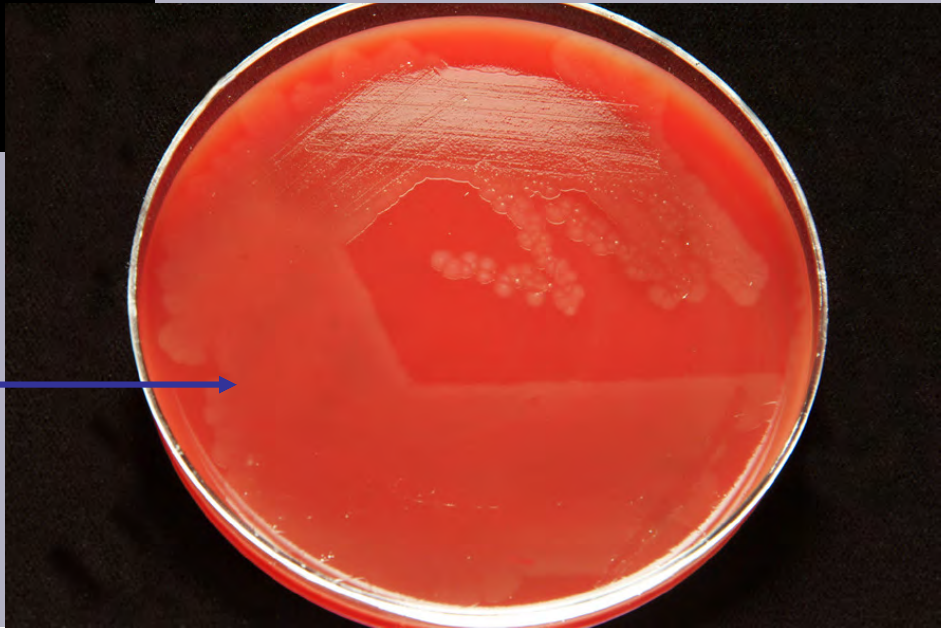
Cells can stain Gram variable & are very coccoid – always check with penicillin challenge & String Test





Important feature is the colony appearance

Colonies are clear & tiny @ 24hrs, pitting and non-pitting forms do occur

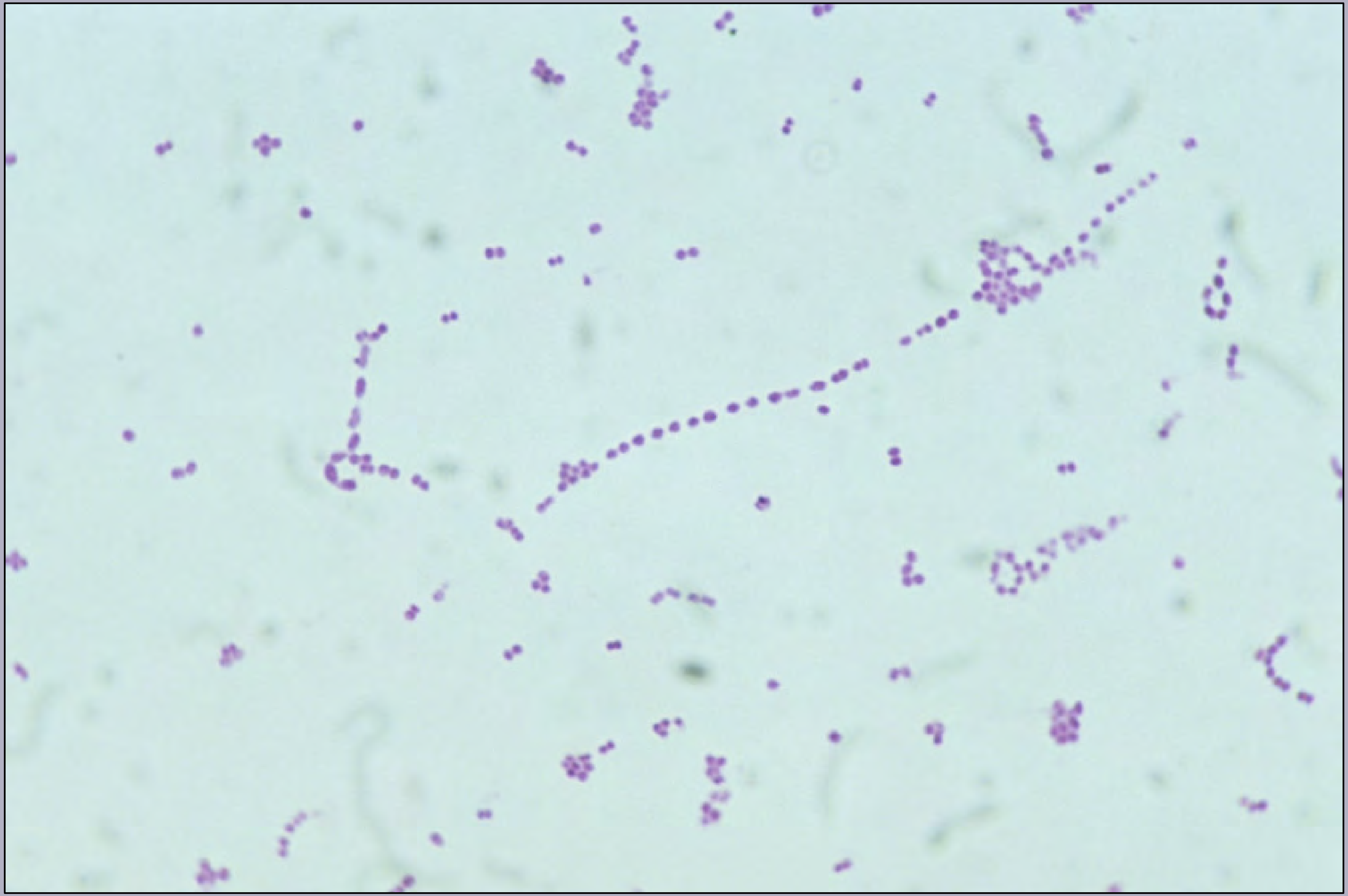


Moraxella atlantae @ 4 days showing spreading flat growth

Roseomonas gilardii

Key features

- Plump coccoid to oval rods in pairs or short chains
- Resists decolourising
- Colonies mucoid and pale rose pink
- Most species grow on a broad range of media & at 30-42°C
- Oxidase +w/-, Urease +
- *Roseomonas* spp. are not on database of API 20 NE - identify as *Methylobacterium* spp.
- Confused with *N. gonorrhoeae* - grows on TM medium

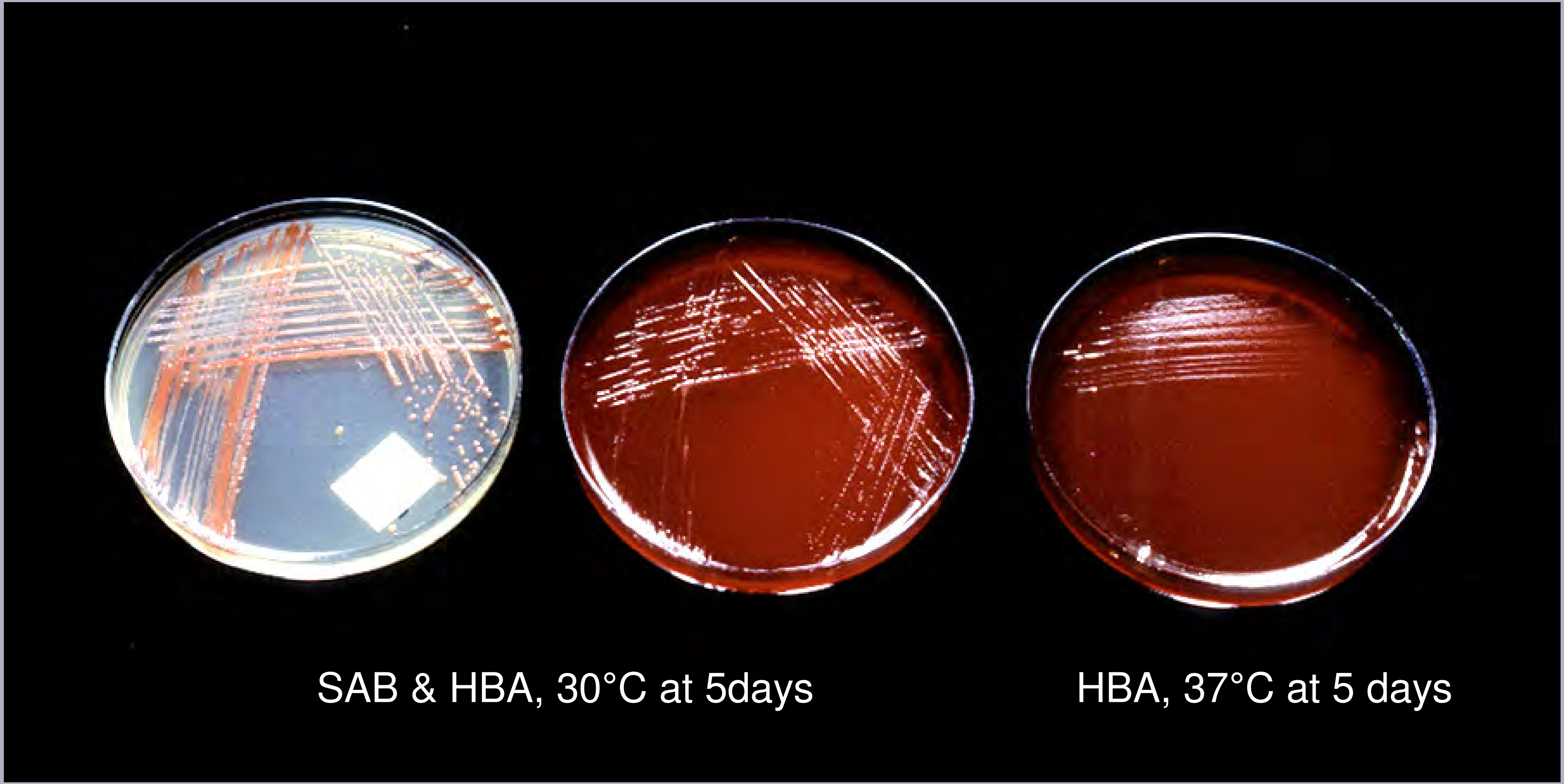


Roseomonas spp. – gs x1000

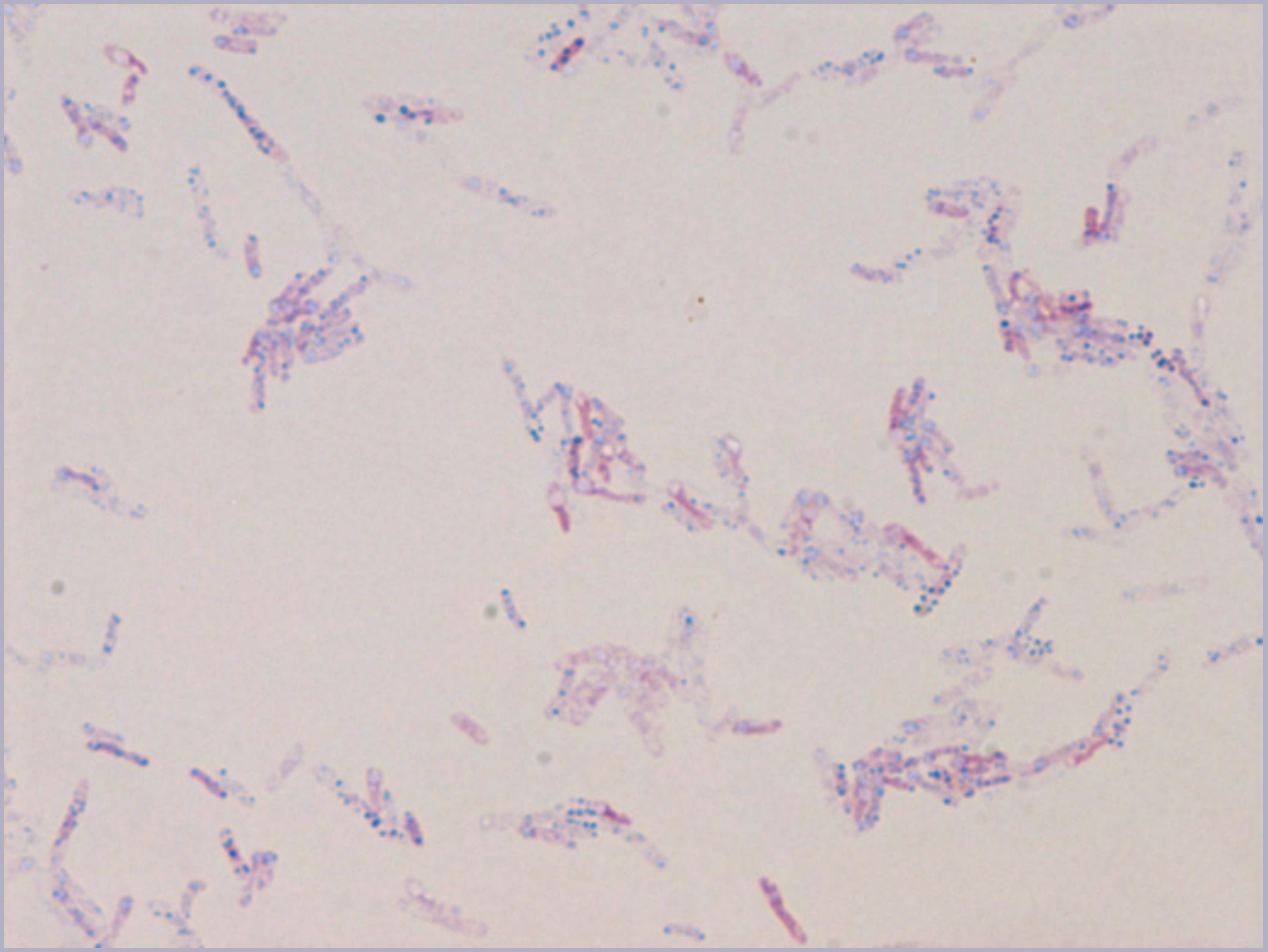
Roseomonas cervicalis

HBA, 48hours

NA plate showing mucoid colonies with pale rose pigment



Methylobacterium spp.



***Methylobacterium* spp. - gs x1000**

Terrible gram – but this is what it can look like!

Capnocytophaga ochracea

Key features

- Slender, fusiform rod, tapered ends
- Capnophilic
- Gliding motility
- Yellow-orange “ear wax” pigment (swab technique)and pink-lilac sheen
- Colonies fringed to spreading, beaten copper look (*C. ochracea*)
- Bluish-grey, entire colonies (*C. canimorsus*)
- Key distinguishing tests: OX, CAT, ADH

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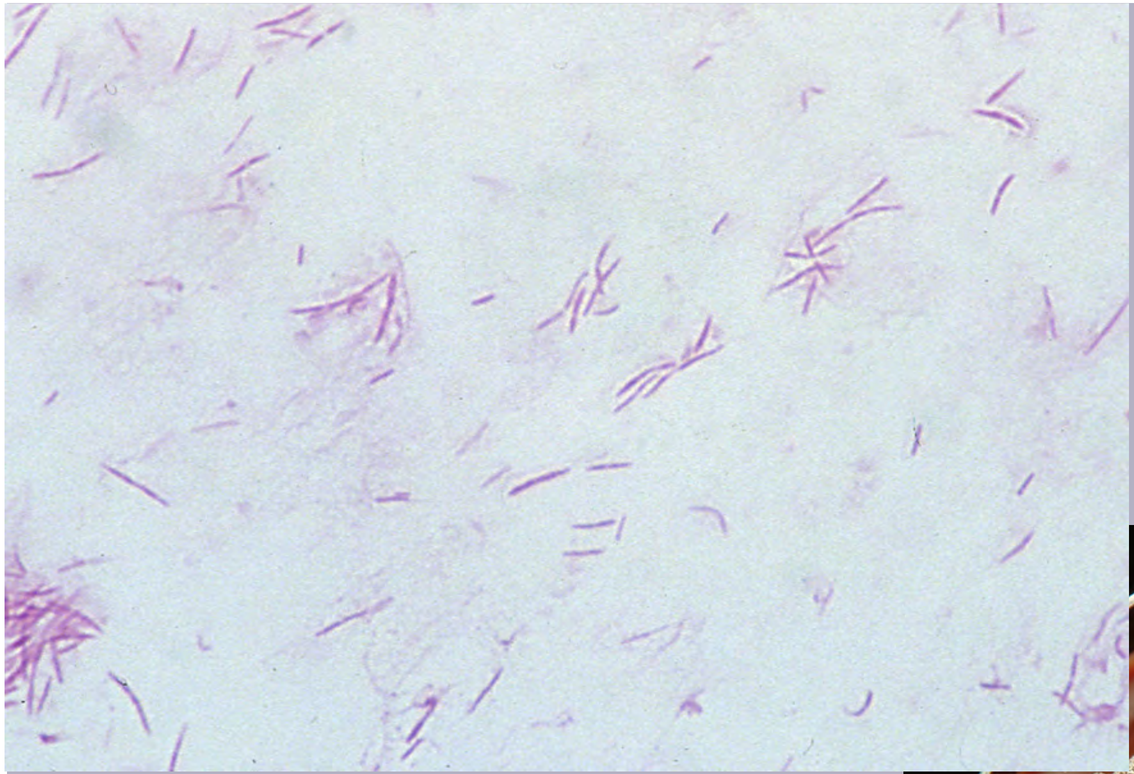
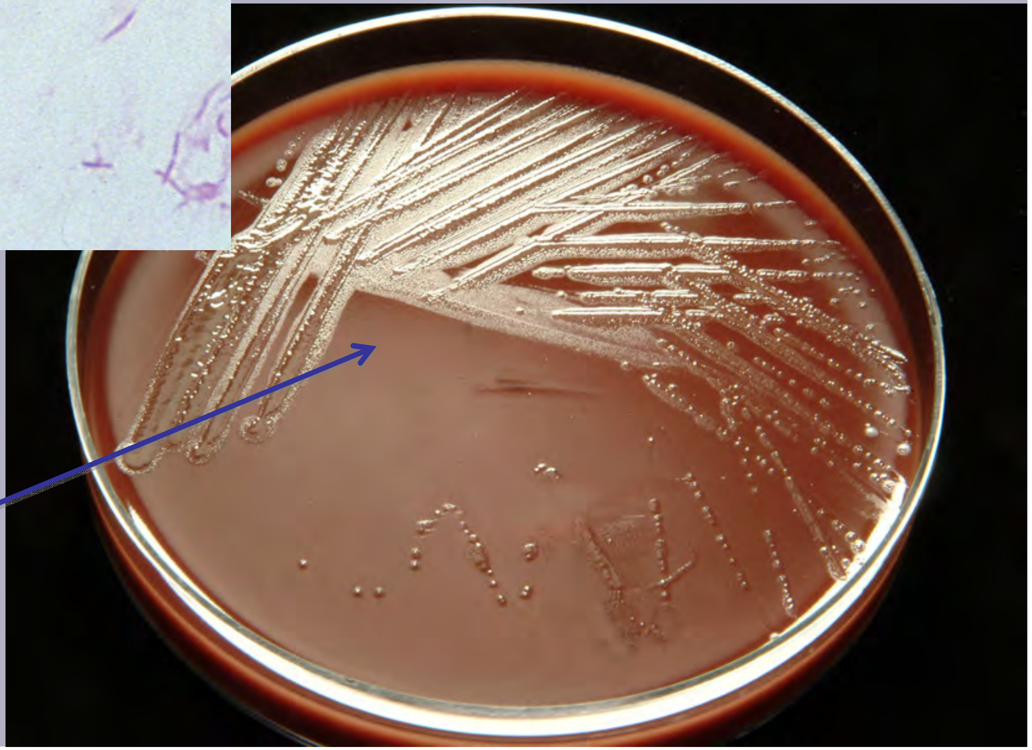


Capnocytophaga ***ochracea***

gs x 1000



Spreading growth, beaten surface of colonies

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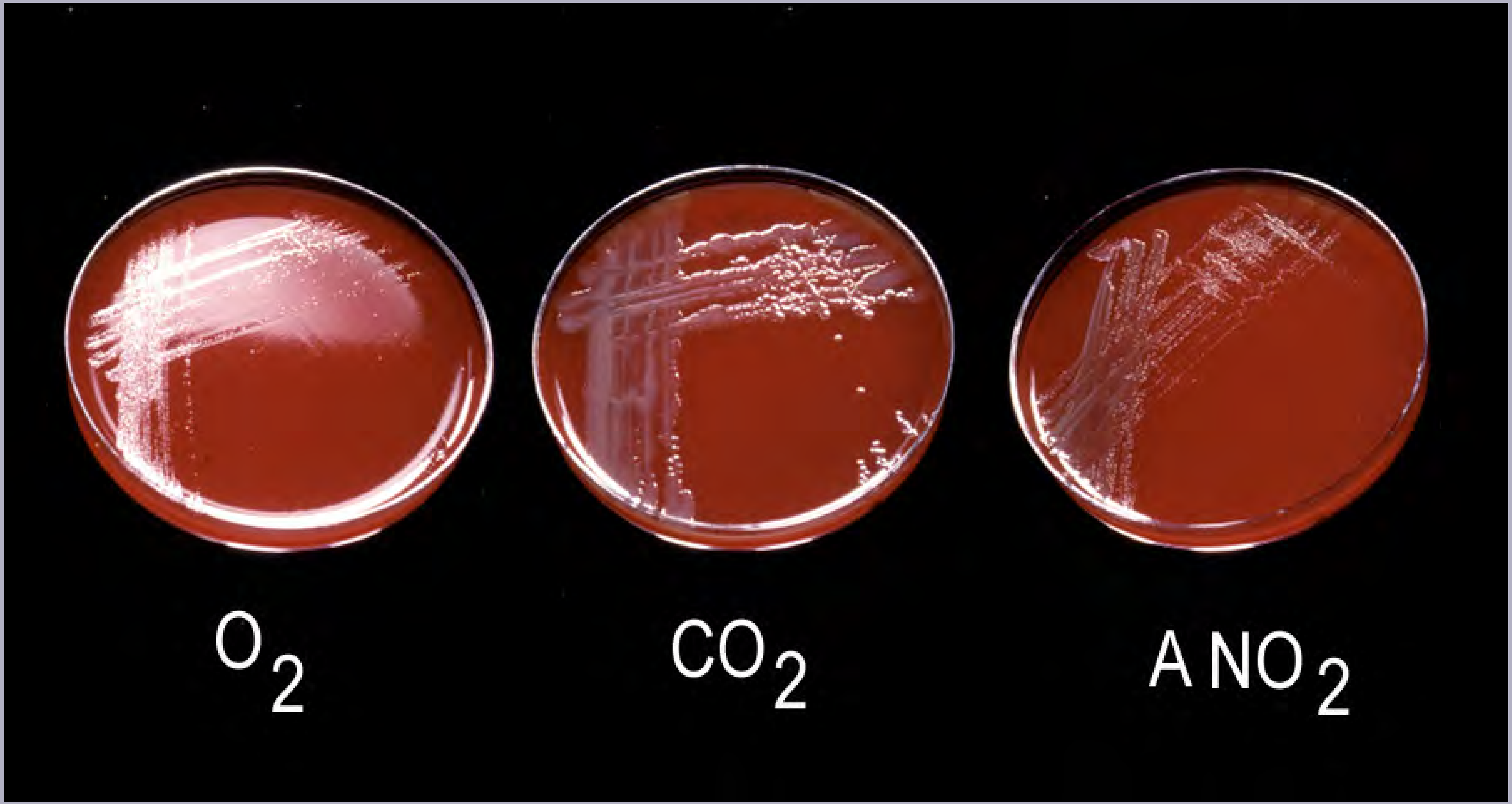

Capnocytophaga ochracea

HBA @ 48hours

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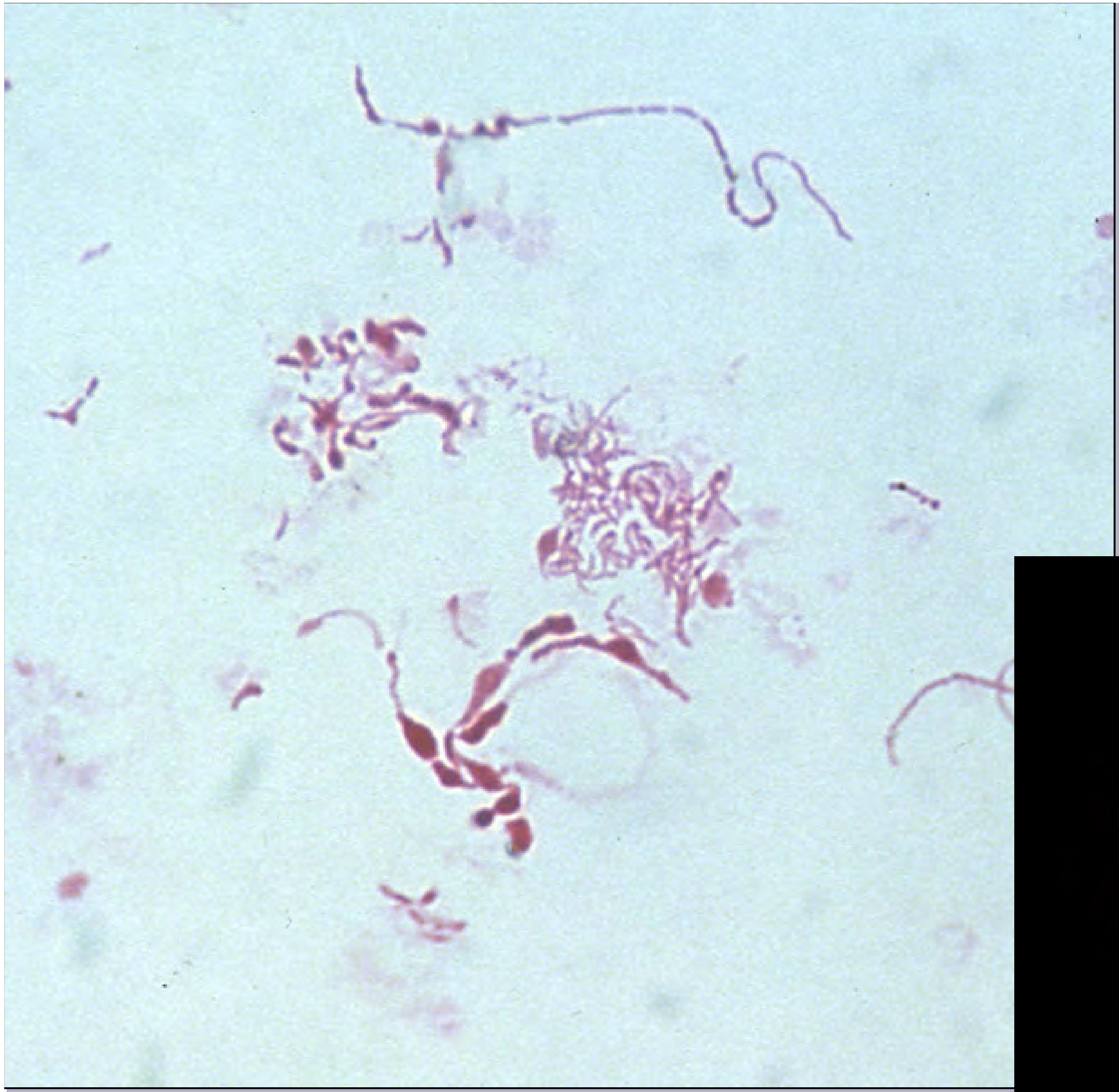
Streptobacillus moniliformis

Key features

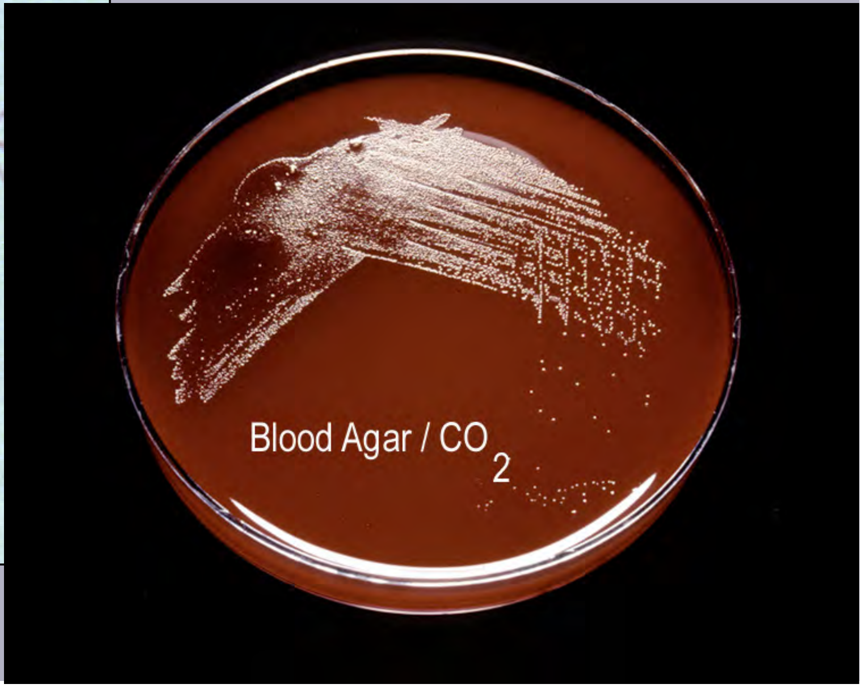
- One of two agents of Rat-bite fever (also *Spirillum minus* – Sodoku)
- Recovered occasionally from blood culture with no added SPS
- Check patient history - ?rat bite, pet rat, drug abuse
- Unusual Gram stain – 'string of pearls', chains, filaments, swellings
- Non-haemolytic to weak alpha with age
- Non-motile
- Fermentative metabolism
- Identification – use a rapid substrate method e.g. ID 32 STREP or similar



Streptobacillus moniliformis

gs x1000

filamentous GNR with swellings



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Vibrio vulnificus

Special features

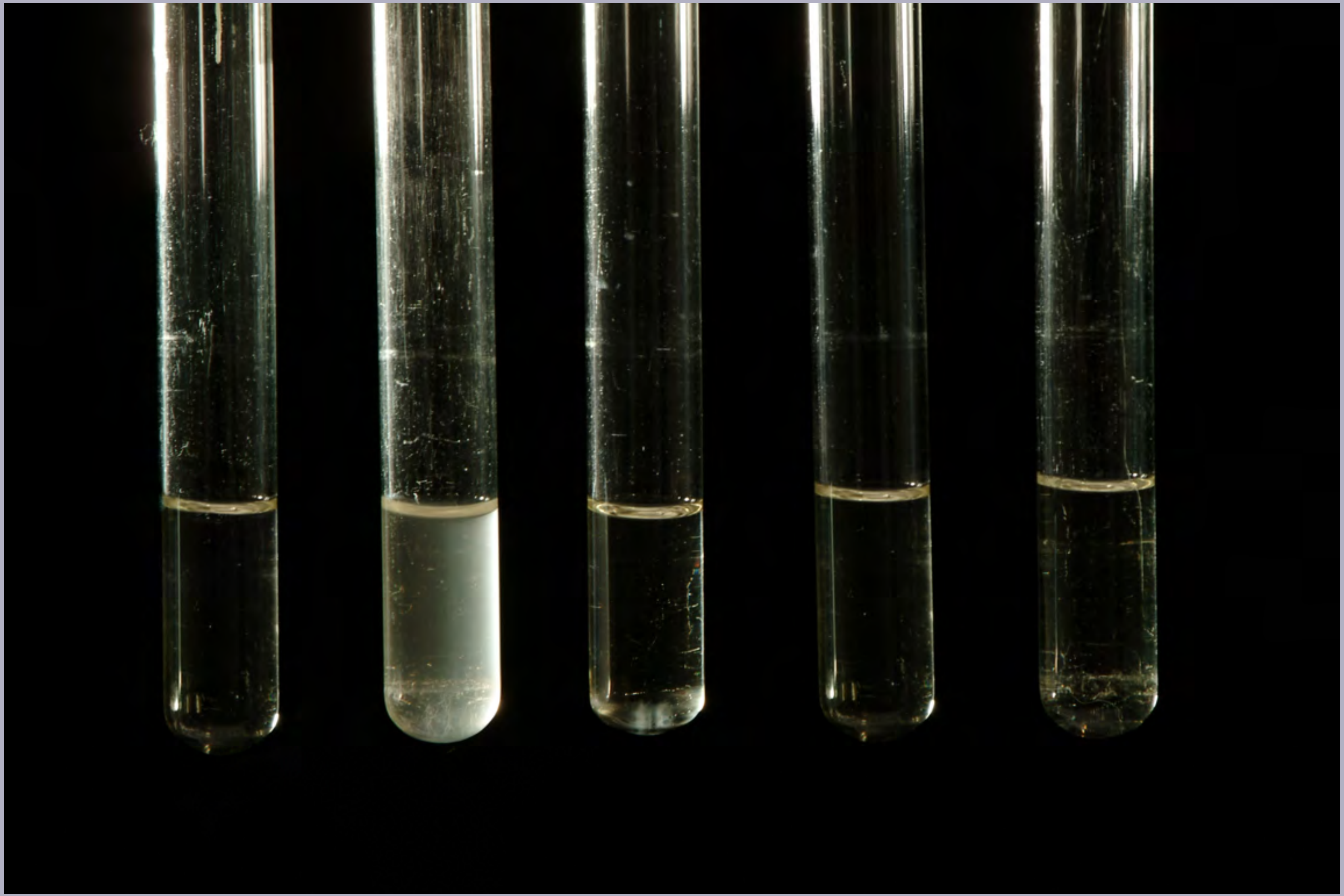
- Cause of wound infections & primary septicaemia following ingestion of oysters
- Curved & straight GNR
- OX+/Fermenter
- Growth on TCBS = green
- Key tests: O/129 = S
- Salt tolerance to 6% (65%)
- Fermentation of sucrose -, salicin +, cellobiose +, lactose + >75%

Vibrio vulnificus

Green colonies on TCBS
at 24 hrs

Vibrio vulnificus
colonies on HBA at 24 hrs

Vibrio vulnificus – Salt Tolerance



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Vibrio parahaemolyticus

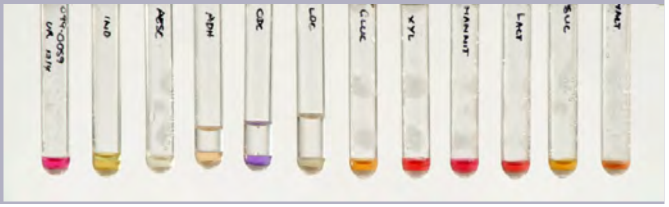
Special features

- Cause of acute GE – consumption of contaminated seafood worldwide
- Curved GNR
- OX+/Fermenter
- TCBS = green
- Key tests: O/129 = S/R, salt tolerance to 8% (most strains), fermentation of sucrose -, salicin -, lactose -, cellobiose -, urea + (50% of strains)

The “HACEK” Group

What defines this group of organisms?

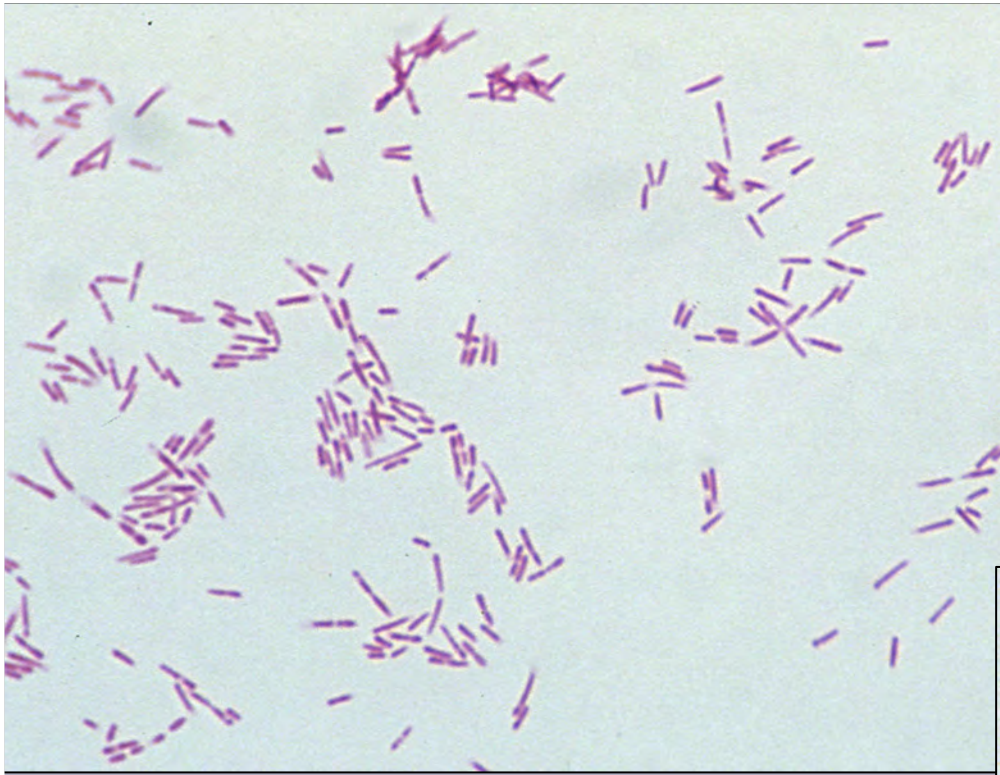
- Gram stain morphology
- Cultural characteristics – e.g. β-haemolysis i.e. *K. kingae,* pitting colonies, no growth on MAC, colony appearance i.e. dry, adherent, mixed appearance, slow growth rate
- Initial requirement for CO_2 and X factor (lost on subculture)
- Key biochemical characteristics – e.g. indole (*C. hominis*)
- Best identified using a rapid substrate method



Cardiobacterium hominis

Key features

- Faintly stained Gram-negative regular rods
- "Comets" and rosette arrangements
- Colonies whitish, shiny, may pit the agar
- Key test: Fermentative, oxidase + cat -, indole +
- Differentiate from indole + *Suttonella (Kingella) indologenes*



Cardiobacterium hominis

gs x1000

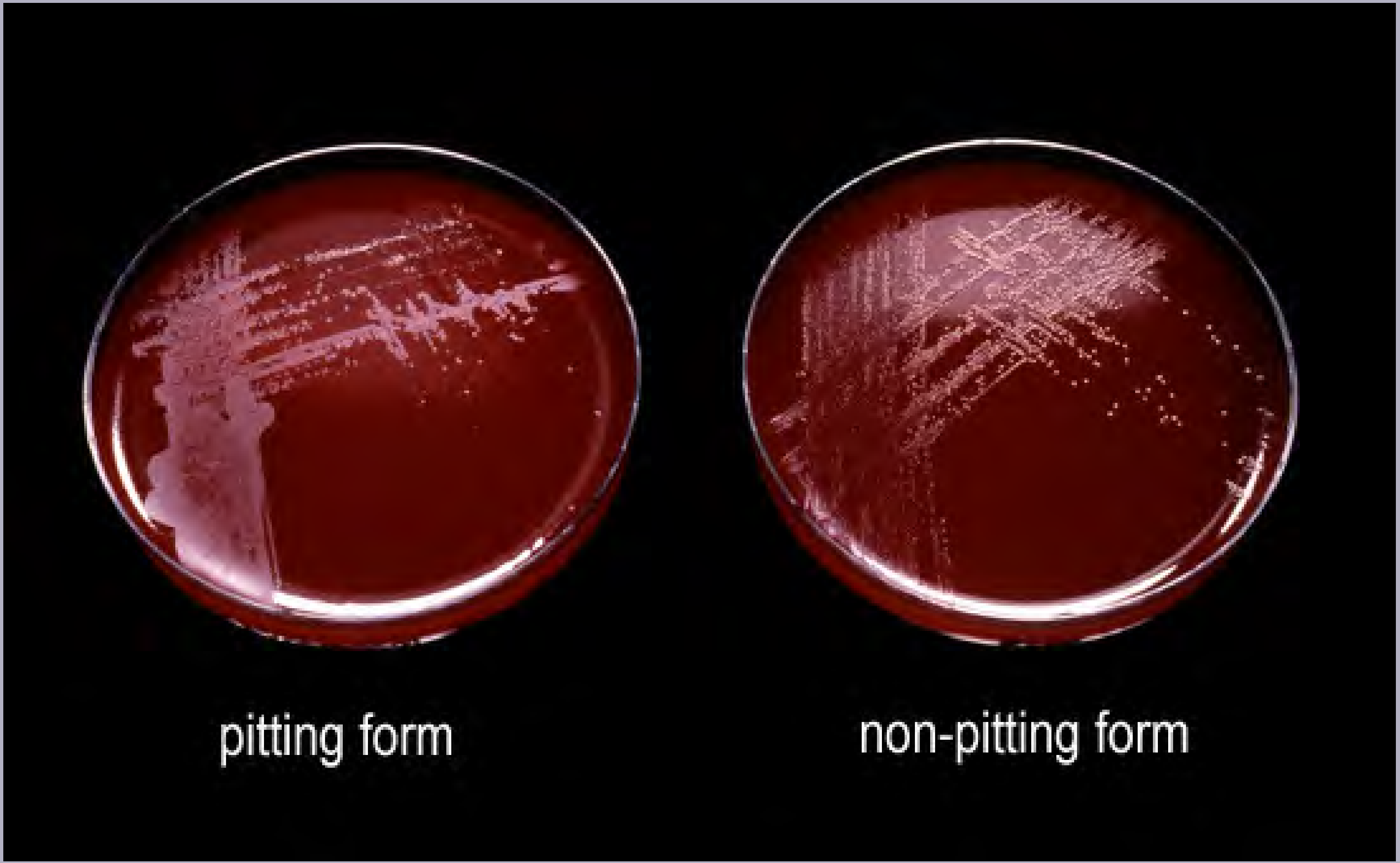
note ‘comet tails’

Eikenella corrodens

Key features

- Faintly stained slender, straight sided, very regular gram-negative rods
- May be X-dependent on isolation (ignore this)
- OX +, CAT -
- Pitting and non-pitting colony forms
- Key tests: Asaccharolytic, NO_3 +, ODC +, LDC +

Eikenella corrodens - gs x1000

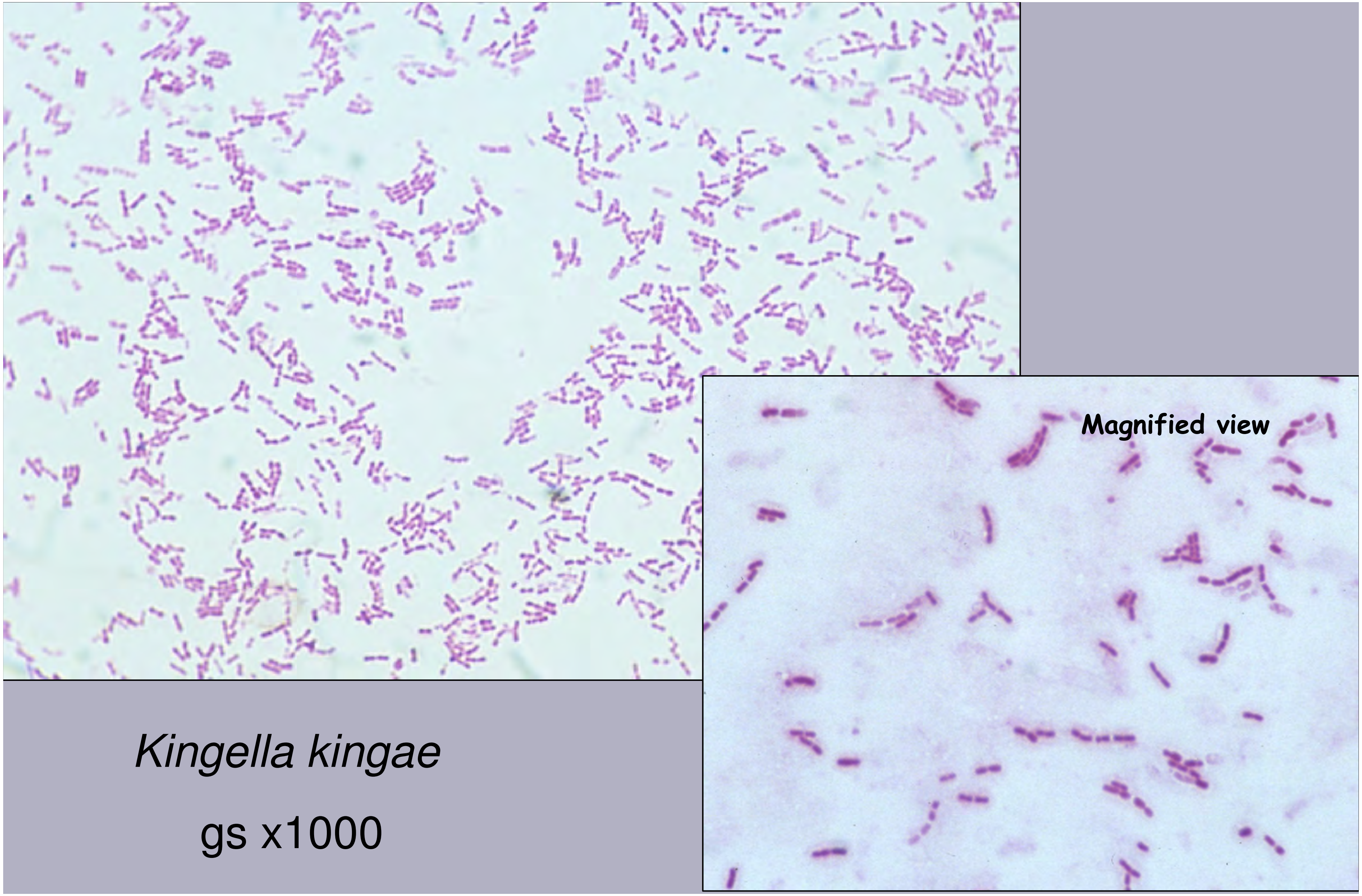


Eikenella corrodens - HBA CO_2 @ 48hrs

Kingella kingae

Key features

- Plump Gram-negative coccobacilli in pairs & short chains, parallel rows & “railway tracks”
- “Soft” β-haemolysis on HBA
- Acid from glucose & maltose – has been confuse with *N. meningitidis*
- Associated with bone and joint disease in children



Kingella kingae

gs x1000

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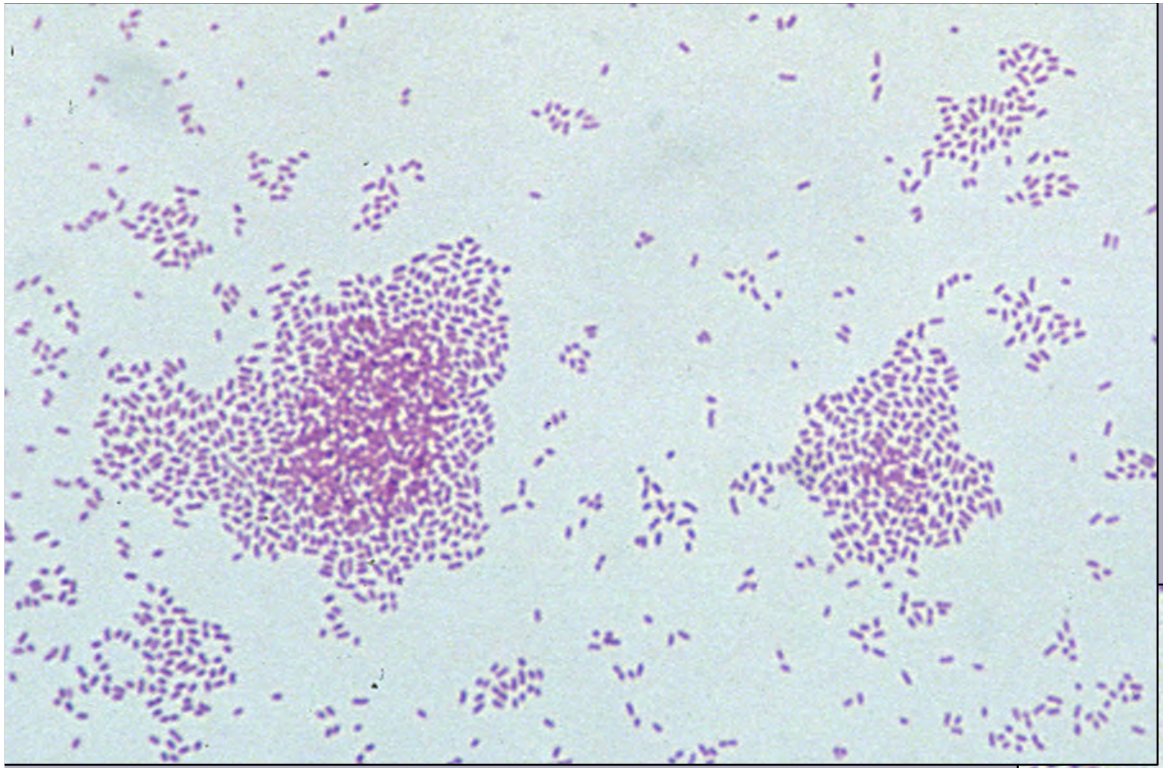
Kingella kingae, HBA, CO_2 at 48hrs

Aggregatibacter aphrophilus

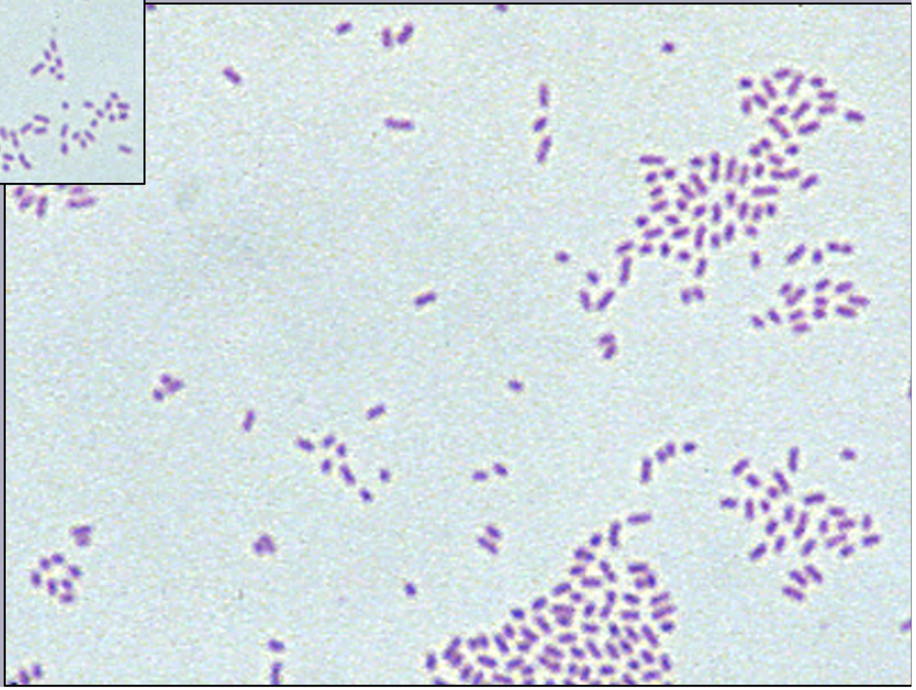
Key features

- Small Gram negative coccobacilli
- Capnophilic
- Strong α-haemolysis on HBA
- Culture may look mixed
- Initial requirement for X factor
- Distinguish *A. actinomycetemcomitans,* catalase +, from *A. aphrophilus*, catalase -



Aggregatibacter aphrophilus

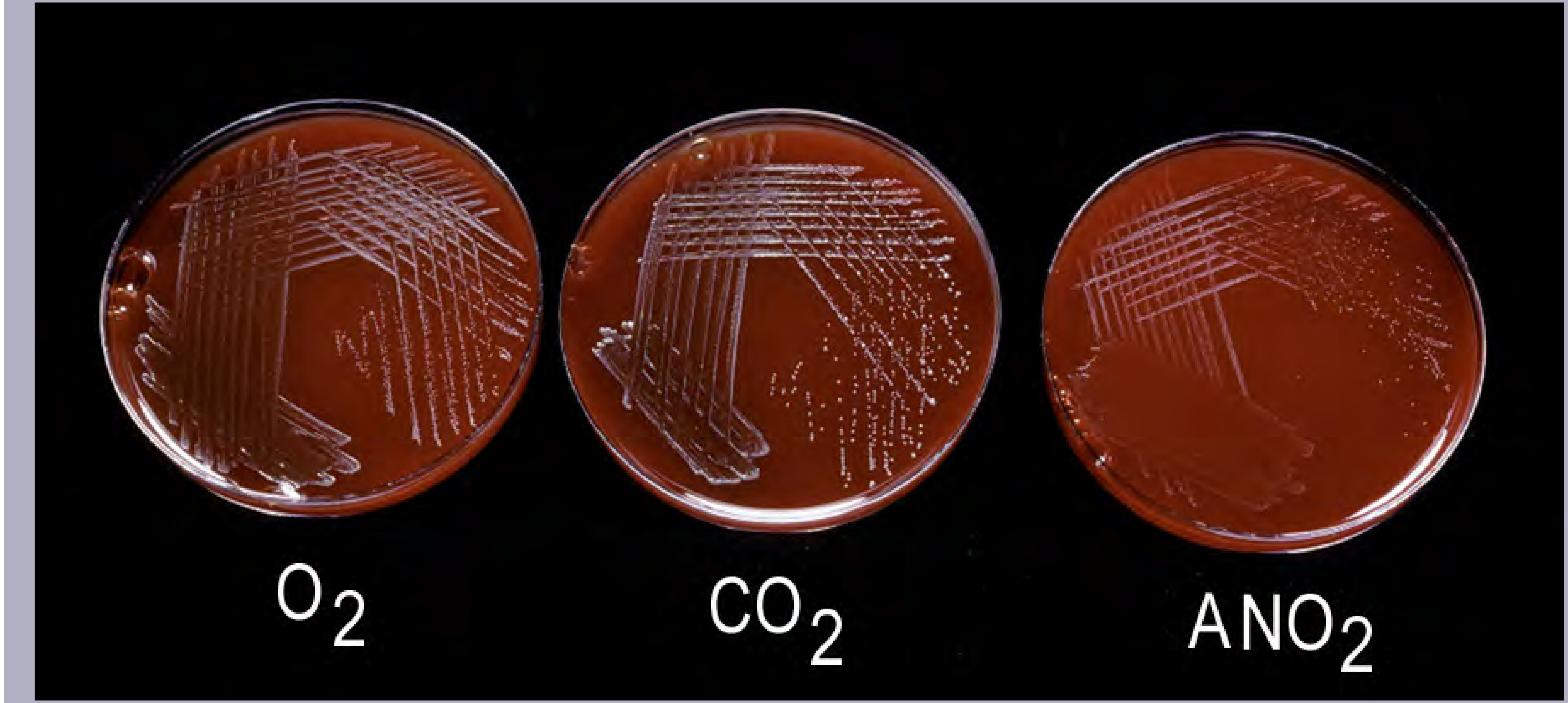
gs x1000



A. aphrophilus now includes

A. paraphrophilus

(V dependent strain)



Aggregatibacter aphrophilus, HBA at 48hrs

Aggregatibacter actinomycetemcomitans

Key features

- Tiny Gram-negative coccobacilli
- Capnophilic
- Colonies adherent, white, dry
- Fermentative
- Distinguish from *Brucella* spp. – similar Gram stain
- Distinguish from *A. aphrophilus* (Catalase -)

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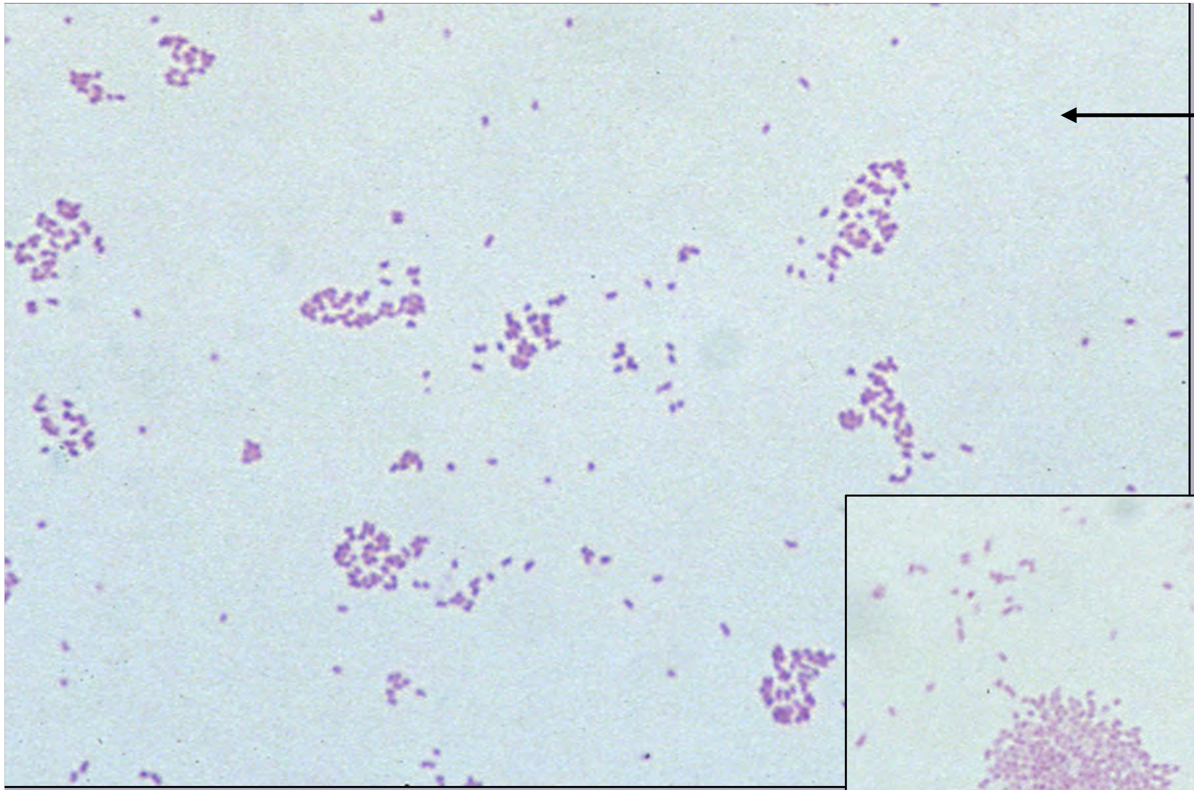
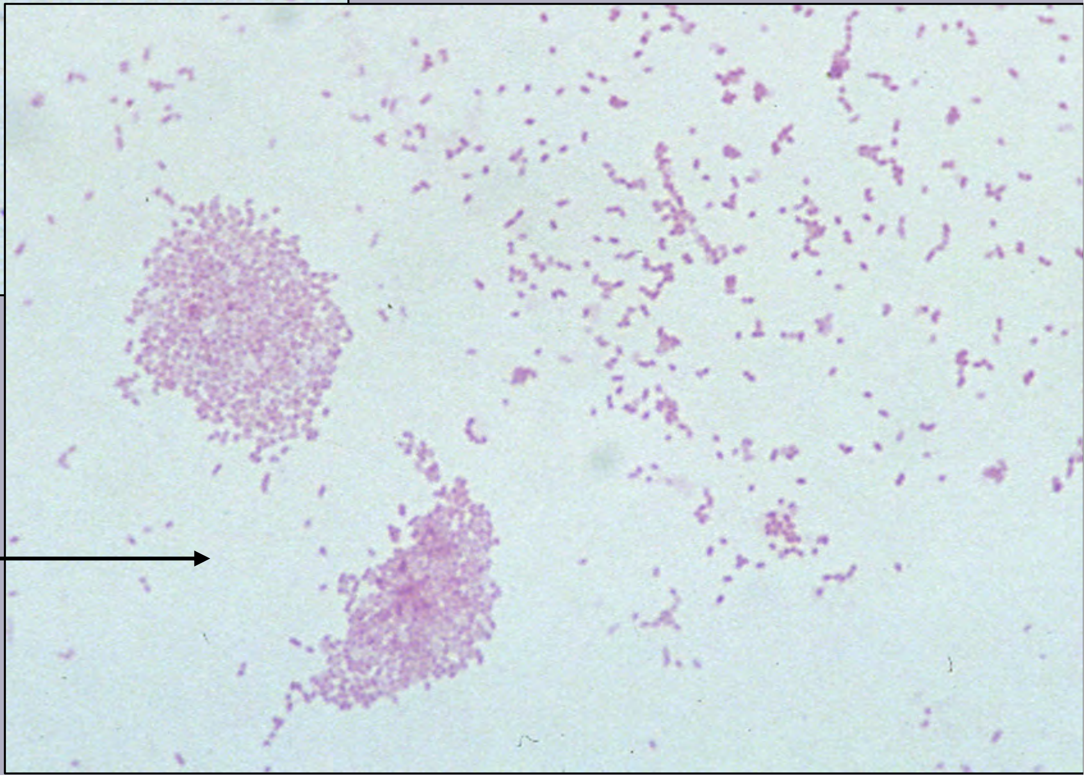


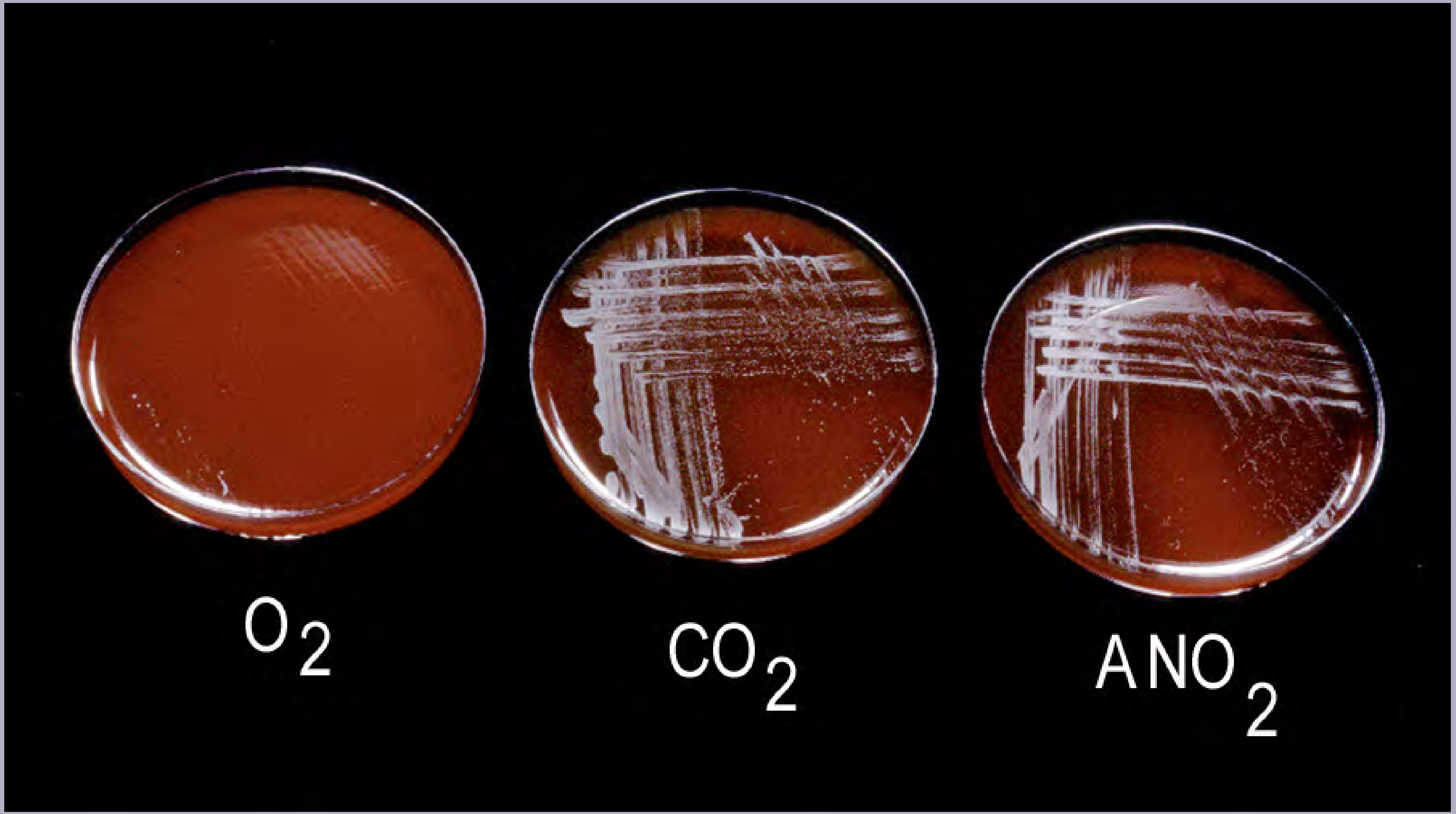
Aggregatibacter actinomycetemcomitans

gs x1000



Brucella melitensis

gs x1000



Aggregatibacter actinomycetemcomitans – HBA plates @ 48hours.
Growth conditions for *B. melitensis* would be reversed – it is a strict aerobe!

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Anaerobiospirillum succiniciproducens

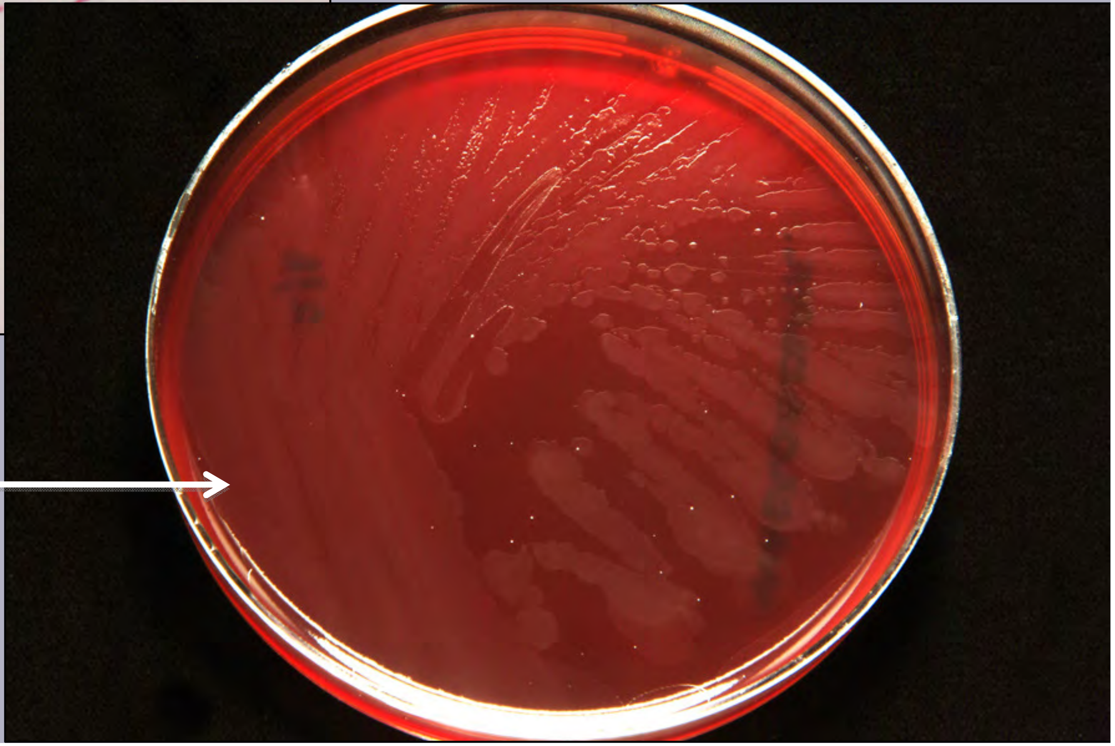
Key features

- Important to recognise that this is not a campylobacter!
- Gram-negative helical rods with rounded ends
- Motile +++ - corkscrew
- Strict anaerobe – not microaerophilic
- Colonies clear, flat, spreading
- Catalase -, Oxidase –
- Key tests: Glucose, indoxyl acetate, nitrate, urea, H_2S in SIM media

Anaerobiospirillum

succiniciproducens

gs x1000



Swarming on HBA

plate after 48 hrs

anaerobic incubation

CIDM

centre for infectious diseases & microbiology

Public Health

RCPA

The Royal College of Pathologists of Australasia

A final cautionary note~

Increasingly sophisticated identification methods are becoming available, improving accuracy and turn around times. We welcome these advancements which result in improved patient outcomes.

However we must not neglect basic microbiology skills in the belief that automation can replace them – we must recognise a microorganism of significance before it can be identified – some detective work and sound microbiology are required.

Thank you for listening

Any Questions?