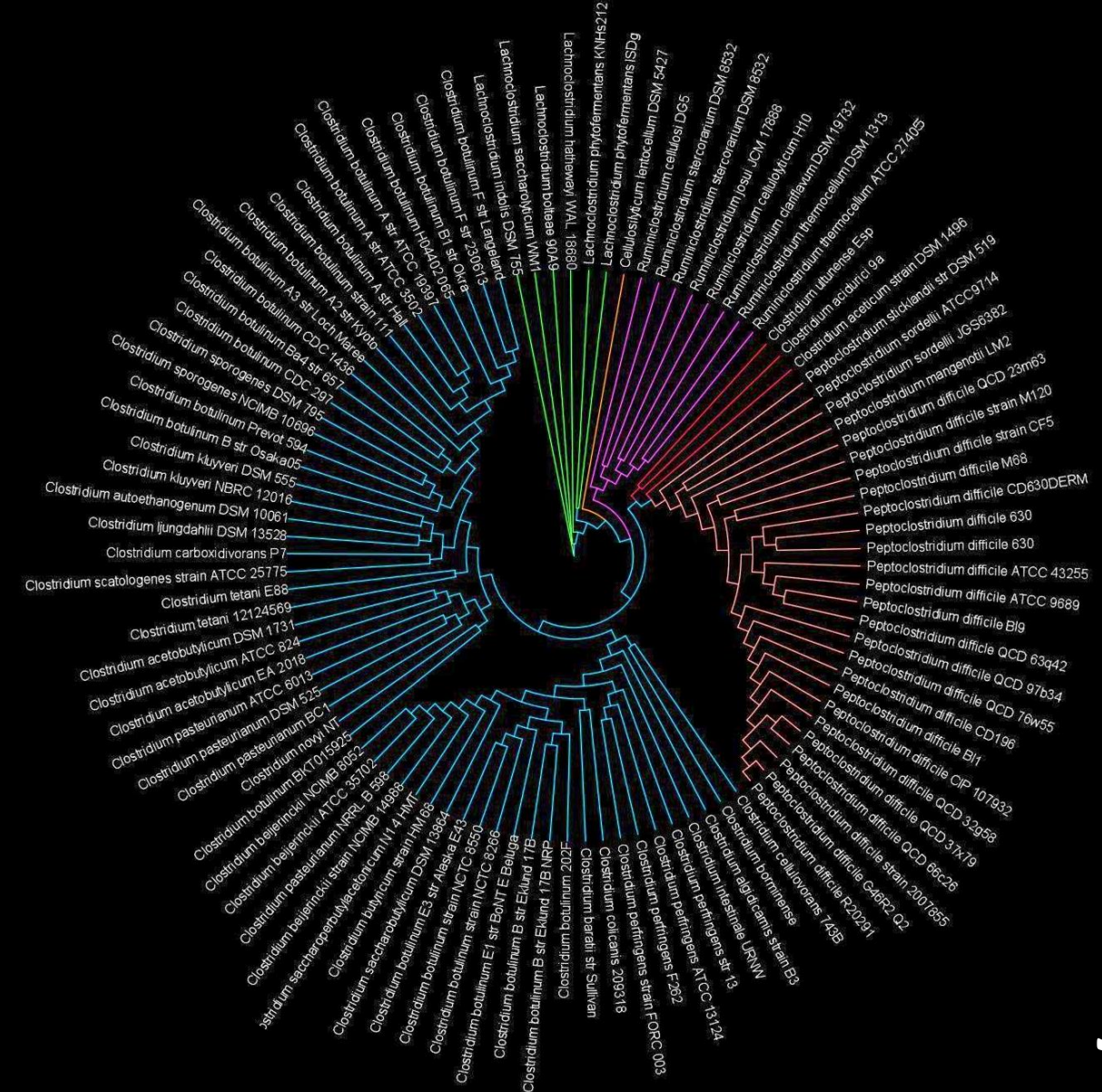

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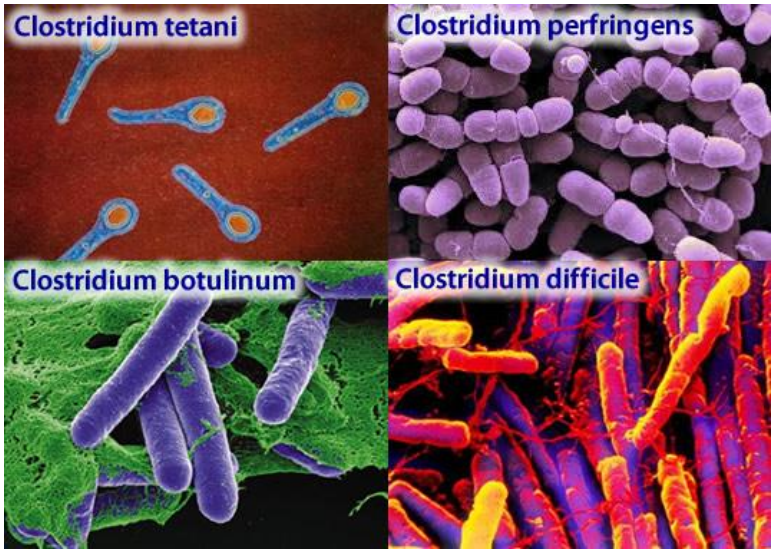
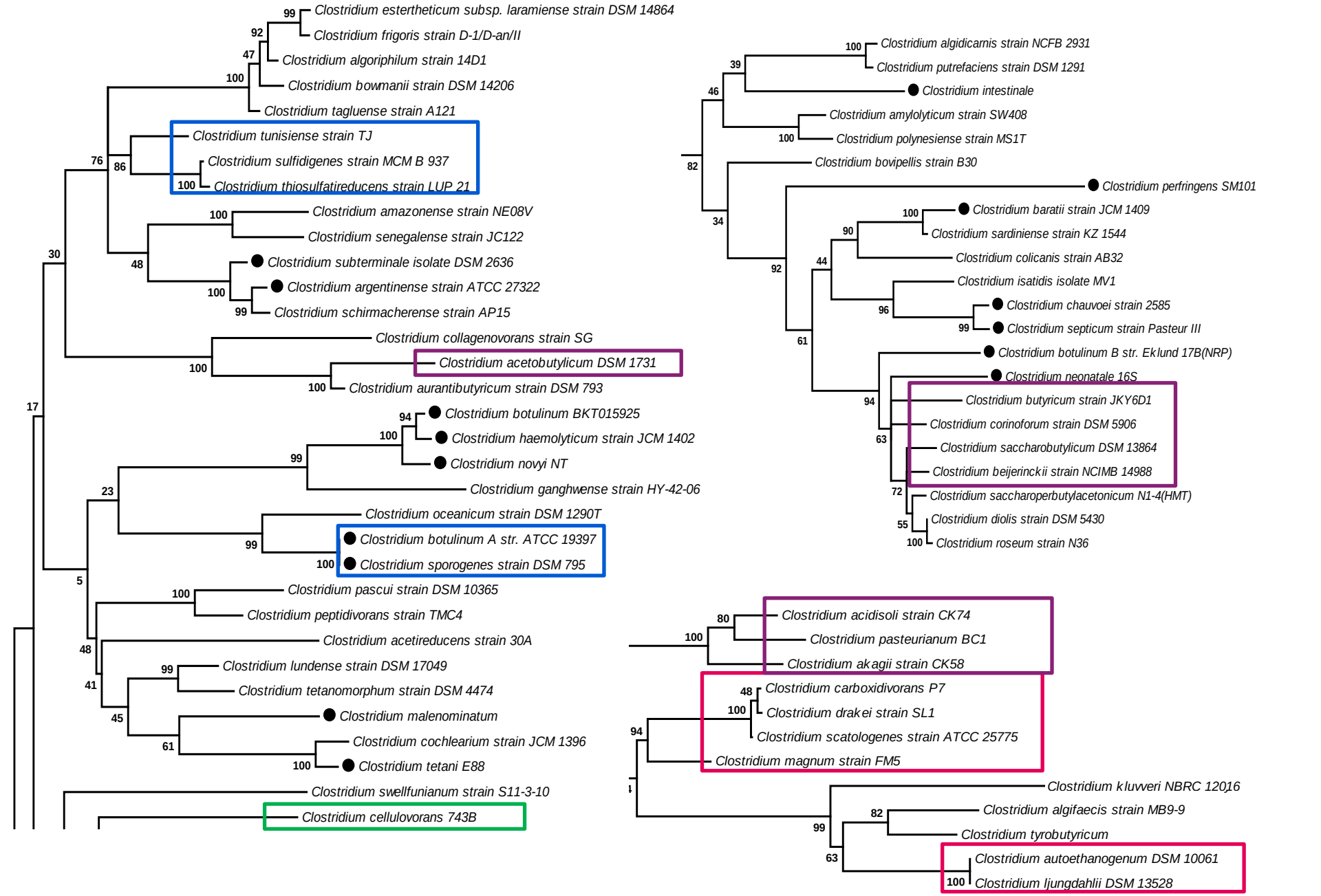
Joseph Palmer

Clostridium in the environment

What is *Clostridium?*

- **Bacteria> Firmicutes > Clostridia > Clostridales > Clostridiaceae > *Clostridium***
- **Contains many species of medical and biotechnological importance**
- **Highly pleomorphic, with heterogeneous phenotype and genotype**
- **Genus subject to recent taxonomic reshuffling**




Clostridium estertheticum subsp. laramiense strain DSM 14864
Clostridium frigoris strain D-1/D-an/II
Clostridium algoriphilum strain 14D1
Clostridium bowmanii strain DSM 14206
Clostridium tagluense strain A121
Clostridium tunisiense strain TJ
Clostridium sulfidigenes strain MCM B 937
Clostridium thiosulfatireducens strain LUP 21
Clostridium amazonense strain NE08V
Clostridium senegalense strain JC122
Clostridium subterminale isolate DSM 2636
Clostridium argentinense strain ATCC 27322
Clostridium schirmacherense strain AP15
Clostridium collagenovorans strain SG
Clostridium acetobutylicum DSM 1731
Clostridium aurantibutyricum strain DSM 793
Clostridium botulinum BKT015925
Clostridium haemolyticum strain JCM 1402
Clostridium novyi NT
Clostridium ganghwense strain HY-42-06
Clostridium oceanicum strain DSM 1290T
Clostridium botulinum A str. ATCC 19397
Clostridium sporogenes strain DSM 795
Clostridium pascui strain DSM 10365
Clostridium peptidivorans strain TMC4
Clostridium acetireducens strain 30A
Clostridium lundense strain DSM 17049
Clostridium tetanomorphum strain DSM 4474
Clostridium malenominatum
Clostridium cochlearium strain JCM 1396
Clostridium tetani E88
Clostridium swellfunianum strain S11-3-10
Clostridium cellulovorans 743B
Clostridium algidicarnis strain NCFB 2931
Clostridium putrefaciens strain DSM 1291
Clostridium intestinale
Clostridium amylolyticum strain SW408
Clostridium polynesiense strain MS1T
Clostridium bovipellis strain B30
Clostridium perfringens SM101
Clostridium baratii strain JCM 1409
Clostridium sardiniense strain KZ 1544
Clostridium colicanis strain AB32
Clostridium isatidis isolate MV1
Clostridium chauvoei strain 2585
Clostridium septicum strain Pasteur III
Clostridium botulinum B str. Eklund 17B(NRP)
Clostridium neonatale 16S
Clostridium butyricum strain JKY6D1
Clostridium corinoforum strain DSM 5906
Clostridium saccharobutylicum DSM 13864
Clostridium beijerinckii strain NCIMB 14988
Clostridium saccharoperbutylacetonicum N1-4(HMT)
Clostridium diolis strain DSM 5430
Clostridium roseum strain N36
Clostridium acidisoli strain CK74
Clostridium pasteurianum BC1
Clostridium akagii strain CK58
Clostridium carboxidivorans P7
Clostridium drakei strain SL1
Clostridium scatologenes strain ATCC 25775
Clostridium magnum strain FM5
Clostridium kluyveri NBRC 12016
Clostridium algifaecis strain MB9-9
Clostridium tyrobutyricum
Clostridium autoethanogenum DSM 10061
Clostridium ljungdahlii DSM 13528

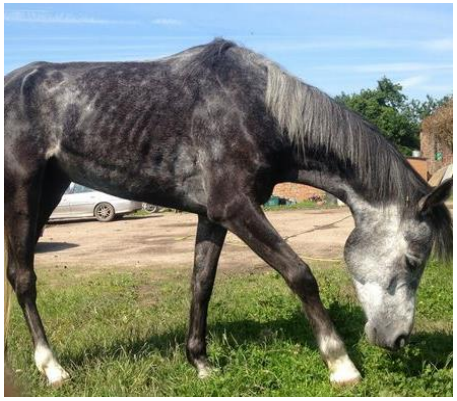
Pathogenesis

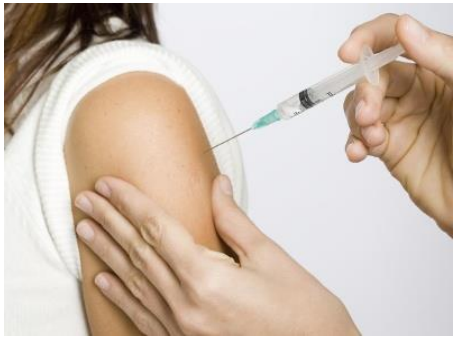
***C. botulinum* .....** botulism, Botox, EGS

***C. tetani* .....** tetanus

***C. perfringens* .....** gas gangrene / food poisoning

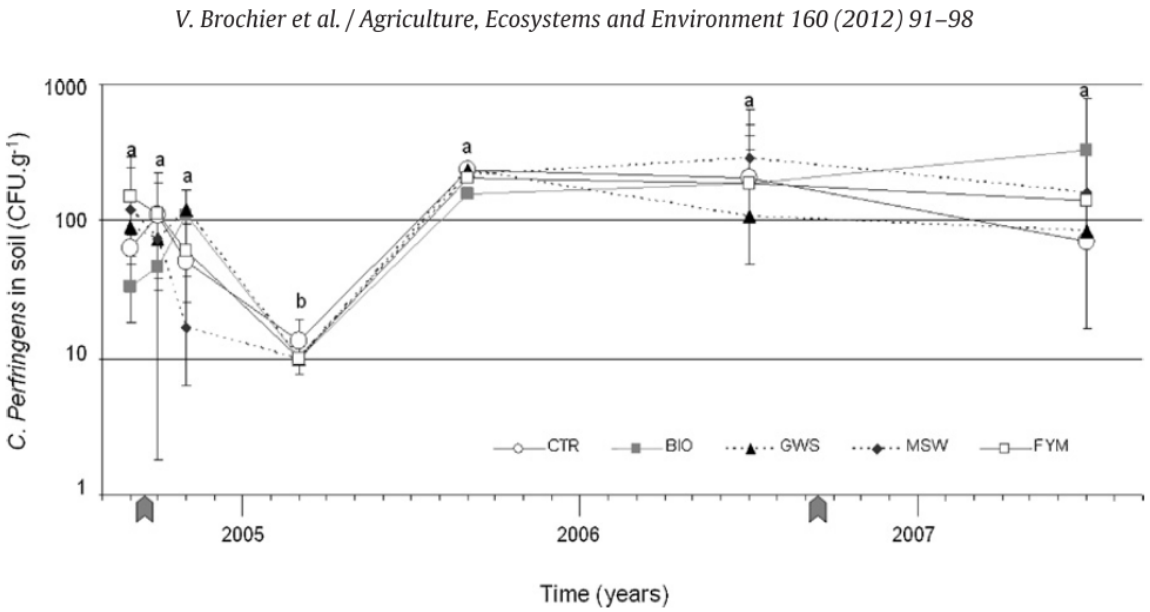
***C.* difficile* .....** antibiotic-associated pseudomembranous colitis





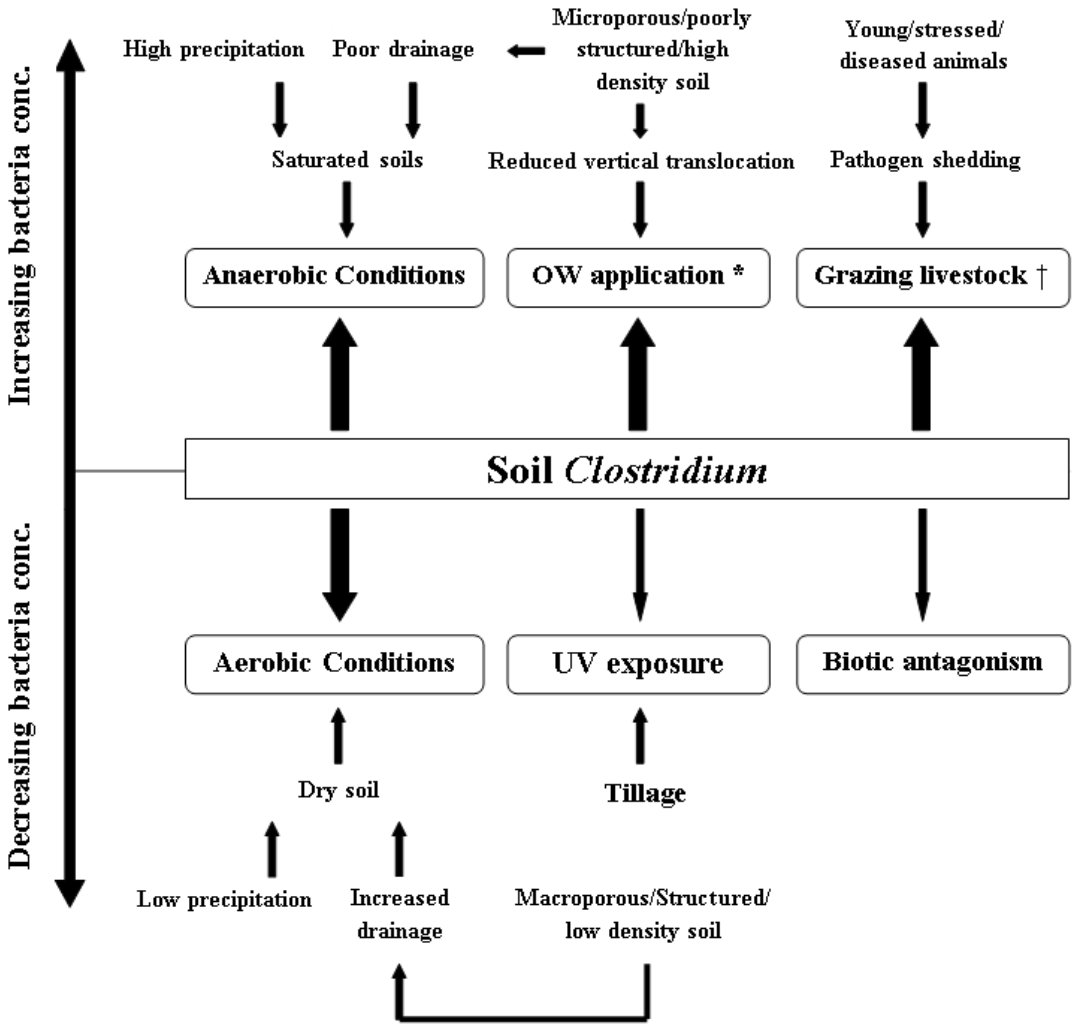
- ***C. septicum, C. chauvoei, C. baratii, C. argentinense, C. haemolyticum, C. intestinalis, C. noyvi, C. subterminale*** **and others…**
- *** *C. difficile* has recently been reclassified as *Peptoclostridium difficile***

Clostridium in the Environment



- **Ubiquitous in many natural environments**
- **Spores- soils, marine & estuarine sediments**
- **Natural intestinal microbiota- faeces, sewage & organic amendments**

Soil Growth Factors



Palmer *et al.* (unpublished work)

What is required?

- More prevalence data on multiple pathogenic spp. across environments
- Key growth data on Clostridium pathogens in the soil

- A molecular tool for multiple *Clostridium* pathogen screening and differentiation
- Determination of what species are truly *Clostridium*

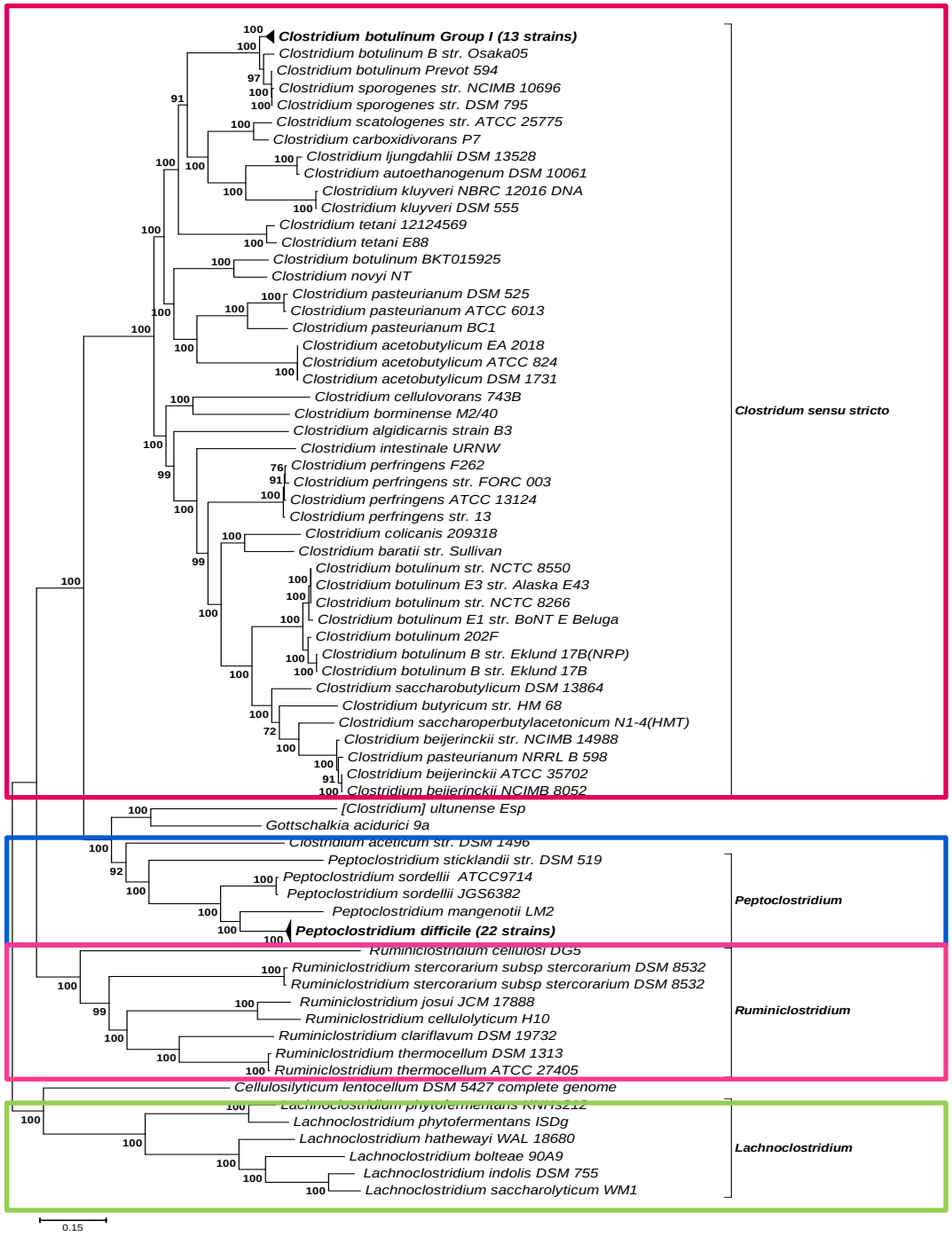
1. Taxonomic Revision

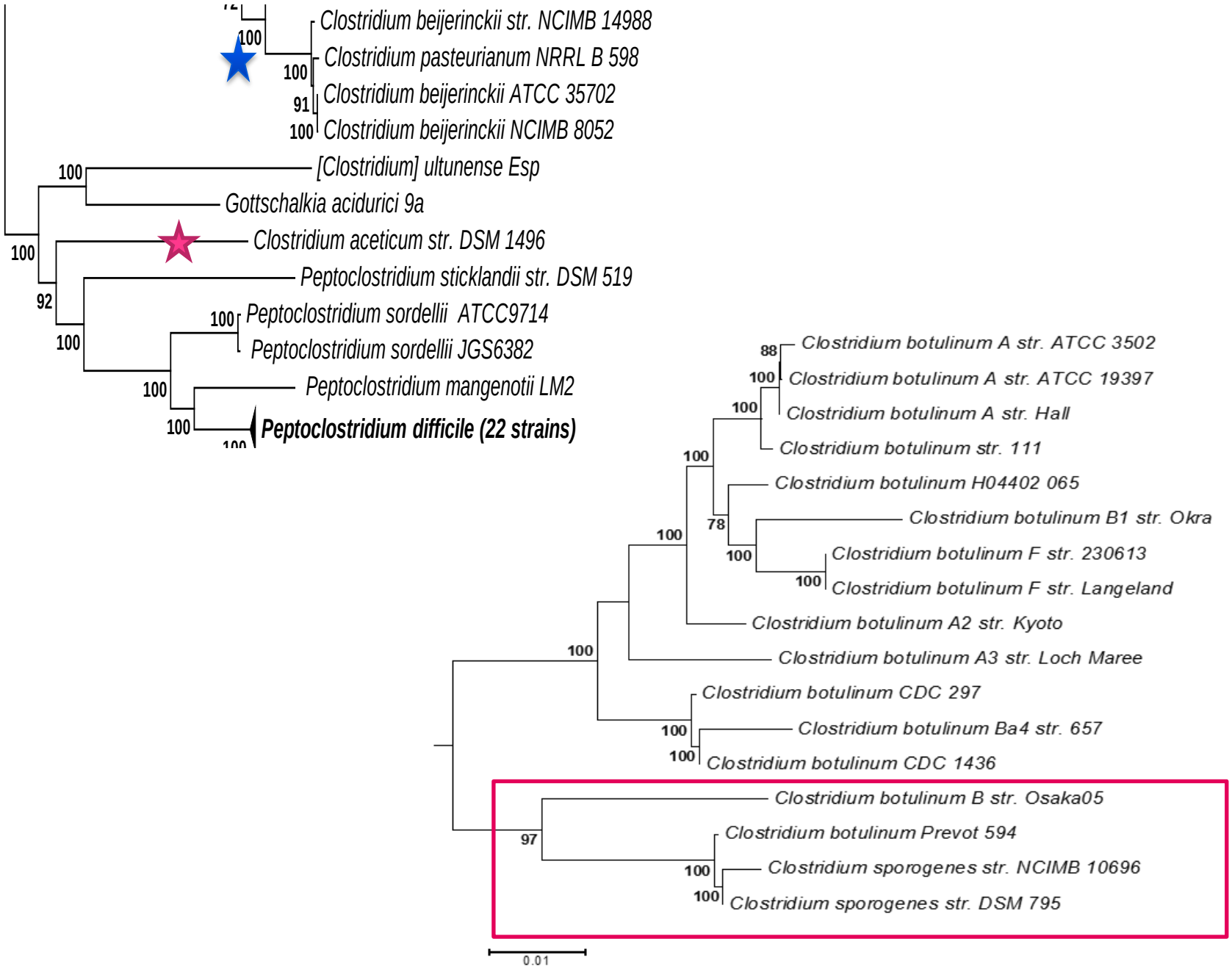
- **Previous Clostridial taxonomy based on phenotypic/ 16S rDNA**
- **Recent research reassigned 78 previous *Clostridium* spp. to other genera[1]**
  - *C. difficile* → *Peptoclostridium difficile*
- **Nomenclature of some spp. differs between resources → imperative to deduce true phylogeny**

[1] Yutin and Galperin (2013). Environ. Microbiol. v15, 10

Method

- Utilised 101 complete genome sequences on NCBI
- Assessed 206 housekeeping genes for their homology amongst clostridial representatives
- 50 suitable genes were aligned and trimmed
- Created 50 housekeeping gene concatenation
- Used NJ, ML & BI models to create phylogeny using CIPRES platform


Clostridium botulinum Group I (13 strains)
Clostridium botulinum B str. Osaka05
Clostridium botulinum Prevot 594
Clostridium sporogenes str. NCIMB 10696
Clostridium sporogenes str. DSM 795
Clostridium scatologenes str. ATCC 25775
Clostridium carboxidivorans P7
Clostridium ljungdahlii DSM 13528
Clostridium autoethanogenum DSM 10061
Clostridium kluyveri NBRC 12016 DNA
Clostridium kluyveri DSM 555
Clostridium tetani 12124569
Clostridium tetani E88
Clostridium botulinum BKT015925
Clostridium novyi NT
Clostridium pasteurianum DSM 525
Clostridium pasteurianum ATCC 6013
Clostridium pasteurianum BC1
Clostridium acetobutylicum EA 2018
Clostridium acetobutylicum ATCC 824
Clostridium acetobutylicum DSM 1731
Clostridium cellulovorans 743B
Clostridium borminense M2/40
Clostridium algidicarnis strain B3
Clostridium intestinale URNW
Clostridium perfringens F262
Clostridium perfringens str. FORC 003
Clostridium perfringens ATCC 13124
Clostridium perfringens str. 13
Clostridium colicanis 209318
Clostridium baratii str. Sullivan
Clostridium botulinum str. NCTC 8550
Clostridium botulinum E3 str. Alaska E43
Clostridium botulinum str. NCTC 8266
Clostridium botulinum E1 str. BoNT E Beluga
Clostridium botulinum 202F
Clostridium botulinum B str. Eklund 17B(NRP)
Clostridium botulinum B str. Eklund 17B
Clostridium saccharobutylicum DSM 13864
Clostridium butyricum str. HM 68
Clostridium saccharoperbutylacetonicum N1-4(HMT)
Clostridium beijerinckii str. NCIMB 14988
Clostridium pasteurianum NRRL B 598
Clostridium beijerinckii ATCC 35702
Clostridium beijerinckii NCIMB 8052
Clostridium sensu stricto
[Clostridium] ultunense Esp
Gottschalkia acidurici 9a
Clostridium aceticum str. DSM 1496
Peptoclostridium sticklandii str. DSM 519
Peptoclostridium sordellii ATCC9714
Peptoclostridium sordellii JGS6382
Peptoclostridium mangenotii LM2
Peptoclostridium difficile (22 strains)
Peptoclostridium
Ruminiclostridium cellulosi DG5
Ruminiclostridium stercorarium subsp stercorarium DSM 8532
Ruminiclostridium stercorarium subsp stercorarium DSM 8532
Ruminiclostridium josui JCM 17888
Ruminiclostridium cellulolyticum H10
Ruminiclostridium clariflavum DSM 19732
Ruminiclostridium thermocellum DSM 1313
Ruminiclostridium thermocellum ATCC 27405
Ruminiclostridium
Cellulosilyticum lentocellum DSM 5427 complete genome
Lachnoclostridium phytofermentans KNHS212
Lachnoclostridium phytofermentans ISDg
Lachnoclostridium hathewayi WAL 18680
Lachnoclostridium bolteae 90A9
Lachnoclostridium indolis DSM 755
Lachnoclostridium saccharolyticum WM1
Lachnoclostridium
0.15


Clostridium beijerinckii str. NCIMB 14988
Clostridium pasteurianum NRRL B 598
Clostridium beijerinckii ATCC 35702
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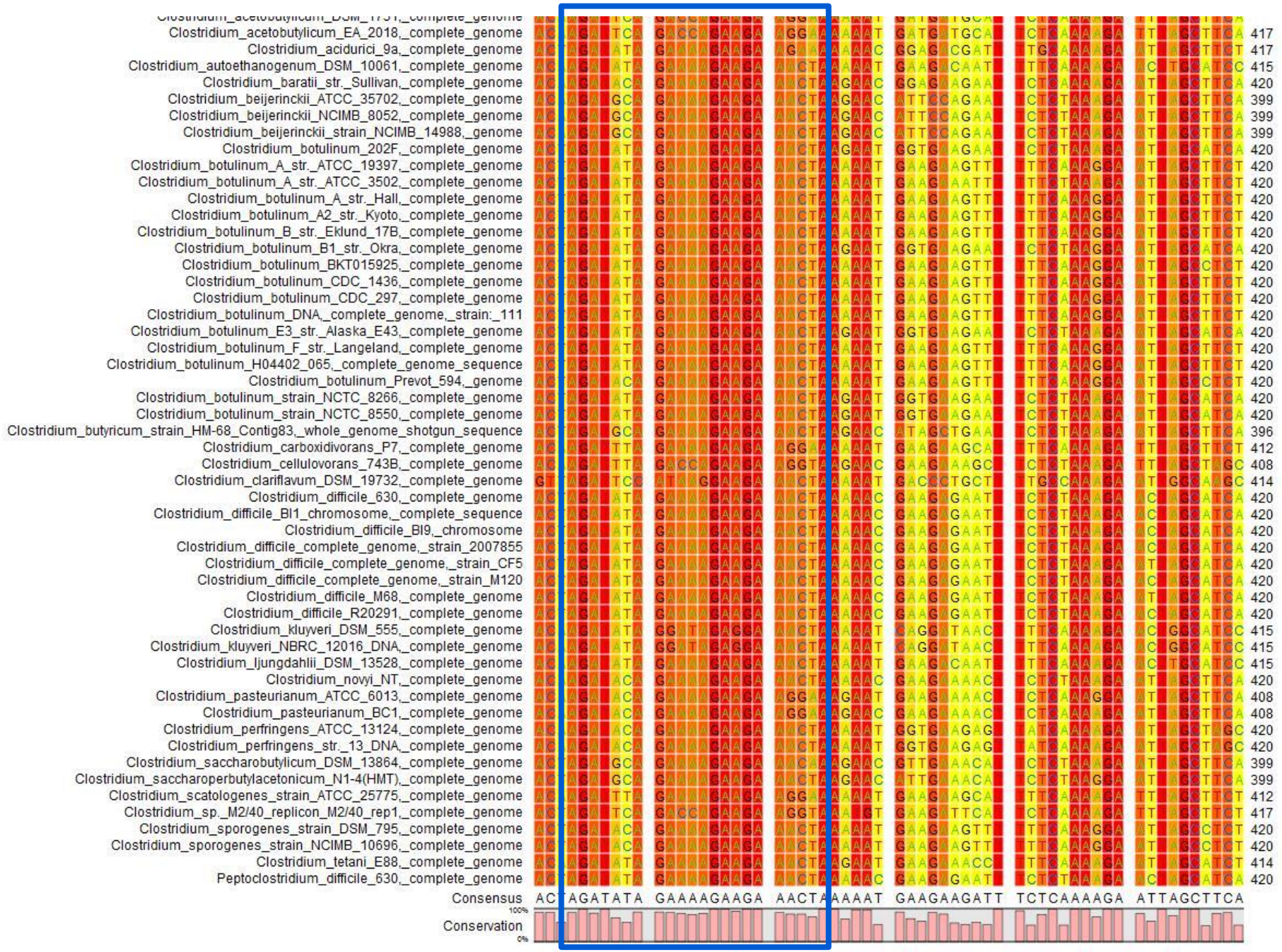
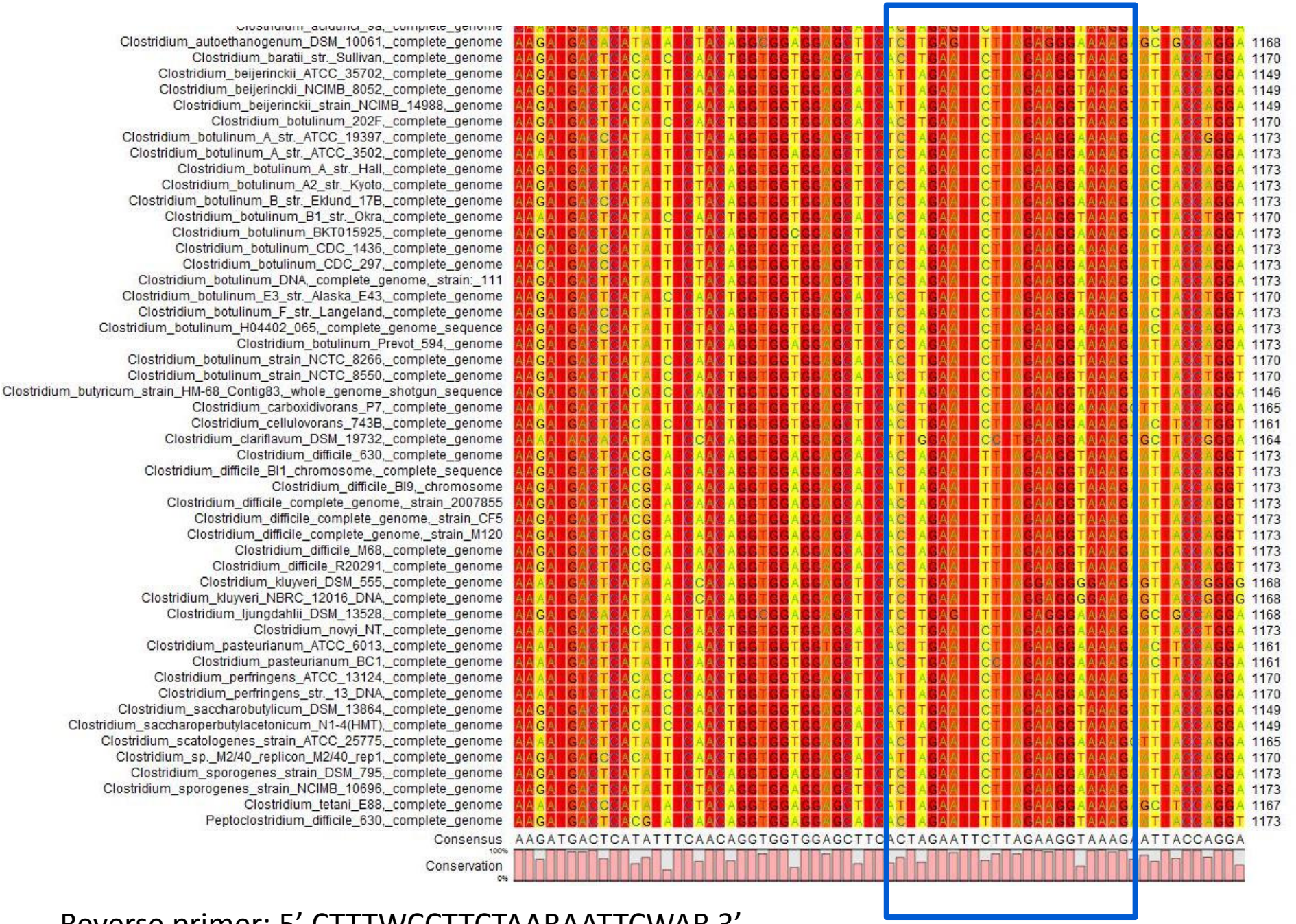
Peptoclostridium mangenotii LM2
Peptoclostridium difficile (22 strains)
Clostridium botulinum A str. ATCC 3502
Clostridium botulinum A str. ATCC 19397
Clostridium botulinum A str. Hall
Clostridium botulinum str. 111
Clostridium botulinum H04402 065
Clostridium botulinum B1 str. Okra
Clostridium botulinum F str. 230613
Clostridium botulinum F str. Langeland
Clostridium botulinum A2 str. Kyoto
Clostridium botulinum A3 str. Loch Maree
Clostridium botulinum CDC 297
Clostridium botulinum Ba4 str. 657
Clostridium botulinum CDC 1436
Clostridium botulinum B str. Osaka05
Clostridium botulinum Prevot 594
Clostridium sporogenes str. NCIMB 10696
Clostridium sporogenes str. DSM 795
0.01

2. Protocol Development

- **Require a rapid, high-throughput molecular method for multi-pathogen detection & enumeration**
- **16S rRNA gene too conserved- can't be specific to *Clostridium &* discriminate between species**
- **Evaluated alternate genes to develop PCR-TRFLP assay to screen for target pathogens**
- **Pathogens present in environmental samples can be individual quantified using qPCR**

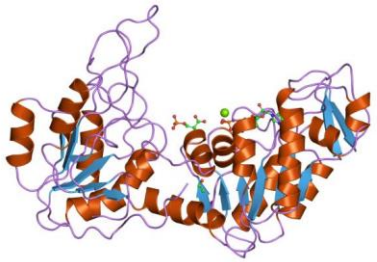


Forward primer: 5’ AGATAYAGAAAAGAAGARACTA 3’

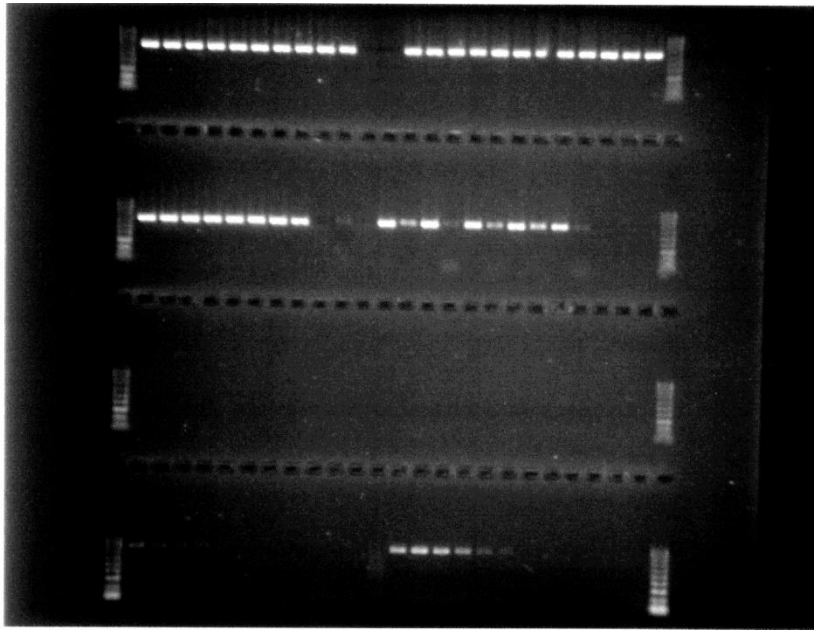


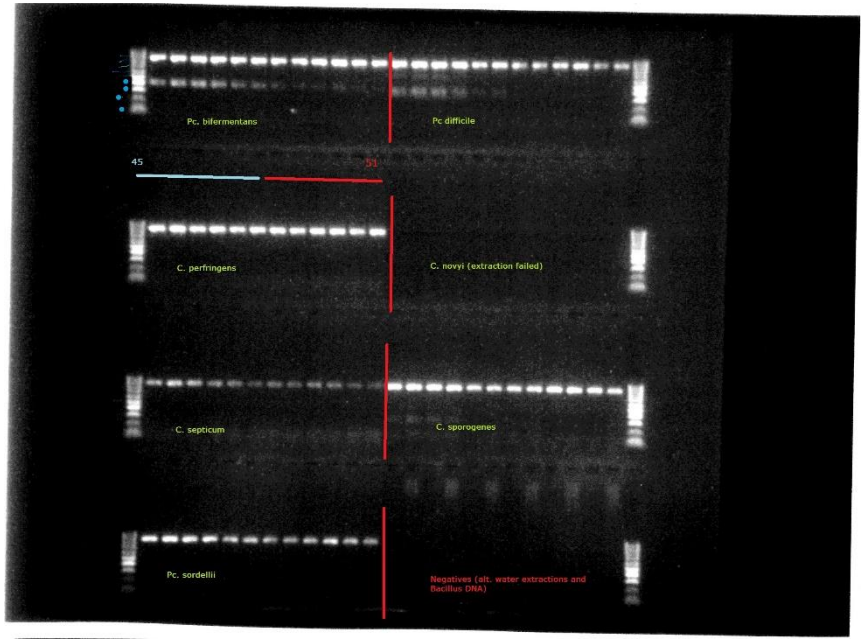
Reverse primer: 5’ CTTTWCCTTCTAARAATTCWAR 3’

In-silico analysis



- **Gene analysis of 70+ housekeeping genes: *PGK* gene (Phosphoglycerate kinase) was selected**
- **A range of F/R primer combination tested to give best specificity & PCR conditions**
- **HIGHLY SPECIFIC : Primer set developed that targets major 10 pathogen groups**
- **Tested on 8 *Clostridum/Peptoclostridium* target pathogens**



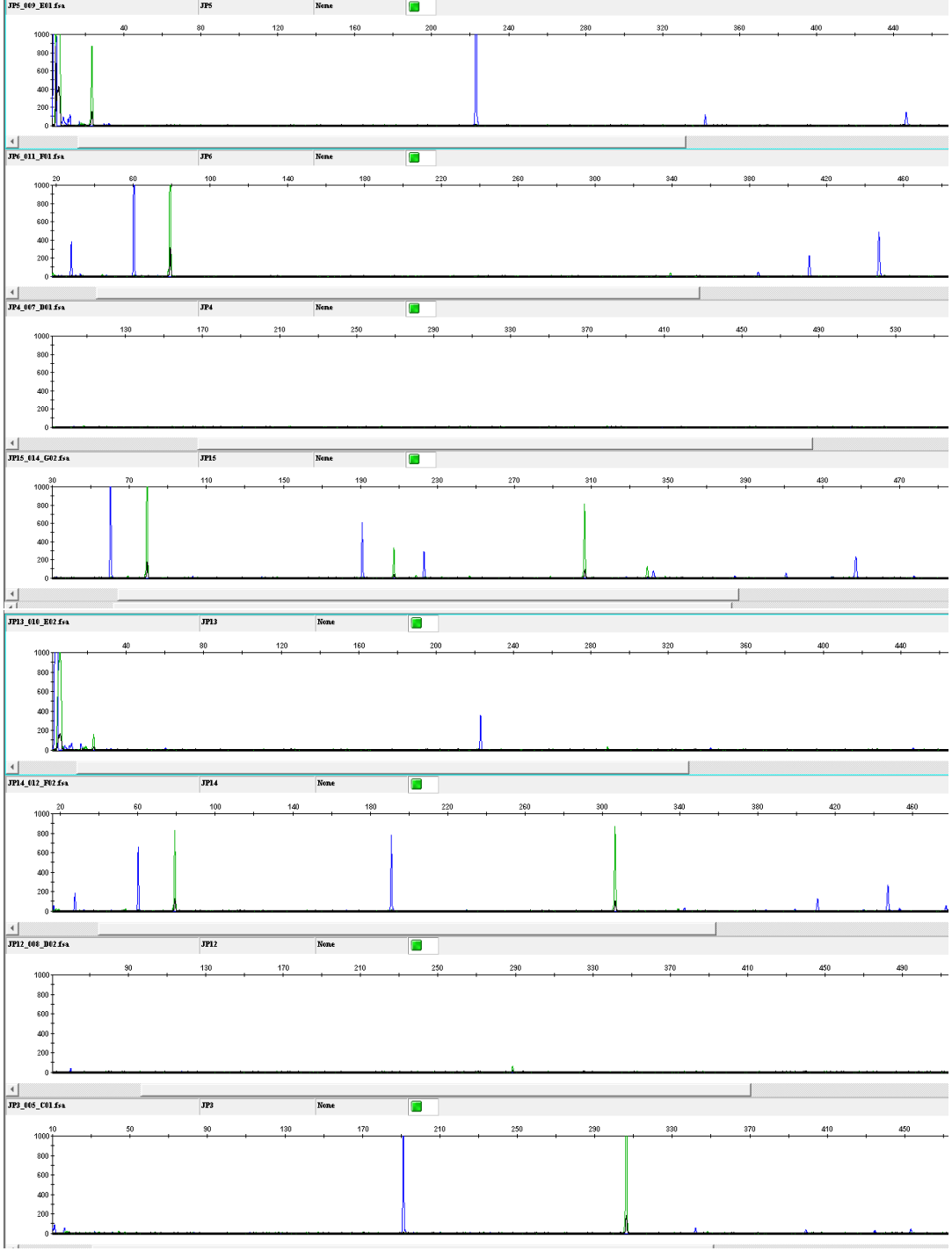

Pc. bifermentans
Pc difficile
45
51
C. perfringens
C. novyi (extraction failed)
C. septicum
C. sporogenes
Pc. sordellii
Negatives (alt. water extractions and Bacillus DNA)

Pathogen differentiation

- Used in-silico restriction digestion software to develop a T-RFLP protocol to differentiate between pathogen groups

- 799 BP amplicon can be digested with restriction enzymes SspI and SfcI to give a unique combination of peaks for each group (using labelled primers)

Clostridium baratii	707	89
Clostridium botulinum Group I	227	39
Clostridium sporogenes	227	39
Clostridium botulinum Group III	491	155
Clostridium haemolyticum	491	155
Clostridium botulinum Group II	458	292
Clostridium colicanis	458	89
Clostridium novyi	440	359
Clostridium perfringens	195	310
Clostridium tetani 12124569	85	591
Clostridium tetani E88	113	39
Peptoclostridium difficile	64	211
Peptoclostridium sordellii	64	85
Peptolostridium bifermentans	64	39



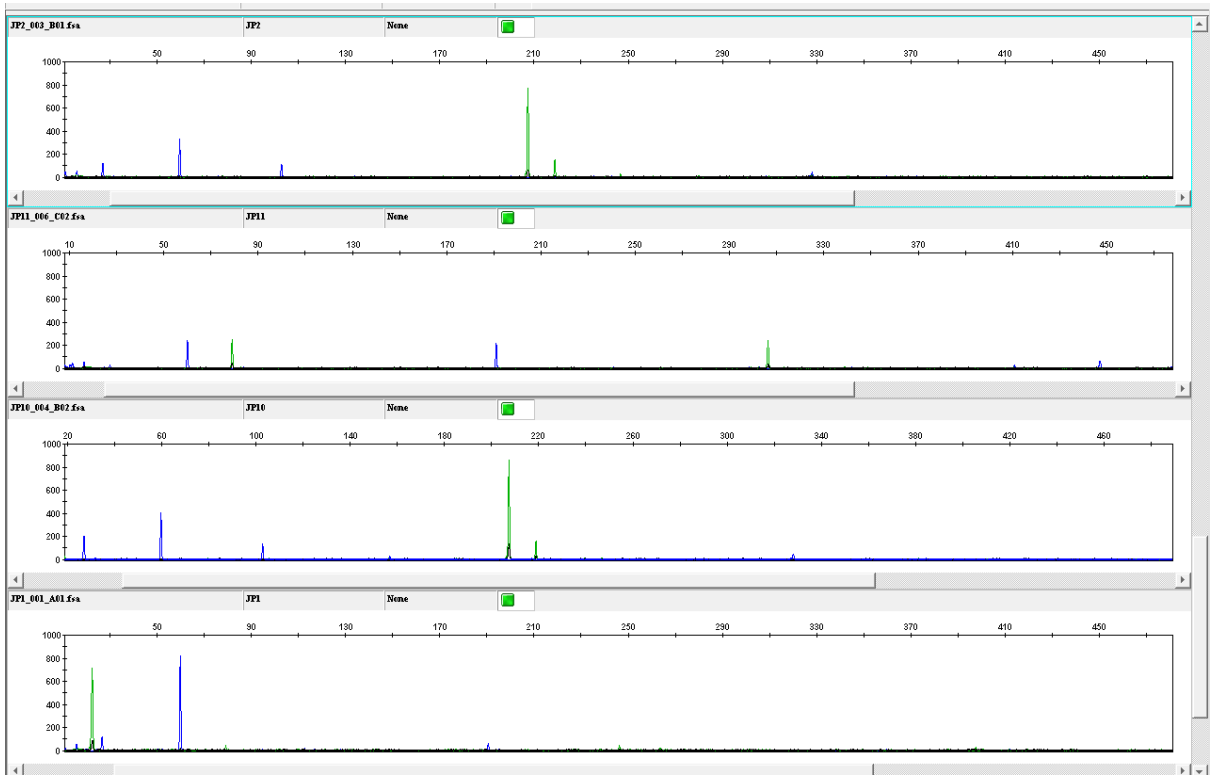
24, 223	*Sporogenes* (50ng)
79, 60	*Sordellii* (50ng)
	Bacillus Neg.
79, 306 60, 191, 447	Mix. (100ng)
24, 223	*Sporogenes* (25ng)
79, 207, 301, 60, 191, 223	Mix (200ng)
	Septicum (25ng)
306, 191	*Perfringens* (50ng)

207, 60

306, 191

207, 60

23, 60



***Difficile* (50ng)**

***Perfringens* (25ng)**

***Difficile* (25ng)**

***Bifermentans* (50ng)**

Results & Refinement

- **PCR: highly specific, only targets pathogens of interest. Works on 5 species tested to date**
- **T-RFLP: On pure DNA, produced unique peak profile depending on pathogen species**
- **Further strains to be tested, sensitivity analysis to be performed**

Species	forward		reverse	
P. bifermentans	39	23	64	60
P. difficile	211	207	64	60
P. sordellii	85	79	64	60
C. perfringens	310	306	195	191
C. sporogenes	39	24	227	223

Environmental sample Screening

- Screen subset of NSIS soil DNA for pathogens using protocol
- Screen estuarine sediments DNA extracts and digestates
- If present, increase the subset size. Quantify pathogen abundance with qPCR
- Running a year long field trial at farms to determine pathogen survival

Microcosm studies

- **Started microcosm studies to manipulate key soil/ environmental parameters and the effect on multiple pathogen species survival**

- **First one investigate the effect of water concentration and soil type**

- **Use the growth data to identify potential hotspots on agricultural land**

Conclusions

- **Developed a robust analytical pipeline for phylogenetic assignment of strains and spp.**
- **Identified the incorrect taxonomy of some strains and spp.**
- **Developed a simple PCR-TRFLP protocol that detects multiple significant pathogens**

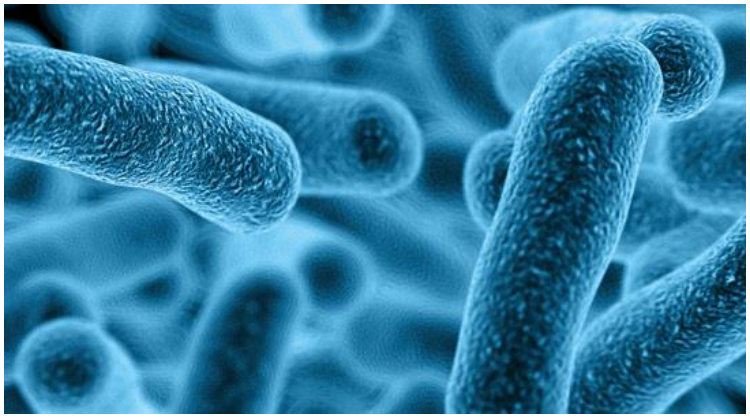
Acknowledgments

- **Supervisors**

  Dr Lisa Avery

  Dr Rupert Hough

  Dr Helen West



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