Update on the pathogenesis of *Clostridioides difficile* infections: does biofilm formation play a role?

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What is a biofilm ?

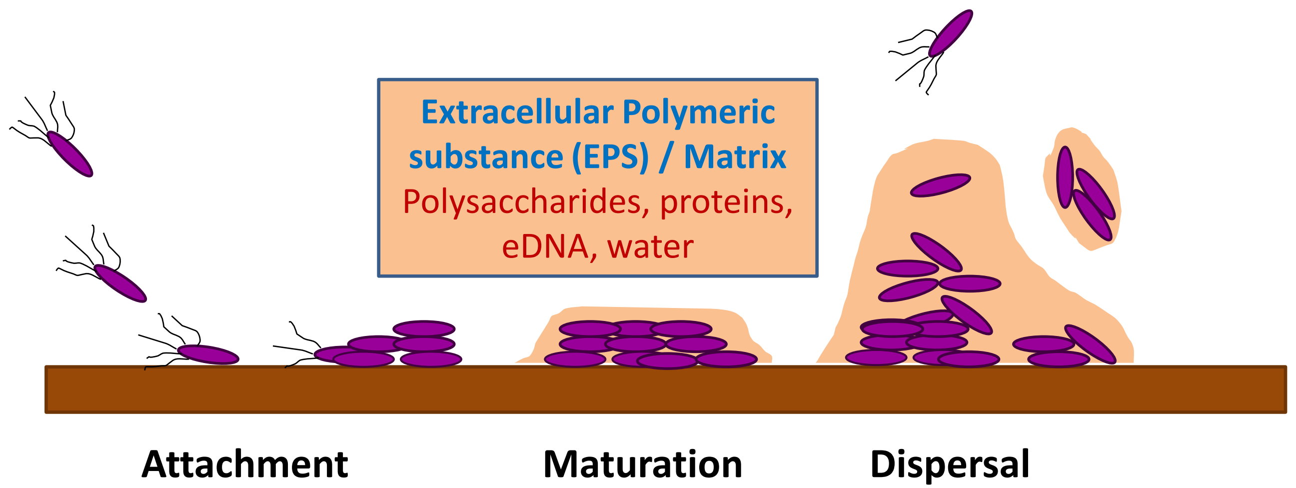
(Costerton et al., Science, 1999; Donné & Dewilde, Adv Microb Physiol, 2015)

Bacterial biofilms are structured communities of bacterial cells enclosed in a self-produced polymer matrix that is attached to an inert or living surface

- **A biofilm may be mono- or polymicrobial**
- **Most bacteria in their natural habitats are sessile and attached as biofilms to a surface**

Structure and main properties of a biofilm

Main steps of biofilm formation in vitro
(adapted from *Donné & Dewilde, Adv Microb Physiol, 2015)*

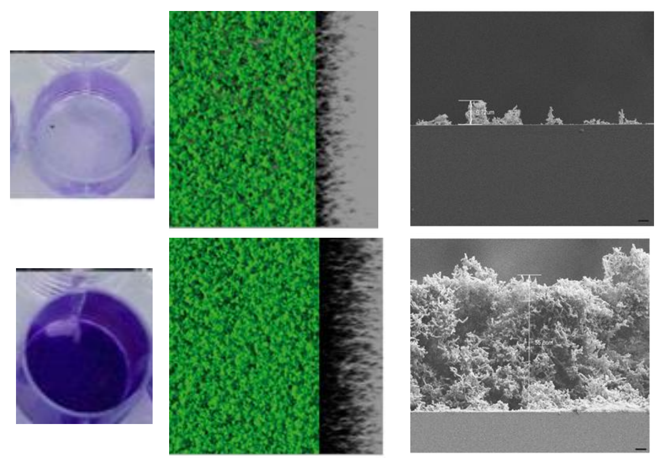


- **Common caracteristics of biofilms**
  - ✓ **Resistance against different forms of environmental stresses:** biocides, antibiotics, dessication, host defences (*in vivo* biofilms)
  - ✓ Highly regulated process in response to environmental signals

Characterization of biofilms

	Quantification		Architecture		Bacterial physiology	Sampling
	Enumeration of viable bacteria	Crystal violet staining	CLSM*	SEM*	Omics	
In vitro	x	x	x	x	x	Easy
In vivo	x		x	x	x	Difficult

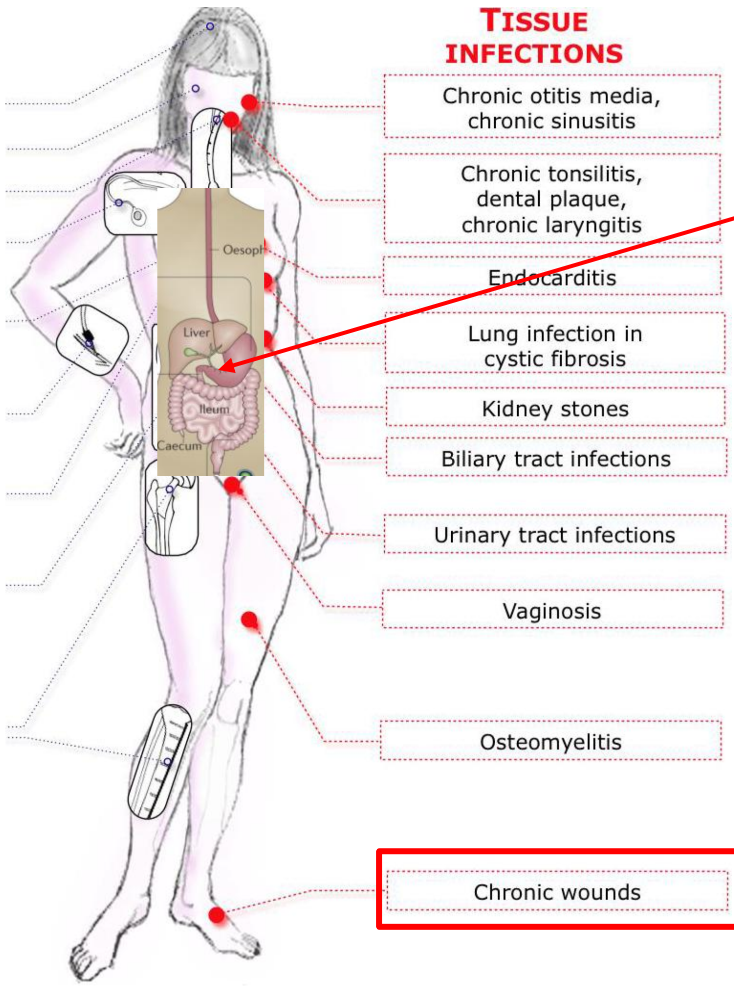
** CLSM: Confocal laser scanning microscopy*
** SEM: Scanning electron microscopy*



Visualization by different methods of in vitro biofilms formed by *C. difficile* strains 630Δ*erm* and mutant Cwp84 KO

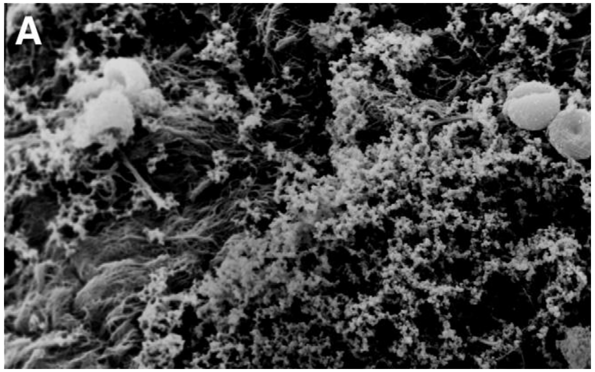
Pantaleon et al., Plos One, 2015

Biofilms in human infections



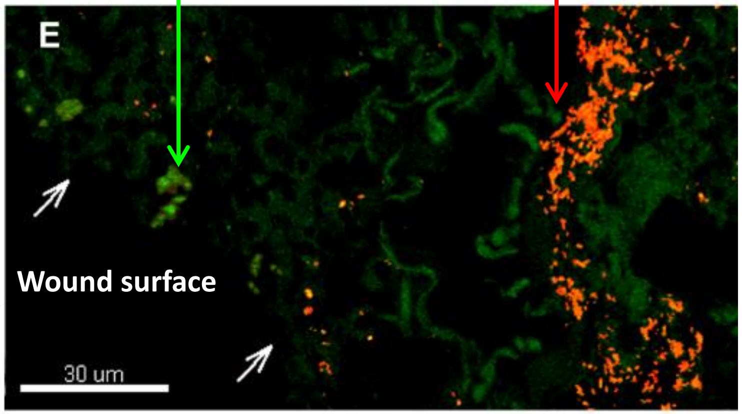
Human (chronic) infections related to *in vivo* biofilms
(Lebeaux, Pathogens, 2013)

***Helicobacter pylori* infection**



SEM on gastric biopsy specimens
(Cammarota et al., Clin Gastroenterol Hepatol, 2010)

Staphylococcus aureus ***Pseudomonas aeruginosa***



Examination of chronic wound sample by dual FISH combined with CLSM *(Fazli et al., J Clin Microbiol, 2009)*

Features of in-vivo biofilms

Bjarnsholt et al., Trends Microbiol, 2013; Burmolle et al., FEMS Immunol Med Microbiol , 2010; Burmolle et al., Trends Microbiol, 2014; Donné & Dewilde, Adv Microb Physiol, 2015; Hall-Stoodley & Stoodley, Cell Microbiol, 2009

- **Main characteristics**
  - ✓ **Resistance** properties to biocides, environmental stresses, host defences
  - ✓ Generally **smaller in spatial extension** than *in vitro* biofilms
  - ✓ **Matrix can include host material**

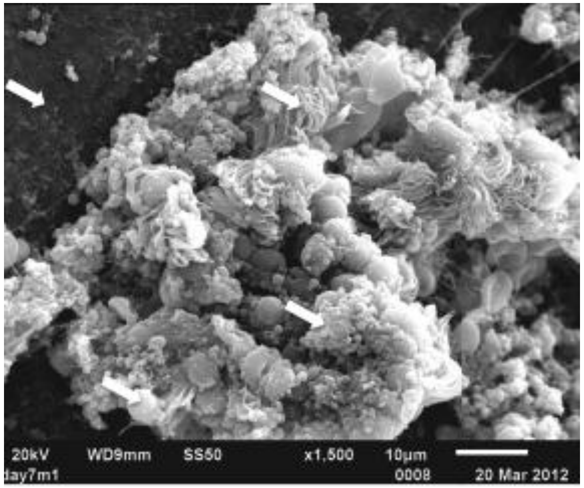
- **Diagnostic criteria for biofilm-associated infections**
  - ✓ Pathogenic bacteria are **associated with a surface**
  - ✓ Direct examination of infected tissue demonstrates **aggregated bacteria** embedded in a matrix
  - ✓ Infection is confined to a particular site in the host
  - ✓ **Recalcitrance to antibiotic treatment** in spite of susceptibility demonstrated by standard testing

- **More than 80% of all microbial infections are biofilm-associated (US NIH)**

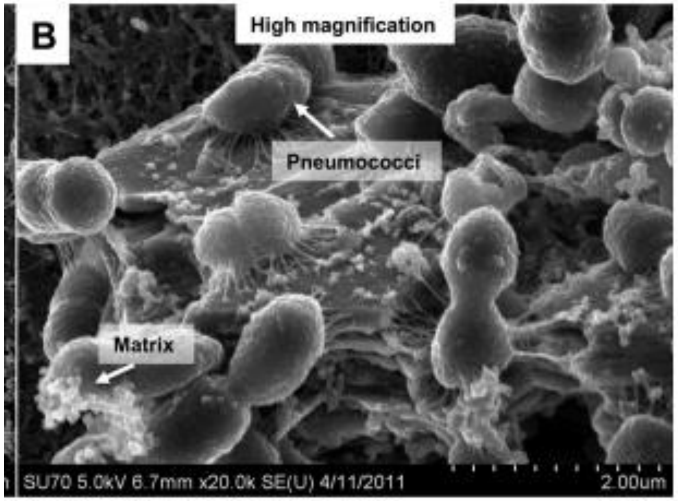
Biofilms associated with asymptomatic carriage

Marks et al., Infect Immun, 2012; Blanchette-Cain et al., mBio, 2013

- **Example of nasopharyngeal carriage of *Streptococcus pneumoniae***



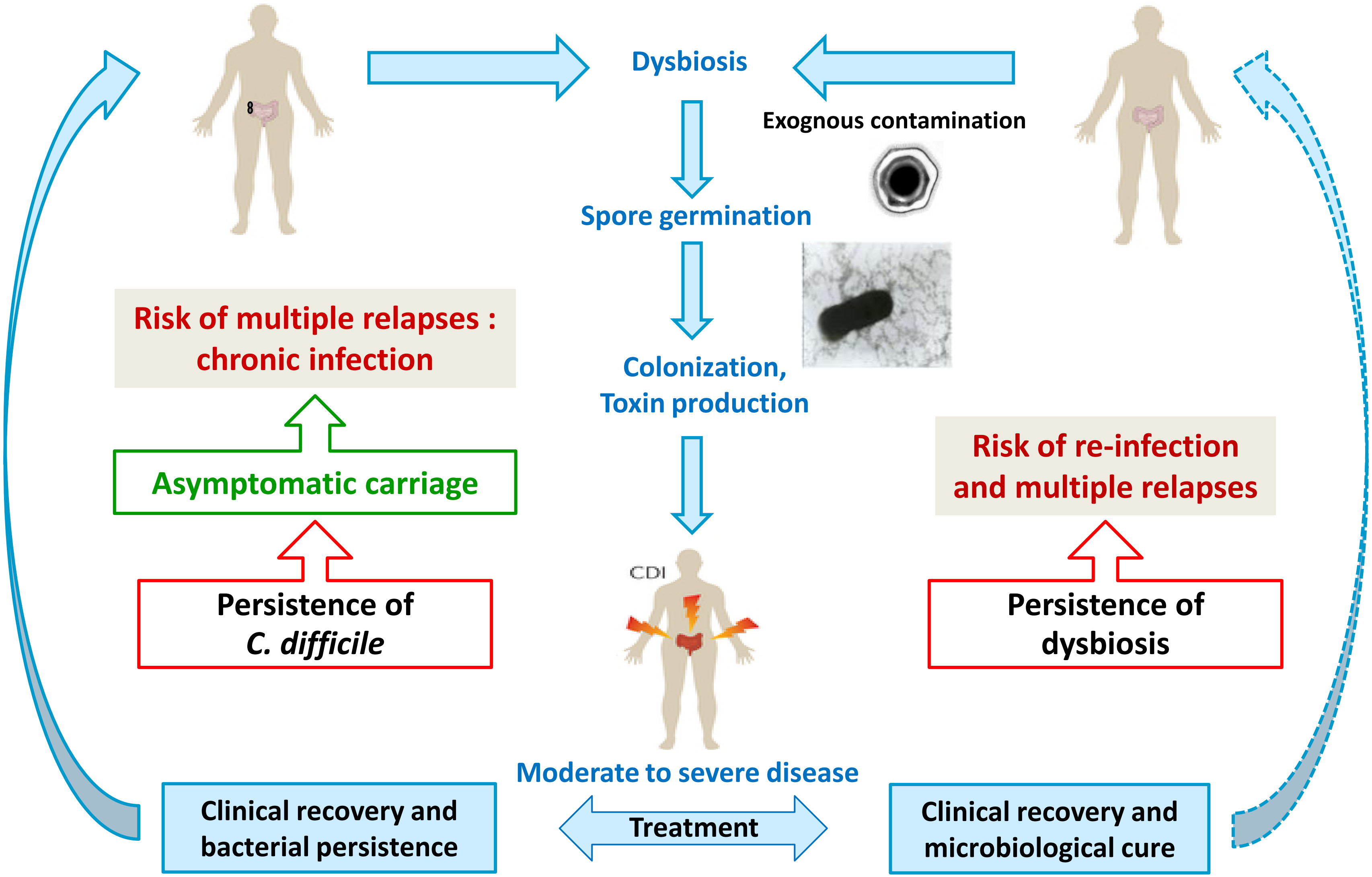
Mouse model of colonization: SEM examination of the nasopharynx



SEM examination of biofilm formed on human respiratory cells

➡ Visualization of aggregated bacteria embedded in a matrix

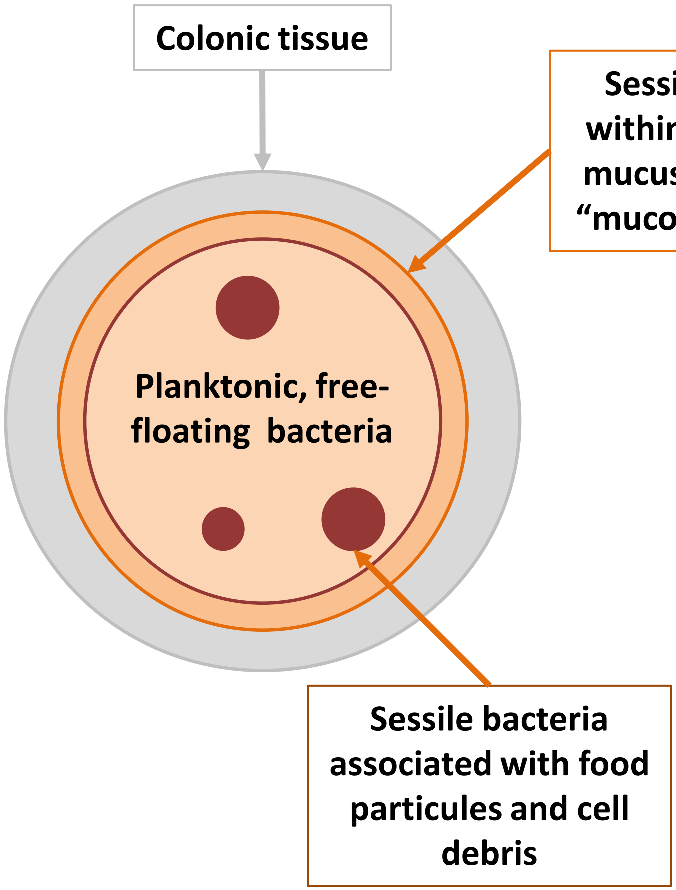
Pathogenesis model of *C. difficile* infection (CDI)

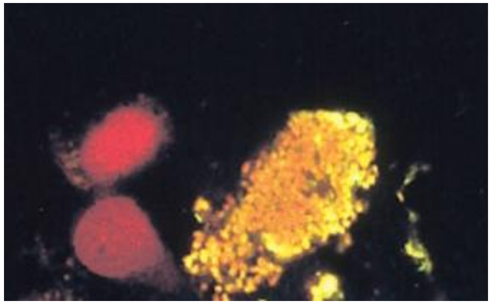


In which form is *C. difficile* able to persist:
spore , single biofilm, polymicrobial mucosal biofilm?

Spatial organization of the microbiota

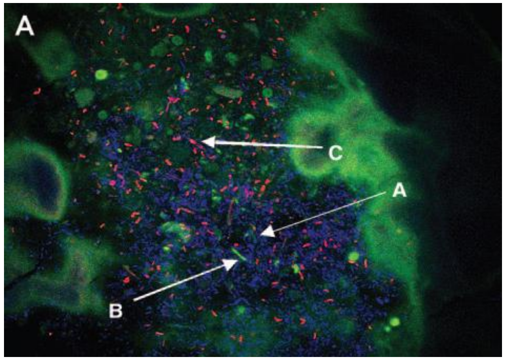
De Vos, Npj Biofilms Microbiomes, 2015; Donaldson et al., Nat Rev Microbiol, 2016; De Weirdt & Van de Wiele, Npj Biofilms Microbiomes, 2015; Nava et al., Isme J, 2011; Swidinski et al., J Clin Microbiol, 2005





CLSM of a bacterial microcolony on healthy rectal mucosa (IBD patient)

(MacFarlane et al., Clin Infect Dis, 2004)



Microscopic examination after FISH on fresh feces

A: all bacteria, B: enterobacteria, C: bifidobacteria

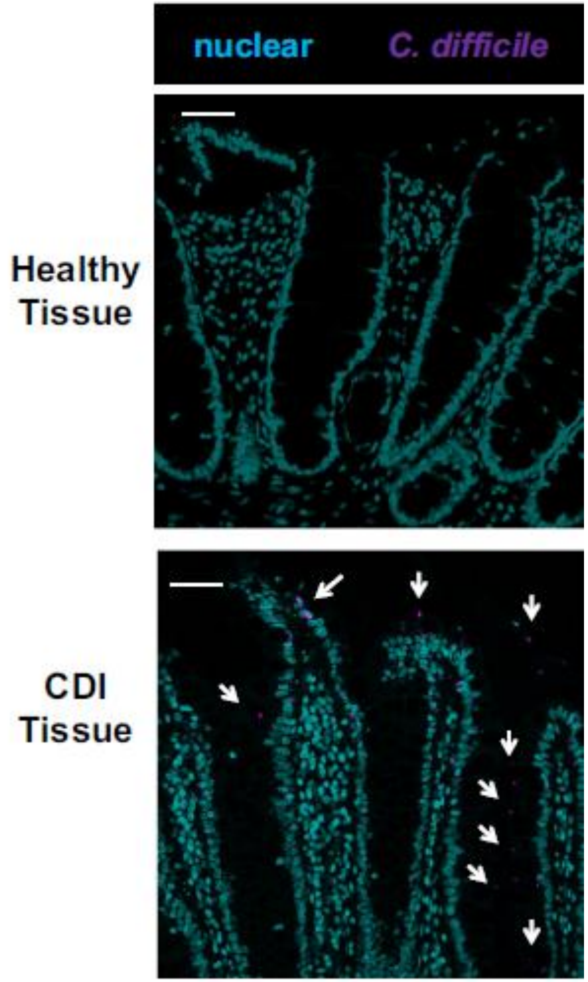
(MacFarlane & MacFarlane, Appl Environ Microbiol, 2006)

What do we know about spatial distribution of *C. difficile* in vivo ?

Spatial distribution of *C. difficile* during human infections

Engevik et al., Am J Physiol Gastrointest Liver Physiol, 2015

- **CLSM observation of colon biopsy specimens**
  - ✓ Formalin fixed colon biopsies specimens
  - ✓ Healthy volunteers or patients with **recurrent CDI**
- **Two main results**
  - ✓ Modification of the mucus composition in patients with recurrent CDI:
    - decreased MUC2 expression
    - modification of the oligosaccharide residues of mucins
  - ✓ *C. difficile* detected as single cells in the mucus of CDI patients (white arrows)

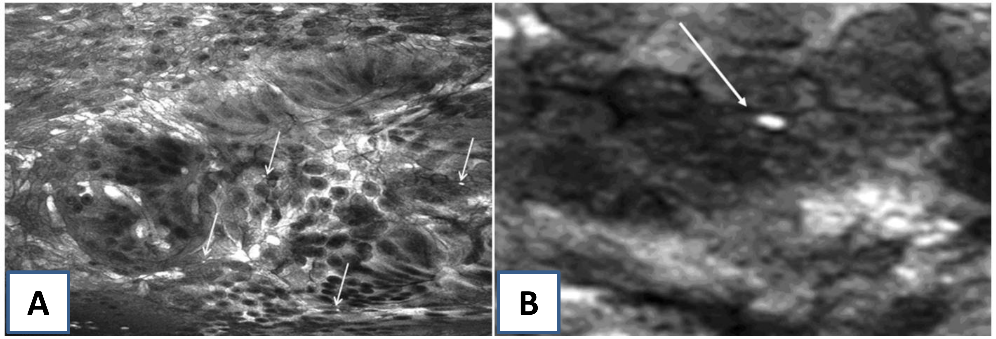


CLSM examination of gut tissues after *C. difficile* labeling with specific Ab and Hoechst couterstaining

Spatial distribution of *C. difficile* during human infections

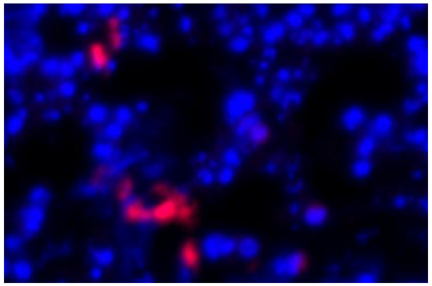
Neumann et al., Plos One, 2013

- **Colonoscopy in patients with diarrhea**
- **Direct *in vivo* detection of *C. difficile* by confocal laser endomicroscopy after topical application of acriflavine hydrochloride**



Fluorescence confocal image below the surface of the colonic mucosa after topical application of acriflavine hydrochloride identified single bacteria (A). 10,000 fold digital magnification (B)

- **Biopsies examined by FISH**
  - ✓ Confirmation of the presence of intra-mucosal *C. difficile*



Microscopic examination after FISH with specific CD probe (red) and couterstaining by Hoechst

Spatial distribution of *C. difficile* during human infections

Bjarnsholt et al., Trends Microbiol, 2013; Burmolle et al., FEMS Immunol Med Microbiol , 2010; Burmolle et al., Trends Microbiol, 2014; Donné & Dewilde, Adv Microb Physiol, 2015; Hall-Stoodley & Stoodley, Cell Microbiol, 2009

- **Diagnostic criteria for biofilm-associated infections**

  ✓ Pathogenic bacteria are associated with a surface

  ✓ Direct examination of infected tissue demonstrates aggregated bacteria embedded in a matrix

  ✓ Infection is confined to a particular site of the host

  ✓ **Recalcitrance to antibiotic treatment** in spite of susceptibility demonstrated by standard testing

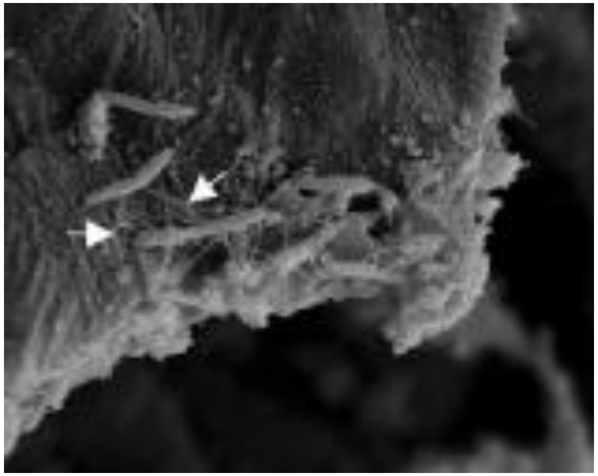
Data from the hamster model of acute infection

Buckley et al., J Med Microbiol, 2011; Spencer et al., Gut Microb, 2014

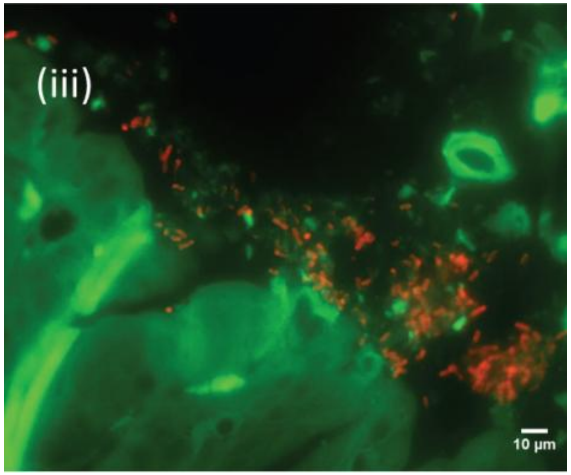
- **Mimicks some features of human infection but causing more severe disease**

- **SEM analysis**
  - ✓ Observation of aggregates of *Clostridium*-like bacteria on the tissue surface forming microcolony plaques associated over the crypt crevasses
  - ✓ Intimate tissue-bacterial and bacterial-bacterial interactions via specific appendages

- **Immunofluorescence microscopy**
  - ✓ Bacterial aggregates **associated with the epithelial barrier**
  - ✓ Presence of other micro-organisms (green)



SEM examination of the caecum of a hamster infected by the strain R20291 36h p.i.

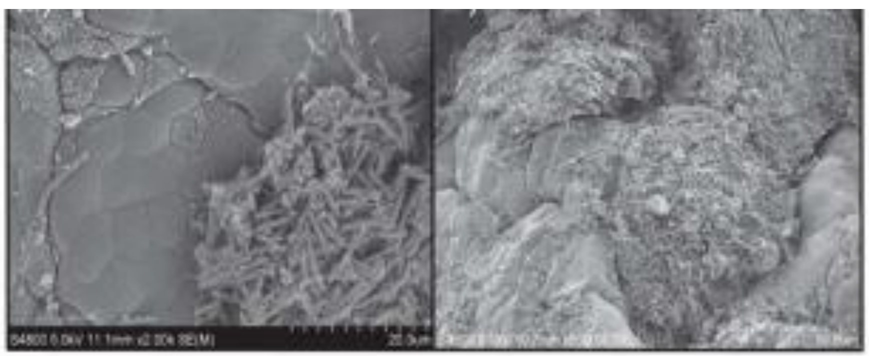


Immunofluorescence of a non-toxigenic strain (in red) in hamster tissues 24 h p.i.

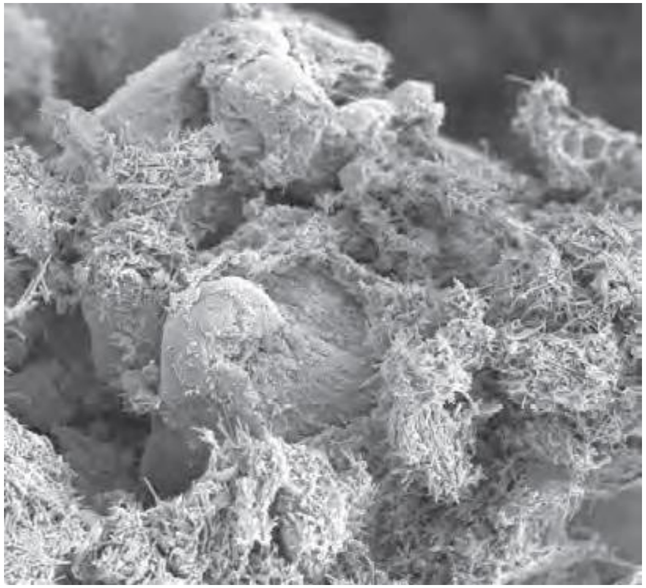
Data from conventionally reared mice -1

Lawley et al. Infect Immun, 2009; Lawley et al., Plos Pathog, 2012

- **Model of long-lasting colonization: mice are infected and then made "C. difficile supershedder" by clindamycin**
  - ✓ Examination by SEM of cecal tissue



SEM examination of mouse intestinal mucosa after infection with the BI strain illustrating the presence of *C. difficile* microcolonies (left) and biofilm-like structures (right) on the intestinal mucosal surface

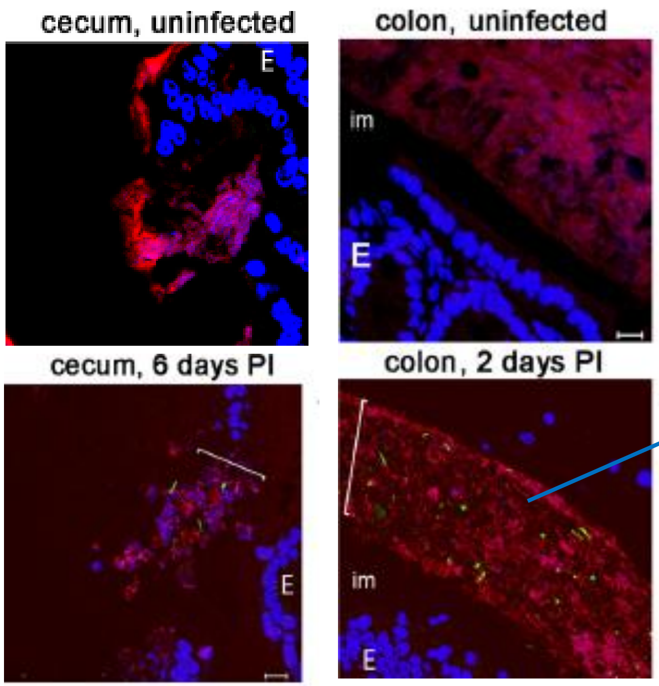


Large aggregates of *C. difficile* covering the damaged cecal epithelium, observed in supershedder mice infected with strain M68 (4 to 7 d p.i.)

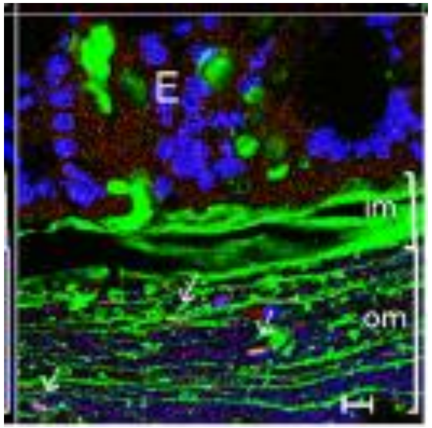
Data from conventionally reared mice -2

Semenuyk et al., Infect Immun, 2015

- **Colonization and infection model: mice are made susceptible with a strong antibiotic cocktail**
  - ✓ Kinetic examination of cecum and colon by FISH combined with CLSM



FISH-CLSM examination of sections of gut tissue from mice infected with *C. difficile* (green) 027/BI17. Other bacteria are labeled in red.



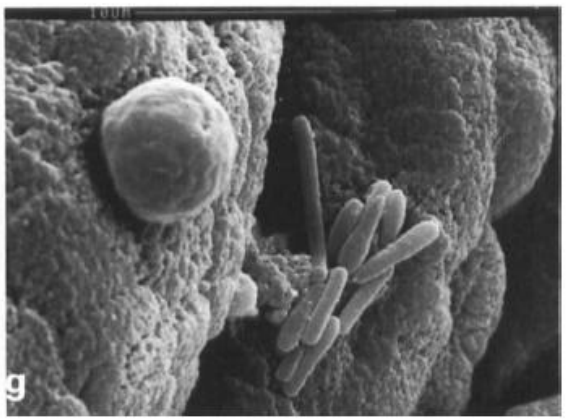
FISH -CLSM examination of sections of colon (6d p.i.) from mice infected with *C. difficile* (yellow) followed by immunodetection of mucin with anti-Muc2 Ab (green).

In this model, *C. difficile* is found within the mucus and associated with other bacteria: mucosal biofilm?

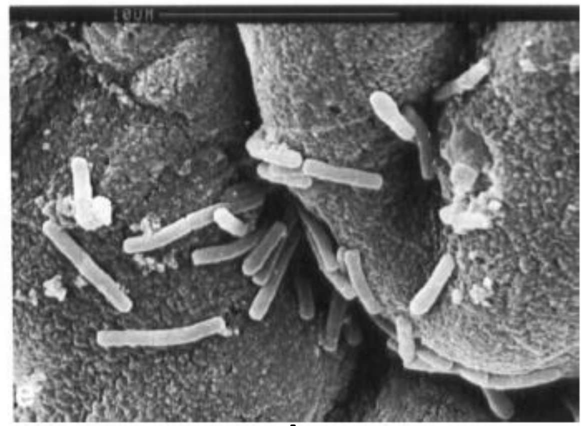
Data from monoxenic mice: colonization model -1

Castex et al., J Med Microbiol, 1994

- **Infection with a low toxin-producing strain of *C. difficile***
  - ✓ Examination of washed tissue by SEM, **30 h p.i.**



cecum



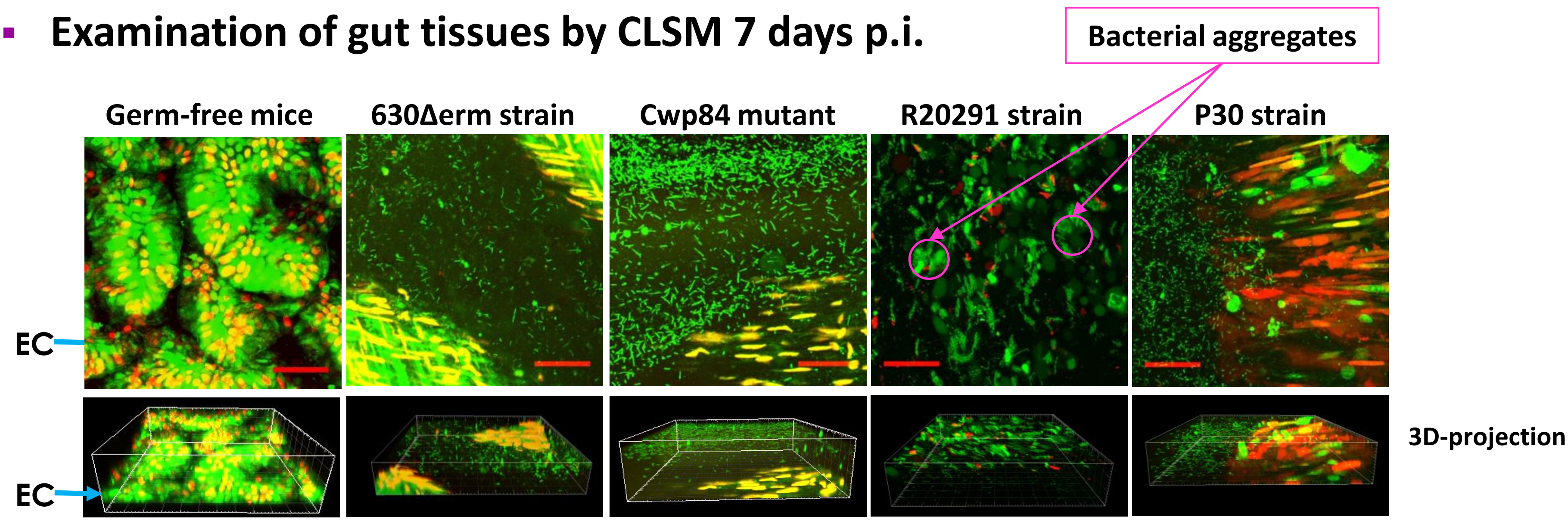
colon

Visualization of adherent single cells of *C. difficile*

Data from monoxenic mice: colonization model -2

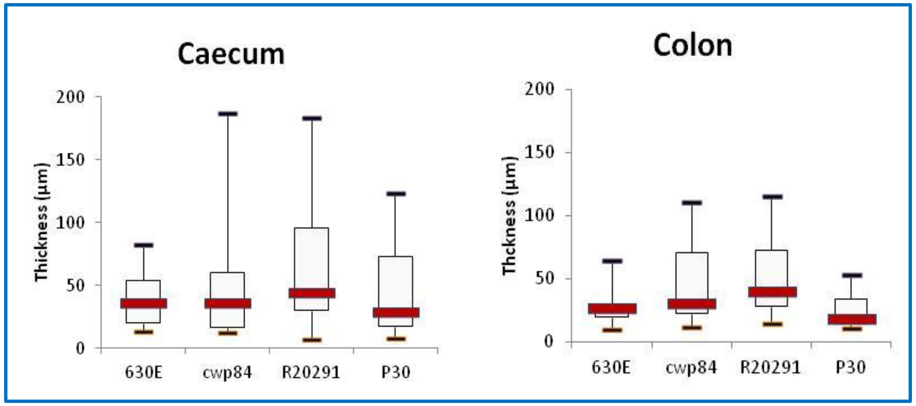
Soavelomandroso et al., Front Microbiol, 2017

- **Examination of gut tissues by CLSM 7 days p.i.**



CLSM examination of cecum of mice infected by different strains of *C. difficile*, 7 days p.i. (live/dead labeling)

- ✓ Heterogeneous distribution, **bacteria organized in 3D-structures**
- ✓ Spores and vegetative cells tightly associated with the mucosa



Thickness of 3D-structures

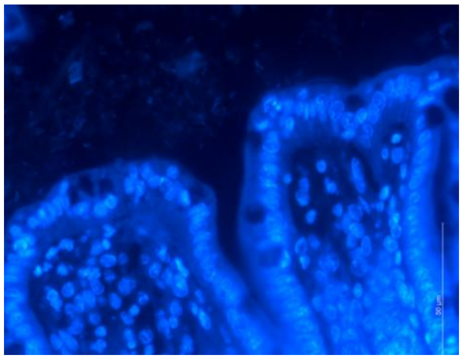
Data from monoxenic mice: colonization model -2

Soavelomandroso et al., Front Microbiol, 2017

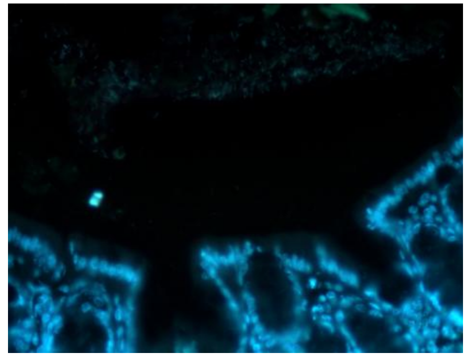
- **Immunohistochemistry on gut sections (after 3 rinses)**

R20291 strain

cecum



colon

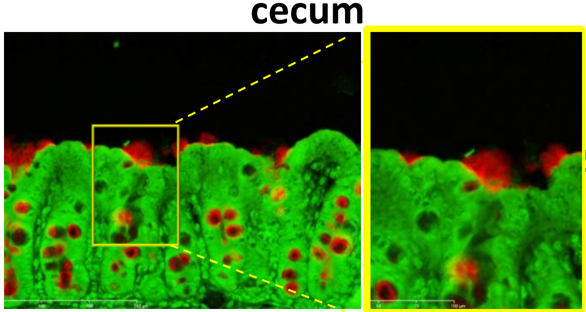


CLSM examination of gut tissue after Hoechst staining

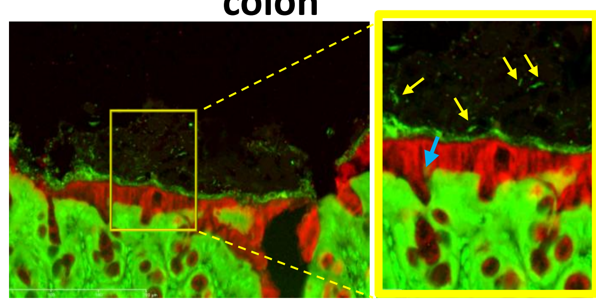
Bacteria are not in direct contact with enterocytes

R20291 strain

cecum



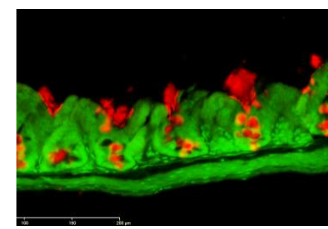
colon



Anti-Muc2 labeling (red) of gut sections with coutertaining by Syto®9

Most of the bacteria are embedded in a 3D-structure adherent to the mucus

cecum



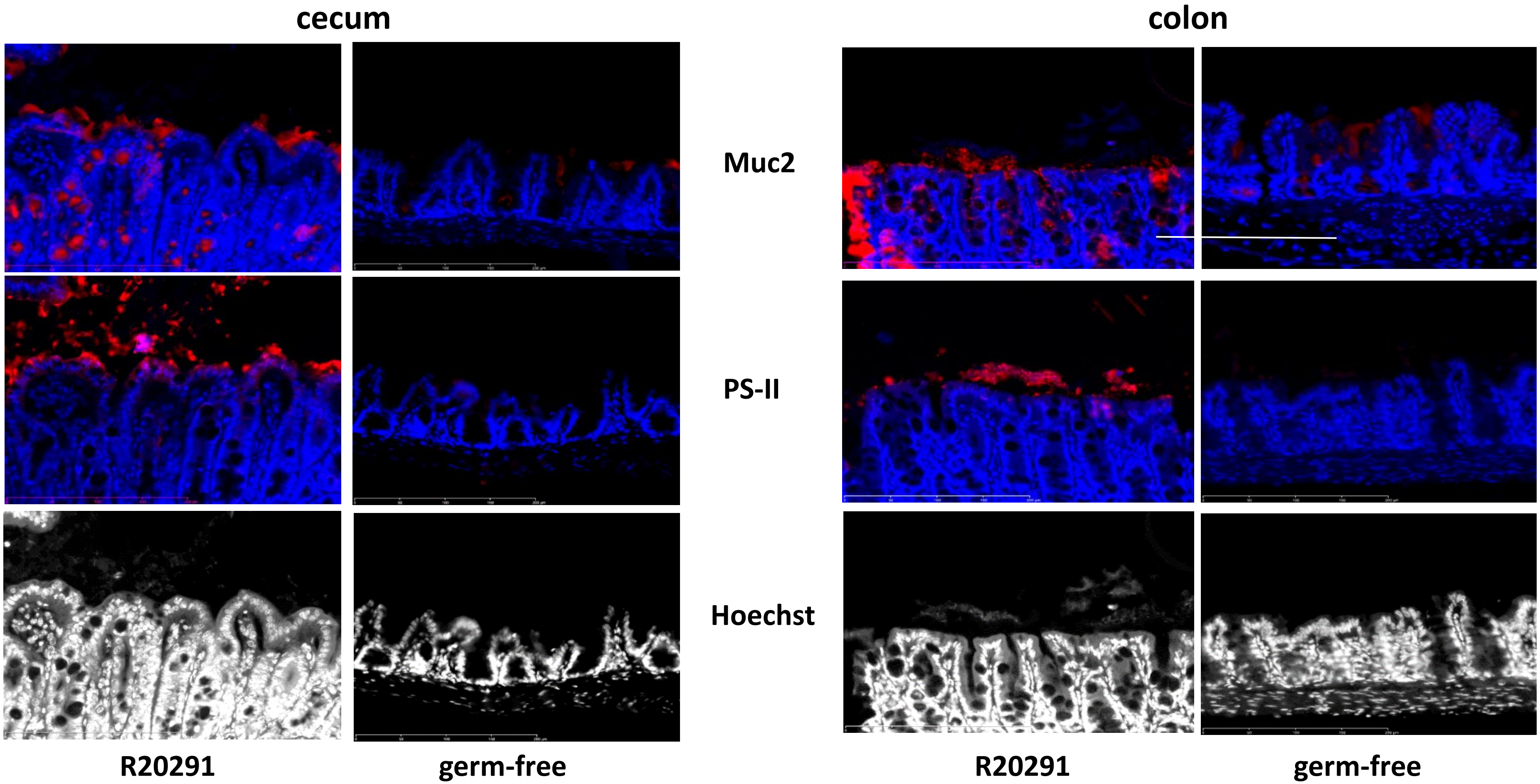
colon

Tissues from germ-free mice

Data from monoxenic mice: colonization model -2

Soavelomandroso et al., Front Microbiol, 2017

- **Serial sections labelled with Muc2 and bacterial PSII antibodies**

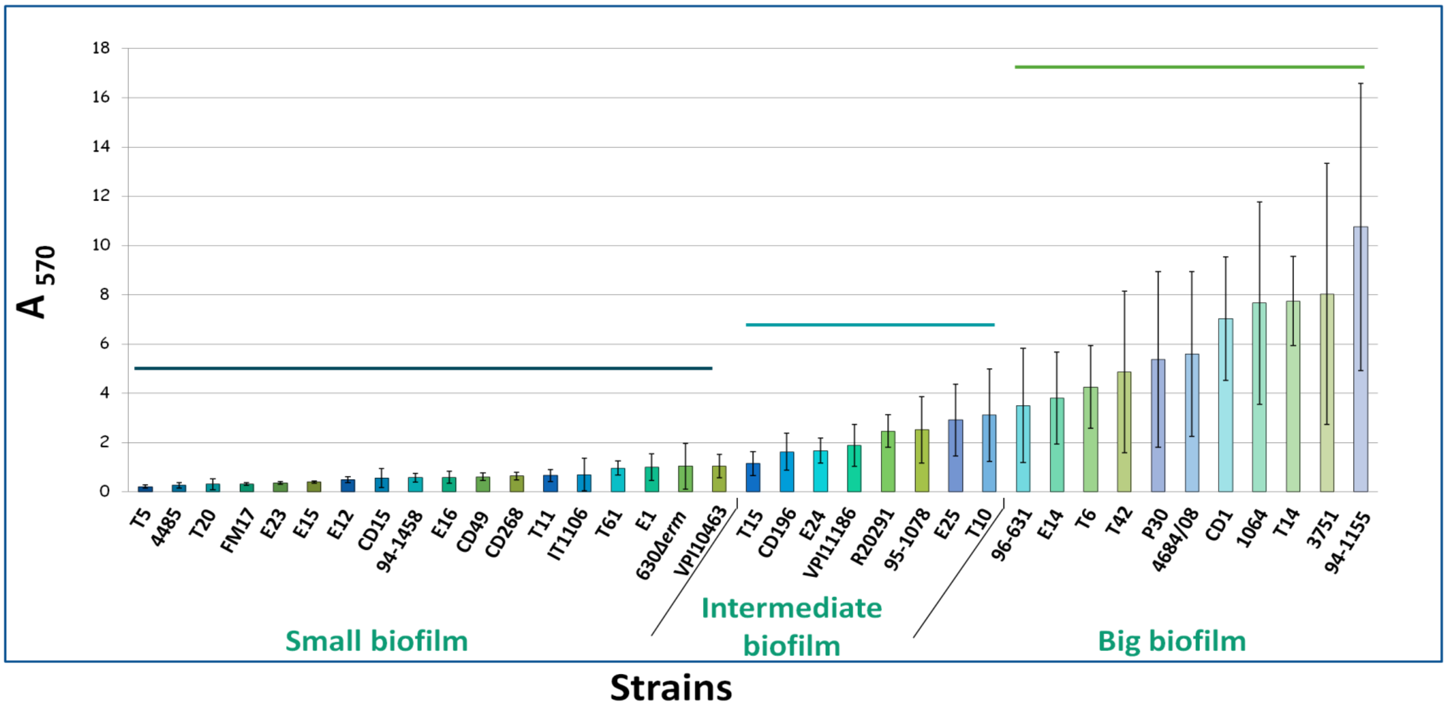


The strain R20291 forms patchy biofilms at the surface of the mucus layer

What is the relevance of information obtained in vitro for the understanding of biofilm formation in vivo

Crowther et al., PlosOne, 2014; Donelli et al., FEMS Immunol Med Microbiol, 2012; Semenuyk et al., Plos One, 2014; Pantaleon et al., Anaerobe , 2018; Piotrowski et al., Eur J Clin Microbiol Infect Dis, 2017

- ***C. difficile*** **is able to form single-species or polymicrobial biofilm in vitro**
  - ✓ Strain-dependant biofilm-producing ability



  - ✓ Mixed biofilm: cooperation of *C. difficile* with *Finegoldia magna*
  - ✓ *C. difficile* forms part of a multi-species biofilm in an in-vitro dynamic model of the human gastrointestinal tract

What is the relevance of information obtained in vitro for the understanding of biofilm formation in vivo

Dapa et al., J Bacteriol, 2013; Crowther et al., PlosOne, 2014;; Pantaleon et al., Plos One, 2015; Semenuyk et al., Plos One, 2014; Mathur et al., Gut Pathog, 2016; Piotrowski et al., Eur J Clin Microbiol Infect Dis, 2017; Poquet et al., Front Microbiol, 2018;

- **Toxins are components of the biofilm matrix**
  - ✓ Variable levels of toxin among strains
  - ✓ *tcdA* is underexpressed in a dynamic in-vitro biofilm model as compared to planktonic conditions

- **Resistance**
  - ✓ To antibiotics, especially those used to treat CDI
  - ✓ To oxygen stress

What is the relevance of information obtained in vitro for the understanding of biofilm formation in vivo? cdt

Crowther et al., J Antimicrob Chemother, 2014; Dapa et al., J Bacteriol, 2013; Dawson et al., PLos One, 2012; Semenuyk et al., Plos One, 2014; Pizarro-Guajardo et al., Appl Environ Microbiol, 2016; Vuotto et al., Adv Exp Med Biol, 2018; Pickering et al., Anaerobe, 2018

- **Sporulation within biofilms**

Study	Strain	Growth medium	Model	Sampling time	Results
Dapa et al., 2013	630, R20291	BHIS	Static, polystyrene plates Mono-species	3 days	↓↓ vs. planktonic suspension
Dawson et al., 2012	630Δ*erm* R20291	BHIS	Static, polystyrene flasks Mono-species	3 days	1% spores 10% spores
Semenuyk et al., 2014	Several strains	Tryptic soy agar	Static, polycarbonate filters (micro-colonies)	6 days	Strain-dependant level of sporulation
Crowther et al., 2014	027 strain		Dynamic, glass rods Polymicrobial		Pronounced prevalence of spores

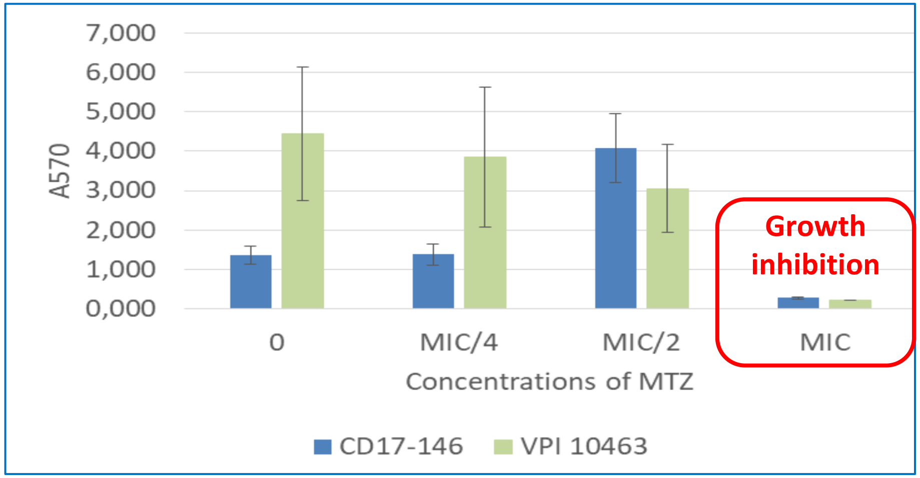
➡ Various results according to growth medium, surface, sampling time, and type of biofilm

- **Properties of sessile *vs*. planktonic spores**
  - ✓ Dormant spores, **recalcitrance to germination**
  - ✓ Differences in exosporium types
  - ✓ Increased heat tolerance

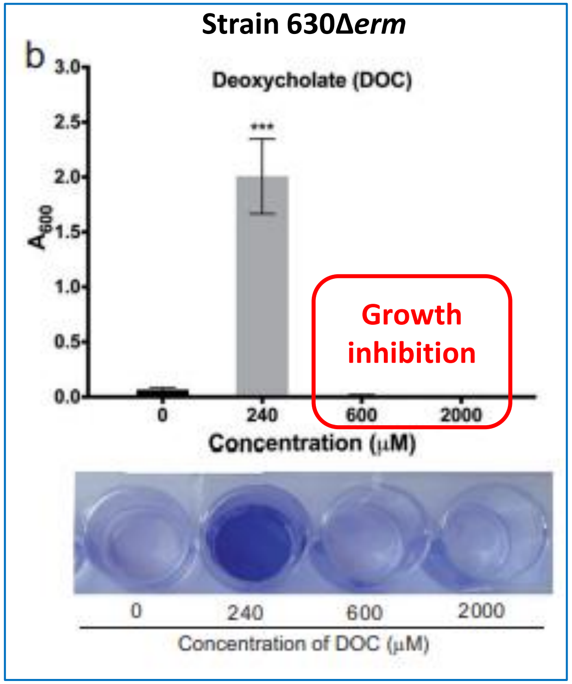
What is the relevance of information obtained in vitro for the understanding of biofilm formation in vivo? cdt

Dapa et al., J Bacteriol, 2013; Vuotto et al., FEMS Pathog Dis, 2016; Dubois et al., Npj Biofilms, 2019; our unpublished data

- **Known environmental inducers of biofilm formation**
  - ✓ **Antibiotics**
    - Moderate increase by vancomycin
    - Important increase by subinhibitory concentrations of metronidazole



  - ✓ **Secondary bile salt deoxycholate**

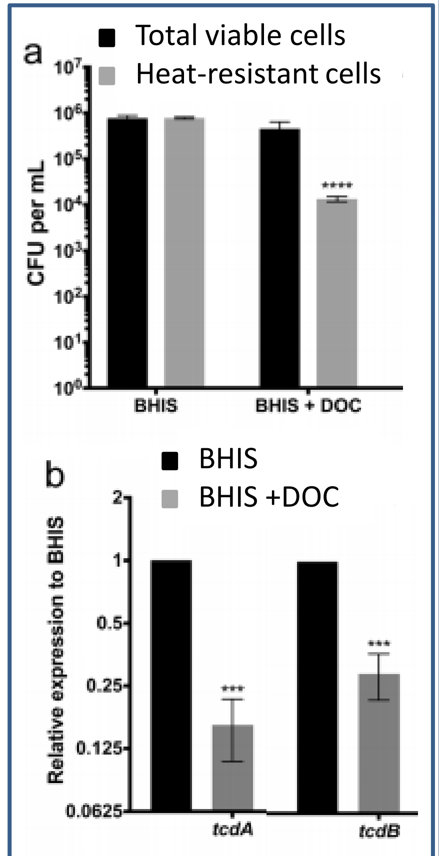


Measurement of biofilm biomass by crystal violet staining in different conditions of induction

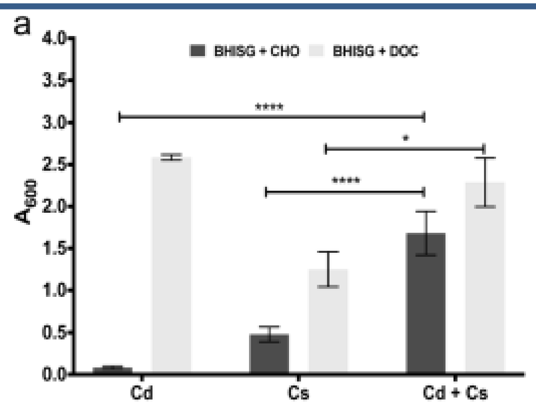
C. difficile biofilm production is induced by DOC

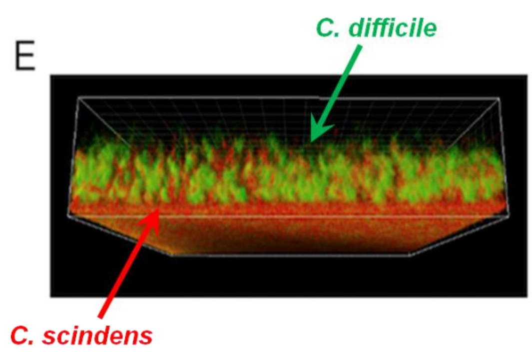
Dubois et al., Npj Biofilms, 2019

- **Characteristics of *in vitro* DOC-induced *C. difficile* biofilms**
  - ✓ Increased *C. difficile* viability
  - ✓ Decreased spore and toxin production



- **In vivo, DOC is produced by dehydroxylation of cholate by commensal bacteria, such as *Clostridium scindens***
  - ✓ In vitro, in presence of cholate, *C. scindens* induced formation of dual biofilm

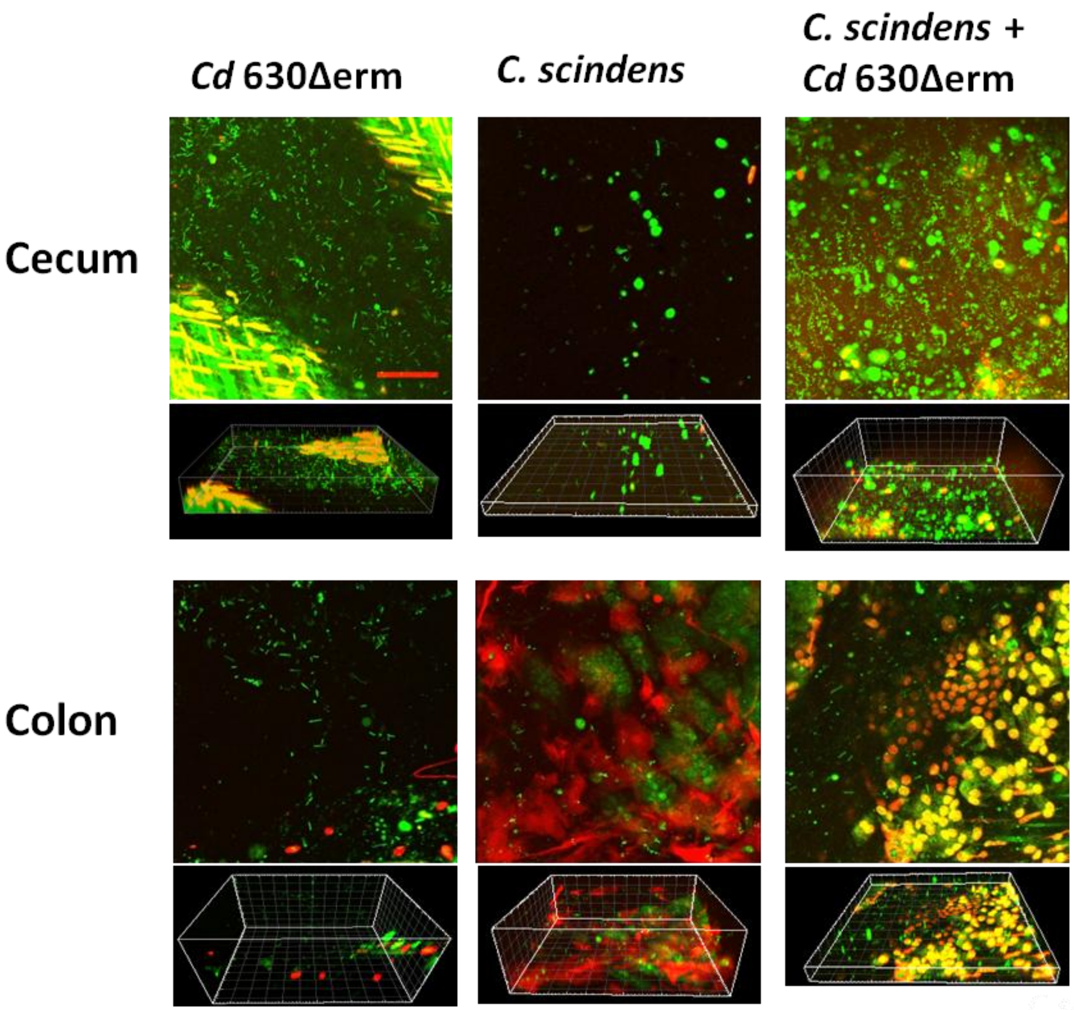


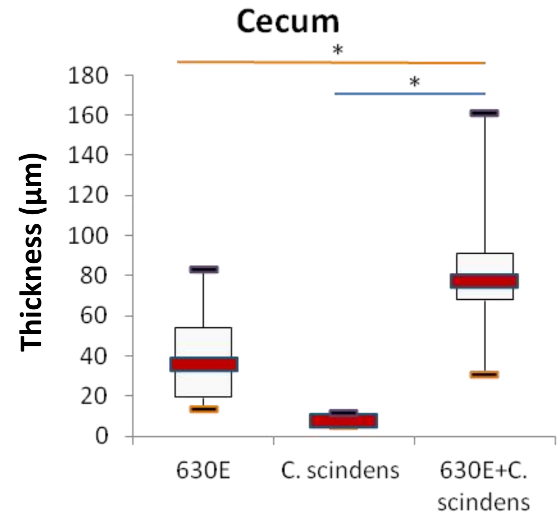


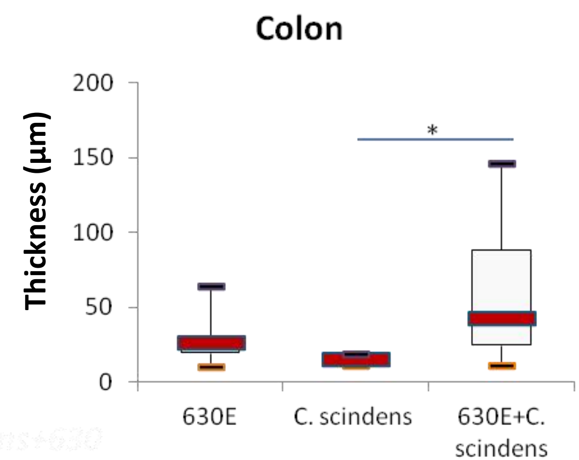
CV quantification and CLSM examination of dual biofilm *Cd* and *Cs* in BHIS + cholate

In murine axenic model, *the production of C. difficile* biofilm is enhanced by *C. scindens*

Our unpublished data in collaboration with B. Dupuy's group







To conclude

- **In humans, scarce data, not in favor of formation of a "true" biofilm in the gut BUT biopsy specimen sampling during acute infections:**
  - ✓ Maybe not the right time!
  - ✓ Bias due to the bowel preparation
  - ✓ ***C. difficile*** **has been found in polymicrobial true subgingival biofilms** *(Colombo et al., Microb pathog, 2016)*
- **In animal models, several arguments in favor of biofilm formation for long-lasting persistence of *C. difficile* in gnotoxenic or complex microbiota models**
- **From in vitro studies, interesting hypotheses regarding properties of biofilm and inducing conditions for their formation BUT needs to be confirmed in vivo!**
- **A lot of remaining questions…**

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Romain Briandet

And all of you for your attention

Features of in-vivo biofilms

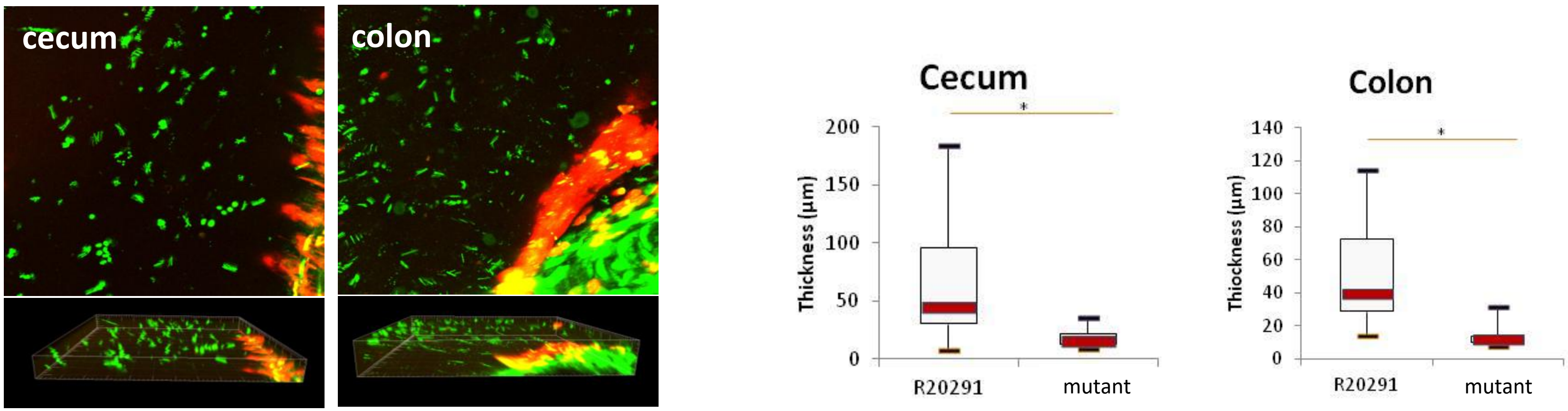
Bjarnsholt et al., Trends Microbiol, 2013; Burmolle et al., FEMS Immunol Med Microbiol , 2010; Burmolle et al., Trends Microbiol, 2014; Donné & Dewilde, Adv Microb Physiol, 2015; Hall-Stoodley & Stoodley, Cell Microbiol, 2009

- **Diagnostic criteria for biofilm-associated infections**
  - ✓ Pathogenic bacteria are **associated with a surface**
  - ✓ Direct examination of infected tissue demonstrates **aggregated bacteria** embedded in a matrix
  - ✓ Infection is confined to a particular site in the host
  - ✓ **Recalcitrance to antibiotic treatment** in spite of susceptibility demonstrated by standard testing
  - ✓ Culture-negative results in spite of strong clinical suspicion of infection
  - ✓ Evidence of ineffective bacterial clearance by the host

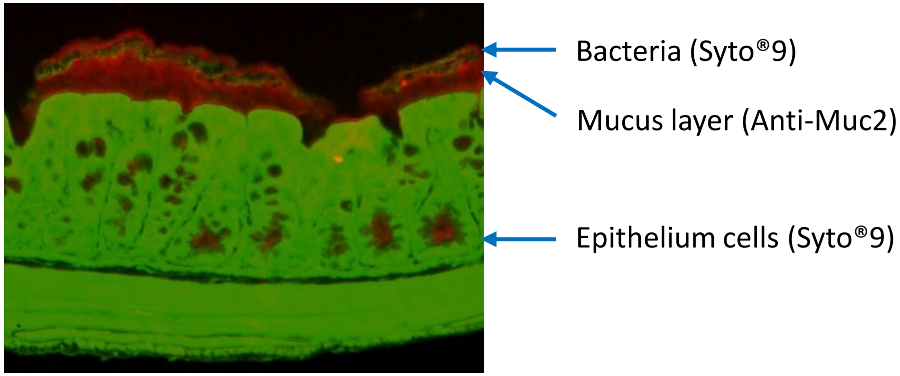
Data from monoxenic mice: colonization model -2

Our unpublished data

- **In the mono-associated mouse model, the spatial distribution of bacteria is strain-dependent**



Examination by CLSM of gut tissue infected with a mutant of the strain R20291 and thickness of 3D-structures in gut tissue



Immunohistochemistry on colonic section

Pathogenicity model for *C. difficile* infection (CDI)