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**Determinants of biofilm formation in
the strict anaerobic symbionts
Bacteroides thetaiotaomicron and
Veillonella parvula.**

Par Nathalie Béchon

Thèse de doctorat de **Microbiologie**

Dirigée par Jean-Marc Ghigo

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Title : Determinants of biofilm formation in the strict anaerobic symbionts *Bacteroides thetaiotaomicron* and *Veillonella parvula*.

Abstract :

The human gut microbiota constitutes a dense multi-species anaerobic biofilm-like structure in which adhesion has been proposed to play a key role for bacterial colonization, maintenance and function. However, classical models of biofilm formation are either minor members of the gut microbiota community, often studied in presence of oxygen, or pathogenic gut bacteria. During my thesis, I have used genetic screens to identify determinants of *in vitro* biofilm formation in two relevant strict anaerobe gut symbionts, *Bacteroides thetaiotaomicron* and *Veillonella parvula*.

B. thetaiotaomicron is one of the most abundant Gram-negative symbionts of the human gut microbiota. Although several clinical *B. thetaiotaomicron* isolates were shown to produce biofilms, the genetically amenable reference strain VPI 5482 is a poor biofilm former *in vitro*. I showed that deletion of capsule 4, one of the 8 capsular polysaccharides (CPS) of *B. thetaiotaomicron*, leads to the genesis of a subpopulation of acapsulated cells that have increased biofilm capacity. Whereas expression of most *B. thetaiotaomicron* capsules masked biofilm formation, the expression of capsule 8 induced biofilm formation, suggesting it might directly promote adhesion. Taken together, these results show that CPS expression is a major determinant of *B. thetaiotaomicron* biofilm formation.

I also showed that addition of either bile extract or tauroolithocholate induced biofilm formation in most *B. thetaiotaomicron* isolates. Bile-induced biofilm formation involved a putative secreted DNase. Consistently, addition of a commercial DNase to the culture medium complemented the lack of bile-induced biofilm formation in the DNase mutant.

V. parvula is an atypical Gram-negative Firmicute and it is a member of the oral and intestinal microbiota. Its ability to co-aggregate with multiple bacteria and to form biofilms is critical for colonization of its niches. However, molecular analysis in this species have been hampered by the lack of efficient genetic tools. Using a recently described naturally competent strain, I used random transposition and site-directed mutagenesis to show that trimeric autotransporters are the main adhesins of *V. parvula* SKV38, mediating cell-cell and cell-surface adhesion. Moreover, I identified a putative HD phosphohydrolase that is involved in autotransporter-mediated biofilm formation.

The identification of biofilm formation determinants in the non-models anaerobic bacteria *B. thetaiotaomicron* and *V. parvula* constitutes a first step that will allow us to address the contribution of biofilms to gut microbiota colonization and function.

Keywords :

Gut microbiota, biofilms, anaerobes, bile, capsule, autotransporters

Titre : Déterminants de la formation de biofilm chez les symbiotes anaérobies strictes *Bacteroides thetaiotaomicron* et *Veillonella parvula*.

Résumé :

Le microbiote intestinal humain constitue une structure anaérobie multi-espèces dense semblable à un biofilm où il a été supposé que l'adhésion soit cruciale pour la colonisation, l'entretien et le fonctionnement des bactéries. Cependant, les modèles classiques de formation de biofilms sont soit des membres mineurs du microbiote intestinal, souvent étudiés en présence d'oxygène, soit des bactéries intestinales pathogènes. Au cours de ma thèse, j'ai utilisé des cribles génétiques pour identifier les déterminants de la formation de biofilms *in vitro* chez deux symbiotes intestinaux strictement anaérobies, *Bacteroides thetaiotaomicron* et *Veillonella parvula*.

B. thetaiotaomicron est l'un des symbiotes à Gram-négatifs les plus abondants du microbiote intestinal humain. Bien que plusieurs isolats cliniques de *B. thetaiotaomicron* produisent des biofilms, la souche de référence génétiquement manipulable VPI 5482 forme peu de biofilm *in vitro*. J'ai montré que la délétion de la capsule 4, un des 8 polysaccharides capsulaires (CPS) de *B. thetaiotaomicron*, conduit à la genèse d'une sous-population de cellules acapsulées qui ont une forte capacité de formation de biofilms. Alors que l'expression de la plupart des capsules de *B. thetaiotaomicron* masque la formation de biofilms, l'expression de la capsule 8 induit la formation de biofilms, ce qui suggère qu'elle pourrait directement favoriser l'adhésion. Collectivement, ces résultats montrent que l'expression des CPS est un déterminant majeur de la formation de biofilms chez *B. thetaiotaomicron*.

J'ai également montré que l'ajout d'extrait de bile ou de tauroolithocholate induisait la formation d'un biofilm chez la plupart des isolats de *B. thetaiotaomicron*. La formation de biofilm induite par la bile implique une DNase sécrétée potentielle. De façon cohérente, l'ajout d'une DNase commerciale dans le milieu de culture restaure la formation de biofilm en présence de bile du mutant délété pour la DNase.

V. parvula est un Firmicute à Gram-négatif atypique et il fait partie du microbiote oral et intestinal. Sa capacité à coagrèger avec de multiples bactéries et à former des biofilms est essentielle pour sa colonisation. Cependant, chez cette espèce l'analyse moléculaire a été entravée par le manque d'outils génétiques efficaces. En utilisant une souche naturellement compétente récemment décrite, j'ai utilisé la mutagénèse par transposition aléatoire et dirigée pour montrer que les autotransporteurs trimériques sont les principales adhésines de *V. parvula* SKV38, assurant l'adhésion cellule-cellule et cellule-surface. De plus, j'ai identifié une phosphohydrolase HD supposée être impliquée dans la formation de biofilms médiée par les autotransporteurs.

L'identification des déterminants de la formation de biofilms dans les bactéries anaérobies non modèles *B. thetaiotaomicron* et *V. parvula* constitue une première étape qui nous permettra d'aborder la contribution des biofilms à la colonisation et au fonctionnement des microbiotes intestinaux.

Mots clefs :

Microbiote intestinal, biofilms, anaérobies, bile, capsule, autotransporteurs

RESUME DETAILLE

Les biofilms bactériens sont des communautés de bactéries adhérentes à elles-mêmes ou à une surface et entourées d'une matrice extracellulaire, en général composée de polysaccharides, de protéines et d'ADN extracellulaire. De par leur structure, les biofilms expriment des propriétés physiologiques différentes de celles observées dans les cultures planctoniques, notamment une résistance accrue aux stress, une augmentation des transferts d'ADN et de la communication entre cellules et des modifications métaboliques. Le microbiote intestinal humain est une communauté de bactéries, d'archées, d'eucaryotes et de virus dense très importante pour la santé humaine. Il est notamment impliqué dans l'acquisition de nutriments, la maturation des systèmes nerveux et immunitaire et de l'épithélium intestinal et la défense contre les micro-organismes pathogènes. Il a été observé par différentes équipes que le microbiote intestinal, au moins pour sa composante bactérienne, est une structure dense, spatialement organisée, enchâssée dans le mucus intestinal et qui exprime des propriétés différentes de celles observées chez les mêmes communautés planctoniques. De plus, des communautés adhérentes de bactéries ont été décrites à la surface des particules de nourriture dans le lumen et cette adhésion semble modifier le métabolisme bactérien pour favoriser la dégradation des sucres complexes. Pour toutes ces raisons, il a été proposé que le microbiote intestinal ressemblerait plus à un biofilm multi-espèces qu'à une communauté planctonique. De plus, le gros intestin est un environnement essentiellement anaérobie, bien qu'on puisse observer un gradient d'oxygène latéral diffusant depuis l'épithélium intestinal. Il est donc très important d'étudier la formation de biofilm par les membres du microbiote intestinal en absence d'oxygène pour mieux comprendre leur physiologie au sein de l'intestin et déterminer dans quelle mesure l'adhésion contribue à la colonisation, au maintien et à la physiologie du microbiote intestinal. Or la majorité des études sur la formation de biofilm par des bactéries intestinales est basée sur l'emploi de bactéries pathogènes ou de bactéries anaérobies facultatives souvent étudiées en condition aérobie. Lors de ma thèse, j'ai utilisé des cribles génétiques pour déterminer les déterminants de la formation de biofilms chez deux anaérobies strictes intestinales pertinentes, *Bacteroides thetaiotaomicron* et *Veillonella parvula*.

B. thetaiotaomicron est l'un des symbiotes à Gram-négatif les plus abondants du microbiote intestinal humain, impliqué notamment dans la dégradation des polysaccharides

complexes, la maturation du système immunitaire et la défense contre certains pathogènes. La souche de référence génétiquement manipulable VPI 5482 forme peu de biofilm *in vitro*, bien qu'il ait été montré que plusieurs isolats cliniques de *B. thetaiotaomicron* produisaient des biofilms *in vitro*. Une mutagenèse par transposon aléatoire de *B. thetaiotaomicron* VPI 5482 suivie d'une sélection positive a permis d'identifier plusieurs classes de mutants présentant une formation accrue de biofilm.

L'une de ces classes de mutants correspond à des insertions dans le locus de biosynthèse de la capsule 4 (CPS4), l'un des 8 locus codant pour un polysaccharide capsulaire de *B. thetaiotaomicron*. La suppression de CPS4, la capsule la plus exprimée dans nos conditions *in vitro*, conduit à l'apparition d'une sous-population de bactéries acapsulées aillant une forte capacité à co-agréger, augmentant la formation de biofilm. Les capsules 1, 2, 3, 5 et 6 masquent toutes la formation de biofilm lorsqu'elles sont exprimées, comme CPS4, tandis que l'expression de la capsule 8 (CPS8) induit la formation d'un biofilm. Cela suggère que CPS8 pourrait être directement impliquée dans l'adhésion. De façon intéressante, CPS8 est le seul locus de polysaccharide capsulaire contenant deux homologues de FimA, une pilin impliquée dans la formation de pilus de type V, des structures d'adhésion chez *Bacteroides* et *Porphyromonas*.

De plus, le criblage d'une banque de mutants de transposition aléatoire de la souche Δ CPS4 a permis d'identifier deux gènes ayant un impact sur la production de CPS et donc sur la formation de biofilm. En effet, l'homologue de UpxZ de l'opéron CPS4 est un inhibiteur transcriptionnel de l'opéron CPS2 et que la production de capsules augmente lorsque la voie de glycosylation des protéines est entravée, ce qui laisse supposer que ces deux processus pourraient être en concurrence l'un avec l'autre. En conclusion, j'ai montré qu'en plus de leur implication critique dans les interactions avec le système immunitaire, la colonisation intestinale et la résistance aux bactériophages, les polysaccharides capsulaires de *B. thetaiotaomicron* sont des déterminants majeurs de la formation de biofilm chez cette bactérie.

J'ai également recherché des conditions de croissance spécifiques qui induiraient la formation d'un biofilm par *B. thetaiotaomicron* VPI 5482. En effet, il a été démontré que l'ajout de plusieurs molécules typiquement présentes dans l'intestin induisait la formation de biofilm chez un certain nombre de symbiotes et d'agents pathogènes intestinaux. Je montre que l'ajout d'extrait de bile au milieu à de concentrations physiologiques augmente fortement la formation de biofilm de VPI 5482, mais aussi de presque tous les isolats cliniques de *B. thetaiotaomicron* testés et de plusieurs autres espèces de Bacteroidales telles que *B. fragilis*, *B. ovatus* et *Parabacteroides distasonis*.

J'ai ensuite identifié le locus *BT3560-3563* par mutagenèse par transposition aléatoire comme étant requis pour observer une induction de la formation de biofilm en présence de bile. Ce locus contient notamment deux DNAses sécrétées supposées. De façon cohérente, l'ajout de DNAses commerciales dans le milieu de croissance rétabli la formation de biofilm en présence de bile par le mutant $\Delta BT3560-3563$, ce qui suggère que la sécrétion de DNAses extracellulaires est nécessaire pour induire la formation de biofilm en présence de bile. De plus, l'ajout de bile augmente la quantité d'ADN extracellulaire mesurée dans le surnageant de culture, un composant connu de la matrice extracellulaire du biofilm de nombreuses bactéries. D'autres expériences sont néanmoins nécessaires pour évaluer la relation précise entre les DNAses extracellulaires, l'ADN extracellulaire et la formation d'un biofilm en présence de bile.

La bile est un mélange complexe de biliverdine, de cholestérol et d'acides biliaries dérivés du cholestérol. Parmi les acides biliaries testés, j'ai montré que seul le tauroolithocholate, un acide biliaire secondaire conjugué, pouvait induire à lui seul à la formation de biofilm par *B. thetaiotaomicron* VPI 5482 et par plusieurs isolats naturels. Cependant, cette induction ne dépend pas de la présence du locus *BT3560-3563*, ce qui montre que plusieurs composants de la bile - ou une combinaison de ceux-ci - peuvent induire la formation d'un biofilm par différents mécanismes. En conclusion, j'ai montré que la bile et certains composants biliaries spécifiques sont des médiateurs importants de la formation de biofilm dans presque tous les *B. thetaiotaomicron* testés, ce qui suggère que la formation de biofilms par la bile pourrait être une propriété importante de la physiologie des *B. thetaiotaomicron*, soit comme système d'induction de la formation de biofilm dans l'intestin soit comme système de défense contre l'activité antimicrobienne de la bile.

Veillonella parvula est une coque anaérobie à Gram-négatif, bien que faisant phylogénétiquement parti des Firmicutes, un phylum à majorité de Gram-positif, qui habite communément les microbiotes humains et animaux dont le microbiote intestinal et la plaque dentaire. Il est considéré comme un organisme symbiotique et en tant que colonisateur précoce du microbiote intestinal il participerait à l'éducation immunitaire des nourrissons. *V. parvula* est un colonisateur précoce clé de la plaque dentaire lors de l'établissement de communautés microbiennes sessiles, favorisant la croissance de biofilms multi-espèces par sa capacité d'adhésion à de nombreuses autres bactéries et couplant le métabolisme des membres de la communauté par la consommation d'acide lactique. Toutefois, *V. parvula* peut se comporter comme un pathogène opportuniste et a été associé à diverses infections. Bien que la capacité de *V. parvula* à former des biofilms multi-espèces soit probablement la clé de son établissement

dans la plaque dentaire, et potentiellement dans l'intestin, le manque d'outils génétiques pour cette bactérie a sérieusement limité les études mécanistiques.

J'ai développé et adapté des outils génétiques pour la mutagenèse de *V. parvula* en utilisant une souche naturellement compétente récemment décrite, SKV38. En utilisant à la fois la mutagenèse par transposition aléatoire et la mutagenèse dirigée, j'ai caractérisé les adhésines de *V. parvula* responsables de la formation de biofilm *in vitro*. En particulier, je montre que les adhésines sécrétées de type V, les autotransporteurs, sont les déterminants principaux de la formation de biofilm par *V. parvula*, en assurant la médiation des interactions entre bactéries et entre bactéries et surfaces. De plus, je montre que l'auto-agrégation médiée par l'autotransporteur VtaA contribue différemment au biofilm en fonction des conditions : en condition statique, l'auto-agrégation promeut la formation de biofilm, alors qu'en condition de flux constant elle l'entrave. J'ai également identifié une nucléotidase avec un domaine HD qui empêcherait la formation de biofilm médiée par les autotransporteurs et qui pourrait être un régulateur de la formation de biofilm nouvellement décrit.

Plus généralement, j'ai montré que les génomes des Veillonellaceae possèdent un arsenal d'adhésines riche et diversifié, ce qui suggère une évolution rapide des propriétés d'adhésion de la cellule. Cette étude constitue la première étude complète des adhésines de *Veillonella parvula* et la première description de différents outils génétiques facilement mis en œuvre pour cet important symbiote.

Au cours de ma thèse, j'ai caractérisé quatre conditions favorisant la formation de biofilm par *B. thetaiotaomicron* : absence de capsule, expression de CPS8 et induction en présence d'extrait de bile ou de tauroolithocholate. De plus, j'ai identifié la principale famille d'adhésines de *V. parvula* SKV38, les autotransporteurs et j'ai développé des outils qui feront probablement de SKV38 la nouvelle souche de référence de *V. parvula*. L'identification des déterminants de la formation de biofilms dans les bactéries anaérobies non modèles *B. thetaiotaomicron* et *V. parvula* constitue une première étape qui nous permettra d'aborder la contribution des biofilms à la colonisation et au fonctionnement des microbiotes intestinaux.

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ABBREVIATIONS

AhpCF: alkylhydroperoxyde reductase
BMI: body mass index
BSAP: Bacteroides secreted antimicrobial peptides
BSH: bile salt hydrolase
c-diGMP: cyclic di-GMP
CDI: C. difficile infection
cDNA: Complementary DNA
CPS: capsular polysaccharides
DGR: diversity generating retro-element
DNA: desoxyribonucleic acid
ECF: extracytoplasmic function
ECM: Extracellular matrix
eDNA: Extracellular DNA
EECs: enteroendocrine cells
EPS: extracellular polymeric substances
FISH: Fluorescence in situ hybridization
FMT: Fecal microbiota transplantation
FnBPs: fibronectin-binding proteins
GI: gastrointestinal
GLP-1: glucagon-like peptide-1
GPR 41/43: G-protein coupled receptors 41 and 43
HGT: horizontal gene transfer
HTCS: hybrid two component system
IBD: Inflammatory bowel disease
IgA: immunoglobulin A
IGF-1: insulin-like growth factor-1
IUPAC: International Union of Pure and Applied Chemistry
KOW: Kyprides-Ouzounis-Woese
LPS: lipopolysaccharide
MACPF: Membrane Attack Complex/Perforin

NIH: National institute for health
OTU: operational taxonomy units
PCR: polymerase chain reaction
PGA: polymeric beta-1,6-N-acetyl-D-glucosamine
PIA: polysaccharide intercellular adhesin
PSM: β -subclass of phenol-soluble modulins,
PUL: polysaccharide utilization loci database
PULDB: polysaccharide utilization loci database
PYY: polypeptide YY
rRNA: ribosomal ribonucleic acid
S-layer: surface layer
SAAT: self-associating autotransporters
SCFA: Short chain fatty acid
SGBP: cell surface glycan binding protein
sIgA: Secreted immunoglobuline A
Sus: starch utilization system
T5SS: Type V secretion system
T6SS: Type 6 secretion system
TBDT: TonB-dependent transporter
TEM: Transmission electron microscopy
TLR: Toll-like receptor
TNF- α : tumor necrosis factor α
tRNA: transfer ribonucleic acid
UC: ulcerative colitis
WHO: World health organization
WT: Wild type

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BIBLIOGRAPHIC INTRODUCTION

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I- BACTERIAL BIOFILMS

A. What is a biofilm?

1. Generality

Although this chapter will focus exclusively on bacterial biofilms, similar structures have been described in archaea, eukaryotes and even viruses (Harding, Marques, Howard, & Olson, 2009; Orell, Fröls, & Albers, 2013; Thoulouze & Alcover, 2011). The international union of pure and applied chemistry (IUPAC) defines biofilms generically as “aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface”. However, different authors use slightly different definitions in the literature. To cite only a few, a bacterial biofilm is “a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface” (J. W. Costerton, Stewart, & Greenberg, 1999), “a functional consortium of microorganisms organized within an extensive exopolymer matrix” (Elder *et al.* 1995) or “an aggregate of microbial cells surrounded by a self-produced polymer matrix” (Niels Høiby, 2017). Two components are key in these definitions: adhesion, either between bacterial cells or to a biotic or abiotic surface; and presence of an extracellular matrix, in the strictest sense, at least partially produced by the bacteria themselves.

Antoni van Leeuwenhoek, a Dutch scientist, was the first to observe biofilms using one of his own microscope to look at the communities of “*animalcula*” of the surface of the tooth, what would later become known as the dental plaque (van Leeuwenhoek, 1684) (Figure 1). However, it is only in the 1920-30s that the study of sessile bacterial communities, named “film”, “glycocalyx” or “slime”, first developed, mostly on environmental aquatic bacteria (Henrici, 1933; Zobell & Allen, 1935) and the term “biofilm” was first coined in 1975 (Mack, Mack, & Ackerson, 1975). In the 1970s-90s, the observation of biofilms in the lungs of cystic fibrosis patients and on medical implants by Niels Høiby and Bill Costerton introduced the study of biofilms in biomedical research (N. Høiby *et al.*, 1977; Lam, Chan, Lam, & Costerton, 1980; Marrie, Nelligan, & Costerton, 1982) and this led to the development of the genetics of

biofilms, building up most of our basic understanding of biofilm formation and physiology (Niels Høiby, 2017; Lappin-Scott, Burton, & Stoodley, 2014). The idea that biofilms are important, and even the predominant lifeform in the environment was proposed early on (Geesey, Mutch, Costerton, & Green, 1978; Geesey, Richardson, Yeomans, Irvin, & Costerton, 1977). Nowadays the ubiquity of biofilm is widely recognized (Flemming & Wuertz, 2019). Bacterial biofilms can be found in all environments on natural and human-made abiotic surfaces (on the hulls of ships, inside pipes, implants and catheters, etc.) and biotic surfaces (plant roots, human skin, tooth, etc.) (J. W. Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995; Karatan & Watnick, 2009). In fact, studies suggest that biofilms are more abundant than planktonic cells in all habitats except for the ocean (Flemming & Wuertz, 2019). Moreover, the National institute for health (NIH) estimates that 65% of microbial infections and 80% chronic infections involve a biofilm (Jamal *et al.*, 2018). Infections involving biofilms are of two types: tissue-related, such as infection of cystic fibrosis patients, urinary tract infections, endocarditis, etc. and device-associated infections, linked with the implantation of a foreign material within the body such as catheters or prosthesis, and they often prove challenging to treat because of their high resistance to antibiotics (Lebeaux *et al.*, 2014).

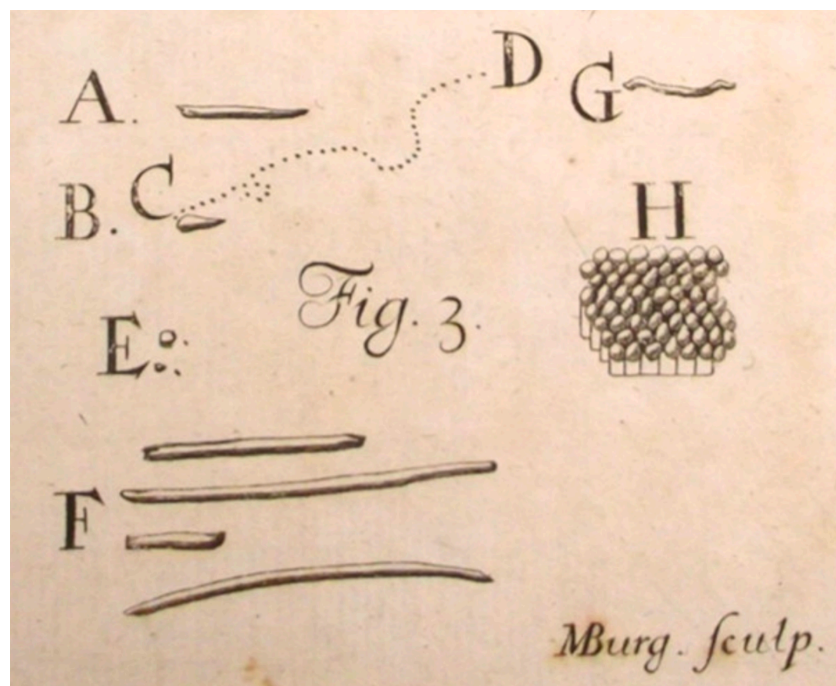


Figure 1: First observation of the “animalcula” of the oral biofilm. From (van Leeuwenhoek, 1684).

Multiple *in vitro* biofilm models are used for their study, including air-liquid interface ring biofilms, colony growing on agar plates, microtiter plates, continuous-flow microfermenters, microfluidic devices, etc. which complicates the comparison of studies performed in different

labs (Azeredo *et al.*, 2017; McBain, 2009) (Figure 2). Classically, the vast majority of studies on single-species biofilms were done with four model organisms: *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (López, Vlamakis, & Kolter, 2010), although consistent literature exist on pathogenic bacteria such as *Vibrio cholerae*, *Acinetobacter baumannii*, *Enterococcus faecalis* and *Klebsiella pneumoniae*, all facultative anaerobes usually studied in aerobic conditions. Except a few exceptions such as *Clostridium difficile*, strict anaerobes are comparatively understudied, despite being highly relevant organisms in many important ecological and medical contexts. In the following chapter, I will present an overview of the general principles of bacterial biofilm formation identified in the main facultative anaerobes model organisms, and then focus on what is known in anaerobic conditions.

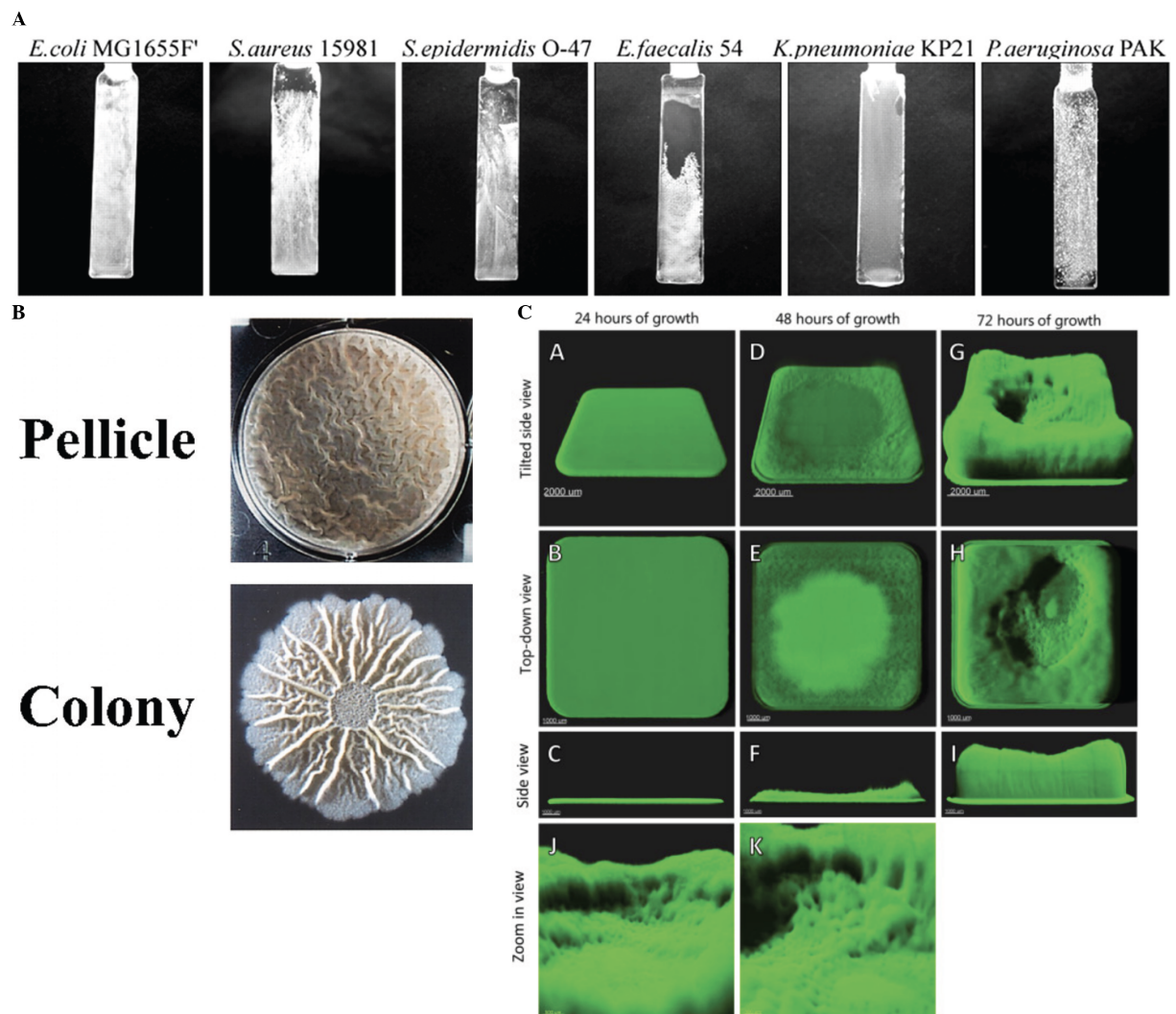


Figure 2: *In vitro* biofilm models.

A. Continuous-flux glass microfermentor biofilms of multiple species. From (Valle *et al.*, 2006). **B.** *B. subtilis* biofilm formation at the liquid-air interface (pellicle) and on agar plates (colony). From (Branda *et al.*, 2004). **C.** Microtiter plate

biofilm formation of *P. aeruginosa* observed by confocal microscopy. Images represent undisturbed biofilms in microtiter plate wells at 24, 48 and 72h. A-I 100x magnification, J-K Magnification of the biomass observed at 72h. From (Kragh, Alhede, Kvich, & Bjarnsholt, 2019).

2. From initial adhesion to a mature biofilm

a. The different stages of biofilm formation

Biofilm formation classically involves three steps: initial adhesion, maturation and dispersal (Figure 3).

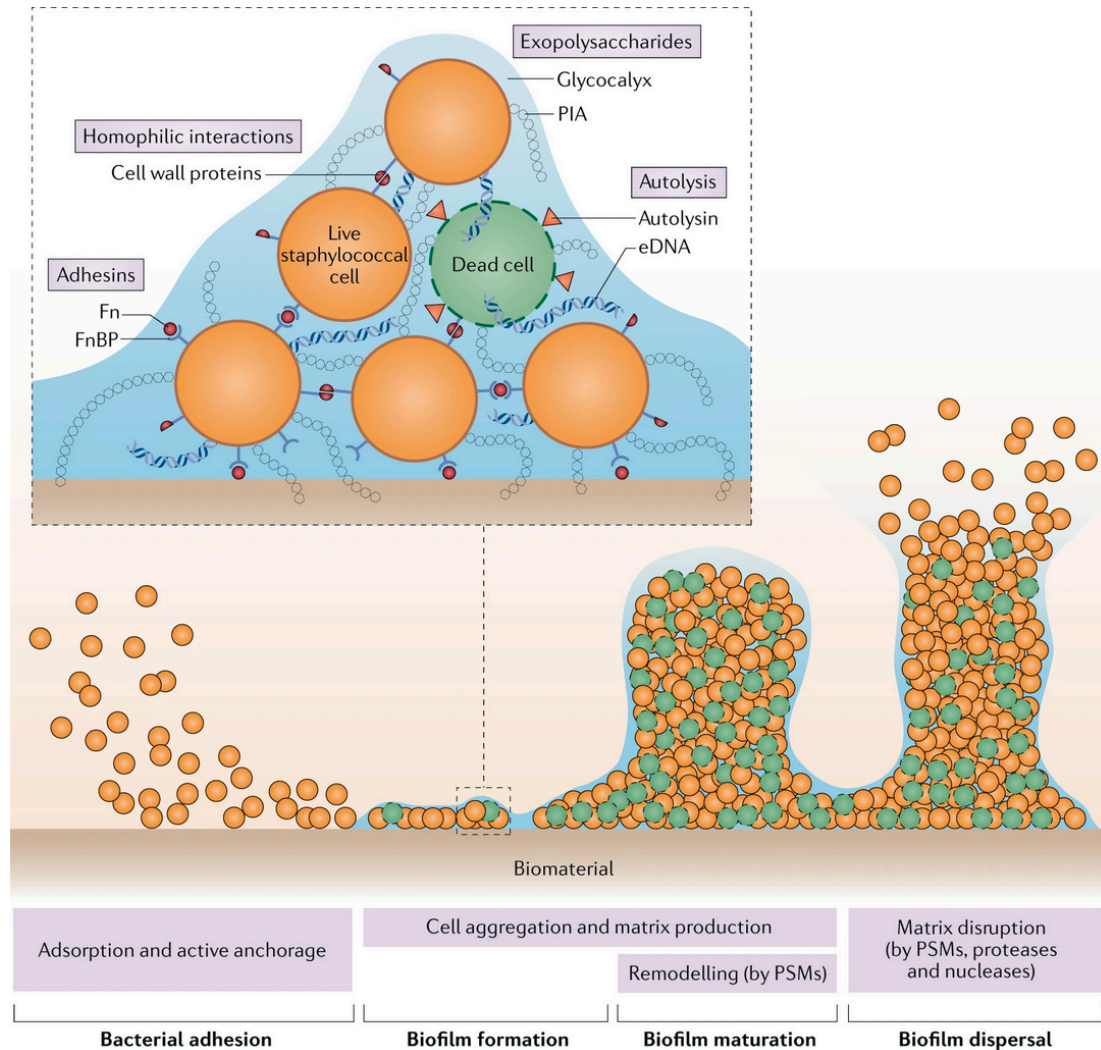


Figure 3: Stages of biofilm formation.

From (Arciola, Campoccia, & Montanaro, 2018). Schematic representation of the classical steps of biofilm formation with the example of *S. aureus*. FnBPs: fibronectin-binding proteins, promote bacterial aggregation. PIA: polysaccharide intercellular adhesin and eDNA: extracellular DNA are components of the extracellular matrix. PSM: β -subclass of phenol-soluble modulins, participates to the formation of water channels in mature biofilms and biofilm dispersal.

Initially, bacteria can reversibly adhere to surfaces if the sum of attractive forces is greater than the sum of repulsive forces between the surface and the bacteria. These forces can include Van der Waals, electrostatic, steric hindrance and hydrophobic forces (Karatan & Watnick,

2009). Both the composition of the bacterial cell envelope, such as its net charge (usually negative) or presence of pili or flagellum, and the surface properties, such as its hydrophobicity or roughness, can impact this adhesion (Cecile Berne, Ellison, Ducret, & Brun, 2018; Faille *et al.*, 2002; Salerno, Logan, & Velegol, 2004). Moreover, any molecule in solution can potentially be adsorbed on the surface, forming a conditioning film that can promote adhesion. For instance, conditioning of a catheter by human blood was shown to enhance biofilm formation of *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Pantoea agglomerans in vitro* (Murga, Miller, & Donlan, 2001). Alginate conditioning film impacted *P. aeruginosa* biofilm formation: it improved non-motile *P. aeruginosa* adhesion, but not so much motile *P. aeruginosa* adhesion and this improvement was strongly dependent on ionic strength of the media (De Kerchove & Elimelech, 2007). After initial contact, the adhesion of bacteria can become irreversible through the action of specific proteinaceous or polysaccharidic adhesins. In *Caulobacter crescentus*, this transition has been especially well studied. Reversible adhesion is mediated by the polar pili and the flagellum. Upon contact with the surface, some cells switch to irreversible adhesion by synthesizing a holdfast, a polysaccharidic adhesive structure. Single-cell labelling of holdfast production showed that cells usually reversibly contacted the surface multiple times before switching to irreversible adhesion (Hoffman *et al.*, 2015).

The biofilm then develops by multiplication of the adhering bacteria and recruitment of planktonic cells through bacteria-to-surface and bacteria-to-bacteria interactions leading to the genesis of a complex 3D structure (J. Costerton, 1987). As the biofilm matures, bacteria produce an extracellular matrix (ECM), a hydrated gel made of polysaccharides, proteins, DNA, ions and other molecules that protects them from stresses and allows spatial organization of the biofilm. It is estimated that only 2-30 % of the biofilm mass is made of cells, the rest being the matrix and empty space (Lawrence, Korber, Hoyle, Costerton, & Caldwell, 1991).

At some point, bacteria start to leave the biofilm to colonize other surfaces, either as planktonic cells or as small aggregates of bacteria. This shift can be caused by detachment, in which bacterial cells are passively removed from the biofilm due to external forces such as shear stress, or by dispersal, in which bacteria respond to an environmental cue to actively release cells from the biofilm (Petrova & Sauer, 2016; Rumbaugh & Sauer, 2020).

b. Adhesins mediate bacteria-to-bacteria and bacteria-to-surface interactions

Bacterial adhesion is mediated by various structures collectively known as adhesins, that are classically either proteinaceous or polysaccharidic. Proteinaceous adhesins include flagella,

type IV and type V pili, curli, chaperone-usher fimbriae, type V secretion system autotransporters, amyloid fibers and various surface proteins (Cécile Berne, Ducret, Hardy, & Brun, 2015; Korea, Ghigo, & Beloin, 2011; Meuskens, Saragliadis, Leo, & Linke, 2019) (Figure 4).

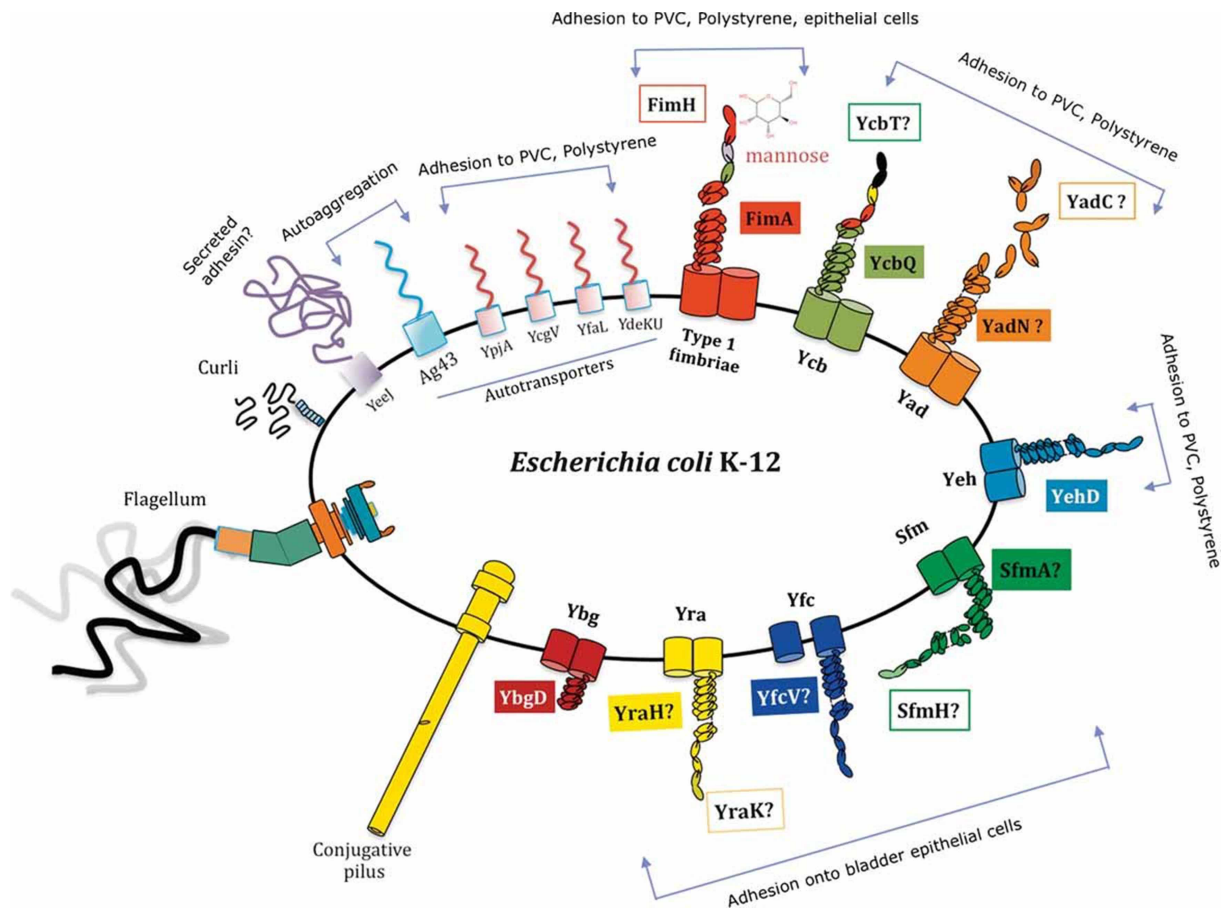


Figure 4: *E. coli* K12 proteinaceous adhesins.

From (Korea *et al.*, 2011)

Many species produce polysaccharidic adhesins. *E. coli* produces polymeric beta-1,6-N-acetyl-D-glucosamine (PGA) that is involved in surface binding, cell-cell adhesion and stabilization of the biofilm structure (X. Wang *et al.*, 2005), and PGA homologs also mediate biofilm formation in many other species such as *S. epidermis*, *S. aureus*, *B. subtilis*, *Y. pestis* and *P. fluorescens* (Itoh, Wang, Joseph Hinnebusch, Preston, & Romeo, 2005; López *et al.*, 2010). *C. crescentus* produces a polysaccharidic holdfast that mediates its irreversible attachment to surfaces (Hoffman *et al.*, 2015). *P. aeruginosa* produces three different polysaccharidic adhesins: alginate, a polymer of mannuronate and guluronate, Pel, a glucose-rich polymer and Psl, a mannose-rich polymer. Alginate is mostly produced by mucoid strains

isolated from cystic fibrosis patients whereas Pel and Psl production is found in non-mucoid strains to different levels (Colvin *et al.*, 2012).

A single bacterium usually encodes multiple adhesins, and even between strains of the same species the arsenal of adhesin can be widely different. For instance, *E. coli* K12 encodes a flagellum, curli, a conjugative pilus, several autotransporters, and several chaperone-usher fimbriae and also produces PGA (Berne *et al.*, 2015; Korea *et al.*, 2011; X. Wang *et al.*, 2005) (Figure 4). These adhesins mediate binding to different substrates, although some appear to be redundant: the autotransporter antigen 43 is involved in cell-cell adhesion, Fim chaperone-usher type I fimbriae binds mannose and mediates epithelial cells adhesion and multiple other adhesins including type I fimbriae and autotransporters mediate adhesion to plastic (Korea *et al.*, 2011). Moreover, these adhesins can be involved at specific stages of biofilm formation: in fact, *E. coli* curli and pili are important for initial binding whereas antigen 43 is involved in biofilm maturation (C. Beloin, Roux, & Ghigo, 2008). Some of these structures pre-exist at the surface of the bacteria, such as flagellum or pili, while others can be induced upon cell contact, such as *C. crescentus* holdfast (Hoffman *et al.*, 2015) or in different environmental conditions.

The multiplicity of adhesins and their tight regulation allow bacteria to modulate precisely their adhesion capacity and to colonize different surfaces.

c. The extracellular matrix

The extracellular matrix (ECM) is a highly hydrated structure, usually produced by the embedded bacteria themselves. Estimated to make up 73 to 98 % of the biofilm mass (Lawrence *et al.*, 1991), it contributes to the adhesive properties of the biofilm and to several other aspects (Flemming & Wingender, 2010; Schlafer & Meyer, 2017). Polysaccharides of the ECM retain water, which maintains a highly hydrated environment and prevent desiccation of the biofilm. More generally, the ECM has been shown to provide protection against many stresses such as osmotic stress, oxygen, antibiotics, host immune defenses, and grazing protozoa. Furthermore, it maintains the structure of the biofilm, allowing genesis of a complex 3D architecture made of pillars, pores and channels (Lawrence *et al.*, 1991). The ECM also limits diffusion, thereby leading to retention of ions, nutrients and enzymes within the biofilm. This spatial structure coupled with impaired diffusion generates gradients of nutrients, oxygen and wastes that define complex micro-niches and drive bacterial heterogeneity. Last, by keeping cells close to each other, it favors DNA exchange and cell-cell communication (Flemming & Wingender, 2010; Schlafer & Meyer, 2017).

The composition of the ECM is highly variable depending on the bacterial species present and the environmental conditions. Polysaccharides were long believed to be the main component of the ECM, and they indeed have been shown to be very important for adhesion, cohesion of the biofilm, retention of various compounds including water and protection against stresses (Arciola, Campoccia, Ravaioli, & Montanaro, 2015). However, it is now acknowledged that the ECM contains many other components such as nucleic acids, proteins, lipids or ions (Flemming & Wingender, 2010; Schlafer & Meyer, 2017) (Figure 5).

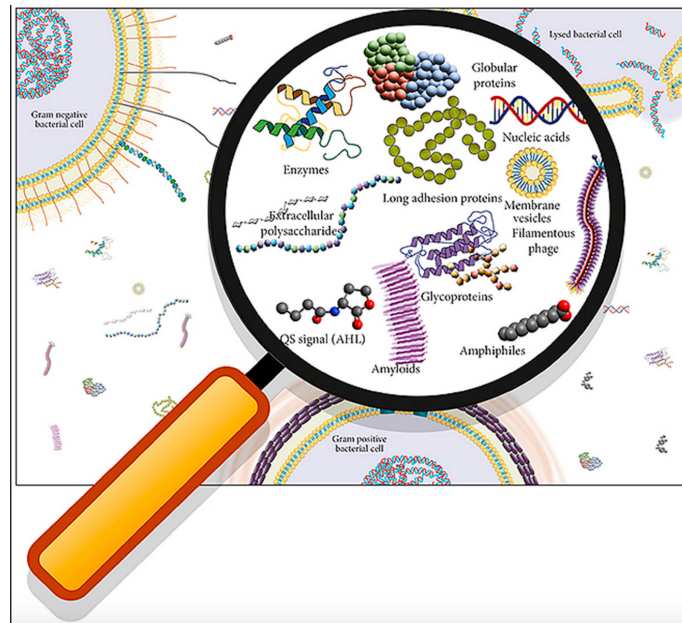


Figure 5: The extracellular matrix.

From (Seviour *et al.*, 2019)

For instance, extracellular DNA (eDNA) was first believed to be a simple waste product of cell lysis. However, the observation that addition of DNases to *P. aeruginosa* biofilms led to a biofilm dispersal showed it played an important role in biofilm formation (Whitchurch, Tolker-Nielsen, Ragas, & Mattick, 2002). Following this work, many others have shown that addition of DNases either prevented biofilm formation or dispersed already formed biofilms of many species (Ibáñez de Aldecoa, Zafra, & González-Pastor, 2017). Moreover, eDNA release within the biofilm has been repeatedly shown to be a controlled process. For instance, *Enterococcus faecalis* produces two proteases, GeIE, that mediates cells lysis and SprE, that prevents GeIE-mediated lysis, both contributing to the tight regulation of eDNA production and biofilm formation (V. C. Thomas, Thurlow, Boyle, & Hancock, 2008). Similarly, *S. aureus* encodes *cidA* and *lrg* that were shown to respectively enhance or prevent cell lysis and eDNA release. Mutation in either of these genes disrupted proper biofilm formation, showing controlled expression of both was necessary (Mann *et al.*, 2009). Many other mechanisms of eDNA release

have been described including bacteriophage-mediated cell lysis or secretion through vesicles or type IV secretion system (Ibáñez de Aldecoa *et al.*, 2017).

Moreover, proteins are abundant within the matrix, in some cases even more abundant than polysaccharides (Frølund, Palmgren, Keiding, & Nielsen, 1996). In particular, many enzymes are confined within the biofilm. They are often involved in the degradation of biopolymers, notably the components of the ECM, allowing usage of this matrix as a nutrient source and for remodeling or dispersal of the biofilm (Flemming & Wingender, 2010). Non-enzymatic proteins include polysaccharide binding proteins that participate to biofilm stabilization by binding ECM structural polysaccharides and proteinaceous adhesins such as amyloid fibers, pili, fimbriae or flagella (Flemming & Wingender, 2010). For instance, the TasA protein, which organizes in amyloid fibers, was found to be a major component of *B. subtilis* ECM (Branda, Chu, Kearns, Losick, & Kolter, 2006; Romero, Aguilar, Losick, & Kolter, 2010).

d. Signals governing biofilm formation

Biofilm formation is a complex process that is tightly regulated by multiple signals. First, mechanical sensing of the surface can lead to transcriptional changes and allow progression of the biofilm formation. For instance, as I previously mentioned, *C. crescentus* surface contact promotes the protease-mediated shedding of the flagellum and induces holdfast production to irreversibly adhere to the surface (Cecile Berne *et al.*, 2018; Hoffman *et al.*, 2015). Moreover, numerous environmental cues have been shown to induce biofilm formation such as glucose, indole, polyamines, iron, bile acids, antimicrobials, etc., although how these signals are sensed and integrated by bacteria is not always fully understood (Karatan & Watnick, 2009).

Despite the variety of signals that can influence biofilm formation, cellular mechanisms controlling biofilm formation very often involve the production of the secondary messenger cyclic di-GMP (c-diGMP). c-diGMP was first identified as a regulator of cellulose production in *Gluconacetobacter xylinus* (Ross *et al.*, 1987). Nowadays, the importance of c-diGMP in mediating biofilm formation was shown for numerous bacteria including *E. coli*, *P. aeruginosa*, *Salmonella* and *V. cholerae* (Hengge, 2020; Jenal, Reinders, & Lori, 2017; Karatan & Watnick, 2009). c-diGMP is synthesized from two GTP moieties by diguanylate cyclases containing the conserved GGDEF domain and is degraded by phosphodiesterases containing either EAL or HD-GYP domains. Many of these proteins contain both a GGDEF and an EAL domain, showing they can function as both a synthetase and a phosphodiesterase, together with sensor domains, showing they can directly respond to environmental signals. Although

phosphodiesterase and diguanylate synthase are often numerous in genomes and little is known about their precise regulation, in most cases, low levels of c-diGMP are linked with motility and induction of biofilm dispersal whereas high levels of c-diGMP are linked with adhesin production and biofilm formation (Hengge, 2020; Jenal *et al.*, 2017; Karatan & Watnick, 2009). Although c-diGMP seems to be the main secondary messenger regulating biofilm formation, cyclic di-AMP has also been shown to be involved (Yin *et al.*, 2020), either inducing it (Peng, Zhang, Bai, Zhou, & Wu, 2016) or repressing it depending on the species (Gundlach, Rath, Herzberg, Mäder, & Stülke, 2016).

Moreover, quorum sensing is a mechanism by which bacteria sense the density of bacterial cells around them by reacting to the concentration of small molecules called autoinducers produced by their neighbors. The higher the density, or the lower the diffusion rate, the higher the concentration of autoinducer. Such a mechanism is particularly important in biofilms, where high density of cells and low diffusion rates are reached. Indeed, it has been shown to regulate adhesion, maturation and dispersal of biofilm in different species (Parsek & Greenberg, 2005; Passos da Silva, Schofield, Parsek, & Tseng, 2017; Saxena, Joshi, Rawat, & Bisht, 2019).

B. Biofilm emergent properties

The distinct microenvironments created within a biofilm drive the emergence of biofilm-specific properties. In the following part, we will review some of them, including gene expression modification, cell-cell interactions and tolerance to antimicrobials (Figure 6).

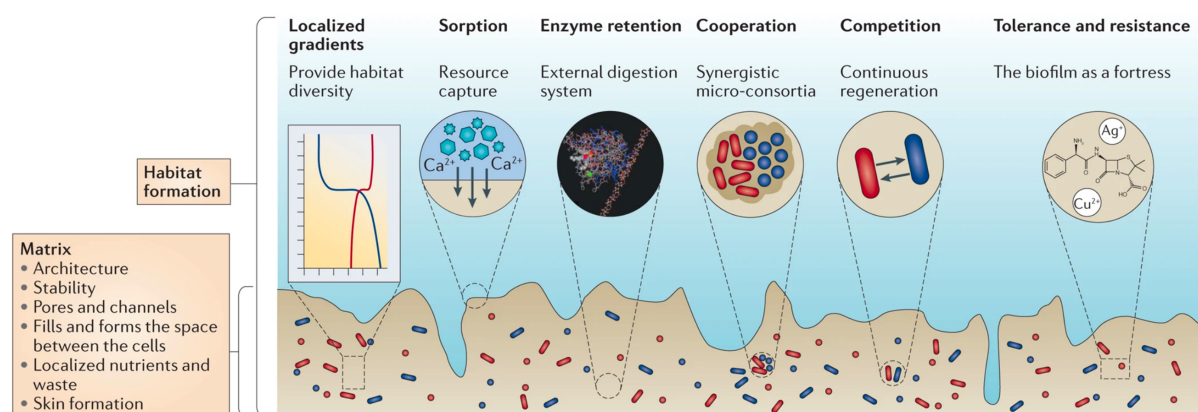


Figure 6: *Biofilm emergent properties.*

From (Flemming *et al.*, 2016)

1. Biofilm-specific gene expression patterns

Several studies have assessed the transcriptomic changes associated with biofilm formation in different bacteria, but there are strong inconsistencies between studies. In fact, between 2 to 60% of *E. coli* genes were shown to be differentially expressed in biofilms compared to planktonic cultures depending on the study considered, and there is little redundancy amongst the genes identified between the different studies (Bayramoglu, Toubiana, & Gillor, 2017; Christophe Beloin *et al.*, 2004; Schembri, Kjærsgaard, & Klemm, 2003). Therefore, the identification of a universal biofilm-specific gene expression pattern has so far remained elusive, in part because multiple biofilm systems are used, preventing data reproducibility. However, biofilms seem to share several properties. As expected, adhesin production is modified, with flagellum expression usually being repressed in biofilms and other adhesins' expression increasing, such as type I fimbriae and antigen 43 in *E. coli* (Schembri *et al.*, 2003). Moreover, many genes induced in biofilms are also associated with stationary phase cultures and with stress responses, probably owing to the specific environment of the biofilm with limited access to nutrients, accumulation of waste and mechanical constraints, etc. (Christophe Beloin & Ghigo, 2005).

2. Cell-cell interactions and heterogeneity

Biofilms are highly heterogenous structures. The ECM limits the diffusion of many compounds, and the balance between production, consumption and diffusion creates complex gradients of nutrients, oxygen, waste, pH and ions. Therefore, bacteria are exposed to different environmental conditions within the biofilm, generating physiological adaptations (Rani *et al.*, 2007; Stewart & Franklin, 2008) (Figure 7). In particular, lack of oxygen and nutrients within the deep layers of the biofilm correlate with low metabolic activity of these cells that enter a state of dormancy, a property which participates to biofilm-mediated antibiotic tolerance (Niels Høiby, Bjarnsholt, Givskov, Molin, & Ciofu, 2010; Rani *et al.*, 2007).

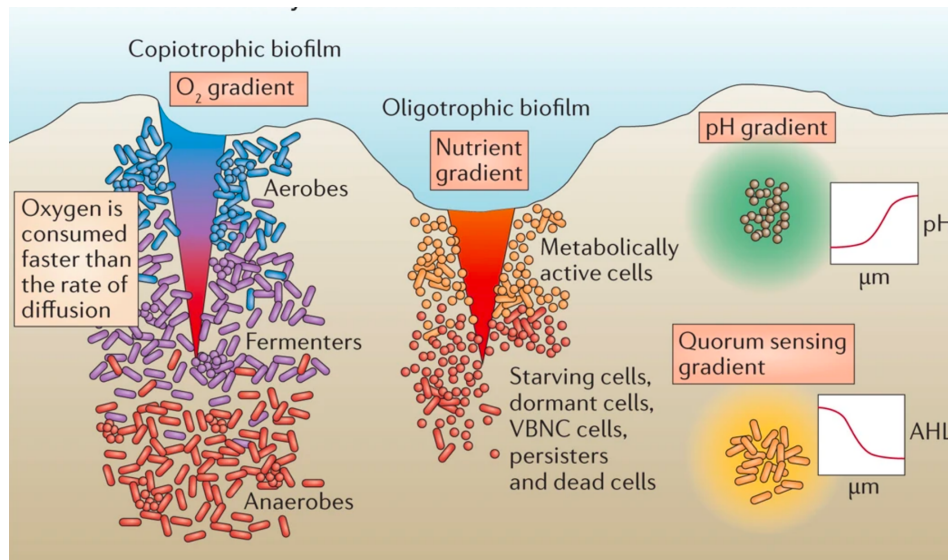


Figure 7: Micro-niches within the biofilm.

From (Flemming *et al.*, 2016). The biofilm is characterized by gradients of oxygen, nutrients, waste products, pH and quorum sensing signals, driving bacterial physiological adaptations.

The close interactions between cells in biofilms also promotes social interactions including communication (quorum sensing, electrical communication), cooperation (sharing of public goods or trophic networks) and competition (killing of other members, cheating) in multi-species biofilms.

In particular, biofilm formation is classically considered to enhance horizontal gene transfer (HGT) notably thanks to the close proximity of cells (Madsen, Burmølle, Hansen, & Sørensen, 2012). Indeed, the rate of conjugative plasmid transfer was shown to be higher in biofilms compared to planktonic cultures (Sørensen, Bailey, Hansen, Kroer, & Wuertz, 2005; Wuertz, Hendrickx, Kuehn, Rodenacker, & Hausner, 2001). However, other studies suggested that plasmid transfer only occurs in the outer layer of the biofilm, where oxygen is present and cells are in a high metabolic state (Król *et al.*, 2011; Merkey, Lardon, Seoane, Kreft, & Smets, 2011). Therefore, the spatial structure of the biofilm is expected to have a strong impact on the local efficiency of plasmid conjugation. Moreover, *E. coli* competence was shown to be induced in biofilms allowing transfer of non-conjugative plasmids by transformation (Maeda *et al.*, 2006). Transformation efficiency might even be further enhanced by the production of eDNA within biofilm. Interestingly, HGT can also enhance biofilm formation. By essence, conjugation requires cell contact and can therefore be expected to promote adhesion. Moreover, conjugative plasmids have been shown to encode biofilm promoting factors (Ghigo, 2001). For instance, IncF conjugative plasmid induced curli and colonic acid production in *E. coli*, thereby increasing biofilm formation (May & Okabe, 2008).

3. Antibiotic tolerance

Biofilms commonly harbor an increased tolerance to multiple stresses such as antibiotic, osmotic stress and oxidative stress, host immune defenses, and grazing protozoa (Flemming & Wingender, 2010). Tolerance describes the capacity of a bacterium to transiently survive lethal stress conditions, contrary to resistance that refers to an inheritable capacity acquired through mutation or HGT to grow in presence of high concentrations of the stressor (Figure 8).

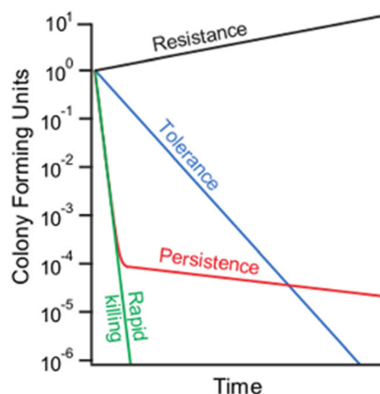


Figure 8: Antibiotic killing kinetics of bacterial cultures.

From (Kaldalu, Hauryliuk, & Tenson, 2016)

In particular, biofilms of multiple species have been shown to be able to tolerate antibiotic concentrations sometimes as high as 100-times the minimal inhibitory concentration (J. W. Costerton *et al.*, 1999; Stewart, 2015). Initially, antibiotic tolerance was thought to be the result of impaired antimicrobial diffusion within the biofilm. Indeed, negatively charged polymers such as eDNA or alginate were shown to interact with positively charged antibiotics, limiting or delaying their diffusion through *P. aeruginosa* biofilms (Chiang *et al.*, 2013; Hentzer *et al.*, 2001; Tseng *et al.*, 2013). Vancomycin was also shown to interact with the polysaccharides of *S. aureus* biofilms leading to a lower diffusion rate. However, this interaction did not prevent antibiotic penetration through the biofilm, merely delayed it, leading the authors to propose that a slower build-up of antibiotic concentration would lead to transcriptional changes, allowing bacteria to better tolerate vancomycin (Jefferson, Goldmann, & Pier, 2005). Despite these studies, it is clear that slower diffusion alone cannot account for the observed increased tolerance as most antibiotics do not interact with ECM components and can diffuse through the biofilm (Daddi Oubekka, Briandet, Fontaine-Aupart, & Steenkeste, 2012; M. C. Walters, Roe, Bugnicourt, Franklin, & Stewart, 2003).

Some ECM components were shown to directly mediate bacterial cells antibiotic tolerance. In particular, eDNA was shown to acidify the ECM and chelate divalent metal cation, which in turn modifies bacterial gene expression and increases *P. aeruginosa* tolerance against different stresses, including antibiotic treatment (Lewenza, Johnson, Charron-Mazenod, Hong, & Mulcahy-O'Grady, 2020; Mulcahy, Charron-Mazenod, & Lewenza, 2008; Wilton, Charron-Mazenod, Moore, & Lewenza, 2016).

Another mechanism that was proposed is the implication of stress responses that are often activated in biofilms and could mediate cross-protection against antimicrobials. Thus, *E. coli* biofilms were shown to have an increased tolerance to fluoroquinolones through activation of the DNA damage SOS response (Bernier *et al.*, 2013).

However, altered bacterial metabolic state is now believed to be the main driver of antibiotic tolerance. Indeed, gradients of nutrients and waste lead to a lowered metabolic activity and even dormancy in the deep layers of the biofilm (Rani *et al.*, 2007; K. D. Xu, Stewart, Xia, Huang, & McFeters, 1998). As many antibiotics target cellular processes that are expressed in growing cells, such as cell wall or protein synthesis, they can only impair metabolically active cells (Yan & Bassler, 2019). Consistently, antibiotic tolerance of *S. aureus* was shown to be similar in stationary phase planktonic cultures and biofilms (Waters, Rowe, O'Gara, & Conlon, 2016), although this was not the case for *Burkholderia cepacia* (Desai, Bühler, Weller, & Bown, 1998). Analysis of *P. aeruginosa* biofilms showed that tobramycin and ciprofloxacin both penetrated the biofilm but killing was only observed in the surface layer of the biofilm where the oxygen concentration and the metabolic activity was high, confirming altered metabolism rather than impaired diffusion was responsible for antimicrobial tolerance (M. C. Walters *et al.*, 2003). Similar observations were made for *Klebsiella pneumoniae* (Anderl, Zahller, Roe, & Stewart, 2003). *P. aeruginosa* mutants impaired for the stringent response, which mediates metabolic dormancy induced by nutrient starvation, showed a 1000-fold reduction of biofilm survival to antimicrobials (Nguyen *et al.*, 2011). Interestingly, the stringent response is also known to be involved in persister formation.

Although a general reduced metabolism is observed in the deep layers of biofilms, a subpopulation of cells, the persisters, can enter into an extreme state of dormancy (Figure 7). Persisters are well-known phenotypic variants that arise partially stochastically within a population and are highly tolerant to antimicrobials (Balaban, Merrin, Chait, Kowalik, & Leibler, 2004). They are believed to be responsible for the chronic state of many biofilm infections, as they serve as a reservoir of surviving cells that are extremely hard to kill and that can resume growth at any time, leading to a relapse of infection (Lewis, 2008).

C. Oxygen within the biofilms

1. Biofilm-associated oxygen gradients

Most studies mentioned in this chapter focused on facultative anaerobe model organisms in presence of oxygen. In these conditions, measurements of oxygen by microelectrode consistently revealed a gradient of oxygen concentration within the biofilm: oxygen concentration was high at the interface with liquid and low or even null in the deeper parts of the biofilm (Rani *et al.*, 2007; Stewart & Franklin, 2008) (Figure 9). This gradient arises through two mechanisms: the lowered oxygen diffusion rate within biofilms, that is estimated to be approximately 60 % of the rate measured in water based on observation for similar compounds, and the rapid consumption of oxygen by bacteria close to the surface (Stewart & Franklin, 2008). It is not a linear gradient, but rather follows the architecture of the biofilm that is made of a complex assembly of cell clusters encased in ECM and void spaces (de Beer, Stoodley, Roe, & Lewandowski, 1994) (Figure 9).

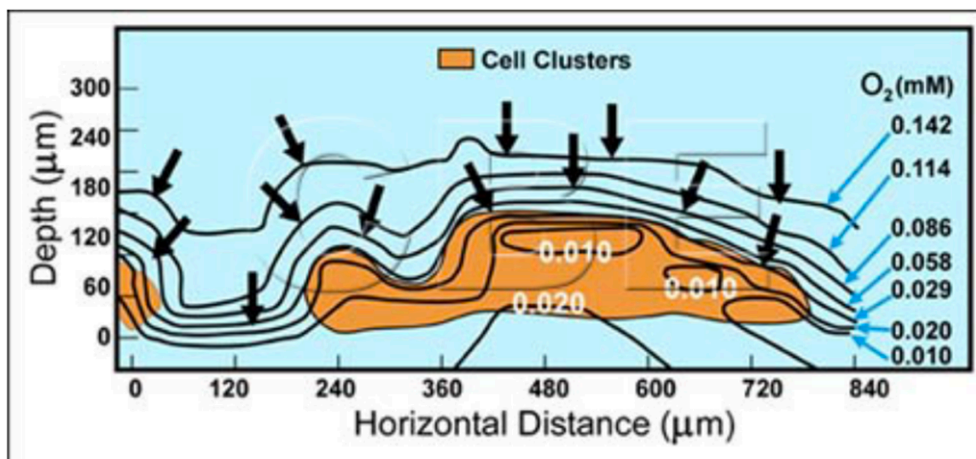


Figure 9: Oxygen gradients within the biofilm.

From (de Beer *et al.*, 1994). Cell clusters are shown in orange and were determined by microscopic observation. Oxygen gradients are represented by vectors, numbers within the figure and on the right margin indicate the local oxygen concentrations (mM).

The oxygen distribution correlates with metabolic activity: metabolism is higher in oxygen-rich regions of the biofilm where respiration can take place (Rani *et al.*, 2007; K. D. Xu *et al.*, 1998). As oxygen is depleted faster than other nutrients, fermentation can take place in the middle layers where oxygen is absent but other nutrients are still present. In the deeper part of the biofilm, bacteria switch to anaerobic metabolism and the metabolic activity is at its lowest, some cells even entering dormancy (Rani *et al.*, 2007; Stewart & Franklin, 2008) (Figure 7). Consistently, *Shewanella oneidensis* cells in the deeper parts of the biofilms expressed genes that are typically upregulated in anaerobic condition (Teal, Lies, Wold, & Newman, 2006). In multi-species biofilms, the different members of the community are

spatially organized according to their metabolic requirements, with aerobes closer to the surface and strict anaerobes persisting in the deeper layers of the biofilm where conditions are fully anaerobic (Stewart & Franklin, 2008). Such an organization has been proposed as the basis of strict anaerobe colonization of the oral microbiota (Kenney & Ash, 1969). It is to be noted that bacteria do not seem to organize in layers, but are rather mixed together in complex spatial arrangements (Stewart & Franklin, 2008) (Figure 10).

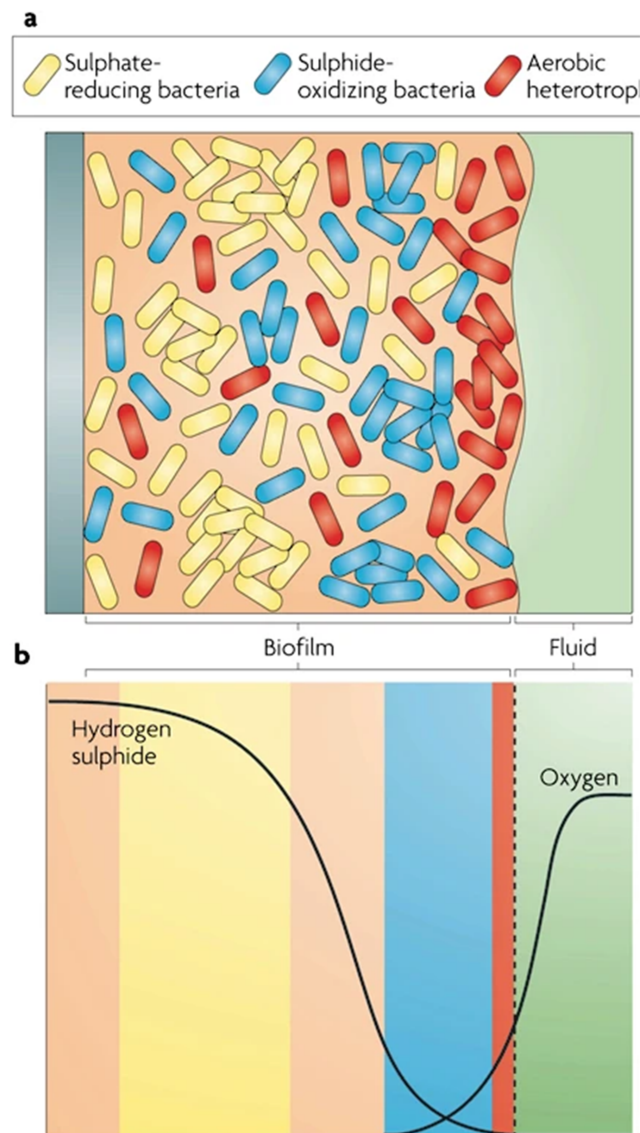


Figure 10: Biofilm organization of species with different oxygen requirements.

Three groups of microorganisms are distributed within a biofilm (a), and each experiences a range of chemical microenvironments (b). In panel b, shaded zones denote the regions of oxygen respiration (red), sulphide oxidation (blue) and sulphate reduction (yellow), and the black curves describe the concentration profiles for hydrogen sulphide and oxygen. From (Stewart & Franklin, 2008).

2. Gradients of oxygen impact bacterial physiology within biofilms

The presence of an oxygen gradient has been shown to drive many phenotypes associated with biofilm formation. Expression of adhesins such as alginate in *P. aeruginosa* and curli and type I fimbriae in uropathogenic *E. coli* is impacted by oxygen concentration, driving differential expression of adhesins within the biofilm (Bayer, Eftekhari, Tu, Nast, & Speert, 1990; Floyd *et al.*, 2015). Morphogenesis of *P. aeruginosa* biofilms was also linked to the redox state of cells and to wrinkles formation, which are expected to maximize oxygen availability within the biofilm (Dietrich *et al.*, 2013). Oxygen concentration also impacted *agfD* promoter activity, the major regulator of multicellularity and biofilm formation of *S. typhimurium* (Gerstel & Römling, 2001). Additionally, in *E. coli* biofilms, expression of quinol oxidases also correlated with the oxygen gradient, a spatial organization important for the regulation of ECM components secretion, with consequences on biofilm architecture and tolerance to stresses (Beebout *et al.*, 2019).

Moreover, oxygen was found to impact classical emergent properties of the biofilm. Oxygen concentration impacts conjugation efficiency, leading to plasmid transfer being restricted to the outer layers of the biofilm (Król *et al.*, 2011). Additionally, several studies showed that the oxygen gradient was involved in antimicrobial tolerance. Oxygen limitation within mature *P. aeruginosa* biofilms was linked with reduced metabolic activity, leading to an increased tolerance to multiple antibiotics compared to early biofilms in which the oxygen gradient was not yet developed. Oxygen limitation contributed to 70% of the observed antimicrobial tolerance in mature biofilms (Borriello *et al.*, 2004; M. C. Walters *et al.*, 2003). Aside from lowering the metabolic activity of cells, hypoxia was shown to modify multidrug efflux pump expression, increasing *P. aeruginosa* tolerance to antimicrobials (Schaible, Taylor, & Schaffer, 2012).

Last, oxygen depletion and anaerobic metabolism products were shown to promote biofilm dispersal (Karatan & Watnick, 2009). In *S. oneidensis*, this hypoxia-induced biofilm dispersal involved transcriptional regulators (Thormann, Saville, Shukla, & Spormann, 2005). Reactive nitrogen intermediates such as nitric oxide formed by anaerobic metabolism were shown to induce biofilm dispersal of *P. aeruginosa*. These reactive nitrogen species were detected in mature biofilms and mutants defective of nitric oxide production failed to disperse (Barraud *et al.*, 2006).

3. Anaerobic biofilm formation

Within aerobic biofilms, the oxygen gradient drives a lot of phenotypes. Although many relevant ecological and medical biofilms are formed in anaerobic conditions, in which no oxygen gradient exists, anaerobic biofilm formation has been comparatively poorly studied.

Anaerobic biofilm formation was assessed in classical facultative anaerobe models grown in absence of oxygen. Absence of oxygen was found to increase biofilm formation of *Streptococcus mutans* in an autolysin A (AtlA)-dependent manner (Ahn & Burne, 2007). Similarly, anaerobic conditions were associated with higher polysaccharide adhesin production of *S. aureus* (Cramton, Ulrich, Götz, & Döring, 2001) and higher cell lysis, increasing eDNA production and ultimately leading to increased biofilm formation of *S. aureus* (Mashruwala, van de Guchte, & Boyd, 2017).

In anaerobic conditions, *E. coli* formed patchier and thinner biofilms than in aerobic conditions. Transcriptome profiling showed that many genes were repressed in anaerobic biofilms compared to aerobic biofilms, including genes involved in metabolism, showing a global reduction of metabolic activity in anaerobic biofilms. Only 3 genes were upregulated in anaerobic biofilms, *safA*, encoding a two-component system connector and two genes of unknown function. However, surprisingly the EPS of anaerobic and aerobic biofilms were quite similar in terms of content and genes regulating EPS production were not expressed differently between the two conditions, showing that while metabolism of the biofilm was altered in absence of oxygen, its structure might be quite similar to aerobic biofilms (Bayramoglu *et al.*, 2017).

Biofilms formed in the lung of cystic fibrosis patients were shown to be mostly anaerobic. Therefore, increasing attention has been given to *P. aeruginosa*, one of the major pathogens responsible for infections of cystic fibrosis patients, anaerobic biofilm formation. Contrary to *E. coli*, *P. aeruginosa* was found to form stronger biofilms in anaerobic compared to aerobic condition. Mutants incapable of synthesizing a type IV pilus formed strong aerobic biofilms but they were severely impacted for anaerobic biofilm formation, whereas mutants incapable of flagellar synthesis formed poor biofilms regardless of oxygenation, showing both flagellum and type IV pilus are important adhesins in anaerobic conditions. Expression of factors to prevent accumulation of nitric oxide was necessary to preserve cell viability within anaerobic biofilms, showing anaerobic metabolism was associated with specific stress conditions. Last, proteomic analysis revealed several proteins that were enriched in anaerobic biofilms compared to aerobic biofilms, including two proteins, the 50S ribosomal protein L9

and OprF, a channel-forming porin, that were present exclusively in anaerobic biofilms (Yoon *et al.*, 2002).

Anaerobic biofilm formation has also been assessed in strict anaerobes such as *Clostridium difficile* as well as in different strictly anaerobic oral microbiota members. *In vivo*, *C. difficile* was found to be associated with the mucus layer both in mono-associated mice and conventional mice (Semenyuk *et al.*, 2015; Soavelomandroso *et al.*, 2017). In particular, strain R20291 formed biofilm-like 3D structures on the top of the outer mucus layer and produced the extracellular polysaccharide PS-II typically found in the ECM of *in vitro* biofilms (Soavelomandroso *et al.*, 2017). *In vitro*, biofilm formation was shown to be widespread within *C. difficile* species (Pantaléon *et al.*, 2018). Biofilm formation was dependent, among other things, on flagella production, quorum sensing, and the regulator of sporulation Spo0A. Moreover, *C. difficile* ECM contained proteins, eDNA and polysaccharides and *C. difficile* biofilms showed an increased tolerance to vancomycin and metronidazole (Dapa *et al.*, 2013; Semenyuk *et al.*, 2014). Therefore, anaerobic biofilms formed by *C. difficile* shared many properties with classical models of aerobic biofilms, suggesting that common properties are associated to sessile lifestyle independently of the oxygen gradient.

In the next chapter, I will show that the gut microbiota has a biofilm-like organization and that it is a mostly anoxic environment, prompting the need for common gut microbiota symbiont models of anaerobic biofilm formation.

II- IS THE COLON BACTERIAL MICROBIOTA A BIOFILM?

A. The colon bacterial microbiota

1. The gastro-intestinal tract

The gastrointestinal (GI) tract is composed of several interconnected parts that are responsible for the digestion of food and absorption of nutrients (Savage, 1977). From the apical end to distal end we can distinguish, based on anatomical and functional characteristics, the esophagus, the stomach, the small intestine (composed of the duodenum, the jejunum and the ileum), the large intestine (the cecum and ascending, transverse and descending colon) and the rectum. The adult human GI tract is approximately 7m long in total and it has a surface of approximately 300 m² (E. G. Zoetendal, Rajilić-Stojanović, & De Vos, 2008).

The small intestine is characterized by a low pH that progressively increases, a high concentration of simple nutrients that are rapidly absorbed by the epithelium, and an oxygen concentration that gradually decreases (Figure 11). The large intestine, and in particular the colon, is almost devoid of simple nutrients and rather contains undigested nutrients such as fibers and complex carbohydrates. It is a mostly anoxic environment, although there is a lateral oxygen gradient diffusing from the epithelium to the lumen (Pereira & Berry, 2017) (Figure 11). The colon epithelium is composed of a single layer of cells tightly bound together to maintain barrier integrity that forms villi to increase the available exchange surface with the lumen. Cells are produced in the intestinal crypts and then migrate progressively as they differentiate to reach the tip of the villus, where cells are shed in the lumen, allowing for a constant renewal of the epithelium every 4-5 days (van der Flier & Clevers, 2009).

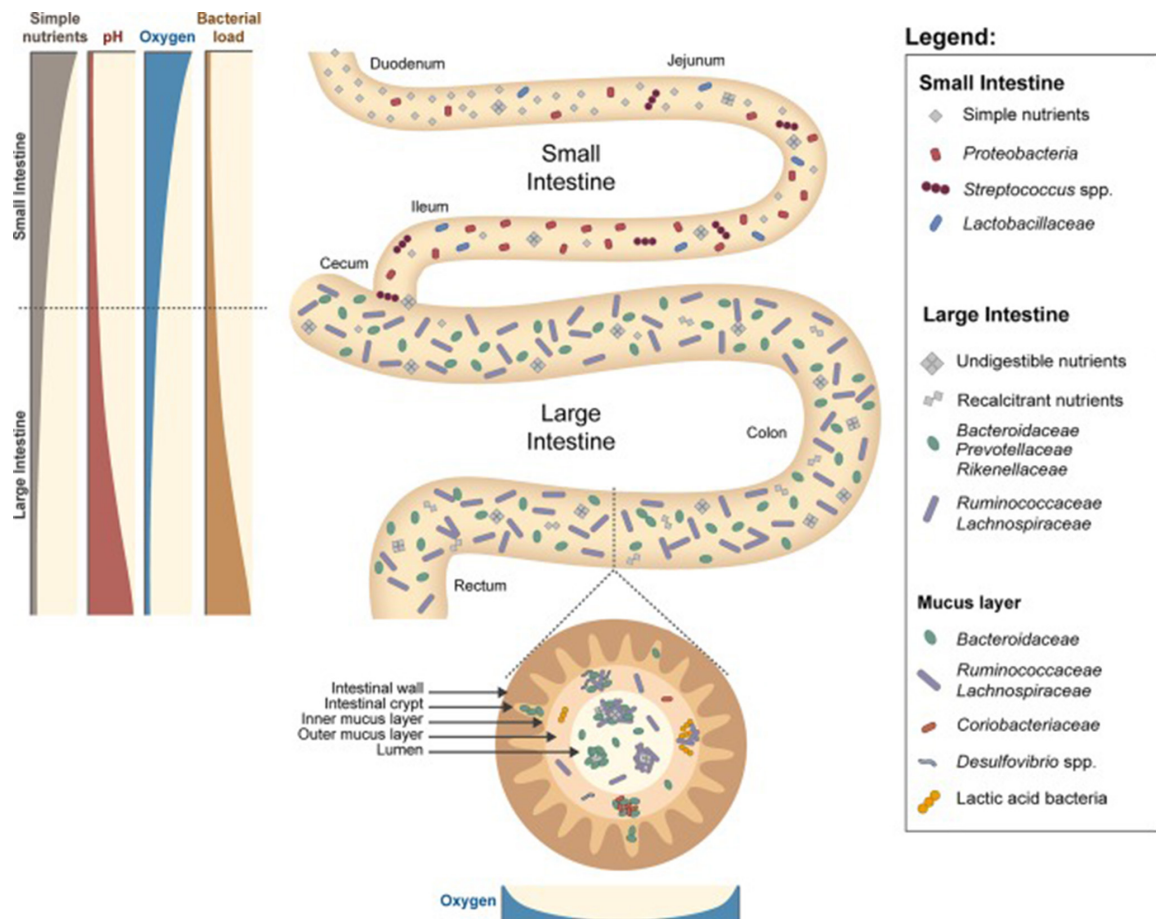


Figure 11: Spatial heterogeneity in the human gut.

Different bacterial niches with the small and large intestine. Gradients of nutrients, pH and oxygen impact the bacterial load and composition. From (Pereira & Berry, 2017).

The intestine represents a very large surface of interaction between the host and the outside environment that is protected by several mechanisms. First, the epithelium is covered by a mucus layer, thin in the small intestine and larger in the large intestine, which lubricates

the intestine and protects the epithelium. This mucus is a gel-like structure composed of mucins, glycoproteins secreted by goblet cells. In the colon, it is composed of two layers: the inner layer is a dense gel attached to the epithelium and it is hardly colonized by micro-organisms, physically preventing contacts between the luminal content and the epithelium. The outer layer is looser, through the action of proteases secreted by the host or by bacteria and is home to a dense community of microorganisms. Moreover, immune cells are extremely abundant in the intestine and there is a constant secretion of antimicrobial peptides and immunoglobulin A (IgA) that prevent micro-organisms from getting too close to the epithelium (Adak & Khan, 2019).

2. Composition of the colon bacterial microbiota

The GI tract is home to a dense community of micro-organisms, composed of bacteria but also viruses, archaea and eukaryotes (Eckburg *et al.*, 2005). The density of micro-organisms increases along the tract to reach its highest density in the colon, where there are low levels of acid and O₂ and where the retention time is high. For the rest of this introduction, I will only consider the bacterial microbiota, with a specific focus on the large intestine microbiota.

The gut microbiota is home to 10¹³-10¹⁴ bacteria, the same order of magnitude as human cells in the body according to the most recent estimate (Sender, Fuchs, & Milo, 2016). Two non-culture-based techniques are widely used to assess community composition. Sequencing of the 16s rRNA genes -or only of the 9 variable regions of this gene- is generally used to define operational taxonomy units (OTU), which are defined as a collection of organisms with more than 97-99 % 16s rRNA sequence identity, depending on the studies, and that are a proxy for species composition. More recently, the development of metagenomics has allowed the characterization of the microbiota in terms of both taxonomy and gene content.

To date, more than 1000 OTUs have been described in the gut microbiota, of which only several hundreds of species could be cultivated. The composition of the microbiota is highly variable between individuals but it is stable for years in adults (Jones *et al.*, 2018; Rajilić-Stojanović, Heilig, Tims, Zoetendal, & de Vos, 2013). While variable in species and strain composition, the distal gut microbiota is always dominated by Firmicutes (genus *Clostridium*, *Eubacterium* and *Ruminococcus*) and Bacteroidetes (genus *Bacteroides* and *Prevotella*) (Figure 12), which make up more than 90 % of the community. Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia and Cyanobacteria are also represented, but not dominant (Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005). The anaerobes outnumber the aerobes

by 100-1000 times in the colon, in keeping with this mostly anoxic environment (Adak & Khan, 2019). The vast majority of studies are performed on stool samples, which are easy to acquire and are believed to be a proxy for distal colonic microbiota. However, it was shown that feces composition only partially recapitulated colon community composition, and that the mucosal and luminal colonic microbiota were also different, highlighting the importance of taking the biogeography of the gut into account when assessing community composition (Jones *et al.*, 2018; Stearns *et al.*, 2011; Zmora *et al.*, 2018) (Figure 12).

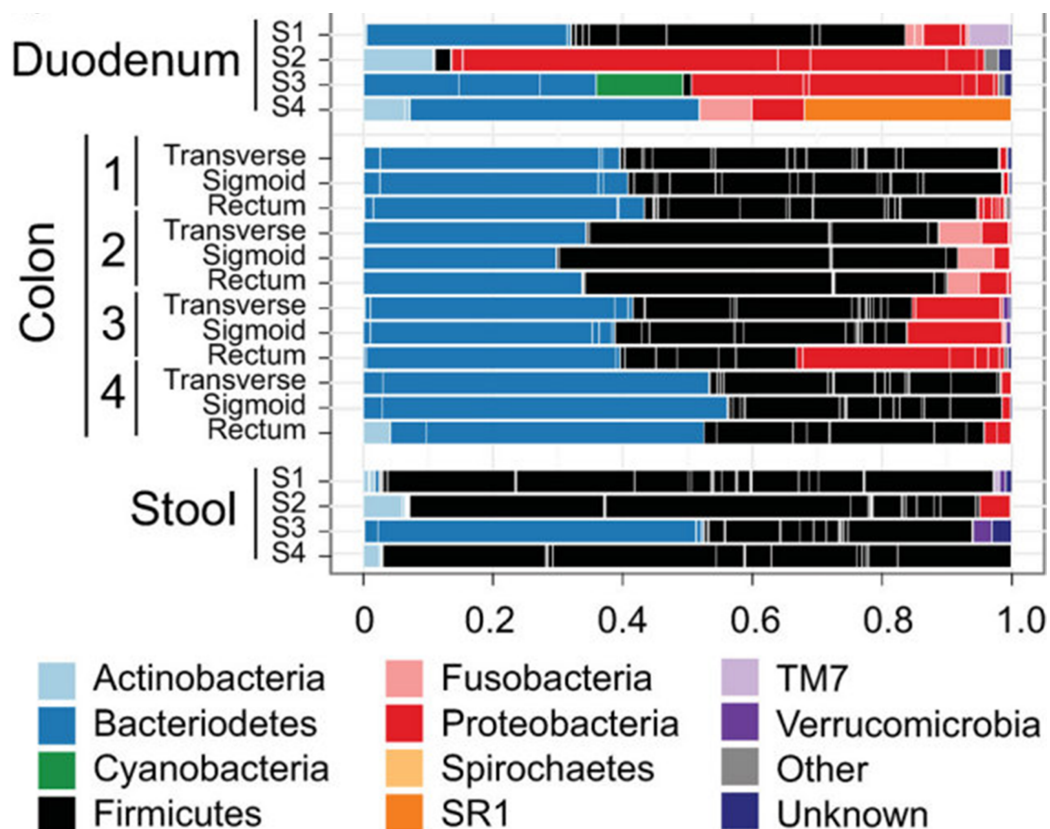


Figure 12: Human gut microbiota composition in different niches.

Adapted from (Stearns *et al.* 2011).

Although the interindividual composition is high, analysis of 39 samples from 6 countries led to the description of three main microbial organizations, called enterotypes, that did not correlate with country of origin. Each one is defined by a predominant genus compared to the others: *Bacteroides* for enterotype 1, *Prevotella* for enterotype 2 and *Ruminococcus* for enterotype 3. It was proposed these enterotypes could be used to predict how individuals would respond to some treatments (Arumugam *et al.*, 2011). However, this concept is still debated, as some studies failed to observe enterotype 3 and some authors suggest that the repartition of

microbiota composition between individuals is continuous rather than stratified (Jeffery, Claesson, O'Toole, & Shanahan, 2012).

More recently, omics techniques have been used to characterize the gut microbiota in terms of composition and function. Metagenomics assay have shown that the microbiome -the collective gene content of the microbiota- is 100 times greater than the human gene number. Moreover, the gene content seems to be more conserved between individuals than the taxonomic composition of the microbiota, suggesting common functionalities are conserved but not performed by the same members of the community (Qin *et al.*, 2010; Turnbaugh *et al.*, 2009). Functional characterization of the microbiota has also been assessed by metatranscriptomics and metaproteomics. In particular, it was shown that the microbiota metaproteome was stable in time, and a core metaproteome of 1,000 proteins was described, composed mainly of proteins involved in carbohydrate metabolism and transport, and surface proteins (Kolmeder *et al.*, 2012).

3. Establishment of the gut microbiota

Two paradigms have been debated in recent years regarding the establishment of gut microbiota. The first one is the sterile womb hypothesis, that states the infant gut is sterile *in utero* and that colonization starts during parturition. It is supported by several studies that failed to detect bacteria in the placenta or in the feces of newborn babies by culturing, microscopy or in some cases PCR amplification. The second hypothesis is the *in utero* colonization that states the placenta is not a sterile environment and colonization of the gut starts during pregnancy. This was proposed after several independent studies detected bacterial DNA to low levels in the placenta of healthy pregnancies by PCR. This second hypothesis is very debated because until recently 1) even though bacterial DNA was detected, no bacteria could be cultivated, suggesting no live bacteria were present 2) PCR based techniques are very sensitive to contamination, especially when low amounts of DNA have to be detected and 3) the successful production of axenic mammals would theoretically not be possible if colonization started *in utero* (Perez-Muñoz, Arrieta, Ramer-Tait, & Walter, 2017). However, a recent study identified low levels of bacteria within the gut of fetus recovered after terminated abortions that they managed to grow *in vitro*, providing the first strong evidence for bacterial colonization *in utero* (Rackaityte *et al.*, 2020).

Although there is no clear scientific consensus between the sterile womb and *in utero* hypothesis, it is accepted that the infant gut is colonized by bacteria from the mother or the

environment during parturition. Delivery mode was shown to strongly impact initial gut microbiota composition, as children vaginally delivered had a gut community close to their mother's vaginal and gut community; whereas babies born by C-section had a gut community closer to their mother's skin microbiota such as *Staphylococcus*, *Corynebacterium* and *Propionibacterium spp* (Dominguez-Bello *et al.*, 2010). In early life, the gut microbiota has a low diversity and is very unstable, with sequential bacterial colonization. Classically, it starts with facultative anaerobes that will consume the oxygen and allow colonization by strict anaerobes such as *Bifidobacterium* (Nogacka *et al.*, 2018; Palmer, Bik, DiGiulio, Relman, & Brown, 2007). During the suckling period, microbiota composition is impacted by the feeding type: breast-fed babies are dominated by *Bifidobacterium* and *Lactobacillus sp.* whereas formula fed baby have a more complex microbiota dominated by *Clostridium*, *Granulicatella*, *Citrobacter*, *Enterobacter* and *Bilophila*, and these differences disappear after weaning (Bäckhed *et al.*, 2015). The potential consequences on health of this transient difference are still not well understood. Progressively, the microbiota diversifies with the introduction of *Bacteroides* and *Clostridia*, especially after weaning and stabilizes at 2-3 years to reach an adult-like composition, although some changes can still occur until adolescence (Nogacka *et al.*, 2018).

4. Gut microbiota composition is highly impacted by environmental parameters

Although it was shown that part of the gut microbiota composition was directed by host genetics, as is especially the case for *Christensenellaceae* (Goodrich *et al.*, 2014), the strongest parameter shaping the community seems to be the environment. Twin studies showed that while twins carried similar gut microbiota, this similarity was identical between monozygotic twins and dizygotic twins, suggesting that the common environment during early life between siblings was responsible for the observed similarity and not genetics (Turnbaugh *et al.*, 2009). Moreover, the heritable part of the microbiota was estimated to be below 10 % in twin studies, and unrelated individuals sharing the same household were shown to have more similar microbiota compared to relatives not sharing the same household (Rothschild *et al.*, 2018).

Several environmental parameters can influence microbiota composition including geography, diet, drugs, stress, smoking, or circadian clock perturbations but I will focus on the two main ones: diet and drugs.

Long-term diet shapes the gut microbiota, and changes in diet can rapidly alter gut microbiota composition. European children (following a Western diet, characterized by high fat-low fiber) were shown to have a lower Bacteroidetes/Firmicutes ratio compared to Burkinabè children (following a traditional high fiber diet) (De Filippo *et al.*, 2017). Wu and colleagues also noticed that long-term dietary habits were correlated to enterotypes, high protein and fat associated to *Bacteroides* enterotype, and carbohydrates rich associated to *Prevotella* enterotype and that 24 h of diet change in controlled feeding experiments were enough to see community composition changes, although not strong enough to change the original dominant enterotype (Wu *et al.*, 2011). Vegan vs. omnivore individuals were shown to have only modest differences in gut microbiota composition but stronger differences in bacterial metabolites production, suggesting diet can impact the composition but also the function of the gut microbiota (Wu *et al.*, 2016). More generally, several studies on both humans and mice showed that carbohydrate, oligosaccharide or fat intake could influence the abundance of precise species in the gut (reviewed in (Bibbò *et al.*, 2016)).

Antibiotics have been shown to affect microbiota composition durably even after short exposure. A seven-day treatment with clindamycin in adult was sufficient to reduce *Bacteroides* population within the gut for at least two years (Jernberg, Löfmark, Edlund, & Jansson, 2007), and macrolide use in Finnish children was associated to a decrease in Actinobacteria and an increase in Bacteroidetes and Proteobacteria for at least two years (Korpela *et al.*, 2016). Use of ciprofloxacin led to a decreased diversity of the gut microbiota in only a few days, which was only partially recovered after the end of the treatment. These changes were individual dependent, showing the difficulty of predicting how a microbiota will react to a precise stress (Dethlefsen & Relman, 2011). The impact of antibiotics on gut microbiota depends on the class of antibiotic used, the duration of the treatment and the age of the individual, amongst others. For instance, clindamycin is a broad-spectrum antibiotic that is effective against anaerobic bacteria, which are the most abundant in the distal gut. Consistently, clindamycin use has been strongly associated to *Clostridium difficile* infection following antibiotic treatment (C. Thomas, Stevenson, & Riley, 2003). Early use of antibiotics has been shown to modify community composition with consequences on the incidence of asthma, while adult use of antibiotics changed the composition but did not increase the risk of developing asthma (Nogacka *et al.*, 2018). Moreover, non-antibiotic drugs have recently been shown to frequently exhibit antimicrobial activities against specific gut bacteria. A quarter of the 1000 drug tested inhibited the growth of at least one gut bacteria tested (Maier *et al.*, 2018). This suggests that the drug

history of individuals -and not just antibiotics- could have a strong impact on microbiota composition.

Because the gut microbiota carries important functions for its human host, any change in the gut microbiota composition or function can be expected to have consequences on health.

B. The gut microbiota is involved in health and disease

The gut microbiota is involved in many processes required for human health, including nutrient acquisition, immune and nervous system maturation, and homeostasis and protection from pathogens (Figure 13). As such, improper bacterial colonization during infancy or community imbalance generated by diet or drugs are expected to have a strong impact on health and disease.

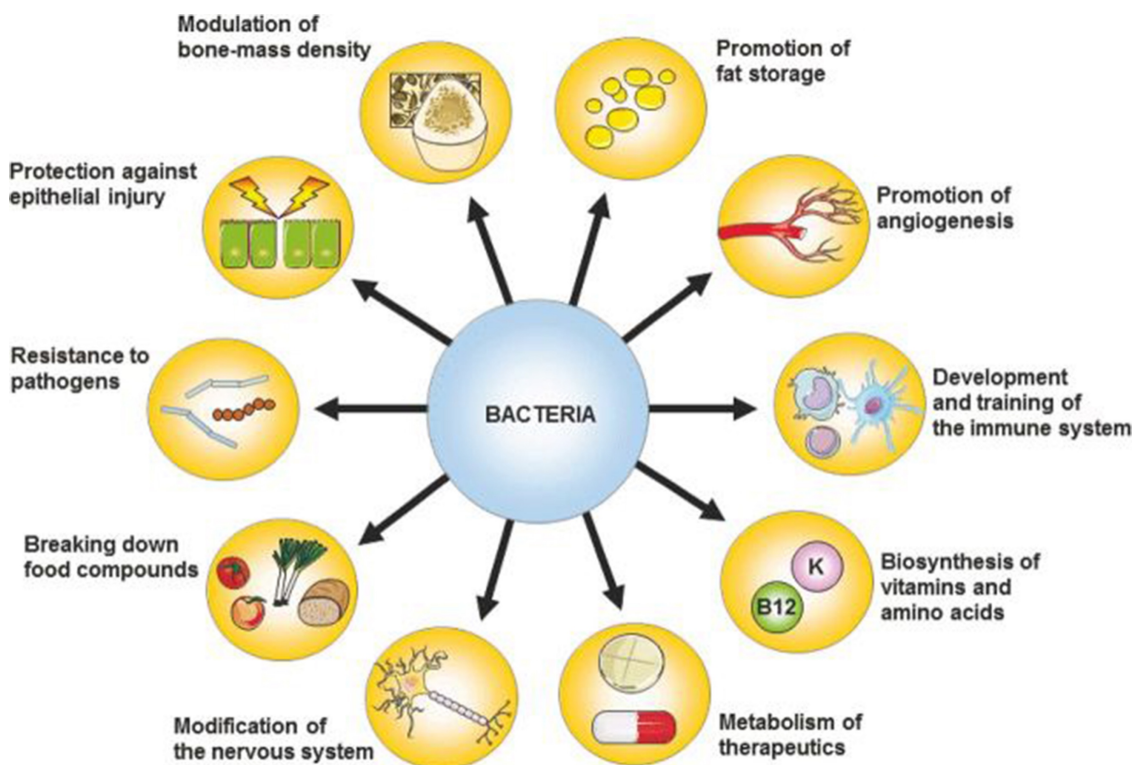


Figure 13: The gut microbiota is involved in health and disease.

From (Laukens, Brinkman, Raes, Vos, & Vandenabeele, 2016).

1. Microbiota in health

a. Nutrition

The observation that conventional mice had a 42 % higher body fat than germ-free animals even though they consumed 29 % less food suggested that the gut microbiota might be critical

for energy intake and fat metabolism. Accordingly, re-conventionalization of germ-free mice led to a body fat increase and a reduction in food consumption. Re-conventionalization also led to an increased leptin production, a hormone known to regulate fat storage and hunger, an increased harvesting of monosaccharide and a stimulation of hepatic lipogenesis (Bäckhed *et al.*, 2004). Moreover, colonization of germ-free mice with obese donor microbiota led to a higher increase in body fat in two weeks than colonization with lean donor microbiota. As these two microbiota differed in composition, it suggests that energy uptake varies depending on the community (Turnbaugh *et al.*, 2006).

Part of the energy intake mediated by the microbiota comes from the fermentation of various nutrients such as complex polysaccharides or mucins, but also proteins and fat. Gut bacteria, especially within Firmicutes and Bacteroidetes, are rich in enzymes catabolizing sugars which are lacking in the human genome, thereby providing the host with the ability to digest otherwise refractory nutrients. The fermentation of carbohydrates leads to the production of short chain fatty acid (SCFA), metabolites that are key to the host-microbiota crosstalk. In humans, SCFA are responsible for 10 % of the caloric intake (Bergman, 1990) and their production depends on the microbiota composition and substrates availability. The three main SCFA are butyrate, acetate and propionate. Butyrate is the main energy source of colonocytes where it is degraded by β -oxidation, ensuing oxygen consumption and maintenance of an anoxic environment in the gut (Litvak, Byndloss, & Bäumlér, 2018). Acetate provides food for other gut bacteria and peripheral tissues. Acetate and propionate can both localize to the liver where they stimulate lipogenesis and cholesterol production (Adak & Khan, 2019). Propionate and butyrate can also induce intestinal gluconeogenesis, which aids in proper energy homeostasis (De Vadder *et al.*, 2014). Moreover, some essential vitamins cannot be synthesized by the host and must be either scavenged through food or synthesized by the gut microbiota. For instance, some *Bifidobacteria* were shown to be able to synthesize folate and *Lactobacillus reuteri* to synthesize vitamin B12 (LeBlanc *et al.*, 2013).

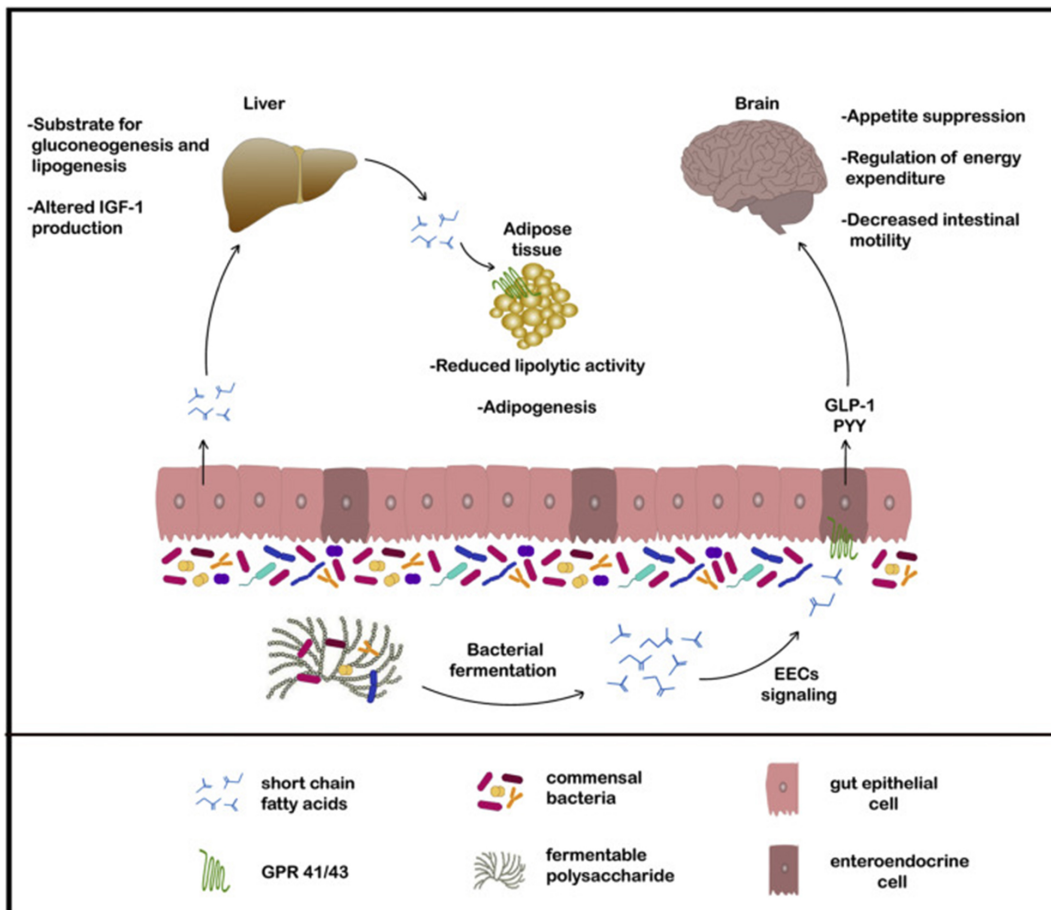


Figure 14: Short chain fatty acids and host energy metabolism.

SCFAs may modulate energy metabolism by stimulating GLP-1 and PYY production. These gut peptides can act in endocrine and/or paracrine fashion and contribute to appetite suppression, regulation of energy expenditure, regulation of glucose homeostasis and decreased intestinal motility. SCFAs can be used as substrate for gluconeogenesis and lipogenesis and may alter IGF-1 production in the liver. In vivo and in vitro models linked SCFAs with both reduced lipolytic activity and adipogenesis. Abbreviations: EECs, enteroendocrine cells; GLP-1, glucagon-like peptide-1; GPR 41/43, g-protein coupled receptors 41 and 43; IGF-1, insulin-like growth factor-1; PYY, polypeptide YY; SCFAs, short chain fatty acids. From (Pekmez, Dragsted, & Brahe, 2019)

b. Crosstalk with the host

Observations of germ-free mice revealed that the gut microbiota might be involved in more processes than originally believed, and that its crosstalk with the host might not be limited to food digestion and nutrient intake.

Compared to conventional mice, germ-free mice have a larger cecum, which is in part caused by accumulation of mucus that is usually degraded by the gut microbiota; however, the total intestinal surface area is lower as villi tend to be smaller (Wostmann, 1981) and the epithelium renewal is two times slower (Savage, Siegel, Snellen, & Whitt, 1981) showing that the gut microbiota broadly influences gut epithelium maturation.

Germ-free mice were also shown to have a deficient immune system: they have hypoplastic Peyer patches, key components of the gut immune system and fewer IgA producing

cells and CD4⁺ T-cells (Macpherson & Harris, 2004). Peyer patches size and IgA-producing cells numbers increased upon addition of *Morganella morganii*, a mouse symbiont (Shroff, Meslin, & Cebra, 1995), showing re-conventionalization of adult mice was enough to rescue the immune deficit. Moreover, deficit in CD4⁺ T-cells was shown to be rescued by addition of *Bacteroides fragilis*, an abundant human gut symbiont, or by addition of only purified *B. fragilis* capsular polysaccharide A, showing that specific symbiont molecules can have strong impact on host physiology (Mazmanian, Cui, Tzianabos, & Kasper, 2005).

More generally, the gut microbiota was shown to impact both the innate and the adaptive immune system, to modulate inflammation and to stimulate the production of IgA or host-produced antimicrobial peptides (Rooks & Garrett, 2016). This very important relationship between immune system and bacterial colonization in the gut was proposed as being key in the observed increased incidence of immune system disorder-related diseases such as asthma or allergy. This “hygiene hypothesis” stipulates that increased hygiene in modern societies resulted in a lower exposure of children to environmental bacteria leading to improper immune system maturation (Macpherson & Harris, 2004).

Finally, germ-free animals were shown to be more sensitive to stress than conventional animals, a phenotype alleviated by recolonization with *Bifidobacterium infantis* (Sudo *et al.*, 2004), suggesting gut microbiota can impact the nervous system. The gut possesses an enteric nervous system, made of millions of neurons connected to the central nervous system, that was proposed to be a “second brain”. Bacteria have been shown to be able to interact both with the central and the enteric nervous systems in a process known as the gut-brain axis. Microbiota mediation of the nervous system includes neuron excitability modulation, production of neuroactive compounds such as neurotransmitters or SCFA, modulation of the availability of neurotransmitters precursors and immune system-mediated neuronal modifications (Kim, Yun, Oh, & Choi, 2018). Consequently, perturbations of the gut microbiota have been implicated in the development of neurodegenerative diseases (Chen *et al.*, 2016; Minter *et al.*, 2016).

c. Colonization resistance

A third function of the gut microbiota is the protection of the host from pathogen colonization, a process known as colonization resistance (Figure 15). Two aspects have to be distinguished: modulation of the host defenses, and direct interactions between symbionts and pathogens. Modulation of the host defenses mainly involves modulation of the immune system, for instance by increasing production of IgA or antimicrobials, modulation of the mucus barrier

or modification of host-produced molecules. In particular, bile acids, antimicrobial molecules produced by the host, can be modified by gut bacteria, thereby modifying their activity. Some *Clostridia* can produce the secondary bile acids deoxycholic and lithocholic acids via 7α -dehydroxylation. Secondary bile acid production by *Clostridium scindens* was shown to delay colonization and infection by *Clostridium difficile* in a gnotobiotic mouse model (Studer *et al.*, 2016).

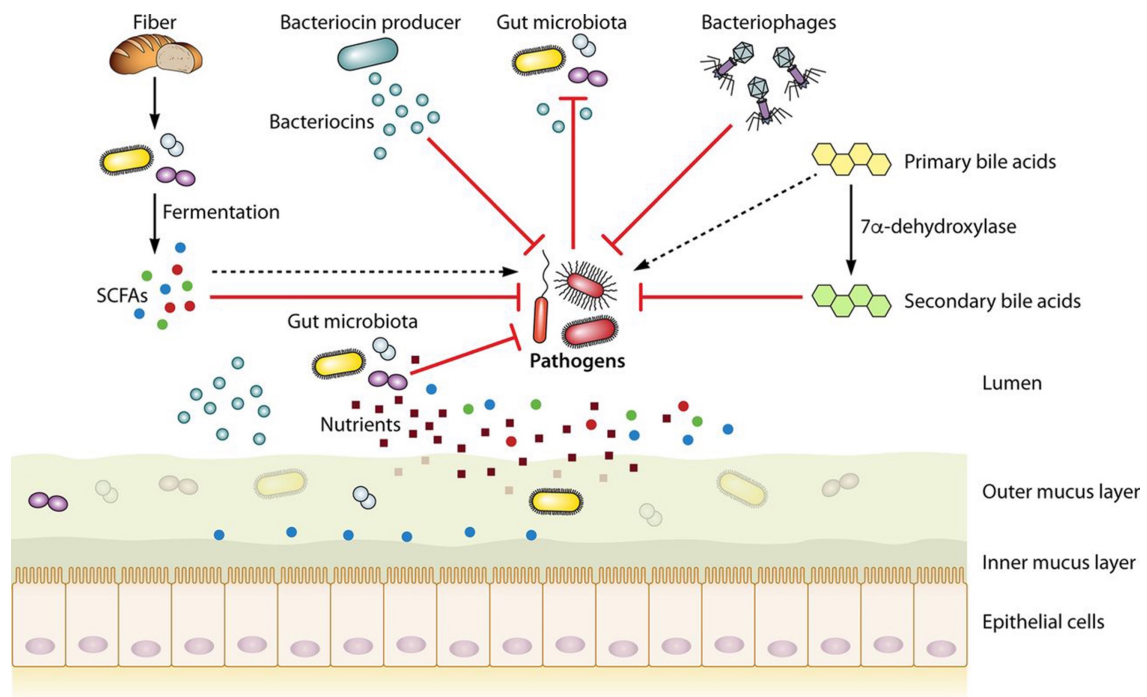


Figure 15: Colonization resistance.

From (Ducarmon *et al.*, 2019)

On the other hand, competition between bacteria in the gut comes in two flavors: exploitative competition that relies on direct competition for space or resources, or interference competition which involves active mechanisms of inhibition. Competition for nutrients often involves closely related bacteria as they tend to have more similar metabolic requirements. High proline-using *E. coli* strains from the microbiota were shown to suppress pathogenic *E. coli* O157:H7 strain growth in caecal suspension, and this inhibition was alleviated by proline addition to the media. This *in vitro* inhibition correlated with lack of *E. coli* O157:H7 colonization in gnotobiotic mice carrying the proline-consuming strains (Momose, Hirayama, & Itoh, 2008). Two symbiotic *E. coli* strains were also shown to consume a complementary set of sugars, thereby allowing co-occurrence in the mouse gut but preventing pathogenic *E. coli* O157:H7 EDL933 colonization by sugar consumption (Maltby, Leatham-Jensen, Gibson, Cohen, & Conway, 2013). Non-pathogenic *E. coli* Nissle 1917 was also shown to be able to prevent

Salmonella enterica serovar Typhimurium colonization by iron scavenging (Deriu *et al.*, 2013). These different studies show that a wide range of nutrients can be involved in exploitative competition.

Gut bacteria produce of host of antimicrobial systems that include secreted antimicrobial peptides bacteriocins and metabolites inhibiting growth or adhesion of pathogens. Bacteriocins are a very diverse family of proteins, and many studies have shown the ability of symbiont-produced bacteriocins to inhibit pathogens such as *Listeria monocytogenes*, *Clostridium difficile*, *Yersinia enterocolitica* and *Salmonella enterica* serovar Typhimurium (Ducarmon *et al.*, 2019). In particular, thuricin, a bacteriocin produced by *Bacillus thuringiensis*, was shown to inhibit the growth of *C. difficile* in a human distal colon model as efficiently as antibiotics, but without the downsides of impacting the microbiota composition, suggesting narrow-spectrum bacteriocins might be good therapeutical candidates (Rea *et al.*, 2011).

Moreover, other bacterial molecules can also impact pathogens. *Bifidobacterium*-secreted products were shown to not only inhibit growth but also adhesion to Caco-2 cells of *C. difficile* and *E. coli* O157:H7 (Gagnon, Kheadr, Le Blay, & Fliss, 2004; Trejo, Minnaard, Perez, & De Antoni, 2006) and SCFA were also shown to be able to inhibit *E. coli* O157:H7 growth in a pH-dependent manner (Shin, Suzuki, & Morishita, 2002).

Colonization resistance prevents the implantation of exogenous bacteria within the gut, but protection can also be conferred by lowering the virulence of pathogenic bacteria. *Vibrio cholerae* type 6 secretion system (T6SS) is an important virulence factor expressed in the mammalian gut. Resident bacteria have been shown to lower *V. cholerae* T6SS killing via bile modifications (Bachmann *et al.*, 2015). Extracellular metabolites produced by gut microbiota were also shown to inhibit *E. coli* O157:H7 main virulence factor, Shiga toxin 2, production (De Sablet *et al.*, 2009).

As we have seen, the gut microbiota is implicated in many processes important for host health. As such, it is expected that imbalances in the community could lead to disease.

2. Microbiota in disease: dysbiosis

a. What is dysbiosis?

Defining an “unhealthy microbiota” requires the definition of a “healthy microbiota”, which would be the community carried by healthy individuals. However, health is complex concept, encompassing social, mental and physical aspects, as defined by the World Health organization, and very few studies on gut microbiota assessed all these parameters in their donors. Moreover,

inter-individual variability in terms of microbiota composition makes it harder to define specific components linked with health or disease.

Nonetheless, several parameters seem to be key to characterize imbalances in the microbiota, known as dysbiosis. In particular, perturbations of the microbiota often lead to a loss of diversity, defined as the richness (the number of species in an environment) and evenness (the relative abundance of the different species) that perturbs microbiota function (Kriss, Hazleton, Nusbacher, Martin, & Lozupone, 2018). In general, “healthy microbiota” have been shown to be diverse, stable with a high Bacteroidetes/Firmicutes ratio and low Proteobacteria (Chassaing, Vijay-Kumar, & Gewirtz, 2017). Last, biogeography of the gut has to be considered, as an increased proximity between gut microbiota and intestinal epithelium, by thinning of the mucus layer or invasion of the mucus, might promote disease even if the community composition is identical (Sicard, Bihan, Voegelé, Jacques, & Harel, 2017).

Dysbiosis arises when environmental parameters perturbate the gut microbiota, such as diet or antibiotic treatment, as discussed previously, but also infection by pathogens (Beatty *et al.*, 2017). The gut microbiota has been implicated in at least 25 diseases or syndromes. More often than not, this implication has only been correlative, with no information on causality or mechanism. 10 of these diseases are supported by strong evidence including multiple independent studies and in some case animal-based validation: inflammatory bowel disease (IBD) (both Crohn’s disease and ulcerative colitis), irritable bowel syndrome, enteric pathogen infection such as *C. difficile* infection, colorectal cancer, allergy, celiac disease, type 1 diabetes, type 2 diabetes and obesity (de Vos & De Vos, 2012). In the following part I will focus on three of them: IBD, *Clostridium difficile* infection (CDI) and obesity.

b. Consequences on health

i. C. difficile infections

C. difficile is a Gram-positive sporulating pathogen. In 2011, *C. difficile* was estimated to have infected almost half a million people in the United States of America, and to be responsible for 29,000 deaths. 65 % of these infections were health-care related (Lessa *et al.*, 2015). Following *C. difficile* infection (CDI) and subsequent antibiotic treatment, around one patient out of five develops recurring CDI, defined as a symptomatic episode arising in the weeks following treatment of the first one, and among those a third to two thirds will experience several recurring episodes (Johnson, 2009). In adults, it is the leading cause of bacterial antibiotic-associated diarrhea worldwide. Cephalosporin and clindamycin are the antibiotics

associated to the greatest risk of developing CDI (Deshpande *et al.*, 2013; Slimings & Riley, 2014). Antibiotics perturbate the gut microbiota, thus limiting colonization resistance and allowing pathogen implantation.

Several mechanisms have been proposed to allow *C. difficile* colonization after gut microbiota perturbation. *C. difficile* first arrives in the gut in the forms of spores, that must germinate to reach the vegetative pathogenic state. This germination, and the survival of the vegetative cell, was shown to be impacted by bile acids. Bile acids derivative of cholate induce spore germination, while chenodeoxycholic acid and its derivatives repress it. Upon antibiotic treatment, shifts in the gut microbiota have been shown to be linked to shifts in bile acid pool in the gut, by reducing the number of bacteria able to dehydroxylate primary bile acids into secondary bile acids, with consequences on gut permissibility to CDI (Theriot *et al.*, 2014). In animal models, competition for nutrients and SCFA production were also shown to be involved in limiting CDI, and the contribution of gram-positive targeting bacteriocin production such as thuricin might also contribute (Collins & Auchtung, 2017).

Because colonization resistance seems to be very important in preventing CDI, and because antibiotic treatments are often not sufficient to cure CDI, an increasing amount of attention has been given to alternative treatments by restoring the gut microbiota to a healthier state. Fecal microbiota transplantation (FMT) in particular has shown to be extremely effective against recurring CDI, in some clinical trials showing as much as 90% efficacy, which is consistent with a strong impact of the gut microbiota imbalance on CDI development. However, several cases of disease transfer after FMT have been described, and not enough is known about how FMT works or what precise gut microbiota features are involved in disease onset to screen donors effectively (Papanicolas, Gordon, Wesselingh, & Rogers, 2020). To limit this risk, several labs are developing defined communities of a few bacteria that would reproducibly cure CDI in a more controlled setting (Collins & Auchtung, 2017).

ii. Inflammatory bowel disease (IBD)

IBD regroups two diseases, Crohn's disease (CD) and ulcerative colitis (UC) that are both characterized by a chronic recurring intestinal inflammation. IBD etiology is not well understood, but it seems to originate from genetic, environmental and microbiota-related factors (Figure 16).

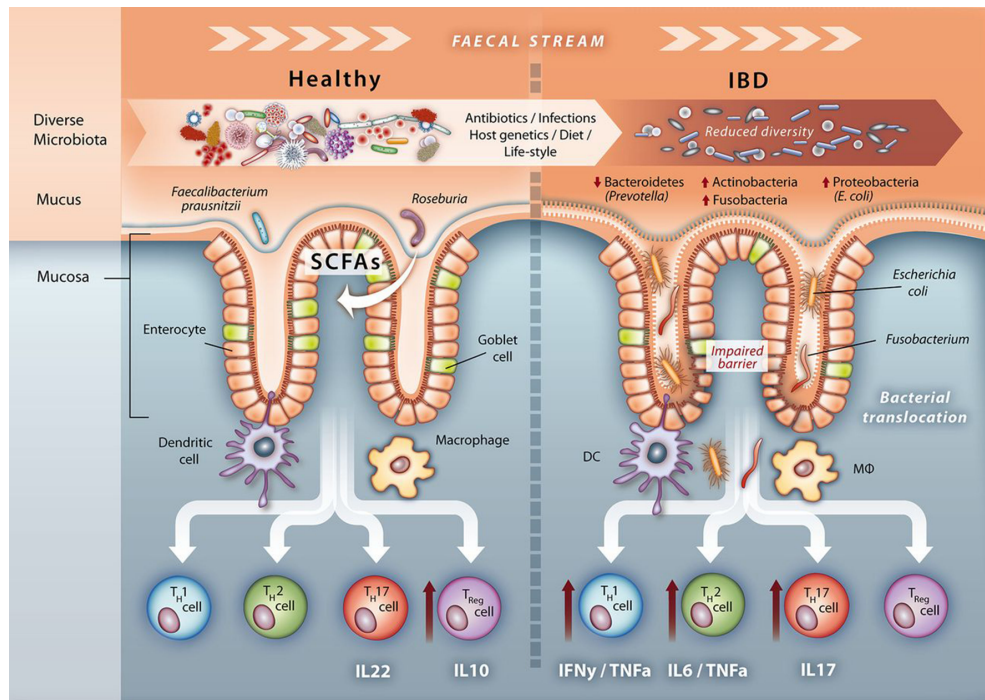


Figure 16: Dysbiosis of the gut microbiota is linked with immune system imbalances in IBD.

From (Sommer *et al.*, 2017)

The hypothesis that IBD involved the gut microbiota arose from different observations. In a genetically predisposed mouse model, IBD does not occur in germ-free animals (Kühn, Löhler, Rennick, Rajewsky, & Müller, 1993). Genome-wide association studies on CD and UC patients have led to the characterization of 163 loci that were associated with IBD. CD predisposition loci were enriched in genes involved in bacterial recognition and processing, UC in genes involved in the mucus barrier (Jostins *et al.*, 2012). Finally, the gut microbiota of IBD patients was found to be dysbiotic, with a consistent loss of diversity compared to healthy subjects, and in most cases a decrease in Firmicutes and an increase in Proteobacteria (Matsuoka & Kanai, 2015). Some specific bacteria were also shown to be associated to IBD. For instance, CD patients were found to have lower levels of the anti-inflammatory *F. prausnitzii* (Sokol *et al.*, 2008; Willing *et al.*, 2010) and higher levels of mucin degrading *Ruminococcus* (Png *et al.*, 2010; Willing *et al.*, 2010). The gut microbiota of IBD patients was also found to be less stable than the microbiota of healthy individuals (Martinez *et al.*, 2008). However, all of these studies are strictly correlative, and the causality link between dysbiosis and intestinal inflammation in IBD still has to be assessed in humans. One study addressed this issue in mice. They identified *Bacteroides* species that conferred IBD to genetically susceptible mice, but not to non-susceptible mice, whereas a control with *Enterobacteriaceae* did not. Interestingly, *Enterobacteriaceae* were found to be enriched in IBD patients gut microbiota,

but *Bacteroides* were not, showing correlation studies are not predictive of causality (Bloom *et al.*, 2011).

Although no clear proof that dysbiosis causes IBD in humans exists, therapeutic interventions targeting the gut microbiota rather than the intestinal inflammation are being investigated. Antibiotics have been proposed for IBD treatment, but clinical trials with antibiotics are showing mixed results. A systematic review of randomized clinical trials involving antibiotics in IBD treatment showed that antibiotics were significantly better than placebo in treating both UC and CD, although the studies compared used different drugs, making it harder to draw decisive conclusions (Khan *et al.*, 2011). Inspired by CDI treatment by FMT to reduce dysbiosis, several clinical trials of FMT to cure IBD have been reported, but the results were not concordant. Some studies showed no effect of FMT on clinical signs (Angelberger *et al.*, 2013; Kump *et al.*, 2013), others a high improvement rate (Kunde *et al.*, 2013). Whether these differences arise from differences in donor or receiver microbiota, genetics or other factors remains to be addressed. However moderate, the impact of microbiota-targeting strategies in IBD treatment gives additional credence to the role of dysbiosis in IBD onset and deserves further study.

iii. Obesity

Obesity is defined by World health organization (WHO) as an unhealthy and excessive accumulation of fat. It can be assessed by the body mass index (BMI): an individual is considered obese if his/her BMI is 30 or more. In 2016, more than 650 million people were obese. Obesity is a burden in both developed and developing countries, associated to millions of deaths each year (“Obesity and overweight,” n.d.).

Obesity is a lifestyle-related disease, but studies suggest a role for the gut microbiota (Figure 17). The gut microbiota of obese mice and humans was shown to have a low Bacteroidetes/Firmicutes ratio compared to lean individuals. Transplantation of the gut microbiota of obese mice in germ-free mice led to a higher body fat intake after re-conventionalization than transplantation of the microbiota of lean mice, suggesting that the “obese microbiota” was more efficient for energy harvesting and could be a causative agent of obesity (Turnbaugh *et al.*, 2006). Moreover, the Bacteroides/Firmicutes ratio of obese humans increased upon weight loss mediated by dietary intervention (Ley, Turnbaugh, Klein, & Gordon, 2006). However, the lowered Bacteroidetes/Firmicutes ratio of the obese microbiota was not consistently observed between studies, showing this ratio could not be used as a predictive

marker of obesity (W. A. Walters, Xu, & Knight, 2014). Analysis of the microbiota of lean and obese human twins also showed that the obese microbiota was characterized by a general lower diversity, a low amount of Bacteroidetes and a higher amount of Actinobacteria compared to lean twins. Several genes were found to be more represented in obese microbiome, including many genes involved in carbohydrate, lipid and amino acid metabolism, suggesting the obese microbiota was enriched in nutrient processing functions (Turnbaugh *et al.*, 2009). Specific bacteria were also proposed to have an impact on obesity. In particular, *A. muciniphila* was shown to be associated with better clinical outcomes after dietary intervention and a general healthier metabolism (Dao *et al.*, 2016).

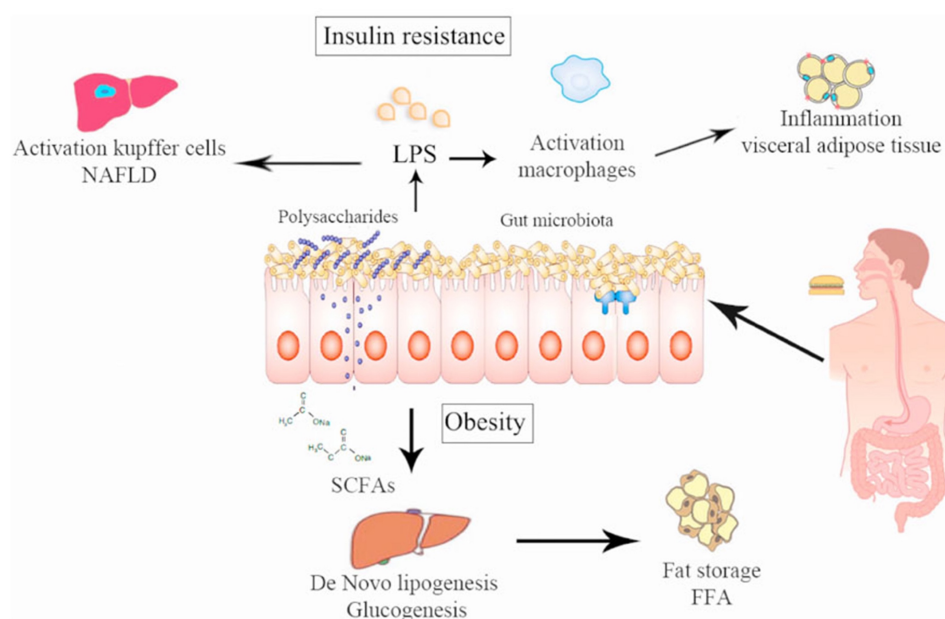


Figure 17: The gut microbiota is implicated in obesity by secretion of pro-inflammatory molecules and modification of fat metabolism.

From (Moreno-Indias, Cardona, Tinahones, & Queipo-Ortuño, 2014).

Interestingly, antibiotic use in children, which are known to modify gut microbiota composition, have been linked with obesity, further confirming the implication of the gut microbiota in obesity development. It has been known for a long time that low levels of antibiotics promoted growth in different animals, which has led to their increased use in animal husbandry (G. Cheng *et al.*, 2014). In mice, fecal transplant showed that the microbiota from antibiotic-treated mice could transfer the growth-promotion phenotype to germ-free mice (Cox *et al.*, 2014). More recently, epidemiological studies in humans seem to confirm a correlation between antibiotic use in children and increased risk of obesity (Ajslev, Andersen, Gamborg, Sørensen, & Jess, 2011; Bailey *et al.*, 2014; Murphy *et al.*, 2014).

Obesity is characterized by a low-grade inflammation. On top of the higher energy harvesting of the obese microbiota, it was proposed that the microbiota could modulate this inflammation through lipopolysaccharide (LPS) production, an abundant Gram-negative molecule known to be recognized by toll-like receptors (TLR). An increase in LPS translocation through the epithelium would be linked to modifications of the gut barrier integrity (Gérard, 2016).

Most of the studies mentioned in this part were done on fecal microbiota, which only partially recapitulates colon microbiota, and does not take the gut biogeography into account at all. However, several observations show that luminal and mucosal communities are dissimilar in both composition and function. In the next part, I will address the role of biofilm-like mucosal communities in microbiota function.

C. Evidence for biofilms in the gut

1. Definition

Microscopy imaging of healthy animals and human intestines suggest that the large intestine microbiota of mammals is commonly organized in dense and structured biofilm-like communities. Electronic microscopy of baboon biopsies showed a layered community, in which cells close to the epithelium were smaller than cells close to the lumen, consistent with an heterogenous structure typical of biofilms (Palestrant *et al.*, 2004). Rat biopsies also showed that cells tended to be orientated in the direction of the flow and not randomly, suggesting a structured community. Finally, humans, rats and baboon biopsies showed that bacteria were encased in porous material reminiscent of an extracellular matrix (ECM), although simple observation could not indicate whether it was self-produced or host-produced (Palestrant *et al.*, 2004). The gut biofilm-like structures were lost when samples were prepared using common techniques, suggesting protocol variation can be responsible for lack of reproducibility between laboratories (Bollinger, Barbas, Bush, Lin, & Parker, 2007; Palestrant *et al.*, 2004). Electronic microscopy and fluorescent light microscopy also showed that in the human GI tract, bacteria often occur as aggregates or microcolonies in different micro-habitats associated to the mucus, the epithelium or food particles (S. Macfarlane, Hopkins, & Macfarlane, 2000). Finally, fluorescent in situ hybridation (FISH) of a defined 15-member consortia of bacteria in gnotobiotic mouse model showed that the community was not randomly distributed, but rather spatially structured, a process which might be mediated by adhesion to mucus, epithelium or between bacteria (Mark Welch, Hasegawa, McNulty, Gordon, & Borisy, 2017) (Figure 18A).

Several studies also showed that bacteria within the gut exhibited specific phenotypes: sugar metabolism of adhering and non-adhering bacteria was different (S. Macfarlane, McBain, & Macfarlane, 1997) and the rate of plasmid exchange in the gut was found to be similar to what is observed in *in vitro* biofilms (Licht, Christensen, Krogfelt, & Molin, 1999).

There is still some debate about whether these structures are proper biofilms. First, some laboratories fail to replicate biofilm-like structure observations, probably because of sample preparation variation (Bollinger *et al.*, 2007). Moreover, in the strictest sense, a structure can only be called a biofilm if it is encased in a self-produced ECM, something that is extremely hard to show in the gut because sample preparation tends to destroy delicate polysaccharidic structures, and because the presence of a host-produced gel-like matrix, the mucus, makes it harder to identify a bacteria-produced ECM. Mucosal communities were shown to form biofilm *ex vivo* and to secrete ECM, but whether or not this occurs *in vivo* remains to be addressed (Buret, Motta, Allain, Ferraz, & Wallace, 2019) (Figure 18B). *L. reuteri* was shown to form biofilms in the forestomach of rats, where no mucus layer is present and the bacteria directly adhered to the epithelium and produced ECM. However, no such structure exists in humans, as the GI tract is continuously protected by mucus. Interestingly, only *L. reuteri* isolates from rats adhered to the rat epithelium. Strains from humans did not, but they showed adhesion to mucus, hinting that adhesion and biofilm formation could be common between strains of different hosts but mediated by different binding ability (Frese *et al.*, 2013). Some authors argue that the relatively short retention time in the gut and the fast shedding of mucus makes it impossible for a biofilm to form. However, other theoretical work suggests that, on the contrary, biofilm formation is required to escape shedding in the luminal content and to be maintained in the gut (J. L. Sonnenburg, Angenent, & Gordon, 2004). Indeed, bacteria unable to colonize the mucus layer were shown to be deficient for gut colonization (J. L. Sonnenburg *et al.*, 2004).

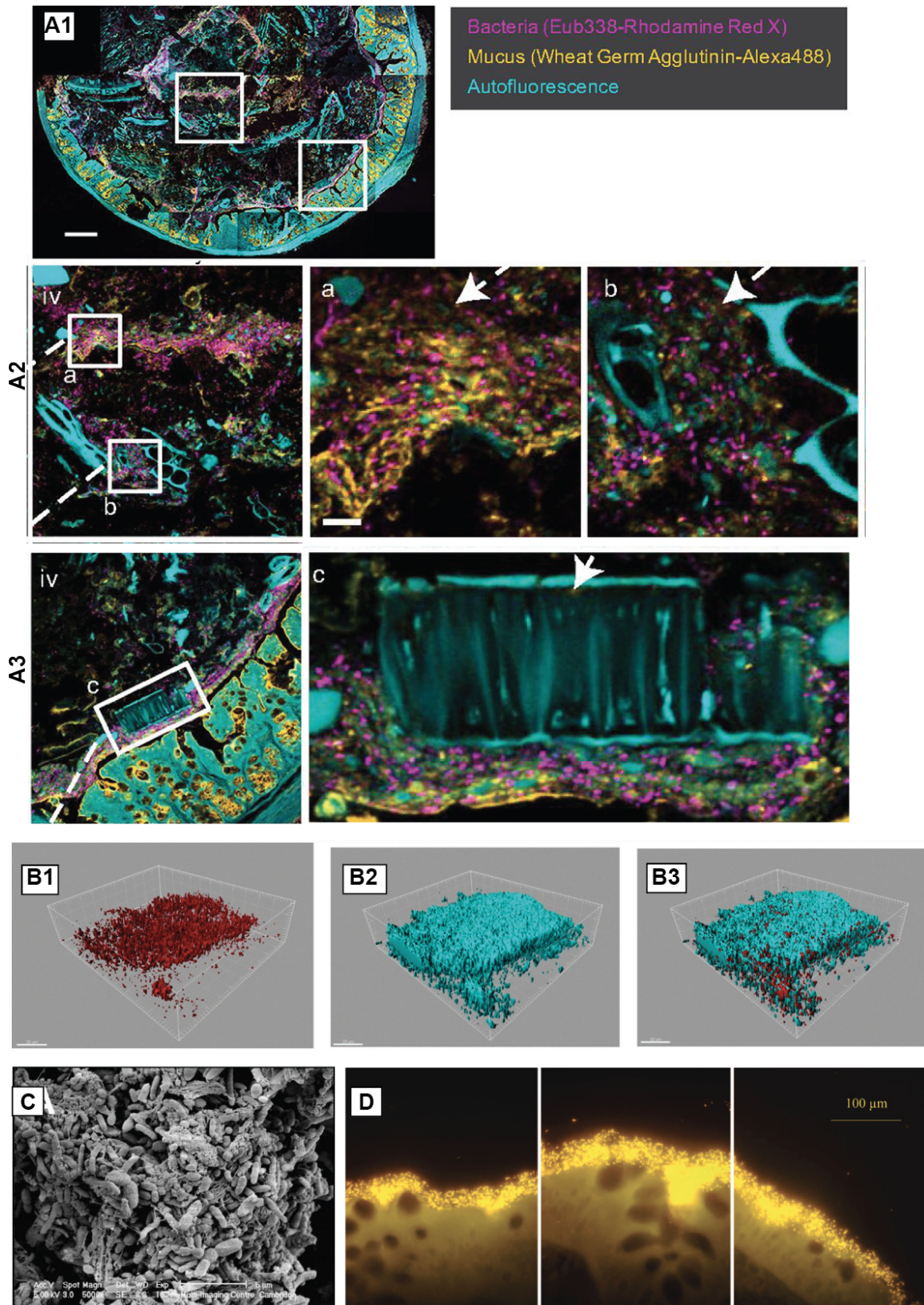


Figure 18: The gut microbiota is a “biofilm-like” structure.

A. Mucus-associated biofilm. A 15-member consortia of human gut bacteria colonizing a gnotobiotic mouse gut. Dense bacterial aggregations occupy mucus-rich regions. A1. Cross-section of colon highlighting location of panels A2 and A3. Bacteria appear in purple, mucus and exopolysaccharide in yellow and tissue and food particles are autofluorescent and appear in blue. A2. In the lumen high density of mucus correlates with abundant bacteria. Enlargement of the two indicated regions are shown in the middle and right panels. A3. The mucus layer covering the epithelium houses a very dense microbial

community. An enlargement of the indicated zone is presented in the right panel. Scale bars: A1, 200 μm ; A2 and A3: right panel, 50 μm ; middle and left panel, 10 μm . Adapted from (Mark Welch *et al.*, 2017) **B. In vitro gut microbiota biofilm.** Confocal laser microscopy of the colonic microbiota from a healthy human donor grown *in vitro* on the Calgary Biofilm Device™. B1. Live bacteria B2. Wheat germ agglutinin staining labelling the exopolysaccharide coating bacteria. B3. Merge. Scale bar represent 20 μm . From (Buret *et al.*, 2019). **C. Food particle-associated biofilm.** Scanning electronic microscopy of a biofilm growing on food particles recovered from human feces. Scale bar represent 5 μm . From (Sandra Macfarlane & Macfarlane, 2006). **D. Mucus-invasive biofilm.** Micrographs of a human colon from a patient with Crohn's disease. Bacteroides are labelled with Bfra-Cy3 probe and appear in yellow, the epithelium autofluoresces. Bacteroides form a dense community in contact with the epithelium. Scale bar represent 100 μm . From (Swidsinski, Weber, Loening-Baucke, Hale, & Lochs, 2005).

A recurring problem in the literature is the lack of a consistent definition for a biofilm in the gut. Some authors concluded to biofilm formation so long as they observed aggregates of cells, while others strictly differentiated between aggregates, microcolonies and biofilms. Some authors only considered communities as biofilms if they reached a specific width, or only if they could observe direct contact with the epithelium. The continuum between adhesion to aggregate to biofilm is easily distinguishable *in vitro* and in some model systems that were originally highly used to study biofilm, but much harder in an environment like the human gut. The self-production of an ECM that is a hallmark of biofilm formation *in vitro* might not be so relevant in a context where host-produced mucus can display exactly the same properties as classical bacteria-produced ECM. Considering to the observed density and spatial structure of bacteria within the gut microbiota, their known adhesive properties to mucus or food particles and their specific phenotypes compared to freely diffusing non-adherent bacteria, I propose that the gut microbiota is a biofilm-like structure even in healthy condition, and that studies made on microbiota bacteria should take their adhesion properties into account.

2. Gut signals mediate *in vitro* biofilm formation

Consistently with the idea that biofilm formation is an important property of symbionts to be able to colonize the gut, several relevant gut molecules have been shown to induce *in vitro* biofilm formation.

Bile is a complex mix of proteins such as biliverdin and cholesterol-derived bile acids. It is produced in the liver, stored in the gall bladder and secreted during meals to favor the emulsification of dietary lipids (Figure 19). Bile and bacteria have a complex relationship: bile has antimicrobial properties, can be used as a food source and its composition is impacted by gut bacteria, notably through two processes: deconjugation, upon which the taurine or glycine conjugates are removed from conjugated bile acids and dehydroxylation of primary bile acids into secondary bile acids (Molinero, Ruiz, Sánchez, Margolles, & Delgado, 2019). Moreover, bile acids have a strong impact on bacterial physiology. I have previously discussed their role

in mediating colonization resistance and sporulation of *C. difficile*. Bile has also been shown to induce *in vitro* biofilm formation of several bacteria. Bile acids induce transcriptional changes and biofilm formation of *V. cholerae*, thus increasing its bile tolerance (Hung, Zhu, Sturtevant, & Mekalanos, 2006). Bile induced fimbria-like structures production, aggregation, biofilm formation, HT-29 human cells adhesion and antibiotic tolerance of *B. fragilis* (Pumbwe *et al.*, 2007). Deoxycholate, a secondary bile acid produced by commensal-mediated 7 α -dehydroxylation of cholate, can induce biofilm formation of *C. difficile* and thus increase its antibiotic tolerance (Dubois *et al.*, 2019).

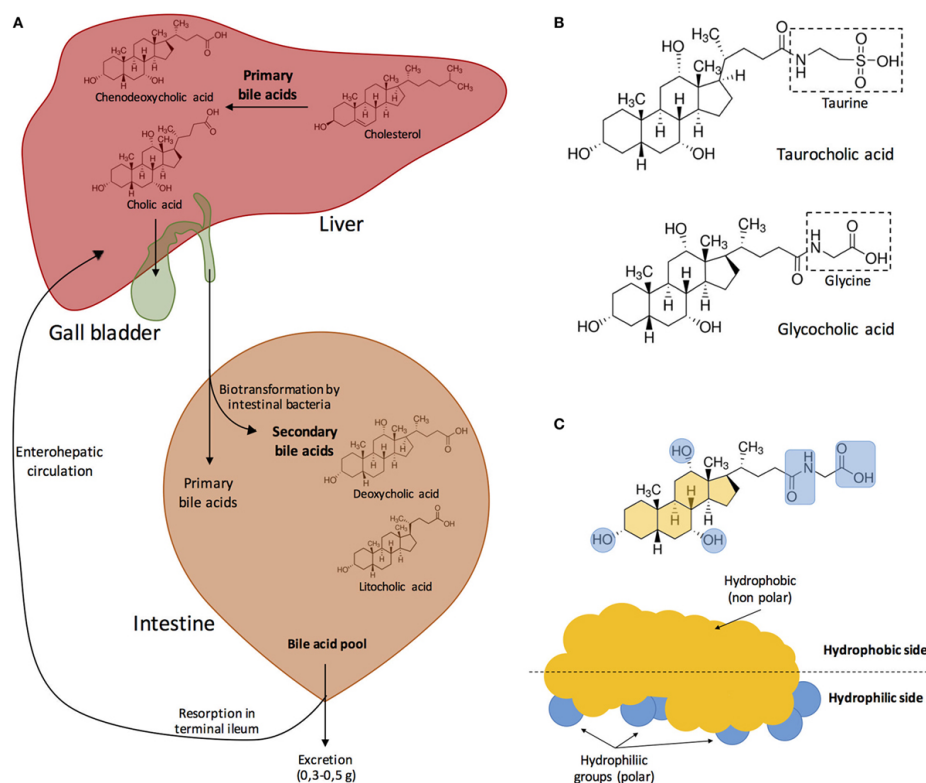


Figure 19: Bile acids.

A. Primary bile acids are synthesized in the liver and stored in the gall bladder. After food ingestion, bile released into the small intestine contains 5–15 g of bile acids. Bile acids are dehydroxylated into secondary bile acids in the intestine by gut symbionts. Both primary and secondary bile acids are reabsorbed in the terminal ileum into the blood stream and returned to the liver where they can be conjugated to either taurine or glycine residues. **B.** Example of conjugated bile acids: taurocholic acid and glycocholic acid. **C.** Bile acids are amphipathic, and their hydrophobic side associates with the surface of lipid droplets while the polar groups interact with water creating a stable emulsion of small, mixed micelles. From (Urdaneta & Casadesús, 2017).

Mucins are glycoproteins making up the protective mucus layer of the GI tract. Like bile, the mucus layer has a complex interaction with the gut microbiota: it can be used as a food source by bacteria, it prevents close contact between the gut epithelium and the microbiota and it provides a colonization niche for dense communities of bacteria (Sicard *et al.*, 2017). Several gut bacteria have been shown to be able to adhere to the O-glycan of mucins *in vitro* including

E. coli, *Lactobacillus B. fragilis*, *Bifidobacteria* and several pathogenic bacteria. This interaction was mediated by different structures including pili, flagellum, polysaccharides... (Sicard *et al.*, 2017). Adhesion to mucins has been proposed to be key for gut colonization, allowing colonization of the mucus outer-layer to avoid wash out with the luminal content (J. L. Sonnenburg *et al.*, 2004). Moreover, agarose gels can be used to mimic biofilm formation within mucus, suggesting the gel-like properties of mucus are enough to induce biofilm formation (Jouenne, Tresse, & Junter, 1994; Pabst, Pitts, Lauchnor, & Stewart, 2016).

Secreted IgA (sIgA) are part of the immune defense preventing contact between the gut epithelium and bacteria. sIgA bind bacteria and trap them in aggregates, preventing breaching of epithelium, a process known as immune exclusion. However, this binding was also shown to facilitate biofilm formation by *E. coli* through type 1 pilus/sIgA interactions (Bollinger *et al.*, 2006; Randal Bollinger *et al.*, 2003).

These *in vitro* works provide an interesting tool to mechanistically study biofilm formation in gut-mimicking conditions, and suggest that bacteria have evolved strategies to induce biofilm in these conditions, potentially to facilitate gut colonization and maintenance.

3. *In vivo* gut microbiota biofilm formation

In vivo, I will consider three niches for biofilm formation: food particle associated biofilm, the outer mucus community and the mucus-invasive epithelium-associated biofilm.

a. Food particle-associated biofilm

Food particles transiting through the GI tract are rapidly colonized by bacteria in the cecum and the colon. 5% of the luminal bacteria were found to be strongly attached to food particle, mainly *Bacteroides* and *Bifidobacteria*, forming microcolonies (Sandra Macfarlane & Macfarlane, 2006) (Figure 18C). The composition of the bacterial community recovered in feces from soluble material, a proxy for non-adhering bacteria, and insoluble material, corresponding to undigested fiber-adhering bacteria, differed. In particular, the Bacteroidetes/Firmicutes ratio was lower in the fiber-associated community and *Ruminococcus* was found to be strongly associated to undigested fibers, showing a specific community was associated to food-particles (Walker *et al.*, 2008). Different insoluble plant polysaccharides are colonized by distinct communities of bacteria in continuous-flow fermenters inoculated with fecal microbiota, and the diversity of each community is lower than that of the inoculum, suggesting colonization of food particles involves a specific set of bacteria. Moreover, the initial

inoculum composition, which is very variable between individuals, had a strong impact on the food-particles community composition, showing that the same substrate can be colonized by different communities between individuals (Leitch, Walker, Duncan, Holtrop, & Flint, 2007). On top of community composition, the metabolic activity of food-particle communities is specific. Food particle-associated community recovered from feces digested polysaccharides such as arabinogalactan and xylan more efficiently and produced mainly acetate, whereas their free-living counterparts digested oligosaccharides faster and produced more butyrate (S. Macfarlane *et al.*, 1997; Sandra Macfarlane & Macfarlane, 2006). These studies suggest food particles are colonized by a specific subset of bacteria in the gut, and this adhesion induces metabolic changes increasing the efficiency of complex polysaccharide degradation.

b. Mucus-associated biofilms

Several studies showed that the mucus-associated microbiota (Figure 18A), also referred to as the mucosal community, differed in composition from the fecal community (Eckburg *et al.*, 2005; Erwin G Zoetendal *et al.*, 2002). Abundant mucosal genus include *Bacteroides*, *Faecalibacterium*, *Roseburia*, *Blautia* and lactic acid bacteria (De Weirdt & Van De Wiele, 2015). Moreover, use of laser microdissection of mice colon allowed the comparison of mucosal and luminal communities. Mucosal communities had a lower Bacteroidetes/Firmicutes ratio, and were in particular enriched in Lachnospiraceae and Ruminococcaceae compared to luminal communities (Nava, Friedrichsen, & Stappenbeck, 2011). Several properties might contribute to mucus colonization (De Weirdt & Van De Wiele, 2015). As I previously described, bacteria can adhere to the O-glycan of mucins (Sicard *et al.*, 2017). Interestingly, the composition of this O-glycan is variable between species, suggesting a possible mechanism to select host-specific strains as colonizers (Johansson, Holmén Larsson, & Hansson, 2011). Moreover, several bacteria have the ability to degrade mucin-bound oligosaccharides such as *A. muciniphila*, *Bacteroides thetaiotaomicron* or *Ruminococcus*, although *in vivo* mucin degradation seems to implicate a consortium of bacteria rather than a single species (Sicard *et al.*, 2017; J. L. Sonnenburg *et al.*, 2004). Last, bacterial shape and motility was shown to determine bacterial penetration in gels of different viscosity, and to correlate with spatial organization of bacteria within the mucus layer, suggesting local viscosity of the mucus could select specific bacterial shapes, rather than species (Swidsinski *et al.*, 2007). Interactions between host factors, such as the immune system or oxygen diffusing through the

epithelium, and bacteria are also key to allow colonization of the mucus layer (Van den Abbeele, Van de Wiele, Verstraete, & Possemiers, 2011).

In vitro studies mimicking the gut environment have allowed the functional characterization of mucus-associated versus luminal communities. *In vitro* colonization of mucus gel by fecal microbiota in fermenters led to rapid colonization of mucus. Mucus-colonizing and planktonic communities differed in both taxa representation and sugar metabolism, although only one fermenter was followed for each tested condition, making it hard to draw definitive conclusion (Sandra Macfarlane, Woodmansey, & Macfarlane, 2005). Using an *in vitro* chemostat system mimicking the lumen and mucosal compartments of the gut, McDonald and colleagues showed that the planktonic and mucus-associated biofilm communities differed in composition and in reaction to antibiotic perturbation (McDonald *et al.*, 2015). These studies point to the importance of accounting for gut biogeography when studying commensals' metabolism and reaction to stress.

c. Mucus-invasive biofilms

In healthy conditions, very few bacteria are found in the inner mucus layer, and very few are in contact with the gut epithelium. However, in a diseased state, mucus-invasive biofilms have been described. Swidsinski and colleagues strictly defined mucus-invasive biofilms as carpets of bacteria of more than 10^6 bacterial/mL that spanned at least $50 \mu\text{m}$ and observed within $1 \mu\text{m}$ of the epithelium (Figure 18D). Only 35 % of the healthy individuals had mucus-invasive biofilms, whereas more than 90 % of subjects suffering IBD or self-limiting colitis did. *B. fragilis* group bacteria (comprised of *B. fragilis*, *B. thetaiotaomicron*, *B. eggerthii* and *B. vulgatus*) accounted for more than 60 % of the biofilm mass of IBD patients (Swidsinski *et al.*, 2005). *In vitro* biofilm cultivation of mucosal communities from IBD patients or healthy donor in anaerobic conditions showed that IBD-mucosal communities formed thicker biofilms than healthy-mucosal communities. Bacteria dispersed from these biofilms were more invasive into Caco-2 cells, and translocated across epithelia more often, than bacteria dispersed from the healthy control. Moreover, IBD-biofilm dispersed cells led to an increased inflammatory response of Caco-2 cells and *Caenorhabditis elegans*, showing these biofilms were pro-inflammatory (Motta *et al.*, 2018). Correlation between increased biofilm formation and IBD is not causation, however the demonstration that communities from IBD patients are pro-inflammatory seems to suggest that the development of a mucus-invasive biofilm might be important in IBD onset.

In another study, mucus-invasive biofilms were defined similarly as bacterial communities of more than 10^6 bacteria/mL in the mucus layer, spanning at least a linear distance of 200 μ m of the epithelial surface. Biofilms were detected in 50 % and 67 % of biopsies from patients suffering from colorectal cancer and adenomas respectively, but only in 15 % of healthy individuals. This correlation varied depending on the colon site considered: biofilms were detected in 87 % of biopsies of ascending colon containing tumors, but only in 13 % of biopsies of transverse and descending colon containing tumors. Mucus-invasive biofilms also correlated with known pro-oncogenesis marker apparition (Dejea *et al.*, 2014). Inoculation of biopsies from mucus-invasive biofilm positive humans into three different mouse colon tumor models led to biofilm formation, inflammation and carcinogenesis whether the samples came from healthy or tumor-positive individuals, whereas inoculation of biofilm-negative samples from healthy humans never induced tumor apparition nor biofilm formation, suggesting mucus-invasive biofilms are carcinogenic, even in healthy individuals (Tomkovich *et al.*, 2019).

Thus, biofilm formation in the gut can be associated to both health and disease. The density of bacteria and their localization in respect to the gut epithelium seem key to distinguish between healthy biofilms and disease-associated biofilms.

In this chapter, I have shown that the gut microbiota can be considered an anaerobic biofilm-like structure. However, most studies on single-species biofilm are done on pathogenic bacteria that are often only minor members of the microbiota composition. It is therefore of critical importance that we develop the study of biofilm formation in major anaerobic symbionts. In the next two chapters, I will present the two model organisms we have chosen for this study, *Bacteroides thetaiotaomicron* and *Veillonella parvula*.

III-B. THETAOTAOMICRON IS A MODEL GUT SYMBIONT.

A. *B. thetaiotaomicron* is one of the most abundant gut mutualists.

1. *B. thetaiotaomicron* is a major member of the gut microbiota

Bacteroides are Gram-negative, rod-shaped, non-spore forming bacteria that represent circa 25 % of the fecal microbiota. The most represented species are *B. vulgatus*, *B. thetaiotaomicron* and *B. distasonis* (now reclassified *Parabacteroides distasonis*). *B. fragilis*, *B. ovatus*, *B. eggerthii* and *B. uniformis* are also abundant, but less frequently found (Salysers, 1984). *B. thetaiotaomicron* is an early colonizer of the gut microbiota that is passed from mother

to child by vertical transmission (Palmer *et al.*, 2007; Rotimi & Duerden, 1981). Its prevalence increases after weaning, and in the adult gut microbiota it represents 6 % of all feces microbiota, and 12% of all gut Bacteroidetes (Eckburg *et al.*, 2005). Thus, it has been proposed as a model symbiont organism and it was the first *Bacteroides* species to be sequenced in 2003 (J. Xu *et al.*, 2003). *B. thetaiotaomicron* was originally described in 1912 under the name *Bacillus thetaiotaomicron* (Distaso, 1912), and moved to the *Bacteroides* genus in 1919 (Castellani & Chalmers, 1919). The name “thetaitaomicron” allegedly comes from the observations that *B. thetaiotaomicron* vacuoles were shaped like the theta, iota and omicron Greek letters (Distaso, 1912). It has been described in the outer mucus layer, free-floating in the lumen and attached to food particles (Sandra Macfarlane & Macfarlane, 2006; Mark Welch *et al.*, 2017; J. L. Sonnenburg *et al.*, 2005) (Figure 20).

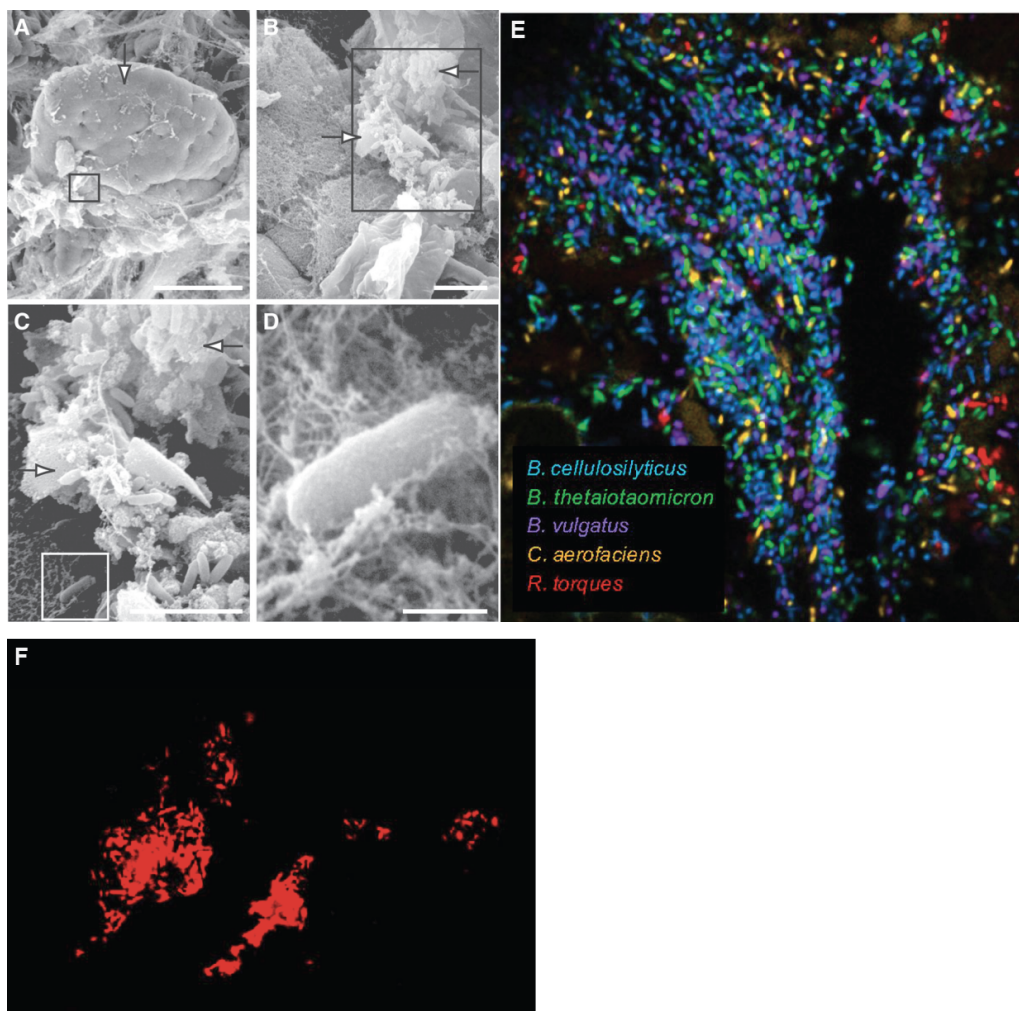


Figure 20: *B. thetaiotaomicron* in the gut.

A-D Scanning electron microscopy images of *B. thetaiotaomicron* colonizing mono-associated gnotobiotic mice gut from (J. L. Sonnenburg *et al.*, 2005). **A**. Distal small intestine of showing a villus (arrow) viewed from above. **B-C** Higher power images of *B. thetaiotaomicron* associated with luminal contents (food particles, shed mucus) (arrows in **B** and **C**) and close-up on *B. thetaiotaomicron* embedded in the mucus layer overlying the epithelium in **D**. Scale bars represent 50 μm in **A**, 5 μm in **B-C**,

0.5 μm in **D. E.** A 15-member consortium of human gut bacteria colonizing a gnotobiotic mouse gut. Among the 15-member consortium, 5 bacterial species were labelled: *B. cellulosilyticus*, *B. thetaiotaomicron*, *B. vulgatus*, *B. aerofaciens* and *R. torque*, showing spatial organization between the different members and *B. thetaiotaomicron* presence in dense multi-species communities in the mouse gut. From (Welch, Hasegawa, McNulty, Gordon, & Borisy, 2017). **F.** Fluorescence microscopy image of a *Bacteroides* microcolony growing on the human rectal mucosa hybridized with a specific 16S rRNA probe labelled with Cy3. Complementary studies indicate that common species associated to the mucosa in *B. vulgatus* and *B. thetaiotaomicron*. From (Macfarlane, M. J. Hopkins, G. T. Ma, 2000).

The most well-studied *B. thetaiotaomicron* strain is VPI 5482 (ATCC29148), a strain isolated from human feces. It was the first strain of *B. thetaiotaomicron* to be sequenced and the one for which many genetic tools are available (Goodman *et al.*, 2009; Martens, Roth, Heuser, & Gordon, 2009; J. Wang, Shoemaker, Wang, & Salyers, 2000a), making it an excellent tool to study the physiology of one of the dominant species of the gut microbiota. Sequencing of this strain revealed that it contained a 6.26 Mb chromosome encoding 4,646 proteins, 15 rRNA and 70 tRNA and a 0.03 Mb plasmid encoding an additional 41 proteins. It is particularly rich in genes encoding proteins involved in polysaccharide uptake and degradation, capsular polysaccharides, environmental sensing and DNA mobilization, suggesting these functions may be important to stably colonize the gut microbiota (J. Xu *et al.*, 2003) (Figure 20).

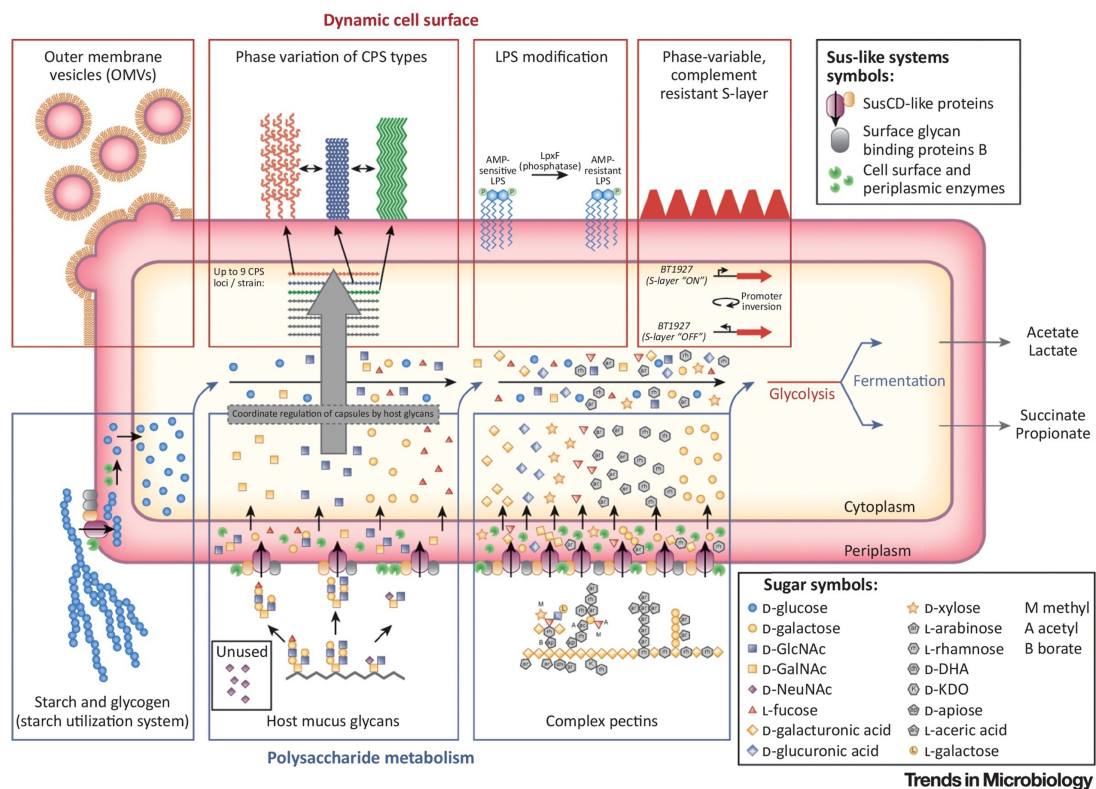


Figure 21: *B. thetaiotaomicron*.

From (Porter, Luis, & Martens, 2018). *B. thetaiotaomicron* main features are represented graphically, including the discussed capsular polysaccharides, S-layer and polysaccharide utilization loci.

When the gut barrier is breached, *B. thetaiotaomicron* can behave as an opportunistic pathogen inducing abscess formation in multiple body sites. It is the second most isolated *Bacteroides* species from sites of infection, behind *B. fragilis*, even though it is present 10-100-times more in the gut microbiota than *B. fragilis* (Wexler, 2007). However, this is a minor aspect of *B. thetaiotaomicron* interaction with the host and many studies have shown that *B. thetaiotaomicron* is a very important symbiont that provides the host with several benefits. In the next part, I will discuss some examples of *B. thetaiotaomicron* beneficial interactions with the host.

2. *B. thetaiotaomicron* interactions with the host

B. thetaiotaomicron colonization provides several benefits for the host including increased nutrient intake, mucosal maturation, immune system modulation and colonization resistance.

a. Nutrition

As previously discussed, gut bacteria participate to host nutrition by performing metabolic reactions that the host cannot perform, including metabolizing complex polysaccharides to produce easily consumed SCFA or synthesizing vitamins. *B. thetaiotaomicron* contains one of the largest glycan utilization capacities described in a bacteria and has been shown to be able to degrade a vast array of complex and simple sugars (Lora V. Hooper, Midtvedt, & Gordon, 2002; Rakoff-Nahoum, Coyne, & Comstock, 2014b; J. Xu & Gordon, 2003). However, comparison of germ-free mice and *B. thetaiotaomicron* mono-associated mice have shown that bacterial colonization also impacts host nutrition through modifications of the host physiology. Indeed, colonization of germ-free mice by *B. thetaiotaomicron* induces expression of host genes involved in lipid, micronutrient and glucose absorption, and reduces expression of fasting-induced adipose factor (L. V. Hooper *et al.*, 2001). Thus, *B. thetaiotaomicron* colonization induces a general increase in nutrient intake by the host.

b. Maturation of the intestinal mucosa

Moreover, *B. thetaiotaomicron* colonization impacts the development of the intestinal mucosa. *B. thetaiotaomicron* stimulates angiogenesis, increasing vascularization of the gut (Stappenbeck, Hooper, & Gordon, 2002). It also strongly induces the expression of *spr2a*, a

gene involved in cell junctions, contributing to epithelial barrier integrity. Unlike *B. thetaiotaomicron*, challenges with *E. coli* or *Bifidobacterium infantis* only led to a small increase of *spr2a* expression, showing at least part of *B. thetaiotaomicron* impact on host physiology is specific to this important symbiont (L. V. Hooper *et al.*, 2001). It stimulates mucus production by inducing mucus-producing goblet cell differentiation and mucus-related gene expression via production of the SCFA acetate (L. V. Hooper *et al.*, 2001; Wrzosek *et al.*, 2013). Last, it modifies the pattern of glycosylation of epithelial cells through the addition of terminal α -fucose to host glycans. This occurs during the weaning period and is maintained in adult animals. However, in germ-free mice, this fucosylation is not maintained past weaning. Addition of a microbiota or *B. thetaiotaomicron* alone at any time is sufficient to restore α -fucose production. Interestingly, induction of α -fucose production by *B. thetaiotaomicron* was shown to be dependent on the production of a secreted molecule regulated by FucR. FucR also regulates the bacterial ability to degrade fucose as a carbon source, thereby linking bacterial metabolic requirement with host modifications (Bry, Falk, Midtvedt, & Gordon, 1996; L V Hooper *et al.*, 1999). A *B. thetaiotaomicron* secreted molecule was also able to modulate HT29-MTX cells galactosylation pattern (Freitas *et al.*, 2001). Host glycoconjugates are very diverse, both intra- and inter-species, and little is known about the function of the outer-glycans of glycoconjugates. These studies suggest that selection of beneficial bacteria might be one role of outer-glycans and might explain their diversity (Gagneux & Varki, 1999).

c. Immune system modulation

Degradation of polysaccharides by *B. thetaiotaomicron* produces SCFA, metabolites that were shown to modulate the immune system (Smith *et al.*, 2013). Moreover, colonization of the gut with *B. thetaiotaomicron* induces the production of antimicrobial molecules by Paneth cells such as angiogenin 4, a bactericidal protein that targets the Gram-positive pathogens *Listeria monocytogenes* and *Enterococcus faecalis*, but not *B. thetaiotaomicron* itself (Lora V. Hooper, Stappenbeck, Hong, & Gordon, 2003) and matrilysin, an enzyme involved in the production of antimicrobial pro-defensins (López-Boado *et al.*, 2000). The presence of *B. thetaiotaomicron* also induces the production of sIgA by the gut epithelium in rats (Scharek, Hartmann, Heinevetter, & Blaut, 2000). Thus, *B. thetaiotaomicron* colonization has a strong impact on enteric immune system maturation. Moreover, *B. thetaiotaomicron* participates in the maintenance of the immune system homeostasis. Indeed, *B. thetaiotaomicron* dampens inflammation by targeting the RelA subunit of NF- κ B, a key transcription factor involved in

the immune response (Kelly *et al.*, 2004). Recently, a pirin-like protein was found to mediate *B. thetaiotaomicron* inhibition of NF- κ B signaling in Caco-2 cells and to have anti-inflammatory effect in a rat model (Delday, Mulder, Logan, & Grant, 2019). *B. thetaiotaomicron* colonization also reduces damage to the intestinal epithelium mediated by two cytokines: tumor necrosis factor (TNF)- α and interferon- γ (Resta-Lenert & Barrett, 2006). Last, diet was shown to modify *B. thetaiotaomicron* interactions with immune T-cells by impacting the expression of surface antigens, providing a mechanism by which the environment can influence immunity through modifications of gut bacteria (Wegorzewska *et al.*, 2019).

d. Gut microbiota stabilization

B. thetaiotaomicron colonization indirectly impacts the host through interactions with other bacteria. *B. thetaiotaomicron* digestion of complex polysaccharides leads to the production of intermediary compounds that can be used by other gut bacteria as a carbon source, thus favoring the colonization of other symbionts (Rakoff-Nahoum, Coyne, & Comstock, 2014a). Moreover, *B. thetaiotaomicron* can confer protection from pathogens by directly interacting with them or reducing their pathogenicity. Indeed, *B. thetaiotaomicron* secretes a molecule that inhibits shiga toxin 2 production, one of the main virulence factor of *E. coli* O157:H7 (De Sablet *et al.*, 2009) and prevents pathogenic *Citrobacter rodentium* colonization by nutrient competition (Kamada *et al.*, 2012).

B. thetaiotaomicron colonization provides the host with several benefits, as described above. On the other hand, the host provides *B. thetaiotaomicron* with nutrients, such as α -fucose. As both partners benefit for the interaction, *B. thetaiotaomicron* should be considered a mutualist bacterium rather than a commensal or symbiont.

3. *B. thetaiotaomicron* colonization factors

B. thetaiotaomicron success as an intestinal symbiont relies on several properties that allow an efficient colonization: tolerance to gut-related stresses such as oxygen or bile, and competitiveness against other bacteria.

a. B. thetaiotaomicron is adapted to the gut environment.

i. Oxygen tolerance

The distal gut is a mostly anoxic environment dominated by obligate anaerobe members of the gut microbiota, although some oxygen diffuses from the epithelium, creating a lateral oxygen gradient (cf chapter 2 and figure 11). *B. thetaiotaomicron* does not grow in presence of oxygen but is highly tolerant to it. Several *Bacteroides* isolates were found to be able to survive up to 72h to atmospheric levels of oxygen (Tally, Stewart, Sutter, & Rosenblatt, 1975), showing oxygen tolerance is widespread in *Bacteroides*. The related *B. fragilis* was even shown to be able to replicate in presence of up to 0.05 % oxygen, and a single mutation in *oxe* was sufficient to allow growth in presence of 2 % oxygen, showing the acquisition of microaerophilia can be very rapid (Meehan, Baughn, Gallegos, & Malamy, 2012). Iron-Sulphur enzymes fumarase and pyruvate-ferredoxin oxidoreductase involved in *B. thetaiotaomicron* central metabolism and production of SCFA were shown to be sensitive to aeration (Pan & Imlay, 2001). Whereas the catalytic activity of these enzymes was severely reduced in presence of oxygen, their folding was not impacted, resulting in rapid growth resumption once the oxygen was removed (Pan & Imlay, 2001), although high levels of hydrogen peroxidase can lead to irreversible fumarase inactivation (Lu & Imlay, 2019). Moreover, aerated *B. thetaiotaomicron* cultures produce 15 times more endogenous reactive oxygen species than *E. coli* cultures (Lu, Sethu, & Imlay, 2018). Taken together, these studies propose a mechanism by which oxygen can arrest growth in *B. thetaiotaomicron*. For protection against oxidative stress, *B. thetaiotaomicron* genome encodes several anti-oxidative stress proteins, including a catalase, rubrerythrins 1 and 2, an alkylhydroperoxyde reductase (AhpCF) and several superoxide dismutases (Mishra & Imlay, 2013; Pennington & Gregory, 1986). Rubrerythrins 1 and 2 were found to have peroxidase activity at low oxygen concentrations but to become inactivated as the oxygen concentration increased, whereas AhpCF and the catalase took over at higher oxygen concentrations (Mishra & Imlay, 2013). In general several anti-oxidant proteins were secreted upon stressing *B. thetaiotaomicron* cultures, suggesting that *B. thetaiotaomicron* could have anti-inflammatory properties (Hochart-Behra *et al.*, 2008; Hochart-Behra, Drobecq, Tourret, Dubreuil, & Behra-Miellet, 2014). All these proteins participate to the remarkable oxygen tolerance of *B. thetaiotaomicron*.

ii. Bile tolerance

Bile acids are secreted in the duodenum after food consumption to participate in lipid absorption. However, they also have strong antimicrobial effects: as detergents, they can damage lipidic membranes and they were also shown to damage DNA, alter protein folding and chelate iron and calcium, thereby limiting bacterial growth (Urdaneta & Casadesús, 2017). In particular, bile acids were shown to decrease membrane integrity and cell viability of *B. thetaiotaomicron* (Watanabe, Fukiya, & Yokota, 2017). Gram-negative bacteria are naturally more resistant against this stress than Gram-positive bacteria, and one study showed that the O-antigen of lipopolysaccharides might be involved (Picken & Beacham, 1977). Bile resistance is a common property of *Bacteroides*, so much so that bile esculin agar was described as a selective media for *Bacteroides* isolation (Wexler, 2007), however little is known about the mechanisms that underlie it. Common mechanisms of bile resistance include production of bile salt hydrolase (BSH) that catalyzes the removal of the taurine or glycine residues of conjugated bile acids, production of efflux pumps to get rid of cytoplasmic bile acid, general stress responses and cell wall modifications (Bustos, Font de Valdez, Fadda, & Taranto, 2018). *B. fragilis* has been shown to produce a bile salt hydrolase (Stellwag & Hylemon, 1976) and to enhance production of RND-efflux pumps upon challenge with bile acids (Pumbwe *et al.*, 2007). Like many other *Bacteroides*, *B. thetaiotaomicron* also possesses a BSH (Shindo & Fukushima, 1976; Yao *et al.*, 2018). *B. thetaiotaomicron* BSH is produced *in vivo* in mice and it specifically targets chenodeoxycholic acid derivatives but not cholic acid derivatives (Yao *et al.*, 2018). *B. thetaiotaomicron* was also shown to produce a 7 α -hydroxysteroid dehydrogenase, an enzyme that catalyzes the oxidation of the 7 α -hydroxy group of primary bile acids (Sherrod & Hylemon, 1977). *B. thetaiotaomicron* VPI 5482 type strain encodes multiple efflux pumps including 19 RND and 13 MATE drug efflux pump (Aires, 2016) but their contribution to bile tolerance has scarcely been assessed. Recently, a tripartite multidrug efflux system was shown to mediate bile tolerance of *B. thetaiotaomicron*. This system is induced by bile and specifically mediates bile but not antibiotic tolerance (H. Liu *et al.*, 2019).

b. B. thetaiotaomicron competes with other commensals for colonization

i. Interference competition

To stably colonize the human gut, *Bacteroides* must compete against the other gut microbiota members. This competition is expected to be stronger between closely related strains, as they have closer metabolic requirements. Bacteriocins are peptidic antimicrobial molecules

produced by bacteria. *Bacteroides* species have been shown to produce several bacteriocins. Among the best characterized *Bacteroides* secreted antimicrobial peptides (BSAP) are proteins of the Membrane Attack Complex/Perforin (MACPF) family. Proteins from this family are pore-forming toxins and an important part of the host immune system. *B. fragilis*, *B. uniformis*, *B. vulgatus* and *B. thetaiotaomicron* natural isolates were all shown to encode MACPF bacteriocins that targeted sensitive strains of the same species exclusively. The sensitive strains encoded a sensitive variant of the receptor of the toxin, an outer membrane protein or LPS, whereas the producing strain possessed a resistant variant (Chatzidaki-Livanis, Coyne, & Comstock, 2014; McEneaney, Coyne, Chatzidaki-Livanis, & Comstock, 2018; Roelofs, Coyne, Gentyala, Chatzidaki-Livanis, & Comstock, 2016). Moreover, a *B. thetaiotaomicron* isolate was shown to secrete a bacteriocin produced *in vivo* that was active against all *Bacteroides*, *Parabacteroides* and most *Prevotella* tested. This bacteriocin resembles *Lactobacillus* type IIa bacteriocin and is the first characterized member of a new family of type II bacteriocins. Homologs of this toxin are widely found in *Bacteroides* strains from the human gut (Coyne *et al.*, 2019).

Bacteroides are known to encode T6SS, a secretion system mediating contact-dependent bacterial killing through the delivery of bacterial effectors inside the target cell. Although these systems are widely found in *Bacteroides*, none were identified so far in the currently studied *B. thetaiotaomicron* strains (Coyne, Roelofs, & Comstock, 2016).

ii. Sugar consumption

Bacteroides are well-known for their remarkable ability to degrade a vast array of complex and simple sugars (Martens, Chiang, & Gordon, 2008; J. Xu & Gordon, 2003). During the suckling period, *B. thetaiotaomicron* grows on host glycans and milk oligosaccharides and then shifts to complex carbohydrate degradation after weaning (Bjursell, Martens, & Gordon, 2006). Moreover, as discussed above, it can induce production of fucose glycoconjugates by host cells (Bry *et al.*, 1996; L V Hooper *et al.*, 1999) and stimulate mucus production (Wrzosek *et al.*, 2013), two sources of sugar usable by *B. thetaiotaomicron*. *B. thetaiotaomicron* was also shown to be able to degrade mannan from yeast, an organism present in human diet and in the natural gut microbiota, showing it can even graze on other gut microbiota members (Cuskin *et al.*, 2015). In *Bacteroides*, polysaccharide degradation is performed by polysaccharide utilization loci (PUL) that are defined *a minima* as a pair of a TonB-dependent transporter (TBDT) and a lipidated cell surface glycan binding protein (SGBP). In most cases, it also

contains an array of enzymes involved in sugar degradation such as glycosyl transferases, polysaccharide lyases and carbohydrate esterases and it can include proteases, sulfatases or phosphatases. Moreover, it often contains regulatory genes (Grondin, Tamura, Déjean, Abbott, & Brumer, 2017). The polysaccharide utilization loci database PULDB (<http://www.cazy.org/PULDB>) regroups described or predicted PUL. According to this database, *B. thetaiotaomicron* encodes 101 pairs of TBDT/SGBP, 269 glycoside hydrolases, 87 glycosyl transferases, 15 polysaccharide lyases, and 19 carbohydrate esterases organized in 88 PULs (some PULs contain more than one TBDT/SGBP pair), encompassing 18% of its genome, one of the largest glycobiome described to date (Martens *et al.*, 2008).

The best described PUL is the starch utilization system (*sus*) (Figure 22). It is composed of a cluster of 8 genes named *susRABCDEFG*. *SusD* is the SGBP responsible for binding starch, assisted by auxiliary SGBP *SusE* and *SusF* lipoproteins. Once bound, starch can be degraded by outer membrane *SusG* and the resulting oligosaccharides are transported through the outer membrane by the TBDT *SusC*. In the periplasm, neopullulanase *SusA* and α -glucosidase *SusB* further degrade the oligosaccharides to maltose and glucose that are then transported to the cytosol. Last, *SusR* is a transcription factor regulating the *sus* system. All PULs encode a homolog of *SusC* (the TBDR) and *SusD* (the SGBP), that can be used for automatic PUL prediction. Substrate binding does not always involve auxiliary SGBP, but when it is the case, these SGBP tend to share little homology with *SusE* or *SusF*, and thus are sometimes called “*SusE* positioned” or “*SusF* positioned”. The enzymes involved in polysaccharide breakdown, both located at the outer membrane and in the periplasm, vary greatly depending on the type of substrate (Grondin *et al.*, 2017).

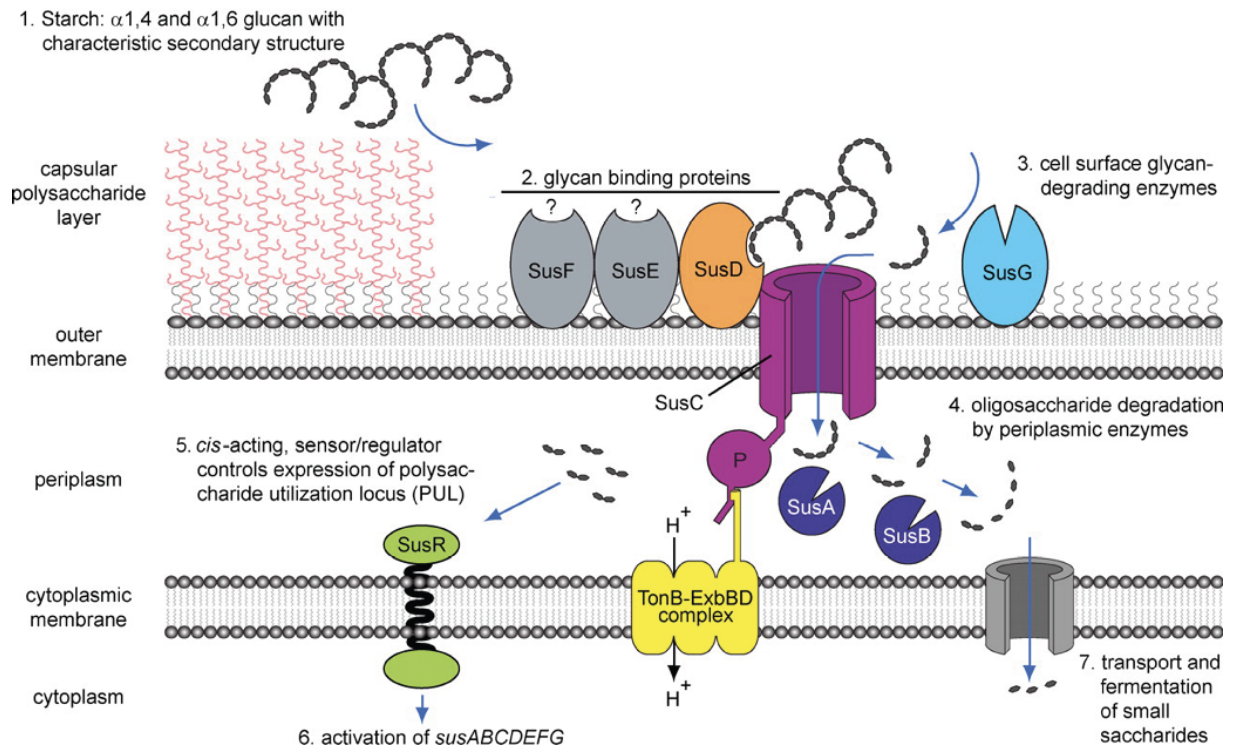


Figure 22: The Sus system.

Adapted from (Martens, Koropatkin, Smith, & Gordon, 2009). Starch is bound by SusDEF and degraded by SusG. The resulting oligosaccharides are transported through the outer membrane by SusC. In the periplasm, they are degraded to glucose and maltose by SusA and SusB and transported to the cytoplasm. The inner membrane SusR regulator detects periplasmic maltose and consequently induce expression of the *susA* and *susBCDEF* operons.

Three main regulation systems of PUL expression have been described: the SusR-like transcription factors, the hybrid two component system (HTCS) and the extracytoplasmic function (ECF) sigma/anti sigma factors (Ravcheev, Godzik, Osterman, & Rodionov, 2013). SusR is located in the inner membrane. It contains a periplasmic sensor domain that recognizes maltose and a cytoplasmic DNA binding domain that can bind next to the *sus* operons (*susA* and *susBCDEF*) promoters and induce expression (D'Elia & Salyers, 1996). Although it was the first PUL regulator described, only 5 homologs of *susR* are present in *B. thetaiotaomicron* genome, and only 3 co-localize with a SusC-D pair (Ravcheev *et al.*, 2013). Two-component systems are classically composed of a membrane sensor kinase and a cytoplasmic response regulator. HTCSs incorporate both functions in a single protein: they contain a periplasmic carbohydrate-sensing domain and conserved cytoplasmic domains including a histidine kinase domain and a DNA-binding response regulator domain (E. D. Sonnenburg *et al.*, 2006). *B. thetaiotaomicron* encodes 36 HTCS, which is more than any other sequenced organism, and 25 of them are found next to a SusC-D pair (Ravcheev *et al.*, 2013). Last, extracytoplasmic function (ECF) sigma/anti sigma factors are composed of two effectors. The membrane-

associated anti-sigma factor is linked to a specific SusC homolog through its periplasmic domain. Its cytoplasmic domain sequesters the ECF sigma factor. Upon stimulation, the sigma factor is liberated and promotes gene expression by recruiting the RNA polymerase (Ravcheev *et al.*, 2013). *B. thetaiotaomicron* encodes 50 ECF sigma/antisigma factors, 20 of them are present next to a SusC-D pair.

Presence of specific PULs are predictive of colonization capacity when the cognate polysaccharide is present in the diet and defects in PUL production can lead to colonization defects, showing the importance of PUL as colonization factors (Q. Cheng, Hwa, & Salyers, 1992; E. D. Sonnenburg *et al.*, 2010). Moreover, mice colonized by *B. fragilis* are resistant to colonization by a second *B. fragilis* but not to colonization by another *Bacteroides* species and this species-specific colonization resistance is mediated by a PUL (Lee *et al.*, 2013), demonstrating the importance of the range of sugars usable by a bacteria to define a colonization niche. Consistently, although *Bacteroides* generally encode numerous PULs, different species of *Bacteroides* such as *B. thetaiotaomicron* and *B. ovatus* have very few PULs in common, allowing them to colonize different niches and cohabit in the host. In particular, *B. thetaiotaomicron* was shown to encode several PULs dedicated to mucin O-glycan degradation that were absent in *B. ovatus*, and *B. ovatus* encodes PULs dedicated to hemicellulosic polysaccharides which are not present in *B. thetaiotaomicron* (Martens *et al.*, 2011).

Altogether, *B. thetaiotaomicron* versatile sugar consumption, tightly regulated by environmental cues, is an important colonization factor contributing to *B. thetaiotaomicron* success in the human gut.

***B. B. thetaiotaomicron* surface structures**

Besides PULs, *B. thetaiotaomicron* encodes several other surface structures including capsules, surface layer and pili that play a considerable role in mediating interactions with the host (Figure 21).

1 Capsular polysaccharides

a. Organization and synthesis

Capsular polysaccharides (CPS) are tightly packed polysaccharidic structures that surround the cell in a protective layer. Gut Bacteroidales encode multiple CPS, whereas other Bacteroidales do not, suggesting multiple CPS production is an important gut colonization

factor (Coyne & Comstock, 2008). *B. thetaiotaomicron* VPI 5482 encodes 8 CPS operons, named *cps1-8* (Coyne & Comstock, 2008; J. Xu *et al.*, 2003), composed of 15 to 31 genes, that always begin with one or two regulatory genes (Figure 23). However, the CPS composition of different *B. thetaiotaomicron* varied strongly between strains: only *cps2* and *cps6* loci were found in more than half of tested *B. thetaiotaomicron*. 49 unique CPS loci were identified in 14 *B. thetaiotaomicron* strain, showing the very important diversity of CPS within this species (Porter, Canales, Peterson, & Martens, 2017).

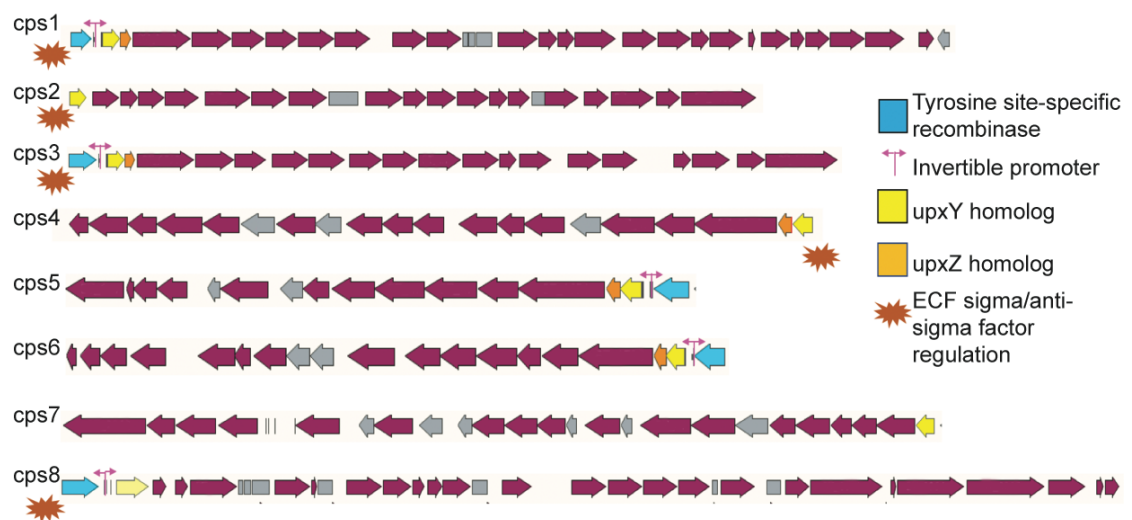


Figure 23: *B. thetaiotaomicron* VPI 5482 capsular polysaccharides encoding loci.

CPS production in *Bacteroides* involves the *wzx/wzy* system (Fletcher, Coyne, Villa, Chatzidaki-Livanis, & Comstock, 2009). Sugar moieties are assembled in the cytoplasm on a lipid carrier of undecaprenyl phosphate at the inner membrane by a set of glycosyl transferases. The resulting capsular subunit is then flipped across the inner membrane to reach the periplasm by the Wzx flippase. In the periplasm, subunits are assembled by Wzy and then exported outside the cell to form the capsule. This assembly mechanism closely resembles that of protein glycosylation, except protein glycosylation does not involve a Wzy polysaccharide polymerase (Fletcher *et al.*, 2009; Singh, Adams, & Brown, 2019) (Figure 24).

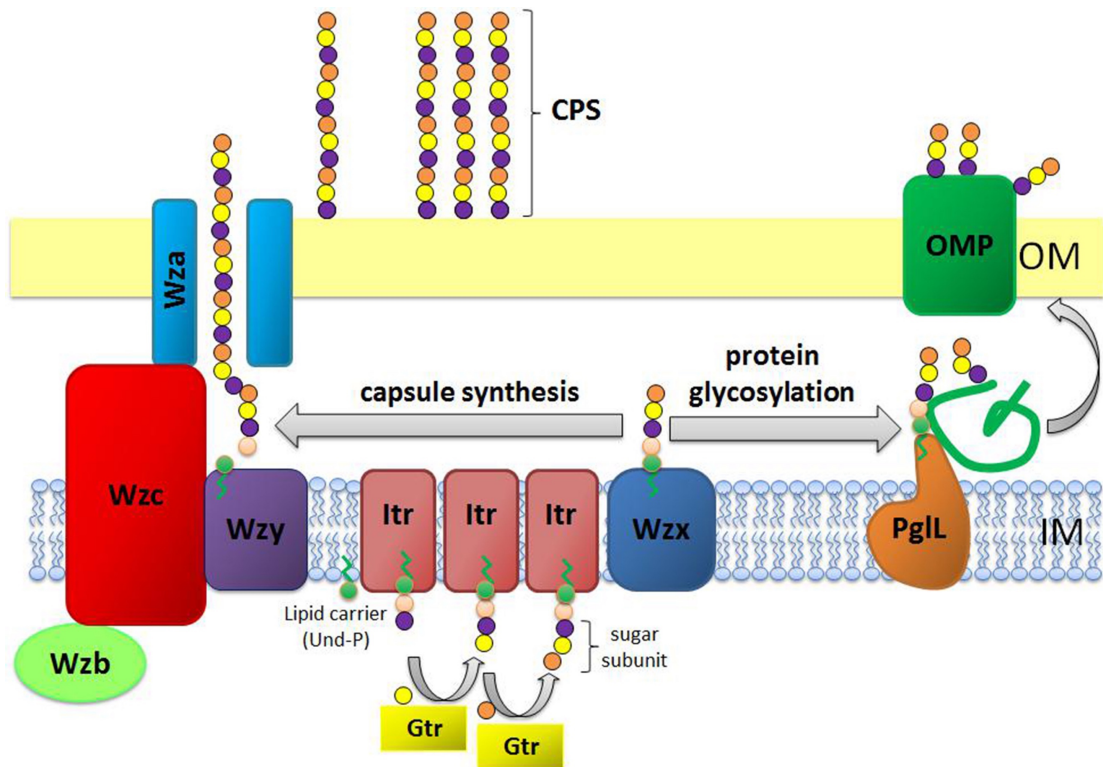


Figure 24: Capsular polysaccharide synthesis and protein glycosylation of *Acinetobacter baumannii*.

From (Singh *et al.*, 2019). *Bacteroides* use a similar *wzx/wzy* export system. A set of glycosyl transferase transfer sugar moieties to an undecaprenyl phosphate carrier on the cytosolic side of the inner membrane. The resulting glycosidic subunit is flipped by *Wzx* in the periplasm where subunits are polymerized by *Wzy*. After export through the outer membrane, the capsule assembles outside the cell.

b. Regulation

Bacteroides capsule regulation involves transcriptional regulators, flipping promoters and ECF sigma/anti sigma factors (Figure 23 and 25). *UpxY* and *UpxZ* are transcriptional regulators of capsules located at the beginning of CPS operons (Figure ZB-C). They were characterized in the related *B. fragilis*. *B. fragilis* also produces 8 different CPS named PSA-PSH encoded in 8 loci. The first gene of each locus is a *upxY* homolog. *UpxY* is a NusG-like protein that prevents premature transcription termination and allows complete transcription of its cognate CPS locus. It contains a Kyprides-Ouzounis-Woese (KOW) motif required for activity and a specificity motif that determines which capsule it regulates (Chatzidaki-Livanis, Coyne, & Comstock, 2009). The second gene of the CPS locus is a *upxZ* homolog. *UpxZ* was shown to inhibit the synthesis of non-cognate capsules by interacting with the corresponding *UpxY* homolog of the target capsule. The different *UpxZ* have different inhibitory patterns, leading to a hierarchical regulation of capsule expression that prevents the formation of chimeric capsules (Chatzidaki-Livanis, Weinacht, & Comstock, 2010). All *B. thetaiotaomicron*

CPS loci encode a *upxY* homolog, but only CPS1, 3, 4, 5 and 6 encode a *upxZ* homolog (J. Xu *et al.*, 2003).

Moreover, 7 out of the 8 *B. fragilis* capsular polysaccharide loci are under the control of an invertible promoter. Invertible repeats surround the promoter region allowing reversible inversion of DNA which switches ON or OFF capsule production (Krinos *et al.*, 2001; Patrick *et al.*, 2003). In *B. fragilis*, these inversions are all mediated by a single serine site-specific recombinase, Mpi, that also regulates the inversion of 6 other non-capsular loci (Coyne, Weinacht, Krinos, & Comstock, 2003). *B. thetaiotaomicron* CPS1, 3, 5, 6 and 8 possess an invertible promoter. Interestingly, each capsule locus with an invertible promoter is preceded by a tyrosine site-specific recombinase, a family of recombinases that was shown to specifically catalyze flipping of DNA immediately downstream of the recombinase encoding gene in *B. fragilis* (Weinacht *et al.*, 2004), suggesting that *B. thetaiotaomicron* capsular operon flipping promoters are each regulated by a different tyrosine-site specific recombinase, rather than by a global recombinase.

CPS1, 2, 3, 4 and 8 were also shown to be co-regulated with PULs by common ECF sigma/anti-sigma factors. This co-regulation required the PUL locus itself to be present. However, no clear link between the type of sugar degraded by the PUL and the composition of the capsule it is co-regulated with could be observed, so the reason for this co-regulation is still unclear (Martens, Roth, *et al.*, 2009).

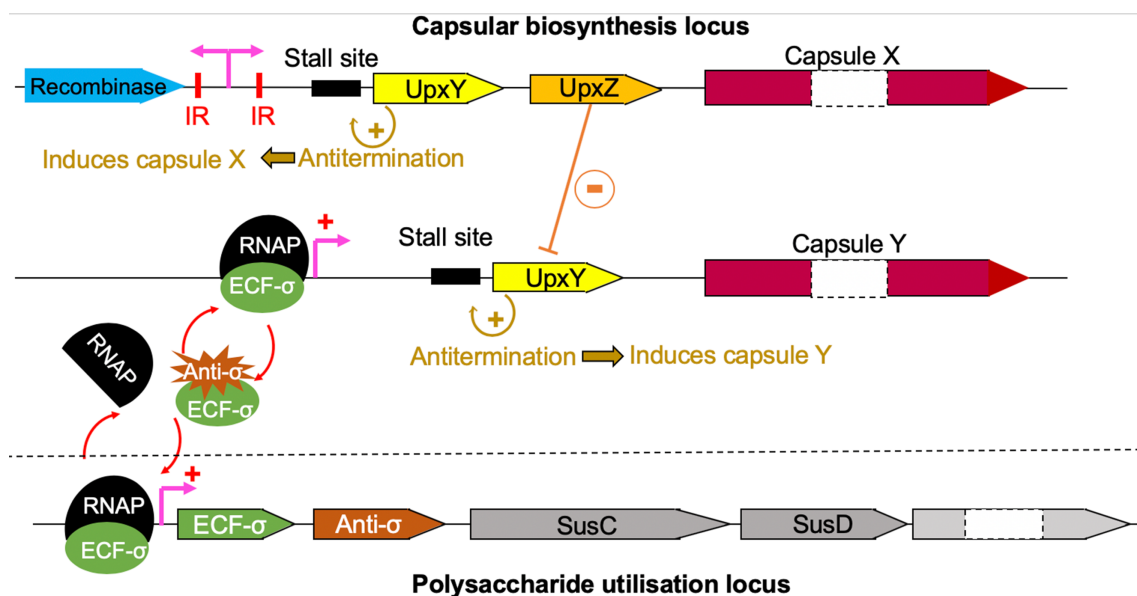


Figure 25: *B. thetaiotaomicron* CPS regulation.

Two hypothetical CPS encoding loci are represented. The first one contains an invertible promoter that can be flipped ON or OFF by the adjacent tyrosine-site specific recombinase (in blue). It also contains a *upxY* homolog (in yellow) required for expression of the cognate capsular polysaccharide encoding locus by preventing premature transcription termination and a

upxZ homolog (in orange) that inhibits the upxY homolog of another capsular polysaccharide encoding locus. This second hypothetical locus is also regulated by an ECF sigma/anti-sigma factor pair encoded by a PUL locus. The PUL encodes the sigma factor that recruits the RNA polymerase and allow transcription of both the PUL and the capsular polysaccharide encoding locus and the anti-sigma factor that binds to the sigma factor and prevent its action. For simplification purposes, the membrane is not represented here but the anti-sigma factor is an inner membrane protein.

This complex regulation ensures that at any time the population of *B. thetaiotaomicron* expresses different capsules, but that each cell only expresses one. In the next part, I will discuss several biological functions that were linked with multiple capsule production.

c. CPS expression in vivo

The expression of the 8 *B. thetaiotaomicron* CPS was assessed in the mouse gut in different conditions shortly after colonization or diet change (10-30 days). *In vivo*, the most expressed CPS is either CPS4 or CPS5, although the relative abundance of each CPS expression varied depending on diet, community composition or mice immune response (Porter *et al.*, 2017) (Figure 26). In particular, CPS4 was more expressed during the weaning period compared to the suckling period (Bjursell *et al.*, 2006). CPS4 was also upregulated when mice were fed a high fiber diet, whereas CPS5 was upregulated when mice were fed a simple sugar-rich diet (J. L. Sonnenburg *et al.*, 2005). Moreover, CPS4 was found to be strongly immunogenic. When *B. thetaiotaomicron* colonized mice producing CPS4-targeting IgA, CPS4 was much less expressed, demonstrating the ability of the adaptive immune system to modulate CPS expression (Peterson, McNulty, Guruge, & Gordon, 2007). Only moderate differences of CPS expression were seen in different part of the large intestine (Porter *et al.*, 2017). Follow up of CPS expression in *B. thetaiotaomicron* mono-associated mice for 5 months showed the CPS expression varied dynamically over time, although the general trend was conserved. Interestingly, regardless of the condition, the population was always heterogenous, with all 8 CPS expressed to some levels, showing that expressing multiple CPS might be beneficial for *B. thetaiotaomicron* colonization.

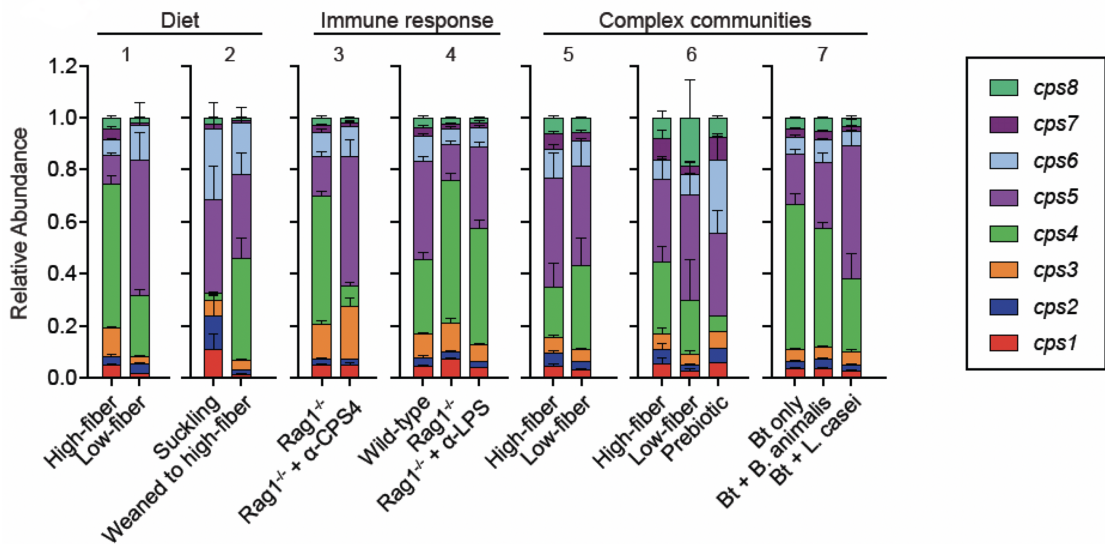


Figure 26: *B. thetaiotaomicron* 8 CPS expression in different in vivo conditions.

From (Porter *et al.*, 2017).

d. CPS-mediated functions

Competition experiments in gnotobiotic mice colonized with a mix of 9 *B. thetaiotaomicron* mutants, 8 mutants each expressing a single CPS and an acapsular mutant, demonstrated that the CPS5-only strain outcompeted all other strains. In $Rag^{-/-}$ mice deficient for adaptive immune response, CPS4-only and CPS6-only also persisted to high levels, confirming the immune system can select against some CPS-expressing cells (Porter *et al.*, 2017). Although CPS5-only was broadly more abundant, other CPS-only strains persisted to different levels depending on the longitudinal and transversal position in the gut. When a similar competition was done including WT strain on top of the 9 mutants, WT was found to colonize mice only a little better than CPS5-only strain. RNAseq experiments showed that WT still expressed several CPS, and not just CPS5. However, when submitted to stress, such as antibiotic treatment, the WT strain always outcompeted all single-CPS expressing strains, showing that multiple CPS expression might be more advantageous than expression of only CPS5 in changing environments (Porter *et al.*, 2017). The acapsular strain was outcompeted very fast in all conditions tested, and was only found to persist to low levels in some mice, demonstrating the importance of capsule expression during mouse gut colonization (Porter *et al.*, 2017). Colonization of germ-free mice with a saturating collection of *B. thetaiotaomicron* transposon mutants showed that mutants with transposon inserted in *cps4* operon, but not *cps5* operon, were outcompeted in all conditions tested, suggesting CPS4 might be an important colonization factor. The competition defect of mutants lacking a functional CPS4 was not

observed *in vitro*, showing this locus is important specifically *in vivo* (Goodman *et al.*, 2009). Why a similar phenotype was not observed for mutants lacking a functional CPS5 is not known, but it might come from technical differences.

B. thetaiotaomicron CPSs were also shown to modulate the host immune system. All single CPS-expressing strains stimulated the innate immune system to produce cytokines and regulated the activation of T-cells, but to different levels. Most single CPS-expressing strains were anti-stimulatory and only weakly activated the innate immune system, whereas CPS8-only and acapsular strain were pro-stimulatory (Hsieh *et al.*, 2020). Moreover, CPSs mediate phage-*B. thetaiotaomicron* interactions. Some CPS act as phage receptors or co-receptors and thus confer sensitivity to some phages, whereas other CPS can provide protection by preventing phage entry (Porter *et al.*, 2019). Last, CPS expression was found to impact co-aggregation between *B. thetaiotaomicron* and *Lactobacillus rhamnosus* (Rielinger, 2019; Schotten, 2016).

2. S-layer

On top of CPS, *B. thetaiotaomicron* was shown produce a surface layer (S-layer). An S-layer is a two-dimensional protein layer surrounding bacterial or archaeal cells and often conferring resistance to stress (Sleytr, Schuster, Egelseer, & Pum, 2014). In *B. thetaiotaomicron* VPI 5482, the S-layer is composed of a hexagonally tessellated array of BT1927 protein. *BT1927* is under the control of an invertible promoter, so that circa 1 cell out of 1,000 express it. Expression of this S-layer confers resistance to complement-mediated killing by the host (Taketani, Donia, Jacobson, Lambris, & Fischbach, 2015). The S-layer was also found to participate in phage/*B. thetaiotaomicron* interactions. Depending on the phage, it could either improve or prevent phage infection (Porter *et al.*, 2019).

3. Type V pilus

Pili are cell surface appendages composed of assembled pilin subunits that can mediate several phenotypes including adhesion, DNA transfer or motility. 4 major pathways of pili synthesis have been commonly described: chaperone-usher, alternate chaperone-usher, curli biogenesis and type IV pathway (Proft & Baker, 2009). Although pili have been described at the surface of *B. fragilis* cells (van Doorn, Oudega, & MacLaren, 1992; van Doorn, Mooi, Verweij-van Vught, & MacLaren, 1987), relatively few studies have been done on gut Bacteroidales pili synthesis and most of the literature on Bacteroidales pilus focused on the oral pathogen *Porphyromonas gingivalis*, in which pili are known to be important virulence factors,

mediating adhesion to host cells and other bacteria (Enersen, Nakano, & Amano, 2013). A structural analysis of putative pilin subunits found in different Bacteroidales led to the identification of a novel pilus synthesis pathway, the type V, which relies on protease-mediated polymerization (Q. Xu *et al.*, 2016) (Figure 27). The pilus assembles for tip to stalk. Tip and major stalk subunits are synthesized as lipidated protein at the cell surface where they are cleaved by a protease to remove both the lipid moiety and part of the N-terminal region of the protein, thereby uncovering the N-terminal groove involved in polymerization. Anchor pilin are also lipidated proteins, but they do not undergo cleavage and thus stay attached to the outer membrane (Coyne & Comstock, 2016; Shibata *et al.*, 2020; Q. Xu *et al.*, 2016). Bacteroidales pilin share little sequence homology with pilin of other bacteria, but a search for homologs of these newly described FimA homologs in Bacteroidales genomes led to the identification of multiple pilin genes among gut Bacteroidales. In particular, *B. thetaiotaomicron* VPI 5482 was shown to encode 32 FimA-like homologs in 13 different loci (Q. Xu *et al.*, 2016). Several homologs of FimA were also identified in *B. fragilis*. Interestingly, several of these pilin-encoding loci had an invertible promoter and were previously shown to be regulated by the global invertase Mpi that regulates *B. fragilis* capsule production (Coyne & Comstock, 2016).

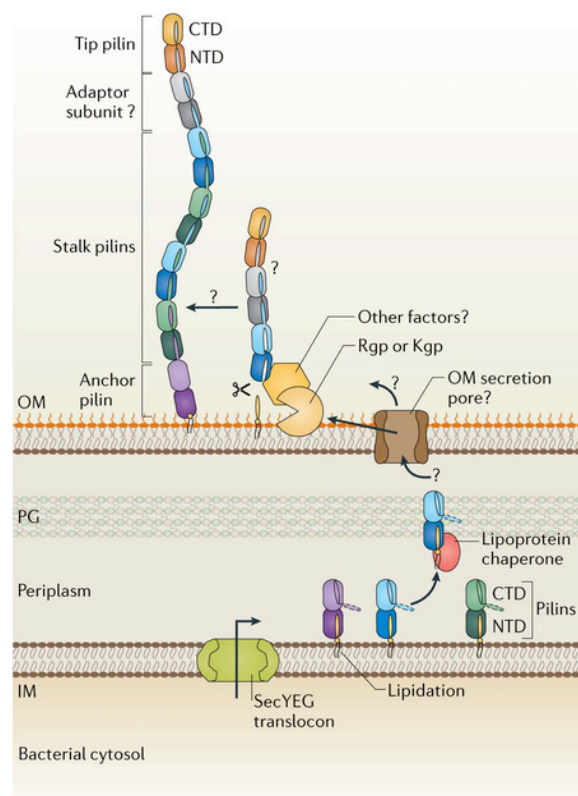


Figure 27 : Type V pilus biogenesis.

From (Hospenthal, Costa, & Waksman, 2017). Lipidated pilin precursors are translocated through the inner membrane by the Sec machinery. After cleavage of the signal sequence, prepilins are shuttled to the outer membrane by chaperones. Gingipain R (Rgp) or K (Kgp) cleave the prepilin at the outer membrane to produce a mature pilin that polymerizes through a strand-exchange mechanism. Anchor pilins do not undergo this second cleavage step and remain membrane associated. The tip pilin C-terminal end does not allow further polymerization. Question marks represent parts of the pathway that involve unknown effectors or mechanisms.

One of these phase variable pilin-encoding regions was previously shown to be involved in *B. fragilis* aggregation when the promoter was locked on (Coyne & Comstock, 2016; Weinacht *et al.*, 2004). Moreover, deletion of the last 9 amino acids of a FimA homolog of *B. thetaiotaomicron* was shown to induce pilus polymerization and biofilm formation (Mihajlovic *et al.*, 2019) (Annex 1). Interestingly, another *B. thetaiotaomicron* VPI 5482 FimA-encoding locus, *BT2318-2316* is followed by a diversity generating retro-element (DGR) (Doulatov *et al.*, 2004; Schillinger, Lisfi, Chi, Cullum, & Zingler, 2012). DGR are composed of a reverse transcriptase, a template repeat and a variable repeat that lies within a protein coding gene (in this case a FimA homolog). The reverse transcriptase is error prone. It synthesizes an imperfect cDNA from the template repeat mRNA and this cDNA is exchanged with the variable repeat DNA, thereby generating mutations in the C-term of the targeted protein (Schillinger *et al.*, 2012). This process might naturally generate variation in the C-term of a FimA homolog in *B. thetaiotaomicron*, potentially impacting polymerization of a type V pilus and adhesion.

Surface structures of *B. thetaiotaomicron* include PULs, type V pili, capsules and S-layer. Several of these components are phase-variable, thereby generating a huge diversity of cell surface architectures in a single *Bacteroides* isolate. Moreover, these different surface structures modulate various processes including stress resistance, immune system interaction and adhesion to substrates. However, with the exception of type V pili, for which information is still scarce, no classical adhesin surface structure has yet been described in *B. thetaiotaomicron*.

In the next part, I will consider what is known about adhesion and biofilm formation in *B. thetaiotaomicron*.

C. *B. thetaiotaomicron* biofilm formation

1. *In vitro* biofilm formation

B. fragilis was shown to rapidly adhere to and colonize mucus gels *in vitro* (Sandra Macfarlane *et al.*, 2005). Moreover, environmental conditions impact *B. fragilis* biofilm

formation: bile was shown to induce *B. fragilis* biofilm formation, adhesion to human cells and production of fimbria-like structures (Pumbwe *et al.*, 2007) and sub-minimal inhibitory concentrations of antibiotics modulated biofilm formation of *B. fragilis* clinical isolates. In particular, imipenem and metronidazole inhibited biofilm formation whereas enrofloxacin induced it (Silva *et al.*, 2014). Quorum sensing regulation involving orthologs of *luxR* also mediated *B. fragilis* biofilm formation (Pumbwe, Skilbeck, & Wexler, 2008).

Comparatively, little is known about *B. thetaiotaomicron* biofilm formation. This is in part due to the poor biofilm formation of the reference strain *B. thetaiotaomicron* VPI 5482. However, analysis of *B. thetaiotaomicron* clinical isolates showed variable *in vitro* biofilm formation capacity, with 14 isolates out of 34 that formed significantly more biofilm than VPI 5482 reference strain, showing this ability was widespread among *B. thetaiotaomicron* clinical isolates (Mihajlovic *et al.*, 2019) (Annex 1). *B. thetaiotaomicron* VPI 5482 formed *in vitro* biofilm in chemostat on carbon paper after 8 days of growth, consistent with a poor *in vitro* biofilm formation (TerAvest *et al.*, 2014). Transcriptomic comparison between biofilms and planktonic cultures showed that 1,154 genes were differentially expressed between the two conditions. In particular, PULs involved in host-derived mucin O-antigen degradation were upregulated in biofilm condition. Moreover, CPS1, 3, 4 and 6 were downregulated and CPS8 and CPS7 were upregulated in biofilms. Interestingly, the transcriptome of *in vitro* biofilms was closer to that of bacteria colonizing the gnotobiotic mouse gut than to planktonic cells, showing that *in vitro* biofilm formation might be a better proxy for *in vivo* phenotypes than planktonic cultures (TerAvest *et al.*, 2014).

None of these studies, either in *B. fragilis* or *B. thetaiotaomicron*, identified adhesins responsible for biofilm formation. Most classical adhesin families are absent from *B. thetaiotaomicron* genome, making identification of such structures more difficult (J. Xu *et al.*, 2003). As mentioned in the previous part, deletion of the last 9 amino acids of a FimA homolog led to polymerization of a pilus and biofilm formation in *B. thetaiotaomicron*, providing the first characterization of a putative adhesin in *B. thetaiotaomicron* (Mihajlovic *et al.*, 2019) (Annex 1).

2. *In vivo* biofilm formation?

As previously mentioned, one can distinguish three *in vivo* biofilm-like structures: bacteria adhering to food particles, mucus-associated bacteria and mucus-invasive biofilms. *B. thetaiotaomicron* showed rapid adhesion to food particles and colonization of the mucus layer

in the mouse gut (Figure 20A-D)(Sandra Macfarlane & Macfarlane, 2006; J. L. Sonnenburg *et al.*, 2005).

In gnotobiotic mouse guts, *B. thetaiotaomicron* was shown to be part of dense communities of bacteria (Figure 20E) that were not randomly distributed but rather spatially organized (Mark Welch *et al.*, 2017). In general, *Bacteroides* are abundant mucus-associated bacteria (De Weirdt & Van De Wiele, 2015) and they were shown to form microcolonies on the rectal mucosa (Figure 20F) (Macfarlane, M. J. Hopkins, G. T. Ma, 2000). Moreover, *B. thetaiotaomicron* possess several PULs involved in mucin O-glycan degradation that could mediate adhesion to mucins (Martens *et al.*, 2008).

Last, as previously mentioned, *B. fragilis/B. thetaiotaomicron/B. egghertii/B. vulgatus* make up 60 % of bacteria in mucus-invasive biofilms associated to IBD (Swidsinski *et al.*, 2005).

Taken together, these observations suggest that *B. thetaiotaomicron* adhesion capacity could be highly important for *in vivo* colonization and that *in vitro* biofilms might be a better proxy for *B. thetaiotaomicron* physiology than planktonic cultures.

IV- VEILLONELLA PARVULA IS A SYMBIONT OF ORAL AND INTESTINAL MICROBIOTA

A. Generalities

1. Introduction

Veillonella parvula is a small, non-motile, non-spore forming anaerobic Gram-negative coccus (circa 400nm) that was first described by Veillon and Zuber in 1898 (Veillon & Zuber, 1898) and was used to observe an outer membrane by electronic microscopy for the first time in 1964 (H. A. Bladen & Mergenhagen, 1964). Although *V. parvula* is a strict anaerobe, it is highly tolerant to oxygen, allowing it to protect other strict anaerobes from the community from oxidative stress (Zhou, Li, Huang, & Qi, 2017). *V. parvula* is unable to degrade carbohydrates, including glucose, and relies on organic acid fermentation, such as lactate, as a carbon source (Delwiche, Pestka, & Tortorello, 1985). Thus, Veillonella is found in close association with lactate producing bacteria such as *Streptococci* or *Actinomyces* (Delwiche *et al.*, 1985).

2. The Negativicutes

V. parvula belongs to the Negativicutes, one of the two clades of diderm bacteria among the Firmicutes. These two clades, the Negativicutes and the Halanaerobiales, present an

interesting evolutionary conundrum: a group of diderm bacteria deeply embedded within a clade of textbook monoderm organisms, the Firmicutes (Megrian, Taib, Witwinowski, Beloin, & Gribaldo, 2020; Vesth *et al.*, 2013) (Figure 28). They are therefore particularly interesting organisms to study the transition between monoderm and diderm bacteria, one of the big unanswered questions in evolutionary microbiology. Two hypotheses have been proposed: the ancestor of the Firmicutes was a monoderm bacteria, and the Negativicutes and the Halanaerobiales acquired an outer membrane, possibly through two independent horizontal gene transfer events; conversely, the ancestor of the Firmicutes was a diderm bacteria and the outer membrane was lost in some clades during evolution. Recently, extensive phylogenomic analyses showed that the most likely hypothesis was that within the Firmicutes, the presence of the outer membrane is an ancestral character and that it was lost several independent times to give rise to the Firmicutes monoderm phyla, although the mechanism for the loss of the outer membrane is not known. One hypothesis is that outer membrane loss occurred accidentally during sporulation and was tolerated well by the bacteria (Antunes *et al.*, 2016; Megrian *et al.*, 2020) (Figure 28). Moreover, the outer membrane of Negativicutes presents several differences compared to the classical diderm models such as Proteobacteria. For instance, outer membrane tethering involves OmpM rather than Braun's lipoprotein in Negativicutes (Megrian *et al.*, 2020), making it an interesting model to study novel properties of the outer membrane of non-canonical diderm bacteria.

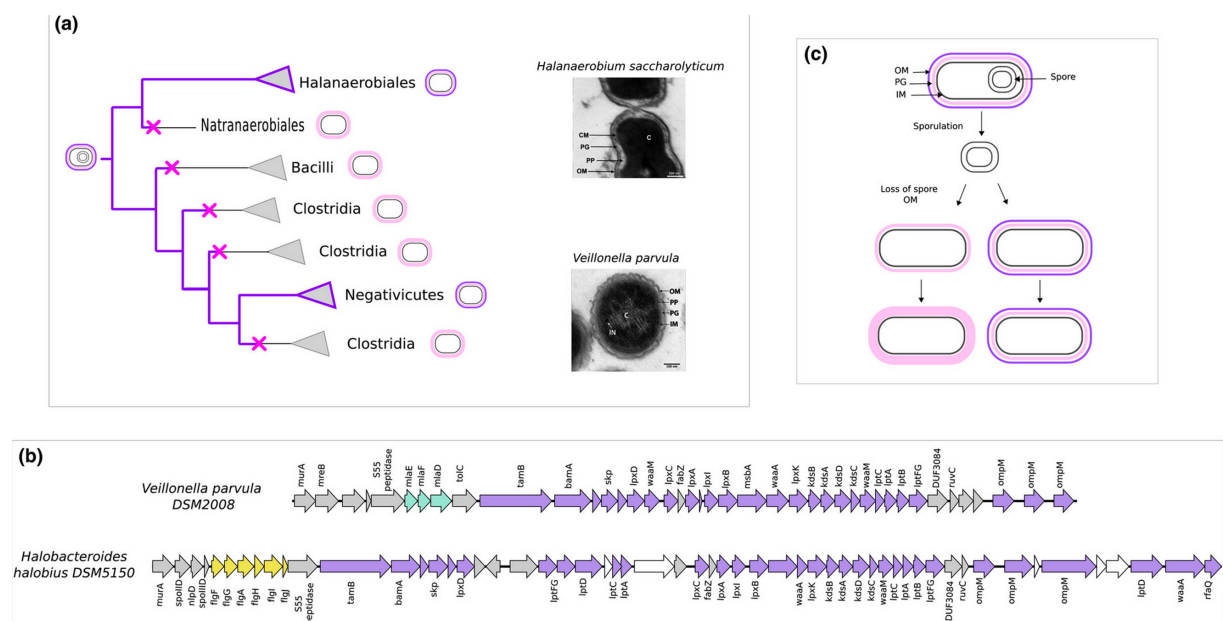


Figure 28: Diderm Firmicutes.

(a) Schematic representation of the Firmicutes phylum-level phylogeny. The diderm/monoderm phenotype of each branch is indicated. Crosses indicate each of the 5 independent outer membrane loss events. Electron microscopy images of a

representative species of each diderm Firmicutes phyla is shown on the right: *Veillonella parvula* (Negativicutes) and *Halanaerobium saccharolyticum* (Halanaerobiales) (b) Outer membrane gene cluster of the diderm Firmicutes. Genes for LPS synthesis and transfer, OMP biogenesis and OM tethering are in purple; Flagellar genes are in yellow; and genes for lipid asymmetry are in green. (c) Hypothetical scenario of OM loss in Firmicutes. The ancestor of Firmicutes was a sporulating diderm. The OM was lost multiple times during sporulation giving rise to the monoderm phenotype while it was retained in diderm Firmicutes. From (Megrian *et al.*, 2020).

3. Genetic tools

V. parvula reference strain, DSM 2008, was sequenced in 2010 (Gronow *et al.*, 2010). Moreover, some genetic tools have been described for this species including an *E. coli*-*Veillonella* shuttle plasmid (J. Liu, Merritt, & Qi, 2011; J. Liu, Xie, Merritt, & Qi, 2012), although production of mutants remained difficult within the type strain. Recently, a highly competent natural isolate was described (Knapp *et al.*, 2017), which will greatly ease mutagenesis in this species. Thus, *V. parvula* is one of the most promising Negativicutes species for molecular studies.

B. *Veillonella parvula* colonizes multiple microbiota

1. *V. parvula* is a symbiont of multiple microbiota

V. parvula is a symbiont of multiple microbiota including oral, genitourinary, respiratory, and intestinal microbiota of humans and animals (Delwiche *et al.*, 1985).

a. *Veillonella* in the oral microbiota

The oral cavity is composed of different sites carrying a microbiota, including the tongue, the saliva and the teeth. Each microbiota has a different composition, and it is estimated that each individual carries hundreds of species in their oral microbiota (Aas, Paster, Stokes, Olsen, & Dewhirst, 2005; Dewhirst *et al.*, 2010). *V. parvula* can be found in different location in the mouth, including buccal mucosa and saliva, but it is most abundant within the dental plaque. *Veillonella* make up to 5% of the initial dental plaque community and *V. parvula* constitutes over 90% of the *Veillonella* recovered from subgingival plaque (Hughes, Kolenbrander, Andersen, & Moore, 1988; Welch, Rossetti, Rieken, Dewhirst, & Borisy, 2016). The dental plaque microbiota is a multi-species biofilm and it has been used as a model of biofilm-like microbiota. It is established sequentially: the pioneering species, such as *Streptococcus mitis* that comprise 80% of the early dental plaque community, directly adhere to the salivary proteins coating the tooth. The early colonizers can then directly adhere to the initial colonizers and the late colonizers adhere to the early colonizers (Figure 29A). *V. parvula* is an early colonizer of the dental plaque. As such, it is well-known for its ability to bind both

pioneering species such as *Streptococci* but also late colonizers such as *Porphyromonas gingivalis* (Kolenbrander, Palmer, Periasamy, & Jakubovics, 2010; Kolenbrander *et al.*, 2006; Nyvad & Kilian, 1987)(Figure 29AB). This physical association is also linked with a metabolic one: *Streptococci* are lactic acid producers, the major carbon source of *V. parvula*. Consumption of lactic acid by *Veillonella* prevents the acidification of the environment, thereby protecting the *Streptococci* (Distler & Kröncke, 1980, 1981; Zhou, Liu, Li, Takahashi, & Qi, 2015). *V. parvula* was found to be unable to grow on its own in saliva but its addition to multi-species communities strongly enhanced the growth of each members, demonstrating its importance in the community (Kolenbrander, 2011; Periasamy & Kolenbrander, 2010). The association of *Veillonella* with *Streptococci* was found to reduce the genesis of caries in mouth models compared to the colonization of mice mouths by only *Streptococci* (Mikx, van der Hoeven, König, Plasschaert, & Guggenheim, 1972). Moreover, *Streptococci* co-cultivated with *V. parvula* showed increased resistance to antimicrobial agents (Luppens *et al.*, 2008), modified biofilm formation (Mashima & Nakazawa, 2014) and an altered carbohydrate metabolism (Mutha *et al.*, 2019).

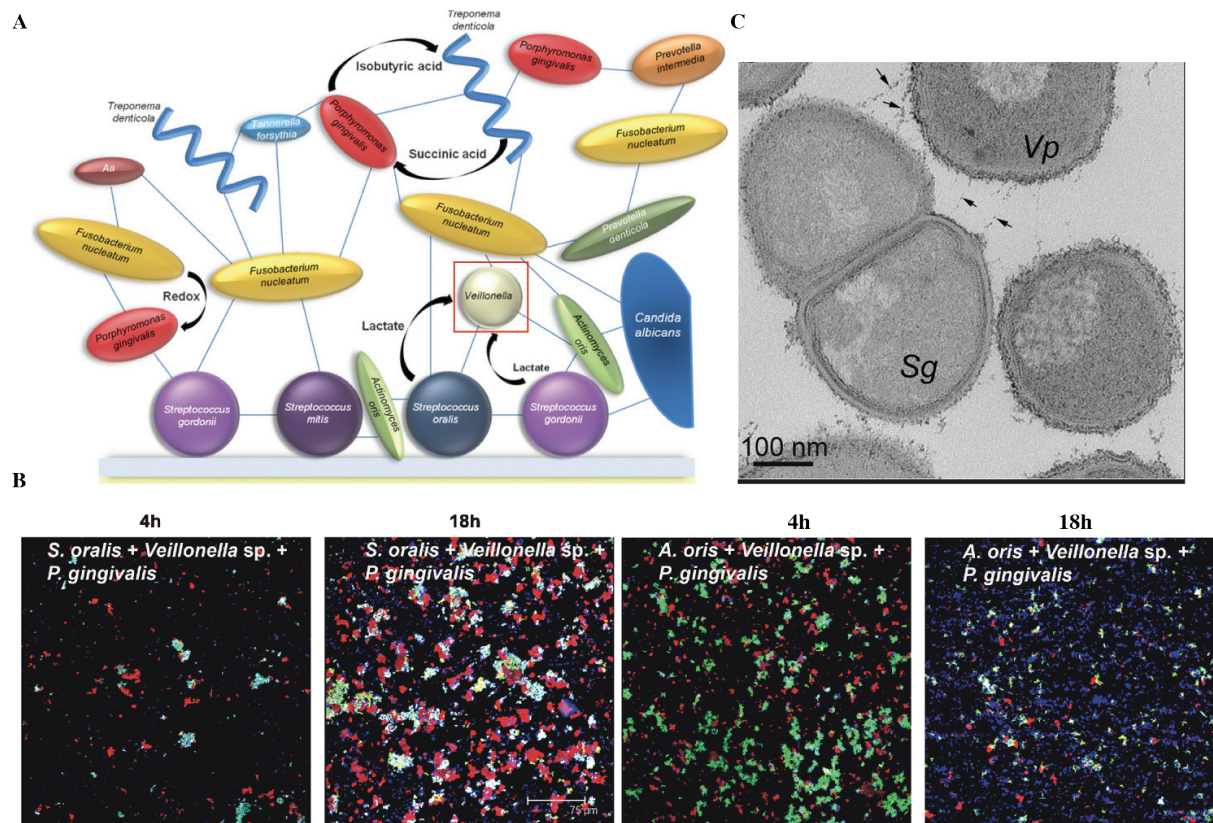


Figure 29: *V. parvula* is a bridging species within the dental plaque.

A. Bacterial cell-to-cell interactions within the dental plaque. *Veillonella* is framed by a red rectangle. Adapted from (Wright *et al.*, 2013). **B.** Confocal microscopy image of three-species biofilms grown in flow cells in saliva at 4 h and at 18 h showing the close association of *Veillonella sp.* (blue) with *S. oralis* (green) and *P. gingivalis* (red) (left panels) and of *Veillonella sp.* (blue) with *A. oris* (green) and *P. gingivalis* (red) (right panels). From (Periasamy & Kolenbrander, 2010). **C.** Transmission electron microscopy image of co-aggregates of *S. gordonii* (Sg) and *V. parvula* (Vp) in human saliva. Nearby cells appeared to be connected by extracellular material or fibrils (arrows). Adapted from (Mutha *et al.*, 2019)

b. *Veillonella* in the gut microbiota

V. parvula is also a member of the human gut. As an early colonizer, it is believed to participate in the immune maturation of the infant gut by producing LPS (Arrieta *et al.*, 2015). Moreover, a recent study identified an increased abundance of *Veillonella* in the gut of athletes after running a marathon. They isolated a strain of *V. atypica*, a close relative of *V. parvula*, that increased mice running performance when administrated to mice via gavage by consuming the excess lactic acid produced during a physical effort (Scheiman *et al.*, 2019). The abundance of *Veillonella* in the gut has also been shown to be impacted by the mode of feeding in infants *ie* breast-feeding or formula-feeding, although not all studies give consistent results (Guaraldi & Salvatori, 2012; J. Wang, Shoemaker, Wang, & Salyers, 2000b).

2. *V. parvula* is an opportunistic pathogen

Although *V. parvula* is an important member of several microbiota, participating in community assembly, it has also been associated to several disease states. Increased abundance of *Veillonella* has been correlated to the development of caries in children (Cherkasov *et al.*, 2019) and type I diabetes (Murri *et al.*, 2013). Decreased abundance of *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* in children was associated with a higher risk of developing asthma (Arrieta *et al.*, 2015). *V. parvula* can also behave as an opportunistic pathogen by itself, and has been shown to be associated with infections including osteomyelitis, endocarditis, spondylodiscitis, abscesses as well as systemic infections (Gouze *et al.*, 2019; Hirai *et al.*, 2016; Hyo, Fukushima, Harada, & Hara, 2019; Li *et al.*, 2017; Wellens, Casteels, & Huygens, 2019). Moreover, *V. parvula* is frequently isolated from the lung of cystic fibrosis patients, and it was shown to enhance the growth of *Pseudomonas aeruginosa* in the lung of mice, thereby worsening the clinical state of the infected mice (Pustelny *et al.*, 2015).

C. Adhesion and biofilm formation of *V. parvula*

1. *V. parvula* adhesion capacity is critical for its ability to colonize multiple microbiota

V. parvula is a member of several microbiota, environments in which the adhesion capacity is expected to be critical. In particular, it was shown that micro-organisms that could not adhere to host surfaces or other bacteria failed to colonize the oral microbiota and were washed away (Scannapieco, 1994). The contribution of adhesion to gut microbiota stability has already been discussed in chapter 2. *V. parvula* has a well-known ability to co-aggregate with multiple species including various *Streptococci*, *Actinomyces* and *Fusobacterium* (H. Bladen, Hageage, Pollock, & Harr, 1970; Delwiche *et al.*, 1985; Hughes *et al.*, 1988; Wright *et al.*, 2013) (Figure 29BC). Both lactose-dependent and lactose-independent aggregation to *Streptococci* were described (Hughes, Andersen, & Kolenbrander, 1992). This ability to co-aggregate with other bacteria is critical for *Veillonella* establishment in the oral microbiota. Thus, *V. parvula* was shown to be unable to attach to nichrome steel wires *in vitro* when grown in monoculture, but they attached to preformed plaques of diphtheroides (H. Bladen *et al.*, 1970). Similarly *in vivo*, *V. parvula* (former *V. alcalescens*) was shown to be unable to colonize mouse teeth on its own, but it colonized the dental plaque when co-cultured with a mixture of ten representative oral symbionts (Gibbons, Socransky, & Kapsimalis, 1964) or when co-cultured with strains of *Streptococcus mutans* with which it could co-aggregate *in vitro*. However, co-culture with non-aggregative *S. mutans* led to a very important decrease in the ability of *V. parvula* to colonize the teeth (McBride & Van der Hoeven, 1981). Additionally, *Veillonella* isolated from different buccal microbiota, such as the tongue or the dental plaque, were shown to predominantly co-aggregate with other bacteria- *Streptococci* and *Actinomyces*- from the same location but not to bacteria from other parts of the mouth colonization (Hughes, Kolenbrander, Andersen, & Moore, 1988). This showed that co-aggregation might have strong implication in niche colonization (Hughes, Kolenbrander, Andersen, & Moore, 1988).

The transcriptome of *V. parvula*-*S. gordonii* co-aggregates (Figure 29C) showed that presence of *S. gordonii* had a major impact on gene expression of *V. parvula* and a more moderate impact on *S. gordonii* gene expression. In particular, *V. parvula* strongly increased its oxidative stress response whereas *S. gordonii* modified its carbohydrate metabolism (Mutha *et al.*, 2019).

2. Adhesins of *V. parvula*: the autotransporters.

Type V family of secreted proteins (T5SS) are typical adhesins found in diderm bacteria (Meuskens *et al.*, 2019). The T5SS autotransporter family is represented by three distinct groups: (i) the monomeric autotransporters (ii) the trimeric autotransporters, and (iii) the two-partner secretion systems (Figure 30). All T5SS proteins share common structural and functional features: a Sec-dependent signal peptide, a passenger domain providing the protein function, and a β -barrel domain that allows secretion of the passenger domain (Cécile Berne *et al.*, 2015). Most proteins from this family are described as adhesins, although some monomeric autotransporters were shown to possess protease or lipase domains (Van Ulsen, Rahman, Jong, Daleke-Schermerhorn, & Luirink, 2014). For instance, self-associating autotransporters (SAATs) have been extensively studied in classical Proteobacteria, such as *E. coli*, and were shown to be critical for biofilm formation. These include monomeric autotransporters such as *E. coli* Ag43, AidA, TibA or EhaA and trimeric autotransporters such as *E. coli* EibD, EhaG, UpaG or, Saa (Ageorges *et al.*, 2019; Klemm, Vejborg, & Sherlock, 2006; Leo *et al.*, 2011; Paton, Srimanote, Woodrow, & Paton, 2001; Totsika *et al.*, 2012; Valle *et al.*, 2008; Wells *et al.*, 2008).

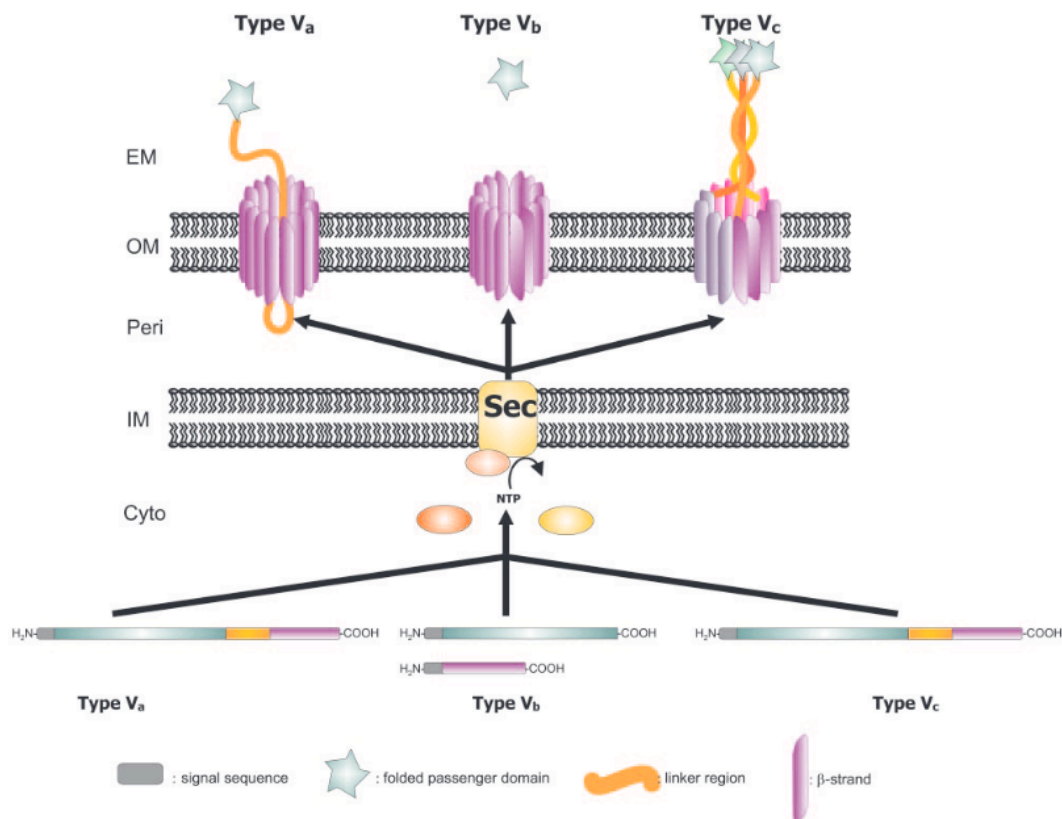


Figure 30: Type V secretion system.

Type Va: autotransporter system, type Vb: two-partner system, type Vc: trimeric autotransporter system. The four functional domains of the proteins are shown: the signal sequence, the passenger domain, the linker region, and the domain. The autotransporter polyproteins are synthesized and generally exported through the cytoplasmic membrane via the Sec machinery. Once through the inner membrane, the signal sequence is cleaved and the domain inserts into the outer membrane in a barrel structure that forms a pore in the outer membrane. After formation of the-barrel, the passenger domain inserts into the pore and is translocated to the bacterial cell surface, where it may or may not undergo further processing. (Henderson, Navarro-Garcia, Desvaux, Fernandez, & Ala'Aldeen, 2004).

A hemagglutinin of the YadA-like trimeric autotransporter family, Hag1, was shown to be responsible for co-aggregation between *V. atypica* and *Streptococci*, *P. gingivalis* and human buccal cells. Its binding partner in *S. gordonii* was identified as a sialic acid binding protein, Hsa, previously shown to be involved in *S. gordonii* colonization of the heat tissue (Zhou, Liu, Li, *et al.*, 2015). Hag1 is the only characterized adhesin of a *Veillonella* to date (Zhou, Liu, Merritt, & Qi, 2015). The outer membrane proteome of *V. parvula* DSM 2008 was found to contain ten different YadA-like trimeric autotransporters out of the 12 putative autotransporters encoded in the genome (Figure 31). 6 of these autotransporters were part of a genomic cluster of adhesins (Poppleton *et al.*, 2017). Moreover, two monomeric autotransporters and one filamentous hemagglutinin Fha-like proteins of the two-partner secretion system were also detected (Poppleton *et al.*, 2017) (Figure 31), showing *V. parvula* expresses multiple putative adhesins of the T5SS family. Their contribution to adhesion, co-aggregation and biofilm formation remains to be addressed. Interestingly, one of the monomeric autotransporter identified, *vpar_0330*, was shown to be up-regulated in a tumor colonization model in mice compared to *in vitro* growth and in caries compared to healthy teeth (Do, Sheehy, Mulli, Hughes, & Beighton, 2015; Pustelny *et al.*, 2015).

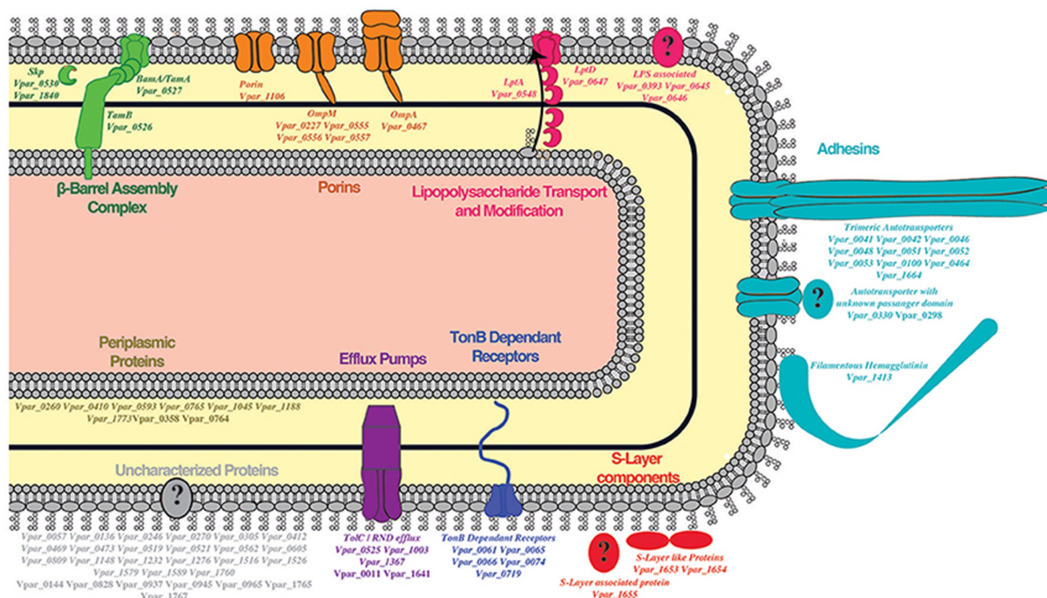


Figure 31: *V. parvula* DSM 2008 outer membrane proteome.

From (Poppleton *et al.*, 2017)

Even though *V. parvula* adhesion capacity is expected to be crucial for the development of the dental plaque and might be involved in colonization of the various niches it inhabits, so far, the molecular characterization of *V. parvula* adhesins has been very limited by the difficulty of making mutants within this species. The recent description of naturally competent isolates of *V. parvula* can be expected to resolve this issue and greatly facilitate mutagenesis in this species. Moreover, *V. parvula* belongs to the Firmicutes, a clade of textbook monoderms, but it possesses an outer membrane that is different from the well-studied Proteobacteria outer membrane. Thus, *V. parvula* might encode original adhesins compared to classical model bacteria. Therefore, the second part of this thesis work was centered around the use of a genetic screen to identify *V. parvula* genes involved in biofilm formation and adhesion (Result chapter 3).

**THESIS PRESENTATION
AND
OBJECTIVES**

THESIS PRESENTATION AND OBJECTIVES

Although many clinically and ecologically important processes take place in absence of oxygen, biofilms formed in anaerobic conditions have not been studied as extensively as aerobic biofilms. However, recent focus on human gut microbiota highlighted the predominance of anaerobes organized as multi-species biofilm-like communities, in which adhesion, aggregation and biofilm formation might be critical for assembly and maintenance of the community and expression of microbiota-related functions.

The aim of this thesis work was to characterize the molecular determinants of biofilm formation in two important anaerobes of the gastro-intestinal tract, *Bacteroides thetaiotaomicron* and *Veillonella parvula*. In the following chapters, I will present the results obtained during my PhD thesis corresponding to two first-author published articles and one in preparation.

- I first participated to the completion of a study characterizing a first class of biofilm-forming *B. thetaiotaomicron* mutants. Indeed, whereas the genetically amenable reference strain *B. thetaiotaomicron* VPI 5482 is a poor *in vitro* biofilm-former, a previous PhD student (Jovana Mihajlovic), used random transposon mutagenesis followed by positive selection and revealed several classes of *B. thetaiotaomicron* VPI 5482 mutants with increased ability to form biofilms.

This study showed that deletion of the last 9 amino acids of the FimA homolog *BT3147* allowed polymerization of *BT3147* and increased biofilm formation (Article presented in [Annex 1](#)).

Mihajlovic J, **Béchon N**, Ivanova C, Chain F, Almeida A, Langella P, **Beloin C**, and Ghigo JM. (2019). A putative type V pilus contributes to *Bacteroides thetaiotaomicron* biofilm formation capacity. *Journal of Bacteriology*. 2019 Mar 4. pii: JB.00650-18. doi: 10.1128/JB.00650-18.

- I then characterized a second class of mutants and showed that the expression of capsules 1, 2, 3, 4, 5 and 6 hinders *B. thetaiotaomicron* biofilm formation, whereas capsule 8 expression favors biofilm formation ([Result chapter 1](#)).

Béchon N*, Mihajlovic J*, Vendrell-Fernandez S, Chain F, Langella P, Beloin C, Ghigo JM (2020) Capsular polysaccharides cross-regulation modulates *Bacteroides thetaiotaomicron* biofilm formation. *mBio* May-June 2020 11(3): e00729-20. * equal contribution

Moreover, I show bile extract and taurocholic acid are widespread inducers of biofilm formation in *Bacteroides thetaiotaomicron* isolates (Result chapter 2).

Béchon N, Mihajlovic J, Vendrell-Fernandez S, , Martin-Verstrate I, Dupuy B, Ghigo JM. 2020. Bile is a widespread inducer of biofilm formation in *Bacteroides*. *In preparation*

Finally, I will describe new genetic tools to mutagenize *V. parvula* and show how they were used to identify the major adhesins of *V. parvula*, the secreted type V autotransporters (Result chapter 3).

Béchon N, Jiménez-Fernández A, Witwinowski J, Bierque E, Taib N, Cokelaere T, Ma L, Ghigo JM, Gribaldo S, Beloin C. 2020. Autotransporters drive biofilm formation and auto-aggregation in the diderm Firmicute *Veillonella parvula*. *Journal of Bacteriology*, in press.

These results opened several new axes of research, some of which are already in progress in the laboratory. They will be discussed in the perspective section of the thesis manuscript.

RESULTS

RESULTS

CHAPTER 1: *B. THETAIOAOMICRON* CAPSULES ARE IMPORTANT BIOFILM FORMATION DETERMINANTS.

Random transposon mutagenesis followed by positive selection of the poor biofilm forming strain *B. thetaiotaomicron* VPI 5482 led to the identification of several classes of mutants displaying increased biofilm formation (see Annex 1). One of this class of mutants corresponded to insertions in the capsule biosynthesis locus 4 (CPS4), one of the 8 capsule biosynthesis locus of *B. thetaiotaomicron*.

Deletion of CPS4, the most expressed capsule in our *in vitro* conditions, led to the apparition of a subpopulation of acapsulated bacteria with strong cell-to-cell interactions, increasing biofilm formation. Capsules 1, 2, 3, 5 and 6 all masked biofilm formation when expressed similar to CPS4, whereas capsule 8 expression induced biofilm formation.


Moreover, screening of a random transposon mutant library of Δ CPS4 strain led to the identification of two genes impacting CPS production and biofilm formation. Indeed, I show that the UpxZ homolog of CPS4 operon is a transcriptional inhibitor of CPS2 operon and that capsules production increases when the protein glycosylation pathway is hindered, hinting that these two processes might be competing with each other.

In conclusion, I showed that in addition to its critical implication in mediating interactions with the immune system, gut colonization and bacteriophage resistance, the complex regulation of *B. thetaiotaomicron* capsular polysaccharides is a major determinant of biofilm formation.

Béchon N*, Mihajlovic J*, Vendrell-Fernandez S, Chain F, Langella P, Beloin C, Ghigo JM (2020) Capsular polysaccharides cross-regulation modulates *Bacteroides thetaiotaomicron* biofilm formation. *mBio* May-June 2020 11(3): e00729-20. * equal contribution



Capsular Polysaccharide Cross-Regulation Modulates *Bacteroides thetaiotaomicron* Biofilm Formation

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Nathalie Béchon and Jovana Mihajlovic contributed equally this work. Author order was determined alphabetically.

ABSTRACT *Bacteroides thetaiotaomicron* is one of the most abundant gut symbiont species, whose contribution to host health through its ability to degrade dietary polysaccharides and mature the immune system is under intense scrutiny. In contrast, adhesion and biofilm formation, which are potentially involved in gut colonization and microbiota structure and stability, have hardly been investigated in this intestinal bacterium. To uncover *B. thetaiotaomicron* biofilm-related functions, we performed a transposon mutagenesis in the poorly biofilm-forming reference strain VPI-5482 and showed that capsule 4, one of the eight *B. thetaiotaomicron* capsules, hinders biofilm formation. We then showed that the production of capsules 1, 2, 3, 5, and 6 also inhibits biofilm formation and that decreased capsulation of the population correlated with increased biofilm formation, suggesting that capsules could be masking adhesive surface structures. In contrast, we showed that capsule 8 displayed intrinsic adhesive properties. Finally, we demonstrated that *BT2934*, the *wzx* homolog of the *B. thetaiotaomicron* glycosylation locus, competes with capsule production and impacts its adhesion capacity. This study therefore establishes *B. thetaiotaomicron* capsule regulation as a major determinant of *B. thetaiotaomicron* biofilm formation, providing new insights into how modulation of different *B. thetaiotaomicron* surface structures affects *in vitro* biofilm formation.

IMPORTANCE The human gut harbors a complex bacterial community that plays important roles in host health and disease, including nutrient acquisition, maturation of the immune system, and resistance to infections. The capacity to adhere to surfaces and form communities called biofilms is believed to be important for niche colonization and maintenance of gut bacteria. However, little is known about the adhesion capacity of most gut bacteria. In this study, we investigated biofilm formation in *Bacteroides thetaiotaomicron*, one of the most abundant bacteria of the normal mammalian intestine. We identified that *B. thetaiotaomicron* capsules, a group of eight surface-exposed polysaccharidic layers mediating important interactions with the gut environment, are also major determinants of biofilm formation that mask or unmask adhesion factors. Studying how *B. thetaiotaomicron* regulates its adhesion properties will allow us to better understand the physiology and specific properties of this important gut symbiont within anaerobic biofilms.

KEYWORDS capsule, *Bacteroides thetaiotaomicron*, biofilm

B*acteroides thetaiotaomicron* is an abundant bacterial symbiont of the normal mammalian intestine that contributes to shaping the nutrient environment of the gut microbiome through degradation of complex polysaccharides and production of short-

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chain fatty acids (1–5). *B. thetaiotaomicron* was also shown to stimulate the development of gut immunity (6), attenuate intestinal inflammation (7), and strengthen the intestinal protective barrier (8, 9). Consistently, a decrease in the abundance of *B. thetaiotaomicron* and other *Bacteroides* species has been correlated with gut inflammation and disease emergence, underlining the importance of the gut microbiota for host intestinal physiology and health (10). In contrast, microbial functions involved in the establishment and maintenance of a healthy gut microbiota are still not well understood. It is speculated that the ability of symbiont bacteria to form biofilms could contribute to microbiota stability (11, 12). However, although bacterial biofilm formation has been studied in various facultative symbiotic and pathogenic anaerobes, information on this widespread lifestyle is still scarce in *B. thetaiotaomicron* (13–15). Whereas comparative gene expression profiling between biofilm and planktonically grown *B. thetaiotaomicron* showed biofilm-associated upregulation of polysaccharide utilization systems and capsule 8, one of the eight *B. thetaiotaomicron* capsule synthesis loci (15, 16), there is still no direct proof of the contribution of these surface structures to adhesion and biofilm formation. We recently showed that although biofilm capacity is widespread among *B. thetaiotaomicron* isolates, the widely used reference strain VPI-5482 is a poor biofilm former. Nevertheless, use of transposon mutagenesis followed by a positive selection procedure revealed mutants with significantly improved biofilm capacity due to alteration of the structure of a putative type V pilus (13). Here, we identified two mechanisms of capsule regulation in *B. thetaiotaomicron*, involving UpxZ-mediated capsule transcription inhibition and competition between protein glycosylation and capsule production. We showed that capsule masking or unmasking of adhesive structures is a major determinant of *B. thetaiotaomicron* biofilm formation. This study therefore provides new insights into the roles of capsular polysaccharides in *B. thetaiotaomicron* and their impact on the physiology and biofilm formation of a prominent gut symbiont.

RESULTS

Transposon insertion in the capsule 4 biosynthesis operon promotes *B. thetaiotaomicron* biofilm formation. Among the previously identified transposon mutants displaying increased *in vitro* biofilm formation capacity compared to the wild-type *B. thetaiotaomicron* VPI-5482 (WT) (13), five of them corresponded to insertions within capsule 4 (*CPS4*) synthesis operon *BT1358-1338*, encoding one of the eight capsular polysaccharides of *B. thetaiotaomicron* (Fig. 1A and B; see Table S1 in the supplemental material) (16, 17). To confirm the increased biofilm phenotype of the transposon mutants, we deleted all 19 *CPS4* structural genes located downstream of the regulators *BT1358-1357*. Crystal violet staining of *in vitro* biofilm formed in 96-well microtiter plates showed that the resulting $\Delta BT1356-1338$ mutant (here named $\Delta CPS4$) displayed a significant increase in biofilm formation compared to the wild-type *B. thetaiotaomicron* VPI-5482 (Fig. 1C).

***B. thetaiotaomicron* biofilm formation is modulated by capsule cross-regulation.** To uncover the mechanism of increased biofilm formation in a $\Delta CPS4$ strain, we performed a random transposon mutagenesis in $\Delta CPS4$ and identified 6 mutants out of 4,650 with reduced biofilm formation capacity compared to the parental $\Delta CPS4$ (Fig. 2A). Five of these mutants corresponded to transposons inserted in the *BT1358-1357* region just upstream of the *CPS4* operon (Fig. 1A and Fig. 2B; Table S1). *BT1358* codes for an UpxY-like homolog and *BT1357* codes for a UpxZ-like homolog, two regulatory genes located at the beginning of most capsule synthesis operons in *B. thetaiotaomicron* and *Bacteroides fragilis* (18, 19). UpxY-like proteins positively regulate their cognate capsular operon by preventing premature transcription termination in the untranslated region, thus facilitating the otherwise abortive transcription of the downstream capsular genes (18). In contrast, UpxZ-like proteins are repressors of transcription of nonadjacent capsular systems (19). We first showed that deletion of *upxY^{BT1358}* in *B. thetaiotaomicron* $\Delta CPS4$ did not impact biofilm formation, which is consistent with its role as a positive regulator of the expression of capsule 4 genes, all

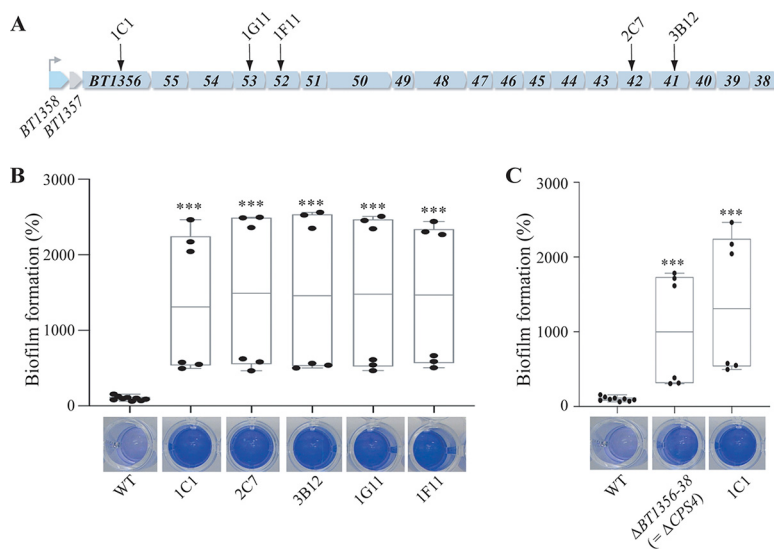


FIG 1 Capsule 4 inhibits biofilm formation in *B. thetaiotaomicron* VPI-5482. (A) Organization of *B. thetaiotaomicron* capsular operon 4 (*CPS4*). The first two genes (*BT1358* and *BT1357*) code regulators of capsular biosynthesis. *BT1356-1338* code the enzymes involved in *Cps4* capsular polysaccharide biosynthesis. The arrows indicate 5 individual transposon insertions within the *CPS4* operon. (B) The 96-well plate biofilm assay after 48 h of growth in BHIS. The Mean of the WT is adjusted to 100%. In the min-max boxplot of 6 biological replicates for each strain, each replicate is the mean of two technical replicates. ***, $P < 0.0005$; Mann-Whitney test, comparing the indicated mutant to the WT. (C) The 96-well plate biofilm assay after 48 h of growth in BHIS. The mean of the WT is adjusted to 100%. In the min-max boxplot of 6 to 9 biological replicates for each strain, each replicate is the mean of two technical replicates. ***, $P < 0.0005$; Mann-Whitney test, comparing the indicated mutant to the WT. The images shown under each boxplot correspond to representative CV-stained microtiter wells after resuspension of the biofilm.

missing in the $\Delta CPS4$ mutant (Fig. 2B and C). We then hypothesized that transposon insertion in $upxY^{BT1358}$ (located upstream of $upxZ^{BT1357}$) could have a polar effect on the expression of the repressor $upxZ^{BT1357}$, leading to the derepression of one or more of the 7 other *B. thetaiotaomicron* capsular polysaccharides. Indeed, in-frame deletion of $upxZ^{BT1357}$ or $upxY^{BT1358}$ - $upxZ^{BT1357}$ in a $\Delta CPS4$ background did not affect growth but led to loss of biofilm capacity (Fig. 2C and Fig. S1A). This phenotype could be

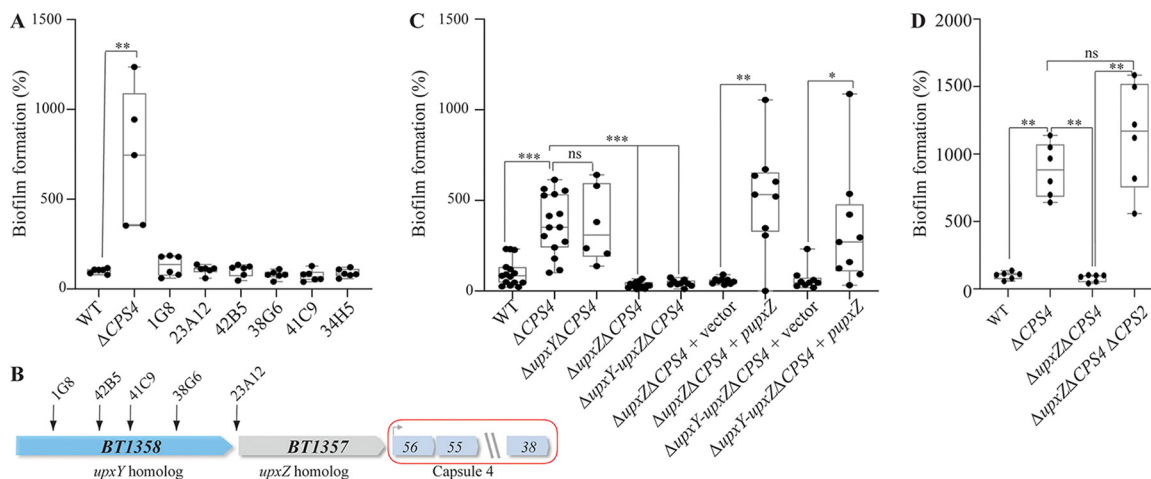


FIG 2 Capsule cross-regulation modulates biofilm formation in *B. thetaiotaomicron*. (A) The 96-well plate crystal violet biofilm assay after 48 h of growth in BHIS. (B) Organization of *B. thetaiotaomicron* capsular operon 4 (*CPS4*) with identified transposon insertion points in the first two genes of the operon (*BT1358* and *BT1357*), coding regulators of capsular biosynthesis. (C) The 96-well plate crystal violet biofilm assay after 48 h of growth in BHIS. The Mean of the WT is adjusted to 100%. (D) The 96-well plate crystal violet biofilm assay after 48 h of growth in BHIS. In panels A, C, and D, the mean of the WT is adjusted to 100%. In the min-max boxplot of 6 to 9 biological replicates for each strain, each replicate is the mean of two technical replicates. **, $P < 0.005$; Mann-Whitney test. *upxY*, *upxY^{BT1358}*; *upxZ*, *upxZ^{BT1357}*.

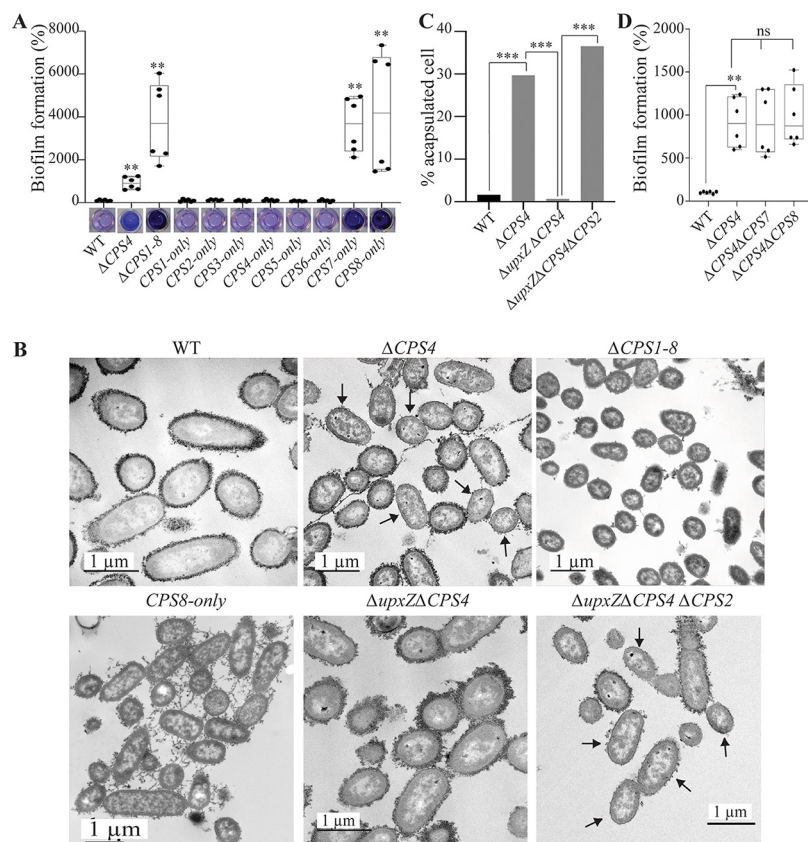


FIG 3 Capsule expression in *B. thetaioaomicron* is heterogenous and has consequences for biofilm formation. (A and D) The 96-well plate biofilm assay after 48 h of growth in BHIS. The mean of the WT is adjusted to 100%. In the min-max boxplot of 6 biological replicates for each strain, each replicate is the mean of two technical replicates. **, $P < 0.005$; Mann-Whitney test, comparing the indicated mutant to the WT. The pictures shown under the boxplot in panel A correspond to representative CV-stained microtiter wells after resuspension of the biofilm. (B) Transmission electron microscopy (TEM) images of overnight cultures fixed with ferritin. Arrows indicate some examples of acapsulated cells. (C) Percentage of acapsulated cells of indicated strains counted on TEM pictures. For each strain, at least 100 cells were counted. ***, $P < 0.0005$; prop.test (R). *upxY*, *upxY^{BT1358}*; *upxZ*, *upxZ^{BT1357}*.

complemented in *trans* by introducing *upxZ^{BT1357}* expressed from a constitutive promoter in the 5' untranslated region of the tRNA-Ser chromosomal locus, in either a Δ *upxZ^{BT1357}* Δ CPS4 or Δ *upxY^{BT1358}*-*upxZ^{BT1357}* Δ CPS4 *B. thetaioaomicron* background (Fig. 2C). To identify which capsules were repressed by *upxZ^{BT1357}*, we used reverse transcription-quantitative PCR (qRT-PCR) to monitor the expression of each capsular operon, and we observed an increased transcription of capsule 2 (CPS2) in *B. thetaioaomicron* Δ *upxZ^{BT1357}* Δ CPS4 compared to the *B. thetaioaomicron* Δ CPS4 single mutant (Fig. S2). Consistently, deletion of the CPS2 operon in the *B. thetaioaomicron* Δ *upxZ^{BT1357}* Δ CPS4 background restored biofilm formation capacity (Fig. 2D) Thus, expression of either CPS4, or CPS2 in the absence of CPS4, hinders biofilm formation.

Expression of capsule 8 and lack of any capsules both induce biofilm formation. To assess the contribution of all capsules, besides inhibition by CPS4 or CPS2, to *B. thetaioaomicron* biofilm formation, we used a recently described set of strains expressing only one of the eight *B. thetaioaomicron* capsular types (20). We observed that derivative strains expressing only capsule 1, 2, 3, 4, 5, or 6 formed as little biofilm as wild-type (WT) *B. thetaioaomicron* VPI-5482. Interestingly, strains only expressing CPS7 or CPS8 formed over 35 times more biofilm than the WT strain (Fig. 3A). However, all CPS7-only bacteria seemed to be acapsulated, which is consistent with previous observations suggesting that capsule 7 may not be expressed in tested laboratory conditions (Fig. S3A and B) (20). Indeed, similar to a CPS7-only strain, a strain deleted

for all 8 capsule operons ($\Delta CPS1-8$) formed 40 times more biofilm than the WT (Fig. 3A) and showed a strong aggregation phenotype in overnight cultures (Fig. S3B). In contrast, India ink staining confirmed the presence of a capsule in biofilm-forming (but not aggregating) CPS8-only bacteria, suggesting that capsule 8 could have intrinsic adhesive properties (Fig. S3A and B). These results showed that, except for CPS8 and potentially CPS7, the expression of all capsules hinders *B. thetaiotaomicron* biofilm formation and that acapsulated cells display cell-to-cell aggregation capacity likely driving the observed increased biofilm phenotype.

Deletion of capsule 4 leads to a heterogeneously capsulated bacterial population. To determine whether lack of capsule or expression of the biofilm-promoting capsule 8 was responsible for the observed increased biofilm formation in the $\Delta CPS4$ strain, we used transmission electron microscopy (TEM) and showed that whereas WT *B. thetaiotaomicron* bacteria were almost all capsulated (>98%), ca. 30% of $\Delta CPS4$ cells lacked a visible capsule (Fig. 3B and C). Considering that $\Delta CPS1-8$ formed 4 times more biofilm than $\Delta CPS4$, this suggested a correlation between the increased frequency of noncapsulated cells in the population and the increased ability to form biofilms (Fig. 3A and C). To determine whether capsulated cells in the $\Delta CPS4$ population contributed to adhesion, we deleted, in the $\Delta CPS4$ background, either *CPS8*, the only biofilm-promoting capsule of *B. thetaiotaomicron*, or *CPS7*, for which we could not ascertain the biofilm formation potential using a single CPS-expressing strain. Both the $\Delta CPS4 \Delta CPS7$ and $\Delta CPS4 \Delta CPS8$ mutants had similar biofilm capacity compared to a $\Delta CPS4$ mutant, showing that neither capsule 7 nor 8 contribute to biofilm formation in the absence of capsule 4 (Fig. 3D). Moreover, TEM imaging showed that the non-biofilm-forming $\Delta upxZ^{BT1357} \Delta CPS4$ double mutant was entirely capsulated (due to induction of *CPS2*; Fig. S2), supporting a correlation between increased biofilm formation (Fig. 3A) and the presence of a subpopulation of acapsulated cells in the $\Delta CPS4$ strain (Fig. 3B and C). Consistently, deletion of *CPS2* in the $\Delta upxZ^{BT1357} \Delta CPS4$ background led to the emergence of 37% of acapsulated bacteria in a $\Delta upxZ^{BT1357} \Delta CPS4 \Delta CPS2$ population (Fig. 3B and C) and restored biofilm formation (Fig. 2D).

Identification of BT2934 as a new *B. thetaiotaomicron* inhibitor of capsule expression. In addition to mutation in $\Delta upxZ^{BT1357}$ capsule repressor, we also identified a biofilm-deficient $\Delta CPS4$ transposon mutant (34H5) with an insertion in *BT2934* (Fig. 3A and 4A). The *BT2934-2947* region corresponds to a *B. thetaiotaomicron* protein glycosylation locus (21, 22), in which *BT2934* encodes a homolog of the transmembrane oligosaccharide flippase Wzx (Fig. 4A). We deleted *BT2934* and the 4 putative glycosyl transferase genes, *BT2935-2938*, located in the same operon and confirmed the role of *BT2934-2938* in protein glycosylation, as several bands disappeared from a protein glycosylation profile in the $\Delta CPS4 \Delta BT2934-2938$ and 34H5 mutants compared to $\Delta CPS4$ (Fig. S4). The double mutant $\Delta CPS4 \Delta BT2934-2938$ had no growth defect and displayed a 2-fold decrease in biofilm formation compared to $\Delta CPS4$ (Fig. 4B; Fig. S1B). However, it still formed more biofilm than the original 34H5 transposon mutant in *BT2934*. To determine the origin of this discrepancy, we only deleted *BT2935-2938* glycosyl transferases genes and did not observe reduced biofilm capacity compared to the $\Delta CPS4$ strain. Although we did not succeed in deleting *BT2934* alone, introduction of *pBT2934*, constitutively expressing *BT2934*, in the 34H5 transposon mutant and $\Delta CPS4 \Delta BT2934-38$ mutant restored biofilm formation but still showed an altered protein glycosylation profile (Fig. 4C; Fig. S4). These results suggested that the impact of *BT2934* on biofilm formation did not involve *BT2935-2938* and might not directly involve protein glycosylation. Finally, we showed that while $\Delta CPS4$ and $\Delta CPS4 \Delta BT2935-2938$ bacteria displayed similar level of acapsulated cells (30% and 28%, respectively), $\Delta CPS4 \Delta BT2934-2938$ cells showed full, wild-type levels of capsulation (Fig. 4D and E), reduced back down to over 50% of capsulated cells upon complementation by *pBT2934* (Fig. 4D and E). To identify whether *BT2934* directly inhibited capsule production, we overexpressed *BT2934* in each strain only expressing a single CPS type. We hypothesized that overexpression of *BT2934* in each of these strains could inhibit capsule expression and lead to acapsulation of the whole population, thus leading to aggregation in overnight cultures.

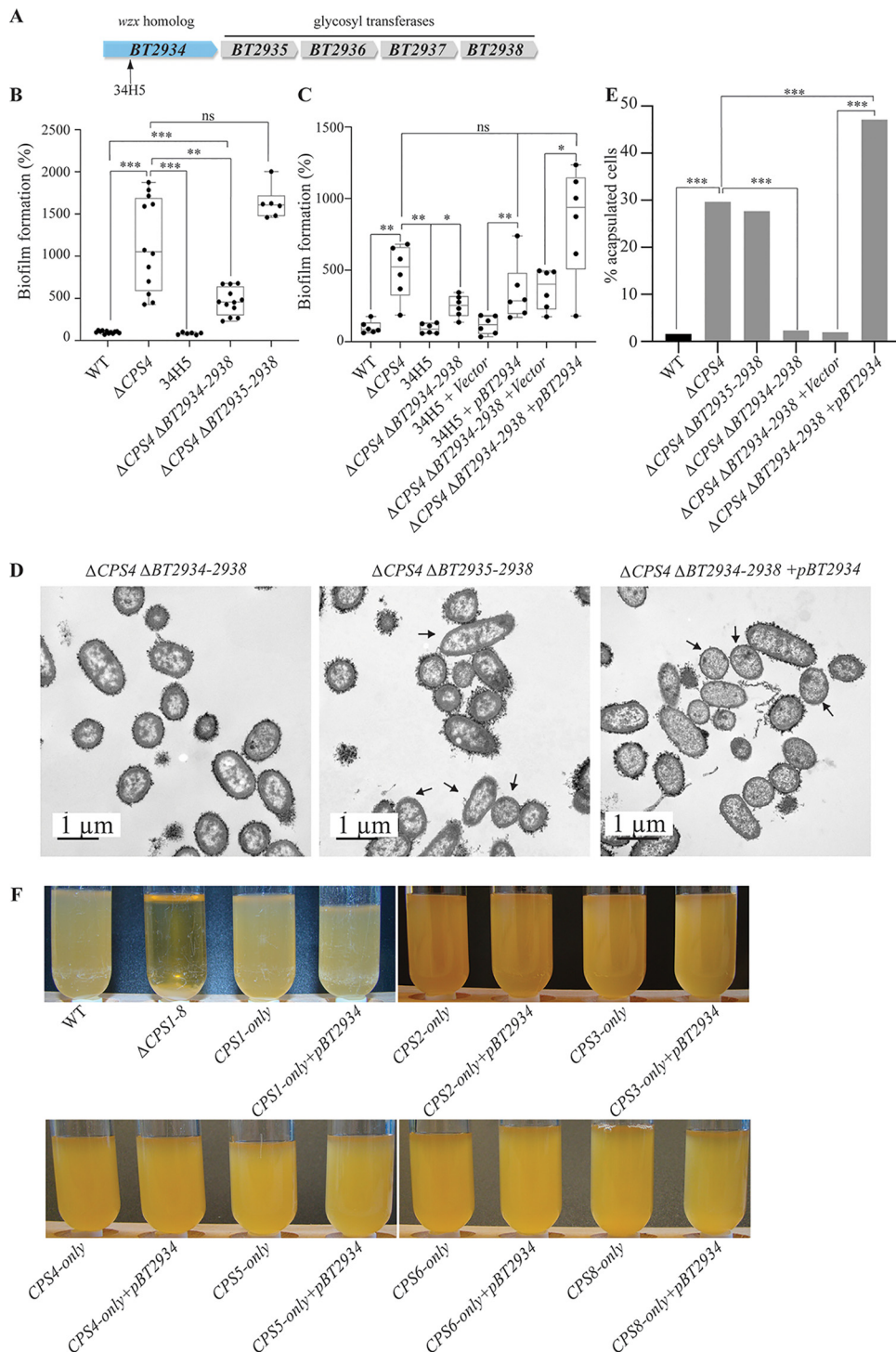


FIG 4 *BT2934* is a novel capsule inhibitor. (A) Organization of the *B. thetaiotaomicron* protein glycosylation *BT2934* locus with identified transposon insertion point. (B and C) The 96-well plate crystal violet biofilm assay after 48 h of growth in BHIS. The mean of the WT is adjusted to 100%. In the min-max boxplot of 6 to 12 biological replicates for each strain, each replicate is the mean of two technical replicates. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; Mann-Whitney test. (D) TEM images of Δ CPS4 Δ BT2934-2938, Δ CPS4 Δ BT2935-2938, and Δ CPS4 Δ BT2934-2938 + pBT2934 overnight cultures fixed with ferritin. The arrows indicate some acapsulated cells as examples. (E) Percentage of acapsulated cells in overnight cultures counted on TEM pictures. For each strain, at least 100 cells were counted. ***, $P < 0.0005$; prop.test (R). (F) Overnight cultures of indicated strains in BHIS. Only Δ CPS1-8 showed aggregation.

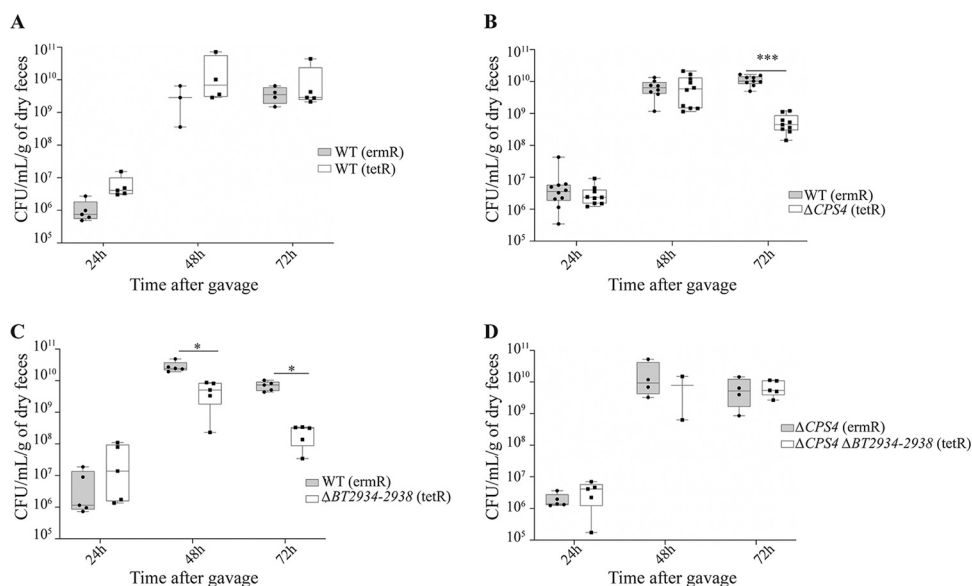


FIG 5 *BT2934* and *CPS4* contribute to *in vivo* colonization in axenic mice. Min-max boxplot of CFU/ml/dry weight of feces, numbered from feces from 5 to 10 axenic mice after cocolonization with indicated strains. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; Mann-Whitney test. (A) WT (ermR) versus WT (tetR). (B) WT (ermR) versus Δ CPS4 (tetR). (C) (ermR) versus Δ BT2934-2938 (tetR). (D) Δ CPS4 (ermR) versus Δ CPS4 Δ BT2934-2938 (tetR).

However, none of the resulting strains aggregated, suggesting that no capsules were directly inhibited by an overexpression of *BT2934*. Taken together, these results suggest that *BT2934* indirectly impacts capsule production in *B. thetaiotaomicron*, with consequences affecting its ability to form biofilm.

Biofilm-forming *CPS4* and *BT2934* mutants are outcompeted by the wild-type strain *in vivo*. *CPS4* and *BT2934* have previously been shown to be important for *in vivo* colonization in the presence of a complex mix of *B. thetaiotaomicron* transposon mutants (23). To test whether unmasking *B. thetaiotaomicron* biofilm formation capacity could contribute to *in vivo* colonization, we used intragastric gavage to inoculate axenic mice with erythromycin-resistant WT-*erm*- and tetracycline-resistant Δ CPS4-*tet* or Δ BT2934-38-*tet* in a 1:1 mix ratio and measured the abundance of each strain in feces for 8 days using erythromycin and tetracycline resistance to discriminate between the strains. We verified that the *erm* and *tet* resistance markers did not impact *in vivo* colonization of erythromycin-resistant WT (WT-*erm*) and tetracycline-resistant WT (WT-*tet*) (Fig. 5A). We then showed that both the Δ CPS4 and Δ BT2934-38 mutants were outcompeted by the WT strain in two-strain cocolonization experiments (Fig. 5B and C), even though both Δ CPS4 and Δ BT2934-38 formed more biofilm than the WT (Fig. 1C; see also Fig. S5 in the supplemental material). When we tested colonization of the double mutant Δ CPS4 Δ BT2934-2938 against the Δ CPS4 mutant, we found that they colonized mice similarly (Fig. 5D), indicating that *BT2934* is necessary for colonization only in the WT but not in the Δ CPS4 background. Taken together, these results showed that increased *in vitro* biofilm formation capacity is not predictive of *in vivo* colonization capacity.

DISCUSSION

In contrast to oral *Bacteroidales*, intestinal *Bacteroidales* species possess numerous capsular polysaccharide loci that play important beneficial roles during gut colonization, ranging from protecting bacteria from stresses to mediating interactions with the host immune system (17, 20, 24–26). In this study, we showed that deletion of one of *B. thetaiotaomicron*'s 8 capsular polysaccharides, CPS4, promotes biofilm formation *in vitro*, indicating that capsules mediate yet another important aspect of bacterial physiology.

Bacterial capsular polysaccharides are known to negatively affect biofilm formation by masking surface structures involved in adhesion in many bacteria (27–31). It was shown, for instance, that *Escherichia coli* capsular polysaccharides inhibit adhesion and auto-aggregation by masking the short autotransporter adhesin antigen 43 as well as the type III secretion system required for attachment in enteropathogenic *E. coli* (EPEC) (32, 33). The fact that CPS4 is the most expressed capsule in the tested laboratory conditions and *in vivo* (20) probably explains why it was the only capsule of *B. thetaiotaomicron* we identified by random transposon mutagenesis screening for increased biofilm formation.

In an adhering Δ CPS4 strain, 30% of the bacteria are acapsulated, indicating that occurrence of only a subpopulation of acapsulated cells is enough to induce biofilm formation. In *Bacteroides fragilis*, acapsular cells were previously shown to aggregate (17, 34), and we also observed that a completely acapsular strain of *B. thetaiotaomicron* lacking all 8 capsules (Δ CPS1-8) displays a strong aggregation phenotype. Aggregation and cell-to-cell adhesion is an essential part of the biofilm formation maturation process, and free-floating aggregates and air-liquid interface pellicles formed in the absence of any solid surface are considered bona fide biofilms (35). Although the capacity of acapsular bacteria to bind surfaces was not addressed in this work, we hypothesized that, in the absence of a capsule, cell-to-cell interactions are strong drivers of *B. thetaiotaomicron* biofilm formation. However, due to the protective roles of *Bacteroides* capsules, acapsular strains are rapidly outcompeted by WT strain in axenic mouse colonization (17, 34, 36). Monitoring the expression of the 8 *B. thetaiotaomicron* capsules by qRT-PCR does not allow detection of acapsular bacteria, as there is no marker of acapsular cells. However, colonization of axenic mice with a mix composed of an acapsular mutant and 8 strains each expressing a single capsule showed that a small amount of acapsular cells was found to persist in the lumen of the small intestine of two out of five mice, potentially due to a decreased immune system pressure allowing the acapsular cells to survive (20). Whether the presence of an acapsular population of bacteria naturally arises and persists in the mammalian gut remains to be demonstrated, and it is still unclear whether increased adhesion capacity of acapsular bacteria is a relevant mechanism by which *B. thetaiotaomicron* can modify its biofilm formation capacity and biofilm-associated resistance to stress.

Bacteroides sp. capsular loci are regulated by a complex transcriptional network involving stochastic inversion of some capsule promoters (17, 37), transcriptional cross-regulation between capsular regulators UpxY and UpxZ (18, 19), and cross talk between polysaccharide utilization loci and capsules through common sigma factors (37). It is also impacted by a range of environmental parameters such as diet, community composition, and host physiology (20, 38, 39). In particular, expression of capsule 4 in mice has been shown to be increased *in vivo* compared to *in vitro* in a high-fiber diet, but it is decreased in the suckling period compared to the weaned period (38, 39), and it is strongly impacted by the immune system (20). Moreover, a transcriptional analysis comparing planktonic cells with biofilms grown on chemostats for 8 days previously showed that CPS4 is downregulated in *B. thetaiotaomicron* biofilms (15).

Random transposon mutagenesis in the Δ CPS4 strain identified capsule regulation as the main parameter governing biofilm formation in our conditions. We show that *BT1357*, encoding the UpxZ homolog of *CPS4*, represses transcription of *CPS2*. UpxZ proteins repress the transcription of nonadjacent capsular operon by interacting with the antiterminator UpxY proteins, necessary for the full transcription of their cognate capsules (18, 19). *BT1357* therefore most likely only interferes with *BT0462*, the UpxY homolog of *CPS2*. Whereas the complex interplay between UpxY and UpxZ homologs of *B. fragilis* was very well described, it is, to our knowledge, the first description of the precise inhibition pattern of a *B. thetaiotaomicron* UpxZ homolog (18, 19).

In addition to *BT1357*, we have identified that deletion of *BT2934* impacted capsule production. *BT2934-2947* is the protein O-glycosylation locus of *B. thetaiotaomicron* (21, 22). This locus is composed of a *wzx* oligosaccharide flippase (*BT2934*) and glycosyl transferases, and its homolog in *B. fragilis*, the *BF4298-4306* locus, was shown to be

required for both *in vivo* and *in vitro* fitness in *B. fragilis* (21, 22). Accordingly, *BT2934* was previously shown to be important in both *in vitro* and *in vivo* competition experiments between complex communities of *B. thetaiotaomicron* transposon mutants (23) and was recently described as a putative essential gene (40). Our results confirm both the role of the $\Delta BT2934$ -2938 locus in protein glycosylation and the decreased colonization capacity of a $\Delta BT2934$ -2938 mutant in axenic mice in competition with the WT strain. However, deletion of *BT2934*-2938 in the $\Delta CPS4$ background had no effect on the colonization capacity of this strain. Although we never succeeded in deleting *BT2934* alone, deletion of *BT2934*-2938 in the WT and the $\Delta CPS4$ background did not lead to any growth defect *in vitro*, suggesting that deleting *BT2935*-2938 might somehow alleviate the fitness cost associated with the loss of *BT2934*.

We showed that deletion of *BT2934* impacted capsule production independently of protein glycosylation, as complementation by *BT2934* is sufficient to restore the $\Delta CPS4$ biofilm formation phenotype but not the lack of protein glycosylation. The mechanism by which *BT2934* impacts capsule production remains to be elucidated. Because overexpression of *BT2934* in each strain only expressing a single CPS type did not lead to general acapsulation, we hypothesize that *BT2934* does not directly inhibit capsule production. *BT2934* catalyzes the flipping of an oligosaccharide bound to an undecaprenyl-phosphate molecule across the membrane. As oligosaccharide flipping is also required for lipopolysaccharide and capsular synthesis, we speculate that these three processes might compete for undecaprenyl-phosphate or sugar moiety availability. Thus, limiting protein glycosylation by removing *BT2934* could favor the production of some capsules.

While our random transposon mutagenesis in $\Delta CPS4$ was not saturating, it is surprising that all identified biofilm-deficient mutants corresponded to insertions affecting capsule production rather than a putative adhesion factor unmasked in acapsulated bacteria. This could be indicative of the role played by purely electrostatic interactions between acapsulated bacteria or mediated by multiple and potentially redundant adhesive surface structures.

We show that expression of all capsular polysaccharide of *B. thetaiotaomicron* hindered biofilm formation, except for CPS8 that, rather, promoted biofilm formation. Consistently, CPS8 expression was shown to be upregulated in 8-day chemostat-grown biofilms (15), while capsules 1, 3, 4, and 6 were downregulated. CPS8 might be either an adhesive capsule or a loose capsule that does not mask adhesion factors. However, if CPS8 did not mask adhesion factors, we would expect the CPS8-only strain to adhere like $\Delta CPS1$ -8, but CPS8-only formed less biofilm than $\Delta CPS1$ -8, and it did not aggregate overnight. This suggests that capsule 8 could be a capsule providing adhesion capacity on its own. Interestingly, CPS8 is the only capsular locus of *B. thetaiotaomicron* containing homologs of FimA, the major component of type V pilus (41). Type V pili are widely found in *Bacteroidetes*, and they were shown to mediate adhesion in *Porphyromonas gingivalis* (42, 43). Moreover, we previously showed that another homolog of FimA, BT3147, mediated biofilm formation in *B. thetaiotaomicron* upon truncation of the last 9 amino acids (13). CPS8 is expressed to low levels in axenic mice mono-colonized with *B. thetaiotaomicron*, and to slightly higher levels in mice colonized with complex communities, suggesting it might confer an advantage to *B. thetaiotaomicron* when competing with other bacteria for colonization. Whereas a strain expressing only CPS8 is rapidly outcompeted by the WT in the *in vivo* competition experiment, some population of CPS8-only bacteria can be found in the lumen of the small intestine in some mice, reminiscent of the acapsular strain localization (20).

Although several studies described biofilm-like structures in the intestine (44, 45), the relevance of a biofilm organization of the gut microbiota is still controversial. It was nevertheless suggested that surface adhesion, the initial step leading to biofilm formation, could provide benefits for host colonization. Compared to free-floating bacteria, bacteria adhering to food particles could contribute to digestion by optimizing the degradation of complex sugars (46, 47). Moreover, many bacteria were shown to be able to adhere to mucins, the main component of the host mucus layer (48), and this

adhesion is believed to be critical to prevent fast shedding of the bacteria with luminal content (49). Finally, both aggregate and biofilm formation were shown to increase bacterial resistance to several stresses and thus might contribute to bacterial survival in the harsh gut environment (35). In this study, we found no evidence that higher *in vitro* adhesion would lead to better colonization of axenic mice. However, we assessed the abundance of each strain by enumerating bacteria in the feces of mice, even though feces composition only partially recapitulates gut microbiota composition (50). In particular, we can imagine that cells with higher adhesion would not be shed in the feces as much as cells with low adhesion, mimicking a colonization defect. Moreover, besides biofilm formation, *CPS4* and *BT2934* participate in other significant processes, i.e., interactions with the immune system and protein glycosylation, respectively. Therefore, we cannot exclude that the loss of *in vivo* fitness of Δ *CPS4* and Δ *BT2934-38* strains might be due to the consequence of these deletions on non-biofilm-related functions, overriding any benefit coming from increased biofilm formation.

In this study, we have shown that capsule regulation is a major determinant of biofilm formation. We described a transcriptional inhibition of *CPS2* by the *UpxZ* homolog of the *CPS4* locus and showed that competition between protein glycosylation and capsule production could constitute another layer of an already very complex capsule regulatory system. Further investigation of the mechanisms of biofilm formation in the gut commensal *B. thetaiotaomicron* will allow us to address the physiological adaptations of these bacteria within an anaerobic biofilm.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table S2. *B. thetaiotaomicron* was grown in brain heart infusion salt (BHIS) broth (51) supplemented with erythromycin (15 μ g/ml [erm]), tetracycline (2.5 μ g/ml [tet]), gentamicin (200 μ g/ml [gent]), or 5'-fluoro-2'-deoxyuridine (200 μ g/ml [FdUR]) when required and incubated at 37°C in anaerobic conditions using jars with anaerobic atmosphere generators (GENbag anaerobic; bioMérieux, ref. 45534) or in a C400M Ruskinn anaerobic-microaerophilic station. *Escherichia coli* S17 λ pir was grown in Miller's lysogeny broth (LB) (Corning) supplemented with ampicillin (100 μ g/ml) when required and incubated at 37°C with 180 rpm shaking. Cultures on solid medium were done in BHIS broth with 1.5% agar, and antibiotics were added when needed. Bacteria were always streaked from glycerol stock on BHIS agar before being grown in liquid cultures. All media and chemicals were purchased from Sigma-Aldrich unless indicated otherwise. All experiments and genetic constructions of *B. thetaiotaomicron* were made in the VPI-5482 Δ *tdk* strain, which was developed for a 2-step selection procedure of unmarked gene deletion by allelic exchange as previously described (52). Therefore, the VPI-5482 Δ *tdk* is referred to as the wild type in this study.

96-well crystal violet biofilm formation assay. Overnight culture was diluted to an optical density at 600 nm (OD_{600}) of 0.05 in 100 μ l BHIS broth and inoculated in technical duplicates in polystyrene Greiner round-bottom 96-well plates. The wells at the border of the plates were filled with 200 μ l water to prevent evaporation. Incubation was done at 37°C in anaerobic conditions for 48 h. Then, 25 μ l of Bouin solution (picric acid 0.9%, formaldehyde 9%, and acetic acid 5%; HT10132, Sigma-Aldrich) was added directly to each well before removal of the supernatant to prevent the washing out of large aggregates. Cultures were fixed for 10 min, and then the wells were washed once with water by immersion and flicking, and the biofilm was stained with 125 μ l 1% crystal violet (V5265; Sigma-Aldrich) for 10 min. Crystal violet solution was removed by flicking, and biofilms were washed twice with water. Stained biofilms were resuspended in a 1:4 acetone:ethanol mix, and absorbance at 575 nm was measured using a Tecan infinite M200 PRO plate reader.

Targeted mutagenesis. Deletion mutants were constructed using the previously described vector for allelic exchange in *B. thetaiotaomicron*—pExchange-*tdk* (52). A list of all the primers used in this study can be found in Table S3. Briefly, a 1-kb region upstream and downstream of the target sequence and pExchange-*tdk* were amplified by PCR using Phusion Flash high-fidelity PCR master mix (Thermo Fischer Scientific; F548). All three fragments were ligated using a Gibson assembly as follows: the inserts and the plasmids were mixed with Gibson master mix 2 \times (100 μ l 5 \times ISO buffer, 0.2 μ l 10,000 U/ml T5 exonuclease [NEB catalog number M0363S], 6.25 μ l 2,000 U/ml Phusion HF polymerase [NEB catalog number M0530S], 50 μ l 40,000 U/ml *Taq* DNA ligase [NEB catalog number M0208S], and 87 μ l dH₂O for 24 reactions) and incubated at 50°C for 35 min. The resulting mix was transformed in *E. coli* S17 λ pir that was used to deliver the vector to *B. thetaiotaomicron* by conjugation. Conjugation was carried out by mixing exponentially grown cultures (OD_{600} = 0.6) of the donor and the recipient strain in a 2:1 ratio. The mixture was spotted on BHIS-agar plates and incubated at 37°C in aerobic conditions overnight. The mix was then streaked on BHIS agar supplemented with antibiotic—for selection of *B. thetaiotaomicron* transconjugants that had undergone the first recombination event—and gentamicin to ensure exclusion of any *E. coli* growth. Eight of the resulting colonies were grown overnight in BHIS broth with no antibiotic to allow a second recombination event, and the culture was plated on BHIS agar plates

supplemented with FdUR to select for loss of plasmid. The resulting deletion mutants were confirmed by PCR and sequencing.

We used the pNBU2-bla-erm vector (53) for complementation, which inserts in the 5' untranslated region of the tRNA-Ser, in which we previously cloned the constitutive promoter of *BT1311* encoding the sigma factor RpoD (13). We constructed a pNBU2-bla-tet vector by replacing the erythromycin resistance gene by a tetracycline resistance gene from the pExchange-tet plasmid using Gibson assembly (see above). Target genes were amplified by PCR using Phusion Flash high-fidelity PCR master mix from start codon to stop codon, and they were cloned after *BT1311* promoter by Gibson assembly. The Gibson mix was transformed in *E. coli* S17λpir, and the resulting *E. coli* was used to transfer the plasmid to *B. thetaiotaomicron* by conjugation (see above).

Transposon mutagenesis. pSAMbt, the previously published tool for random mariner-based transposon mutagenesis in *B. thetaiotaomicron* (23), was conjugated in *B. thetaiotaomicron* as described above. After streaking on BHIS-erm-genta agar plates, isolated colonies were resuspended in 100 μl BHIS broth in 96-well plates, grown overnight, and tested for biofilm formation as described above. The selected clones were then streaked on a fresh BHIS-erm-genta agar plate, and 3 isolated colonies were tested for biofilm formation to ensure that no mix of transposon mutants had occurred during preparation of the library. The genomic DNA of the validated clones was extracted using a DNeasy blood and tissue kit (Qiagen) and sent for whole-genome sequencing at the Mutualized Platform for Microbiology of the Institut Pasteur.

Electronic microscopy and numbering of acapsulated bacteria. Overnight cultures were adjusted to 1 ml and an OD₆₀₀ of 1.5. Cells were treated as described by Jacques and Foiry (54) for capsule observation as follows: cultures were resuspended in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and incubated at room temperature for 2 h. Cells were then washed three times in 0.1 M cacodylate buffer (pH 7.2) and fixed 30 min in 1 mg/ml ferritin in 0.1 M cacodylate buffer (pH 7.2). Cells were washed one last time in 0.1 M cacodylate buffer (pH 7.2) and sent for transmission electronic microscopy at the electronic microscopy platform IBISA of the University of Tours (<https://microscopies.med.univ-tours.fr/>). Acapsulated cells were counted by hand using the Fiji cell counter plugin.

India ink stain. For the India ink stain, 5 μl of overnight cultures were mixed with 3 μl India ink directly on a Superfrost Plus glass microscopy slide (Thermo Fischer Scientific) and left to dry for 2 min. The excess liquid was removed with a paper towel after addition of the coverslip, and the cells were observed with a photonic microscope at ×1000 magnification.

Observation of aggregates by light microscopy. First, 1 μl of the bottom of an overnight sedimented culture were mixed with 3 μl phosphate buffered saline (PBS) buffer and dropped on a Superfrost Plus glass microscopy slide (Thermo Fischer Scientific), and then it was covered with a coverslip. The samples were observed with a photonic microscope at ×400 magnification.

RNA extraction. Overnight cultures were mixed with RNAprotect bacteria reagent (Qiagen) at a 1:2 volume ratio. The mix was incubated for 5 min at room temperature and then spun down for 10 min at 5,000 × g. The pellet was kept at -80°C. RNA was extracted from the pellet using a FastRNA Pro Blue kit (MP). The pellet was resuspended in 1 ml RNAPro and mixed with lysing matrix B. Cells were broken using FastPrep instrument at 40 s, speed 6, twice at 4°C. The lysate was centrifuged for 10 min at 4°C at 12,000 × g, and the supernatant was collected and mixed with 300 μl of chloroform. After 5 min of incubation at room temperature, the mix was centrifuged at 12,000 × g at 4°C for 5 min. The upper phase was transferred to a tube containing 500 μl cold 100% ethanol, and the nucleic acids were precipitated for 1 h at -20°C. The tubes were centrifuged at 12,000 × g at 4°C for 15 min, and the pellet was washed in 500 μl cold 75% ethanol. After centrifugation at 12,000 × g and 4°C for 5 min, the ethanol was removed, and the pellet was air-dried. Then, 60 μl of RNA-free water was added to resuspend the nucleic acid, and we treated it with Turbo DNase from the Turbo DNA-free kit (Thermo Fischer Scientific; product number AM1907) for 1.5 h. Then, the enzyme was inactivated using Turbo DNase inactivator for 2 min at room temperature, and the extracted RNA was kept at -20°C.

qRT-PCR. We performed reverse transcription using the first-strand cDNA synthesis kit for RT-PCR (AMV) (Sigma-Aldrich) and the protocol described by the supplier. Briefly, 500 μg RNA previously boiled for 15 min at 65°C was mixed with 2 μl 10× reaction buffer, 4 μl MgCl₂ 25 mM, 2 μl dNTP mix at 10 mM each, 1 μl 3' primer 20 μM, 1 μl RNase inhibitor 50 U/μl, and 0.8 AMV reverse transcriptase and water. The mix was incubated at 42°C for 1.5 h, and the enzyme was inactivated by heating to 99°C for 5 min. qPCR was performed using SYBR green PCR master mix (Life Technologies). cDNA was mixed with SYBR green master mix as described by the supplier and with corresponding primers in technical duplicates in 384-well plates. The qPCR was performed using the QuantStudio 6 Flex real-time PCR system (Thermo Fischer Scientific) and the "ΔΔCt method" program. We followed 16s rRNA and RpoB as housekeeping genes for normalization.

Staining of glycosylated proteins. Overnight cultures were adjusted to 1 ml at an OD₆₀₀ of 1, spun down and resuspended in 100 μl 1× Laemmli-β-mercaptoethanol lysis buffer (Bio-Rad), and boiled for 5 min at 95°C. Then, 10 μl was run on Mini-PROTEAN TGX stain-free TM precast gels (Bio-Rad) in 1× TGX buffer for 40 min at 170 V. The gel was then stained using a Pro-Q Emerald 300 staining for glycoproteins kit (Invitrogen) following the procedure described by the supplier.

Cocolonization of axenic mice. Animal experiments were done at the Animalerie Axénique de MICALIS (ANAXEM) platform (Microbiologie de l'Alimentation au Service de la Santé [MICALIS], Jouy-en-Josas, France) according to official authorization number 3441-2016010614307552 delivered by the French Ministry of Education Nationale, Enseignement Supérieur et Recherche. The protocol was approved by a local ethics committee on animal experimentation (committee number 45). All animals were housed in flexible-film isolators (Getinge-La Calhène, Vendôme, France) with controlled environ-

mental conditions (light/dark cycle of 12 h/12 h, temperature between 20 and 22°C, humidity between 45% and 55%). Mice were provided with sterile tap water and a gamma-irradiated standard diet (R03-40; S.A.F.E., Augy, France) *ad libitum*. Their bedding was composed of wood shavings, and they were also given cellulose sheets as enrichment.

For each combination performed in separate isolators, groups of 5 male C3H/HeN germfree mice (6 to 11 weeks old) were gavaged with 200 μ l bacterial suspensions containing 100 cells of each of the two strains we coinoculated. One of the strains was conjugated with the pNBU2-bla-erm plasmid, and the other, with the pNBU2-bla-tet plasmid so that they contained a different antibiotic resistance marker for later distinction. One of the combinations (WT [ermR] versus Δ CPS4 [tetR]) was performed twice (10 mice total). We plated the mix used for gavage onto BHIS agar plates with erythromycin or with tetracycline to check the initial ratio. When it was not 1:1, we corrected the measured abundance according to the ratio we had effectively used. At 24, 48, and 72 h after inoculation, feces were collected, split into two tubes, and weighed. One of the tubes was homogenized in 1 ml of BHIS, and serial dilutions were plated onto BHIS agar plates with erythromycin or with tetracycline. The abundance of each strain in the feces was measured by counting the CFU growing on each type of plate using an automated plater (easySpiral; Interscience, France) and counter (Scan500; Interscience, France). The other tube was dried in a speed vac concentrator (Savant, USA) and weighed. This allowed us to calculate the percentage humidity of the feces we were using for each mouse and each condition and to infer the dry weight of the feces used for CFU numbering. We divided the number of bacteria obtained by CFU counting by the dry weight of the feces they were collected from.

Growth curve. Overnight cultures were diluted to 0.05 OD₆₀₀ in 200 μ l BHIS, which had previously been incubated in anaerobic conditions overnight to remove dissolved oxygen, in Greiner flat-bottom 96-well plates. A plastic adhesive film (adhesive sealing sheet; Thermo Fisher Scientific, AB0558) was added on top of the plate inside the anaerobic station, and the plates were then incubated in a Tecan Infinite M200 Pro spectrophotometer for 24 h at 37°C. The OD₆₀₀ was measured every 30 min after 900 s orbital shaking of 2 mm amplitude.

Statistical analyses. Statistical analyses were performed using either R and Rstudio software or GraphPad Prism 8 for Mac OS (GraphPad Software, Inc.). We used only nonparametric tests. For *in vivo* experiments, 5 to 10 mice were used in 1 or 2 independent experiments. For all other experiments, at least 6 biological replicates in at least 2 independent experiments were used. A cutoff *P* value of 5% was used for all tests. *, *P* < 0.05; **, *P* < 0.05; ***, *P* < 0.005.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 2.5 MB.

FIG S2, TIF file, 2.5 MB.

FIG S3, TIF file, 2.4 MB.

FIG S4, TIF file, 2.6 MB.

FIG S5, TIF file, 2.7 MB.

TABLE S1, PDF file, 0.1 MB.

TABLE S2, PDF file, 0.2 MB.

TABLE S3, PDF file, 0.1 MB.

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N.B., J.M., S.V.-F., and F.C. performed the experiments; N.B., J.M., C.B., and J.-M.G. designed the experiments and analyzed the data. J.-M.G. and P.L. provided resources and funding. N.B., J.M., and J.-M.G. wrote the manuscript.

We declare no competing financial interests.

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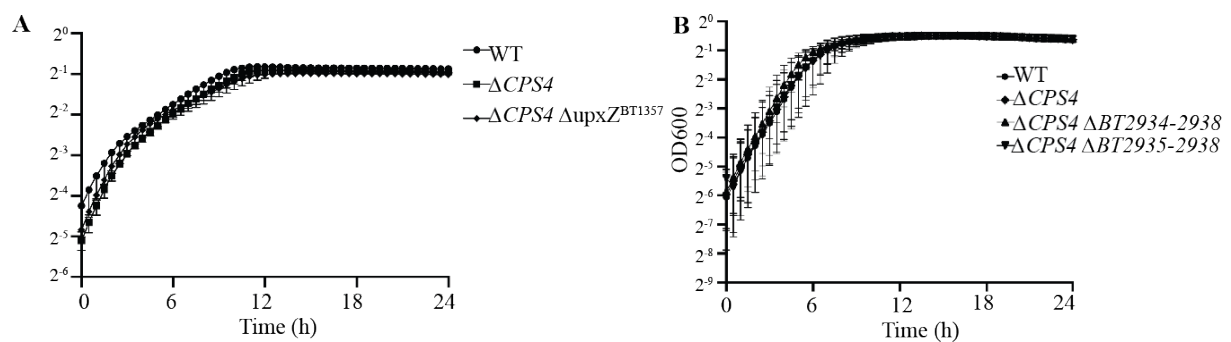
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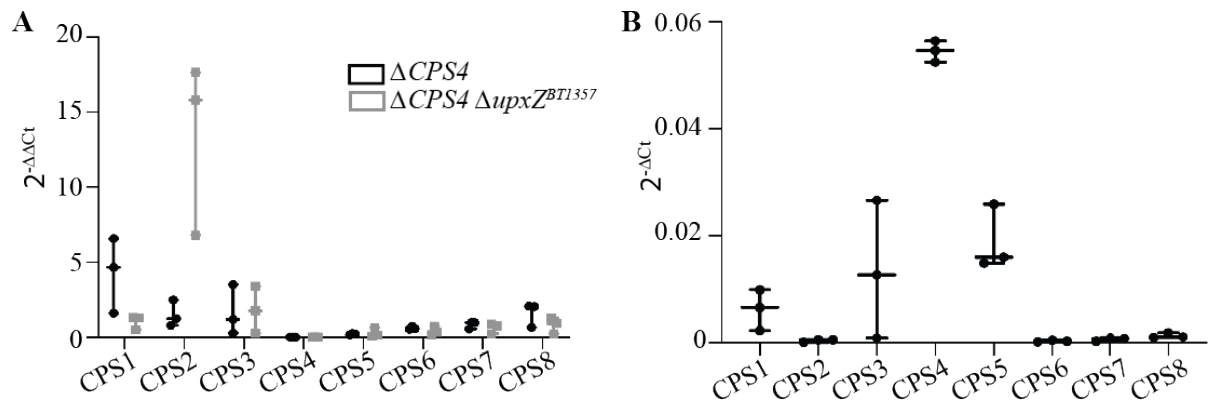
Capsular polysaccharides cross-regulation modulates *Bacteroides thetaiotaomicron* biofilm formation.

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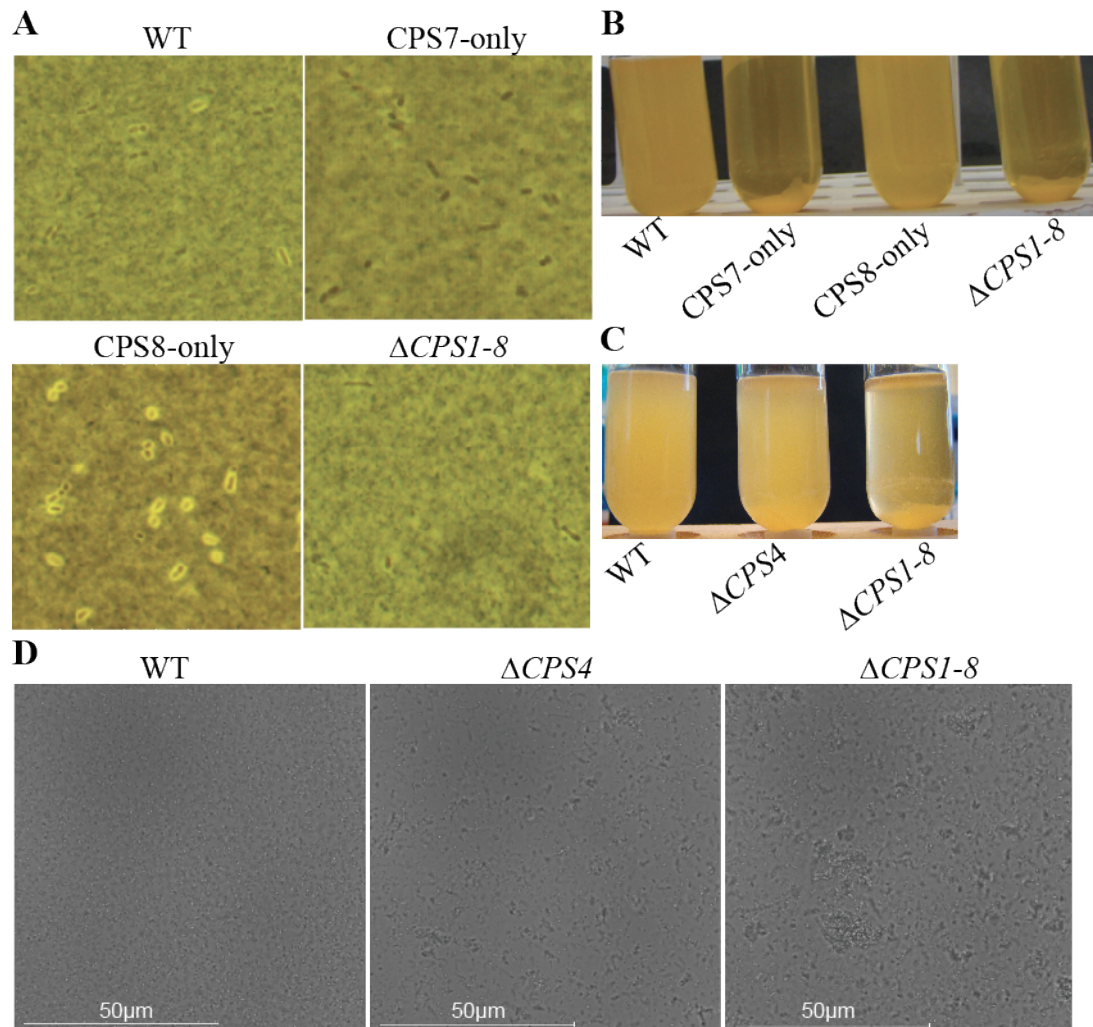
SUPPLEMENTARY FIGURES



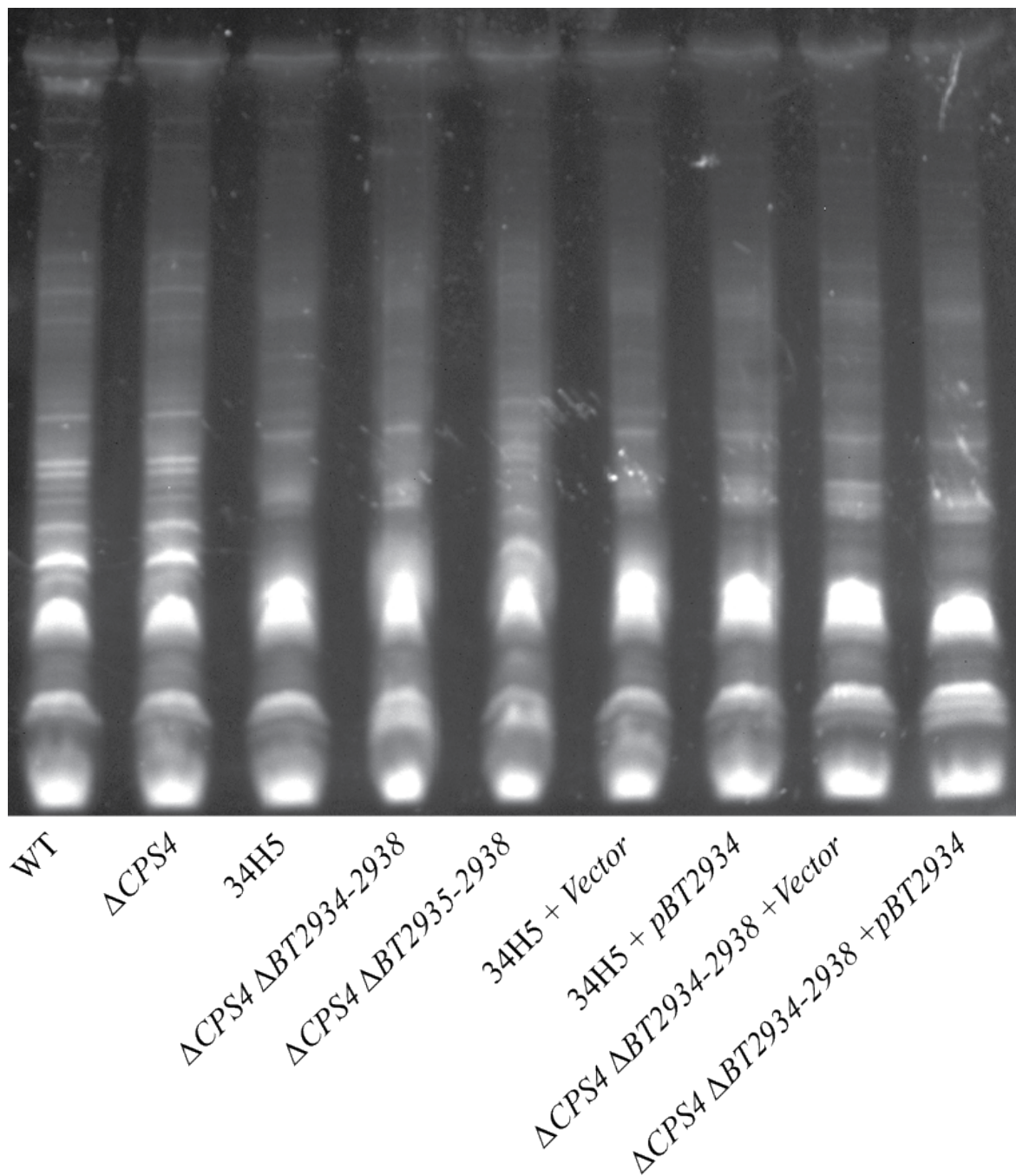
Supplementary Figure S1. **The mutants considered in this study were not affected for growth.** Planktonic growth of indicated mutants for 24h in BHIS in 96-well plates. OD600 was measured every 30min. Each dot is the median of 6 biological replicates. Error bars represent 95% confidence interval. **A.** Impact of *BT2934-2938* on growth. **B.** $\Delta CPSI-8$ transposon mutants.



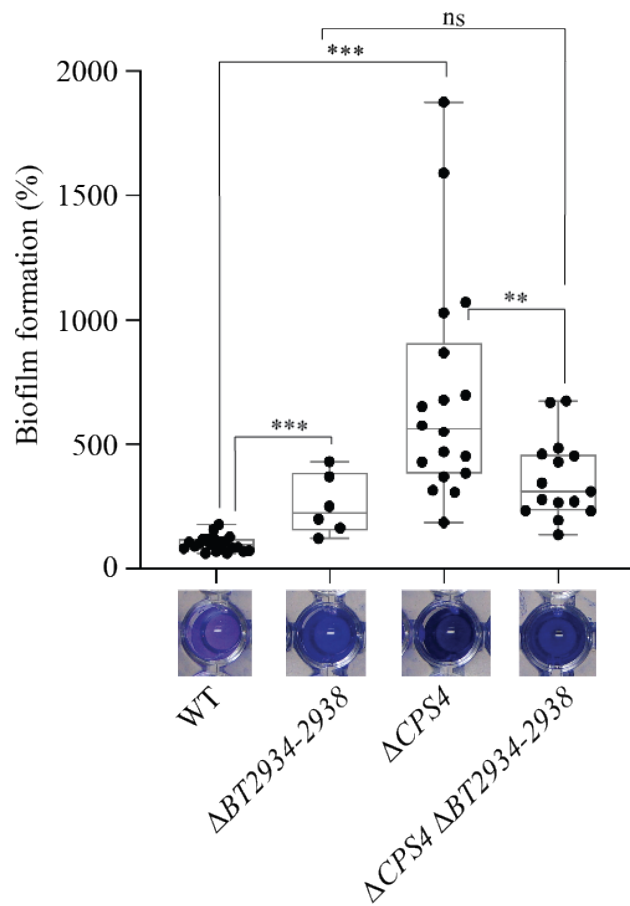
Supplementary Figure S2. ***BT1357* is a repressor of capsule 2 transcription.** qRT-PCR of 3 biological replicates of overnight planktonic cultures of WT, $\Delta CPS4$, $\Delta CPS4 \Delta upxZ^{BT1357}$ following one gene of each capsular operon and 16sRNA and *rpoB* housekeeping genes. **A.** Expression levels of each capsules were controlled within each strain using the expression of 16s rRNA and *rpoB* housekeeping genes, and they were normalized between strains using the expression of WT as a reference. Y-axis represent fold-change of expression ($2^{-\Delta\Delta Ct}$) for each of the 8 capsules of *B. thetaiotaomicron*. **B.** Expression levels of each capsule normalized by 16sRNA and *rpoB* genes in WT. Y-axis represent fold-change of expression within one strain ($2^{-\Delta Ct}$) for each of the 8 capsules of *B. thetaiotaomicron*.



Supplementary figure S3. Acapsulated *B. thetaiotaomicron* aggregate in BHIS. **A.** India ink stained cultures observed under the phase contrast microscope, 1000X. **B.** and **C.** Aggregation in overnight cultures of *B. thetaiotaomicron* in BHIS. **D.** Observation of bacterial aggregates with a phase contrast microscope, 400X.



Supplementary Figure S4. **BT2934-2938 is involved in protein glycosylation.** ProQ Emerald 300 staining of glycosylated proteins on whole cell extract of overnight cultures.



Supplementary figure S5. ***BT2934-2938* impacts biofilm formation.** A. 96-well plate crystal violet biofilm assay after 48h growth in BHIS. Mean of WT is adjusted to 100 %. Min-max boxplot of 6-18 biological replicates for each strain, each replicate is the mean of two technical replicates. * p-value<0.05, ** p-value <0.005, *** p-value <0.0005 Mann-Whitney test. The pictures shown under boxplot C correspond to representative CV-stained microtiter wells after resuspension of the biofilm.

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SUPPLEMENTARY TABLES

Supplementary Table S1. Transposon insertion targets.

Name	Insertion	Gene annotation on NCBI	Function	Reference
Transposon mutant in WT				
1C1	<i>BT1356</i>	capsule polysaccharide export	CPS4 synthesis	Xu <i>et al.</i> ,
2C7	<i>BT1342</i>	protein UDP-glucuronic acid	CPS4 synthesis	2003 Xu <i>et</i>
3B12	<i>BT1341</i>	epimerase	CPS4 synthesis	<i>al.</i> , 2003 Xu
1G11	<i>BT1353</i>	UDP-glucose 6-	CPS4 synthesis	<i>et al.</i> , 2003
1F11	<i>BT1352</i>	dehydrogenase	CPS4 synthesis	Xu <i>et al.</i> ,
		glycosyltransferase		2003
		glycosyltransferase		Xu <i>et al.</i> , 2003
Transposon mutant in Δ CPS4				
1G8	<i>BT1358</i>	transcriptional regulator UpxY homolog	capsule regulation	Chatzidaki-Livanis <i>et al.</i> , 2010
23A12	<i>BT1358 // BT1357</i> intergenic region	transcriptional regulator UpxY homolog // UpxZ homolog	capsule regulation	Chatzidaki-Livanis <i>et al.</i> , 2010
42B5	<i>BT1358</i>	transcriptional regulator UpxY homolog	capsule regulation	Chatzidaki-Livanis <i>et al.</i> , 2010
38G6	<i>BT1358</i>	transcriptional regulator UpxY homolog	capsule regulation	Chatzidaki-Livanis <i>et al.</i> , 2010
41C9	<i>BT1358</i>	transcriptional regulator UpxY homolog	capsule regulation	Chatzidaki-Livanis <i>et al.</i> , 2010
34H5	<i>BT2934</i>	wzx homolog, LPS biosynthesis transmembrane transport protein, Multidrug and toxin extrusion (MATE) domain	protein O- glycosylation	Fletcher <i>et al.</i> , 2009

1. Xu J, *et al.* (2003) A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis.
2. Chatzidaki-Livanis M, Weinacht KG, & Comstock LE (2010) Trans locus inhibitors limit concomitant polysaccharide synthesis in the human gut symbiont *Bacteroides fragilis*. *Proceedings of the National Academy of Sciences of the United States of America* 107(26):11976-11980.
3. Fletcher CM, Coyne MJ, Villa OF, Chatzidaki-Livanis M, & Comstock LE (2009) A general O-glycosylation system important to the physiology of a major human intestinal symbiont. *Cell* 137(2):321-331.
4. Martens EC, Roth R, Heuser JE, & Gordon JI (2009) Coordinate regulation of glycan degradation and polysaccharide capsule biosynthesis by a prominent human gut symbiont. *The Journal of biological chemistry* 284(27):18445-18457.

Supplementary Table S2. **List of strains and plasmids used in this study.**

Name in this paper	Genotype	Reference
<i>Bacteroides thetaiotaomicron</i>		
WT	VPI 5482 <i>Δtdk</i>	Koropatkin <i>et al.</i> , 2008
1C1	VPI 5482 <i>Δtdk</i> - BT1356::Tn	This study
2C7	VPI 5482 <i>Δtdk</i> - BT1342::Tn	This study
3B12	VPI 5482 <i>Δtdk</i> - BT1341::Tn	This study
1G11	VPI 5482 <i>Δtdk</i> - BT1353::Tn	This study
1F11	VPI 5482 <i>Δtdk</i> - BT1352::Tn	This study
<i>ΔCPS4</i>	VPI 5482 <i>Δtdk_ΔBT1356-38</i>	This study
CPS1-only	VPI 5482 <i>Δtdk_Δcps2-8 cps1-lock</i>	Porter <i>et al.</i> 2017
CPS2-only	VPI 5482 <i>Δtdk_Δcps1_Δcps3-8</i>	Porter <i>et al.</i> 2017
CPS3-only	VPI 5482 <i>Δtdk_Δcps1-2_Δcps4-8 cps3-lock</i>	Porter <i>et al.</i> 2017
CPS4-only	VPI 5482 <i>Δtdk_Δcps1-3_Δcps5-8</i>	Porter <i>et al.</i> 2017
CPS5-only	VPI 5482 <i>Δtdk_Δcps1-4_Δcps6-8 cps5-lock</i>	Porter <i>et al.</i> 2017
CPS6-only	VPI 5482 <i>Δtdk_Δcps1-5_Δcps7-8 cps6-lock</i>	Porter <i>et al.</i> 2017
CPS7-only	VPI 5482 <i>Δtdk_Δcps1-6_Δcps8</i>	Porter <i>et al.</i> 2017
CPS8-only	VPI 5482 <i>Δtdk_Δcps1-7 cps8-lock</i>	Porter <i>et al.</i> 2017
<i>ΔCPS1-8</i>	VPI 5482 <i>Δtdk_Δcps1-8</i>	Porter <i>et al.</i> 2017
<i>ΔCPS4ΔCPS7</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT2886-62</i>	This study
<i>ΔCPS4ΔCPS8</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT0038-68</i>	This study
1G8	VPI 5482 <i>Δtdk_ΔBT1356-38-BT1358::Tn</i>	This study
23A12	VPI 5482 <i>Δtdk_ΔBT1356-38-BT1358-7 intergenic region::Tn</i>	This study
42B5	VPI 5482 <i>Δtdk_ΔBT1356-38-BT1358::Tn</i>	This study
38G6	VPI 5482 <i>Δtdk_ΔBT1356-38-BT1358::Tn</i>	This study
41C9	VPI 5482 <i>Δtdk_ΔBT1356-38-BT1358::Tn</i>	This study
34H5	VPI 5482 <i>Δtdk_ΔBT1356-38-BT2934::Tn</i>	This study
<i>ΔupxZ^{BT1357}ΔCPS4</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT1357</i>	This study
<i>upxY^{BT1358}ΔCPS4</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT1358</i>	This study
<i>ΔupxY^{BT1358}_ΔupxZ^{BT1357}ΔCPS4</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT1358-7</i>	This study
<i>ΔupxZ^{BT1357}ΔCPS4ΔCPS2</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT1357_ΔBT0463-82</i>	This study
<i>ΔupxY^{BT1358}_ΔupxZ^{BT1357}ΔCPS4 + vector</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT1358-7 + pNBU2-bla-erm-p1311</i>	This study
<i>ΔupxY^{BT1358}_ΔupxZ^{BT1357}ΔCPS4 + pupxZ^{BT1357}</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT1358-7 + pNBU2-bla-erm-p1311-BT1357</i>	This study

<i>ΔupxZ^{BT1357}ΔCPS4 + vector</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT1357 + pNBU2-bla-erm-p1311</i>	This study
<i>ΔupxZ^{BT1357}ΔCPS4 + pupxZ^{BT1357}</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT1357 + pNBU2-bla-erm-p1311-BT1357</i>	This study
<i>ΔCPS4ΔBT2934-2938</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT2934-8</i>	This study
<i>ΔCPS4ΔBT2935-2938</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT2935-8</i>	This study
<i>ΔCPS4ΔBT2934-2938 + Vector</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT2934-8+ pNBU2-bla-tet-p1311</i>	This study
<i>ΔCPS4ΔBT2934-2938 + pBT2934</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT2934-8+ pNBU2-bla-tet-p1311-BT2934</i>	This study
<i>34H5 + Vector</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_BT2934::Tn+ pNBU2-bla-tet-p1311</i>	This study
<i>34H5 + pBT2934</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_BT2934::Tn+ pNBU2-bla-tet-p1311-BT2934</i>	This study
<i>WT-ery</i>	VPI 5482 <i>Δtdk+ pNBU2-bla-ery-p1311</i>	This study
<i>WT-tet</i>	VPI 5482 <i>Δtdk+ pNBU2-bla-tet-p1311</i>	This study
<i>ΔCPS4-ery</i>	VPI 5482 <i>Δtdk_ΔBT1356-38+ pNBU2-bla-ery-p1311</i>	This study
<i>ΔCPS4-tet</i>	VPI 5482 <i>Δtdk_ΔBT1356-38+ pNBU2-bla-tet-p1311</i>	This study
<i>ΔBT2934-2938</i>	VPI 5482 <i>Δtdk_ΔBT2934-8</i>	This study
<i>ΔBT2934-2938 tet</i>	VPI 5482 <i>Δtdk_ΔBT2934-8 + pNBU2-bla-tet-p1311</i>	This study
<i>ΔCPS4ΔBT2934-2938 tet</i>	VPI 5482 <i>Δtdk_ΔBT1356-38_ΔBT2934-8+ pNBU2-bla-tet-p1311</i>	This study
<i>CPS1-only+pBT2934</i>	VPI 5482 <i>Δtdk_Δcps2-8 cps1-lock+ pNBU2-bla-tet-p1311-BT2934</i>	This study
<i>CPS2-only+pBT2934</i>	VPI 5482 <i>Δtdk_Δcps1_Δcps3-8+ pNBU2-bla-tet-p1311-BT2934</i>	This study
<i>CPS3-only+pBT2934</i>	VPI 5482 <i>Δtdk_Δcps1-2_Δcps4-8 cps3-lock+ pNBU2-bla-tet-p1311-BT2934</i>	This study
<i>CPS4-only+pBT2934</i>	VPI 5482 <i>Δtdk_Δcps1-3_Δcps5-8+ pNBU2-bla-tet-p1311-BT2934</i>	This study
<i>CPS5-only+pBT2934</i>	VPI 5482 <i>Δtdk_Δcps1-4_Δcps6-8 cps5-lock+ pNBU2-bla-tet-p1311-BT2934</i>	This study
<i>CPS6-only+pBT2934</i>	VPI 5482 <i>Δtdk_Δcps1-5_Δcps7-8 cps6-lock+ pNBU2-bla-tet-p1311-BT2934</i>	This study
<i>CPS8-only+pBT2934</i>	VPI 5482 <i>Δtdk_Δcps1-7 cps8-lock+ pNBU2-bla-tet-p1311-BT2934</i>	This study
<i>Escherichia coli</i>		
<i>S17λpir_pSAM-bt</i>	<i>S17λpir_pSAM-bt</i>	This study
<i>S17λpir_pExchange-tdk</i>	<i>S17λpir_pExchange-tdk</i>	This study
<i>S17λpir_pExchange-BT1356-38</i>	<i>S17λpir_pExchange-BT1356-38</i>	This study
<i>S17λpir_pExchange-BT2886-62</i>	<i>S17λpir_pExchange-BT2886-62</i>	This study
<i>S17λpir_pExchange-BT0038-68</i>	<i>S17λpir_pExchange-BT0038-68</i>	This study
<i>S17λpir_pExchange-BT1357</i>	<i>S17λpir_pExchange-BT1357</i>	This study
<i>S17λpir_pExchange-BT1358</i>	<i>S17λpir_pExchange-BT1358</i>	This study
<i>S17λpir_pExchange-BT1358-7</i>	<i>S17λpir_pExchange-BT1358-7</i>	This study
<i>S17λpir_pExchange-BT0463-82</i>	<i>S17λpir_pExchange-BT0463-82</i>	This study

S17λpir_pExchange-BT2934-8	S17λpir_pExchange-BT2934-8	This study
S17λpir_pExchange-BT2935-8	S17λpir_pExchange-BT2935-8	This study
S17λpir_pNBU2-bla-erm-p1311	S17λpir_pNBU2-bla-erm-p1311	This study
S17λpir_pNBU2-bla-erm-p1311-BT1357	S17λpir_pNBU2-bla-erm-p1311-BT1357	This study
S17λpir_pNBU2-bla-tet-p1311	S17λpir_pNBU2-bla-tet-p1311	This study
S17λpir_pNBU2-bla-tet-p1311-BT2934	S17λpir_pNBU2-bla-tet-p1311-BT2934	This study
Name	Description	Reference
Plasmids		
pSAM-bt	Random transposon mutagenesis	Goodman <i>et al.</i> , 2009
pExchange-tdk	Site-directed unmarked gene deletion	Martens <i>et al.</i> , 2008
pExchange-BT1356-38	Site-directed unmarked gene deletion of BT1356-38	This study
pExchange-BT2886-62	Site-directed unmarked gene deletion of BT2882-62	This study
pExchange-BT0038-68	Site-directed unmarked gene deletion of BT0038-68	This study
pExchange-BT1357	Site-directed unmarked gene deletion of BT1357	This study
pExchange-BT1358	Site-directed unmarked gene deletion of BT1358	This study
pExchange-BT1358-7	Site-directed unmarked gene deletion of BT1358-7	This study
pExchange-BT0463-82	Site-directed unmarked gene deletion of BT0463-82	This study
pExchange-BT2934-8	Site-directed unmarked gene deletion of BT2934-8	This study
pExchange-BT2935-8	Site-directed unmarked gene deletion of BT2935-8	This study
pNBU2-bla-erm	Complementation	Wang et al 2000
Vector (erm)	Complementation	This study
pupxZ ^{BT1357}	Complementation with BT1357	This study
Vector (tet)	Complementation	This study
pBT2934	Complementation with BT2934	This study

1. Koropatkin NM, Martens EC, Gordon JI, & Smith TJ (2008) Starch catabolism by a prominent human gut symbiont is directed by the recognition of amylose helices. *Structure* 16(7):1105-1115.
2. Porter NT & Martens EC (2017) The Critical Roles of Polysaccharides in Gut Microbial Ecology and Physiology. *Annual review of microbiology* 71:349-369.
3. Goodman AL, *et al.* (2009) Identifying genetic determinants needed to establish a human gut symbiont in its habitat. *Cell host & microbe* 6(3):279-289.
4. Martens EC, Roth R, Heuser JE, & Gordon JI (2009) Coordinate regulation of glycan degradation and polysaccharide capsule biosynthesis by a prominent human gut symbiont. *The Journal of biological chemistry* 284(27):18445-18457.
5. Wang J, Shoemaker NB, Wang G-R, & Salyers AA (2000) Characterization of a Bacteroides Mobilizable Transposon, NBU2, Which Carries a Functional Lincomycin Resistance Gene. *Journal of bacteriology* 182(12):3559-3571.

Supplementary Table S3. Primers used in this study

Construct	Name	Sequence (5'-->3')
pExchange	pEx-ch-F	TGGGAATCCCCTCCACCGC
	pEx-ch-R	GGGGAGAGGACGGACAGAAGAT
	pExchangeR	CGTCGACTCGAATGTTATCTTC
	pExchangeF	TCTAGAGCGGCCGCCACC
pExchange-BT1356-1338	1356-5R	AGAACAATAAAGTAGAATGCTAAAAAGTCGTTGTATTTTC
	1356-5F	GCGGTGACAGAATAACGAATAAGTGTGGGG
	1356-3F	GAAAATACAACGACTTTTTAGCATTCTACTTTTTTGTTCTTTCTTAGAATTGAAATAAACG
	1356-3R	CCCTCTAGAATTCGATCCTACGAATACGACC
pExchange-BT0038-0068	cps8-intF	ACGGCATAGATGTAGCCAAAAG
	cps8-intR	GATCGCCCAACCTAGTGTC
	cps8-3F	TTGATAATTCTTTTAAACATGATGCATGGTCTATTACAACCTGTC
	cps8-3R	GCGGTGGCGGCCGCTCTAGACAAAGAAGCTTTCCTTCTGAC
	cps8-5R	GGTTGTAATAGACCATGCATCATGTTTAAAAGAATTATCAATAGAAACATG
	cps8-5F	AGATAACATTCGAGTCGACGCATAAATTAGTTTGAGCGACG
pExchange-BT2886-2862	cps7-extF	GACTGTTCCGAACATTG
	cps7-extR	AGCCTTGCTCAAAAACCTGG
	cps7-5R	GTCAGAGAGAAATGATAGCAAATAACTCCTCCAATTCTATCATTTAAAG
	cps7-5F	AGATAACATTCGAGTCGACGGCAGAGTGAACCTTATCCCTC
	cps7-3F	ATAGAATTGGAGGAGTTATTTGCTATCATTCTCTCTGACAGATG
	cps7-3R	GCGGTGGCGGCCGCTCTAGATCCTTAGTCCCTGTACCC
pExchange-BT1358	1358-5F	GCGGTGGCGGCCGCTCTAGACAGCAGCCTCATTCTTTAGTGC
	1358-5R	TTTCACTTATATTTAAACCCCTATTTACCCACACTTATTTCGT
	1358-3F	AATAAGTGTGGGGTAAATAGGGGTTTAAATATAAGTGAACAAGCA

	1358-3R	AGATAACATTCGAGTCGACGGTTGCATGCACTCATCCG
pExchange-BT1357	1357-3R	AGATAACATTCGAGTCGACGTGATTGTAATCTTGATATG CTAGCAAAG
	1357-3F	ATTAAGTGAATAAAAGGGTCTTTTTTATTGTCAATGA AAATACAACGAC
	1357-5R	TTCATTGACAAATAAAAAAGACCCCTTTTATTCACTTAA TGCTTG
	1357-5F	GCGGTGGCGGCCGCTCTAGATGTCAAGTTCAGGTTTCA GGTC
pExchange-BT1358- 1357	1357-58 3F	AATAAGTGTGGGGTAAATAGCTTTTTTATTGTCAATGA AAATACAACGAC
	1357-58 5R	TTCATTGACAAATAAAAAAGCTATTTACCCACACTTAT TCGT
pExchange-BT0463- 0482	cps2-3R	GCGGTGGCGGCCGCTCTAGAGATTGAAAGTGGCGGCAG
	cps2-3F	TGAGTACTAATAACAAATCATCCGGTCTAAGAATAAA CCTGAG
	cps2-5R	GGTTTATTCTTAGAACCGGATGATTTGTTATTAGTACTC AAGATTTGAG
	cps2-5F	AGATAACATTCGAGTCGACGTGAAAGCTGTCAGGAAGC TAAG
pExchange-BT2934- 2938	2934-5F	AGATAACATTCGAGTCGACGGGAAAGACTTCCAGGCAC G
	2934-5R	GCATTTGGGGACTTCACCGGATTATTATAATCGGTGAA GGAGAGTG
	2938-3F	CCTTACCGATTATAATAATCCGGTGAAGTCCCCAAATG C
	2938-3R	GCGGTGGCGGCCGCTCTAGATCTCTATGAATGTCCTGTT TTCGT
pExchange-BT2935- 2938	2935-5F	AGATAACATTCGAGTCGACGGGATAGCTCCGCGAGCTC
	2935-5R	GCATTTGGGGACTTCACCGGTCATGATATCTTTTCTTTT TAATGAACTG
	35-2938-3F	AAAAGAAAAAGATATCATGACCGGTGAAGTCCCCAAAT GC
pNBU2-bla	pNBU-chR	GCCAATGCACAAATGCTGTCC
	pNBU-chF	CAGGTGTATTCCCATCCGG
	pNBU-F	CGACGTCGACTAATTGCC
	pNBU-R	ATGTTAAAAACAGATTTGGAGTGC

pNBU2-bla-tetR	pNBU-del-eryF	ATGTCATCAAAATAAAAACAATAGGCCACATGCAAC
	pNBU-del-eryR	ATTTATAATATTCATTATAACCTCTCCTTAATTTATTG
	tetQ-F	ATTAAGGAGAGGTTATAATGAATATTATAAAATTTAGGA ATTCTTGCTC
	tetQ-R	TTGCATGTGGCCTATTGTTTTTATTTTGATGACATTGATT TTTGG
	tetQ-NBU-chF	CGGGATGAACCATGAGTAC
	tetQ-NBU-chR	GTACCGAGGACGCGTAAAC
pNBU2-bla-erm-BT1357	pNBU-1357F	TCCAAATCTGTTTTTAACATATGGTGAGTTTTTACTAC AAG
	pNBU-1357R	TAGGCAATTAGTCGACGTCGTTAGTTGGTTTCACACAGT TCC
pNBU2-bla-tet-p1311-BT2934	NBU-2934-F	CACTCCAAATCTGTTTTTAACATATGAGTGAAGAACAGT CACTGAAAC
	NBU-2934-R	GATAGGCAATTAGTCGACGTCGTCATGATATCTTTTTCT TTTTAATGAACTG
qPCR	16SQF	TCAGCTCGTGTTGTGAAATG
	16SQR	GTAAGGGCCATGATGACTTG
	rpoB-Q-F	CAAATTTACGCCCAAAGTT
	rpoB-Q-R	GTGCGTCAGGAAGATATG
	0381-QF	CGCTTTATCATGTTCGTTGGA
	0381-QR	GTACAAGCCGGAGCTTTTTG
	0463-QF	TCCGATCACAAAAGGAGTGA
	0463-QR	ACGTTTAAAGCCCGGAAGAT
	0602-QF	TTGGGTTACATCGGTCTTCC
	0602-QR	TTTGAAGGTGGTGTGACCTG
	1355-QR	CGCAGGTTCTATCACTGCAA
	1355-QF	AGGAGCGATTGCAAAATGAC
	1653-QF	TGTTCCATTGAAAGCTTCAGG
	1653-QR	CTCCCCATCAATATCCAGCTT
	1722-QR	TGCAAAGACTGGCTTTTCT
	1722-QF	TGAGCAGGAGCGTTTACAGA
	2885-QR	TAGAGGAAGAGTCCCGTTGGT
	2885-QF	GGCAACGCAAATGGATTACTA
	0039-QF	GGCATTGCTTGTTTTATGGA
	0039-QR	AGGGCTGGTCATATCGTTTTCT

CHAPTER 2: BILE IS A WIDESPREAD INDUCER OF *B. THETAIOAOMICRON* BIOFILM FORMATION.

In the **Result chapter 1** section, we used random mutagenesis to identify mutants exhibiting increased biofilm formation capacity compared to WT *B. thetaiotaomicron* VPI 5482. In the **Result chapter 2** section, we searched for specific growth conditions that would induce biofilm formation in WT VPI 5482 strain. In particular, addition of several molecules typically found in the gut to the growth media have previously been shown to induce biofilm formation in a number of gut symbionts and pathogens (see **Introduction chapter 2**).

We showed that addition of bile extract to the growth media was the only molecule tested that strongly increased biofilm formation in *B. thetaiotaomicron* VPI 5482, as well as in almost all *B. thetaiotaomicron* clinical isolates tested and several *Bacteroides* species. I then used random transposon mutagenesis to identify genes required for bile-mediated biofilm formation, and found the *BT3560-3563* locus, encoding two secreted DNAses, is necessary for bile-mediated biofilm formation. Addition of commercial DNAses to the growth medium restored bile-mediated biofilm formation in $\Delta BT3560-3563$ mutant, suggesting that extracellular DNase are required to induce biofilm formation in the presence of bile. Moreover, bile increased eDNA concentration in the supernatant, and addition of gDNA to the growth medium also induced $\Delta BT3560-3563$ biofilm formation in presence of bile. This suggests that both eDNA and DNase production in presence of bile are required to observe biofilm formation. Further experiments are nevertheless needed to assess the precise relationship between eDNA, DNAses and biofilm formation in presence of bile.

Bile is a complex mix of biliverdin, cholesterol and cholesterol-derived bile acids. Among the tested bile acids, I showed that only the conjugated secondary bile acid tauroolithocholate was enough to induce biofilm formation on its own in *B. thetaiotaomicron* VPI 5482, but also several natural isolates. However, this induction was not dependent on the presence of *BT3560-3563* locus, showing that several components of bile-or a combination of them-can induce biofilm formation through different mechanisms.

In conclusion, I showed that bile and some specific bile components are important mediators of biofilm formation in almost all tested *B. thetaiotaomicron*, suggesting bile-mediated biofilm formation might be an important property of *B. thetaiotaomicron* physiology.

Béchon N, Mihajlovic J, Vendrell-Fernandez S, , Martin-Verstrate I, Dupuy B, Ghigo JM. 2020.

Bile is a widespread inducer of biofilm formation in *Bacteroides*. *In preparation*

Bile is a widespread inducer of biofilm formation in *Bacteroides thetaiotaomicron* species.

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Running Title: Bile-induced biofilms of *Bacteroides thetaiotaomicron*

ABSTRACT

Bacteroides thetaiotaomicron is an important gut mutualist involved in polysaccharide digestion and immune system and gut maturation. However, many other aspects of *B. thetaiotaomicron* biology, such as biofilm formation are understudied, even though adhesion and biofilm formation might be critical factor governing *B. thetaiotaomicron* ability to colonize and be maintained in the gut. In our previous work we showed that the best-studied *B. thetaiotaomicron* reference strain VPI 5482 is a poor *in vitro* biofilm former. We suspected that this might be due to the lack of certain environmental cues, so we tested different gut-relevant molecules and showed that addition of bile extract to the growth media triggered biofilm formation of *B. thetaiotaomicron* VPI 5482, most *B. thetaiotaomicron* clinical isolates from our collection and representatives of other *Bacteroides* species. In *B. thetaiotaomicron* VPI 5482, this induction is dependent on *BT3563*, a putative secreted endonuclease. Accordingly, addition of an extracellular DNase rescued the lack of biofilm formation of $\Delta BT3560-3563$ mutant in presence of bile. Moreover, we show that tauroolithocholate is also an inducer of biofilm formation in *B. thetaiotaomicron* and that this induction does not require *BT3563*.

INTRODUCTION

Bacteroides thetaiotaomicron is one of the most abundant gut symbiont, involved in the degradation of complex polysaccharides as well as positively contributing to the development and maturation of host immune system (Eckburg et al., 2005; Martens, Chiang, & Gordon, 2008; Wexler, 2007). Whereas its ability to stably colonize the gut is essential for host health, *B. thetaiotaomicron* functions involved in biofilm formation and adhesion to host and bacterial surfaces are still poorly understood. We have previously shown that the widely used *B. thetaiotaomicron* reference strain VPI 5482 forms poor biofilms *in vitro*, but that use of random transposon mutagenesis and positive selection leads to the identification of mutants with increased biofilm formation capacity (Béchon, Mihajlovic, Vendrell-Fernández, et al., 2020; Mihajlovic et al., 2019).

Biofilm formation can be influenced by environmental conditions. In particular, several stresses have been shown to induce biofilm formation, such as antibiotics, oxygen concentration or high metal concentration (Ahn & Burne, 2007; N. Bollinger, Hassett, Iglewski, Costerton, & McDermott, 2001; Hoffman et al., 2005). Moreover, environmental cues can also modulate biofilm formation. For instance, compounds commonly found in the gut such as mucins, secreted immunoglobulin A, or bile can induce biofilm formation of many bacterial species (R. R. Bollinger et al., 2006; Dubois et al., 2019; Pumbwe et al., 2007; Sicard, Bihan, Vogeleer, Jacques, & Harel, 2017).

In this study, we sought to identify gut-relevant growth conditions triggering biofilm formation in the wild type and poor-biofilm former *B. thetaiotaomicron* VPI 5482. We showed that *B. thetaiotaomicron* exposure to bile extract strongly induced biofilm formation. Moreover, bile-induced biofilm formation was observed for all *B. thetaiotaomicron* clinical isolates tested except for one and several *Bacteroides* species. Using transposon mutagenesis, we identified an operon, *BT3560-3563*, encoding a putative secreted DNase involved in biofilm formation and confirmed that addition of exogenous external DNase rescued a Δ *BT3560-3563* bile-induced biofilm formation defect. Moreover, we show that the conjugated secondary bile acid tauroolithocholate alone could induce biofilm formation of numerous *B. thetaiotaomicron* strains, in a *BT3563*-independent manner.

RESULTS

Bile induces biofilm formation in most *Bacteroides*.

To identify gut-relevant growth condition inducing biofilm formation in *B. thetaiotaomicron* VPI 5482, we supplemented BHIS growth medium with various components of the normal intestinal environment, including sugars (0.5% D-glucose, D-mannose, D-rhamnose), complex polysaccharides (0.5% D-cellobiose and D-maltose), different concentrations of hemin (25 mg/L and 50 mg/L), mucin (0.1% and 0.5%) and bovine - ovine bile extract (0.1%, 0.5%, 1%). Among the tested medium supplements, only addition of bile salts induced a strong increase in biofilm production (Figure 1A), with a maximal induction observed when 0.3 to 1% bile extract were added, which are relevant gut concentrations (Figure 1B). Using the concentration of 0.5% bile extract, we then tested the impact of bile extract supplementation on the biofilm formation capacity of a collection of clinical isolates of *Bacteroides thetaiotaomicron* (Mihajlovic et al., 2019) and found that all tested strains except for jmh71 showed an increased biofilm formation in presence of bile (Figure 1C). Bile was previously shown to induce *B. fragilis* biofilm formation (Pumbwe et al., 2007) and we showed that, in addition to *B. thetaiotaomicron* and *B. fragilis*, bile induced biofilm formation of *Bacteroides ovatus* ATCC8483 and *Parabacteroides distasonis*, two prominent gut commensals (Supplementary Figure 1). These experiments showed that bile extract is a widespread inducer of biofilm formation among *Bacteroides* strains of several species.

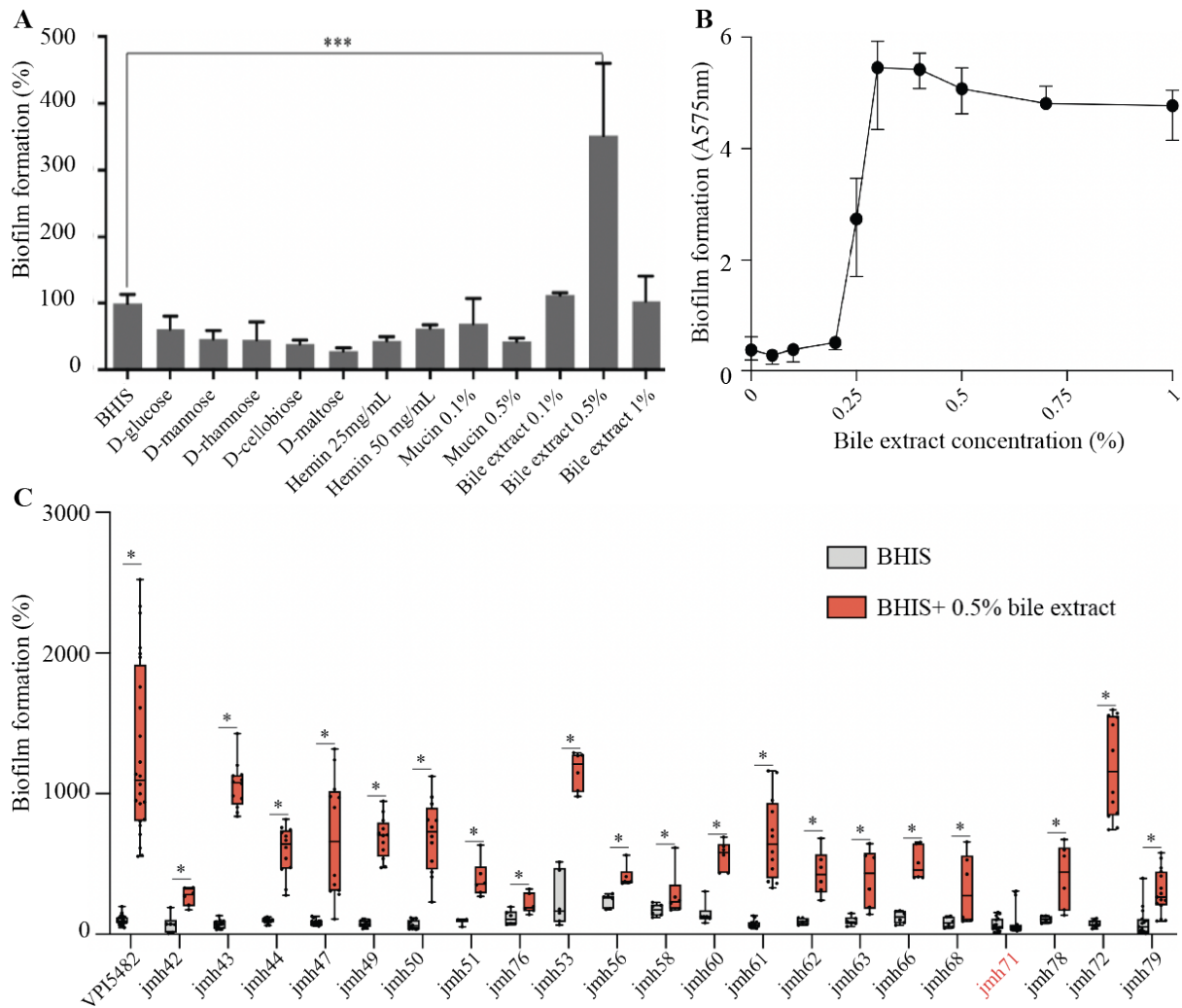


Figure 1. Bile extract induces biofilm formation of several *B. thetaiotaomicron* isolates. 96-well plate crystal violet biofilm assay. **A.** *B. thetaiotaomicron* VPI 5482 after 48h growth in BHIS supplemented with sugars (0.5% D-glucose, D-mannose, D-rhamnose), complex polysaccharides (0.5% D-cellobiose and D-maltose), different concentrations of hemin (25 mg/L and 50 mg/L), mucin (0.1% and 0.5%) or bovine-ovine bile extract (0.1%, 0.5%, 1%). Mean of WT grown in BHIS is adjusted to 100 %. Mean of 6 biological replicates for each strain, error bars represent standard deviation, each replicate is the mean of two technical replicates. *, p-value<0.05, Mann-Whitney test. **B.** *B. thetaiotaomicron* VPI 5482 biofilm formation as a function of bile extract concentration. Mean of 6 biological replicates, error bars represent standard deviation. **C.** Mean of WT in BHIS is adjusted to 100 %. Min-max boxplot of 6-12 biological replicates for each strain, each replicate is the mean of two technical replicates. *, p-value<0.05, Mann-Whitney test.

Screening for bile-insensitive mutants identifies *BT3563* as required for bile-induced biofilm formation.

To identify genes involved in bile-induced biofilm formation, we performed a random transposon mutagenesis in *B. thetaiotaomicron* VPI 5482. We screened for mutants failing to

produce biofilm in presence of bile extract and identified 9 transposon mutants out of the 6510 tested (Figure 2A), 7 of them corresponded to an insertion in *BT3560*, coding for a TonB-dependent receptor and 2 were inserted in *BT3562*, coding for a putative extracellular nuclease/protease (Figure 2B). The genetic organization of the *BT3560* region was suggestive of an operon also including a gene encoding a hypothetical protein, *BT3561*, and a second putative extracellular nuclease, *BT3563* (Figure 2B). Consistently, deletion of the entire $\Delta BT3560-3563$ region led to a lowered biofilm formation in presence of bile (Figure 2C). To determine which gene(s) of the operon were responsible for this induction, we constructed single deletion for each gene and showed that, whereas *BT3561* deletion had no impact on biofilm formation, $\Delta 3560$, $\Delta 3562$ and $\Delta 3563$ mutants showed reduced bile-induced biofilm formation but no growth defect compared to WT (Figure 2C and Supplementary figure 2). However, complementation of *BT3560* or *BT3562* *in trans* under the control of a constitutive promoter in $\Delta BT3560$ or $\Delta BT3562$ strains did not restore bile-induced biofilm formation, suggesting that deletion of *BT3560* and *BT3562* might have a polar effect on downstream *BT3563* gene (Figure 2D). By contrast, addition of *pBT3563* in $\Delta 3563$ led to a full complementation of biofilm formation defect and a significant complementation when introduced in $\Delta BT3560-3563$ (Figure 2D). These results indicated that *BT3563* was the only gene of the operon required for bile-induced biofilm formation.

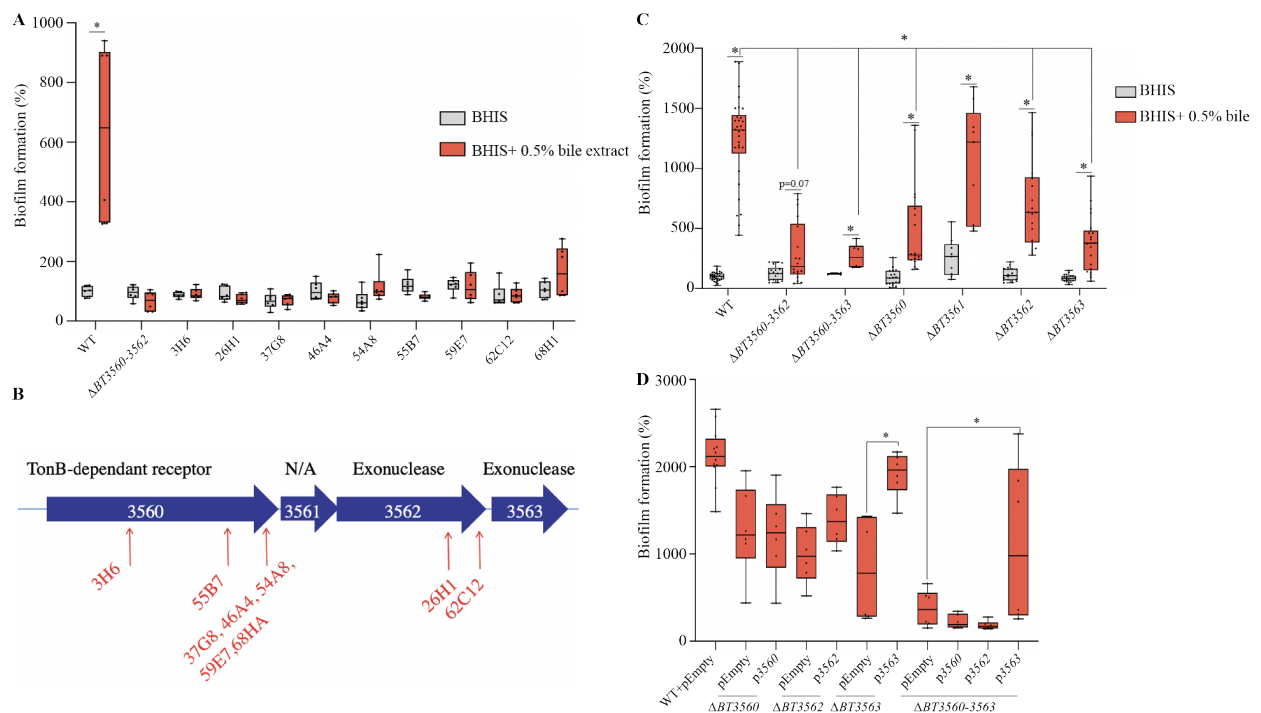


Figure 2. *BT3560-3563* is required for bile-induced biofilm formation. **A.** and **C.** 96-well plate crystal violet biofilm assay after 48h growth in BHIS or BHIS+0.5% bile extract. Mean of WT in BHIS is adjusted to 100 %.

Min-max boxplot of 6 biological replicates for each strain, each replicate is the mean of two technical replicates. * p-value<0.05, Mann-Whitney test. **B.** Schema of the *BT3560-3563* locus. Red arrows indicate transposon insertion point. **D.** 96-well plate crystal violet biofilm assay after 48h growth in BHIS+0.5% bile extract. Mean of WT in BHIS is adjusted to 100 %. Min-max boxplot of 6 biological replicates for each strain, each replicate is the mean of two technical replicates. * p-value<0.05, Mann-Whitney test.

Addition of extracellular DNase induce biofilm formation in presence of bile.

Considering that *BT3563* potentially encodes a predicted extracellular nuclease, we tested whether extracellular addition of an unrelated DNase could complement its function. Indeed, we showed that extracellular addition of the commercial DNase I in the growth medium complemented the observed reduction of bile-induced biofilm formation in *ΔBT3560-3563* (Figure 3A). Moreover, addition of 0.5mM EDTA, a chelator of divalent cations required for DNase activity, inhibited biofilm formation in presence of bile of WT VPI 5482 (Figure 3A). Taken together, this suggests that *BT3563* might encode a secreted DNase that is required for bile-induced biofilm formation. To ascertain whether DNases were also involved in bile-induced biofilm formation of other *B. thetaiotaomicron* isolates, we added DNaseI to *jmh71*, the only tested *B. thetaiotaomicron* strain that did not react to bile and showed that DNaseI increased *jmh71* biofilm formation in presence of bile, (Figure 3B), suggesting a lack of extracellular DNase might be the reason *jmh71* does not form bile-induced biofilms. Moreover, addition of 0.5mM EDTA decreased bile-induced biofilm formation in all the tested clinical isolates (Figure 3C).

The concentration of eDNA measured in the supernatant of overnight cultures is higher in presence of bile, both in WT and in *ΔBT3560-3563* (Figure 3D), showing bile induces eDNA production. Moreover, eDNA concentration was higher in *ΔBT3560-3563* than in WT, which is consistent with the hypothesis that either *BT3562* or *BT3563* encodes a secreted DNase. However, complementation with *BT3563* did not lead to a reduction in eDNA concentration even though it complemented the lack of bile-induced biofilm formation (Figure 3D). It is possible that *BT3563* only cuts eDNA in fragments rather than fully degrades it, so that the total concentration of eDNA remains constant and that small fragments of DNA are necessary for bile-induced biofilm formation. We also observed that addition of genomic DNA extracted from *B. thetaiotaomicron* VPI 5482 to the medium slightly increased *ΔBT3560-3563* biofilm formation in presence of bile (Figure 3A), suggesting both eDNA and DNases might be required for bile-induced biofilm formation.

Taken together, these results suggest that both eDNA, which could be released upon exposure to bile and extracellular DNase, which could partially degrade eDNA into smaller fragments, are required for bile-induced biofilm formation. However, the DNase activity of *BT3563* and the precise contribution of eDNA and DNases to bile-induced biofilm formation remains to be clarified.

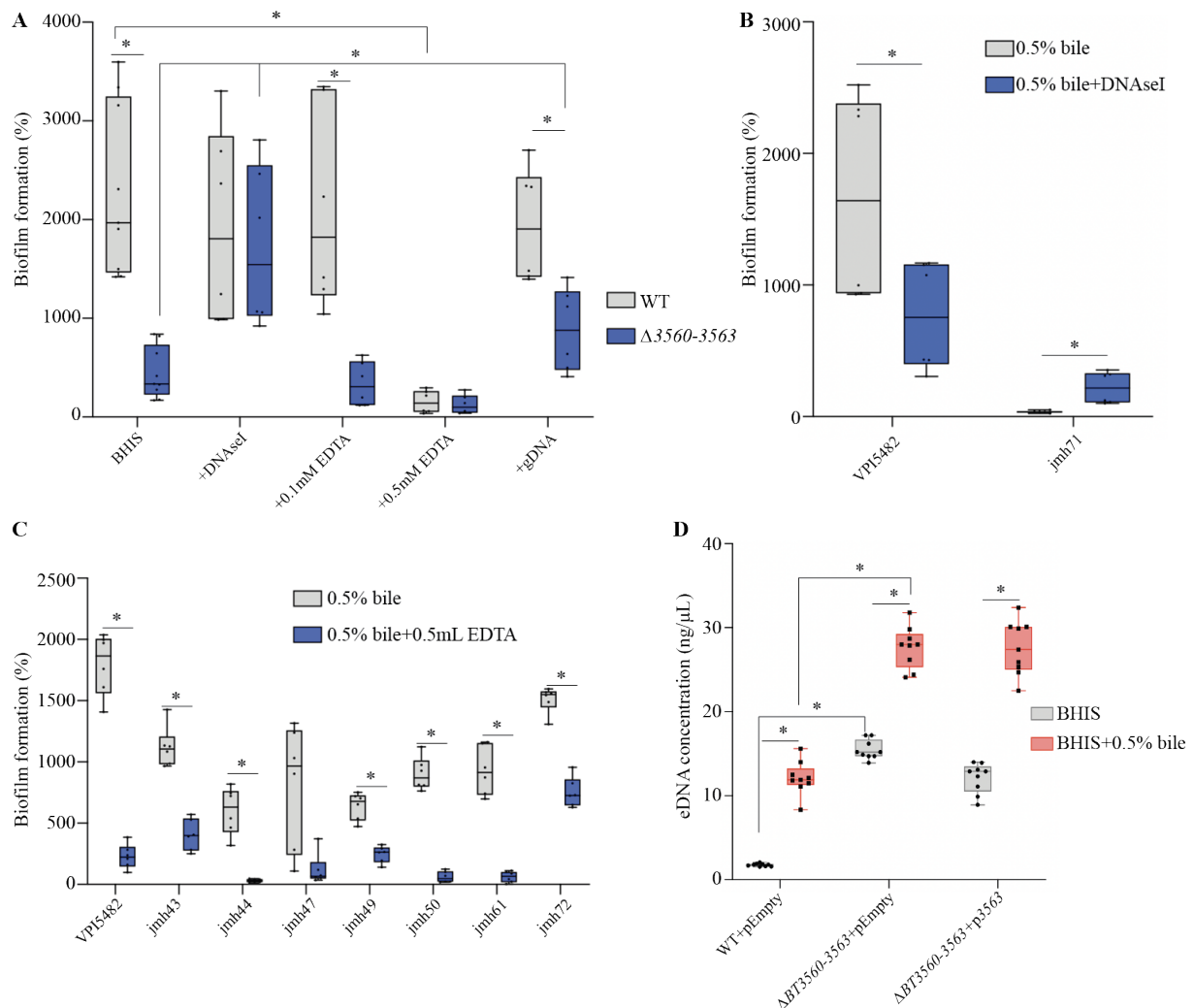


Figure 3. eDNA is involved in bile-induced biofilm formation. **A. B.** and **C.** 96-well plate crystal violet biofilm assay after 48h growth in BHIS+0.5% bile extract with or without DNaseI, EDTA or gDNA. Mean of WT in BHIS is adjusted to 100 %. Min-max boxplot of 6 biological replicates for each strain, each replicate is the mean of two technical replicates. * p-value<0.05, Mann-Whitney test. **D.** eDNA concentration (ng/ μ L) in the supernatant of overnight cultures of indicated strains grown in BHIS or BHIS+0.5% bile extract. Min-max boxplot of 9-12 biological replicates for each strain. * p-value<0.05, Mann-Whitney test.

Taurolithocholate induces biofilm formation in a *BT3563* independent manner.

Bile is a complex mixture of biliverdin, bile acids and cholesterol. To ascertain what component(s) of bile extract was responsible for the observed biofilm formation induction, we tested the impact of 9 bile acids on biofilm formation of *B. thetaiotaomicron* VPI 5482: cholate, chenodeoxycholate, deoxycholate, taurocholate, glycodeoxycholate, taurodeoxycholate, glycolithocholate, tauroolithocholate, taurochenodeoxycholate (Figure 4A). Only tauroolithocholate was able to induce biofilm formation of *B. thetaiotaomicron* VPI 5482, with a peak of induction at 0.025% (Figure 4B). Taurolithocholate also induced biofilm formation in 10 out of 12 clinical *B. thetaiotaomicron* isolates (Figure 4C), but it did not recapitulate the induction observed with bile extract: in particular, jmh71 did not react to bile extract, but it formed more biofilm in presence of tauroolithocholate while jmh49 did not react to tauroolithocholate but reacted to bile extract. This suggested that the mechanisms by which bile extract and tauroolithocholate induce biofilm formation might be different. Consistently, $\Delta 3560-3563$ formed as much biofilm as WT in presence of tauroolithocholate, showing tauroolithocholate-induced biofilm formation is *BT3563*-independent (Figure 4D).

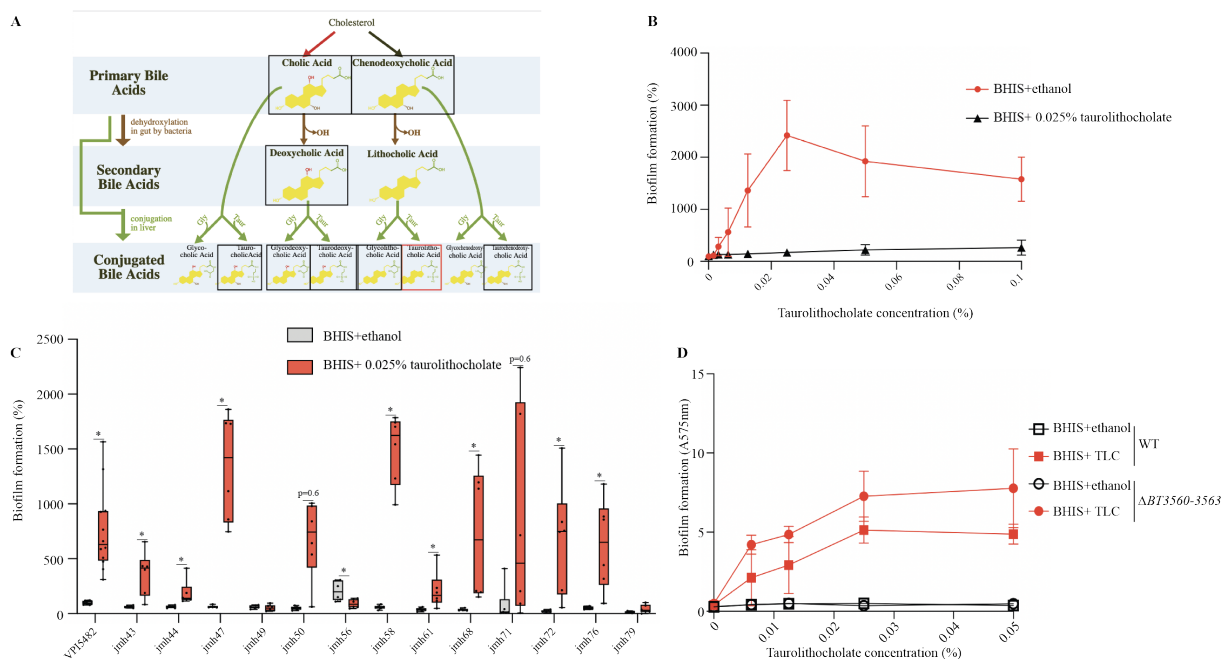


Figure 4. **Taurolithocholate induces biofilm formation in several *B. thetaiotaomicron* strains.** **A.** Schema of the 12 main bile acid composing bile. From (https://commons.wikimedia.org/wiki/File:Bile_acid_differentiation.svg). There are twelve most common bile acids: the primary bile acids cholic acid and chenodeoxycholic acids are produced from cholesterol in the liver. These primary bile acids can be dehydroxylated by gut bacteria to form secondary bile acids deoxycholic acid and lithocholic acid respectively. Moreover, primary and secondary bile acids can be conjugated in the liver with either a taurine or a glycine amino acid to form primary and secondary conjugated bile acids glycocholic acid,

tauroolithocholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid, glycodeoxycholic acid, taurodeoxycholic acid, glycolithocholic acid and tauroolithocholic acid (Martinez-Augustin and Sanchez de Medina 2008; Urdaneta and Casadesús 2017). Tested bile acids for which no biofilm induction was observed are framed in black (data not shown). Tauroolithocholate, the only bile acid tested that induced biofilm formation of *B. thetaiotaomicron*, is framed in red. **B.** 96-well plate crystal violet biofilm assay of *B. thetaiotaomicron* VPI 5482 as a function of tauroolithocholate concentration (red curve). In black is the control experiment where only ethanol, the solvent used for tauroolithocholate solubilization, was added. Mean of 6 biological replicates, error bars represent standard deviation. **C.** 96-well plate crystal violet biofilm assay after 48h growth in BHIS+ethanol or BHIS+0.025% tauroolithocholate. Mean of WT in BHIS is adjusted to 100 %. Min-max boxplot of 6 biological replicates for each strain, each replicate is the mean of two technical replicates. * p-value<0.05, Mann-Whitney test. **D.** 96-well plate crystal violet biofilm assay as a function of tauroolithocholate concentration (red curve). In black is the control experiment where only ethanol, the solvent used for tauroolithocholate solubilization, was added. Mean of 6 biological replicates, error bars represent standard deviation.

DISCUSSION

Bile is a well-known inducer of biofilm formation in various gut symbiont and pathogens such as *C. difficile*, *V. cholerae* and *Bifidobacteria* (Ambalam, Kondepudi, Nilsson, Wadström, & Ljungh, 2014; Dubois et al., 2019; Hung, Zhu, Sturtevant, & Mekalanos, 2006). We showed that bile extract induced biofilm formation of almost all *B. thetaiotaomicron* strains tested, as well as *B. fragilis*, *B. ovatus* and *P. distasonis*, suggesting bile is conserved signal for biofilm formation within the Bacteroidales. Bile concentrations higher than 0.3% are sufficient to induce maximal biofilm formation of *B. thetaiotaomicron* VPI 5482. Bile concentration within the intestine is highly variable depending on the individual, the time of the day, diet and the precise localization within the gut but it is estimated that bile concentration within the human gut can range between 0.2 to 2% (Kristoffersen et al., 2007), consistent with the concentrations required to induce *B. thetaiotaomicron* biofilm formation. The widespread activation of biofilm formation upon exposure to physiological concentrations of bile might be due to the importance of adhesion to stably colonize the gut. Conversely, bile-induced biofilm could be a way to tolerate bile exposure. Indeed, bile has cytotoxic effects, by damaging lipidic membranes and DNA and denaturing proteins (Urdaneta & Casadesús, 2017) and bacteria exposed to bile induce various stress responses and increase production of efflux pumps (Kristoffersen et al., 2007). Biofilms are known to be more tolerant to antimicrobials than planktonic cultures, and might therefore constitute a strategy by which gut bacteria prevent bile-mediated killing (Flemming et al., 2016). The impact that bile might have on *B.*

thetaitotaomicron tolerance to antimicrobials, both via biofilm formation or not, remains to be addressed.

eDNA is a well-known component of the biofilm extracellular matrix in many organism and numerous studies have shown that addition of DNase to the growth media prevented biofilm formation or dispersed pre-formed biofilm in both Gram-positive and Gram-negative bacteria (Okshevsky & Meyer, 2015). However, we show that *B. thetaiotaomicron* VPI 5482 bile-induced biofilm formation was dependent on BT3563, a putative extracellular nuclease. Moreover, addition of a commercial DNase to $\Delta BT3560-3563$ cultures rescued the bile-induced biofilm formation defect of that strain, suggesting eDNA might somehow impair biofilm formation in our conditions. On the other hand, addition of gDNA to $\Delta BT3560-3563$ cultures also increased bile-induced biofilm formation, similarly to DNase addition, showing the relationship between eDNA and *B. thetaiotaomicron* biofilm is not simple. We can hypothesize that depending on the size or shape of the eDNA, its impact on biofilm formation will vary. Partial DNA degradation by BT3563 might be required for proper matrix formation, thereby allowing biofilm formation without impacting the total eDNA concentration. More experimental work, including microscopy to visualize the eDNA within the biofilm, is required to uncover the complex interplay between extracellular DNases and eDNA in bile-mediated biofilm formation. The potential contribution of the other genes of the operon to eDNA production or degradation also remains to be addressed.

Bile addition to the medium led to higher eDNA concentration measured in the supernatant of overnight cultures. Although we do not know the mechanism for this increased eDNA release, we can hypothesize that bile will induce the lysis of a small proportion of the population and therefore release eDNA. Whether this is linked to biofilm formation is not clear. It is interesting to note that we never observed biofilm formation in cultures that did not contain bile, even when we added gDNA or DNases. Therefore, eDNA or DNase release upon bile exposure cannot be the only mechanism by which biofilm formation is induced.

Analysis of the genomes of several *B. thetaiotaomicron* isolates showed that presence/absence of *BT3563* did not correlate with bile-induced formation capacity. Although jmh71, the only *B. thetaiotaomicron* strain that did not react to bile, did not possess a homolog of *BT3563*, neither did jmh43, jmh49 and jmh72 which all formed bile-induced biofilms. However, it is possible that other DNases would carry the same function within these strains. Interestingly, EDTA, a chelator of divalent cation that are required of DNase activity, inhibited

bile-induced biofilm formation in all tested strains. Whereas this is only weak evidence of the implication of DNase in bile-induced biofilm formation of these strains, as EDTA can have pleiotropic effects, it is nevertheless consistent with this hypothesis.

The conjugated secondary bile acid tauroolithocholate is also an inducer of biofilm formation of most *B. thetaiotaomicron* isolates tested. However, not all strains reacted to both bile and tauroolithocholate: jmh71 did not react to bile extract but did react to tauroolithocholate whereas jmh49 reacted to bile but not to tauroolithocholate. Moreover, this induction was not dependent on *BT3563* in VPI 5482 strain, suggesting that bile and tauroolithocholate induction of biofilm formation occur through independent mechanisms. The mechanism of tauroolithocholate-induced biofilm formation is still unknown but the screening of our *B. thetaiotaomicron* VPI 5482 transposon library in presence of tauroolithocholate might give us some clue. None of the other bile acid tested induced biofilm formation, therefore it is still unclear what constituent(s) of bile are responsible for the observed biofilm induction in presence of bile. It is possible that a mix of bile acids, rather than a single one, are necessary to observe bile-induced biofilm formation.

Tauroolithocholate is produced in the liver by conjugating a taurine residue with lithocholate. Production of lithocholate requires bacterial-mediated $7\alpha/\beta$ -dehydroxylation of the primary bile acid chenodeoxycholate in the colon (Urdaneta & Casadesús, 2017). This reaction is mainly performed by members of the class Clostridia. Moreover, numerous bacteria have been shown to be able to hydrolyze the amid bond of conjugated bile acids, for instance converting tauroolithocholic acid into lithocholic acid (Urdaneta & Casadesús, 2017). Therefore, tauroolithocholate concentration is expected to be strongly impacted by other members of the gut microbiota. Such an interaction was previously described for *C. difficile*: *C. scindens* was shown to induce *C. difficile* biofilm formation by converting cholate to deoxycholate, an inducer of *C. difficile* biofilm formation (Dubois et al., 2019). How this type of interactions impacts the function and stability of the gut microbial community remains to be assessed. Moreover, diet can have an impact on the pool of bile acids. For instance, high-fat low-fiber diet and high-aurine diet were shown to increase the amount of taurine conjugated bile acids in bile (Hardison, 1978; Stadler et al., 1988). Therefore, both diet and other microbiota members can impact the pool of tauroolithocholate present in the gut, with potential consequences on *B. thetaiotaomicron* adhesion within the gut.

We showed that bile extract is a widespread inducer of biofilm formation: most *B. thetaiotaomicron* isolates as well as several Bacteroidales species (*B. fragilis*, *B. ovatus* and *P. distasonis*) formed bile-induced biofilms. In *B. thetaiotaomicron* VPI 5482, this induction is dependent on BT3563, encoding a putative extracellular DNase. This suggests that at least partial degradation of eDNA is required for proper biofilm formation, in contrast to what is known in classical biofilm models such as *P. aeruginosa*. Last, we show that tauroolithocholate, a conjugated secondary bile acid, could also induce biofilm formation of numerous *B. thetaiotaomicron* in a BT3563-independent manner. This study demonstrates that gut-relevant environmental cues can strongly increase the adhesion capacity of a very important gut symbiont, *B. thetaiotaomicron*, with potential consequences on its ability to colonize the gut and tolerate various stresses.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains used in this study are listed in Table S1. *B. thetaiotaomicron* and other *Bacteroides* strains were grown in BHIS broth (Bacic & Smith, 2008) supplemented with erythromycin 15 $\mu\text{g/ml}$ (erm), tetracycline 2.5 $\mu\text{g/ml}$ (tet), gentamycin 200 $\mu\text{g/ml}$ (genta), 5'-fluoro-2'-deoxyuridin 200 $\mu\text{g/ml}$ (FdUR), D-glucose, D-mannose, D-rhamnose, D-cellobiose, D-maltose (0,5% w/v), hemin from bovine (25mg/L and 25mg/L), porcine mucin extract (0,1% and 0,5% w/v), bile extract from bovine and ovine at 0.5% unless indicated otherwise, tauroolithocholate at 0.025% unless stated otherwise, DNaseI 98U/mL or EDTA when required. Tauroolithocholate was dissolved in 50% ethanol, therefore all controls were performed in BHIS supplemented with ethanol. Cultures were incubated at 37°C in anaerobic conditions using jars with anaerobic atmosphere generators (GENbag anaero, Biomerieux, ref. 45534) or in a C400M Ruskinn anaerobic-microaerophilic station. *Escherichia coli* S17 λ pir was grown in Miller's Lysogeny Broth (LB) (Corning) supplemented with ampicillin (100 $\mu\text{g/ml}$) when required and incubated at 37°C with 180 rpm shaking. Cultures on solid media were done in BHIS with 1.5% agar and antibiotics were added when needed. Bacteria were always streaked from glycerol stock on BHIS-agar before being grown in liquid cultures. All media and chemicals were purchased from Sigma-Aldrich unless indicated otherwise.

All experiments and genetic constructions of *B. thetaiotaomicron* were made in VPI 5482 Δ tdk (Koropatkin, Martens, Gordon, & Smith, 2008) unless indicated otherwise, which was developed for 2-step selection procedure of unmarked gene deletion by allelic exchange, as

previously described. Therefore, the VPI 5482 Δ *tdk* is referred to as wild type or VPI 5482 in this study.

Genome analysis.

Genomic DNA was prepared from overnight cultures using the DNeasy blood and tissue kit. Sequencing was performed by the P2M platform of Pasteur institute. The obtained genomes were added on MicroScope database of Genoscope and compared using the Gene Phyloprofile tool, using default settings.

96-well crystal violet biofilm formation assay.

Overnight culture was diluted to $OD_{600} = 0.05$ in 100 μ L BHIS and inoculated in technical duplicates in polystyrene Greiner round-bottom 96-well plates. The wells at the border of the plates were filled with 200 μ L water to prevent evaporation. Incubation was done at 37°C in anaerobic conditions for 48h. The supernatant was removed by careful pipetting and the biofilms were fixed using 100 μ L of Bouin's solution (picric acid 0.9%, formaldehyde 9% and acetic acid 5%, HT10132, Sigma-Aldrich) for 10min. Then the wells were washed once with water by immersion and flicking, and the biofilm was stained with 125 μ L 1% crystal violet (V5265, Sigma-Aldrich) for 10 minutes. Crystal violet solution was removed by flicking and biofilms were washed twice with water. Stained biofilms were resuspended in 1:4 acetone: ethanol mix and absorbance at 575nm was measured using TECAN infinite M200 PRO plate reader.

Targeted mutagenesis.

Deletion mutants were constructed using the previously described vector for allelic exchange in *B. thetaiotaomicron*: pExchange-*tdk* (Koropatkin et al., 2008). A list of all the primers used in this study can be found in Table S2. Briefly, 1kb region upstream and downstream of the target sequence and pExchange-*tdk* were amplified by PCR using Phusion Flash High-Fidelity PCR Master Mix (ThermoFischer Scientific, F548). All three fragments were ligated using Gibson assembly: the inserts and the plasmids were mixed with Gibson master mix 2x (100 μ L 5X ISO Buffer, 0.2 μ L 10,000 U/mL T5 exonuclease (NEB #M0363S), 6.25 μ L 2,000 U/mL Phusion HF polymerase (NEB #M0530S), 50 μ L 40,000 U/mL Taq DNA ligase (NEB #M0208S), 87 μ L dH₂O for 24 reactions) and incubated at 50 °C for 35 min. The resulting mix was transformed in *E. coli* S17 λ pir that was used to deliver the vector to *B. thetaiotaomicron* by conjugation. Conjugation was carried out by mixing exponentially grown cultures

(OD₆₀₀=0.6) of the donor and the recipient strain in a 2:1 ratio. The mixture was spotted on BHIS-agar plates and incubated at 37°C in aerobic conditions overnight. The mix was then streaked on BHIS agar supplemented with antibiotic – for selection of *B. thetaiotaomicron* transconjugants that had undergone the first recombination event – and gentamicin to ensure exclusion of any *E. coli* growth. 8 of the resulting colonies were grown overnight in BHIS with no antibiotic to allow a second recombination event, and the culture was plated on BHIS-agar plates supplemented with FdUR to select for loss of plasmid. The resulting deletion mutants were confirmed by PCR and sequencing.

We used the pNBU2-bla-erm vector (Wang, Shoemaker, Wang, & Salyers, 2000) or pNBU2-bla-tet (Béchon, Mihajlovic, Vendrell, et al., 2020) vector for complementation, which inserts in the 5' untranslated region of the tRNA-Ser, in which we previously cloned the constitutive promoter of *BT1311* encoding the sigma factor RpoD (Mihajlovic et al., 2019). Target genes were amplified by PCR using Phusion Flash High-Fidelity PCR Master Mix from start codon to stop codon and they were cloned after *BT1311* promoter by Gibson assembly. The Gibson mix was transformed in *E. coli* S17λpir and the resulting *E. coli* was used to transfer the plasmid to *B. thetaiotaomicron* by conjugation (see above).

Transposon mutagenesis.

pSAMbt, the previously published tool for random mariner-based transposon mutagenesis in *B. thetaiotaomicron* (Goodman et al., 2009) was conjugated in *B. thetaiotaomicron* as described above. After streaking on BHIS-erm-genta agar plates, isolated colonies were resuspended in 100μL BHIS in 96-well plates, grown overnight and tested for biofilm formation as described above. The selected clones were then streaked on a fresh BHIS-erm-genta agar plate and 3 isolated colonies were tested for biofilm formation to ensure no mix of transposon mutants had occurred during preparation of the library. The genomic DNA of the validated clones was extracted using DNeasy blood and tissue kit (Qiagen) and sent for whole genome sequencing at the Mutualized platform for Microbiology of Institut Pasteur.

eDNA measure in supernatant.

Overnight cultures were centrifugated 5min at 6000xg. The supernatant was recovered and filtered. eDNA concentration in the supernatant was measured using QuBit HS double stranded DNA kit.

Growth curve.

2.5 μ L overnight cultures were added to 200 μ L BHIS that had previously been incubated in anaerobic condition overnight to remove dissolved oxygen, in Greiner flat-bottom 96-well plates. A plastic adhesive film (adhesive sealing sheet, Thermo Scientific, AB0558) was added on top of the plate inside the anaerobic station, and the plates were then incubated in a TECAN Infinite M200 Pro spectrophotometer for 24 hours at 37°C. OD600 was measured every 30 minutes, after 900 seconds orbital shaking of 2 mm amplitude.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

ACKNOWLEDGEMENTS

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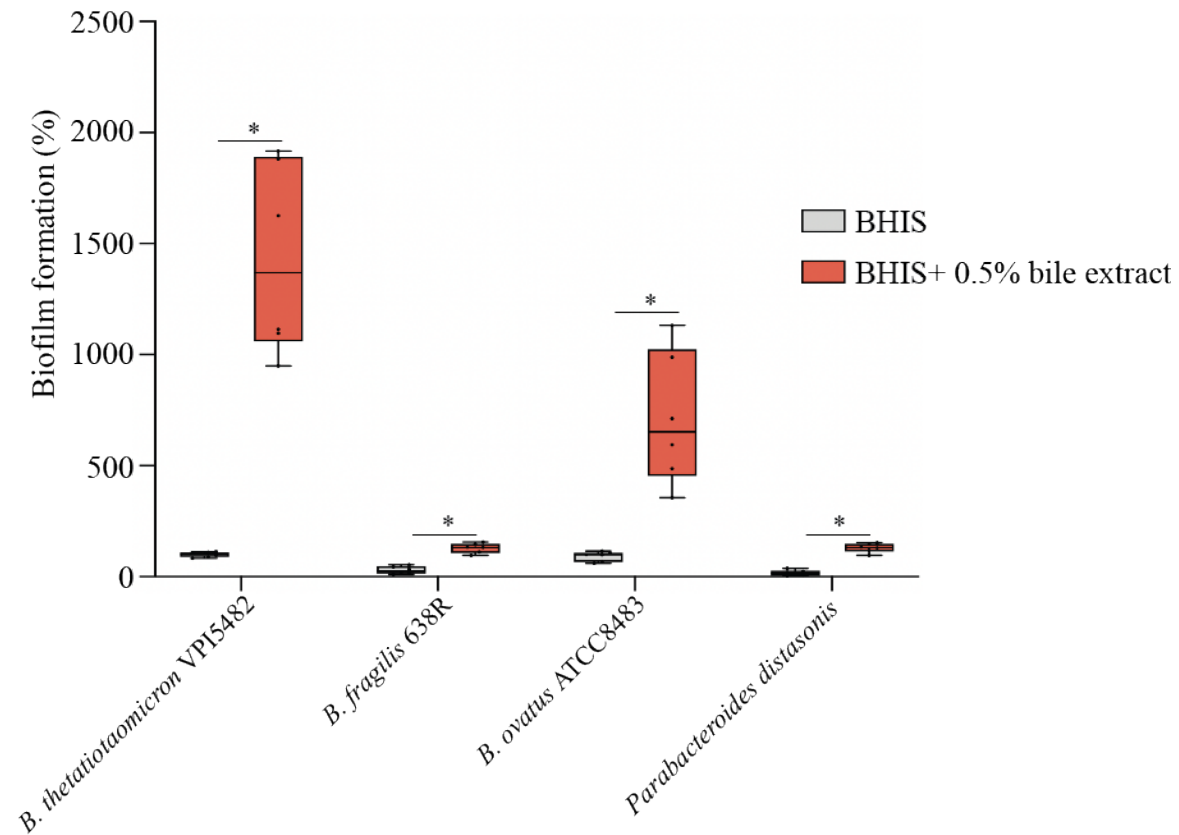
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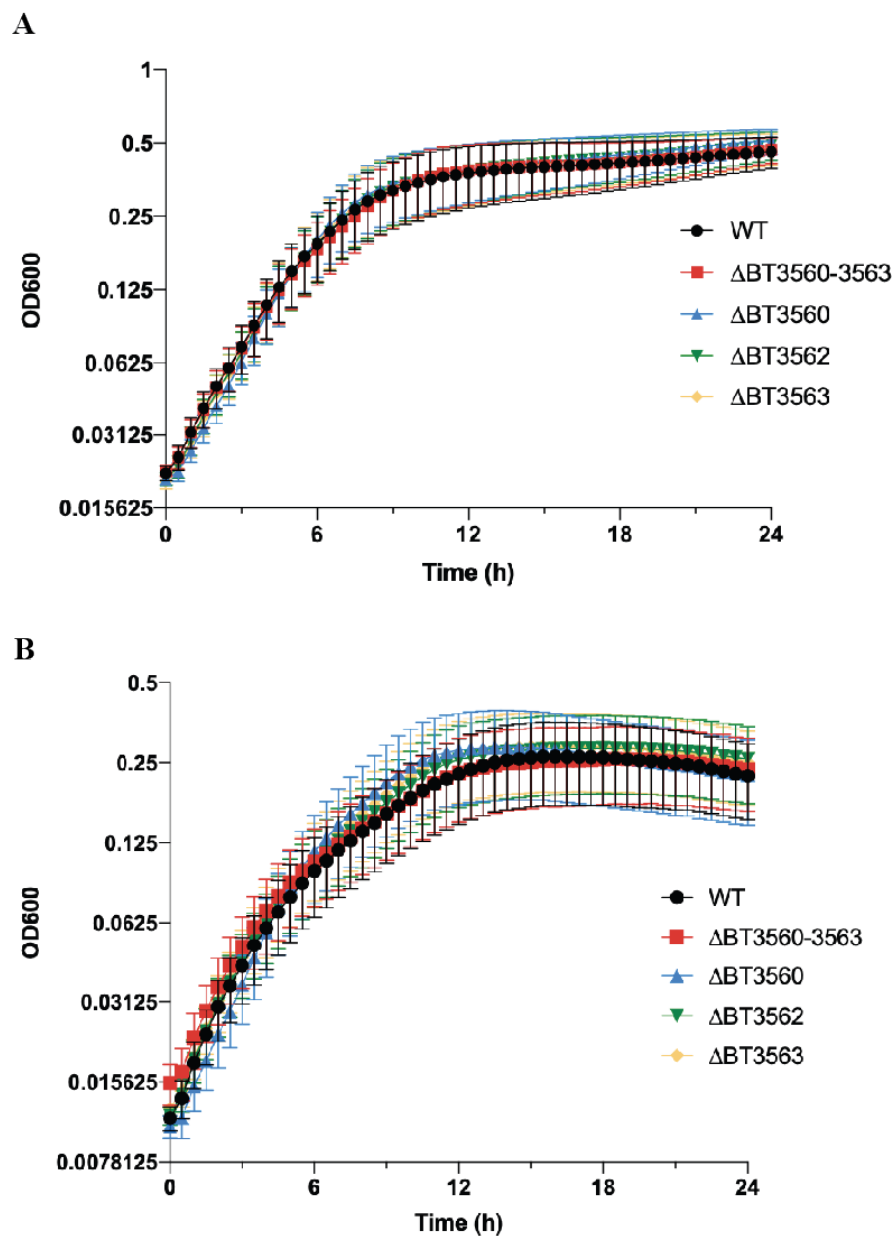
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SUPPLEMENTARY MATERIALS



Supplementary Figure S1. **Bile extract induces biofilm formation of several *Bacteroides* species.** 96-well plate crystal violet biofilm assay after 48h growth in BHIS or BHIS+0.5% bile extract. Mean of *B. thetaiotaomicron* VPI 5482 in BHIS is adjusted to 100 %. Min-max boxplot of 6 biological replicates for each strain, each replicate is the mean of two technical replicates. *, p-value<0.05, Mann-Whitney test.



Supplementary figure 2. **Growth curve.** 24h growth curve of indicated strains in 96-well plate in **A.** BHIS or **B.** BHIS+0.5% bile extract. Mean of 6 biological replicates, error bars represent standard error to the mean (SEM).

Name in this paper	Genotype	Reference
<i>Bacteroides</i> <i>thetaiotaomicron</i>		
WT/VPI 5482	VPI 5482 <i>_Atdk</i>	Koropatkin <i>et al.</i> , 2008
jmh42		Mihajlovic <i>et al.</i> , 2019
jmh43		Mihajlovic <i>et al.</i> , 2019
jmh44		Mihajlovic <i>et al.</i> , 2019

jmh47		Mihajlovic <i>et al.</i> , 2019
jmh49		Mihajlovic <i>et al.</i> , 2019
jmh50		Mihajlovic <i>et al.</i> , 2019
jmh51		Mihajlovic <i>et al.</i> , 2019
jmh76		Mihajlovic <i>et al.</i> , 2019
jmh53		Mihajlovic <i>et al.</i> , 2019
jmh56		Mihajlovic <i>et al.</i> , 2019
jmh58		Mihajlovic <i>et al.</i> , 2019
jmh60		Mihajlovic <i>et al.</i> , 2019
jmh61		Mihajlovic <i>et al.</i> , 2019
jmh62		Mihajlovic <i>et al.</i> , 2019
jmh63		Mihajlovic <i>et al.</i> , 2019
jmh66		Mihajlovic <i>et al.</i> , 2019
jmh68		Mihajlovic <i>et al.</i> , 2019
jmh71		Mihajlovic <i>et al.</i> , 2019
jmh78		Mihajlovic <i>et al.</i> , 2019
jmh72		Mihajlovic <i>et al.</i> , 2019
jmh79		Mihajlovic <i>et al.</i> , 2019
3H6	VPI 5482 <i>_Atdk-</i> BT3560::Tn	This study
26H1	VPI 5482 <i>_Atdk-</i> BT3562::Tn	This study
37G8	VPI 5482 <i>_Atdk-</i> BT3560::Tn	This study
46A4	VPI 5482 <i>_Atdk-</i> BT3560::Tn	This study
54A8	VPI 5482 <i>_Atdk-</i> BT3560::Tn	This study
55B7	VPI 5482 <i>_Atdk-</i> BT3560::Tn	This study
59E7	VPI 5482 <i>_Atdk-</i> BT3560::Tn	This study
62C12	VPI 5482 <i>_Atdk-</i> BT3562::Tn	This study
68H1	VPI 5482 <i>_Atdk-</i> BT3560::Tn	This study
<i>ΔBT3560-3562</i>	VPI 5482 <i>_Atdk_ΔBT3560-3562</i>	This study
<i>ΔBT3560-3563</i>	VPI 5482 <i>_Atdk_ΔBT3560-3563</i>	This study
<i>ΔBT3560</i>	VPI 5482 <i>_Atdk_ΔBT3560</i>	This study
<i>ΔBT3561</i>	VPI 5482 <i>_Atdk_ΔBT3561</i>	This study
<i>ΔBT3562</i>	VPI 5482 <i>_Atdk_ΔBT3562</i>	This study
<i>ΔBT3563</i>	VPI 5482 <i>_Atdk_ΔBT3563</i>	This study

WT+pEmpty	VPI 5482 <i>Δtdk</i> + pNBU2-bla-ery-p1311	This study
<i>ΔBT3560</i> +pEmpty	VPI 5482 <i>Δtdk_ΔBT3560</i> + pNBU2-bla-erm-p1311	This study
<i>ΔBT3560</i> +p3560	VPI 5482 <i>Δtdk_ΔBT3560</i> + pNBU2-bla-erm-p1311_ <i>BT3560</i>	This study
<i>ΔBT3562</i> +pEmpty	VPI 5482 <i>Δtdk_ΔBT3562</i> + pNBU2-bla-erm-p1311	This study
<i>ΔBT3562</i> +p3562	VPI 5482 <i>Δtdk_ΔBT3560</i> + pNBU2-bla-erm-p1311_ <i>BT3562</i>	This study
<i>ΔBT3563</i> +pEmpty	VPI 5482 <i>Δtdk_ΔBT3563</i> + pNBU2-bla-tet-p1311	This study
<i>ΔBT3563</i> +p3563	VPI 5482 <i>Δtdk_ΔBT3560</i> + pNBU2-bla-tet-p1311_ <i>BT3563</i>	This study
<i>ΔBT3560-3563</i> +pEmpty	VPI 5482 <i>Δtdk_ΔBT3560-3563</i> + pNBU2-bla-tet-p1311	This study
<i>ΔBT3560-3563</i> +p3563	VPI 5482 <i>Δtdk_ΔBT3560</i> + pNBU2-bla-tet-p1311_ <i>BT3563</i>	This study
<i>Bacteroides</i>		
<i>Bacteroides fragilis</i> 638R		
<i>Bacteroides ovatus</i> ATCC8483		
<i>Parabacteroides distasonis</i>		
<i>Escherichia coli</i>		
S17λpir_pSAM-bt	S17λpir_pSAM-bt	Goodman <i>et al.</i> , 2009
S17λpir_pExchange-tdk	S17λpir_pExchange-tdk	Koropatkin <i>et al.</i> , 2008
S17λpir_pExchange- <i>BT3560-3562</i>	S17λpir_pExchange- <i>BT3560-3562</i>	This study
S17λpir_pExchange- <i>BT3560-3563</i>	S17λpir_pExchange- <i>BT3560-3563</i>	This study

S17λpir_pExchange-BT3560	S17λpir_pExchange-BT3560	This study
S17λpir_pExchange-BT3561	S17λpir_pExchange-BT3561	This study
S17λpir_pExchange-BT3562	S17λpir_pExchange-BT3562	This study
S17λpir_pExchange-BT3563	S17λpir_pExchange-BT3563	This study
S17λpir_pNBU2-bla-erm-p1311	S17λpir_pNBU2-bla-erm-p1311	This study
S17λpir_pNBU2-bla-erm-p1311-BT3560	S17λpir_pNBU2-bla-erm-p1311-BT3560	This study
S17λpir_pNBU2-bla-erm-p1311-BT3562	S17λpir_pNBU2-bla-erm-p1311-BT3562	This study
S17λpir_pNBU2-bla-tet-p1311	S17λpir_pNBU2-bla-tet-p1311	This study
S17λpir_pNBU2-bla-tet-p1311-BT3563	S17λpir_pNBU2-bla-tet-p1311-BT3563	This study
Name	Description	Reference
Plasmids		
pSAM-bt	Random transposon mutagenesis	Goodman <i>et al.</i> , 2009
pExchange-tdk	Site-directed unmarked gene deletion	Martens <i>et al.</i> , 2008
pExchange-BT3560-3562	Site-directed unmarked gene deletion	This study
pExchange-BT3560-3563	Site-directed unmarked gene deletion	This study
pExchange-BT3560	Site-directed unmarked gene deletion	This study
pExchange-BT3561	Site-directed unmarked gene deletion	This study
pExchange-BT3562	Site-directed unmarked gene deletion	This study
pExchange-BT3563	Site-directed unmarked gene deletion	This study
pNBU2-bla-erm-p1311	Site-directed unmarked gene deletion	Wang et al 2000
pNBU2-bla-erm-p1311-BT3560	Complementation	This study

pNBU2-bla-erm-p1311- <i>BT3562</i>	Complementation	This study
pNBU2-bla-tet-p1311	Complementation	This study
pNBU2-bla-tet-p1311- <i>BT3563</i>	Complementation	This study

Table S1: Strain and plasmid list

Construct	Name	Sequence (5'-->3')
pExchange	pEx-ch- F	TGGGAATTCCCCTCCACCGC
	pEx-ch- R	GGGGAGAGGACGGACAGAAGAT
	pExchan geR	CGTCGACTCGAATGTTATCTTC
	pExchan geF	TCTAGAGCGGCCGCCACC
pExchange- <i>BT3560-3562</i>	3560- 5Fb	AGATAACATTCGAGTCGACGGTACTTTCCTTCTTTTG GAAC
	3560- 5Rb	TACTTTAAAATATGCACCCAAATTTAAATTATTATTAG TGTTAATTTGTAGC
	3562- 3Fb	CACTAATAATAATTTAAATTTGGGTGCATATTTAAAG TACCAGG
	3562-3R	GCGGTGGCGGCCGCTCTAGATTGTGGATGAAGATCAG CAATAGAC
pExchange- <i>BT3560-3563</i>	3560- 5Fb	AGATAACATTCGAGTCGACGGTACTTTCCTTCTTTTG GAAC
	3560- 5Rb	TATAACAAACAAGTTTTATTAATTTAAATTATTATTAG TGTTAATTTGTAGC
	3563- 3Fb	CACTAATAATAATTTAAATTAATAAAACTTGTTTGTTA TAGATATAATTAGACC

	3563-3R	GCGGTGGCGGCCGCTCTAGAAACATATATCTTTGCTG TACAC
	3560- extF	CGCTATACCCATTAAGCCATAC
	3563- extR	GATATTAATGGCAACAACACC
pExchange- <i>BT3560</i>	3560-3F	GATTAGGAATAGTGATTGCAATAAATGGGCAAATTA TACAAGGAAATG
	3560-3R	GCGGTGGCGGCCGCTCTAGACAAGCTTCTGAGTTATC CTGTTTC
	3560-5R	GTATTAATTTGCCATTTATTGCAATCACTATTCCTAA TCTTTGTTTC
	3560-5F	AGATAACATTCGAGTCGACGTTAGCGGATACAGAACA GATGG
pExchange- <i>BT3561</i>	3561-3F	TAATACAAGGAAATGATATGCAAAGAAATAACTAAT AAAATAAATATTCAAC
	3561-3R	GCGGTGGCGGCCGCTCTAGAAGAAGAGCTAGTACTTG TATATTTAAATCC
	3561-5R	TTTTATTAGTTATTTCTTTGCATATCATTTCCTTGATT AATTTGCC
	3561-5F	AGATAACATTCGAGTCGACGTTATCAGGGTGATAACG ATGGAC
pExchange- <i>BT3562</i>	3562-3F	TGATGTTCTGCTCCTTATGTGGGTGCATATTTTAAAG TACCAGG
	3562-3R	GCGGTGGCGGCCGCTCTAGATTGTGGATGAAGATCAG CAATAGAC
	3562-5R	GTACTTTAAAATATGCACCCACATAAGGAGCAGGAAC ATCACTAC
	3562-5F	AGATAACATTCGAGTCGACGTACGAACTTGCGTACTG GCG
S17 λ pir_pExc hange- <i>BT3563</i>	3563-3F	CTTTTTTAACAAAAATGAATTAATAAACTTGTGTT ATAGATATAATTAGACC

	3563-3R	GCGGTGGCGGCCGCTCTAGAAACATATATCTTTGCTG TACAC
	3563-5R	CTATAACAAACAAGTTTTATTAATTCATTTTTGTAAA AAAGGAG
	3563-5F	GAAGATAACATTCGAGTCGACGGCAGATTTGAGTGCT CAAGC
	3563- extF	GATAATACATGGGTATCATTGAC
	3563- extR	GATATTAATGGCAACAACACC
pNBU2-bla	pNBU- chR	GCCAATGCACAAATGCTGTTCC
	pNBU- chF	CAGGTGTATTCCCATCCGG
	pNBU-F	CGACGTCGACTAATTGCC
	pNBU- R	ATGTTAAAAACAGATTTGGAGTGC
pNBU2-bla- erm-p1311- <i>BT3560</i>	NBU- 3560-F	AATCTGTTTTTAACATATGAAACAAAGATTAGGAATA GTGATTGC
	NBU- 3560-R	TAGGCAATTAGTCGACGTCGTTAGAATCTATATCCAA TGTTCAAGAAAGC
pNBU2-bla- erm-p1311- <i>BT3562</i>	NBU- 3562-F	AAATCTGTTTTTAACATATGAAAAAGATTCTAAATGC TTTATTCTTG
	NBU- 3562-R	TAGGCAATTAGTCGACGTCGTTATTCAACGAACTTAT ATTCGGTAGG
pNBU2-bla- tet-p1311- <i>BT3563</i>	NBU- 3563-R	TAGGCAATTAGTCGACGTCGCTATTGCAGTCCGGTCC
	NBU- 3563-F	TCCAAATCTGTTTTTAACATATGAGATTTTTTAATAGA AGTGTTG

Table S2: Strain and plasmid list

CHAPTER 3: AUTOTRANSPORTERS ARE THE MAIN ADHESINS OF *V. PARVULA*.

V. parvula is a key dental plaque colonizer and a member of the human intestinal microbiota, two models of biofilm-like anaerobic microbiota. Although *V. parvula* capacity to form multi-species biofilms is likely to be key for its establishment in both environments, lack of genetic tools for this bacterium severely limited mechanistical studies. In the **Result chapter 3** of my thesis, I developed genetic tools for *V. parvula* cloning and mutagenesis using a recently described naturally competent strain. Using both random transposon and site-directed mutagenesis, I characterized *V. parvula* adhesins responsible for *in vitro* biofilm formation. In particular, I showed that secreted type V adhesins, the autotransporters, are the main determinants of *V. parvula* biofilm formation, mediating both bacteria-to-bacteria and bacteria-to-surface interactions. I also identified a putative HD-domain nucleotidase that prevented autotransporter-mediated biofilm formation and might be a newly described regulator of biofilm formation. More generally, I showed that the genomes of *Veillonellaceae* have a rich and diverse adhesins arsenal, suggesting rapid evolution of the adhesion properties of the cell. This study constitutes the first comprehensive study of *Veillonella parvula* adhesins and the first description of numerous, easily implemented genetic tools for this important symbiont.

Béchon N, Jiménez-Fernández A, Witwinowski J, Bierque E, Taib N, Cokelaere T, Ma L, **Ghigo JM**, Gribaldo S, **Beloin C**. 2020. Autotransporters drive biofilm formation and auto-aggregation in the diderm Firmicute *Veillonella parvula*. **Journal of Bacteriology**, in press.

Autotransporters drive biofilm formation and auto-aggregation in the diderm Firmicute *Veillonella parvula*.

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[§]The authors equally contributed to the work. Author order was determined alphabetically.

Running Title: Autotransporters drive *V. parvula* biofilm.

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ABSTRACT

The Negativicutes are a clade of Firmicutes that have retained the ancestral diderm character and possess an outer membrane. One of the best studied Negativicute, *Veillonella parvula*, is an anaerobic commensal and opportunistic pathogen inhabiting complex human microbial communities, including the gut and the dental plaque microbiota. Whereas adhesion and biofilm capacity of *V. parvula* is expected to be crucial for its maintenance and development in these environments, studies of *V. parvula* adhesion have been hindered by the lack of efficient genetic tools to perform functional analyses in this bacterium. Here, we took advantage of a recently described naturally transformable *V. parvula* isolate, SKV38, and adapted tools developed for the closely related *Clostridia spp.* to perform random transposon and targeted mutagenesis to identify *V. parvula* genes involved in biofilm formation. We show that type V secreted autotransporters -typically found in diderm bacteria- are the main determinants of *V. parvula* auto-aggregation and biofilm formation, and compete with each other for binding either to cells or to surfaces, with strong consequences on *V. parvula* biofilm formation capacity. The identified trimeric autotransporters have an original structure compared to classical autotransporters identified in Proteobacteria with an additional C-terminal domain. We also show that inactivation of the gene coding for a poorly characterized metal-dependent phosphohydrolase HD domain protein conserved in the Firmicutes and their closely related diderm phyla inhibits autotransporter-mediated biofilm formation. This study paves the way for further molecular characterization of *V. parvula* interactions with other bacteria and the host within complex microbiota environments.

IMPORTANCE

Veillonella parvula is an anaerobic commensal and opportunistic pathogen whose ability to adhere to surfaces or other bacteria and form biofilms is critical to inhabit complex human microbial communities such as the gut and oral microbiota. Although the adhesive capacity of *V. parvula* has been previously described, very little is known about the underlying molecular mechanisms due to a lack of genetically amenable *Veillonella* strains. In this study, we took advantage of a naturally transformable *V. parvula* isolate and newly adapted genetic tools to identify surface exposed adhesin called autotransporters as the main molecular determinants of adhesion in this bacterium. This work therefore provides new insights on an important aspect of *V. parvula* lifestyle, opening new possibilities for mechanistic studies of the contribution of biofilm formation to the biology of this major commensal of the oral-digestive tract.

INTRODUCTION

Negativicutes are atypical and poorly studied Firmicute lineages displaying an outer envelope with lipopolysaccharide (1). Among the Negativicutes, *Veillonella* spp. are anaerobic diderm cocci that commonly inhabit the human and animal microbiota. One of their best studied species, *Veillonella parvula* (2), is a natural inhabitant of multiple different microbiota, including the human gut (3, 4). *V. parvula* is considered a commensal organism, and proposed to play a role in the development of immunity through its capacity to colonize the infant gut (5, 6). It is a key early colonizer of the dental plaque during the establishment of sessile microbial communities called biofilms (7), promoting multi-species growth and playing a central role in the metabolism of community members through lactic acid consumption (8). However, *V. parvula* is also described as an opportunistic pathogen and has been associated with diverse infections, including osteomyelitis, endocarditis, spondylodiscitis, abscesses as well as systemic infections (9–13).

The importance of *V. parvula* in the development of microbial community spurred our interest in identifying the determinants of its adhesion and biofilm formation capacities. Moreover, considering the presence of an outer membrane in this atypical Firmicute, we wondered whether *V. parvula* uses known diderm or monoderm biofilm determinants, or currently undescribed adhesion factors. We recently studied *V. parvula* DSM2008 as a model diderm Firmicute strain (14) to investigate its outer membrane (OM) protein composition and detected 78 OM proteins, thirteen of which being potential adhesins belonging to the type V family of secreted autotransporter proteins (T5SS) (15). Autotransporter proteins are specifically found in diderms and all share common structural and functional features: a Sec-dependent signal peptide, a passenger domain providing the protein function, and an outer-membrane β -barrel domain that allows secretion of the passenger domain (16). However, the challenge of genetic manipulation in *V. parvula* DSM2008 severely limited the study of these adhesins in this strain.

Here, we have sequenced and annotated the genome of *V. parvula* SKV38, a recently isolated, naturally transformable and genetically amenable strain (17). We adapted and developed genetic tools for this organism, permitting random and site directed mutagenesis, plasmid complementation and controlled expression using an inducible promoter. This enabled us to identify and characterize factors involved in *V. parvula* biofilm formation. We find that

the main *V. parvula* biofilm modulating determinants are T5SS adhesins, i.e. typical diderm determinants. Interestingly, the identified adhesins possess an additional C-terminal domain compared to the known domain architecture of classical autotransporters. We also show that a locus encoding a metal-dependent phosphohydrolase HD domain protein is involved in biofilm formation, similarly to what was shown in the prototypical monoderm *Bacillus subtilis* (18). Therefore, our results demonstrate that diderm Firmicutes use a mixture of diderm and monoderm factors to modulate their ability to engage into biofilm lifestyle, supporting the idea that monoderm and diderm molecular systems could have co-evolved in these atypical Firmicutes.

RESULTS

Random transposon mutagenesis reveals two *V. parvula* SKV38 genes involved in biofilm formation

In order to obtain a framework for genetic work in the recently described naturally competent *V. parvula* SKV38 isolate, we sequenced it using PacBio technology. We obtained a completely assembled genome of 2.146Mbp, encoding 1,912 predicted protein-encoding open reading frame (ORF), 12 rRNA, 49 tRNA and one tmRNA (see Material and Methods). We performed random transposon mutagenesis in *V. parvula* SKV38 using the pRPF215 plasmid carrying an inducible transposase and a mariner-based transposon previously used to mutagenize *Clostridioides difficile* (19), a close relative of the Negativicutes. We screened 940 individual transposon mutants for biofilm formation using crystal violet staining (CV) static biofilm assay in 96-well microtiter plates and identified eight independent mutants with significant reduction in biofilm formation (Figure 1A). Whole genome sequencing localized the transposons in two loci putatively implicated in biofilm formation (Figure 1B). The most affected mutants correspond to insertions in *FNLLGLLA_00516* (seven mutants), encoding a T5SS type Vc trimeric autotransporter. One transposon mutant was inserted in *FNLLGLLA_01127*, encoding a putative HD phosphatase (Figure 1B).

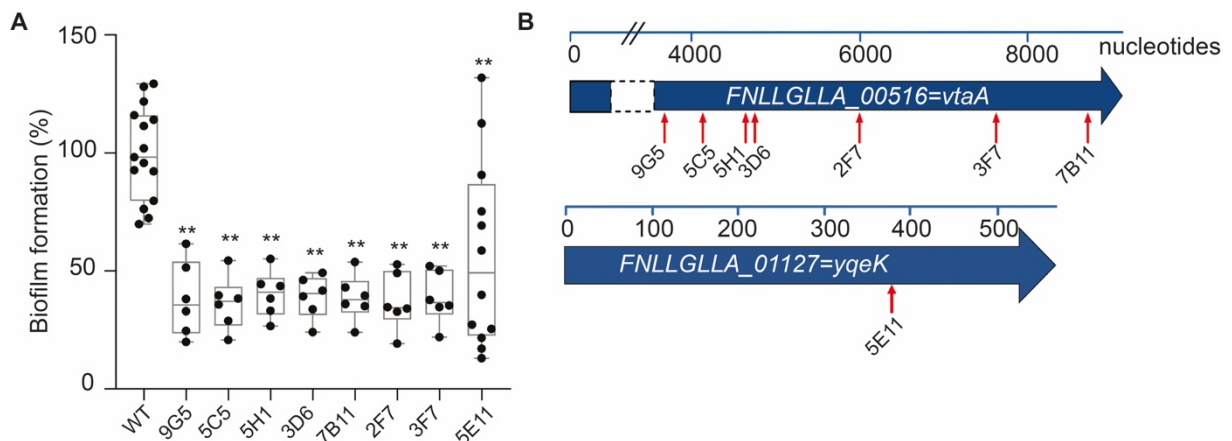


Figure 1: **Random transposon mutagenesis in *Veillonella parvula* SKV38 led to identification of mutants with reduced biofilm formation.** **A.** 96-well polystyrene plate biofilm assay after CV staining of nine transposon mutants identified by random mutagenesis grown 24h in BHILC. Mean of WT is adjusted to 100 %. Min-max boxplot of 6-15 biological replicates for each strain are represented, each replicate is the mean of two technical replicates. * p-value<0.05, ** p-value <0.005, Mann-Whitney test. **B.** Schematic representation of the transposon insertion point identified (red arrow) for the 8 transposon mutants. Blue bar represents the size of the gene in nucleotides.

***FNLLGLLA_00516* encodes a trimeric autotransporter involved in auto-aggregation**

FNLLGLLA_00516 encodes a protein containing several domains usually identified in the T5SS type Vc trimeric autotransporters. Trimeric autotransporters are OM proteins specific of diderm bacteria that have been widely studied for their ability to bind to different surfaces or to other bacteria (20). *FNLLGLLA_00516* is a homolog of *V. parvula* DSM2008 *vpar_0464*, which encodes a protein that was detected in the OM (15). *FNLLGLLA_00516* was annotated by PROKKA as BtaF, a trimeric autotransporter identified in *Brucella suis* involved in adhesion to extracellular matrix and abiotic surfaces (21). Here, we renamed it *Veillonella* trimeric autotransporter A (VtaA), as the first trimeric autotransporter involved in biofilm formation identified in *V. parvula* SKV38. We deleted the *vtaA* coding sequence and showed that $\Delta vtaA$ had no growth defect (Figure S1A) but displayed a marked reduction of biofilm formation in 96-well polystyrene microtiter plate (Figure 2A). Moreover, while *V. parvula* SKV38 cultures strongly aggregated, $\Delta vtaA$ did not (Figure 2B and S2). We constructed the strain *pTet-vtaA*, where the chromosomal *vtaA* gene is placed under the control of a functional tetracycline/anhydrotetracycline (aTc) inducible promoter (Figure S3) and showed that its aggregation capacity and biofilm formation in 96-well polystyrene microtiter plate directly correlated with aTc concentration (Figure 2C-D), demonstrating that VtaA-mediated cell-to-cell interactions are critical for biofilm formation in these conditions. Whereas the microtiter plate assay corresponds to a static biofilm assay, we also used continuous flow glass microfermentors to investigate the contribution of VtaA to biofilm formation in dynamic conditions. Surprisingly, $\Delta vtaA$ formed almost six times more biofilm than the WT strain in these conditions (Figure 2E). Accordingly, scanning electronic microscopy (SEM) images of mature biofilms on microscopic plastic slides in microfermentor showed that $\Delta vtaA$ formed a much thicker biofilm when compared to WT (Figure S4). Altogether, these results suggest that auto-aggregation differentially contributes to biofilm formation in static conditions on hydrophobic surfaces versus continuous flow conditions on hydrophilic surfaces.

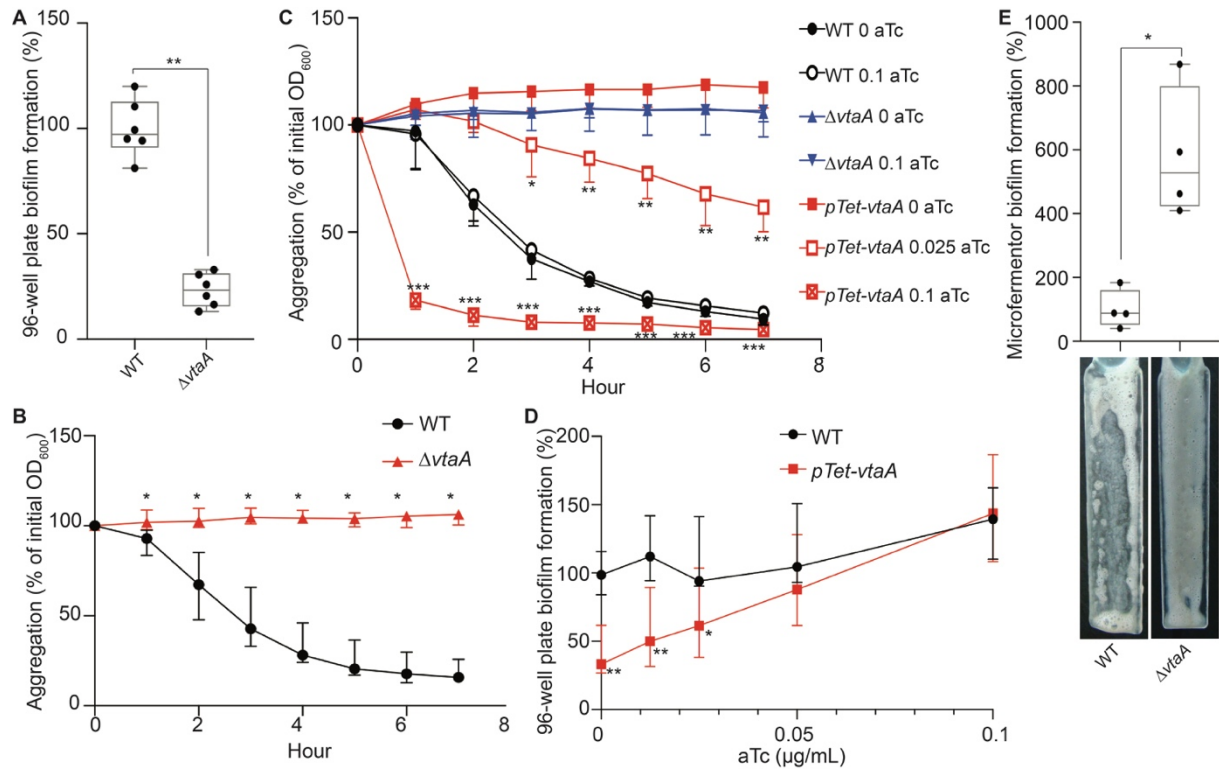


Figure 2: VtaA is an adhesin involved in auto-aggregation and biofilm formation. **A.** 96-well plate biofilm assay after 24h growth in BHILC. Mean of WT is adjusted to 100 %. Min-max boxplot of 6 biological replicates for each strain. * p-value<0.05, ** p-value <0.005, Mann-Whitney test between strains. **B.** and **C.** Aggregation curve in spectrophotometry cuvette of WT and $\Delta vtaA$ (**B**) and of an inducible *vtaA* with 0, 0.025 or 0.1 $\mu\text{g/mL}$ of the inducer aTc (**C**). 100 % represent lack of aggregation, 0 % complete sedimentation of the culture. Median of 6 biological replicates, error bars represent 95% confidence interval. At each time points we computed the Mann-Whitney test between conditions. We applied Bonferroni correction for multiple testing: p-value are only considered significant if *p-value<0.004, **p-value<0.0004, ***p-value<0.00004. Indicated p-values were calculated by comparing in **B**, WT and $\Delta vtaA$, and in **C**, *pTet-vtaA* without aTc and *pTet-vtaA* with different aTc concentrations. **D.** 96-well plate biofilm assay after 24h growth of an inducible *vtaA* in BHILC with different concentrations of aTc. WT without aTc is adjusted to 100 %. Median of 6 biological replicates, each replicate corresponds to the mean of two technical replicates, error bars represent 95% confidence interval. * p-value<0.05, ** p-value <0.005, Mann-Whitney test. **E.** Biofilm formation in continuous flow microfermentor on glass spatula during 48h in BHILC. WT was adjusted to 100 %. Min-max boxplot of 4 biological replicates for each strain. A picture of the spatula before resuspension is shown below each boxplot bar. * p-value<0.05, Mann-Whitney test.

V. parvula SKV38 encodes sixteen putative autotransporters in addition to VtaA

The strong biofilm phenotype displayed by the $\Delta vtaA$ mutant in microfermentor led us to suspect that additional adhesins could modulate *V. parvula* biofilm formation capacity. Indeed, searching the *V. parvula* SKV38 genome revealed multiple genes encoding autotransporters (Table 1): three Va classical monomeric autotransporters with a characteristic PFAM_PF03797 autotransporter β -domain (renamed *Veillonella* monomeric autotransporter A to C : VmA to C), and eight other putative Vc trimeric autotransporters with a characteristic

PFAM_PF03895 YadA _anchor_domain (renamed *Veillonella* trimeric autotransporter B to I: VtaB to I). We also identified several partial autotransporters: *FNLLGLLA_00035*, that only contains a PFAM_PF11924 Ve inverse autotransporter β -domain but no putative α -domain that normally carries the function of the protein, and *FNLLGLLA_00036-37* and *FNLLGLLA_00040-41*, which are homologs of *V. parvula* DSM2008 *Vpar_0041* and *Vpar_0048*, respectively, and that appear to be split in SKV38 (Table 1). Interestingly, domain analysis of all trimeric ATs of *V. parvula* SKV38 showed that they possess an extra C-terminal-domain (SLH or coiled-coil domain) after the YadA anchor domain that is not found in classical trimeric ATs. Among those, six autotransporters plus *FNLLGLLA_00035*, *FNLLGLLA_00036-37* and *FNLLGLLA_00040-41* form a potential genomic cluster coding for adhesins (Figure 3A), whereas the six others are located in different areas of the genome (Figure 3B).

We selected eight *Veillonella* strains to study more precisely the evolution of the adhesin cluster, including SKV38 and DSM2008. The trimeric autotransporter adhesins seem to evolve dynamically with numerous domain swaps, duplications and reductions of gene copies, likely through homologous recombination suggesting rapid evolutionary changes in the repertoire of *Veillonella* adhesins (Figure 4). Duplications and deletions could be eased by the presence of short ORFs annotated as hypothetical proteins presenting a high degree of sequence identity. The most basal strain in the *Veillonella* phylogeny has a minimal cluster of only three adhesins genes. Throughout the *Veillonella* genus, the size of the cluster is very variable with a minimal form in *V. atypica*, with only two adhesins. This specific adhesin locus, immediately upstream of rRNA coding genes, is to our knowledge a peculiar genomic character of the *Veillonella* genus and is not found in other genera of the Veillonellaceae, suggesting it originated in the common ancestor of all *Veillonella* species.

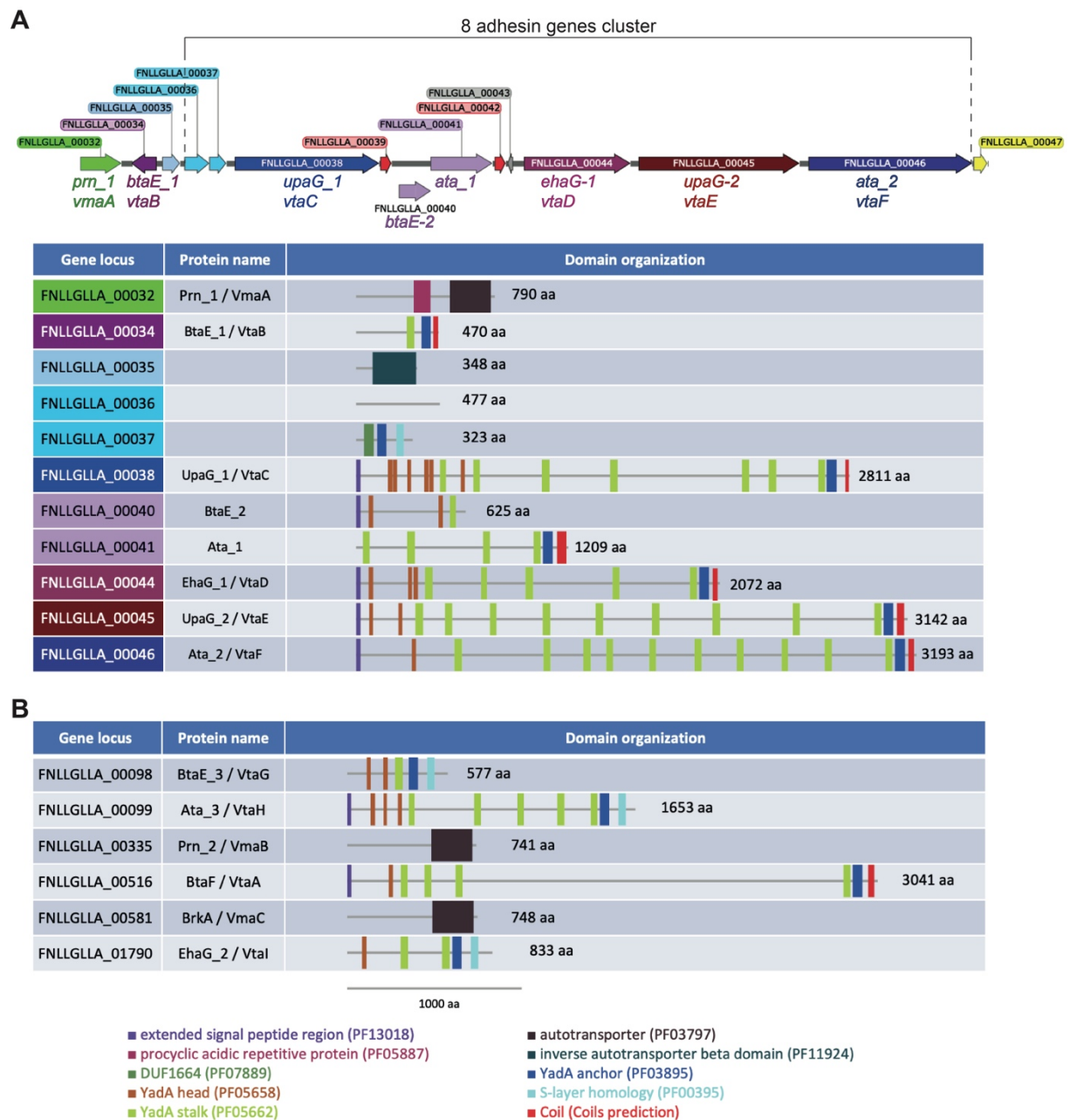


Figure 3: *Veillonella parvula* autotransporters domain organization. **A.** Genetic organization of the *V. parvula* SKV38 autotransporter adhesin gene cluster and the corresponding adhesin domain organization. **B.** Domain organization of the six remaining *V. parvula* SKV38 autotransporter adhesins encoded by genes located outside of the cluster. Domains were detected with the HMMER package (60), only the domains with e-values lower than 10^{-3} are shown. Presence of C-terminal coils structure was determined using the COILS program (https://embnet.vital-it.ch/software/COILS_form.html). All *V. parvula* trimeric ATs display an additional C-terminal domain (a SLH or a coiled coil domain) following the YadA anchor domain as compared to classical trimeric autotransporters.

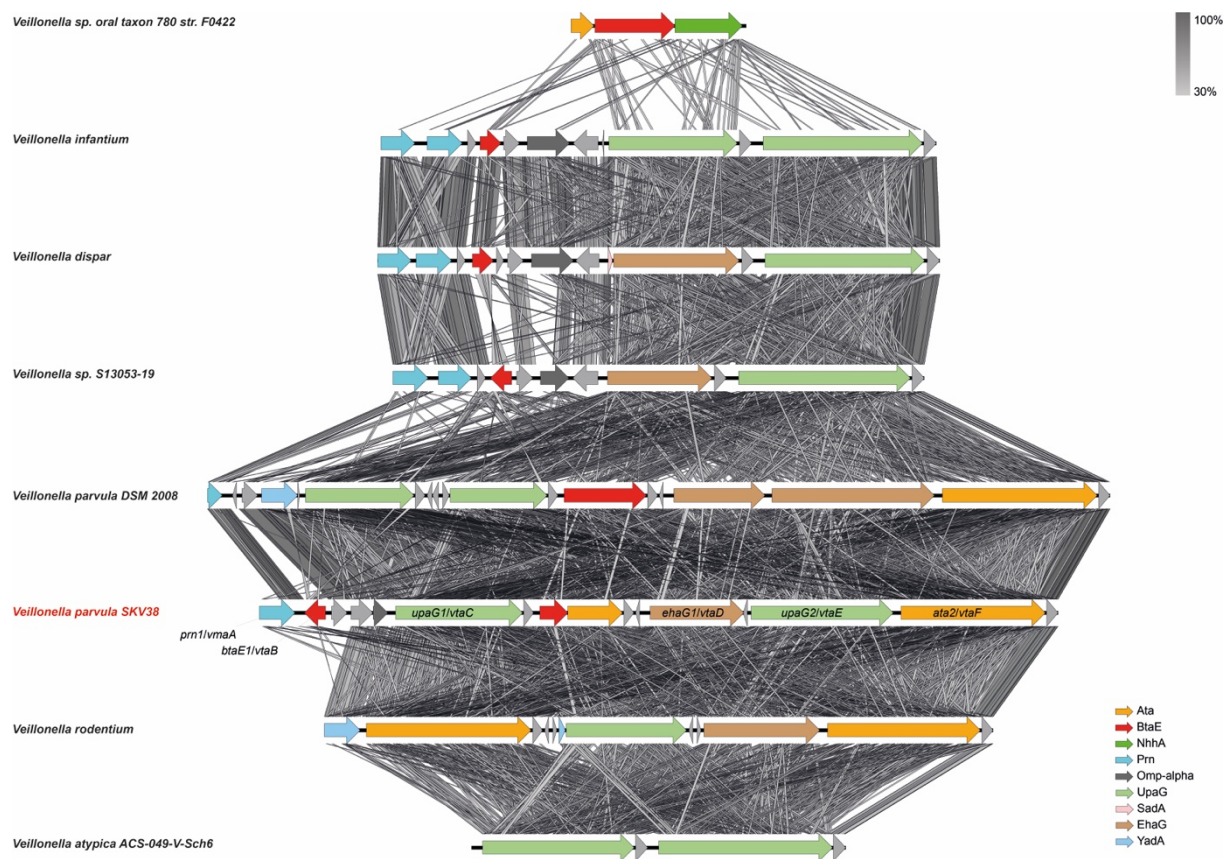


Figure 4: **Synteny of the adhesin gene cluster in a selection of *Veillonella* species.** The synteny of the proteins of the cluster between the closest relatives was assessed using EasyFig (69). Oblique lines between genes represent tblastx identities (program parameters: maximum e-value of 10^{-2} , minimum length of 30, minimum identity of 30). The *V. parvula* SKV38 strain used in this study is presented in red. The annotation of the genes of the cluster is indicated on the right.

The cluster of trimeric autotransporters is involved in surface binding and not aggregation.

To assess the function of the potential adhesins identified in the *V. parvula* SKV38 genome, we constructed -within the cluster of adhesin genes- independent deletion mutants for the two first autotransporters (*vmaA* and *vtaB*) and a large deletion for the eight adjacent genes encoding trimeric autotransporters or partial trimeric autotransporters, hereafter called $\Delta 8$ ($\Delta F_{NLLGLLA_00036}$ to *vtaF*). We also generated independent individual mutants for each of the six additional autotransporters located outside of the cluster. These mutants were all tested for biofilm formation in 96-well polystyrene plate and aggregation capacities. None of the mutants, with the exception of the previously mentioned $\Delta vtaA$ strain, was affected for aggregation capacities (Figure 5A). The $\Delta 8$ mutant was the sole mutant, in addition to the $\Delta vtaA$ mutant, to display lower biofilm formation in 96-well polystyrene microtiter plate (Figure 5B and C), suggesting that the adhesins of this cluster could be involved in biofilm formation

independently of cell-to-cell interactions. When tested in microfermentor, $\Delta 8$ displayed a slight reduced ability to form mature biofilm, however, not statistically different from WT (Figure 5D). This reduced ability to form mature biofilms was actually more visible when observing SEM images, since the $\Delta 8$ mutant only poorly covered the coverslip with sporadic aggregates of cells producing extracellular matrix (Figure S4). Initial adhesion assay to glass spatula showed that both $\Delta vtaA$ and $\Delta 8$ displayed a lower percentage of initial adhesion than WT, suggesting that VtaA-mediated auto-aggregation contributed to initial adhesion of the WT strain while the adhesin cluster is probably directly involved in surface binding (Figure 5E). This also indicates that $\Delta vtaA$ does not adhere to glass better than WT, and so the increased biofilm formation of $\Delta vtaA$ in microfermentor arises during the continuous flow culture step. The effect of deleting *vtaA* and the 8 adhesin genes on initial adhesion was additive since a $\Delta vtaA\Delta 8$ double mutant showed a reduced initial adhesion on microfermentor spatula compared to either WT, $\Delta vtaA$ or $\Delta 8$ (Figure 5E). In addition, $\Delta vtaA\Delta 8$ formed 17 times less biofilm than $\Delta vtaA$ in microfermentor, indicating that in the absence of VtaA, the adhesins encoded by some of these eight genes strongly promote mature biofilm formation in microfermentor (Figure 5D).

Taken together, these results demonstrate the differential contribution of VtaA and part of the cluster of adhesin to *V. parvula* SKV38 adhesion and highlight the existence of potential interference mechanisms between them.

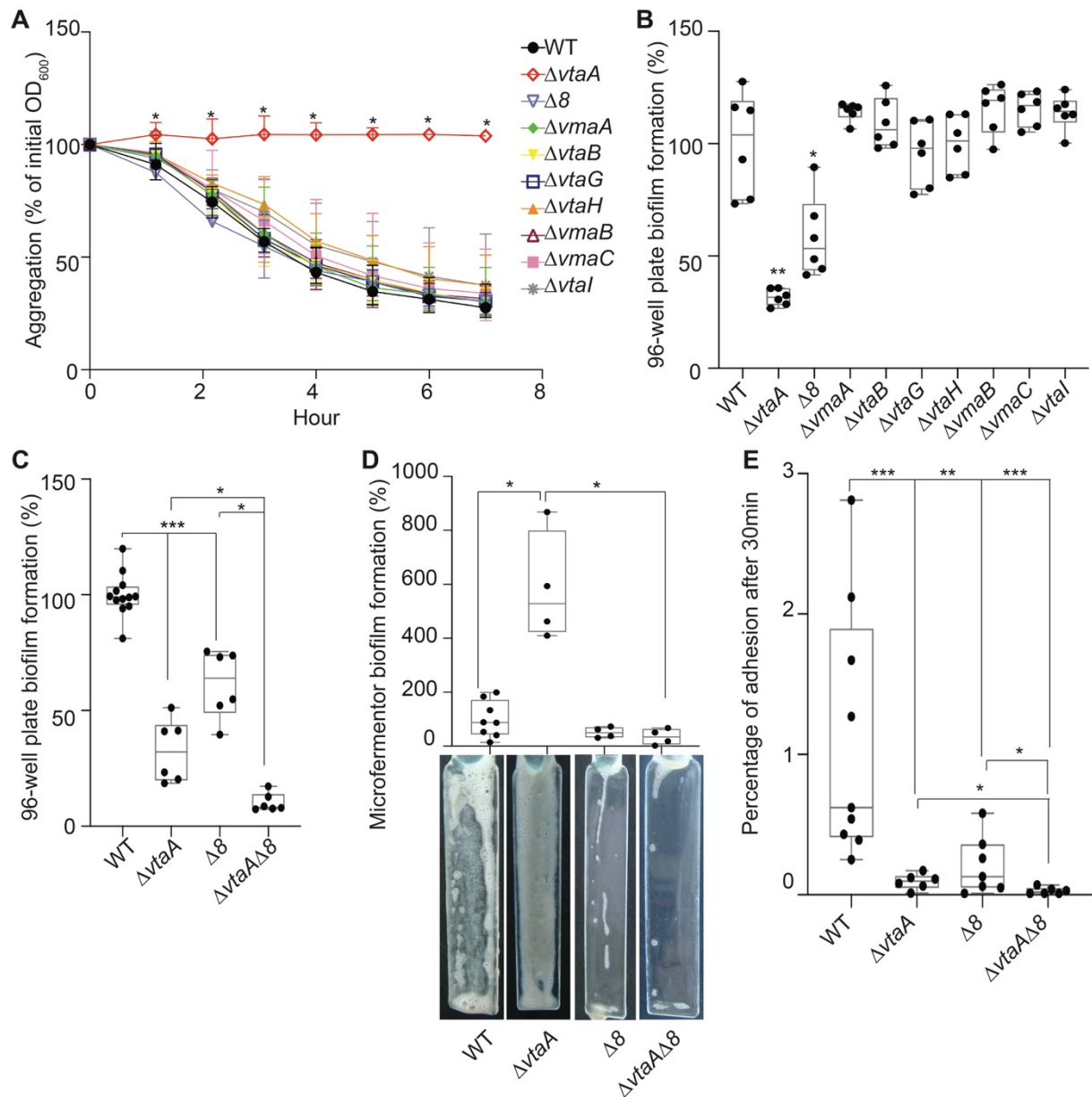


Figure 5: A cluster of eight trimetric autotransporters is involved in surface binding. **A.** Aggregation curve in spectrophotometry cuvette. 100 % represent lack of aggregation, 0 % complete sedimentation of the culture. Median of 6 biological replicates, error bars represent 95% confidence interval. * Mann-Whitney test, corrected for multiple testing with Bonferroni correction: significance is achieved if p-value < 0.007. **B.** and **C.** 96-well plate biofilm assay after 24 h growth in BHILC. Mean of WT is adjusted to 100 %. Min-max boxplot of 6 biological replicates for each strain, each replicate is the mean of two technical replicates. In **B.** we applied a Mann-Whitney; * p-value < 0.05, ** p-value < 0.005. In **C.** we applied Bonferroni correction for multiple testing: tests were called significant only if p-value < 0.01: * p-value < 0.01, ** p-value < 0.001, *** p-value < 0.0001. **D.** Biofilm formation in continuous flow microfermentor on glass spatula during 48h in BHILC. WT was adjusted to 100 %. Min-max boxplot of 4 biological replicates for each strain. * p-value < 0.05, Mann-Whitney test. A picture of spatula before resuspension is shown for each mutant below the boxplot. **E.** Initial adhesion on glass spatula. Percentage of CFU that adhered to the spatula controlled by the number of CFU of the inoculation solution. Min-max boxplot of 6-9 replicates per strain is represented. * p-value < 0.05, ** p-value < 0.005, *** p-value < 0.0005, Mann-Whitney test.

FNLLGLLA_01127 encodes an HD phosphatase that inhibits biofilm formation

In addition to genes encoding potential T5SS proteins, we also identified a transposon mutant in *FNLLGLLA_01127*, encoding a protein of the HD phosphatase superfamily (Figure 1B). The *FNLLGLLA_01127* gene is homologous to YqeK, a putative phosphatase required for pellicle formation and the development of biofilm in *B. subtilis* (18). *FNLLGLLA_01127/yqeK* is found in a cluster of genes (*obg*, *yhbY*, *proB*, *proA*, *nadD*, *yqeK*, *lytR*, and *rsfS*), whose synteny is very well conserved among Negativicutes. This cluster, or part of it, is also well conserved in almost all Firmicutes genomes we analyzed, both monoderm and diderm, as well as in members of other diderm phyla phylogenetically close to the Firmicutes, notably Deinococcus-Thermus (Figure 6 and S5, DataSet 2). A *FNLLGLLA_01127* deletion mutant ($\Delta I127$) had a lower carrying capacity compared to WT, maybe due to higher mortality during the stationary phase (Figure S1) and a moderate 1.5-fold decrease in biofilm formation in microtiter plate after correcting for the growth defect (Figure 7A). This mutant also displayed a slightly faster aggregation rate than the WT during early time points (Figure 7B). The strongest phenotype of this mutant was detected in microfermentor with a 9-fold increase in biofilm formation compared to WT (Figure 7C). Expression of *FNLLGLLA_01127* gene *in trans* (plasmid *p1127*) did not complement the observed growth defect (Figure S1B) but it did complement the increased biofilm formation in microfermentor (Figure 7D), showing that deletion of *FNLLGLLA_01127* might have had polar effects on downstream genes of the operon causing a growth defect, but that *FNLLGLLA_01127* alone was responsible for the observed inhibition of biofilm formation. Scanning electronic microscopy showed that $\Delta I127$, similarly to $\Delta vtaA$, formed a thick layered biofilm, although with fewer filaments and protein deposits compared to WT (Figure 7E). However, contrary to $\Delta vtaA$ or $\Delta 8$ mutants, $\Delta I127$ showed no defect in initial adhesion to a glass spatula (Figure 7F). Interestingly, a $\Delta I127\Delta 8$ double mutant formed almost 20 times less biofilm than $\Delta I127$ in microfermentor (Figure 7C), suggesting that at least some of the autotransporters of the cluster were necessary for $\Delta I127$ observed strong biofilm formation in microfermentor.

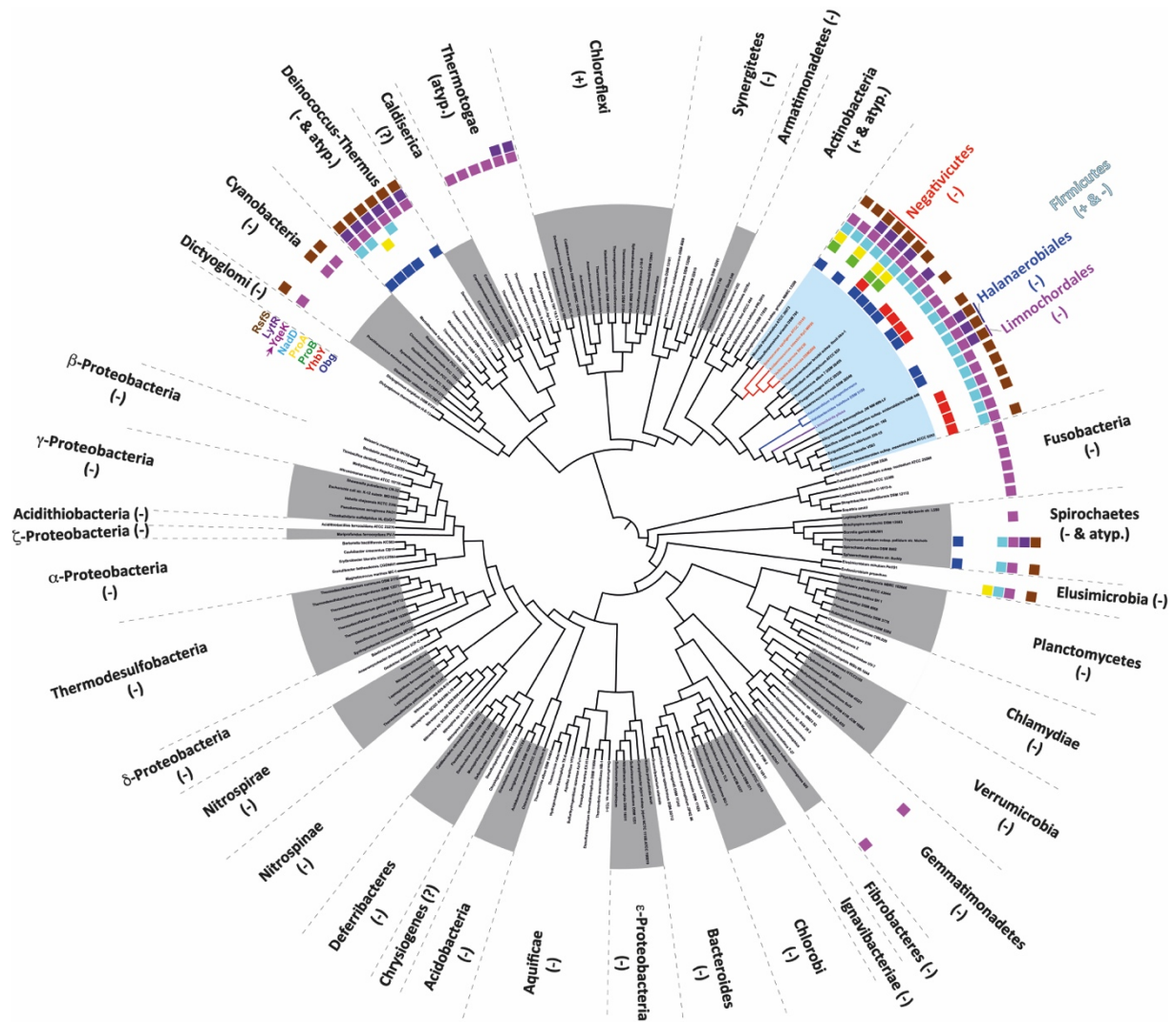


Figure 6: Occurrence and synteny of HD Phosphatase (YqeK) in diderm and monoderm bacteria. A. The presence of the cluster was investigated using MacSyFinder (62) and the results were plotted onto a schematic reference tree of 187 cultivable bacteria among the 390 of the analyzed databank. The cell wall status of each phylum is indicated as: (-) diderm with LPS, (+) monoderm, (atyp.) diderm without LPS, (?) unclear. For the Firmicutes, the diderm lineages are indicated in red (Negativicutes), blue (Halanaerobiales) and purple (Limnochordales).

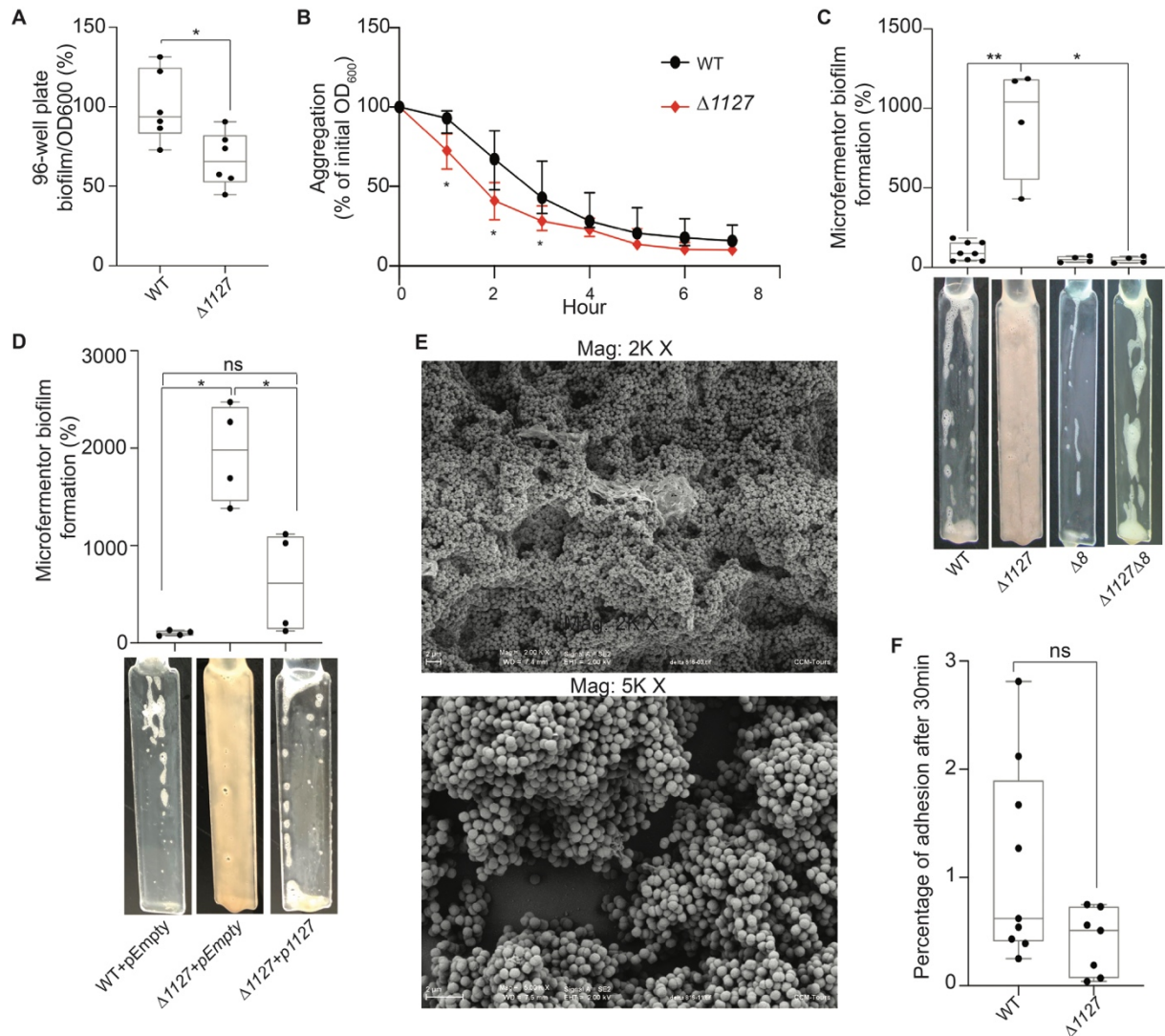


Figure 7: FNNLGLLA_01127 represses biofilm formation in microfermentor. **A.** 96-well plate biofilm assay after 24 h growth in BHILC corrected by OD₆₀₀ after 24 h growth in plate. Mean of WT is adjusted to 100 %. Min-max boxplot of 6 biological replicates for each strain, each replicate is the mean of two technical replicates. * p-value < 0.05, Mann-Whitney test. **B.** Aggregation curve in spectrophotometry cuvette. 100 % represent lack of aggregation, 0 % complete sedimentation of the culture. Median of 6 biological replicates, error bars represent 95% confidence interval. * Mann-Whitney test, corrected for multiple testing with Bonferroni correction: significance is achieved if p-value<0.007. **C.** Biofilm formation in continuous flow microfermentor on glass spatula during 48h in BHILC. Mean of WT is adjusted to 100 %. Min-max boxplot of 4 biological replicates for each strain. * p-value < 0.05, ** p-value<0.005, Mann-Whitney test. A picture of a spatula before resuspension is shown for each strain below the histogram. **D.** Biofilm formation in continuous flow microfermentor on glass spatula during 48h in BHILC+chloramphenicol. Mean of WT+pEmpty is adjusted to 100 %. Min-max boxplot of 4 biological replicates for each strain. * p-value < 0.05, Mann-Whitney test. A picture of a spatula before resuspension is shown for each strain below the boxplot. **E.** Scanning electronic microscopy of Δ1127 biofilm grown under continuous flow of BHILC in microfermentor on a plastic microscopy slide. Magnification 2K and 5K. **F.** Initial adhesion on glass spatula. Percentage of CFU that adhered to the spatula in 30 min controlled by the number of CFU of the inoculation solution. Min-max boxplot of 6-9 replicates per strain. * p-value<0.05, Mann-Whitney test.

DISCUSSION

Originally described as a social organism mostly living in biofilm communities (8), *Veillonella* is a known bacterial member of multiple human microbiota. Biofilm formation and adhesion are important in these niches, but their study in *Veillonella* has been hindered by the lack of efficient genetic tools. Here, we used genetics tools adapted from *Clostridia* to characterize factors promoting biofilm formation in a naturally competent *Veillonella parvula* isolate.

We identified a T5SS type Vc trimeric autotransporter, FNLLGLLA_0516 (VtaA), as an important biofilm factor promoting *V. parvula* SKV38 auto-aggregation. In addition to Hag1, a YadA-like autotransporter identified from the related species *V. atypica* involved in interspecies interactions (22), VtaA represents the second *Veillonella* protein described which is involved in adhesion, and the first involved in abiotic surface adhesion and auto-aggregation in diderm Firmicutes. Beyond the potential impact on *Veillonella* niche colonization, aggregation capacity is known to contribute to bacterial protection from environmental stresses or host responses (23), promotion of host colonization (24), or pathogenesis (25) in various bacterial species. VtaA is homologous to *Brucella suis* trimeric autotransporter BtaF. However, while *B. suis* BtaF promotes biofilm formation *in vitro*, it was not shown to promote aggregation (21), suggesting that these two proteins have different functions.

In diderm bacteria such as *E. coli*, self-associating autotransporters (SAATs) from the type Va family and type Vc trimeric autotransporters were shown to contribute to biofilm formation through their self-recognition properties (26–32). However, in *V. parvula* VtaA-mediated auto-aggregation either promoted (plastic surface and static conditions) or strongly impaired (glass surface and continuous-flow conditions) biofilm formation depending on the model used. $\Delta vtaA$ initially adhered less to glass spatula compared to WT, even though later on it formed much more biofilm, thus we suspect that the material (glass vs. plastic) is not responsible for the observed difference between our two systems. We hypothesize instead that in the WT, VtaA-mediated aggregates are more sensitive to flow than individual cells, and are thus washed out faster of the microfermentor, and that adhesion to surfaces or to the biofilm extracellular matrix is more important than cell-to-cell interactions when the culture is performed under continuous flow.

Interference between cell surface structures is a well-described mechanism by which bacteria modulate their adhesion properties. In *E. coli*, multiple structures, such as chaperone-usher fimbriae, LPS O-antigen or capsules, interfere with the self-recognizing autotransporter Ag43

though unknown mechanisms (33–36). Therefore, it is possible that in *V. parvula*, VtaA could compete with other adhesins through steric hindrance or competition for membrane export and thus limit biofilm formation under continuous-flow conditions. Consistently, $\Delta vtaA$ enhanced biofilm formation in microfermentor was dependent on the presence of eight genes of the cluster of trimeric autotransporters, suggesting a competition between VtaA and adhesin(s) of this cluster. Moreover, we noticed that both VtaA and the 8-gene cluster are necessary for full initial adhesion to glass spatula in an independent manner, suggesting that any competition between them only arises later on, during continuous-flow cultures. The exact contribution of these different trimeric autotransporters to biofilm formation and their interplay with VtaA will require further characterization.

Analysis of *V. parvula* SKV38 genome revealed the presence of seven other potential full-length autotransporters, but no other types of classical diderm adhesins. None of them appeared to be involved in cell-to-cell interactions or biofilm formation on abiotic surfaces, and their function remains to be fully elucidated. As *V. parvula* is present in different microbiota, it is expected that a large arsenal of adhesion factors is necessary to adhere under different mechanical constraints and on different surfaces, such as tooth enamel or various epithelia. Moreover, *Veillonella* is known to co-aggregate with *Streptococci* (37–39), that produce *Veillonella* favored carbon source, lactate (40), and they were shown to specifically co-aggregate with *Streptococci* and *Actinomyces* strains from the same microbiota, showing that co-aggregation could have strong implication in niche colonization of these bacteria (41). *V. parvula* and other *Veillonella* are also associated to different opportunistic infections and the contribution of their adhesins to pathogenicity remains to be addressed. Finally, some autotransporters have been shown to carry non-adhesive functions, including protease activity (42), but we detected no classical protease domain in the *Veillonella* autotransporters.

Trimeric autotransporters possess a characteristic YadA_anchor domain (PF03895) that is found mainly in Proteobacteria, but also in Cyanobacteria, Verrumicrobia, Planctomycetes, Kiritimatiellaeota, Chlorobi, Synergistetes, Fusobacteria and Negativicutes (<https://pfam.xfam.org/family/PF03895>, Dec 2019 (43)). Interestingly, the YadA_anchor of *V. parvula* SKV38 and all *Veillonella* trimeric autotransporters is not at the very end of the C-terminus, where it is usually found in Proteobacteria, but is pre-C-terminal, followed by either a coiled domain or a S-layer homology (SLH) domain (Figure 3, DataSet 1). While the function of the coiled domain is unknown, in some bacteria the periplasmic SLH domain binds to

peptidoglycan (44), suggesting that *Veillonella* trimeric autotransporters could be non-covalently attached to the peptidoglycan. These extra-domains after the YadA_anchor are also found in other Negativicutes (notably the extra SLH domain) and in some other diderm phyla phylogenetically related to the Firmicutes such as Synergistetes and Fusobacteria (DataSet 1). In addition to possessing trimeric autotransporters with an extra coiled C-terminus domain, the Fusobacterium *Streptobacillus moniliformis* ATCC14647 carries eight genes encoding unique trimeric autotransporters with an extra OmpA_family domain (PF00691) at their extreme C-terminus, a domain known to display affinity to peptidoglycan (45) (DataSet 1). These data suggest that a subset of phylogenetically close diderm bacteria have evolved trimeric autotransporters integrating different peptidoglycan binding domains. Whether these domains have an impact on trimeric autotransporters function or exposure to the surface, or more generally on outer membrane stabilization is presently unknown.

Our screening also led to the identification of FNLLGLLA_01127, the homolog of *B. subtilis* YqeK, a putative phosphatase required for pellicle formation and the development of biofilm (18). *Staphylococcus aureus* YqeK was recently shown to be a nucleosidase hydrolyzing diadenosine-tetraphosphate (Ap4A) into ADP (46). In *Pseudomonas fluorescens*, an increased level of Ap4A increases cyclic-di-GMP concentration and enhances cell-surface exposure of a large adhesin LapA, thus inducing biofilm formation (47). c-di-GMP regulates biofilm formation by modulating production of a variety cell-surface appendages or exopolysaccharides in both monoderm and diderm bacteria (48–52). Interestingly, *B. subtilis* YqeK induces the *epsA-O* operon, involved in the production of biofilm matrix-forming polysaccharides (53). Deletion of *V. parvula* FNLLGLLA_01127 only led to a minor decrease in biofilm formation in 96-well plate, but to a strong increase in continuous-flow biofilm formation that was dependent on the presence of the cluster of trimeric autotransporters. Further work is needed to determine whether FNLLGLLA_01127 directly impacts production of the adhesins of the cluster or participates to the production/regulation of an unknown exopolysaccharide, which, contrary to *B. subtilis*, would interfere with the function or exposure of the adhesins of the cluster rather than favor community development.

In this study we have shown that classical diderm trimeric autotransporters and a potential nucleotidase, conserved both in monoderms and diderms are crucial for adhesion both between cells and/or to surfaces in the diderm Firmicute *V. parvula*. Our work also underscores the rapid evolution of a diverse arsenal of trimeric autotransporters in the *Veillonella* genus, both in

numbers and size, probably by efficient recombination favored by gene clustering, allowing rapid adaptation to changing environments. Taken together, our results suggest a complex interplay at the surface of *V. parvula* between different cell surface structures that may have co-evolved for a long time in these atypical Firmicutes. Much remains to be discovered on the regulatory circuits controlling these adhesion factors and their role in diderm Firmicutes biology.

MATERIALS AND METHODS

Genome preparation and sequencing

V. parvula SKV38 genomic DNA was extracted using Qiagen genomic tip 20G kit. It was sequenced to 1,500X coverage using PacBio sequencing of one single molecule real time (SMRT) cell with no multiplexing using the V2.1 chemistry. Only one SMRT cell was used but with no multiplexing, leading to an unusually large amount of subreads: 3 Gbp, meaning about 1,500X coverage assuming a 2.1 Mbp genome. This yielded 338,310 reads with a mean subread length of 9,080 bp and N50 read length of 13,500 bp. The longest subread length is above 70 kbp. We randomly subsampled the data to avoid misassemblies keeping only 100,000 subreads, which resulted in a 430X coverage. The genome was then assembled using Canu version 1.8 (54) keeping the default parameters. In particular, subread below 1,000 bp were dropped. The error correction steps of the Canu algorithm were not tuned, keeping the parameters that control alignment seed length, read length, overlap length and error rates to their default values. We obtained one contig of 2.146 Mbp and an additional contig of only 1,972 bp that was abandoned due to lack of supporting data and was removed by the circularization process. The obtained assembled genome closely matched the genome size (2.1422 Mbp) and GC content (38.7%, expected 38.6%) of the reference *V. parvula* DSM2008 strain. The resulting assembled genome was polished using Pilon (55) but no correction was required. No gaps or drops of coverage was detected based on sequana_coverage output (56, 57). The completeness of the candidate assembly was assessed to be 98% using the bacteria mode and the bacteria_db9 lineage-specific profile library of BUSCO software (58), while the number of complete duplicated or fragmented BUSCOs remains at 0, indicative of complete assembly. Alignment of all reads show that only 4% (13,028) remained unmapped and 80% of their length were below 2 kbp. The remaining reads (2000 reads) map on various species and could not be further assembled. Overall, these analyses indicate that the final genome assembly is complete and of good quality.

Bioinformatic analyses

The *V. parvula* SKV38 genome was annotated using PROKKA (59). The SKV38 annotated genome sequence was deposited in the ENA (European Nucleotide Archive) under the accession number ERZ1303164.

For protein domain visualization, PFAM domains (pfam.xfam.org, Pfam 32.0. (43)) were detected using HMMER (60). Domains with an e-value lower than 10^{-3} were kept and, in case of overlapping domains, the domain having the best e-value was kept. Presence of C-terminal coils structure was determined using the COILS program (https://embnet.vital-it.ch/software/COILS_form.html) (61).

The search for HD phosphatase (YqeK) cluster homologs was conducted as follows: a local databank containing 390 genomes representative of bacterial diversity was mined for the presence of a phosphatase containing HD domain (PF01966) using HMMSEARCH and the --cut_ga option. Protein sequences were then filtered using alignment, functional annotation, protein domains presence and phylogeny. Synteny was investigated in the locus around *yqeK* by looking, using MacSyFinder (62), for the presence of at least one of the 7 genes surrounding *yqeK* in *V. parvula* SKV38, namely *obg* (containing GTP1_OBG domain, PF01018), *yhbY* (containing CRS1_YhbY domain, PF01985), *proB* (containing AA_kinase domain, PF00696), *proA* (containing Aldedh domain, PF00171), *nadD* (containing CTP_transf_like domain, PF01467), *lytR* (containing LytR_cpsA_psr domain, PF03816) and *rsfS* (containing RsfS domain, PF02410), with no more than eight other genes separating them. All HMM profiles were downloaded from the PFAM site (pfam.xfam.org). As YqeK homologs are widespread in the Firmicutes, another local databank containing 230 representative Firmicutes genomes was queried by the MacSyFinder approach as described above. All trees were visualized with ITOL (63). Details of the results are presented in Dataset S2.

Strains and growth conditions

Bacterial strains and plasmids are listed in Table 2. *V. parvula* was grown in either Brain Heart infusion medium (Bacto Brain Heart infusion, Difco) supplemented with 0.1 % L-cysteine and 0.6 % sodium DL-lactate (BHILC) or SK medium (10 g L⁻¹ tryptone (Difco), 10 g L⁻¹ yeast extract (Difco), 0.4 g L⁻¹ disodium phosphate, 2 g L⁻¹ sodium chloride, and 10 mL L⁻¹ 60 % w/v

sodium DL-lactate, described in (17)) and incubated at 37°C in anaerobic conditions in anaerobic bags (GENbag anaero, Biomerieux, ref. 45534) or in a C400M Ruskinn anaerobic-microaerophilic station. *Escherichia coli* was grown in Lysogeny Broth (LB) (Corning) medium under aerobic conditions at 37°C. 20 mg L⁻¹ chloramphenicol (Cm), 200 mg L⁻¹ erythromycin (Ery) or 2.5 mg L⁻¹ tetracycline (Tc) were added to *V. parvula* cultures, 100 mg L⁻¹ carbenicillin (Cb) or 5 mg L⁻¹ tetracycline (Tc) were added to *E. coli* cultures when needed. 100 µg L⁻¹ anhydrotetracycline (aTc) was added to induce the pTet promoter unless stated otherwise. All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Natural transformation

Cells were resuspended in 1 mL SK media adjusted to 0.4-0.8 OD₆₀₀ and 10 µL were dotted on SK-agar petri dishes. On each drop, 0.5-1 µg plasmid or 75-200 ng µL⁻¹ linear dsDNA PCR product was added, or water for the negative control. The plates were incubated 48 hours. The biomass was resuspended in 500 µL SK medium and plated on SK-agar supplemented with the corresponding antibiotic and incubated for another 48 hours. Colonies were streaked on fresh selective plates and the correct integration of the construct was confirmed by PCR and sequencing.

Random mariner transposon mutagenesis

Plasmid pRPF215, described for *Clostridium* mutagenesis (Addgene 106377) (19) was transformed into *V. parvula* SKV38 by natural transformation and selected on Cm supplemented SK-agar medium. An overnight culture of *V. parvula* SKV38-pRPF215 in BHILC was then diluted to 0.1 OD₆₀₀ in the same media, supplemented with aTc and grown 5 hours to induce the transposase. After induction, the culture was diluted and plated on BHILC supplemented with Ery and aTc for selection and incubated for 48 hours. From the resulting colonies, 940 were inoculated in Greiner Bio-one polystyrene flat-bottom 96-well plates (655101) and grown in BHILC supplemented with either Ery and aTc, or Cm, to confirm both the presence of the transposon and the loss of pRPF215 and then kept in 15 % glycerol at -80°C. Selected transposon mutants were grown overnight and the genomic DNA was harvested using DNeasy blood and tissue kit (Qiagen). The genomic DNA was then sent for whole genome sequencing at the Mutualized platform for Microbiology of Institut Pasteur.

Cloning-independent allelic exchange mutagenesis

Site-directed mutagenesis of *V. parvula* SK38 strain was performed as described by Knapp and colleagues (17). Briefly, 1-Kb regions upstream and downstream the target sequence and *V. atypica* tetracycline resistance cassette (*tetM* in pBSJL2) or *catP* resistance cassette from *C. difficile* (*catP* in pRPF185, Addgene 106367, from (64)) were PCR-amplified with overlapping primers using Phusion Flash High-Fidelity PCR Master-Mix (Thermo Scientific, F548). PCR products were used as templates in a second PCR round using only the external primers that generated a linear dsDNA with the tetracycline resistance cassette flanked by the upstream and downstream sequences. This construct was transformed into *V. parvula* by natural transformation and its integration into the genome was selected by plating on Tc or Cm supplemented medium. Positive candidates were further confirmed by a set of PCRs and sequencing around the site. Primers used in this study are listed in Table S1.

Complementation

We replaced the tetracycline resistance gene and its *gyrA* promoter of the shuttle vector pBSJL2 by a chloramphenicol resistance gene, P_{cat} :*cat* from pRPF185 by Gibson assembly. Briefly, the inserts and the plasmids were PCR amplified and then mixed with Gibson master mix 2x (100 μ L 5X ISO Buffer, 0.2 μ L 10,000 U/mL T5 exonuclease (NEB #M0363S), 6.25 μ L 2,000 U/mL Phusion HF polymerase (NEB #M0530S), 50 μ L 40,000 U/mL Taq DNA ligase (NEB #M0208S), 87 μ L dH₂O for 24 reactions) and incubated at 50°C for 30-60 min.

The resulting plasmid pBSJL2-*cat* was digested by Fastdigest *Bam*HI (Thermo scientific) and the band obtained was purified from agarose gel using QIAquick gel extraction kit (Qiagen) to be used as linear plasmid in a second Gibson assembly. The genes and the P_{mth} promoter of *V. parvula* SKV38 were amplified by PCR using PhusionFlash Master-mix and cloned in pBSJL2-*cat* using Gibson assembly. The mix was then transformed in *E. coli* DH5 α and plated on LB with carbenicillin. The plasmid was harvested by miniprep using QIAprep spin miniprep kit (Qiagen) and transformed in *V. parvula* as described above.

Alternatively, the anhydrotetracycline inducible expression cassette of pRPF185, hereafter referred to as *pTet*, (Addgene 106367, (64)) was inserted along with a chloramphenicol marker right before the ATG of the target gene, following the procedure described above for cloning-independent allelic exchange mutagenesis. The functionality of *pTet* in *V. parvula* was previously verified using measurement of the aTc dependent β -glucuronidase activity generated

by the presence of pRPF185 transformed in *V. parvula* SKV38 (Figure S3).

Biofilm formation in 96-well microtiter plates

Overnight cultures in BHILC medium were diluted to 0.05 OD₆₀₀ and transferred to three Greiner Bio-one polystyrene flat bottom 96-well plates adding 150 µL per well. After 24 hours of static incubation, one of the three plates was resuspended by pipetting to measure OD₆₀₀ using a TECAN Infinite-M200-Pro spectrophotometer. The two other plates were used for coloration: cultures were removed by pipetting carefully the supernatant out and biofilms fixed with 150 µL Bouin solution (HT10132, Sigma-Aldrich) for 15 minutes. Bouin solution was removed by inversion and the biofilms were washed once in water. The biofilms were stained with 150 µL crystal violet 1 % (V5265, Sigma-Aldrich) for 15 minutes without shaking, then washed in water twice and left to dry. All washes were made by flicking of the plate. After drying the plate, crystal violet was dissolved with 200 µL absolute ethanol and transferred to a clean 96-well plate for OD₆₀₀ measurement (TECAN Infinite-M200-Pro spectrophotometer).

Biofilm formation in microfermentor

Continuous flow non-bubbled microfermentor containing a removable spatula were used as described in (65, 66) (see <https://research.pasteur.fr/en/tool/biofilm-microfermenters/>). Briefly, a glass spatula was dipped in an overnight culture diluted to 0.5 OD₆₀₀ in 15 mL BHILC for 15 minutes and returned to the fermentor. Biofilm was grown on the spatula for 48 hours at 37°C. BHILC was constantly supplied through a peristaltic pump at 4 rpm. During the last hour, the speed was increased to 10 rpm to remove planktonic bacteria. A mix of filtered 90% nitrogen/5% hydrogen/5% carbon dioxide was also constantly supplied to maintain anaerobic condition. After 48 hours of growth, the spatula was removed, and the biofilm was resuspended by vortexing in 15 mL BHILC. We measured OD₆₀₀ of the resuspended biofilms with Smart Spec Plus spectrophotometer (BioRad).

Aggregation curve

Overnight cultures were diluted to 0.8 OD₆₀₀ in Brain-heart infusion (BHI) media in semi-micro spectrophotometry cuvette (Fisherbrand) and left to sediment on the bench in presence of oxygen, so no growth should occur. OD₆₀₀ was measured every hour in a single point of the cuvette using SmartSpec spectrophotometer (BioRad).

Initial adhesion on glass

Glass spatula from microfermentor (described above) were dipped in overnight cultures diluted to 0.5 OD₆₀₀ in 15 mL Brain-Heart Infusion (BHI) media for 30 minutes to let bacteria adhere. The spatulas were washed once in 15 mL BHI by submersion and the adhering bacteria were resuspended in 15 mL clean BHI by vortexing. The culture used for inoculation, as well as the resuspended bacteria were serially diluted and plated on SK-agar plate for colony forming unit (CFU) counting.

Statistical analysis

Statistical analysis was performed using either R and Rstudio software or GraphPad Prism8 (GraphPad software, Inc.). We used only non-parametric test, and when applicable corrected for multiple testing. For microfermentor experiments, 4 replicates of each condition were used. For all the other experiments, at least 6 biological replicates in at least 2 independent experiment were used. A cut-off of p-value of 5% was used for all tests. * p<0.05; ** p<0.05; *** p<0.005. For growth curve analyses, we computed the growth rate and carrying capacity of each biological replicates using Growthcurver 0.3.0 package in R and we performed a Mann-Whitney test comparing both parameters for each mutant to the corresponding WT.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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AUTHORS CONTRIBUTIONS

C.B., N.B. and A.J.F. designed the experiments. N.B., A.J.F., E.B. and L.M. performed the experiments. J.W., N.T., and T.C. carried out all genomics and phylogeny analyses under the supervision of SG. N.B., C.B. and A.J.F. wrote the paper, with contribution from S.G., J.W., T.C. and JM.G. All authors have read and approved the manuscript.

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TABLES

Locus tag	PROKKA Gene name	Genome position		Gene size (kb)	Strand	Description	DSM2008 homolog	Name	Class
FNLLGLLA_00032	prn 1	39,354	41,723	2,37	forward	Autotransporter	fusion Vpar_0036-0037	VmaA	Va
FNLLGLLA_00034	biaE 1	42,345	43,754	1,41	reverse	Trimeric Autotransporter: YadA like	Vpar_0039	VtaB	Vc
FNLLGLLA_00035	hypothetical protein	44,146	45,189	1,04	forward	Autotransporter (partial)	Vpar_0040		Ve
FNLLGLLA_00036	hypothetical protein	45,453	46,883	1,431	forward	none	split Vpar_0041		?
FNLLGLLA_00037	omp-alpha	46,91	47,878	969	forward	Trimeric Autotransporter/ S-layer homology domain	split Vpar_0041		Vc?
FNLLGLLA_00038	upaG 1	48,397	56,829	8,433	forward	Trimeric Autotransporter: YadA like	Vpar_0042	VtaC	Vc
FNLLGLLA_00040	biaE 2	57,966	59,84	1,875	forward	Trimeric Autotransporter: YadA like (Partial)	split Vpar_0048		?
FNLLGLLA_00041	ata 1	59,837	63,463	3,627	forward	Trimeric Autotransporter: YadA like	split Vpar_0048		Vc?
FNLLGLLA_00044	ehaG 1	65,3	71,515	6,216	forward	Trimeric Autotransporter: YadA like	Vpar_0051	VtaD	Vc
FNLLGLLA_00045	upaG 2	71,995	81,42	9,426	forward	Trimeric Autotransporter: YadA like	Vpar_0052	VtaE	Vc
FNLLGLLA_00046	ata 2	81,941	91,519	9,579	forward	Trimeric Autotransporter: YadA like	Vpar_0053	VtaF	Vc
FNLLGLLA_00098	biaE 3	151,792	153,522	1,731	forward	Trimeric Autotransporter/ S-layer homology domain	Vpar_0100	VtaG	Vc
FNLLGLLA_00099	ata 3	154,024	158,982	4,959	forward	Trimeric Autotransporter/ S-layer homology domain	absent	VtaH	Vc
FNLLGLLA_00335	prn 2	414,666	416,888	2,223	forward	Autotransporter	Vpar_0330	VmaB	Va
FNLLGLLA_00516	biaF	581,236	590,358	9,123	forward	Trimeric Autotransporter: YadA like	Vpar_0464	VtaA	Vc
FNLLGLLA_00581	brkA	668,34	670,583	2,244	forward	Autotransporter	Vpar_1322	VmaC	Va
FNLLGLLA_01790	ehaG 2	1,943,661	1,946,159	2,499	reverse	Trimeric Autotransporter/ S-layer homology domain	Vpar_1664	VtaI	Vc

Table 1 : *V. parvula* SKV38 autotransporters

Strain name	Description	Reference
WT	<i>Veillonella parvula</i> SKV38	(17)
9G5	<i>Veillonella parvula</i> SKV38 <i>FNLLGLLA_00516::Transposon</i>	This study
5C5	<i>Veillonella parvula</i> SKV38 <i>FNLLGLLA_00516::Transposon</i>	This study
5H1	<i>Veillonella parvula</i> SKV38 <i>FNLLGLLA_00516::Transposon</i>	This study
3D6	<i>Veillonella parvula</i> SKV38 <i>FNLLGLLA_00516::Transposon</i>	This study
7B11	<i>Veillonella parvula</i> SKV38 <i>FNLLGLLA_00516::Transposon</i>	This study
2F7	<i>Veillonella parvula</i> SKV38 <i>FNLLGLLA_00516::Transposon</i>	This study
3F7	<i>Veillonella parvula</i> SKV38 <i>FNLLGLLA_00516::Transposon</i>	This study
5E11	<i>Veillonella parvula</i> SKV38 <i>FNLLGLLA_01127::Transposon</i>	This study
Δ vtA	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_00516::tetM</i>	This study
pTet-vtA	<i>Veillonella parvula</i> SKV38 <i>catP-Term(fdx)-Ptet-FNLLGLLA_00516</i>	This study
Δ 8	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_00036-46::tetM</i>	This study
Δ vmaA	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_00032::tetM</i>	This study
Δ vtAB	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_00034::tetM</i>	This study
Δ vtAG	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_00098::tetM</i>	This study
Δ vtAH	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_00099::tetM</i>	This study
Δ vmaB	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_00335::tetM</i>	This study
Δ vmaC	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_00581::tetM</i>	This study
Δ vtAI	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_01790::tetM</i>	This study
Δ vtA Δ 8	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_00516::catP</i> Δ <i>FNLLGLLA_00036-46::tetM</i>	This study
Δ 1127	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_01127::tetM</i>	This study
Δ 1127 Δ 8	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_01127::tetM</i> Δ <i>FNLLGLLA_00036-46::catP</i>	This study
WT+pEmpty	<i>Veillonella parvula</i> SKV38-pBSJL2- <i>catP-pmdh</i>	This study
Δ 1127+pEmpty	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_01127::tetM</i> - pBSJL2- <i>catP-pmdh</i>	This study
Δ 1127+p1127	<i>Veillonella parvula</i> SKV38 Δ <i>FNLLGLLA_01127::tetM</i> - pBSJL2- <i>catP-pmdh-FNLLGLLA_01127</i>	This study
P _{ori} - ϕ	SKV38- pRPF185 Δ <i>gusA</i>	This study

P_{tet} - <i>gusA</i>	SKV38- pRPF185	This study
P_{Cwp2} - <i>gusA</i>	SKV38- pRPF144	This study

Plasmid	Description	Reference
pRPF215	<i>mariner</i> Tn delivery plasmid, $P_{tet}::HimarI$, ITR- <i>ermB</i> -ITR, <i>catP</i> , <i>tetR</i>	(19)
pRPF185	tetracycline inducible expression system fused with β -glucuronidase <i>gusA</i> Term(fdx)- P_{tet} - <i>gusA</i> -Term(slpA), <i>catP</i>	(64)
pRPF185 Δ <i>gusA</i>	pDIA6103, tetracycline inducible expression system Term(fdx)- P_{tet} -Term(slpA), <i>catP</i>	(67)
pRPF144	carries a <i>Clostridium</i> constitutive promoter fused with <i>gusA</i> P_{Cwp2} - <i>gusA</i>	(64)
pBSJL2	<i>E. coli</i> - <i>Veillonella</i> shuttle plasmid, $P_{gyrA}::tetM$	(68)
pBSJL2-cat	<i>E. coli</i> - <i>Veillonella</i> shuttle plasmid, $P_{cat}::catP$, <i>pmdh</i> promoter	This study
p1127	pBSJL2- <i>catP</i> - <i>pmdh</i> -FNLLGLLA_01127	This study

Table 2: Strains and plasmids used in this study

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL AND METHOD

Growth curve

Overnight cultures were diluted to 0.05 OD₆₀₀ in 150 μL BHILC that had previously been left in anaerobic condition overnight to remove dissolved oxygen, in Greiner flat-bottom 96-well plates. A plastic adhesive film (adhesive sealing sheet, Thermo Scientific, AB0558) was added on top of the plate inside the anaerobic station, and the plates were then incubated in a TECAN Infinite M200 Pro spectrophotometer for 24 hours at 37°C. OD₆₀₀ was measured every 30 minutes, after 900 seconds orbital shaking of 2 mm amplitude.

Scanning electronic microscopy

Spatula carrying Thermanox plastic coverslips (10.5 x 22 mm, Nunc Thermo Scientific) were introduced in our microfermentor system described above. After 48 hours of growth of the biofilm, the microscopy coverslips were submerged in fixation solution: volume/volume mix of glutaraldehyde 4 %/ sodium cacodylate trihydrate buffer 0.2M pH=7.4/ Ruthenium red 0.15 % and kept at 4°C. Scanning electronic microscopy was then performed by the *Laboratoire de biologie cellulaire et microscopie électronique* of the University of Tours, France.

β-glucuronidase assay

Overnight cultures were diluted to OD 0.05 and grown for 2-3 hours after which the inducer aTc was added. Induced cultures and controls were cultured for 3.5 hours and then β-glucuronidase activity was assessed. 75 μl of the culture was mixed with 675 μl of Z-buffer (60 mM Na₂HPO₄; 40 mM NaH₂PO₄; 10 mM KCl; 1 mM MgSO₄; 50 mM HO-CH₂-CH₂-SH), 50 μl of CHCl₃ and 25 μl of 0.1% sodium dodecyl sulfate solution, vortexed and incubated 5 min at 37°C. Then 100 μl of 10 mM 4-nitrophenyl-β-D-glucuronide (in water) was added, and the reaction mix was incubated at 37°C until it turned visibly yellow. The reaction was stopped by adding 375 μl of 1M Na₂CO₃ (in water) and the absorbance at 405 nm wavelength was measured after a centrifugation. Then the β-glucuronidase activity expressed in Miller units was calculated as described previously for β-galactosidase (1).

Light microscopy

Cultures were grown overnight with no shaking and the aggregates at the bottom of the tubes

were harvested. 1 μ L of these aggregates were dropped on multitest 12-well slide (MP Biomedicals glass), covered with a coverslip (24 x 60 mm coverslip, Menzel glaser) and observed using light microscopy, 1000X.

References for Supplemental material

1. Miller J. 1972. Beta-galactosidase assay. *Exp Mol Genet* 352:355.

SUPPLEMENTARY FIGURES

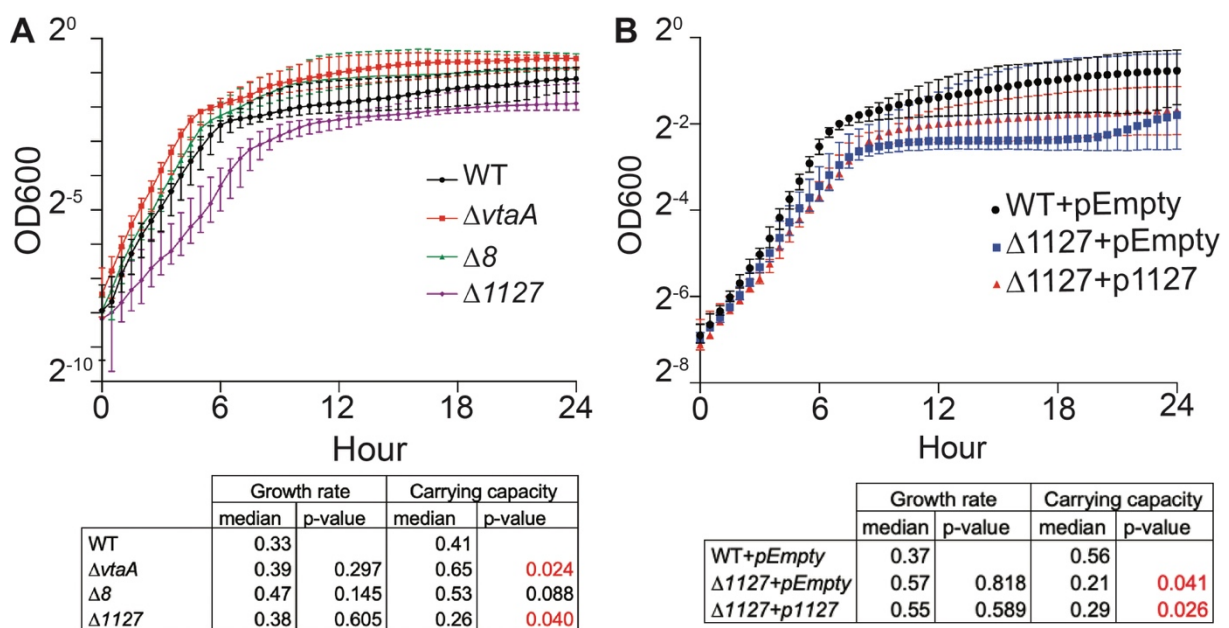


Figure S1: **Growth of *V. parvula* mutants.** 24h growth curve in BHILC in 96-well plates. Each point represents the median of 6 biological replicates. Error bars represent 95% confidence interval. For each biological replicate of each strain, the growth rate and the carrying capacity were computed using R library growthcurver. The median of both parameters is indicated in the table below, as well as the p-value of a Mann-Whitney test comparing growth rate and carrying capacity of the mutant with the corresponding WT or WT+pEmpty. **A.** Selected deletion mutants. **B.** Complementation of the $\Delta 1127$ mutant. pEmpty correspond to pRPF185. p1127 corresponds to pBSJL2-catP-pmdh-FNLLGLLA_01127. See detailed Supplemental Material and Methods.

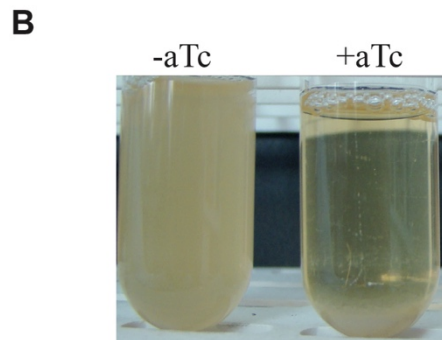
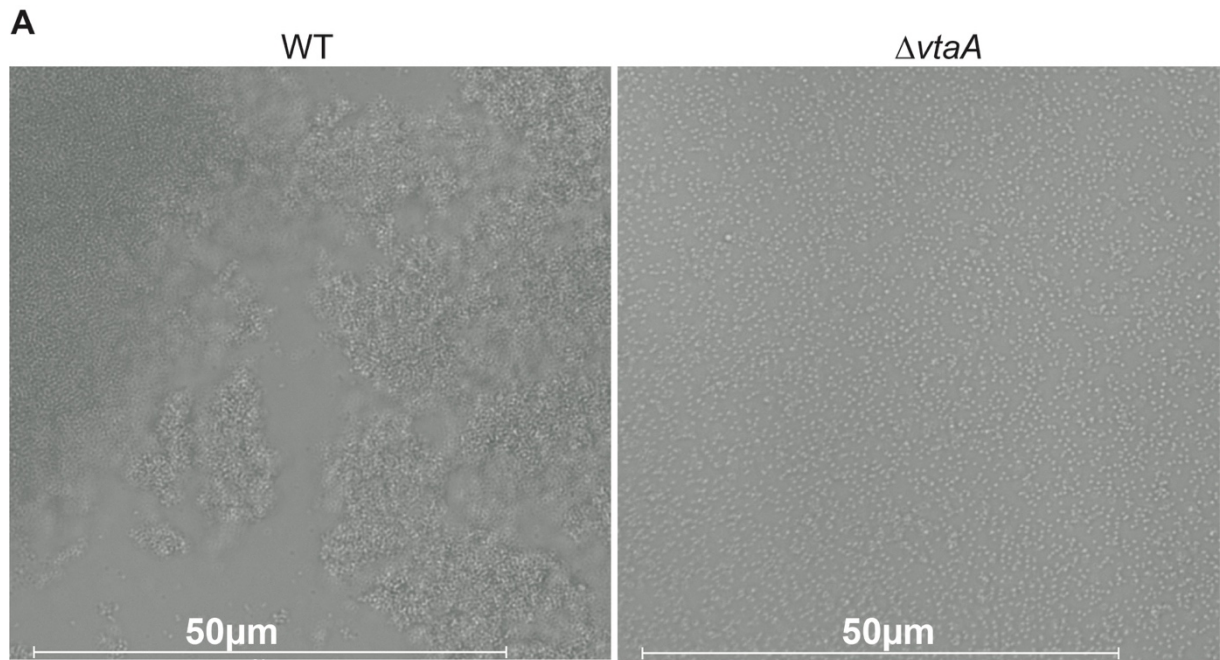


Figure S2: **VtaA mediates auto-aggregation.** **A.** Light microscopy of overnight cultures of WT and $\Delta vtaA$ grown in BHILC, 1000X. Scale bar represent 50 μ m. **B.** Overnight cultures of $pTet-vtaA$ strain, where $vtaA$ is under the control of the inducible pTet promoter, grown in BHILC with or without 0.1 μ g/mL aTc.

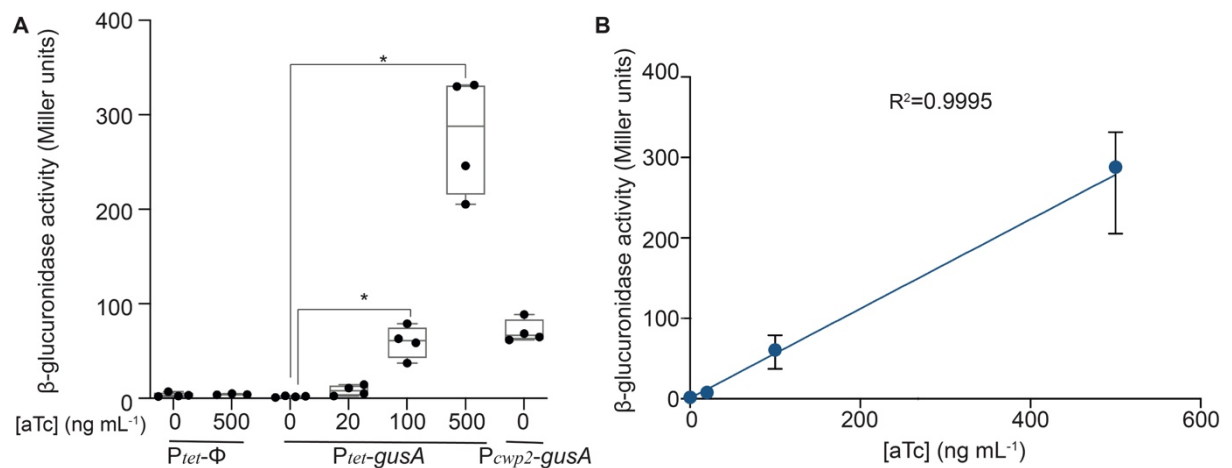


Figure S3. **Tetracycline inducible promoter (P_{Tet}) from *Clostridioides difficile* is active in *V. parvula*.** **A.** β -glucuronidase assay showing the expression of P_{Tet} fused to $gusA$ under different concentrations of aTc. SKV38 was transformed with pRPF185 $\Delta gusA$ (P_{Tet} - ϕ) as a negative control, pRPF185 carrying the P_{Tet} - $gusA$ or pRPF144 carrying a copy of $gusA$ under a constitutive promoter from *Clostridium* (P_{Cwp2} - $gusA$). Min-max boxplot of 4 biological replicates. *p-value<0.05, Mann-Whitney test. **B.** β -glucuronidase activity measured

for SKV38-pRPF185 carrying P_{vt} -*gusA* is represented as a function of the inducer aTc concentration. Each dot is the median of 4 biological replicates, error bars represent 95% confidence interval. The coefficient of correlation R^2 is indicated. See detailed Supplemental Material and Methods.

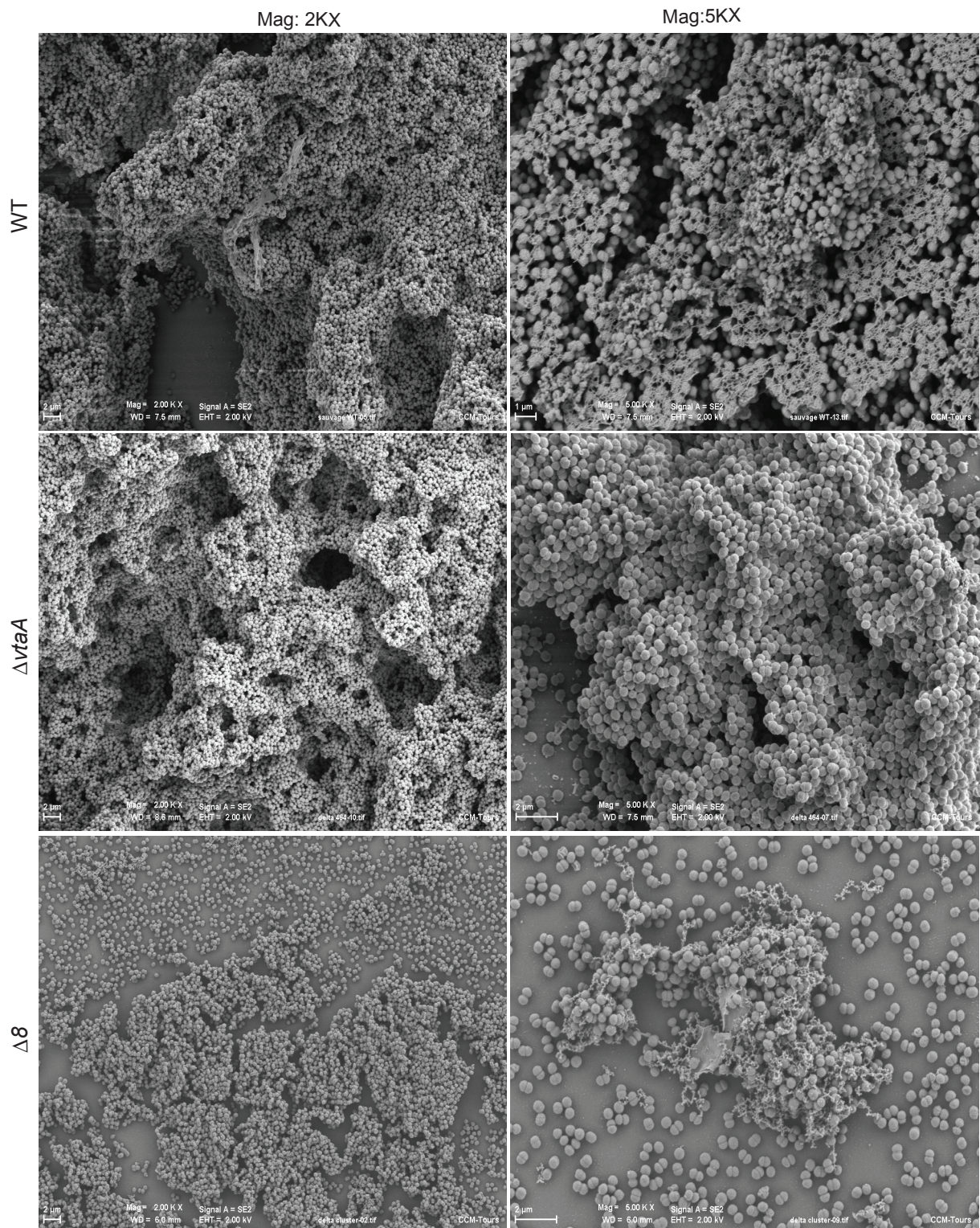


Figure S4: Scanning electronic microscopy (SEM) of biofilms grown under continuous flow in microfermentor on microscopy slide, magnified 2K or 5K times. Biofilms of the *V. parvula* WT, $\Delta vtaA$ and $\Delta 8$ strains were grown for 48h in BHILC medium under anaerobic conditions on Thermanox plastic coverslips. Biofilms were fixed in a glutaraldehyde/cacodylate/ruthenium red solution before scanning electronic microscopy imaging.

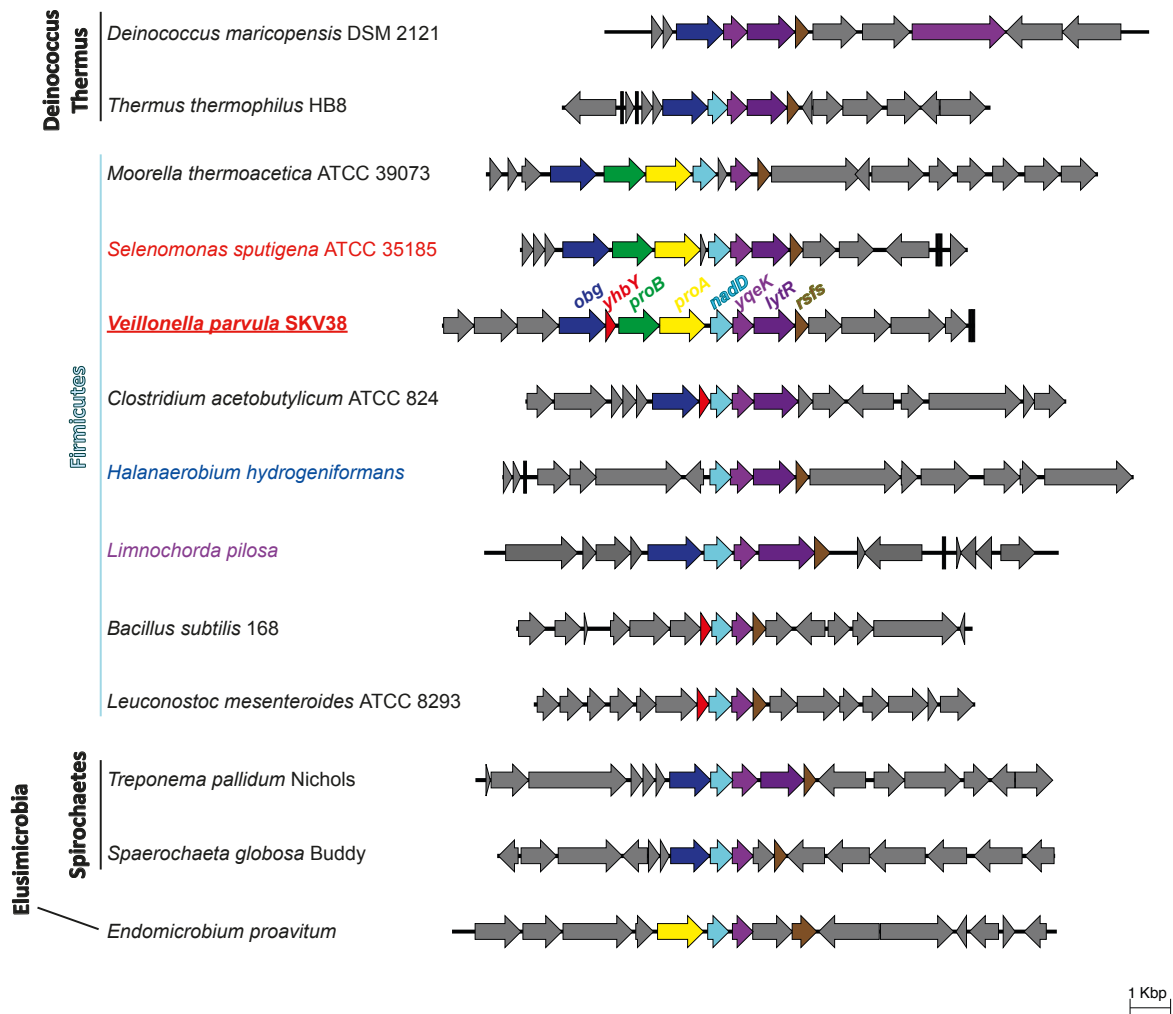


Figure S5: **Conservation of the HD Phosphatase encoding gene (*yqeK*) synteny in selected diderm and monoderm bacteria.** Some species from Figure S5 that contain a minimum of three genes in addition to *yqeK* within the cluster were selected, and the corresponding genetic organizations are represented according to their phylogenetic proximity. The different genes are displayed with different colors. The color code is the same as that of Figure S5. *V. parvula* SKV38 strain is underlined. In this strain *yqeK* corresponds to *FNLLGLLA_01127*.

SUPPLEMENTARY TABLES

Primer name	Sequence
TetM-F	agtaaaatgcaggcgagtgaag
TetM-R	gtggatccacaggacacaat
TetM-outF	agatttgaattaaagtgtaaaggagga
TetM-outR	tcttgtaacagcgcaattcct
catP-F	ggccttttgctcacatgttc
catP-R	cctgaagttaactattatcaattcctgc
catP-chF	cgcagtatgtgacggatttc
catP-chR	gcttcctcgctcactgactc
vtaA-extF	ggctgatatgcatcccac
vtaA-extR	cacttacaaggcccacgact
vtaA-5F	gtagctttagccaatgtggc
vtaA-5R	cttactcgcctgcattttactactttctcctataaactatagtaatattaatgc
vtaA-3F	attgtgtcctgtggatccacgagttgagaactaattttgataatagtc
vtaA-3R	gtatacataccgaaccgctc
cat-vtaA-5R	gaacatgtgagcaaaaggccactttctcctataaactatagtaatattaatgc
cat-vtaF-3F	gataaatagttaacttcaggaggagttgagaactaattttgataatagtc
0036-extF	cagcctatgcaggggactac
0046-extR	tctgtgcccctcatatggg
0036-5F	gtcagggcggttgagatg
0036-5R	cttactcgcctgcattttactttatcctctccattccctaataagaa
0046-3F	attgtgtcctgtggatccactacgttcatactgaacattacgg
0046-3R	tttgacaatttcacggcc
cat-0036-5R	agaacatgtgagcaaaaggccctttatcctctccattccctaataagaa
cat-0046-3F	gataaatagttaacttcagggtacgttcatactgaacattacgg
vmaC-extF	caatccttgatcgcaacgc
vmaC-extR	ctacgtaatttaacgctccc
vmaC-3R	tagtgttccggctaggatg
vmaC-3F	attgtgtcctgtggatccaccggcataatttttaattgaatattg
vmaC-5F	atataaaggcgctccttagg
vmaC-5R	cttactcgcctgcattttactaaaaatctctctttcttacctcgc
vtaH-extF	ggttatggtcactaccataatgc
vtaH-extR	caaatcaacatatggtcacatcc
vtaH-3R	cctgatctgcatcagagc
vtaH-3F	attgtgtcctgtggatccaccgggtaatagaatcgaag
vtaH-5F	cgcattctaccgcctaac
vtaH-5R	cttactcgcctgcattttactcgggttcataaacctcctcc
vtaB-extF	ccgttagacacattttccatgc
vtaB-extR	gagtccaagtaggtcgtgc
vtaB-3R	cgtatacaattggtgcacgc
vtaB-3F	attgtgtcctgtggatccactaagatccctttatggtagtcctatg
vtaB-5F	accagacacaccgcgatac

vtaB-5R	cttcaactgcctgcattttactctcatcgtatacactcctttgtgtac
vmaA-extF	gcttctttcaattgctattagc
vmaA-extR	ggtaaaagtacaagtcacacgc
vmaA-3R	gtacaacatgtatctcaagagg
vmaA-3F	attgtgtcctgtggatccacgcgaattattaatagataggagcg
vmaA-5F	tgcataataaagctattccttc
vmaA-5R	cttcaactgcctgcattttactcataataaacctctcttattcacac
vtaG-extF	caatcccctgatacacgtg
vtaG-extR	catcatcttcagccaccgc
vtaG-3R	cctttgctgcgcattac
vtaG-3F	attgtgtcctgtggatccacgcgtatacggttcccgtataac
vtaG-5F	agctattgctgtagggtctc
vtaG-5R	cttcaactgcctgcattttactctcatcttacaccctcac
vmaB-extF	caagtttcaaggagtactcg
vmaB-extR	gaataaaatattcaaaagcatctcc
vmaB-5F	ttcttctgggaaatgaagttgactacagt
vmaB-5R	cttcaactgcctgcattttactgttatacctcctaacactcaac
vmaB_3F	attgtgtcctgtggatccacttaagggtaaaaatagcagagggc
vmaB-3R	gaaaataatggttgagtctcag
vtaI-extF	cccgcatacaagagcattgg
vtaI-extR	aaacatgcacgatggcccta
vtaI-5F	taataaaactagcaggcattgcg
vtaI-5R	cttcaactgcctgcattttactcgttactattcctccctacac
vtaI_3F	attgtgtcctgtggatccacttactgtcatgaattaaccttttaaac
vtaI-3R	caaagagcattggaatgcc
1127-extF	ggtgttcgattcacggag
1127-extR	gtgccccattatgttccg
1127-5F	gtagctcaatataatggaaaacac
1127-5R	cttcaactgcctgcattttactcatttattcctccatataatggtttc
1127_3F	attgtgtcctgtggatccactggagttttatattgacgaacg
1127-3R	ggtgcaactttcgacatac
pvtaA-3F	gcgtaacagatctgagctccttactatagtttataggagaaagtatg
pvtaA-3R	ctacagaacgactcgcag
pvtaA-5R	aagaacatgtgagcaaaaggccattcataattctatatagcgcac
pvtaA-extF	ctatctattggggcagg
pvtaA-extR	agcattaacattatatacccgc
pTet-chF	cgtaacagatctgagctcc
pTet-chR	ccttgaattgatcatatgcgg
catR-pTet	tagcttgatgcagaattcggcctgaagttaactttatcaattcctgc
cat-pTet-F	gataaatagttaacttcagggcggaattctgcatcaagc
pTet-R	aggagctcagatctgttaac
pBSJL2_ΔtetR	tgtattttatgtttatataaatatg
pBSJL2_ΔtetF	ctgcaggaattcgaatc

cat_pBSJL2_R	catatttatataacaacataaaatacattaactatttatcaattcctgcaattcgt
cat_pBSJL2_F	gatatcgaattcctgcagagtgagctgataccgctcg
pmdh-F	caacaatcactagtggatccatacaaaattcttcaaaaaaac
pmdh-R	cgtaaaacctctttcagaaaatg
mdh-1127-F	tctgaaaagaggtttaacgatgttatcgttgatgaaatacaac
pBSJL2-1127-R	atattgtcctgtggatcctatttacaagattttaatacatcattacg

Table S1. Primers used in this study

**CONCLUSION
AND
PERSPECTIVES**

CONCLUSION AND PERSPECTIVES

During my PhD, I studied the determinants of biofilm formation in two important gastrointestinal tract symbionts, *Bacteroides thetaiotaomicron* and *Veillonella parvula*. In this section, I will discuss the outcomes of my thesis work and their implications for further studies.

Capsules are important determinants of B. thetaiotaomicron biofilm formation

B. thetaiotaomicron VPI 5482 is a poor biofilm former *in vitro*. However, I showed that expression of most capsular polysaccharides mask biofilm formation in this strain, and that emergence of a small subpopulation of acapsulated bacteria was sufficient to lead to biofilm formation. Capsules have been shown to be involved in several important processes in *B. thetaiotaomicron*, including interactions with the immune system and protection from phage attacks (Hsieh *et al.*, 2020; Porter *et al.*, 2017, 2019). It is therefore unknown whether acapsulated bacteria can be found *in vivo*. Increasing adhesion capacity at the expense of exposing antigens to the immune system might be relevant in some conditions, for instance to associate with food particles in the gut lumen, or during mother-to-child transmission. Studies specifically localizing acapsulated *B. thetaiotaomicron* in different parts of the gut will be necessary to determine whether these cells occur *in vivo* and where.

In this work, I also identified CPS8 as the only capsule of *B. thetaiotaomicron* with adhesive properties. Interestingly, CPS8 is also the only capsule containing a homolog of FimA, the main component of type V pilus that are known to mediate adhesion in *Porphyromonas gingivalis* and *B. thetaiotaomicron* (Mihajlovic *et al.*, 2019; Q. Xu *et al.*, 2016). We are currently assessing whether CPS8 contains a functional type V pilus and how the presence of a FimA homolog might contribute to CPS8 adhesion. This would provide the first characterization of a *B. thetaiotaomicron* native adhesive structure.

CPS8 was expressed to low levels in *B. thetaiotaomicron* VPI 5482 overnight cultures and the CPS8-only strain was shown to be strongly outcompeted by WT *B. thetaiotaomicron* in the mouse gut. It is therefore unclear how much CPS8 contributes to *in vivo* biofilm formation. Moreover, CPS8 is not commonly found within *B. thetaiotaomicron* isolates. Indeed, among our collection of clinical isolates, only one out of the thirteen sequenced strains possessed a homolog of CPS8, and previous analysis by Porter and colleagues also showed that CPS8 was found in only 1% of *B. thetaiotaomicron* genomes (Porter *et al.*, 2017). However, data suggest

that CPS8 encoding locus is located within a mobile genetic element and might thus be transferred between strains. Although CPS8 is rare, it is possible that other adhesive capsules could be found among the vast diversity of *Bacteroides* encoded capsules. A better understanding of which features of CPS8 are responsible for adhesion will allow us to screen *B. thetaiotaomicron* published genomes for similar structures and to assess how widespread capsule-mediated biofilm formation is within *B. thetaiotaomicron* species.

Bile extract induces biofilm formation in most Bacteroides strains tested

Bile was shown to induce biofilm formation in at least one representative of all 4 major phylum of the gut microbiota: Firmicutes (*Clostridium difficile*), Bacteroidetes (*Bacteroides fragilis*), Proteobacteria (*Vibrio cholerae*) and Actinobacteria (*Bifidobacterium*) (Ambalam, Kondepudi, Nilsson, Wadström, & Ljungh, 2014; Dubois *et al.*, 2019; Hung *et al.*, 2006; Pumbwe *et al.*, 2007), suggesting that bile is an important gut signal. In this work, I showed that physiological concentrations of bile extract induced biofilm formation of almost all tested *B. thetaiotaomicron* strains, and of representative strains of several *Bacteroides* species. This widespread induction of biofilm formation, conserved among Bacteroidales, by a compound exclusive to the intestinal environment suggests that adhesion might be an important property of *Bacteroides* in the gut. Alternatively, biofilm formation might be a conserved mechanism used by bacteria to protect against bile toxicity. Indeed, biofilms are known to be more tolerant to various antimicrobials, which might include bile.

Bile concentration is highly variable in the gut, both temporally and geographically. Moreover, bile composition changes depending on the location, but also on environmental parameters such as diet and gut microbiota composition. Thus, we can expect that the bile concentration and composition necessary to induce biofilm formation are only reached at certain times or in certain locations in the gut. Whether these bile-induced biofilms occur *in vivo*, where precisely in the gut and how much they contribute to *B. thetaiotaomicron* gut colonization capacity remains to be addressed. Ideally, we should compare *B. thetaiotaomicron* VPI 5482 WT and $\Delta 3560-3563$ colonization of the mouse gut and assess their geographical repartition at different times, for instance right after a meal when bile concentration is high vs after fasting when the concentration should be lower.

BT3560-3563 is the only locus of *B. thetaiotaomicron* VPI 5482 involved in bile-mediated biofilm formation that we identified during our screening. However interestingly, tauroolithocholate was also found to induce biofilm formation in a *BT3560-3563*-independent

manner, suggesting bile extract and tauroolithocholate induce biofilm through different mechanisms. Screening of *B. thetaiotaomicron* VPI 5482 random transposon library in presence of tauroolithocholate will allow the identification of genes specifically required for taurocholate-mediated biofilm formation. Bile and tauroolithocholate are both closely related chemicals and are thus expected to share some properties. Since some *B. thetaiotaomicron* strains reacted to one and not the other, and since *B. thetaiotaomicron* VPI 5482 used different mechanism to mediate bile- and tauroolithocholate-induced biofilm formation, we can expect that the bacteria are not simply reacting to a chemical stress such as detergent or lower pH, but rather that they are specifically sensing the presence of these molecules.

Are secreted DNAses commonly required for bile-induced biofilm formation of B. thetaiotaomicron?

BT3563, which is necessary for bile-induced biofilm formation, encodes a secreted DNase. This was a surprising finding considering most of the literature on the subject shows that DNAses tend to prevent biofilm formation by degrading a very important component of the ECM, eDNA. We are currently assessing the contribution of extracellular DNA to the ECM of *B. thetaiotaomicron* biofilms, and how total or partial degradation by DNAses might impact biofilm formation. However, the precise mechanism of bile-induced biofilm formation and notably the adhesins involved are not yet known.

Although almost all *B. thetaiotaomicron* tested strains induced biofilm formation in presence of bile extract, it is unclear whether the mechanism is conserved. Indeed, several isolates that formed bile-induced biofilms did not possess a homolog of *BT3563*. Assessing bile-induced biofilm formation of *B. thetaiotaomicron* clinical isolates deleted for their *BT3563* homolog would confirm -or not- that *BT3563* is also necessary in these strains. For *B. thetaiotaomicron* strains lacking a *BT3563* homolog, we can hypothesize they either encode another secreted DNase that has no homology with *BT3563* but performs the same function or they use a different mechanism altogether. Screening for other secreted DNAses in *B. thetaiotaomicron* clinical isolated lacking *BT3563*, or more broadly in the different Bacteroidales species tested, is necessary to assess how widely are secreted DNAses responsible for bile-induced biofilm formation of Bacteroidales strains.

Is there a set of genes specifically found in B. thetaiotaomicron forming biofilm in presence of bile?

Although there was no correlation between presence of a BT3563 homolog and bile-induced biofilm formation in our strains, it is possible that genome comparison of bile-induced biofilm formers and non-formers would reveal other genes important in this process. We therefore compared the whole genomes of VPI 5482 and the 8 *B. thetaiotaomicron* clinical isolates we sequenced using MicroScope platform Gene Phyloprofile tool. We identified 200 genes present in VPI 5482, jmh43, jmh44, jmh47, jmh49, jmh50, jmh58, and jmh79 genomes but not in jmh71, the only strain that did not react to bile addition (Annex 2). 69 out of the 200 genes are annotated as hypothetical proteins, the remaining 131 encode very diverse proteins including proteins involved in DNA mobilization (integrase, transposase, recombination...), gene regulation (two component systems, ECF sigma factor...), outer membrane proteins (efflux pumps, transporters...) and metabolism. It is yet unclear whether any of these genes would be required for bile-induced biofilm formation, and considering the number of genes identified, a site-directed mutagenesis approach would be inefficient. However, sequencing other *B. thetaiotaomicron* strains or testing for bile-induced biofilm formation other sequenced *B. thetaiotaomicron* isolates to add them to our analysis would probably allow us to shorten that list to identify promising gene candidates.

B. thetaiotaomicron is a model of gut symbiont biofilm

Understanding the mechanisms of biofilm formation in a major intestinal symbiont will allow us to evaluate the contribution of adhesion to colonization and maintenance in the intestinal microbiota. Moreover, the development of *in vitro* models of biofilm formation by *B. thetaiotaomicron* will also allow us to study its physiological adaptations within biofilms, which might be a better proxy for the physiology of *B. thetaiotaomicron* within the microbiota than planktonic cultures. In particular, biofilms are known to tolerate antibiotic treatments much better than planktonic cultures. While this aspect has been extensively studied in relevant pathogenic bacteria, in order to develop strategies to clear biofilm-mediated infections, little is known on the antibiotic tolerance of common gut symbionts and how it might be impacted by biofilm formation, even though antibiotic-induced dysbiosis of the gut microbiota can have strong consequences on human health. A project on the antibiotic tolerance of *B. thetaiotaomicron* biofilms and their possible contribution to antibiotic-induced dysbiosis has been initiated in the laboratory following my work.

Mixed biofilms with B. thetaiotaomicron?

My thesis work was centered on mono-species biofilm, although the gut microbiota is a very complex multi-species biofilm. Further work should also focus on bacterial-bacterial interactions within mixed biofilms and how they differ from pure *B. thetaiotaomicron* biofilms, either in symbiont-symbiont system or in symbiont-pathogen. In particular, bile-induced biofilms are formed by very diverse gut bacteria, and might be a relevant model of gut-mimicking multi-species biofilm. For instance, *C. difficile* is an opportunistic pathogen that must overcome the gut microbiota-mediated colonization resistance in order to implant in the gut. It would be interesting to study how *C. difficile*, which can form bile-induced biofilms, would colonize a pre-formed *B. thetaiotaomicron* biofilm, and how this colonization would be impacted by pretreatment of *B. thetaiotaomicron* biofilm with antibiotics. Another idea would be to study the impact of biofilm formation on cooperative degradation of complex polysaccharides by multiple bacteria and its relevance in mimicking food-particle associated communities physiology.

Is VPI 5482 representative of the B. thetaiotaomicron species?

VPI 5482 is the first sequenced *B. thetaiotaomicron* strain and the development of genetic tools established this particular isolate as the reference strain. However, results obtained with this strain might not necessary reflect what happens in natural isolates. For instance, we previously showed that a collection of natural *B. thetaiotaomicron* strains taken from different infections exhibited varying biofilm formation capacities, 41% of them forming more biofilm than VPI 5482 (Mihajlovic *et al.*, 2019). Similarly, *BT3563*, which is required for bile-induced biofilm formation, was not found in all the strains reacting to bile addition and CPS8, the only adhesive capsule of *B. thetaiotaomicron* was rare among natural isolates, showing CPS8-mediated biofilm formation might not be so relevant *in vivo*. The development of sequencing and genetic tools to manipulate more *B. thetaiotaomicron* natural isolates might lead to a progressive shift from VPI 5482 study to more biologically relevant strains.

Type V secreted autotransporters are the main adhesins of V. parvula

In this work, I used a recently described naturally competent isolate of *V. parvula*, SKV38 (Knapp *et al.*, 2017) and adapted genetic tools originally developed for *Clostridium* to show that *V. parvula* SKV38 genome encodes 12 full-length type V secreted autotransporters, including three classical autotransporters and 9 trimeric autotransporters. I identified a trimeric

autotransporter, VtaA, involved in cell-to-cell adhesion and a cluster of 8 genes containing 4 full-length trimeric autotransporters involved in surface binding. The function of the remaining 7 autotransporters remains to be addressed. Using the already produced deletion mutants, we will test several phenotypes including adhesion to other bacteria or to different surfaces to identify their function. Bioinformatic analysis already revealed that *V. parvula* trimeric autotransporters were not identical to canonical Proteobacteria trimeric autotransporters as they possess an extra C-terminal domain potentially binding peptidoglycan. Thus, we are developing cryoelectronic microscopy to characterize the structure and the abundance of different types of autotransporters, and to see how they differ from classical Proteobacteria autotransporters. This work provides the first evidence for efficient genetic manipulation in a representative of *Veillonella* genus and should greatly facilitate functional studies in *Veillonella*.

In conclusion, during my thesis, I characterized four biofilm-promoting conditions of *B. thetaiotaomicron*: absence of capsule, expression of CPS8 and induction in presence of bile extract or tauroolithocholate. Moreover, I identified the major family of adhesins in *V. parvula* SKV38 and developed tools that will likely make SKV38 the new *V. parvula* reference strain. This work has already opened new investigations in my laboratory, including the analysis of the physiology of *B. thetaiotaomicron* within different biofilm systems and the molecular characterization of outer membrane homeostasis and function in *V. parvula*.

ANNEXES

ANNEXES

ANNEX 1: A PUTATIVE TYPE V PILUS CONTRIBUTES TO *BACTEROIDES THETAJOTAOMICRON* BIOFILM FORMATION CAPACITY

ANNEX 2: LIST OF GENES PRESENT IN BILE-INDUCED BIOFILM FORMER SEQUENCED STRAINS AND ABSENT IN NON-BIOFILM FORMER JMH71

Label	Product
BACTN_0429	N-acetylmuramoyl-L-alanine amidase (T7 lysozyme) (fragment)
BACTN_3471	Predicted protein
BACTN_4822	conserved protein of unknown function
BT_0084	conjugate transposon protein
BT_0107	hypothetical protein
BT_0219	thioredoxin C-2
BT_0258	Putative glucosamine-6-phosphate deaminase-like protein BT_0258
BT_0358	transposase
BT_0377	hypothetical protein
BT_0459	beta-hexosaminidase
BT_0500	Uncharacterized transporter BT_0500
BT_0526	L-asparaginase I
BT_0543	glutamine synthetase
BT_0597	hypothetical protein
BT_0677	thioredoxin
BT_0680	cation efflux system protein CzcA
BT_0684	putative long-chain-fatty-acid--CoA ligase
BT_0696	hypothetical protein
BT_0721	DNA repair and recombination protein
BT_0732	two-component system response regulator
BT_0918	integrase
BT_0978	ECF-type sigma factor
BT_0980	hypothetical protein
BT_1018	polygalacturonase (Pectinase)
BT_1025	hypothetical protein
BT_1029	hypothetical protein
BT_1073	hypothetical protein
BT_1074	hypothetical protein
BT_1076	two-component system response regulator
BT_1129	hypothetical protein
BT_1130	hypothetical protein
BT_1135	hypothetical protein
BT_1226	putative long-chain-fatty-acid--CoA ligase
BT_1229	Uncharacterized transporter BT_1229
BT_1235	AraC-type transcription regulator
BT_1249	putative multidrug resistance protein
BT_1259	choloylglycine hydrolase

BT_1265	two-component system response regulator
BT_1357	hypothetical protein
BT_1398	putative aminotransferase B
BT_1435	hypothetical protein
BT_1456	thioredoxin
BT_1482	two-component system response regulator
BT_1488	hypothetical protein
BT_1501	major outer membrane protein OmpA
BT_1505	hypothetical protein
BT_1511	outer membrane protein OmpA
BT_1512	putative surface protein
BT_1547	hypothetical protein
BT_1655	hypothetical protein
BT_1666	hydrolase
BT_1705	hypothetical protein
BT_1724	hypothetical protein
BT_1735	chromate transport protein
BT_1736	chromate transport protein
BT_1791	outer membrane protein
BT_1827	transposase
BT_1892	integrase
BT_1923	O-acetylhomoserine (thiol)-lyase
BT_1930	hypothetical protein
BT_1950	Fe ³⁺ ABC transporter ATP-binding protein
BT_1951	Fe ³⁺ ABC transporter permease
BT_1952	Fe ³⁺ ABC transporter periplasmic iron-binding protein
BT_1953	putative TonB-linked outer membrane receptor
BT_1954	putative surface layer protein
BT_1956	putative cell surface protein
BT_1962	hypothetical protein
BT_1970	Glutamate dehydrogenase
BT_1973	glutamate dehydrogenase
BT_1993	putative cell wall-associated protein
BT_2069	hypothetical protein
BT_2083	hypothetical protein
BT_2086	choloylglycine hydrolase
BT_2092	Uncharacterized transporter BT_2092
BT_2094	TonB-dependent receptor
BT_2095	putative surface layer protein
BT_2098	Fe ³⁺ ABC transporter periplasmic iron-binding protein
BT_2099	Fe ³⁺ ABC transporter permease
BT_2100	putative ABC transporter ATP-binding protein

BT_2116	hypothetical protein
BT_2121	putative Na ⁺ -driven multidrug efflux pump
BT_2154	putative long-chain-fatty-acid--CoA ligase
BT_2155	hypothetical protein
BT_2156	putative sugar phosphate isomerase/epimerase
BT_2159	putative oxidoreductase
BT_2165	two-component system response regulator
BT_2229	thioredoxin M
BT_2255	hypothetical protein
BT_2264	outer membrane protein Omp121
BT_2277	transposase
BT_2287	conjugate transposon protein
BT_2288	conjugate transposon protein
BT_2336	hypothetical protein
BT_2359	formamidopyrimidine-DNA glycosylase
BT_2366	hypothetical protein
BT_2387	O-acetylhomoserine (thiol)-lyase
BT_2399	transposase
BT_2404	L-asparaginase I
BT_2427	transposase
BT_2437	hypothetical protein
BT_2438	hypothetical protein
BT_2442	major outer membrane protein OmpA
BT_2444	hypothetical protein
BT_2448	hypothetical protein
BT_2468	hypothetical protein
BT_2472	hypothetical protein
BT_2484	hypothetical protein
BT_2485	major outer membrane protein OmpA
BT_2488	integrase
BT_2495	ABC transporter permease
BT_2496	putative ABC transporter ATP-binding protein
BT_2593	conjugate transposon protein
BT_2609	conjugate transposon protein
BT_2653	hypothetical protein
BT_2674	transposase
BT_2680	beta-galactosidase
BT_2780	putative membrane-associated protein
BT_2788	chromate transport protein
BT_2789	chromate transport protein
BT_2794	putative multidrug resistance protein
BT_2900	arabinan endo-1,5-alpha-L-arabinosidase A

BT_2912	putative glycosylhydrolase
BT_2926	hypothetical protein
BT_3002	hypothetical protein
BT_3064	hypothetical protein
BT_3140	hypothetical protein
BT_3237	hypothetical protein
BT_3238	hypothetical protein
BT_3239	hypothetical protein
BT_3240	hypothetical protein
BT_3241	hypothetical protein
BT_3242	hypothetical protein
BT_3370	putative glycosyltransferase YibD
BT_3371	putative glycosyltransferase YibD
BT_3467	putative glycosylhydrolase
BT_3477	glutaminase A
BT_3481	putative glutaminase A
BT_3482	hypothetical protein
BT_3491	glutaminase A
BT_3492	hypothetical protein
BT_3503	glutaminase A
BT_3504	hypothetical protein
BT_3507	hypothetical protein
BT_3512	glutaminase A
BT_3515	putative glycosylhydrolase
BT_3516	arabinan endo-1,5-alpha-L-arabinosidase A
BT_3517	RNA polymerase ECF-type sigma factor
BT_3521	alpha-1,6-mannanase
BT_3524	alpha-1,6-mannanase
BT_3526	glutaminase
BT_3546	glutaminase
BT_3552	peptide chain release factor RF-2
BT_3634	transposase
BT_3675	endo-1,4-beta-xylanase D
BT_3678	two-component system sensor histidine kinase/response regulator
BT_3737	hypothetical protein
BT_3742	hypothetical protein
BT_3951	transcriptional regulator
BT_3952	hypothetical protein
BT_3953	putative outer membrane protein
BT_3959	putative outer membrane protein
BT_3966	two-component system response regulator
BT_3969	AcrB/AcrD/AcrF family cation efflux system protein

BT_3978	putative cardiolipin synthetase
BT_4030	hypothetical protein
BT_4032	putative non-specific DNA-binding protein
BT_4057	two-component system sensor histidine kinase
BT_4114	hypothetical protein
BT_4115	hypothetical protein
BT_4116	hypothetical protein
BT_4119	hypothetical protein
BT_4121	hypothetical protein
BT_4123	exo-poly-alpha-D-galacturonosidase
BT_4127	Glucosamine-6-phosphate deaminase
BT_4130	putative helicase
BT_4131	hypothetical protein
BT_4138	putative aminotransferase
BT_4170	putative pectate lyase L
BT_4178	two-component system sensor histidine kinase/response regulator
BT_4181	beta-galactosidase
BT_4182	two-component system sensor histidine kinase/response regulator
BT_4183	pectate lyase L
BT_4187	exo-poly-alpha-D-galacturonosidase
BT_4199	hypothetical protein
BT_4200	hypothetical protein
BT_4201	hypothetical protein
BT_4210	Peptide chain release factor 1
BT_4212	hypothetical protein
BT_4339	glutamine synthetase
BT_4446	sugar phosphate isomerase/epimerase
BT_4449	putative dehydrogenase
BT_4450	hypothetical protein
BT_4459	putative transport protein
BT_4479	integrase protein
BT_4480	hypothetical protein
BT_4481	outer membrane protein
BT_4488	formamidopyrimidine-DNA glycosylase
BT_4578	transposase
BT_4636	RNA polymerase ECF-type sigma factor
BT_4775	conjugate transposon protein

Annex 2: Genes present in strains VPI 5482, jmh43, jmh44, jmh47, jmh49, jmh50, jmh58, jmh79 and absent from strain jmh71

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Élément retiré de la version de diffusion de la thèse de Nathalie Béchon

Annexe 1 : A putative type V pilus contributes to *Bacteroides thetaiotaomicron* biofilm formation capacity. *Journal of Bacteriology*. 2019 Mar 4. pii: JB.00650-18. doi: 10.1128/JB.00650-18, [Mihajlovic J](#), [Béchon N](#), [Ivanova C](#), Chain F, Almeida A, Langella P, [Beloin C](#), and [Ghigo JM](#). (2019)

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