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## A Putative Type V Pilus Contributes to *Bacteroides thetaiotaomicron* Biofilm Formation Capacity

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1 **A putative type V pilus contributes to *Bacteroides thetaiotaomicron* biofilm**  
2 **formation capacity**

3

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32

33 **Running Title:** *Bacteroides thetaiotaomicron* biofilm formation

34

35 **ABSTRACT**

36

37 *Bacteroides thetaiotaomicron* is a prominent anaerobe member of the healthy human gut  
38 microbiota. While the majority of functional studies on *B. thetaiotaomicron* addressed its  
39 <sup>[1]</sup><sub>SEP</sub> impact on the immune system and the utilization of diet polysaccharide, *B.*  
40 *thetaiotaomicron* biofilm capacity and its contribution to intestinal colonization are still  
41 poorly characterized. We tested the natural adhesion of 34 *B. thetaiotaomicron* isolates and  
42 showed that, although biofilm capacity is widespread among *B. thetaiotaomicron* strains, this  
43 phenotype is masked or repressed in the widely used reference strain VPI 5482. Using  
44 transposon mutagenesis followed by a biofilm positive selection procedure we identified VPI  
45 5482 mutants with increased biofilm capacity corresponding to an alteration in the C-terminal  
46 region of BT3147, encoded by the *BT3148-BT3147* locus, which displays homology with  
47 mfa-like typeV pili found in many *Bacteroidetes*. We showed that BT3147 is exposed on *B.*  
48 *thetaiotaomicron* surface and that BT3147-dependent adhesion also requires BT3148,  
49 suggesting that BT3148-BT3147 correspond to the anchor and stalk subunits of a new type V  
50 pilus involved in *B. thetaiotaomicron* adhesion. This study therefore introduces *B.*  
51 *thetaiotaomicron* as a model to study proteinaceous adhesins and biofilm-related phenotypes  
52 in this important intestinal symbiont.

53

54

55 **IMPORTANCE**

56

57 Although the gut anaerobe *Bacteroides thetaiotaomicron* is a prominent member of the  
58 healthy human gut microbiota, little is known on its capacity adhere to surfaces and form  
59 biofilms. Here, we identified that alteration of a surface exposed protein corresponding to a  
60 type of pili found in many *Bacteroidetes* increases *B. thetaiotaomicron* biofilm formation.  
61 This study lays the ground for establishing this bacterium as a model organism for *in vitro*  
62 and *in vivo* study of biofilm-related phenotypes in gut anaerobes.

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66 **KEYWORDS**

67 Biofilm; *Bacteroides thetaiotaomicron*; gut commensal, adhesion, typeV pili

68

69 **INTRODUCTION**

70

71 Bacterial biofilms are widespread surface-attached communities, in which reduced diffusion  
72 and a heterogeneous structure favor physical and metabolic contacts between bacteria and  
73 induce novel behaviors as compared to individual planktonic microorganisms (1, 2). In the  
74 past decades, biofilms were often investigated under aerobic conditions using genetically  
75 amenable aerobic bacterial models, and anaerobic biofilms studies mainly focused on oral  
76 anaerobes. Although these studies considerably advanced our understanding of this important  
77 bacterial lifestyle, many clinically and ecologically important processes take place in  
78 anaerobic conditions and are carried out by strict or facultative commensal and pathogenic  
79 anaerobes (3). This is in particular the case of the vertebrate gut microbiota, which is vastly  
80 dominated by anaerobic Firmicutes and Bacteroidetes (4, 5). However, although the intestinal  
81 microbiota provides protection from pathogen colonization and impacts host intestinal  
82 physiology and <sup>[[SEP]]</sup>health (6-11), the contributions of its structural organization and of the  
83 biofilm-formation capacities of gut anaerobes are still poorly <sup>[[SEP]]</sup>understood.

84

85 One of the most abundant and best-studied members of the healthy human gut microbiota is  
86 the strictly anaerobic bacterium *Bacteroides thetaiotaomicron* (12). *B. thetaiotaomicron* was  
87 shown to contribute to the development of the intestinal mucosal layer, modulate the function  
88 of the intestinal immune system and is considered to play a key role in the breakdown of diet  
89 and host-derived polysaccharides (13-16). Whereas biofilm formation could be involved in  
90 several important aspects of *B. thetaiotaomicron* biology, including intestinal colonization,  
91 inter- and intra-species interactions and virulence in extra-intestinal sites (17, 18), studies  
92 investigating *B. thetaiotaomicron* adhesion and biofilm formation are still scarce. A  
93 transcriptomic analysis performed on biofilm formed on carbon paper surface in chemostats  
94 showed an upregulation of several polysaccharide utilization loci (PUL) involved in  
95 polysaccharide foraging, mucin degradation enzymes, as well as of two out of eight *B.*  
96 *thetaiotaomicron* capsular systems (19). This study, however, did not reveal significant  
97 differences in the expression level of genes encoding extracellular appendages usually  
98 associated with bacterial surface-adhesion, leaving open the question whether adhesins  
99 contribute to *B. thetaiotaomicron* biofilm formation.

100 In the present study, we showed that, although *in vitro* biofilm development is widespread  
101 among natural *B. thetaiotaomicron* isolates, the reference *B. thetaiotaomicron* VPI 5482

102 (ATCC 29148) commonly used in human gut microbiota studies forms poor biofilm. We  
103 hypothesized that functions involved in VPI 5482 *in vitro* biofilm formation could be  
104 repressed or masked and use of transposon mutagenesis and a positive selection procedure  
105 allowed us to identify a mutation in the gene *BT3147*, altering the C-terminal region of  
106 *BT3147* and increasing VPI 5482 biofilm formation. We showed that the *BT3148-BT3147*  
107 locus displays homology with the Mfa-like type V pili found in many *Bacteroides* species  
108 (20) and could encode a surface exposed type V pilus involved in *B. thetaiotaomicron*  
109 adhesion. We expect this work to lay the ground for further characterization of *B.*  
110 *thetaiotaomicron* adhesins in VPI5482 and other biofilm-forming isolates, establishing this  
111 species as a model organism for *in vitro* and *in vivo* study of biofilm-related phenotypes in  
112 gut anaerobes  
113  
114

## 115 RESULTS

116

### 117 **The reference strain *B. thetaiotaomicron* VPI 5482 forms poor *in vitro* biofilm**

118 In order to analyze the ability of *B. thetaiotaomicron* VPI 5482 (ATCC 29148) (12) to form  
119 biofilm, we performed *in vitro* biofilm assays, using either a static 96-well plate model  
120 followed by crystal violet staining or dynamic continuous-flow biofilm microfermenters.  
121 Although *B. thetaiotaomicron* VPI 5482 adhesion and biofilm formation was previously  
122 reported in chemostat on carbon paper (19), we observed poor or no biofilm formation in both  
123 biofilm models (Fig. 1A and 1C). We then tested the biofilm capacity of 34 *B.*  
124 *thetaitotaomicron* isolates (Table S1) and found that 41% of them (14 out of 34) produce more  
125 biofilm than VPI 5482 (Fig. 1AB and Fig. S1). Taken together, these results indicate that,  
126 whereas the ability to form biofilm is widespread among natural *B. thetaiotaomicron* isolates,  
127 the widely used reference strain VPI5482 is a poor biofilm former in the tested growth  
128 conditions.

129

### 130 **Selection of *B. thetaiotaomicron* VPI 5482 mutants with increased biofilm capacity**

131 Considering the ability of several *B. thetaiotaomicron* strains to form biofilm, we  
132 hypothesized that functions involved in biofilm formation could be repressed or masked in the  
133 *B. thetaiotaomicron* VPI 5482 strain. To test this hypothesis and identify *B. thetaiotaomicron*  
134 VPI 5482 mutants with increased biofilm formation ability, we generated a library of over  
135 10,000 mariner transposon mutants and subjected them to a positive selection procedure for  
136 biofilm formation. Briefly, we resuspended a mix of *B. thetaiotaomicron* VPI 5482  
137 transposon mutants in a tube with 15 ml of BHIS medium (adjusted to OD<sub>600</sub>=1) and we  
138 placed a microfermenter glass spatula into this solution for 10 minutes, to allow for initial  
139 adhesion (Fig. 2A). The spatula was then washed in BHIS medium to remove non-adherent  
140 bacteria. Subsequently, the spatula potentially carrying the most adherent mutants was  
141 inserted in a continuous-flow biofilm microfermenter to allow surface growth and favor  
142 positive selection of *B. thetaiotaomicron* mutants with increased adhesion capacity. After 8  
143 hours the spatula-associated bacterial population was re-suspended in fresh BHIS medium  
144 and the resulting overnight culture was used as the inoculum for another cycle of positive  
145 selection (Fig. 2A). After 4 cycles of positive selection, the biofilm microfermenter inoculated  
146 with culture potentially enriched in biofilm positive *B. thetaiotaomicron* VPI 5482 mutants  
147 displayed, after 24h, a strong biofilm formation compared to a microfermenter inoculated  
148 with the original wild type VPI 5482 strain (Fig. 2B). After resuspension of the corresponding

149 biofilm, *B. thetaiotaomicron* VPI 5482 mutants were subjected to serial dilution in BHIS and  
150 plated on selective agar plates. We re-isolated the resulting colonies and individually tested  
151 mutants using 96-well plate biofilm assay. This led us to identify several mutants with an  
152 improved biofilm-forming capacity compared to WT VPI 5482.

### 153 **Transposon insertion in the 3' end of the Mfa/type V pilin homolog gene *BT3147*** 154 **increases biofilm formation**

155 We determined that 8 of the identified biofilm positive mutants (Fig. 2C), had a transposon  
156 inserted in *BT3147*, a gene located downstream of *BT3148* and upstream of *BT3146-BT3145*  
157 (Fig. 3A). Although protein sequence analysis only showed weak homologies, search in  
158 structure databases revealed that the N-terminal region (Residues 49 to 192) and the central  
159 region of *BT3147* (residues 171 to 388) contain a pfam 08842 (Mfa2) domain corresponding  
160 to Mfa2/Mfa1 pilin of recently defined type V pili that are ubiquitous in *Porphyromonas*  
161 *gingivalis* and other gut Bacteroidetes (20). All eight transposon insertions were located in the  
162 last 40 codons of *BT3147* and produced three different deletion/insertion events in *BT3147*  
163 coding sequence (Figure 3B). Class 1 mutants (3 mutants 2D7; 2D12; 2B9; see Figure 2C)  
164 correspond to an out of frame transposon insertion at position 1138 bp, deleting the last 9  
165 amino acids from *BT3147* without adding any transposon-derived coding sequence (hereafter  
166 named *BT3147Δ9*). One representative of class 1, 2D7, was chosen for further analysis. Class  
167 2 mutants (3 mutants 1A8; 1H2; 2A12; see Figure 2C) correspond to an in-frame transposon  
168 insertion at position 1047, deleting the last 40 amino acids of *BT3147* and replacing them by  
169 50 amino acids from the transposon sequence leading to a chimeric 398 codon sequence  
170 (*BT3147Δ40+50*). One representative of class 2, 1H2, was chosen for further analysis.  
171 Finally, Class 3 mutants (2 mutants 1H9, 3F12; see Figure 2C) correspond to a 434 amino  
172 acid fusion protein arising from the deletion of the last 3 *BT3147* codons replaced by 49  
173 codons from the transposon sequence (*BT3147Δ3+49*) (Fig. 3B).

174 To confirm the link between biofilm production and transposon insertion in *BT3147*, we  
175 deleted gene *BT3147* in the wild type VPI 5482 strain, however this approach did not lead to  
176 an increase in biofilm formation (Fig. 3C). We then hypothesized that insertion of the  
177 transposon in the 3' extremity of *BT3147* could prevent (by polar effect) or enhance the  
178 expression of the downstream genes *BT3146* and *BT3145* (Fig. 3A). However, deletion of  
179 *BT3146-BT3145* did not lead to significant alteration of biofilm formation compared to WT  
180 VPI 5482 strain (Fig. 3C). In addition, the comparison of VPI 5482 and 1H2 transcription  
181 using RNAseq analysis showed that the insertion of the transposon did not alleviate a

182 potential premature transcription termination in the wild type strain. Moreover, we did not  
183 detect any antisense transcript in the *BT3147-5* region that could be interfering with *BT3147*  
184 transcription (Fig. S2). Nevertheless, deletion of the 1046 base pairs of *BT3147* (1167 bp)  
185 located upstream of the transposon insertion point in the class2 1H2 mutant, the strongest  
186 biofilm former among our 8 mutants (Figure 2C), abolished biofilm formation in this strain  
187 (Fig. 3D). This result demonstrated that the presence of the mariner transposon *per se* does  
188 not promote biofilm formation and that the observed biofilm phenotype requires at least part  
189 of the *BT3147* gene.

190

### 191 **C-terminal truncation of BT3147 promotes BT3148-dependent *B. thetaiotaomicron*** 192 **biofilm formation**

193 We then further investigated the consequences of transposon insertions on the *BT3147*  
194 protein by reproducing one of the genetic configurations created by transposon insertion. We  
195 chose class 1 biofilm-forming mutants, as they correspond to the truncation of *BT3147* nine  
196 last C-terminal amino-acids (*BT3147Δ9*) without added transposon-derived coding sequence.  
197 Since downstream genes *BT3146* and *BT3145* are not involved in the studied phenotype (Fig.  
198 3C, Fig. S2), we constructed a chromosomal *BT3147Δ9 de novo* by removing the VPI 5482  
199 region encompassing the last 9 codons of *BT3147* and the downstream genes *BT3146* and  
200 *BT3145*, replacing it by a stop codon (Fig. 4A). We then compared the resulting strain,  
201 *BT3147Δ9 ΔBT3146-45* with the wild type strain VPI 5482 and showed that it displayed an  
202 increased biofilm formation capacity (Fig. 4A and supplementary Fig.S3 ). Consistently,  
203 whereas chromosomal-based expression of *BT3148-BT3147* displayed a wild type biofilm  
204 phenotype, expression of *BT3148-BT3147Δ9* led to increased biofilm formation compared to  
205 WT VPI 5482 (Fig. 4A).

206 Use of an antiserum raised against *BT3147* enabled the immunodetection of a truncated form  
207 of *BT3147* in whole cell extracts of strain *BT3147Δ9 ΔBT3146-45*, class 1 (2D7), class 2  
208 (1H2) transposon mutants and in the wild type strain constitutively expressing *BT3147Δ9*  
209 (Fig. 4B). However, while WT (40.6 kDa) and truncated versions of *BT3147* (1H2, 41.6 kDa;  
210 2D7 and *de novo* *BT3147Δ9*, 39.6 kDa) have very similar theoretical size, the truncated forms  
211 of *BT3147* migrated at an apparent smaller molecular mass of ~30-35kDa, suggesting an  
212 additional cleavage of these forms (see discussion).

213 Finally, considering that *BT3148*, encoded by the gene located immediately upstream of  
214 *BT3147* (Figure 3A) displays homology with the *B. thetaiotaomicron* Fim2B/Mfa2 homolog  
215 *BT2657* (PDB code c3gf8A), we tested *BT3148* contribution to *BT3147Δ9*-dependent biofilm

216 formation. Deletion of *BT3148* in *B. thetaiotaomicron* *BT3147Δ9 ΔBT3146-45* resulted in a  
217 significant decrease in biofilm formation (Fig. 4C). Taken together, these results  
218 demonstrated that a C-terminal truncation of *BT3147* promote biofilm formation in *B.*  
219 *thetaitotaomicron*.

220

### 221 **Localization of a putative *BT3148-BT3147* type V pilus on *B. thetaiotaomicron* surface**

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223 *BT3148* and *BT3147* display structural similarities with *Mfa1/* type V pili identified in the  
224 anaerobic oral pathogen *P. gingivalis* and other gut Bacteroidetes (20). Two types of pili were  
225 described in *P. gingivalis* – the long *FimA* and the short *Mfa1*. *FimA* filaments are more than  
226 1 μm long, fragile and promote autoaggregation and biofilm formation (21). *FimA*  
227 corresponds to the major structural component of the *FimA* filaments, *FimB* to the anchor  
228 protein, and *FimC-E* to accessory proteins (22). The shorter *Mfa1* type pili are 0.1 – 0.5 μm  
229 long filaments essentially composed of the major stalk protein *Mfa1*, bound to the cell surface  
230 via the membrane-located anchor protein *Mfa2* (Fig. 5 A). In addition to *mfa1* and *mfa2*, the  
231 *P. gingivalis* gene cluster often includes genes *mfa3-5*, which encode accessory proteins.  
232 Since *BT3148* displays homology with *B. thetaiotaomicron* *Fim2B/Mfa2* homolog *BT2657*  
233 (PDB code c3gf8A), we propose that it could be a *B. thetaiotaomicron* VPI 5482 type V pilus  
234 anchor (Fig. 5 A and Fig. S3). *BT3147*, on the other hand displays structural homology with  
235 *Bacteroides eggerthii* DSM 20697 *BegFim1A*-like proteins belonging to the *FimA/Mfa1*  
236 protein family (PDB code 4gpv) and could correspond to the stalk component of Bacteroidia  
237 type V pili. We could not identify candidates for accessory proteins to the putative *BT3148-*  
238 *BT3147* pilus (Fig. 5 A and Fig. S4).

239 Based on these analyses, we hypothesized that *BT3147* could be the major pilin of a structure  
240 exposed at the *B. thetaiotaomicron* cell surface. However, attempts to visualize *BT3147-*  
241 based pilus using immunofluorescence, and immuno-staining coupled to transmission  
242 electron microscopy analyses were unsuccessful, as it is sometimes the case for thin or  
243 masked fimbrial surface structures. Nevertheless, proteinase K sensitivity of *BT3147* on  
244 treated whole cells suggested that this protein is surface exposed (Fig. S5). We therefore tried  
245 tried to detect *BT3147*-containing structures by releasing *B. thetaiotaomicron* protruding  
246 surface appendages in the supernatant using a blender-based shaving procedure that do not  
247 lead to detectable bacterial lysis. We concentrated the protein obtained using this surface  
248 shaving procedure with strain VPI 5482 WT, *BT3147Δ9 ΔBT3146-45*, class 1 transposon

249 mutant (2D7), and class 2 transposon mutant strain (1H2) and we performed an  
250 immunodetection using anti-BT3147 antibodies.

251 We detected BT3147 in all samples, with BT3147 truncated forms migrating lower than WT  
252 as described above (Fig. 5B and Fig. 4B). However, while WT displayed full length and  
253 monomeric protein, biofilm-forming *BT3147Δ9 ΔBT3146-45* and 2D7 and 1H2 transposon  
254 mutants ample showed multiple specific products, potentially indicative of protein  
255 polymerization during pilus formation (Fig. 5B).

256

### 257 ***In vivo* relevance of BT3147-mediated biofilm formation**

258 The demonstration that BT3147 is exposed on *B. thetaiotaomicron* surface as part of a  
259 potential type V pilus structure contributing to *B. thetaiotaomicron* biofilm formation  
260 prompted us to investigate the *in vivo* role of BT3148-BT3147 to *B. thetaiotaomicron*  
261 adhesion or colonization. To this end, we used a germ-free mouse model of intestinal  
262 colonization and performed *in vivo* mixed cultures competition experiments, comparing the  
263 respective colonization capacity of the competing strains administrated intra-gastrically at a  
264 1:1 mixed ratio ( $2 \times 10^7$  cells/ml). We first verified that *B. thetaiotaomicron ΔBT3146-3145*  
265 showed a similar colonization capacity than the wild type VPI 5482 strain indicating that  
266 deletion of *BT3146-3145* does not affect colonization (Fig. 6A). We then found that  
267 *ΔBT3148-BT3145* has the same colonization capacity as the wild type (Fig. 6B), and that the  
268 biofilm-forming strain *BT3147Δ9 ΔBT3146-45* shows similar colonization capacity as the  
269 wild type VPI 5482 strain (Fig. 6C). This analysis therefore showed that BT3148-3147  
270 putative pilus or its biofilm-promoting truncated form does not significantly contribute to *B.*  
271 *thetaitotaomicron* intestinal colonization capacity in the tested conditions.

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278 **DISCUSSION**

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280 In this study we investigated the biofilm formation capacity of the prominent gut  
281 symbiont *B. thetaiotaomicron*. We found that many *B. thetaiotaomicron* isolates displayed an  
282 increased biofilm formation capacity compared to the reference *B. thetaiotaomicron* VPI 5482  
283 strain that is used in most gut microbiota studies. This widespread but highly variable  
284 capacity to form biofilm is observed in many aerobic bacteria, including the facultative  
285 anaerobe *Escherichia coli* (24) and *Staphylococcus epidermidis* (25) and is consistent with a  
286 study performed in *Bacteroides fragilis*, a closely related intestinal anaerobe, which also  
287 displays variable biofilm formation capacity (26). Biofilm formation by pathogenic bacteria is  
288 a major cause of chronic and recurring infections (2) and pathogenicity and abscess formation  
289 by *B. fragilis* is associated with biofilm and adhesion to peritoneal epithelium (27, 28).  
290 Whereas *B. thetaiotaomicron* usually behaves as an intestinal non-pathogenic bacterium, it is  
291 also found associated with abdominal and deep wound infections, representing up to 14% of  
292 the bacteria found at these sites (29). Since 5 out of 14 of the best biofilm-forming strains  
293 identified in our study were isolated from different infection sites (bone biopsies, blood  
294 samples, or abscesses, see Table S1), this suggests that biofilm formation of *B.*  
295 *thetaiotaomicron* could be correlated with opportunistic extra-intestinal infections and future  
296 investigations could provide insights into this aspect of *B. thetaiotaomicron* biology.

297 Our static and dynamic biofilm assays show that *B. thetaiotaomicron* strain VPI 5482  
298 forms poor biofilms *in vitro*, which is consistent with the study performed by TerAvest and  
299 colleagues who obtained a VPI 5482 biofilm only after 8 days of continuous incubation in a  
300 chemostat (19). Whereas we did not observe significant biofilm accumulation in VPI 5482  
301 even after 48h, this result might reflect the fact that VPI 5482 biofilm formation is repressed  
302 in laboratory conditions. For example, uropathogenic *E. coli* is known to cause infections by  
303 adhering to the bladder epithelium using fimbriae, but displays poor biofilm formation *in*  
304 *vitro* (30, 31). Similarly, Huang and colleagues demonstrated that *B. fragilis* poor adherence  
305 is enhanced on mucin-covered plates, which mimics the intestinal mucosa – the natural  
306 habitat of *B. fragilis* (32).

307 Our approach to uncover the biofilm potential and biofilm-related functions in *B.*  
308 *thetaiotaomicron* VPI 5482 resulted in the identification of two putative Mfa1-like proteins  
309 (BT3148 and BT3147) potentially involved in biofilm formation in *B. thetaiotaomicron*.  
310 BT3147 and BT3148 have N-terminal domain homologies with Mfa1 type pili of the closely  
311 related anaerobic oral pathogen *P. gingivalis*, in which two types of pili were described – the

312 long FimA and the short Mfa1, which represent the prototype of a recently defined type V  
313 pilus that are ubiquitous in gut bacteroidetes (20). In VPI 5482, genes *BT3148-3147* appear to  
314 encode a minimal Mfa1 type operon only encoding the potential pilus anchor (BT3148) and  
315 stalk pilin (BT3147) (Fig.5A). Intriguingly, studies of Mfa1-type pili in *P. gingivalis* showed  
316 that the presence of minor pilins is necessary for proper pili function and that deletion of the  
317 minor pili component *mfa4* results in complete loss of function (33, 34). The apparent lack of  
318 *mfa3-5* genes encoding accessory pilins near putative *mfa1*- and *mfa2*-like genes (*BT3147* and  
319 *BT3148* respectively) suggests that *BT3148-BT3147* might correspond to a new minimal type  
320 V pili operon that does not contain or lost its accessory pilins during evolution.

321 The contribution of the putative Mfa-like BT3148-BT3147-based pilus to biofilm formation is  
322 only revealed upon removal of the last 9 C-terminal amino acids of BT3147. The potential  
323 polymerization observed in our immune-detection experiments (Fig.5B) suggested that C-  
324 terminal truncation of the putative stalk pilin BT3147 could lead to the synthesis of an  
325 extended pilus, thus enhancing *in vitro* adhesion. This is surprising, as it has been suggested  
326 that the C-terminus plays a critical role in type V pili assembly (35) and the observed  
327 polymerization could correspond to an artifact introduced upon C-terminal truncation of  
328 BT3147. However, a similar phenotype had been described in *P. gingivalis*, in which deletion  
329 and truncation of the anchor resulted into the production of an unusually long pilus that  
330 promoted aggregation and biofilm formation (36, 37). The exact mechanism of synthesis of  
331 Mfa1 and FimA-like pili has not yet been resolved, however recent structural studies of  
332 crystal structures and cysteine-based crosslinking showed an interaction between beta strands  
333 of Mfa1 N- and C-termini (20, 35), indicating that polymerization could include a strand  
334 exchange between the C-terminal and N-terminal pilin domains (38). In *P. gingivalis*,  
335 maturation of Mfa1 pilins involves a N-terminus proteolytic cleavage step performed by  
336 RgpA/B arginine protease of *P. gingivalis*. The proteolysis takes place in a loop connecting  
337 two N-terminal  $\beta$ -strands, enabling the removal of one strand, freeing a groove for the  
338 insertion of a C-terminal  $\beta$ -strand coming from another Mfa1 pilin subunit. It is speculated  
339 that in this processive assembly process, Mfa1/FimA stalk pilin subunits need to undergo  
340 conformational changes allowing them to connect to the N-terminus of the next pilin unit  
341 (20). The critical role of the Mfa1/FimA C-terminus during type V pili assembly was recently  
342 confirmed by Hall and colleagues, as they showed that presence of the last 6 C-terminal  
343 amino acids is necessary for polymerization (35). The reason why truncation of the putative  
344 Mfa1-like *B. thetaiotaomicron* pilin BT3147 seems to stimulate rather than impair  
345 polymerization and biofilm formation is not clear but could reflect a structural difference in

346 the C-terminus of BT3147 compared to *P. gingivalis* Mfa1. We indeed could not find any  
347 homology associated with BT3147 C-terminus and the Mfa1-type structural model (pdb code  
348 4gpv, Fig. S3). By contrast, Phyre structural model prediction of BT3147 suggests the  
349 presence of a long and unstructured C-terminus in BT3147 (Fig. S4). This suggests that at  
350 least some of *B. thetaiotaomicron* type V pilin structures might differ from *P. gingivalis* ones  
351 and could potentially require additional or different post-translational pili modifications  
352 during the assembly process. In our study, removing the last 9 C-terminal amino acids of  
353 BT3147 might have exposed the A1 C-terminal strand, that was shown to be involved in  
354 interaction with the N-terminus (35), allowing for pilus polymerization, as suggested by the  
355 polymerization pattern observed in the *BT3147Δ9 ΔBT3146-45* strain (Figure 5B). In support  
356 of this possibility, we showed that BT3147Δ9 detected using anti-BT3147 antibodies migrates  
357 at about 10 kDa lower than the WT protein. We therefore hypothesize that the truncation in  
358 the BT3147Δ9 protein could unmask a protease cleavage site and undergo an extra proteolytic  
359 cleavage step. Preliminary results using mass spectrometry analysis of the shorter band  
360 detected in the 1H2 mutant indicated a protein size of 27 kDa, in accordance to the protein  
361 size observed by immunodetection and consistent with a further maturation of the protein.  
362 Alternatively, considering that all type V pilins (major, minor and accessory) share domain  
363 homology in their N-termini, we cannot completely rule out that BT3147 belongs to the group  
364 of accessory pilins (Mfa3-5-like) whose C-terminal structure does not allow further pilus  
365 elongation (20). In this scenario, deletion of the last 9 C-terminal amino acids of BT3147  
366 could remove a peptide normally blocking polymerization, thus resulting in the observed  
367 polymerization in the *BT3147Δ9 ΔBT3146-5* strain.  
368 We could also show that *BT3147* is constitutively expressed in the wild type strain,  
369 suggesting that BT3148-BT3147 genes are not cryptic. However, as we were unable to see  
370 differences of colonization of the intestinal tract of germ-free mice using strains with WT or  
371 the mutated form of BT3147, the *in vivo* function of this locus is yet to be determined. In *P.*  
372 *gingivalis* the Mfa1 type filaments promote heterologous cell-to-cell interactions including  
373 adhesion to an oral pathogen often found in dental plaque *Streptococcus gordonii* (Lamont et  
374 al., 2002), and future work will determine whether a similar function is carried-out by *B.*  
375 *thetaitaomicron* BT3148-BT3147.  
376  
377 Our study demonstrates the widespread ability of *B. thetaiotaomicron* to form biofilm and  
378 brings first evidence for the existence of functional type V pili structures as mediators of  
379 adhesion in *B. thetaiotaomicron in vitro*. We expect this work to contribute to the introduction

380 of *B. thetaiotaomicron* as a new a model to study proteinaceous adhesins and biofilm-related  
381 functions in intestinal colonization and microbiota-host interactions in this anaerobic gut  
382 symbiont.

383

## 384 MATERIALS AND METHODS

385

### 386 *Bacterial strains and growth conditions*

387 Bacterial strains used in this study are listed in Table S2. *B. thetaiotaomicron* was grown in  
388 BHIS broth (39) supplemented with erythromycin (20 µg/ml), tetracycline (2.5 µg/ml),  
389 gentamicin (200 µg/ml) or 5'-fluoro-2'-deoxyuridin (FdUR, 200 µg/ml) when required and  
390 incubated at 37°C in anaerobic conditions using anaerobic jars with anaerobic atmosphere  
391 generators or in a C400M Ruskinn anaerobic-microaerophilic station. *E. coli* S17λpir was  
392 grown in Lysogeny Broth (LB) broth supplemented with ampicillin (100 µg/ml) when  
393 required and incubated at 37°C. All media and chemicals were purchased from Sigma-  
394 Aldrich.

395

### 396 *Biofilm formation in microfermenters*

397 Continuous-flow biofilm microfermenters containing a removable glass spatula were used as  
398 described in (40) (see also <https://research.pasteur.fr/en/tool/biofilm-microfermenters/>), with  
399 or without internal bubbling of a filter-sterilized compressed mix of 90% nitrogen / 5%  
400 hydrogen /5% carbon dioxide (anaerobic biofilm conditions). Biofilm microfermenters were  
401 inoculated by placing the spatula in a culture solution adjusted to OD<sub>600</sub>=0.5 (containing  
402 2.5x10<sup>8</sup> bacteria/ml) for 10 min. The spatula was then reintroduced into the microfermenter.  
403 Flow rate was then adjusted (30 ml/h) so that total time for renewal of microfermenter  
404 medium was lower than bacterial generation time, thus minimizing planktonic growth by  
405 constant dilution of non-biofilm bacteria.

406

### 407 *Transposon mutagenesis and positive selection*

408 We used pSAMbt – the previously published plasmid used for random mariner-based  
409 transposon mutagenesis (41), to generate a library of approximately 10.000 *B.*  
410 *thetaiotaomicron* mutants carrying erythromycin resistant transposons. The mutants were  
411 pooled and subjected to a positive selection procedure using biofilm microfermenters. The  
412 mutants pool was put in contact with the glass microfermenter spatula for 10 minutes and the  
413 spatula was then introduced in the microfermenter and incubated with internal bubbling  
414 agitation to select for the strongly adherent bacteria during 8 hours. Following incubation, the  
415 bacteria attached to the spatula were resuspended in 15 ml of fresh BHIS medium  
416 supplemented with erythromycin and incubated anaerobically overnight. On the next day the

417 overnight culture was used to start another round of positive selection. This procedure was  
418 repeated four times, with the last microfermenter incubation lasting 24 hours instead of 8  
419 hours. The biofilm composed of adherent transposon mutants developing on the spatula was  
420 then recovered and plated out on BHIS-erythromycin agar to obtain single colonies that were  
421 then individually tested for biofilm formation using 96-well crystal violet staining.

422

### 423 ***Biofilm microtiter plate assay***

424 Overnight cultures were diluted to  $OD_{600} = 0.01$  ( $5 \times 10^6$  cells/ml) and inoculated in technical  
425 triplicates in a polystyrene Greiner 96-well plate. Incubation was done at  $37^\circ\text{C}$  in anaerobic  
426 conditions for 24 hours. Supernatant with free-floating bacteria was removed by careful  
427 pipetting and 100  $\mu\text{l}$  of Bouin's solution (picric acid 0.9 %, formaldehyde 9 % and acetic acid  
428 5 %) was applied during 15 minutes to fix the biofilm attached to the well. Next the fixation  
429 solution was washed 3 times with water and biofilm was stained with 1% crystal violet  
430 solution during 15 minutes. After removal of the crystal violet solution, biofilms were washed  
431 3 times with water. For quantification of biofilm formation, dried stained biofilms were  
432 resuspended in a 1:5 acetone:ethanol mixture and absorbance was measured at 575 nm using a  
433 infinite M200 PRO plate reader. Biofilm formation in different *B. thetaiotaomicron* strains is  
434 represented in values normalized to average biofilm formation in the wild type VPI 5482.

435

### 436 ***Construction of B. thetaiotaomicron mutants***

437 All experiments and genetic constructions of *B. thetaiotaomicron* were made in VPI  
438 5482\_Δtdk strain, which was developed for 2-step selection procedure of unmarked gene  
439 deletion by allelic exchange, as previously described (42). Therefore, the VPI 5482\_Δtdk is  
440 referred to as wild type in this study. All primers used in this study are described in Table S3.  
441 For the generation of deletion strains by allelic exchange in *B. thetaiotaomicron*, we used  
442 pExchange-tdk vector (42). Briefly, 1 Kb upstream and downstream regions of the target  
443 sequence were cloned in the pExchange-tdk vector and transformed into *E. coli* S17λpir strain  
444 that was used to deliver the vector to *B. thetaiotaomicron* by conjugation. Conjugation was  
445 carried out by mixing exponentially grown cultures of the donor and the recipient strain at a  
446 2:1 ratio and mating was done by placing the mixture on Millipore 0.45  $\mu\text{m}$  hydrophilic  
447 cellulose ester filter disc (ref. HAWP29325) on top of BHIS agar plates at  $37^\circ\text{C}$  in aerobic  
448 conditions over night. After this, bacteria were recovered by resuspending and vortexing the  
449 filters in fresh BHIS medium and then plated on selective BHIS agar supplemented with  
450 erythromycin for selection of *B. thetaiotaomicron* transconjugants that underwent the first

451 recombination event and gentamicin to ensure exclusion of any *E. coli* growth. Erythromycin  
452 and gentamicin-resistant colonies of *B. thetaiotaomicron* were then subjected to the second  
453 round of selection on BHIS agar plates supplemented with FdUR for selection of double  
454 recombination colonies. The resulting deletion mutants were confirmed by PCR and  
455 sequencing.

456 For gene constitutive expression we used the pNBU2-*bla-erm* vector (43), which inserts itself  
457 in the 5' untranslated region of the tRNA<sup>ser</sup>, thus providing chromosomal based expression.  
458 In order to provide equal expression of different target genes we used the promoter of gene  
459 *BT1311* (coding for sigma factor RpoD), that had been cloned from a PCR fragment into the  
460 multiple cloning site of pNBU2-*bla-erm* to yield pNBU1311. Target genes were cloned (Start  
461 to Stop codon) using the NdeI/NotI sites of pNBU1311 and the resulting vector was then  
462 transformed into *E. coli* S17λpir strain. Transfer of the expression vector to *B.*  
463 *thetaitotaomicron* was done by conjugation as previously described, this time with only one  
464 selection step. The transformed *B. thetaiotaomicron* strains were selected on erythromycin  
465 and vector insertions were confirmed by PCR using pNBU1311-specific primers.

466

#### 467 ***RNAseq analysis***

468 Total RNA was extracted from exponentially grown cultures of the wild type *B.*  
469 *thetaitotaomicron* using MP Biomedicals FastRNA Pro<sup>TM</sup> BLUE KIT by the provider's  
470 manual and treated with Ambion Turbo DNA-free<sup>TM</sup> kit to remove possible DNA  
471 contamination. All samples were checked for residual genomic DNA contamination with the  
472 *DNAPol3-F* and *DNAPol3-R* primer pair (Table S3) and were considered DNA-free if no  
473 amplification was detected at <30 cycles. Ribosomal RNA was removed using Illumina Ribo-  
474 Zero<sup>TM</sup> rRNA Removal Kits and the purified RNA concentration was measured with  
475 NanoDrop and RNA quality was checked by Agilent 2100 BioAnalyzer. The obtained RNA  
476 samples were fragmented and used for cDNA library preparation using TruSeq Stranded  
477 mRNA Library Preparation Kit Set A<sup>TM</sup>. Samples were sequenced with an Illumina  
478 HiSeq2500 sequencer. Sequence quality was analyzed by FastQC  
479 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and reads were aligned against  
480 the reference VPI 5482 genome using the Bowtie2 R package (44).

481

482

#### 483 ***Structural analyses***

484 CD-search analysis was performed using the CDSEARCH/cdd v3.15 database (45). 3D  
485 homology search was performed using Phyre2 program using the intensive mode  
486 (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>)

487

#### 488 ***In vivo mixed cultures competition experiments in germ-free mice***

489 Animal experiments were done at “Animalerie Axénique de MICALIS (ANAXEM)”  
490 platform (Microbiologie de l’Alimentation au Service de la Santé (MICALIS), Jouy-en-Josas,  
491 France) according to an official authorization n°3441-2016010614307552 delivered by a  
492 French ministry (Education nationale, enseignement supérieur et recherche). The protocol was  
493 approved by a local ethic committee on animal experimentation (committee n°45). All  
494 animals were housed in flexible-film isolators (Getinge-La Calhène, Vendôme, France) with  
495 controlled environment conditions (light/dark 12 h/12 h, temperature between 20 and 22°C,  
496 humidity between 45 to 55%). Mice were provided with sterile tap water and a gamma-  
497 irradiated standard diet (R03-40, S.A.F.E., Augy, France), ad libitum. Their bedding was  
498 composed of wood shavings and they were also given cellulose sheets as enrichment.

499 *Methods:* 6 male C3H/HeN germ free mice were gavaged with 200 µl bacterial suspensions  
500 containing 2x10<sup>7</sup> cell/ml consisting of 1:1 ratio mix of the wild type *B. thetaiotaomicron* and  
501 one of the tested strains: ΔBT3146-5, ΔBT3148-5 or BT3147Δ9\_ΔBT3146-45. The wild type  
502 *B. thetaiotaomicron* carried erythromycin resistance gene (VPI 5482\_pNBU2-bla-erm, Table  
503 S1) for later distinction from the erythromycin-sensitive tested strains. Six mice were used for  
504 each condition (wild-type with ΔBT3146-5, wild-type with ΔBT3148-5 and wild-type with  
505 BT3147Δ9\_ΔBT3146-45). At 24, 48 and 96 hours after inoculation, feces were collected,  
506 homogenized in 1 ml of 1X PBS, and serial dilutions were plated onto BHIS agar plates with  
507 and without erythromycin (20 µg/ml). Intestinal colonization capacity of the wild type and  
508 different mutants was estimated by assessing their abundance in the feces using qPCR. Total  
509 bacterial DNA was isolated from the fecal samples with QIAamp® DNA Stool kit and 10 ng  
510 were used as template for qPCR amplification of *gyrA* and *ermG* using Brilliant II SYBR®  
511 Green QPCR Master Mix following the producer’s manual. The reaction was performed in  
512 the BIORAD Thermal cycler and absolute quantities of target genes were determined using  
513 standard-curve quantification method. Gene copy number was calculated from the absolute  
514 quantities using the following formula: (Starting quantity ng \* 60221 \* 10<sup>23</sup> molecules/mole) /  
515 (Amplicon length bp \* 660 g/mole \* 1\*10<sup>9</sup> ng/g). The abundance of the wild type and the  
516 competing mutant strains was estimated by quantifying the copy number of genes coding for  
517 gyrase A subunit (*gyrA* -BT0899) and *ermG* conferring resistance to erythromycin. While

518 *gyrA* is present in the wild type as well as in the mutant strains, *ermG* is only present in the  
519 wild type strain. Therefore, the abundance of wild type strain corresponds to the copy number  
520 of *ermG* and the mutant strain abundance is deduced as difference in the copy number  
521 between *gyrA* (total population) and the *ermG* (wild type).

522

### 523 ***Statistical analysis***

524 Unpaired two-tailed Mann-Whitney non parametric test analyses were performed using Prism  
525 6.0 for Mac OS X (GraphPad Software, Inc.). Each experiment was performed at least 3  
526 times. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

527

### 528 ***Generation of an antiserum against BT3147***

529 Polyclonal antibodies against BT3147 were raised in rabbits injected with a mixture of two  
530 peptides: peptide 1: C-VYDETGKTEVKQETTF-coNH<sub>2</sub> (corresponding to BT3147 amino  
531 acids 54-69) and peptide 2: C-VADGKTTGTESDTKT-coNH<sub>2</sub> (corresponding to BT3147  
532 amino acids 169-183). The design and synthesis of the peptides, as well as the production and  
533 purification of the antibody production were performed at the Covalab Research Institute,  
534 Villeurbanne, France. A mix of peptide 1 and 2 with Freund adjuvant was used for  
535 immunization of rabbits by 3 intradermic injections at day 0, day 21 and day 42 of the  
536 immunization protocol, followed by a subcutaneous injection at day 74. At day 88 of the  
537 protocol anti-BT3147 antibodies were purified from rabbit serum using affinity  
538 chromatography with a mix of peptide 1 and 2 as column ligands.

539

### 540 ***Preparation of protein extracts from shaved bacteria***

541 Overnight cultures in BHIS were washed and resuspended in 1X PBS. ‘Shaving’ of surface  
542 pili was performed as previously described (23). Briefly, 100 ml of culture was grown in  
543 BHIS for 48 h at 37°C without agitation in anaerobic conditions. The culture was centrifuged  
544 (6000 rpm, 10 min) and pellet resuspended in 1X PBS and potential surface appendages were  
545 ‘shaved’ on ice using a mini hand blender using 5 1-minute pulses (19 600 rpm). Next, the  
546 samples were centrifuged at 6000 rpm for 1h at 4°C and proteins from the supernatant were  
547 precipitated using 10% TCA for 48h at 4°C, followed by centrifugation at 20 000 rpm for 1h  
548 at 4°C. The pellet was washed with 100 % acetone and resuspended in 100 µl of 8M urea. 8  
549 µg of ‘shaved’ surface protein was analyzed by western blot using anti-BT3147 antibodies.

550

### 551 ***Western blot analysis***

552 Cultures were grown in BHIS overnight and 1 ml at OD<sub>600</sub>=2 was centrifuged (7000 rpm for 5  
553 min) and resuspended in 100µl of 1X Laemmli buffer with 250U of benzonase nuclease  
554 (Sigma E0114) and incubated for 5 minutes at 95°C. Aliquots of these raw cell extracts or  
555 protein extract from shaved bacteria were run on Mini-PROTEAN TGX Stain-Free™ precast  
556 Gels (BioRad) in 1X TGX buffer and then transferred to nitrocellulose membrane using a  
557 Trans-Blot® Turbo™ Transfer System (BioRad). Blocking was performed during 1 h in a 5%  
558 solution of dry milk and 0.05% Tween –1X PBS (1X PBST). The membranes were then  
559 incubated overnight in 1X PBST with anti-BT3147 antiserum at 1:1000 at 4°C with agitation.  
560 Membranes were washed in 1X PBST and then incubated with the secondary antibody (anti-  
561 rabbit IgG conjugated with horse radish peroxidase at 1:3000, Promega). After washing the  
562 excess second antibody, specific bands were visualized using the ECL prime detection  
563 method (GE Healthcare).

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**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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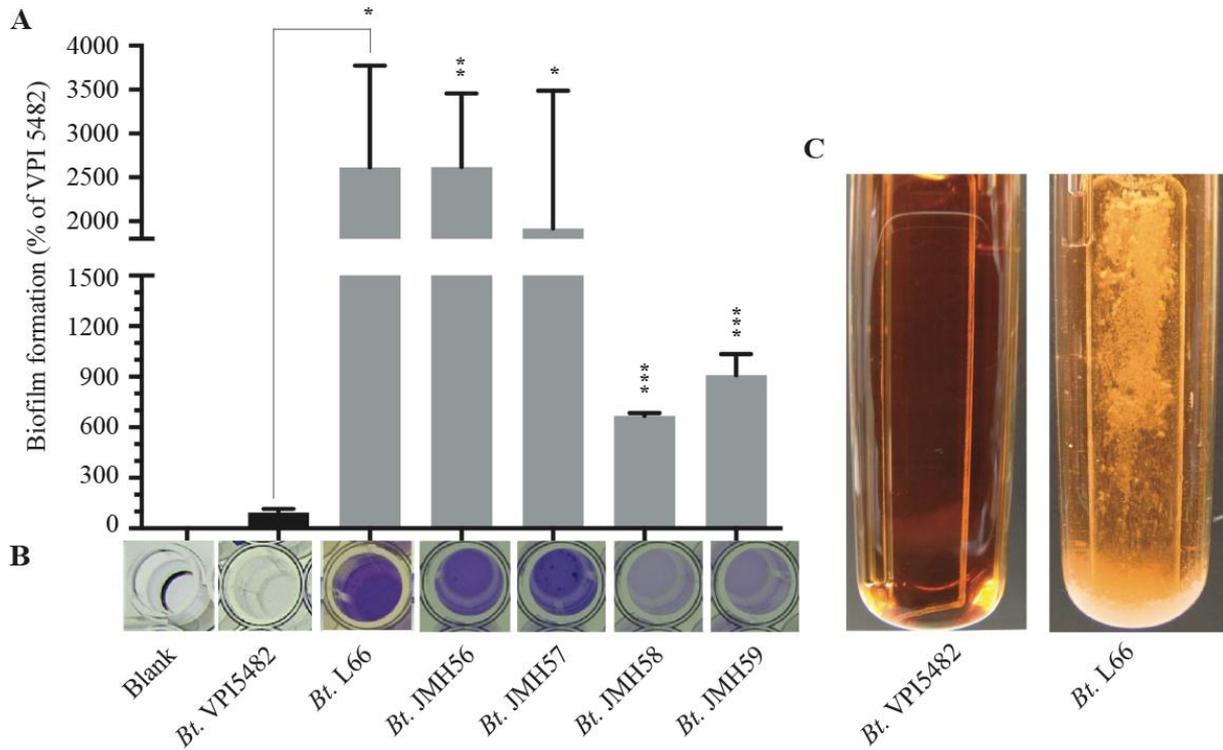
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738 **FIGURE AND FIGURE LEGENDS**

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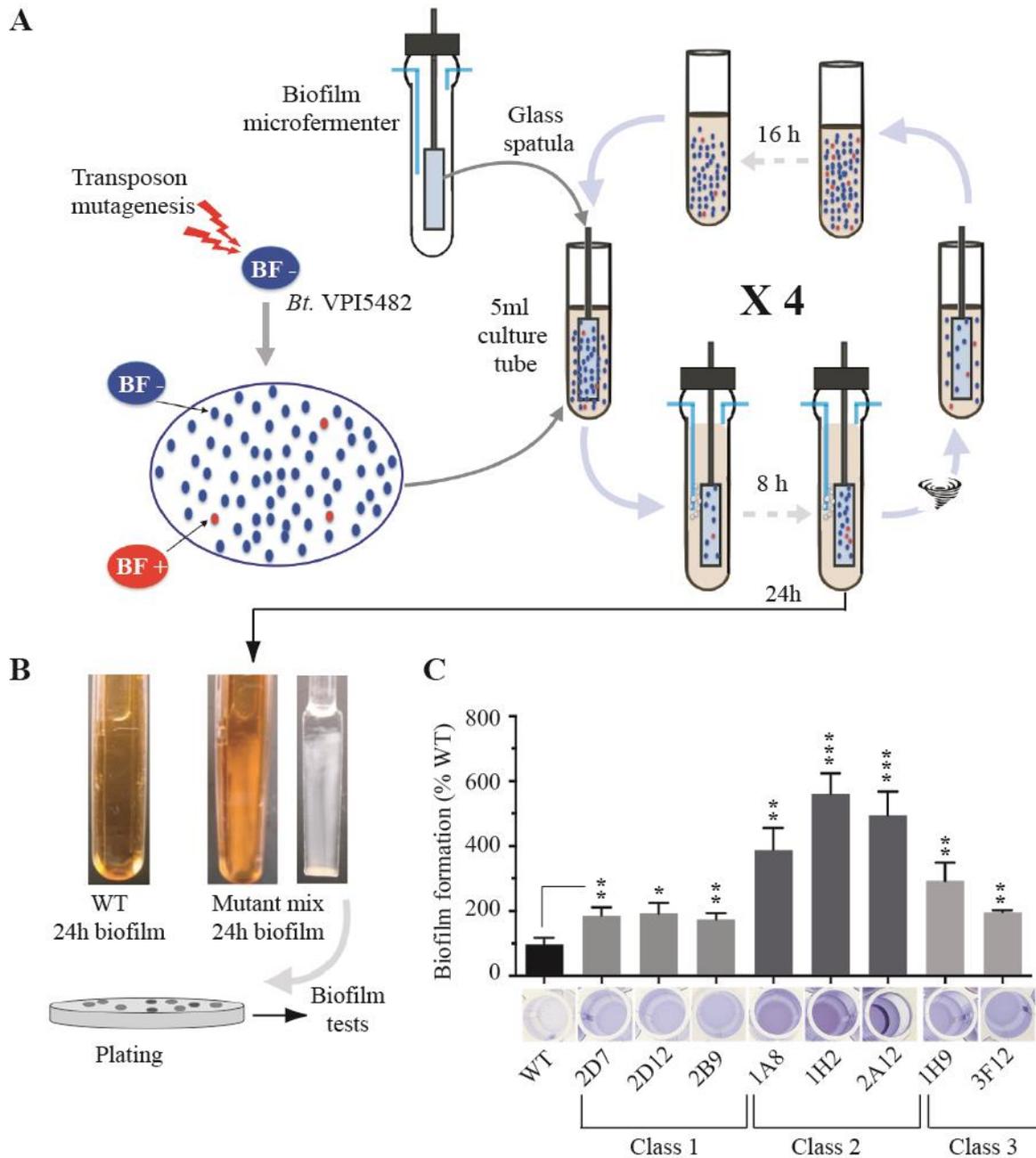
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742 **Figure 1. *B. thetaiotaomicron* VPI 5482 strain forms poor biofilms compared to different**  
743 **isolates. A-B.** Biofilm formation in 96-well plate biofilm assay followed by crystal violet  
744 staining of *B. thetaiotaomicron* VPI 5482 and various biofilm-forming *B. thetaiotaomicron*  
745 isolates (see also Supplementary Figure S1). Error bars indicate standard deviation of 3  
746 technical replicates. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . **C.** Biofilm formation on a glass slide  
747 inserted in continuous-flow biofilm microfermenters. The images were taken 48 h after  
748 inoculation.

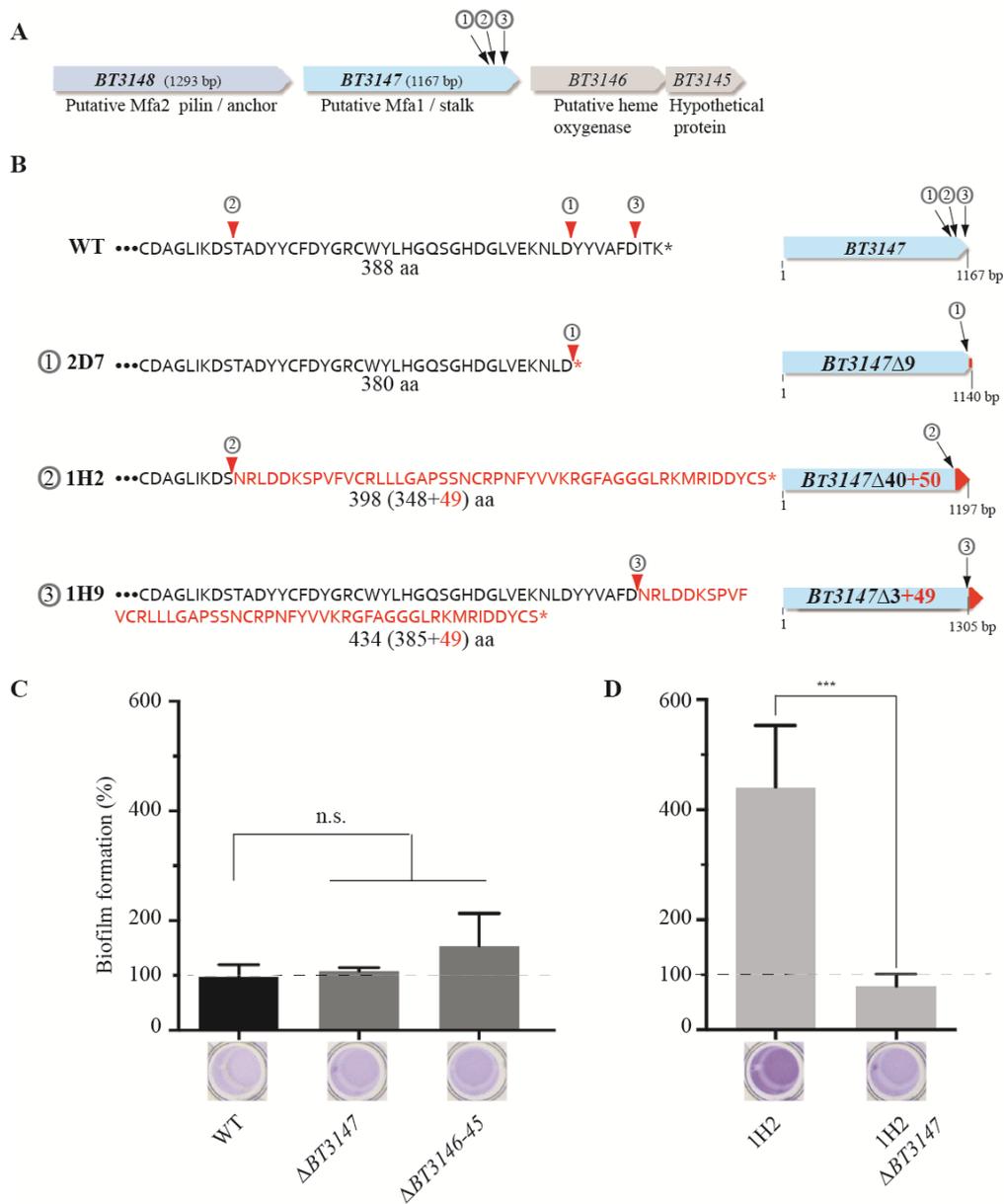
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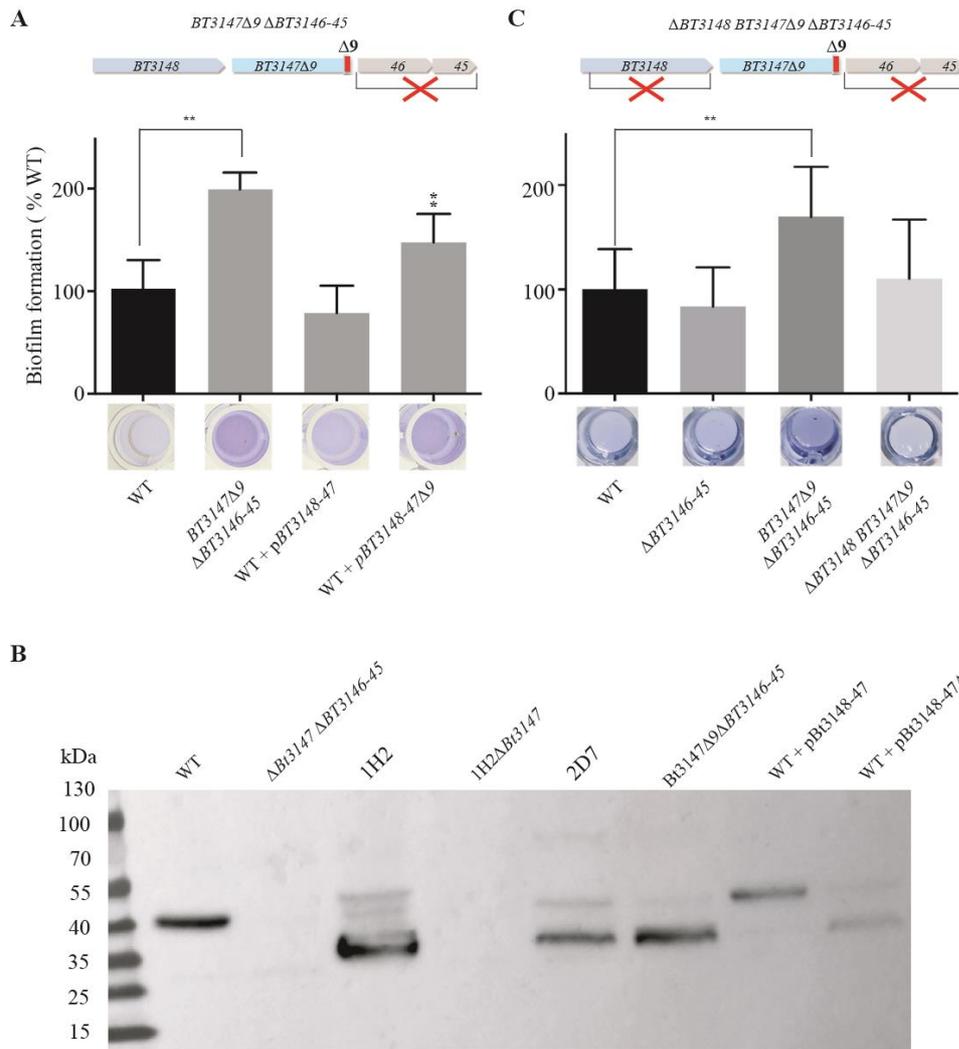
751 **Figure 2. Selection of *B. thtaiotaomicron* VPI 5482 mutants with increased biofilm**  
 752 **capacities.** **A** Schematic representation of the positive selection strategy used to identify  
 753 transposon mutants with an increased biofilm production. **B** Biofilm biomass formation on the  
 754 internal spatula of continuous-flow biofilm microfermenters after 24 h. On the left, wild type  
 755 *B. thtaiotaomicron* VPI 5482, in the middle and on the right: microfermenter and spatula  
 756 inoculated with a culture enriched for biofilm production after 4 cycles of positive selection.  
 757 **C** Comparison of the biofilm formation capacity of *B. thtaiotaomicron* WT and eight  
 758 identified mutants with insertion in gene *BT3147* Bottom: crystal violet staining in 96-well  
 759 microtiter plates. Top: corresponding biomass quantification after resuspension in  
 760 acetone:ethanol mix and absorbance measured at 575 nm . Biofilm formation capacities of  
 761 WT (*VPI 5482*) have been set to 100%. Error bars indicate standard deviation of 3 technical  
 762 replicates. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .





764

765 **Figure 3. Insertions of transposon in the 3'-end of *B. thetaiotaomicron* BT3147 increase**  
 766 **biofilm formation** **A.** Genetic organization of the *BT3148-3145* *B. thetaiotaomicron* locus  
 767 with indication of the three transposon insertion points in the 3' end of *BT3147*. 2D7  
 768 corresponds to class 1 (①) mutants. 1H2 corresponds to class 2 (②) mutant. 1H9 corresponds  
 769 to class 3 (③) mutant. **B.** Schematic representation of the genetic consequences of transposon  
 770 insertion in *BT3147*. **C.** Comparison of biofilm formation capacity of *B. thetaiotaomicron* WT  
 771 (VPI 5482) with class 2 transposon mutant 1H2 and different deletion strains lacking either  
 772 *BT3147* ( $\Delta BT3147$ ), *BT3146* and *BT3145* ( $\Delta BT3146-45$ ). Bottom: crystal violet staining in  
 773 96-well microtiter plates. Top: corresponding quantification after resuspension in  
 774 acetone:ethanol and absorbance measured at 575 nm **D.** Comparison of biofilm formation  
 775 capacity *B. thetaiotaomicron* 1H2 mutant and strain 1H2 $\Delta BT3147$ , derived from the biofilm-  
 776 forming 1H2 transposon mutant after deletion of *BT3147* 1046 bp located upstream of the  
 777 transposon insertion. Biofilm formation capacities of WT (VPI 5482) have been set to 100%.  
 778 Error bars indicate standard deviation of 3 technical replicates. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  
 779  $p < 0.001$ .



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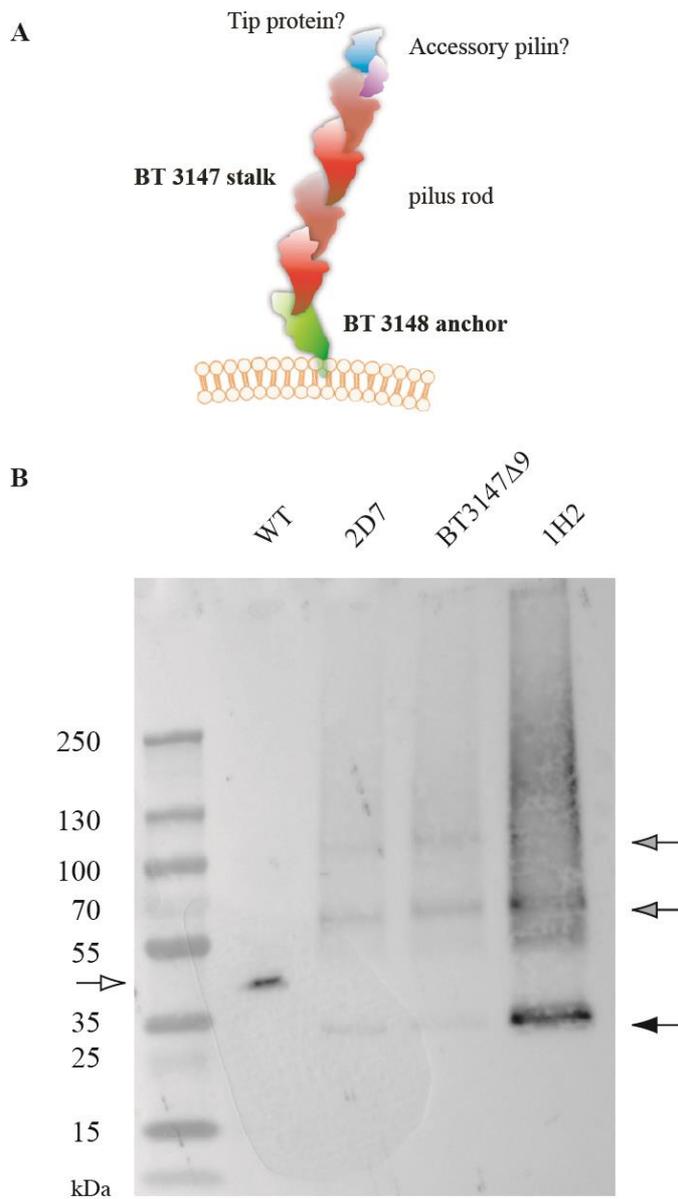
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**Figure 4. C-terminal truncation of BT3147 protein promotes *B. thetaiotaomicron* biofilm formation.** **A.** Top, schematic genetic organization of the *BT3147Δ9 ΔBT3146-45* mutant. Bottom: comparison of the biofilm formation of WT *B. thetaiotaomicron* VPI 5482 and indicated mutant or plasmid-containing strains. Bottom: crystal violet staining in 96-well microtiter plates. Top: corresponding quantification after resuspension in acetone:ethanol and absorbance measured at 575 nm. Biofilm formation capacities of WT (VPI 5482) have been set to 100%. Error bars indicate standard deviation of 3 technical replicates. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . **B.** Immunodetection of BT3147 in WT *B. thetaiotaomicron* VPI 5482 and indicated mutants or complemented strains expressing either full length BT3147 (*p.BT3148-7*) or truncated version of BT3147..



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793 **Figure 5. BT3147 is exposed on *B. thetaiotaomicron* surface as part of a potential type V**  
 794 **pilus.**

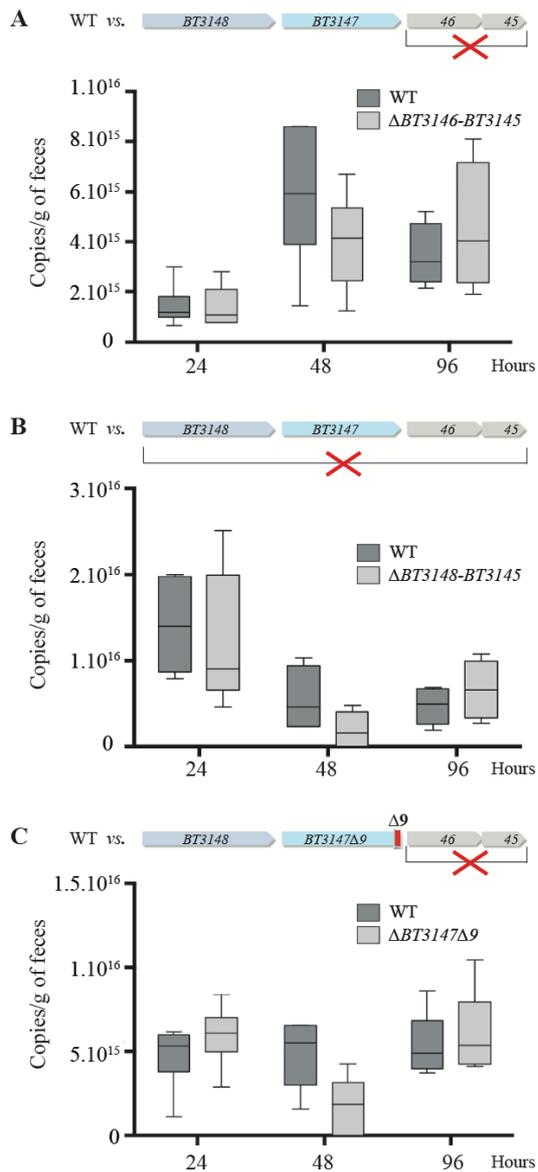
795 **A.** Model of the putative BT3148-7 pilus. **B.** Western blot analysis of BT3147-containing  
 796 structures released after surface shaving of the indicated *B. thetaiotaomicron* strains and  
 797 mutants, followed by immunodetection using anti-BT3147 antibodies. White arrows indicates  
 798 WT, black arrows indicate BT3147Δ9 variant of BT3147 and its potential multimers.

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804 **Figure 6. Contribution of genes *BT3148-5* to *B. thetaiotaomicron* in vivo intestinal**

805 **colonization in germ-free mice. A:** *in vivo* mixed cultures competition experiments,

806 comparing the respective colonization capacity of WT and  $\Delta BT3146-BT3145$  strains. **B:** *in*

807 *in vivo* mixed cultures competition experiments, comparing the respective colonization capacity

808 of WT and  $\Delta BT3148-BT3145$  strain. **C:** *in vivo* mixed cultures competition experiments,

809 comparing the respective colonization capacity of WT and the biofilm-forming *BT3147Δ9*

810 *BT3146-BT3145* strain. All strains were administrated intra-gastrically at 1:1 mixed ratio

811 ( $2 \times 10^7$  cell/ml). Comparisons were performed after collecting feces samples at 24 h, 48 h, and

812 96 h after the initial gavage and strain-specific qPCR on frozen fecal material samples and did

813 not show any significant statistical difference between the tested strains.

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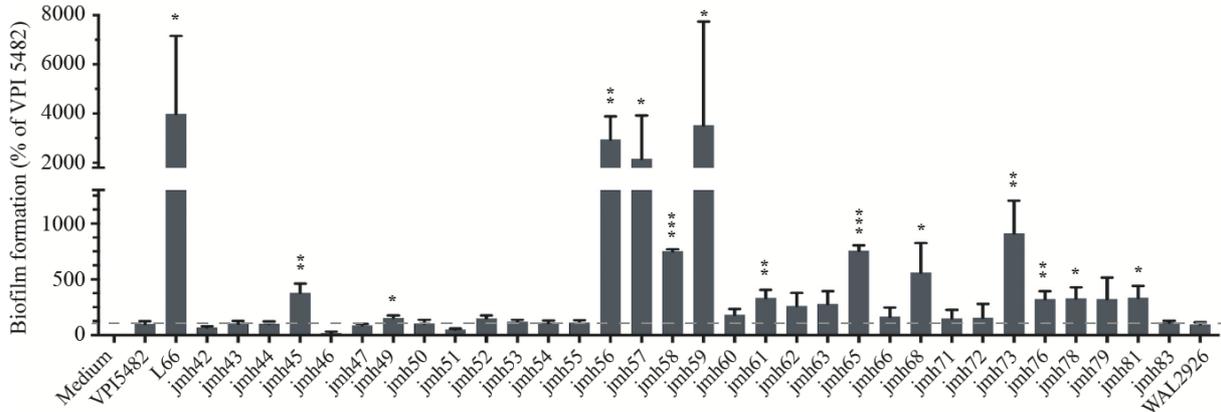
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816 **SUPPLEMENTARY MATERIAL**

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818 **SUPPLEMENTARY FIGURES**

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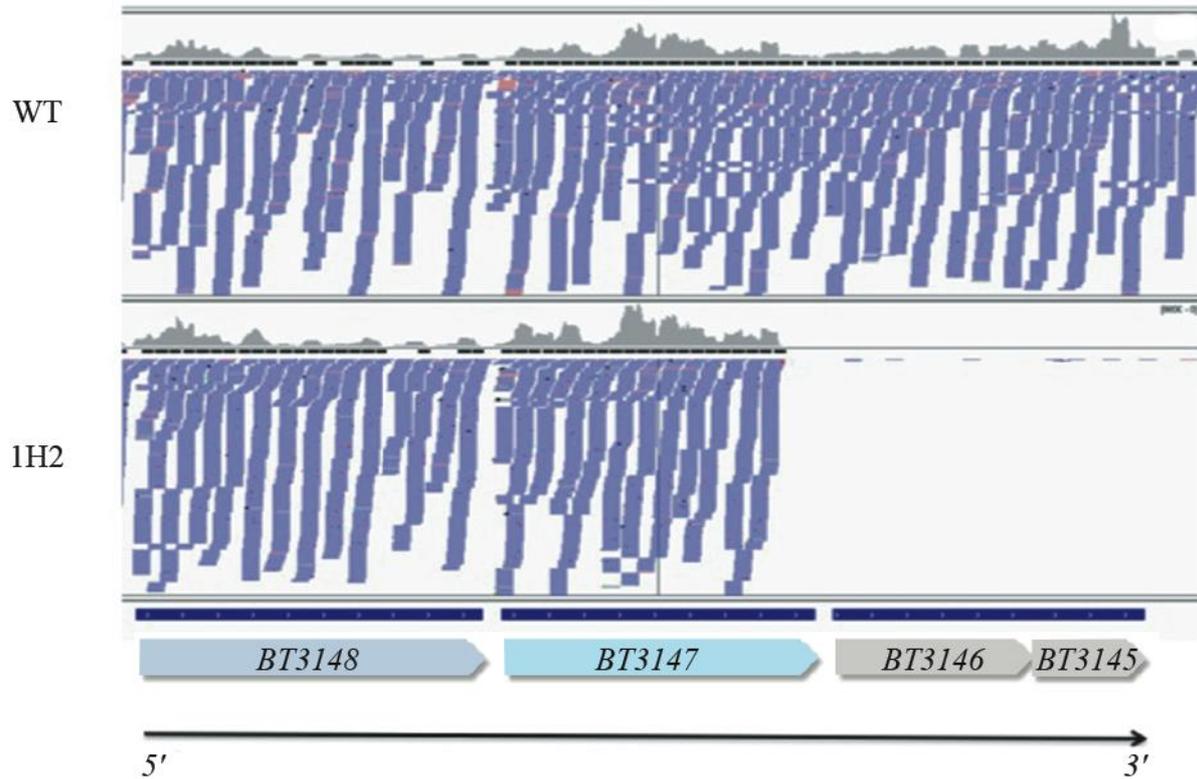
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821 **Supplementary Figure S1. Biofilm formation by 34 *B. thetaiotaomicron* natural isolates.**

822 Comparison of biofilm formation in various *B. thetaiotaomicron* isolates in a 96-well plate  
823 biofilm assay followed by crystal violet staining. VPI 5482 biofilm formation has been set to  
824 100% as represented by the grey dashed lines and used for reference in the statistical test.  
825 These strains were collected from adults by the Microbiology laboratory of Dupuytren  
826 (Limoges) and Dron Tourcoing (Tourcoing) hospitals in France. For a complete list of the  
827 strains, see supplementary Table S3. Error bars indicate standard deviation of 3 technical  
828 replicates. \* p<0.05; \*\* p<0.01; \*\*\* p<0.001.

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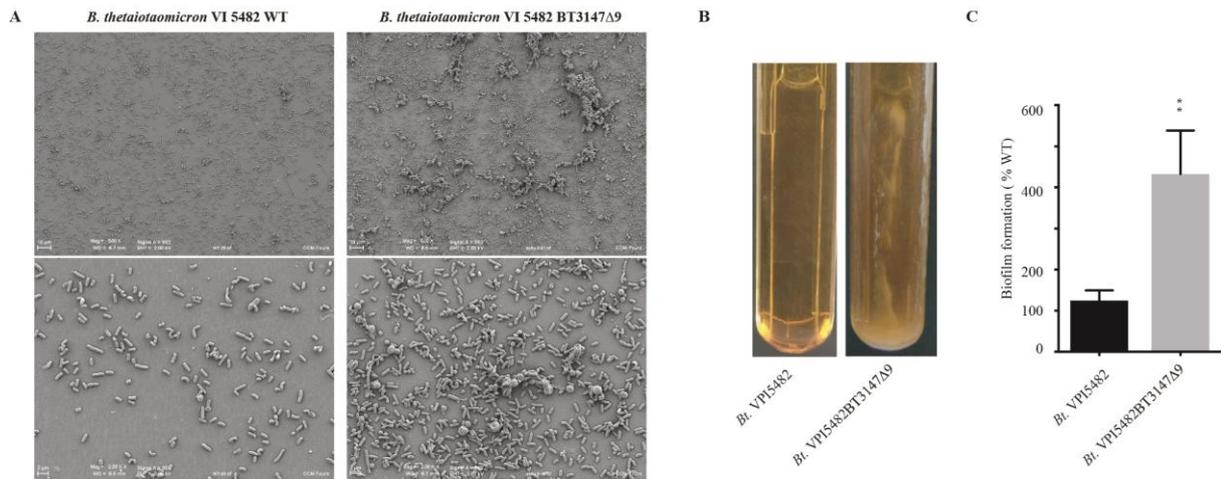
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833 **Supplementary Figure S2. Transcription in *BT3148-5* locus in WT *B. thtaiotaomicron***  
 834 **VPI 5482 and the transposon mutant 1H2.** Reads mapping in the *BT3148-5* region are  
 835 represented in blue, antisense reads are represented in red. Strand-specific mapping shows no  
 836 antisense transcripts arising from the *BT3146-5* towards *BT3147*. *BT3147* transcript is  
 837 uninterrupted in the WT indicating that there are no premature stop codons or transcription-  
 838 stopping secondary structures. Transcription of the last portion of *BT3147* (position 1047 to  
 839 1167 bp) and *BT3146-5* is abolished in 1H2 due to transposon insertion.

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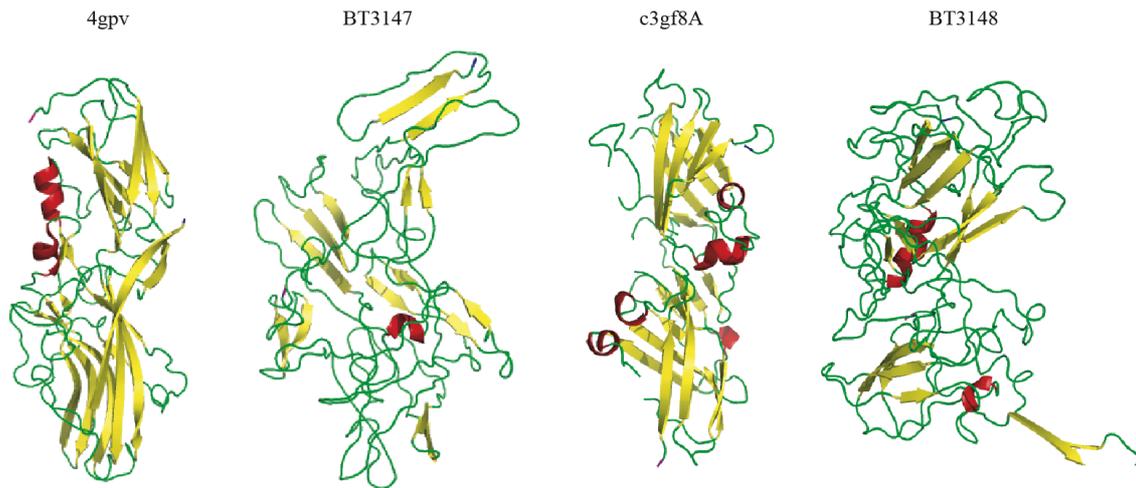
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844 **Supplementary Figure S3. C-terminal truncation of BT3147 increases *in vitro* adhesion**  
845 **and biofilm formation.** **A** Scanning electron microscopy observation of *B. thetaiotaomicron*  
846 WT and *BT3147 $\Delta$ 9* grown in continuous flow biofilm-microfermenter (x500 and x2000  
847 magnification) **B** Biofilm formation *B. thetaiotaomicron* WT and *BT3147 $\Delta$ 9* on a glass slide  
848 inserted in continuous flow biofilm microfermenters. The images were taken 48h after  
849 inoculation. **B** Quantification after resuspension and measure at OD 600 of *B.*  
850 *thetaiotaomicron* WT and *BT3147 $\Delta$ 9* in continuous flow biofilm microfermenters after 48h.

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855 **Supplementary Figure S4. Comparison of structural models of BT3147 and BT3148.**

856 Structural modeling for BT3147 and BT3148 has been performed using Phyre2 web interface  
857 with the intensive mode (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>) and  
858 compared to 3D-structure of Mfa1-type structural model (PDB code 4gpv:) and Fim2B/Mfa2  
859 structural model (PDB code c3gf8A).

860



871 Table S1. Wild type *B. thetaiotaomicron* collection used in this study

Strains and plasmids	Relevant characteristics	References
<i>Bacteroides thetaiotaomicron</i> VPI 5482	Healthy adult human feces	(1)
L66	Healthy adult human feces, INRA	INRA
Jmh 42	Perforated ulcer	CHU Limoges
Jmh 43	Blood	CHU Limoges
Jmh 44	Blood	CHU Limoges
Jmh 45	Liquid from redon drain	CHU Limoges
Jmh 46	Blood	CHU Limoges
Jmh 47	Hepatic abscess	CHU Limoges
Jmh 48	Abscess	CHU Limoges
Jmh 49	Blood	CHU Limoges
Jmh 50	Liquid ascite	CHU Limoges
Jmh 51	Blood	CHU Limoges
Jmh 52	Blood	CHU Limoges
Jmh 53	Peritoneal liquid	CHU Limoges
Jmh 54	Bone biopsy	CHU Limoges
Jmh 55	Skin biopsy	CHU Limoges
Jmh 56	Blood	CHU Limoges
Jmh 57	Bone biopsy	CHU Limoges
Jmh 58	Blood	CHU Limoges
Jmh 59	Blood	CHU Limoges
Jmh 60	Hip implant	CHU Limoges
Jmh 61	DPCA	CHU Limoges
Jmh 62	Peritoneal liquid	CHRU Lille
Jmh 63	Blood	CHRU Lille

Jmh 65	Abdomen	CHRU Lille
Jmh 66	Maxilar pus	CHRU Lille
Jmh 68	Blood	CHRU Lille
Jmh 71	Blood	CHRU Lille
Jmh 72	Anal abscess	CHRU Lille
Jmh 73	Blood	CHRU Lille
Jmh 76	Fat tissue	CHRU Lille
Jmh 78	Abdominal abscess	CHRU Lille
Jmh 79	Intestinal cyste	CHRU Lille
Jmh 81	Blood	CHU Limoges
Jmh 83	Peritoneal liquid	CHRU Lille

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873 genomic view of the human-Bacteroides thetaiotaomicron symbiosis. Science (New York, NY)  
874 299:2074-2076.  
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880 **TABLE S2. Primers used in this study.**

Strain construction and analysis	Name	Sequence
$\Delta BT3147$	<i>BT3147</i> -up-F	5'-GCGGTCGACAATGTGACGGAG GACGGCATCAGC-3'
	<i>BT3147</i> -up-R	5'-CAAAAAAAGCTTTTAAATCATTCTAATTG-3'
	<i>BT3147</i> -down-F	5'-CGAATGGAGCGGAATATACAATGTGTGGATA ATCATGCGTGGACGGAGTATCC-3'
	<i>BT3147</i> -down-R	5'-GCGTCTAGATGAGCCGATGCAACTGTTG GTTGC-3'
$\Delta BT3146-5$	<i>BT3146</i> -up-F	5'-GCGGTCGACTTTCGAAAACAAGCTTTACC-3'
	<i>BT3146</i> -up-R	5'-AATTGTTTACGTTTAAAATTATTGAATGTGTC-3'
	<i>BT3145</i> -down-F	5'-GACACATTCAATAATTTAAACGTAAACA ATTATTGAAAAAAAACAAGCTAGG-3'
	<i>BT3145</i> -down-R	5'-CCC GCGGCCCTACGGCACAGTTGA GAGATTGGAG-3'
<i>BT3147</i> $\Delta 9$	<i>BT3147</i> $\Delta 9$ -up-F	5'-GATAACATTCGAGTCGACAGAACGA GGAGTTGTTACC-3'
	<i>BT3147</i> $\Delta 9$ -up-R	5'-TTAATCTAGATTTTTTCTACTAAACCG-3'
	<i>BT3147</i> $\Delta 9$ -down-F	5'-CGGTTTAGTAGAAAAAATCTAGATTAATGA TTTTCGTCTCCACAAAACGATTG-3'
	<i>BT3147</i> $\Delta 9$ -down-R	5'-CTTATCGATACCCTACGGCACAGTTGA GAGATTGGAG-3'
$\Delta BT3148$	<i>BT3148</i> -up-F	5'- GAAAGAAGATAACATTCGAGGGAAAACGA TGCCTTGGTG -3'
	<i>BT3148</i> -up-R	5'- ATATTTACTTGTACCCTATTCATCGTCTTGGT TCTAC - 3'
	<i>BT3148</i> -down-F	5'- AGACGATGAATACGGTAAACAGTAAATATACA ATTAGAA -3'
	<i>BT3148</i> -down-R	5'- ACCGCGGTGGCGCCGCTTAAACCGTCATGACC ACTTTGA -3'
$\Delta BT3148-5$	<i>BT3148</i> -up-F	5'- GAAAGAAGATAACATTCGAGGGAAAACGA TGCCTTGGTG -3'
	<i>BT3148</i> -up-R	5'-GTTTTGTGGAAGACGCGTATTCATCGTCTTGG TTCTAC-3'
	<i>BT3145</i> -down-F	5'-AGACGATGAATACGCGTCTCCACAAAACGATTG-3'
	<i>BT3145</i> -down-R	5'-ACCGCGGTGGCGCCGCTGAAACGCAGAAGAGG CTTT-3'
$\Delta BT3148-7$	<i>BT3148</i> -up-F	5'- GAAAGAAGATAACATTCGAGGGAAAACGA TGCCTTGGTG -3'
	<i>BT3148</i> -up-R	5'-GTTTTGTGGAAGACGCGTATTCATCGTCTTGG TTCTAC-3'
	<i>BT3147</i> -down-F	5'-AGACGATGAATACGTGACACATTCAATAATTT AAAC-3'
	<i>BT3147</i> -down-R	5'-ACCGCGGTGGCGCCGCTTTCGGCATTGA GGTCATTTA-3'
Insertion of <i>BT1311</i>	<i>BT1311</i> promoter-F	5'-GGGTCTAGATAGTGCCATATGTTAAAAACA

promoter in pNBU2- <i>bla-erm</i>	<i>BT3111</i> promoter-R	GATTTGGAGTGC-3' 5'-CCCGCGGCCGCTGATCTGGAAGAAGCA ATGAAAGC-3'
Cloning <i>BT3148-7</i> in pNBU1311	<i>BT3148-F</i>	5'-CCAAATCTGTTTTTAACATATGCTGGTCAT GTTCTGTGG-3'
	<i>BT3147-R</i>	5'-GTTCTAGATAGTGCTTATTTTGTATAT CAAAAGC-3'
Cloning of <i>BT3146-5</i> in pNBU1311	<i>BT3146-F</i>	5'-CCCCATATGAATGATTTCAAGAATCAATGG-3'
	<i>BT3146-R</i>	5'-CCGTCTAGATCAGCCTTTCTTGTCCACG-3'
Cloning <i>BT3148-7Δ9</i> in pNBU1311	<i>BT3148-7Δ9-F</i>	5'-GTTTTTAACATATGGCACTATATGCTGGTCATG TTCGTGTG-3'
	<i>BT3148-7Δ9-R</i>	5'-AGCCCGGGGATCCACTAGTTTTAATCTAG ATTTTTTCTACTAAACCGTCA-3'
882		
Cloning <i>tetQ</i> into pExchange	<i>tetQ-F</i>	5'-GGCCATATGAATATTATAAATTTAGGAAT TCTTGCTCAC-3'
	<i>tetQ-R</i>	5'-CCCTCTAGATCGTCTATTTTTTATTGCCA AGTTCTAATGC-3'
Deletion of <i>BT3147</i> upstream of Tn	<i>BT3148-up-F</i>	5'-GCGGTCGACAGCAGACCTCTGCTGATAGC-3'
	<i>BT3148-up-R</i>	5'-ACATATCTTCAGATAGATGTATC-3'
	<i>BT3147*-down-F</i>	5'-GATACATCTATCTGAAGATATGTTTCTACC GGCACTTCTGC-3'
	<i>BT3147*-down-R</i>	5'-GCGTCTAGACTCTTTGTTAACCC-3'
Localization of Tn	ARB-bt1	5'-GGCCACGCGTCGACTAGTAC NNNNNNNNNGATAT-3'
	ARB-bt-bis	5'-GGCCACGCGTCGACTAGTAC-3'
	pSCIRerm R	5'-GATTTGAACGTTGCGAAGC-3'
	pSCIRerm R-bis	5'-TTTTTGCGTTTCTACCTGC-3'
	pSCIRerm L	5'-CGTATCGGTCTGTATATCAGC-3'
	pSCIRerm L-bis	5'-CTCATCTTTCTGAGTCCACC-3'
DNA contamination check by RT-qPCR	<i>DNApol3-F</i>	5'-TGTCGCCCGTCTGGTAGATA-3'
	<i>DNApol3-R</i>	5'-CCGCCTCCTTATCCTCACAC-3'

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884

885 **Table S3. List of strains used in this study**

Strains and plasmids	Relevant characteristics	References <sup>a</sup>
<b><i>Escherichia coli</i></b>		
MG1655	K-12 wild-type strain	(2)
S17-1 $\lambda$ pir	(F <sup>-</sup> ) RP4-2-Tc::Mu aphA::Tn7 recA $\lambda$ pir lysogen. Sm <sup>R</sup> , Tp <sup>R</sup>	
S17-1 $\lambda$ pir_pSAM-bt	S17-1 $\lambda$ pir derivative carrying pSAM-bt	This study
S17-1 $\lambda$ pir_pExchange-tdk	S17-1 $\lambda$ pir derivative carrying pExchange-tdk	This study
S17-1 $\lambda$ pir_pNBU2-bla-erm	S17-1 $\lambda$ pir carrying pNBU2-bla-erm	This study
S17-1 $\lambda$ pir_pExchange-BT3147	S17-1 $\lambda$ pir carrying pExchange-BT3147	This study
S17-1 $\lambda$ pir_pExchange-BT3146-5	S17-1 $\lambda$ pir carrying pExchange-BT3146-3145	This study
S17-1 $\lambda$ pir_pExchange-BT3147 $\Delta$ 9	S17-1 $\lambda$ pir carrying pExchange-BT3147 $\Delta$ 9	This study
S17-1 $\lambda$ pir_pExchange-BT3148	S17-1 $\lambda$ pir carrying pExchange-BT3148	This study
S17-1 $\lambda$ pir_pExchange-BT3148-5	S17-1 $\lambda$ pir carrying pExchange-BT3148-3145	This study
S17-1 $\lambda$ pir_pExchange-BT3148-7	S17-1 $\lambda$ pir carrying pExchange-BT3148-3147	This study
S17-1 $\lambda$ pir_pNBU1311	S17-1 $\lambda$ pir carrying pNBU1311	This study
S17-1 $\lambda$ pir_pNBU1311-BT3146-5	S17-1 $\lambda$ pir carrying pNBU1311-BT3146-3145	This study
S17-1 $\lambda$ pir_pNBU1311-BT3148-7	S17-1 $\lambda$ pir carrying pNBU1311-BT3148-3147	This study
S17-1 $\lambda$ pir_pNBU1311-BT3148-7 $\Delta$ 9	S17-1 $\lambda$ pir carrying pNBU1311- BT3148-3147 $\Delta$ 9	This study
S17-1 $\lambda$ pir_pExchange-tetQ-BT3147*	S17-1 $\lambda$ pir carrying pExchange-tetQ-BT3147*	This study
S17-1 $\lambda$ pir_pExchange-tetQ	S17-1 $\lambda$ pir carrying pExchange-tetQ	This study
<b><i>Bacteroides thetaiotaomicron</i></b>		
VPI 5482	Healthy adult human feces	(1)
VPI 5482_ <i>Atdk</i>	Deletion of thymidine kinase in the wild type strain ATCC29148	(3)
VPI 5482_ $\Delta$ BT3147	Deletion of BT3147	This study
VPI 5482_ $\Delta$ BT3146-5	Deletion of BT3146-3145	This study
VPI 5482 $\Delta$ BT3148-5	Deletion of BT3148-3145	This study
VPI 5482 $\Delta$ BT3148-7	Deletion of BT3148-3147	This study
VPI 5482 BT3147 $\Delta$ 9	Deletion of BT3146-3145 and the last 9 C-terminal amino acids of BT3147	This study
VPI 5482 $\Delta$ BT3148 BT3147 $\Delta$ 9	Deletion of BT3148 in VPI 5482_ BT3147 $\Delta$ 9 strain	This study

VPI 5482_p. <i>BT3146-5</i>	Chromosomal-based expression of <i>BT3146-5</i> from pNBU1311	This study
VPI 5482_p. <i>BT3148-7</i>	Chromosomal-based expression of <i>BT3148-7</i> from pNBU1311	This study
VPI 5482_p. <i>BT3148-7</i> $\Delta$ 9	Chromosomal-based expression of <i>BT3148</i> and the 9 amino acid truncated version of <i>BT3147</i> from pNBU1311	This study
VPI 5482_pNBU2- <i>bla-erm</i>	Erythromycin resistant strain	This study
1H2 $\Delta$ <i>BT3147</i> *	1H2 mutant with deletion of <i>BT3147</i> portion upstream of the Tn-insertion	This study

## Plasmids

pSAM-bt	Random transposon mutagenesis	(Goodman <i>et al.</i> , 2009)
pExchange- <i>tdk</i>	Site-directed unmarked gene deletion	(Martens <i>et al.</i> , 2008)
pNBU2- <i>bla-erm</i>	Chromosomal-based gene expression	(Martens <i>et al.</i> , 2008)
pNBU1311	Derivative of pNBU2- <i>bla-erm</i> with cloned constitutive promoter of <i>BT1311</i> for gene expression	This study
pExchange- <i>BT3147</i>	pExchange derivative for unmarked deletion of <i>BT3147</i>	This study
pExchange- <i>BT3146-5</i>	pExchange derivative for unmarked deletion of <i>BT3146</i> and <i>BT3145</i>	This study
pExchange- <i>BT3147</i> $\Delta$ 9	pExchange derivative for unmarked deletion of <i>BT3146-5</i> and last 9 amino acids of <i>BT3147</i> C-terminus	This study
pExchange- <i>BT3148</i>	pExchange derivative for unmarked deletion of <i>BT3148</i>	This study
pExchange- <i>BT3148-5</i>	pExchange derivative for unmarked deletion of <i>BT3148</i> to <i>BT3145</i>	This study
pExchange- <i>BT3148-7</i>	pExchange derivative for unmarked deletion of <i>BT3148</i> and <i>BT3147</i>	This study
pNBU1311- <i>BT3146-5</i>	pNBU1311 derivative for expression of <i>BT3146-5</i>	This study
pNBU1311- <i>BT3148-7</i>	pNBU1311 derivative for expression of <i>BT3148-7</i>	This study
pNBU1311- <i>BT3148-7</i> <sup><math>\Delta</math>9</sup>	pNBU1311 derivative for expression of <i>BT3148-7</i> <sup><math>\Delta</math>9</sup>	This study
pExchange- <i>tetQ</i>	pExchange derivative with <i>erm</i> resistance exchanged for tetracycline	This study
pExchange- <i>tetQ</i> - <i>BT3147</i> *	pExchange- <i>tetQ</i> derivative for deletion of <i>BT3147</i> portion laying upstream of the Tn insertion	This study

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887 <sup>a</sup> Bacterial strains from our laboratory collection unless otherwise specified.

888 CRBIP: *Institut Pasteur, Centre de Ressources Biologiques de l'Institut Pasteur.*

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