



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

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

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19

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

21

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## 23 ABSTRACT

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

## 45 IMPORTANCE

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

56

57 **INTRODUCTION**

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

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

83       Here, we have sequenced and annotated the genome of *V. parvula* SKV38, a recently  
84 isolated, naturally transformable and genetically amenable strain (17). We adapted and  
85 developed genetic tools for this organism, permitting random and site directed mutagenesis,  
86 plasmid complementation and controlled expression using an inducible promoter. This enabled  
87 us to identify and characterize factors involved in *V. parvula* biofilm formation. We find that  
88 the main *V. parvula* biofilm modulating determinants are T5SS adhesins, i.e. typical diderm



89 determinants. Interestingly, the identified adhesins possess an additional C-terminal domain  
90 compared to the known domain architecture of classical autotransporters. We also show that a  
91 locus encoding a metal-dependent phosphohydrolase HD domain protein is involved in biofilm  
92 formation, similarly to what was shown in the prototypical monoderm *Bacillus subtilis* (18).  
93 Therefore, our results demonstrate that diderm Firmicutes use a mixture of diderm and  
94 monoderm factors to modulate their ability to engage into biofilm lifestyle, supporting the idea  
95 that monoderm and diderm molecular systems could have co-evolved in these atypical  
96 Firmicutes.  
97

## 98 RESULTS

### 99 Random transposon mutagenesis reveals two *V. parvula* SKV38 genes involved in biofilm 100 formation

101 In order to obtain a framework for genetic work in the recently described naturally  
102 competent *V. parvula* SKV38 isolate, we sequenced it using PacBio technology. We obtained  
103 a completely assembled genome of 2.146Mbp, encoding 1,912 predicted protein-encoding open  
104 reading frame (ORF), 12 rRNA, 49 tRNA and one tmRNA (see Material and Methods). We  
105 performed random transposon mutagenesis in *V. parvula* SKV38 using the pRPF215 plasmid  
106 carrying an inducible transposase and a mariner-based transposon previously used to  
107 mutagenize *Clostridioides difficile* (19), a close relative of the Negativicutes. We screened 940  
108 individual transposon mutants for biofilm formation using crystal violet staining (CV) static  
109 biofilm assay in 96-well microtiter plates and identified eight independent mutants with  
110 significant reduction in biofilm formation (Figure 1A). Whole genome sequencing localized  
111 the transposons in two loci putatively implicated in biofilm formation (Figure 1B). The most  
112 affected mutants correspond to insertions in *FNLLGLLA\_00516* (seven mutants), encoding a  
113 T5SS type Vc trimeric autotransporter. One transposon mutant was inserted in  
114 *FNLLGLLA\_01127*, encoding a putative HD phosphatase (Figure 1B).

### 116 *FNLLGLLA\_00516* encodes a trimeric autotransporter involved in auto-aggregation

117 *FNLLGLLA\_00516* encodes a protein containing several domains usually identified in  
118 the T5SS type Vc trimeric autotransporters. Trimeric autotransporters are OM proteins specific  
119 of diderm bacteria that have been widely studied for their ability to bind to different surfaces  
120 or to other bacteria (20). *FNLLGLLA\_00516* is a homolog of *V. parvula* DSM2008 *vpar\_0464*,  
121 which encodes a protein that was detected in the OM (15). *FNLLGLLA\_00516* was annotated  
122 by PROKKA as BtaF, a trimeric autotransporter identified in *Brucella suis* involved in adhesion  
123 to extracellular matrix and abiotic surfaces (21). Here, we renamed it *Veillonella* trimeric  
124 autotransporter A (VtaA), as the first trimeric autotransporter involved in biofilm formation  
125 identified in *V. parvula* SKV38. We deleted the *vtaA* coding sequence and showed that  $\Delta vtaA$   
126 had no growth defect (Figure S1A) but displayed a marked reduction of biofilm formation in  
127 96-well polystyrene microtiter plate (Figure 2A). Moreover, while *V. parvula* SKV38 cultures  
128 strongly aggregated,  $\Delta vtaA$  did not (Figure 2B and S2). We constructed the strain *pTet-vtaA*,  
129 where the chromosomal *vtaA* gene is placed under the control of a functional  
130 tetracycline/anhydrotetracycline (aTc) inducible promoter (Figure S3) and showed that its

131 aggregation capacity and biofilm formation in 96-well polystyrene microtiter plate directly  
132 correlated with aTc concentration (Figure 2C-D), demonstrating that VtaA-mediated cell-to-  
133 cell interactions are critical for biofilm formation in these conditions. Whereas the microtiter  
134 plate assay corresponds to a static biofilm assay, we also used continuous flow glass  
135 microfermentors to investigate the contribution of VtaA to biofilm formation in dynamic  
136 conditions. Surprisingly,  $\Delta vtaA$  formed almost six times more biofilm than the WT strain in  
137 these conditions (Figure 2E). Accordingly, scanning electronic microscopy (SEM) images of  
138 mature biofilms on microscopic plastic slides in microfermentor showed that  $\Delta vtaA$  formed a  
139 much thicker biofilm when compared to WT (Figure S4). Altogether, these results suggest that  
140 auto-aggregation differentially contributes to biofilm formation in static conditions on  
141 hydrophobic surfaces versus continuous flow conditions on hydrophilic surfaces.

142

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

144 The strong biofilm phenotype displayed by the  $\Delta vtaA$  mutant in microfermentor led us  
145 to suspect that additional adhesins could modulate *V. parvula* biofilm formation capacity.  
146 Indeed, searching the *V. parvula* SKV38 genome revealed multiple genes encoding  
147 autotransporters (Table 1): three Va classical monomeric autotransporters with a characteristic  
148 PFAM\_PF03797 autotransporter  $\beta$ -domain (renamed *Veillonella* monomeric autotransporter A  
149 to C : VmaA to C), and eight other putative Vc trimeric autotransporters with a characteristic  
150 PFAM\_PF03895 YadA\_anchor\_domain (renamed *Veillonella* trimeric autotransporter B to I:  
151 VtaB to I). We also identified several partial autotransporters: *FNLLGLLA\_00035*, that only  
152 contains a PFAM\_PF11924 Ve inverse autotransporter  $\beta$ -domain but no putative  $\alpha$ -domain that  
153 normally carries the function of the protein, and *FNLLGLLA\_00036-37* and  
154 *FNLLGLLA\_00040-41*, which are homologs of *V. parvula* DSM2008 *Vpar\_0041* and  
155 *Vpar\_0048*, respectively, and that appear to be split in SKV38 (Table 1). Interestingly, domain  
156 analysis of all trimeric ATs of *V. parvula* SKV38 showed that they possess an extra C-terminal-  
157 domain (SLH or coiled-coil domain) after the YadA anchor domain that is not found in classical  
158 trimeric ATs. Among those, six autotransporters plus *FNLLGLLA\_00035*, *FNLLGLLA\_00036-*  
159 *37* and *FNLLGLLA\_00040-41* form a potential genomic cluster coding for adhesins (Figure  
160 3A), whereas the six others are located in different areas of the genome (Figure 3B).

161 We selected eight *Veillonella* strains to study more precisely the evolution of the adhesin  
162 cluster, including SKV38 and DSM2008. The trimeric autotransporter adhesins seem to evolve  
163 dynamically with numerous domain swaps, duplications and reductions of gene copies, likely

164 through homologous recombination suggesting rapid evolutionary changes in the repertoire of  
165 *Veillonella* adhesins (Figure 4). Duplications and deletions could be eased by the presence of  
166 short ORFs annotated as hypothetical proteins presenting a high degree of sequence identity.  
167 The most basal strain in the *Veillonella* phylogeny has a minimal cluster of only three adhesins  
168 genes. Throughout the *Veillonella* genus, the size of the cluster is very variable with a minimal  
169 form in *V. atypica*, with only two adhesins. This specific adhesin locus, immediately upstream  
170 of rRNA coding genes, is to our knowledge a peculiar genomic character of the *Veillonella*  
171 genus and is not found in other genera of the Veillonellaceae, suggesting it originated in the  
172 common ancestor of all *Veillonella* species.

173

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

176 To assess the function of the potential adhesins identified in the *V. parvula* SKV38  
177 genome, we constructed -within the cluster of adhesin genes- independent deletion mutants for  
178 the two first autotransporters (*vmaA* and *vtaB*) and a large deletion for the eight adjacent genes  
179 encoding trimeric autotransporters or partial trimeric autotransporters, hereafter called  $\Delta 8$   
180 ( $\Delta$ FNLLGLLA\_00036 to *vtaF*). We also generated independent individual mutants for each of  
181 the six additional autotransporters located outside of the cluster. These mutants were all tested  
182 for biofilm formation in 96-well polystyrene plate and aggregation capacities. None of the  
183 mutants, with the exception of the previously mentioned  $\Delta$ *vtaA* strain, was affected for  
184 aggregation capacities (Figure 5A). The  $\Delta 8$  mutant was the sole mutant, in addition to the  $\Delta$ *vtaA*  
185 mutant, to display lower biofilm formation in 96-well polystyrene microtiter plate (Figure 5B  
186 and C), suggesting that the adhesins of this cluster could be involved in biofilm formation  
187 independently of cell-to-cell interactions. When tested in microfermentor,  $\Delta 8$  displayed a  
188 slightly reduced ability to form mature biofilm, however, not statistically different from WT  
189 (Figure 5D). This reduced ability to form mature biofilms was actually more visible when  
190 observing SEM images, since the  $\Delta 8$  mutant only poorly covered the coverslip with sporadic  
191 aggregates of cells producing extracellular matrix (Figure S4). Initial adhesion assay to glass  
192 spatula showed that both  $\Delta$ *vtaA* and  $\Delta 8$  displayed a lower percentage of initial adhesion than  
193 WT, suggesting that VtaA-mediated auto-aggregation contributed to initial adhesion of the WT  
194 strain while the adhesin cluster is probably directly involved in surface binding (Figure 5E).  
195 This also indicates that  $\Delta$ *vtaA* does not adhere to glass better than WT, and so the increased  
196 biofilm formation of  $\Delta$ *vtaA* in microfermenter arises during the continuous flow culture step. The

197 effect of deleting *vtaA* and the 8 adhesin genes on initial adhesion was additive since a  $\Delta vtaA\Delta 8$   
198 double mutant showed a reduced initial adhesion on microfermentor spatula compared to either  
199 WT,  $\Delta vtaA$  or  $\Delta 8$  (Figure 5E). In addition,  $\Delta vtaA\Delta 8$  formed 17 times less biofilm than  $\Delta vtaA$   
200 in microfermentor, indicating that in the absence of VtaA, the adhesins encoded by some of  
201 these eight genes strongly promote mature biofilm formation in microfermentor (Figure 5D).

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

#### 206 ***FNLLGLLA\_01127 encodes an HD phosphatase that inhibits biofilm formation***

207 In addition to genes encoding potential T5SS proteins, we also identified a transposon  
208 mutant in *FNLLGLLA\_01127*, encoding a protein of the HD phosphatase superfamily (Figure  
209 1B). The *FNLLGLLA\_01127* gene is homologous to YqeK, a putative phosphatase required for  
210 pellicle formation and the development of biofilm in *B. subtilis* (18). *FNLLGLLA\_01127/yqeK*  
211 is found in a cluster of genes (*obg*, *yhbY*, *proB*, *proA*, *nadD*, *yqeK*, *lytR*, and *rsfS*), whose  
212 synteny is very well conserved among Negativicutes. This cluster, or part of it, is also well  
213 conserved in almost all Firmicutes genomes we analyzed, both monoderm and diderm, as well  
214 as in members of other diderm phyla phylogenetically close to the Firmicutes, notably  
215 *Deinococcus-Thermus* (Figure 6 and S5, DataSet 2). A *FNLLGLLA\_01127* deletion mutant  
216 ( $\Delta 1127$ ) had a lower carrying capacity compared to WT, maybe due to higher mortality during  
217 the stationary phase (Figure S1) and a moderate 1.5-fold decrease in biofilm formation in  
218 microtiter plate after correcting for the growth defect (Figure 7A). This mutant also displayed  
219 a slightly faster aggregation rate than the WT during early time points (Figure 7B). The  
220 strongest phenotype of this mutant was detected in microfermentor with a 9-fold increase in  
221 biofilm formation compared to WT (Figure 7C). Expression of *FNLLGLLA\_01127* gene *in*  
222 *trans* (plasmid *p1127*) did not complement the observed growth defect (Figure S1B) but it did  
223 complement the increased biofilm formation in microfermentor (Figure 7D), showing that  
224 deletion of *FNLLGLLA\_01127* might have had polar effects on downstream genes of the operon  
225 causing a growth defect, but that *FNLLGLLA\_01127* alone was responsible for the observed  
226 inhibition of biofilm formation. Scanning electronic microscopy showed that  $\Delta 1127$ , similarly  
227 to  $\Delta vtaA$ , formed a thick layered biofilm, although with fewer filaments and protein deposits  
228 compared to WT (Figure 7E). However, contrary to  $\Delta vtaA$  or  $\Delta 8$  mutants,  $\Delta 1127$  showed no  
229 defect in initial adhesion to a glass spatula (Figure 7F). Interestingly, a  $\Delta 1127\Delta 8$  double mutant

230 formed almost 20 times less biofilm than  $\Delta I127$  in microfermentor (Figure 7C), suggesting that  
231 at least some of the autotransporters of the cluster were necessary for  $\Delta I127$  observed strong  
232 biofilm formation in microfermentor.

233

234

235

236 **DISCUSSION**

237

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

243 We identified a T5SS type Vc trimeric autotransporter, FNLLGLLA\_0516 (VtaA), as an  
244 important biofilm factor promoting *V. parvula* SKV38 auto-aggregation. In addition to Hag1,  
245 a YadA-like autotransporter identified from the related species *V. atypica* involved in  
246 interspecies interactions (22), VtaA represents the second *Veillonella* protein described which  
247 is involved in adhesion, and the first involved in abiotic surface adhesion and auto-aggregation  
248 in diderm Firmicutes. Beyond the potential impact on *Veillonella* niche colonization,  
249 aggregation capacity is known to contribute to bacterial protection from environmental stresses  
250 or host responses (23), promotion of host colonization (24), or pathogenesis (25) in various  
251 bacterial species. VtaA is homologous to *Brucella suis* trimeric autotransporter BtaF. However,  
252 while *B. suis* BtaF promotes biofilm formation *in vitro*, it was not shown to promote aggregation  
253 (21), suggesting that these two proteins have different functions.

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

266 Interference between cell surface structures is a well-described mechanism by which bacteria  
267 modulate their adhesion properties. In *E. coli*, multiple structures, such as chaperone-usher



268 fimbriae, LPS O-antigen or capsules, interfere with the self-recognizing autotransporter Ag43  
269 though unknown mechanisms (33–36). Therefore, it is possible that in *V. parvula*, VtaA could  
270 compete with other adhesins through steric hindrance or competition for membrane export and  
271 thus limit biofilm formation under continuous-flow conditions. Consistently,  $\Delta vtaA$  enhanced  
272 biofilm formation in microfermentor was dependent on the presence of eight genes of the  
273 cluster of trimeric autotransporters, suggesting a competition between VtaA and adhesin(s) of  
274 this cluster. Moreover, we noticed that both VtaA and the 8-gene cluster are necessary for full  
275 initial adhesion to glass spatula in an independent manner, suggesting that any competition  
276 between them only arises later on, during continuous-flow cultures. The exact contribution of  
277 these different trimeric autotransporters to biofilm formation and their interplay with VtaA will  
278 require further characterization.

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

293  
294 Trimeric autotransporters possess a characteristic YadA\_anchor domain (PF03895) that is  
295 found mainly in Proteobacteria, but also in Cyanobacteria, Verrumicrobia, Planctomycetes,  
296 Kiritimatiellaeota, Chlorobi, Synergistetes, Fusobacteria and Negativicutes  
297 (<https://pfam.xfam.org/family/PF03895>, Dec 2019 (43)). Interestingly, the YadA\_anchor of *V.*  
298 *parvula* SKV38 and all *Veillonella* trimeric autotransporters is not at the very end of the C-  
299 terminus, where it is usually found in Proteobacteria, but is pre-C-terminal, followed by either  
300 a coiled domain or a S-layer homology (SLH) domain (Figure 3, DataSet 1). While the function



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

314 Our screening also led to the identification of FNLLGLLA\_01127, the homolog of *B. subtilis*  
315 YqeK, a putative phosphatase required for pellicle formation and the development of biofilm  
316 (18). *Staphylococcus aureus* YqeK was recently shown to be a nucleosidase hydrolyzing  
317 diadenosine-tetraphosphate (Ap4A) into ADP (46). In *Pseudomonas fluorescens*, an increased  
318 level of Ap4A increases cyclic-di-GMP concentration and enhances cell-surface exposure of a  
319 large adhesin LapA, thus inducing biofilm formation (47). c-di-GMP regulates biofilm  
320 formation by modulating production of a variety cell-surface appendages or exopolysaccharides  
321 in both monoderm and diderm bacteria (48–52). Interestingly, *B. subtilis* YqeK induces the  
322 *epsA-O* operon, involved in the production of biofilm matrix-forming polysaccharides (53).  
323 Deletion of *V. parvula* FNLLGLLA\_01127 only led to a minor decrease in biofilm formation in  
324 96-well plate, but to a strong increase in continuous-flow biofilm formation that was dependent  
325 on the presence of the cluster of trimeric autotransporters. Further work is needed to determine  
326 whether FNLLGLLA\_01127 directly impacts production of the adhesins of the cluster or  
327 participates to the production/regulation of an unknown exopolysaccharide, which, contrary to  
328 *B. subtilis*, would interfere with the function or exposure of the adhesins of the cluster rather  
329 than favor community development.

330

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

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

341

342

343 **MATERIALS AND METHODS**344 **Genome preparation and sequencing**

345 *V. parvula* SKV38 genomic DNA was extracted using Qiagen genomic tip 20G kit. It was  
346 sequenced to 1,500X coverage using PacBio sequencing of one single molecule real time  
347 (SMRT) cell with no multiplexing using the V2.1 chemistry. Only one SMRT cell was used but  
348 with no multiplexing, leading to an unusually large amount of subreads: 3 Gbp, meaning about  
349 1,500X coverage assuming a 2.1 Mbp genome. This yielded 338,310 reads with a mean subread  
350 length of 9,080 bp and N50 read length of 13,500 bp. The longest subread length is above 70  
351 kbp. We randomly subsampled the data to avoid misassemblies keeping only 100,000 subreads,  
352 which resulted in a 430X coverage. The genome was then assembled using Canu version 1.8  
353 (54) keeping the default parameters. In particular, subread below 1,000 bp were dropped. The  
354 error correction steps of the Canu algorithm were not tuned, keeping the parameters that control  
355 alignment seed length, read length, overlap length and error rates to their default values. We  
356 obtained one contig of 2.146 Mbp and an additional contig of only 1,972 bp that was abandoned  
357 due to lack of supporting data and was removed by the circularization process. The obtained  
358 assembled genome closely matched the genome size (2.1422 Mbp) and GC content (38.7%,  
359 expected 38.6%) of the reference *V. parvula* DSM2008 strain. The resulting assembled genome  
360 was polished using Pilon (55) but no correction was required. No gaps or drops of coverage  
361 was detected based on sequana\_coverage output (56, 57). The completeness of the candidate  
362 assembly was assessed to be 98% using the bacteria mode and the bacteria\_db9 lineage-specific  
363 profile library of BUSCO software (58), while the number of complete duplicated or  
364 fragmented BUSCOs remains at 0, indicative of complete assembly. Alignment of all reads  
365 show that only 4% (13,028) remained unmapped and 80% of their length were below 2 kbp.  
366 The remaining reads (2000 reads) map on various species and could not be further assembled.  
367 Overall, these analyses indicate that the final genome assembly is complete and of good quality.

368

369 **Bioinformatic analyses**

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

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

378 The search for HD phosphatase (YqeK) cluster homologs was conducted as follows: a local  
379 databank containing 390 genomes representative of bacterial diversity was mined for the  
380 presence of a phosphatase containing HD domain (PF01966) using HMMSEARCH and the --  
381 cut\_ga option. Protein sequences were then filtered using alignment, functional annotation,  
382 protein domains presence and phylogeny. Synteny was investigated in the locus around *yqeK*  
383 by looking, using MacSyFinder (62), for the presence of at least one of the 7 genes surrounding  
384 *yqeK* in *V. parvula* SKV38, namely *obg* (containing GTP1\_OBG domain, PF01018), *yhbY*  
385 (containing CRS1\_YhbY domain, PF01985), *proB* (containing AA\_kinase domain, PF00696),  
386 *proA* (containing Aldedh domain, PF00171), *nadD* (containing CTP\_transf\_like domain,  
387 PF01467), *lytR* (containing LytR\_cpsA\_psr domain, PF03816) and *rsfS* (containing RsfS  
388 domain, PF02410), with no more than eight other genes separating them. All HMM profiles  
389 were downloaded from the PFAM site ([pfam.xfam.org](http://pfam.xfam.org)). As YqeK homologs are widespread in  
390 the Firmicutes, another local databank containing 230 representative Firmicutes genomes was  
391 queried by the MacSyFinder approach as described above. All trees were visualized with ITOL  
392 (63). Details of the results are presented in Dataset S2.

393

#### 394 **Strains and growth conditions**

395 Bacterial strains and plasmids are listed in Table 2. *V. parvula* was grown in either Brain Heart  
396 infusion medium (Bacto Brain Heart infusion, Difco) supplemented with 0.1 % L-cysteine and  
397 0.6 % sodium DL-lactate (BHILC) or SK medium (10 g L<sup>-1</sup> tryptone (Difco), 10 g L<sup>-1</sup> yeast  
398 extract (Difco), 0.4 g L<sup>-1</sup> disodium phosphate, 2 g L<sup>-1</sup> sodium chloride, and 10 mL L<sup>-1</sup> 60 %  
399 w/v sodium DL-lactate, described in (17)) and incubated at 37°C in anaerobic conditions in  
400 anaerobic bags (GENbag anaero, Biomerieux, ref. 45534) or in a C400M Ruskinn anaerobic-  
401 microaerophilic station. *Escherichia coli* was grown in Lysogeny Broth (LB) (Corning)  
402 medium under aerobic conditions at 37°C. 20 mg L<sup>-1</sup> chloramphenicol (Cm), 200 mg L<sup>-1</sup>  
403 erythromycin (Ery) or 2.5 mg L<sup>-1</sup> tetracycline (Tc) were added to *V. parvula* cultures, 100 mg  
404 L<sup>-1</sup> carbenicillin (Cb) or 5 mg L<sup>-1</sup> tetracycline (Tc) were added to *E. coli* cultures when needed.

405 100  $\mu\text{g L}^{-1}$  anhydrotetracycline (aTc) was added to induce the pTet promoter unless stated  
406 otherwise. All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

407

#### 408 **Natural transformation**

409 Cells were resuspended in 1 mL SK media adjusted to 0.4-0.8 OD<sub>600</sub> and 10  $\mu\text{L}$  were dotted on  
410 SK-agar petri dishes. On each drop, 0.5-1  $\mu\text{g}$  plasmid or 75-200 ng  $\mu\text{L}^{-1}$  linear dsDNA PCR  
411 product was added, or water for the negative control. The plates were incubated 48 hours. The  
412 biomass was resuspended in 500  $\mu\text{L}$  SK medium and plated on SK-agar supplemented with the  
413 corresponding antibiotic and incubated for another 48 hours. Colonies were streaked on fresh  
414 selective plates and the correct integration of the construct was confirmed by PCR and  
415 sequencing.

416

#### 417 **Random mariner transposon mutagenesis**

418 Plasmid pRPF215, described for *Clostridium* mutagenesis (Addgene 106377) (19) was  
419 transformed into *V. parvula* SKV38 by natural transformation and selected on Cm  
420 supplemented SK-agar medium. An overnight culture of *V. parvula* SKV38-pRPF215 in  
421 BHILC was then diluted to 0.1 OD<sub>600</sub> in the same media, supplemented with aTc and grown 5  
422 hours to induce the transposase. After induction, the culture was diluted and plated on BHILC  
423 supplemented with Ery and aTc for selection and incubated for 48 hours. From the resulting  
424 colonies, 940 were inoculated in Greiner Bio-one polystyrene flat-bottom 96-well plates  
425 (655101) and grown in BHILC supplemented with either Ery and aTc, or Cm, to confirm both  
426 the presence of the transposon and the loss of pRPF215 and then kept in 15 % glycerol at -  
427 80°C. Selected transposon mutants were grown overnight and the genomic DNA was harvested  
428 using DNeasy blood and tissue kit (Qiagen). The genomic DNA was then sent for whole  
429 genome sequencing at the Mutualized platform for Microbiology of Institut Pasteur.

430

#### 431 **Cloning-independent allelic exchange mutagenesis**

432 Site-directed mutagenesis of *V. parvula* SK38 strain was performed as described by Knapp and  
433 colleagues (17). Briefly, 1-Kb regions upstream and downstream the target sequence and *V.*  
434 *atypica* tetracycline resistance cassette (*tetM* in pBSJL2) or *catP* resistance cassette from *C.*  
435 *difficile* (*catP* in pRPF185, Addgene 106367, from (64)) were PCR-amplified with overlapping

436 primers using Phusion Flash High-Fidelity PCR Master-Mix (Thermo Scientific, F548). PCR  
437 products were used as templates in a second PCR round using only the external primers that  
438 generated a linear dsDNA with the tetracycline resistance cassette flanked by the upstream and  
439 downstream sequences. This construct was transformed into *V. parvula* by natural  
440 transformation and its integration into the genome was selected by plating on Tc or Cm  
441 supplemented medium. Positive candidates were further confirmed by a set of PCRs and  
442 sequencing around the site. Primers used in this study are listed in Table S1.

443

#### 444 **Complementation**

445 We replaced the tetracycline resistance gene and its *gyrA* promoter of the shuttle vector pBSJL2  
446 by a chloramphenicol resistance gene,  $P_{cat}:cat$  from pRPF185 by Gibson assembly. Briefly, the  
447 inserts and the plasmids were PCR amplified and then mixed with Gibson master mix 2x  
448 (100 $\mu$ L 5X ISO Buffer, 0.2  $\mu$ L 10,000 U/mL T5 exonuclease (NEB #M0363S), 6.25  $\mu$ L 2,000  
449 U/mL Phusion HF polymerase (NEB #M0530S), 50  $\mu$ L 40,000 U/mL Taq DNA ligase (NEB  
450 #M0208S), 87  $\mu$ L dH<sub>2</sub>O for 24 reactions) and incubated at 50°C for 30-60 min.

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

458 Alternatively, the anhydrotetracycline inducible expression cassette of pRPF185, hereafter  
459 referred to as *pTet*, (Addgene 106367, (64)) was inserted along with a chloramphenicol marker  
460 right before the ATG of the target gene, following the procedure described above for cloning-  
461 independent allelic exchange mutagenesis. The functionality of *pTet* in *V. parvula* was  
462 previously verified using measurement of the aTc dependent  $\beta$ -glucuronidase activity generated  
463 by the presence of pRPF185 transformed in *V. parvula* SKV38 (Figure S3).

464

#### 465 **Biofilm formation in 96-well microtiter plates**

466 Overnight cultures in BHILC medium were diluted to 0.05 OD<sub>600</sub> and transferred to three  
467 Greiner Bio-one polystyrene flat bottom 96-well plates adding 150  $\mu$ L per well. After 24 hours  
468 of static incubation, one of the three plates was resuspended by pipetting to measure OD<sub>600</sub>

469 using a TECAN Infinite-M200-Pro spectrophotometer. The two other plates were used for  
470 coloration: cultures were removed by pipetting carefully the supernatant out and biofilms fixed  
471 with 150  $\mu$ L Bouin solution (HT10132, Sigma-Aldrich) for 15 minutes. Bouin solution was  
472 removed by inversion and the biofilms were washed once in water. The biofilms were stained  
473 with 150  $\mu$ L crystal violet 1 % (V5265, Sigma-Aldrich) for 15 minutes without shaking, then  
474 washed in water twice and left to dry. All washes were made by flicking of the plate. After  
475 drying the plate, crystal violet was dissolved with 200  $\mu$ L absolute ethanol and transferred to a  
476 clean 96-well plate for OD<sub>620</sub> measurement (TECAN Infinite-M200-Pro spectrophotometer).

477

#### 478 **Biofilm formation in microfermentor**

479 Continuous flow non-bubbled microfermentor containing a removable spatula were used as  
480 described in (65, 66) (see <https://research.pasteur.fr/en/tool/biofilm-microfermenters/>). Briefly,  
481 a glass spatula was dipped in an overnight culture diluted to 0.5 OD<sub>600</sub> in 15 mL BHILC for 15  
482 minutes and returned to the fermentor. Biofilm was grown on the spatula for 48 hours at 37°C.  
483 BHILC was constantly supplied through a peristaltic pump at 4 rpm. During the last hour, the  
484 speed was increased to 10 rpm to remove planktonic bacteria. A mix of filtered 90% nitrogen/5%  
485 hydrogen/5% carbon dioxide was also constantly supplied to maintain anaerobic condition.  
486 After 48 hours of growth, the spatula was removed, and the biofilm was resuspended by  
487 vortexing in 15 mL BHILC. We measured OD<sub>600</sub> of the resuspended biofilms with Smart Spec  
488 Plus spectrophotometer (BioRad).

489

#### 490 **Aggregation curve**

491 Overnight cultures were diluted to 0.8 OD<sub>600</sub> in Brain-heart infusion (BHI) media in semi-micro  
492 spectrophotometry cuvette (Fisherbrand) and left to sediment on the bench in presence of  
493 oxygen, so no growth should occur. OD<sub>600</sub> was measured every hour in a single point of the  
494 cuvette using SmartSpec spectrophotometer (BioRad).

495

#### 496 **Initial adhesion on glass**

497 Glass spatula from microfermentor (described above) were dipped in overnight cultures diluted  
498 to 0.5 OD<sub>600</sub> in 15 mL Brain-Heart Infusion (BHI) media for 30 minutes to let bacteria adhere.  
499 The spatulas were washed once in 15 mL BHI by submersion and the adhering bacteria were  
500 resuspended in 15 mL clean BHI by vortexing. The culture used for inoculation, as well as the  
501 resuspended bacteria were serially diluted and plated on SK-agar plate for colony forming unit  
502 (CFU) counting.



503

504 **Statistical analysis**

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

513



514

515 **COMPETING FINANCIAL INTERESTS**

516 The authors declare no competing financial interests.

517

518

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531

532 **AUTHORS CONTRIBUTIONS**

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

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746 **TABLES**

747

748

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	btaE 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	btaE 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	btaE 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	btaF	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

749 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
$\Delta$ vtaA	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_00516::tetM</i>	This study
pTet-vtaA	<i>Veillonella parvula</i> SKV38 <i>catP-Term(fdx)-Ptet-FNLLGLLA_00516</i>	This study
$\Delta$ 8	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_00036-46::tetM</i>	This study
$\Delta$ vmaA	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_00032::tetM</i>	This study
$\Delta$ vtaB	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_00034::tetM</i>	This study
$\Delta$ vtaG	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_00098::tetM</i>	This study
$\Delta$ vtaH	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_00099::tetM</i>	This study
$\Delta$ vmaB	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_00335::tetM</i>	This study
$\Delta$ vmaC	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_00581::tetM</i>	This study
$\Delta$ vtaI	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_01790::tetM</i>	This study
$\Delta$ vtaA $\Delta$ 8	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_00516::catP</i> $\Delta$ <i>FNLLGLLA_00036-46::tetM</i>	This study
$\Delta$ 1127	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_01127::tetM</i>	This study
$\Delta$ 1127 $\Delta$ 8	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_01127::tetM</i> $\Delta$ <i>FNLLGLLA_00036-46::catP</i>	This study
WT+pEmpty	<i>Veillonella parvula</i> SKV38-pBSJL2- <i>catP-pmdh</i>	This study
$\Delta$ 1127+pEmpty	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_01127::tetM</i> - pBSJL2- <i>catP-pmdh</i>	This study
$\Delta$ 1127+p1127	<i>Veillonella parvula</i> SKV38 $\Delta$ <i>FNLLGLLA_01127::tetM</i> - pBSJL2- <i>catP-pmdh-FNLLGLLA_01127</i>	This study
P <sub>tet</sub> - $\phi$	SKV38- pRPF185 $\Delta$ <i>gusA</i>	This study
P <sub>tet</sub> - <i>gusA</i>	SKV38- pRPF185	This study
P <sub>Cwp2</sub> - <i>gusA</i>	SKV38- pRPF144	This study
Plasmid	Description	Reference

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

751 **Table 2: Strains and plasmids used in this study**

752

753

754

755 **FIGURES LEGEND**

756

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

765

766 **Figure 2: *VtaA* is an adhesin involved in auto-aggregation and biofilm formation.** **A.** 96-  
767 well plate biofilm assay after 24h growth in BHILC. Mean of WT is adjusted to 100 %. Min-  
768 max boxplot of 6 biological replicates for each strain. \* p-value<0.05, \*\* p-value <0.005,  
769 Mann-Whitney test between strains. **B.** and **C.** Aggregation curve in spectrophotometry cuvette  
770 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  
771 (**C**). 100 % represent lack of aggregation, 0 % complete sedimentation of the culture. Median  
772 of 6 biological replicates, error bars represent 95% confidence interval. At each time points we  
773 computed the Mann-Whitney test between conditions. We applied Bonferroni correction for  
774 multiple testing: p-value are only considered significant if \*p-value<0.004, \*\*p-value<0.0004,  
775 \*\*\*p-value<0.00004. Indicated p-values were calculated by comparing in **B**, WT and  $\Delta vtaA$ ,  
776 and in **C**, *pTet-vtaA* without aTc and *pTet-vtaA* with different aTc concentrations. **D.** 96-well  
777 plate biofilm assay after 24h growth of an inducible *vtaA* in BHILC with different  
778 concentrations of aTc. WT without aTc is adjusted to 100 %. Median of 6 biological replicates,  
779 each replicate corresponds to the mean of two technical replicates, error bars represent 95%  
780 confidence interval. \* p-value<0.05, \*\* p-value <0.005, Mann-Whitney test. **E.** Biofilm  
781 formation in continuous flow microfermentor on glass spatula during 48h in BHILC. WT was  
782 adjusted to 100 %. Min-max boxplot of 4 biological replicates for each strain. A picture of the  
783 spatula before resuspension is shown below each boxplot bar. \* p-value<0.05, Mann-Whitney  
784 test.

785

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

795

796 **Figure 4: Synteny of the adhesin gene cluster in a selection of *Veillonella* species.** The  
797 synteny of the proteins of the cluster between the closest relatives was assessed using EasyFig  
798 (69). Oblique lines between genes represent tblastx identities (program parameters: maximum  
799 e-value of  $10^{12}$ , minimum length of 30, minimum identity of 30). The *V. parvula* SKV38 strain

800 used in this study is presented in red. The annotation of the genes of the cluster is indicated on  
801 the right.

802

803 **Figure 5: A cluster of eight trimeric autotransporters is involved in surface binding. A.**  
804 Aggregation curve in spectrophotometry cuvette. 100 % represent lack of aggregation, 0 %  
805 complete sedimentation of the culture. Median of 6 biological replicates, error bars represent  
806 95% confidence interval. \* Mann-Whitney test, corrected for multiple testing with Bonferroni  
807 correction: significance is achieved if p-value < 0.007. **B.** and **C.** 96-well plate biofilm assay  
808 after 24 h growth in BHILC. Mean of WT is adjusted to 100 %. Min-max boxplot of 6 biological  
809 replicates for each strain, each replicate is the mean of two technical replicates. In **B.** we applied  
810 a Mann-Whitney; \* p-value<0.05, \*\* p-value <0.005. In **C.** we applied Bonferroni correction  
811 for multiple testing: tests were called significant only if p-value<0.01: \* p-value<0.01, \*\* p-  
812 value <0.001, \*\*\* p-value <0.0001. **D.** Biofilm formation in continuous flow microfermentor  
813 on glass spatula during 48h in BHILC. WT was adjusted to 100 %. Min-max boxplot of 4  
814 biological replicates for each strain. \* p-value<0.05, Mann-Whitney test. A picture of spatula  
815 before resuspension is shown for each mutant below the boxplot. **E.** Initial adhesion on glass  
816 spatula. Percentage of CFU that adhered to the spatula controlled by the number of CFU of the  
817 inoculation solution. Min-max boxplot of 6-9 replicates per strain is represented. \* p-  
818 value<0.05, \*\* p-value <0.005, \*\*\* p-value <0.0005, Mann-Whitney test.

819

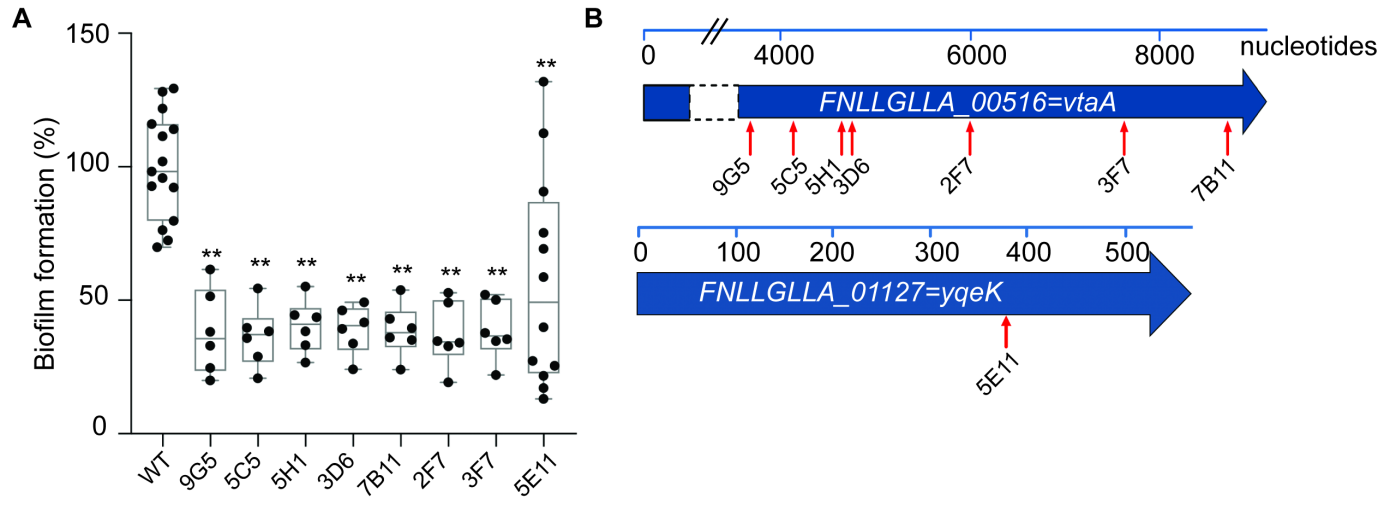
820 **Figure 6: Occurrence and synteny of HD Phosphatase (YqeK) in diderm and monoderm**  
821 **bacteria. A.** The presence of the cluster was investigated using MacSyFinder (62) and the  
822 results were plotted onto a schematic reference tree of 187 cultivable bacteria among the 390  
823 of the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

827

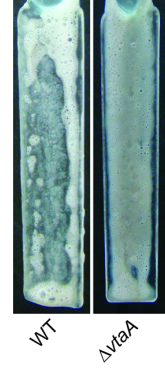
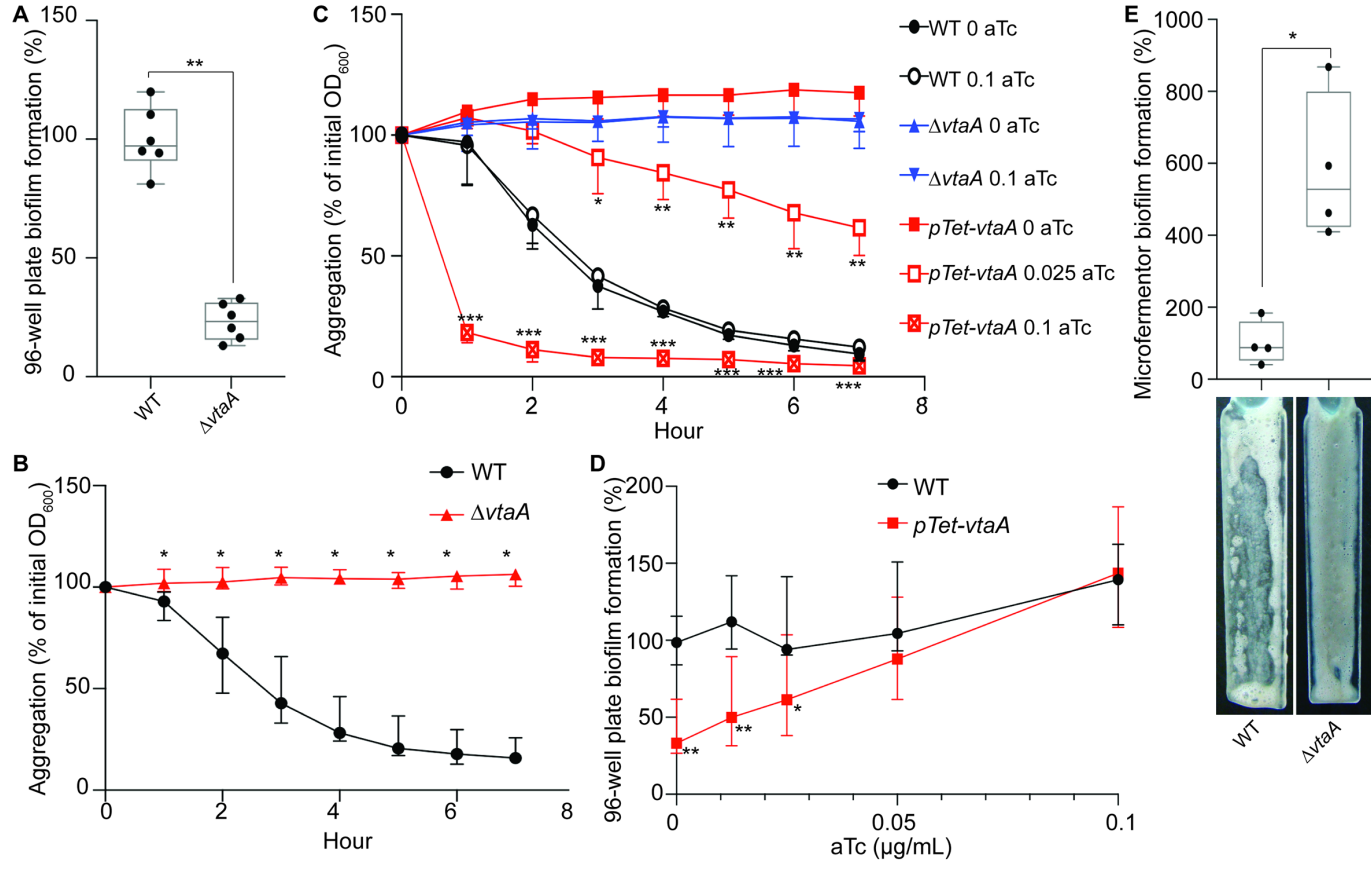
828 **Figure 7: FNLLGLLA\_01127 represses biofilm formation in microfermentor. A.** 96-well  
829 plate biofilm assay after 24 h growth in BHILC corrected by OD<sub>600</sub> after 24 h growth in plate.  
830 Mean of WT is adjusted to 100 %. Min-max boxplot of 6 biological replicates for each strain,  
831 each replicate is the mean of two technical replicates. \* p-value < 0.05, Mann-Whitney test. **B.**  
832 Aggregation curve in spectrophotometry cuvette. 100 % represent lack of aggregation, 0 %  
833 complete sedimentation of the culture. Median of 6 biological replicates, error bars represent  
834 95% confidence interval. \* Mann-Whitney test, corrected for multiple testing with Bonferroni  
835 correction: significance is achieved if p-value<0.007. **C.** Biofilm formation in continuous flow  
836 microfermentor on glass spatula during 48h in BHILC. Mean of WT is adjusted to 100 %. Min-  
837 max boxplot of 4 biological replicates for each strain. \* p-value < 0.05, \*\* p-value<0.005,  
838 Mann-Whitney test. A picture of a spatula before resuspension is shown for each strain below  
839 the histogram. **D.** Biofilm formation in continuous flow microfermentor on glass spatula during  
840 48h in BHILC+chloramphenicol. Mean of WT+pEmpty is adjusted to 100 %. Min-max boxplot  
841 of 4 biological replicates for each strain. \* p-value < 0.05, Mann-Whitney test. A picture of a  
842 spatula before resuspension is shown for each strain below the boxplot. **E.** Scanning electronic  
843 microscopy of  $\Delta$ 1127 biofilm grown under continuous flow of BHILC in microfermentor on a  
844 plastic microscopy slide. Magnification 2K and 5K. **F.** Initial adhesion on glass spatula.  
845 Percentage of CFU that adhered to the spatula in 30 min controlled by the number of CFU of

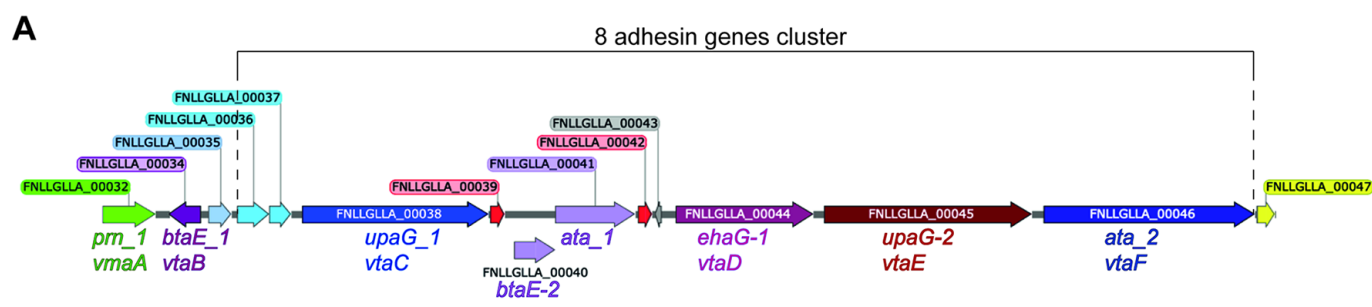
846 the inoculation solution. Min-max boxplot of 6-9 replicates per strain. \* p-value<0.05, Mann-  
847 Whitney test.

848









Gene locus	Protein name	Domain organization
FNLLGLLA_00032	Prn_1 / VmaA	790 aa
FNLLGLLA_00034	BtaE_1 / VtaB	470 aa
FNLLGLLA_00035		348 aa
FNLLGLLA_00036		477 aa
FNLLGLLA_00037		323 aa
FNLLGLLA_00038	UpaG_1 / VtaC	2811 aa
FNLLGLLA_00040	BtaE_2	625 aa
FNLLGLLA_00041	Ata_1	1209 aa
FNLLGLLA_00044	EhaG_1 / VtaD	2072 aa
FNLLGLLA_00045	UpaG_2 / VtaE	3142 aa
FNLLGLLA_00046	Ata_2 / VtaF	3193 aa

**B**

Gene locus	Protein name	Domain organization
FNLLGLLA_00098	BtaE_3 / VtaG	577 aa
FNLLGLLA_00099	Ata_3 / VtaH	1653 aa
FNLLGLLA_00335	Prn_2 / VmaB	741 aa
FNLLGLLA_00516	BtaF / VtaA	3041 aa
FNLLGLLA_00581	BrkA / VmaC	748 aa
FNLLGLLA_01790	EhaG_2 / VtaI	833 aa

- 1000 aa
- extended signal peptide region (PF13018)
  - procyclic acidic repetitive protein (PF05887)
  - DUF1664 (PF07889)
  - YadA head (PF05658)
  - YadA stalk (PF05662)
  - autotransporter (PF03797)
  - inverse autotransporter beta domain (PF11924)
  - YadA anchor (PF03895)
  - S-layer homology (PF00395)
  - Coil (Coils prediction)

