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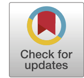
Short communication

Insights into *Streptococcus agalactiae* PI-2b pilus biosynthesis and role in adherence to host cells



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The core PI-2b pilus present in "hypervirulent" ST-17 *Streptococcus agalactiae* strains consists of three pilin subunits (Spb1, Ap1 and Ap2) assembled by sortase SrtC1 and cell-wall anchored by Srt2. Spb1 was shown to be the major pilin and Ap2 the anchor pilin. Ap1 is a putative adhesin. Two additional genes, *orf* and *lep*, are part of this operon. The contribution of Lep and Ap1 to the biogenesis of the PI-2b pilus was investigated. Concerning the role of PI-2b, we found that higher PI-2b expression resulted in higher adherence to human brain endothelial cells and higher phagocytosis by human THP1 macrophages.

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Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a major cause of invasive neonatal infections, sepsis and meningitis, that may occur during the first week of life (0–6 days) or up to 3 months of age (7–89 days), designated as early (EOD) and late (LOD) onset diseases, respectively [1]. GBS strains belonging to the capsular serotype III and sequence type ST-17 are of particular interest since they have been shown to be responsible for more than 75% of LOD cases, with septicaemia and meningitis as the most common clinical manifestations. Comparative genomics of unrelated GBS isolates revealed that ST-17 strains exhibit a few specific genes such as *srr2* and *hvgA* [2]. These two genes code for surface proteins, which enhance the capacity of these strains to adhere to intestinal cells and to cross the blood–brain barrier [3,4].

Pili are generally considered as important colonization factors. Genome mining allowed the identification of two types of pilus islands in GBS, PI-1 and PI-2, the latter having two alleles, PI-2a and PI-2b [5,6]. Although ubiquitous in the ST-17 lineage, the PI-2b pilus is also found in a few non-ST17 human isolates such as strain A909 and in most bovine strains [7,8].

The PI-2b locus encodes three pilin subunits: the major pilin Spb1, two accessory pilins (Ap1 and Ap2); and two sortases (SrtC1 and Srt2) (Fig. S1). SrtC1 is necessary for covalent assembly of pilin subunits whereas Srt2 was shown to be involved in the anchoring of the polymerized pilus to the cell wall [9]. The *spb1* gene encoding the major pilin was originally cloned by subtractive hybridization and shown to slightly promote invasion in various human epithelial cell lines (pulmonary A549, cervix HeLa, and colonic C2BBe1) [10]. Later, Chattopadhyay et al. [11] showed that the presence of Spb1 in clinical strains enhanced phagocytosis of *S. agalactiae* by both murine and human macrophages.

We recently showed that expression of the PI-2b pilus locus is about 5-fold lower in the ST-17 strain BM110 than in the non-ST-17 A909 isolate [8]. In BM110, presence of a 43-base pair hairpin structure located in the intergenic region between the upstream locus coding for the group B carbohydrate (antigen B) and the promoter region of the PI-2b locus contributes to this lower expression. Absence of this structure in A909 WT, as well as in the BM110Δ43 mutant where this hairpin has been deleted, was responsible for an increased expression of PI-2b by a read-through transcription from the upstream antigen B operon [8].

In contrast to the previously characterized PI-2a and PI-1 pilus loci in *S. agalactiae*, the PI-2b genetic locus contains two additional

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overlapping genes, named *orf* and *lep*. *orf* is potentially coding for a 90 amino acid-long peptide of unknown function and *lep* is coding for a putative signal peptidase.

In this work, we have studied the role of *lep* and *ap1* in pilus biosynthesis through construction of in-frame deletion mutants in *S. agalactiae* strains A909 and BM110. Our results indicate that *lep* is involved in the efficient processing of the Spb1 signal peptide and its incorporation into an Ap1-containing pilus polymer. To determine the role of PI-2b, we tested the binding of these deletion mutants to extracellular matrix components, human intestinal cells, brain derived endothelial cells, and to the human macrophage cell line THP-1. Overall, our results showed that higher expression of PI-2b pilus polymer results in higher phagocytosis by human monocyte-derived THP-1 macrophages.

1. Materials and methods

1.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *S. agalactiae* strain A909, capsular serotype Ia ST-7 and strain BM110 capsular serotype III ST-17 are well-characterized isolates from human invasive infections [12,13]. *Escherichia coli* DH5α was used for cloning experiments.

S. agalactiae strains were cultured in Todd Hewitt (TH) broth or agar (Difco Laboratories, Detroit, MI) at 37 °C in standing filled flasks and *E. coli* in Luria–Bertani (LB) medium. Antibiotics were used at the following concentrations: for *E. coli*, erythromycin, 150 $\mu g\ ml^{-1}$, kanamycin, 50 $\mu g\ ml^{-1}$; for *S. agalactiae*, erythromycin, 10 $\mu g\ ml^{-1}$.

1.2. Construction of mutants

In frame deletion mutants of *ap1* and *lep* in A909 were constructed by using splicing-by-overlap-extension PCR as previously described [14]. The primers used for the construction of deletion alleles are listed in Supplementary Table 2. Each deletion was confirmed by PCR and sequencing on the genomic DNA of the mutants.

1.3. Cell culture and adherence assays

The human colon adenocarcinoma Caco-2 cell line was cultured in DMEM (Dulbecco Modified Eagle Medium) supplemented with 10% FBS. The human brain endothelial cell line hCMEC/D3, provided by Pierre-Olivier Couraud (INSERM, Paris, France), were grown in EndoGRO™ medium (Millipore). The human cell line THP-1 was differentiated from monocyte state to macrophage-like phenotype by using phorbol-12-myristate-13-acetate (PMA). Cells were seeded at 3×10^5 cells ml^{-1} in RPMI 1640, fetal calf serum 10%, β-mercaptoethanol 0.05 mM and 200 nM of PMA. After 3 days, cells were washed with PBS to remove PMA and further incubated in complete media without PMA for 5 days. Adherence assays were performed in RPMI at a MOI of 10 bacteria/cell. Bacteria grown to the mid-log phase were added to confluent monolayers and centrifugated 5 min at 1000 RPM 179 g to synchronize infection. After 1 h of incubation at 37 °C under a 5% CO_2 atmosphere, monolayers were washed 4 times with PBS to remove non-adherent bacteria, cells were then lysed with cold water and plated to enumerate cell-associated bacteria. The percent of adherence was calculated as follows: (CFU on plate count/CFU in inoculum) × 100. Assays were performed in triplicate and were repeated at least three times.

2. Results and discussion

2.1. Role of Lep and Ap1 in PI-2b pilus biosynthesis

As previously reported [6,9], the PI-2b locus is comprised of 3 genes coding for the pilus structural subunits (*spb1*, *ap1* and *ap2*), 2 genes encoding sortases (*srtC1* and *srt2*) and two additional overlapping genes (*orf* and *lep*) encoding a small conserved gene of unknown function and a putative signal peptidase, respectively (Fig. S1). *In silico* analysis predicted that Lep is a functional type I signal peptidase of 189 aa belonging to the S26A family displaying all the conserved residues, in particular the Ser–Lys dyad, found in all bacterial type I signal peptidases [15] (Fig. S2). To investigate the contribution of *lep*, *ap1* and *spb1* to PI-2b biosynthesis, in-frame deletion mutants of these three genes were constructed in A909 (non ST-17) and BM110 (ST-17) backgrounds. Phenotypic characterization of the mutants did not reveal any significant differences in the general morphology of these mutants compared to the wild-type strains (Fig. S1). Only a slightly prolonged lag phase was observed for Δ*lep* mutants in THY broth (data not shown).

Pilus polymerization was then studied by western blotting of cell wall extracts using specific antibodies against the two pilin subunits Ap1 and Spb1 (Fig. 1A). Antisera raised against Ap1 and Spb1 were found to be highly specific as demonstrated by the absence of reactive bands in the cell wall extracts of the corresponding mutants, Δ*ap1* and Δ*spb1*, respectively (Fig. 1A). The antiserum raised against Ap1 recognized a band of approximately 160 kDa in the non-piliated Δ*spb1* mutant, which corresponds to the predicted size of Ap1 monomer (160.43 kDa). Lower molecular weight (LMW) reactive bands are likely to correspond to Ap1 degradation products. While high molecular weight (HMW) species, most likely representing Ap1-containing polymers, were clearly visible in the wild-type strain A909 (WT), their presence was strongly reduced in the isogenic Δ*lep* mutant (Fig. 1A). The antiserum raised against the major pilin Spb1 recognized several reactive species in the cell wall extracts of WT, Δ*lep* and Δ*ap1* mutants (Fig. 1A). Of note, two reactive bands above 50 kDa were visible in the Δ*lep* mutant, a higher MW band corresponding to the predicted size of Spb1 monomer (53.45 kDa) and a slightly lower band possibly corresponding to processed Spb1 (Spb1 without the signal peptide). Spb1-containing HMW polymers were decreased in Δ*lep* and Δ*ap1* mutants as compared to the wild-type (Fig. 1A). Since pilus expression cannot be quantified by western blotting as HMW polymers cannot enter the gel, flow-cytometry experiments were carried out. As shown in Fig. 1B, surface display of Spb1 is slightly reduced in Δ*lep* and Δ*ap1* mutants. Similarly, Ap1 display is reduced in the Δ*spb1* mutant in accordance with its localization at the tip of the pilus and its potential masking by the capsule as shown previously for the PI-2a adhesin [16]. Finally, complementation of Δ*lep* mutant with a plasmid overexpressing *lep* under a strong constitutive promoter restored the formation of highest Ap1-containing polymers whereas overexpression of *spb1* alone could not (Fig. 1C). Overexpression of *spb1* in Δ*lep* mutant resulted in the accumulation of equivalent amount of processed and unprocessed monomeric forms of Spb1 (Fig. 1C). Of note, beside Lep, A909 carries two other predicted type I signal peptidases (SAK_1038; SAK_1731) that could potentially process the Spb1 pilin in the Δ*lep* mutant. Indeed, Spb1 polymerization is not abolished in the Δ*lep* mutant, probably because the major pilin Spb1 contains a N-terminal signal peptide with a AxA amino acid sequence recognized by canonical type I signal peptidases [17]. Surprisingly, no signal peptide sequence can be found in the minor Ap1 pilin primary sequence but only a N-terminal transmembrane domain. Thus, it is tempting to speculate that Lep is required for the cleavage of the potentially non-canonical peptide sequence of Ap1, and also

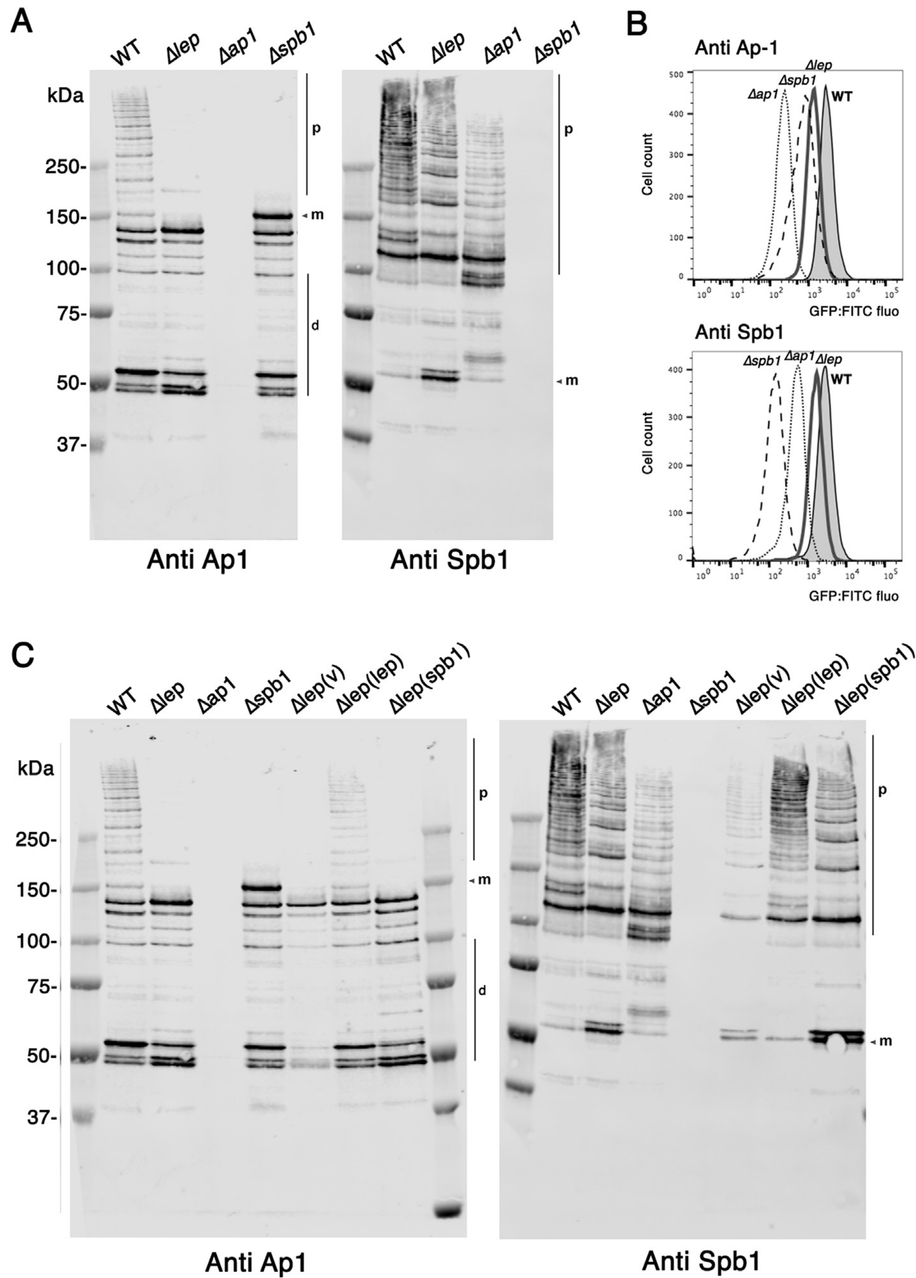

Fig. 1. PI-2b biosynthesis is altered in Δ*lep* and Δ*ap1* mutant strains. (A) Cell wall protein extracts were prepared from *S. agalactiae* A909 WT and its isogenic mutants Δ*lep*, Δ*ap1*, and Δ*spb1*, separated on gradient 4–12% Criterion XT SDS-PAGE gels and detected by immunoblotting as described previously [8] with specific polyclonal antibodies raised against Ap1 and Spb1. Equivalent amounts of total cell wall proteins (50 μg) were loaded. (B) Flow-cytometry analysis of Ap1 and Spb1 expression in A909 WT and its isogenic Δ*lep*, Δ*ap1*, and Δ*spb1* mutants as described previously [8]. (C) Same analysis as in (A) with additional strains A909Δ*lep* complemented with empty plasmid Δ*lep*(v) or with plasmid containing *lep* or *spb1*. Monomers (**m**); polymers (**p**); and degradation products (**d**) are indicated.

for its incorporation in the pilus fiber. This result strongly suggests that Ap1 is the substrate of Lep and cannot be cleaved by other signal peptidases present in A909. Indeed, as shown in Fig. 1A, no Ap1 containing polymers could be seen in the Δ*lep* mutant. Of note, Ap1 monomer can be clearly detected in Spb1 mutant and in A909 WT while in Δ*lep* it appears degraded suggesting that Lep may contribute to Ap1 stability. In *Streptococcus suis*, a pilus-specific signal peptidase was previously shown to be involved in the efficient polymerization of FctF pilus [18]. Lazzarin et al. previously showed that the PI-2b locus encodes two sortases, SrtC1 which is required for pilin polymerization and Srt2, a non-canonical sortase, responsible for the anchoring of the PI-2b pilus to the cell wall by targeting the minor anchor pilin (Ap2) [9]. Altogether, these results indicate that the PI-2b locus is an autonomous module containing all structural (*spb1*, *ap1*, and *ap2*) and non-structural genes (*lep*, *srtC1*, and *srt2*) required for its own biosynthesis and anchoring to the cell wall. Indeed, analysis of single pilus subunits in a surrogate host, such as *Lactococcus lactis*, resulted in poor display on the bacterial surface (our unpublished observations).

2.2. Role of Spb1 in adhesion to extracellular matrix and to intestinal cells

To identify the biological role of the PI-2b pilus, especially in the context of so-called "hypervirulent" ST-17 strains, we compared the binding of BM110 WT and of isogenic Δ*ap1* and Δ*spb1* mutants to

various extracellular matrix components (fibronectin, fibrinogen, and mucus). Since PI-2b expression is relatively low in BM110 WT, as compared to other ST-17 strains or A909 [8], we also tested the BM110Δ43 mutant which overexpresses the PI-2b pilus as shown previously [8]. No differences were found between BM110 WT and the various mutants (Fig. S3). These results were confirmed in the higher PI-2b expressing, non ST-17 A909 background (data not shown).

Since Spb1 was first described as an ST-17-specific invasin [9], we also tested adhesion and invasion of BM110 WT, Δ43, Δ*ap1* and Δ*spb1* strains to various human colonic cell lines such as Caco-2, T84 and HT-29. No significant differences were found between WT and isogenic mutants neither in BM110 nor in A909 backgrounds (data not shown). Since ST-17 strains exhibit a strong neuronal tropism, the adherence of BM110 WT, Δ*43*, Δ*ap1* and Δ*spb1* strains to human brain-derived endothelial cells hCMEC/D3 was tested. As shown in Fig. 2, the BM110Δ43 mutant overexpressing PI-2b pilus exhibits a stronger adherence to these cells as compared to BM110 WT. Our findings fit well with a recent report showing defective adhesion of COH1Δ*bp1* (or Δ*spb1*) to brain derived endothelial cells as compared to the wild-type COH1 strain, another broadly studied ST-17 strain [19].

2.3. Role of Spb1 in bacterial uptake by THP-1 macrophages

It was previously shown that isolates possessing *spb1* were phagocytosed better than *spb1*-deficient mutants by J774A.1 murine macrophages as well as by human U937 monocyte-derived macrophages [11]. To evaluate if higher expression of *spb1* correlates with higher phagocytosis, we first tested phagocytosis of GBS strains BM110 and A909 in human THP-1 macrophages. As shown in Fig. 3A, GBS strain A909, which express about 3- to 5- fold higher level of *spb1* as compared to BM110, was phagocytosed more efficiently. We next tested phagocytosis of BM110 WT and its various isogenic mutants (Δ43, Δ*ap1*, and Δ*spb1*). Phagocytosis of BM110Δ43, expressing about 5-fold higher amounts of Spb1, was significantly higher as compared to BM110 WT, Δ*ap1* or Δ*spb1* (Fig. 3B). Furthermore, the mutant strains Δ*spb1* and Δ*ap1* were less phagocytosed than BM110 WT but the differences were not statistically significant (Fig. 3B). Survival of the various mutants in THP-1 macrophages was tested and did not show any significant differences 2 h post-phagocytosis (data not shown).

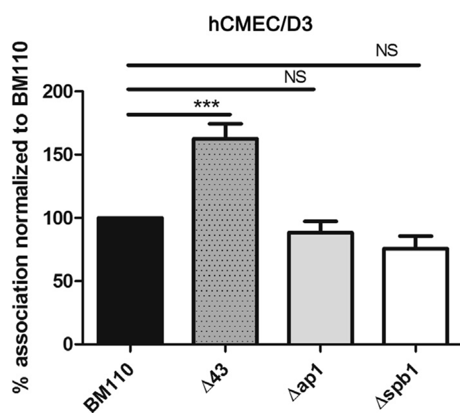

Fig. 2. Adhesion of BM110 and its isogenic deletion mutants to human brain-derived endothelial cells hCMEC/D3. Exponentially growing bacteria were added to confluent monolayers at a multiplicity of infection of 10 bacteria/cell. The levels of adhesion were calculated as follows: (CFU on plate count/CFU in inoculum) × 100, and were normalized to BM110. Results are means ± SD from at least four independent cultures in triplicate. Asterisks represent statistical differences relative to WT strain BM110 with $^{**}p \le 0.01$; $^{***}p \le 0.001$ as assessed by using two-way ANOVA with Bonferroni's post-test in GraphPad Prism version 5. NS, non-significant difference.

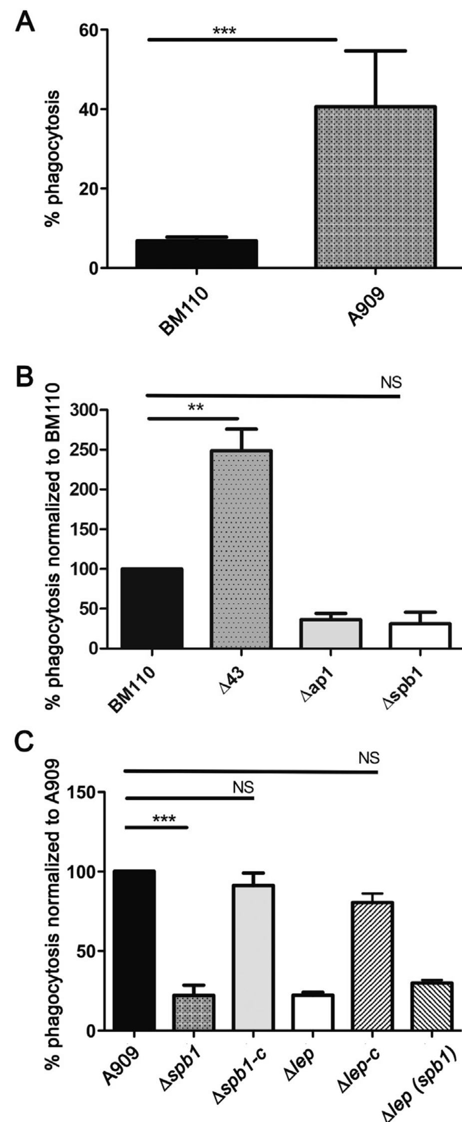

Fig. 3. Increased PI-2b levels enhances phagocytosis by human macrophages THP-1. Macrophages were infected at a MOI of 10 in RPMI 1640 and centrifuged 5 min at 179 RCF to synchronize infection. After 45 min at 37 °C under a 5% CO_2 atmosphere, monolayers were washed with PBS and incubated for 30 min at 37 °C in RPMI 1640 supplemented with penicillin (30 U ml^{-1}) and streptomycin (30 μg ml^{-1}). After extensive rinsing with PBS, macrophages were lysed with fresh water. GBS were quantified by colony counts. The percent of phagocytosis was calculated as follows: (CFU on plate count/CFU in inoculum) × 100. Assays were performed in triplicate and were repeated at least three times. (A) Phagocytosis of BM110 (WT ST-17) versus A909 (WT ST-9) by human monocyte-derived THP-1 macrophages. (B) Phagocytosis of BM110 WT, BM110Δ43, BM110Δ*ap1* and BM110Δ*spb1* by THP-1 macrophage. (C) Phagocytosis of A909 WT, A909Δ*lep*, A909Δ*spb1* and the complemented strains A909Δ*lep* (*lep*) and A909Δ*spb1* (*spb1*). Results are means ± SD from at least three independent cultures in triplicate. Asterisks represent statistical differences relative to WT strain BM110 with $^{**}p \le 0.01$; $^{***}p \le 0.001$ as assessed by using two-way ANOVA with Bonferroni's post-test in GraphPad Prism version 5.

We similarly analyzed the role of *spb1* in deletion mutants in A909 background, where the decrease in phagocytosis of A909Δ*spb1* and A909Δ*lep* as compared to A909 wild-type was statistically significant (Fig. 3C). Furthermore, complementation of A909Δ*spb1* mutant with a plasmid overexpressing *spb1* gene restored phagocytosis to WT level. Similarly, complementation of A909Δ*lep* with a plasmid overexpressing *lep* restored phagocytosis almost to WT level, whereas overexpression of *spb1* in Δ*lep* mutant did not (Fig. 3C). Of note, Δ*lep* cells overexpressing *spb1* display Spb1-containing polymers but not Ap1-containing HMW polymers.

These results indicate that Lep plays an important role in the biosynthesis of functional PI-2b polymers. Taken together, these results indicate that the integrity of PI-2b pilus polymers is required for efficient GBS uptake by THP-1 macrophages.

3. Conclusions

Bioinformatic and genetic evidences indicate that Lep is likely a type I signal peptidase necessary for the efficient maturation of Spb1 pilin and for the incorporation of Ap1 minor pilin to Spb1-polymers. We also showed that enhanced pilus expression results in increased uptake by THP-1 macrophages. The contribution of PI-2b to the virulence of ST-17 strains has been recently evaluated in a murine model of hematogenous meningitis [19]. The authors demonstrated that the PI-2b pilus mediates adhesion and invasion of pulmonary epithelial and brain endothelial cells and contributes to the translocation across the blood–brain barrier. We thus speculate that fine-tuning of PI-2b expression *in vivo* will be key to optimize adherence to host tissues while mitigating host immune responses.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2018.10.004.

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