The GBS PI-2a Pilus Is Required for Virulence in Mice Neonates

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Abstract

Background: *Streptococcus agalactiae* (Group B Streptococcus) is a leading cause of sepsis and meningitis in newborns. Most bacterial pathogens, including gram-positive bacteria, have long filamentous structures known as pili extending from their surface. Although pili are described as adhesive organelles, they have been also implicated in many other functions including thwarting the host immune responses. We previously characterized the pilus-encoding operon PI-2a (*gbs1479-1474*) in strain NEM316. This pilus is composed of three structural subunit proteins: PilA (*Gbs1478*), PilB (*Gbs1477*), and PilC (*Gbs1474*), and its assembly involves two class C sortases (SrtC3 and SrtC4). PilB, the *bona fide* pilin, is the major component whereas PilA, the pilus associated adhesin, and PilC the pilus anchor are both accessory proteins incorporated into the pilus backbone.

Methodology/Principal Findings: In this study, the role of the major pilin subunit PilB was tested in systemic virulence using 6-weeks old and newborn mice. Notably, the non-piliated *ΔpilB* mutant was less virulent than its wild-type counterpart in the newborn mice model. Next, we investigated the possible role(s) of PilB in resistance to innate immune host defenses, i.e. resistance to macrophage killing and to antimicrobial peptides. Phagocytosis and survival of wild-type NEM316 and its isogenic *ΔpilB* mutant in immortalized RAW 264.7 murine macrophages were not significantly different whereas the isogenic *ΔsodA* mutant was more susceptible to killing. These results were confirmed using primary peritoneal macrophages. We also tested the activities of five cationic antimicrobial peptides (AMP-1D, LL-37, colistin, polymyxin B, and mCRAMP) and found no significant difference between WT and *ΔpilB* strains whereas the isogenic *dltA* mutant showed increased sensitivity.

Conclusions/Significance: These results question the previously described role of PilB pilus in resistance to the host immune defenses. Interestingly, PilB was found to be important for virulence in the neonatal context.

Introduction

*Streptococcus agalactiae* (also referred to as Group B Streptococcus, GBS) is a gram-positive encapsulated bacterium responsible for life-threatening infections in newborns, elderly, and adults with underlying diseases [1,2]. Two distinct clinical syndromes, early-onset disease (EOD) or late-onset disease (LOD) have been described in neonates and young infants [3]. For EOD, the main route of infection is assumed to be a vertical transmission from the maternal vaginal or amniotic fluid, resulting in subsequent systemic infection after translocation across the respiratory epithelium. For LOD, the mode of transmission and the infection route still remains unclear. Once into the bloodstream, *S. agalactiae* can cause septicemia and then cross the blood-brain barrier to cause meningitis.

Bacterial pili have recently been recognized in major human pathogens such as *S. agalactiae*, *Streptococcus pyogenes* (GAS), and *Streptococcus pneumoniae* (for reviews see [4,5,6,7,8,9]). Sortase-mediated pilus assembly was first demonstrated in *Corynebacterium diphtheriae* [10,11] and these pioneer studies revealed the existence of three conserved motifs within the major pilin subunit that are necessary for pilus formation: i) the pilin motif (WxxxVxVYPK); ii) the E-box domain (YxLxETxAPxGY); and iii) the cell wall sorting signal (LPxTG followed by a hydrophobic domain and a positively charged tail). The current model for pilus assembly is as follows: the major subunit is assembled to form a pilus by a signal (LPxTG followed by a hydrophobic domain and a positively charged tail). The current model for pilus assembly is as follows: the major subunit is assembled to form a pilus by a covalent attachment of the conserved threonyl residue of the pilin motif of one subunit with the conserved lysyl residue of the LPxTG motif of another subunit. In addition, one or more accessory subunits are incorporated into the
pilus by an as yet unknown mechanism that requires the pilus-specific sortase. The crystallographic structures of two major pilins have now shown that the E-box domain is involved in the formation of intramolecular isopeptide bond conferring higher stability to the pilin monomer [12,13]. Then, during the final step, the pilus fiber is covalently linked to the peptidoglycan by either the pilus-specific or the housekeeping sortase. This mechanism of pilus assembly catalyzed by class C sortases has now been characterized in several gram-positive pathogens using similar genetic and biochemical analyses [14,15,16,17,18,19,20,21,22].

Three genomic loci (PlI-1, PlI-2a, and PlI-2b) have been described in GBS strains [23], the latter two being mutually exclusive as they are located at the same chromosomal location. In a survey of 289 GBS clinical isolates, PI-1, PI-2a, and PI-2b were detected in 72%, 75%, and 27% of the strains, the combination of PI-1 + PI-2a being the most frequent [24]. We and others previously carried out a detailed structural and functional analysis of the pilus locus PlI-2a (gbs1479–1474) in GBS strain NEM316 [16,23]. This locus encodes the three structural pilus subunits PilA (Gbs1478), PilB (Gbs1477), and PilC (Gbs1474) whose assembly involves two class C sortases (SrtC3 and SrtC4). PilB, the bona fide pilin, is the major component; PilC is a minor associated component mainly localized at the base of the pilus [25]; and PilA is the adhesin located at intervals along the pilus backbone [16]. The PlI-2a GBS pili have also been implicated in mediating attachment to human epithelial cells [16,26,27], in biofilm formation [26,28], in the adhesion and invasion of brain microvascular endothelial cells [29], and in promoting transepithelial migration [30].

Intriguingly, the pilin subunit PilB of PI-2a was also reported to mediate resistance to cathelicidin antimicrobial peptide and phagocyte killing, to increase bloodstream survival, and to confer virulence in a mouse challenge model [31].

Here, we re-investigate the contribution of PilB in the virulence of strain NEM316 using two different mice models and in resistance to innate host immune defenses by testing GBS survival to killing by macrophages or antimicrobial peptides.

**Results**

**PilB mutant is attenuated for virulence in a neonatal mouse infection model**

To investigate the role of the pilus in invasive disease, we made use of the previously described in-frame deletion mutant of gbs1477, encoding the backbone protein PilB in GBS strain NEM316 [16] in combination with the heterologous expression of PilB under the constitutive lactococcal p23 promoter in the non-pathogenic host Lactococcus lactis strain NZ9000. As shown by Western blotting using anti-PilB polyclonal antibody, expression of pilB in L. lactis strain NZ9000 was associated with the presence in the cell wall extracts of a band of 75 kDa corresponding to PilB monomer that was missing in the control strain L. lactis harboring the cloning vector without DNA insert (Fig. 1). As previously shown [16], PilB appears mainly as a polymer in GBS strain NEM316 (Fig. 1) whereas PilB monomers are directly anchored to the cell wall in L. lactis.

S. agalactiae and lactococcal strains were then tested in parallel for 6-weeks old C57BL/6 mice were challenged intravenously with two doses (10⁴ or 5×10⁵ CFU) of GBS NEM316 and ApilB mutant, and with a higher dose (5×10⁶ CFU) of L. lactis strains NZ9000 expressing or not pilB, and blood was collected 24 h later to enumerate viable bacterial CFUs. As shown in Fig. 2A and 2B, no significant differences were found between strains expressing or not pilB in these conditions. It is worth noting that the inter-animal variability is quite important in these experiments.

We then tested the role of PilB in a neonatal sepsis model. BALB/c mice (≤24 h-old) were infected subcutaneously with low (17 to 30 CFU) and high (30 to 60 CFU) GBS challenge inoculum (Fig. 2C and 2D, respectively). At low dose, only 60% of the mice infected with 17 CFU of WT strain survived whereas 100% of mice infected with 30 CFU of ApilB mutant survived (Fig 2C). More strikingly, only 40% of the mice infected with 30 CFU of WT strain survived whereas all the mice survived when infected with 60 CFU of ApilB mutant (Fig. 2D). Thus in both experiments, we consistently observed that a higher number of mice survived when challenged with ApilB mutant as compared to WT strain after 24 h post-infection demonstrating the importance of PilB pili of S. agalactiae NEM316 in the neonatal context.

**PilB does not promote bacterial survival in murine macrophages**

The survival of GBS strain NEM316 and its isogenic non-piliated mutant ApilB was compared in the widely used murine macrophage cell line RAW 264.7. We also included as control the NEM316ΔsodA mutant which was previously shown to display increased susceptibility to bacterial killing by macrophages [32]. As shown in Fig. 3A, the wild-type strain NEM316 (WT) and the ApilB mutant displayed a similar survival kinetic in this phagocytic cell line. In contrast, survival of the isogenic ΔsodA mutant was significantly reduced compared to the wild-type strain.

Since phagocytic cell lines are considered to be less harmful for bacteria than primary macrophages, we performed similar
experiments using thioglycolate-elicitated murine peritoneal macrophages. *S. agalactiae* and lactococcal strains were tested in parallel for survival in murine peritoneal macrophages (Fig. 3B). Whereas piliated *S. agalactiae* NEM316 WT and non-piliated *ΔpilB* mutant survived similarly, a dramatic phagocytic killing of both lactococcal strains was observed (20-fold decreased as compared to GBS strains). No gain of function was noticed for the *L. lactis* strain expressing *pilB* when compared to the *L. lactis* strain expressing the vector alone.

Altogether these results indicate that NEM316 PilB is not involved in resistance to bacterial killing by macrophages.

PilB does not confer resistance to antimicrobial peptides

It has been shown by others that GBS NCTC10/V4 *ΔpilB* exhibited enhanced susceptibility to various cationic antimicrobial peptides CAMPs (mCRAMP, polymyxin B, and LL-37). We thus tested the activities of four cationic molecules having similar net charges such as AMP-1D (+6), LL-37 (+6), colistin (+5) and polymyxin B (+5) by determining the minimal inhibitory concentration required to inhibit the growth of 90% of the bacteria (MIC<sub>90</sub>). As shown in Table 1, no difference was found between GBS WT and the isogenic *ΔpilB* mutant. In contrast, the *ΔdltA* mutant showed increased sensitivity to these CAMPs, as shown previously [33]. As shown in Table 1, no difference was found between GBS WT and the isogenic *ΔpilB* mutant. In contrast, the *ΔdltA* mutant showed increased sensitivity to these CAMPs, as shown previously [33]. For consistency with our animal models, we also tested the effect of various concentrations of the murine cathelicidin mCRAMP (+6) on the growth curve of WT, *ΔpilB*, and *ΔdltA* in TH medium (Fig. 4). Again, no significant difference was found between WT and the *ΔpilB* mutant. Similar results were obtained using this experimental condition for the three other tested CAMPs: colistine, AMP-1D, and polymyxin B (data not shown). We also observed that expression of *pilB* in *L. lactis* NZ9000 did not modify the MIC<sub>90</sub> towards these antibiotics (Table 1), nor the bacterial growth (Fig. S1). Collectively, our results do not support a role for the pilus in resistance to cationic antimicrobial peptides.

**Discussion**

The goal of the present work was to evaluate the contribution of the pilin subunit PilB of the GBS pilus-encoding operon PI-2a to bacterial virulence. This was done either by deleting the corresponding gene *pilB* in the WT serotype III strain NEM316 or by expressing it in the food grade bacterium *L. lactis* NZ9000. In a 6 weeks old CD1 septicemic mouse model, we observed that PilB was dispensable for bacterial virulence in both genetic backgrounds. These results conflict with those of Maisey et al. (2008) who reported that PilB of GBS NCTC10/V4, a highly hemolytic serotype V strain [34], conferred virulence to the parental GBS strain and to *L. lactis*, as assessed in a similar animal model. Moreover, over-expression of the *pilB* gene alone in the non-pathogenic *L. lactis* was found to enhance resistance to phagocyte killing, increased bloodstream survival, and conferred virulence in a mouse model [31]. The latter observation was intriguing as it suggested that PilB is an essential GBS virulence factor, being sufficient to turn the unencapsulated and non-pathogenic bacterium *L. lactis* into an invasive extracellular bacteria.

The molecular basis of PilB-associated virulence is thought to reside in its ability to confer resistance to CAMP and phagocytosis,
and in consequence to bloodstream survival. To exert antimicrobial activity, CAMPs must bind to the bacterial surface, whether they act by the inhibition of biosynthetic processes on the bacterial surface, pore formation in the cytoplasmic membrane, or yet other mechanisms. The bacterial surface is negatively charged owing to the production of anionic polymers. Moreover, the outer and inner leaflets of the bacterial cytoplasmic membrane are also negatively charged. In Gram-positive bacteria, resistance to CAMPs is mainly due to an increase of the positive surface charge through increase in D-alanylation of the LTAs or incorporation of L-lysine into membrane phosphatidylglycerol, more rarely to specific proteolytic degradation [35]. PilB from GBS strains NEM316 and NCTC10/84 display 84.5% of sequence identity but the pilin subunit from NEM316 is positively charged (+2; pKi 8.44) at neutral pH whereas that from NCTC10/84 is slightly electronegative (−2; pKi 6.57). These observations, combined with the fact that GBS pili are not evenly distributed along the bacterial surface, strongly argue against their involvement in CAMP resistance by electrostatic repulsion and it is worth noting that neither proteins contain any known proteolytic

| S. agalactiae | L. lactis |
|--------------|----------|
| AMP-1D       | 8        | 8        |
| LL-37        | >64      | >64      |
| Colistin     | >256     | >256     |
| Polymyxin B  | 128      | 128      |

Values are expressed in µg/ml.

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Figure 3. Bacterial survival in cultured and primary murine macrophages. A- Immortalized RAW264.7 were cultured in vitro and exposed to S. agalactiae NEM316 (WT), the non-piliated mutant (ΔpilB), and the sodA mutant (NEM1640) at an m.o.i. of 10 bacteria per cell in 24-wells plate. Error bars represent the standard deviation of three independent experiments done in duplicate for each strain studied. B- Survival of S. agalactiae and L. lactis strains in thioglycolate-elicited primary murine peritoneal macrophages. Bacteria in exponential phase (OD600 of about 0.6) were added to macrophages (m.o.i. of 10) for 30 min and then survival was measured after 2 h30 at 37°C. Error bars represent the standard deviation of a representative experiment done in triplicate for each strain studied.

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Role of PilB in GBS Virulence

Materials and Methods

Ethics statement

All of the animal experiments described in the present study were conducted at the Mectchnikoff Department of the University of Messina according to the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.html) and were approved by the relevant national authority (Istituto Superiore di Sanita’ of Italy).

Bacterial strains, media, and growth conditions

Streptococcus agalactiae NEM316 was responsible for a fatal septicemia and belongs to the capsular serotype III. The complete genome sequence of this strain has been determined [38]. S. agalactiae were grown in Todd-Hewitt (TH) medium (Difco-BD). Lactococcus lactis strain NZ9000 [39] was grown in M17 medium supplemented with 1% glucose (GM17). For antibiotic selection in L. lactis, 5 µg/ml erythromycin was added to GM17. Heterologous expression of pilB in L. lactis strain was realized as follows: The full-length pilB gene was amplified from genomic DNA of strain NEM316 and subcloned in the laccoccovector pIL253::P23 [40], a high copy number erythromycin resistance plasmid expressing the cloned gene from the strong constitutive promoter P23 [41]. The primers used for pilB amplification were: 1477a (5’-ATG GCC CCA TGA AAT ATA CTG CAG CCT - 3’) and 1477b (5’- ATA CTG CAG CAG AAA TAA TGG CTC TTG ATG ATG - 3’). The 2.1-kb PCR product was then cut by Apal and PstI (New England Biolabs), purified, and cloned into pIL253::P23 cut with the same enzymes resulting in pIL253::P23pilBl477 (pVES5616). This ligation mixture was transformed to electrocompetent L. lactis IL1403. After verification by DNA sequencing, pVES5616 was transferred into L. lactis NZ9000.

General DNA techniques

Standard recombinant techniques were used for nucleic acid cloning and restriction analysis [42]. Plasmid DNA from E. coli was prepared by rapid alkaline lysis using the Nucleospin Plasmid kit (Macherey-Nagel). Genomic DNA from S. agalactiae was prepared using the DNeasy Blood and Tissue kit (Qiagen). PCR was carried out with the High-Fidelity Phusion DNA polymerase as recommended by the manufacturer (Finnzymes).

Immunoblotting

Cell wall protein extracts were prepared by harvesting 50 ml of bacteria in early stationary phase (OD600 = 1). The bacterial pellet was washed once in Tris-HCl buffer (50 mM, pH 7.3) and once in the mutanolysin digestion buffer (Tris-HCl 50 mM pH 7.3 supplemented with 20% sucrose and a complete protease inhibitor cocktail (Roche®)). Mutanolysin (Sigma) dissolved to 5,000 U ml\(^{-1}\) in potassium phosphate buffer (pH 6.2) was then added to the bacterial suspension to give a final concentration of 100 U ml\(^{-1}\) and samples were rotated for 2 h at 37°C. After centrifuging at 13,000 g for 15 min at 4°C, supernatants corresponding to the cell wall extracts were analyzed on SDS-PAGE or kept frozen at −20°C. For western blotting, proteins were boiled in Laemmli...
sample buffer, resolved on Tris-Acetate Criterion XT gradient gels 4–12% SDS-PAGE gels (BioRad) and transferred to nitrocellulose membrane (Hybond-C, Amersham). PilB was detected using polyclonal antibodies and horseradish peroxidase (HRP)-coupled anti-rabbit secondary antibodies (Zymed) and the Western pico chemiluminescence kit (Thermo scientific).

**Adult mouse infection model**

Two groups of male CD-1 mice (6- weeks old) were injected intravenously (i.v.) via the tail vein with the indicated amounts of early logarithmic-phase of GBS or L. lactis strains. Mice were monitored daily for survival. Bacteremia was assessed at 24 h by blood collection and enumeration of CFUs on TH or M17 agar plates.

**Neonatal mouse model of GBS sepsis**

Neonatal BALB/c mice were used for virulence studies. Randomized groups of 7 to 10 mice pups were inoculated subcutaneously with a dilution of mild-log-phase strain NEM316 or ApilB (0.03 ml of each strain in 0.9% NaCl). Under these conditions, from 17 to 60 CFU/mouse, GBS are contained at the inoculation site or spread systematically, depending on the bacterial intrinsic virulence factor and the ability of the host immune system to prevent bacterial growth. Mice were observed daily for up to 8 days post-infection. In this model, deaths are rarely observed after Day 5.

**Assay for GBS intracellular survival in macrophages**

Cells were infected at a multiplicity of infection (m.o.i) of 10 bacteria per cell for 1 h at 37°C in 10% of CO2. The monolayers were then washed four times with PBS and fresh medium containing gentamicin (100 µg/ml) was added to kill extracellular bacteria (time zero of the assay). To quantify intracellular GBS at different times post-infection, the supernatants were removed and the cells were disrupted by the addition of 1 ml sterile deionized ice-cold water and repeated pipetting. Serial dilutions of the lysate were plated on TH agar for counting of viable bacteria.

**Determination of the Minimal Inhibitory Concentration (MIC)**

The MICs (µg/ml) of *S. agalactiae* towards antimicrobial peptides (AMP-1D, LL-37, colistin, and polymyxin B) were performed by a dilution method in 96 wells polypropylene microplates (Costar, Cambridge, MA) containing Todd-Hewitt (TH) broth buffered with 100 mM HEPES using two biological replicates in duplicate. Polymyxin B and colistin were purchased from Sigma whereas LL-37 and AMP-1D were synthesized in the Department of Biological Chemistry (Rehovot, Israel). Bacteria (approx. 10^5 CFU) were added to wells containing increasing concentrations of the antimicrobial peptides. Plates were incubated overnight at 37°C and were then read at OD_{600 nm} with a microplate reader BioTek Synergy for bacterial growth. The MIC_{90} was considered and expressed as the lowest peptide concentration inhibiting growth of 90% of the bacterial cells. The same experimental procedure was used for *L. lactis* except that bacteria were grown at 30°C in M17 broth supplemented with glucose (1%) and erythromycin (5 µg/ml).

**Growth curves in the presence of mCRAMP**

Overnight cultures of GBS in TH broth buffered with HEPES (100 mM) were diluted in fresh media to give approximately 10^7 cfu/ml and 150 µl were distributed in 96 wells plate without (control wells) or with mCRAMP at selected concentrations (test wells). The microplate was incubated at 37°C with constant shaking in the BioTek Synergy plate reader and the OD_{600 nm} was recorded every 20 min for 12 h. The same experimental procedure was used for *L. lactis* except that bacteria were grown at 30°C in M17 broth supplemented with glucose (1%) and erythromycin (5 µg/ml). mCRAMP was purchased from TEBU.

**Supporting Information**

**Figure S1 Effect of the murine cathelicidin mCRAMP on bacterial growth.** Overnight culture of the control strain *L. lactis* NZ9000/vec (A) or the *L. lactis* NZ9000/vecPilB strain expressing the GBS PilB protein (B) were diluted in M17 broth supplemented with glucose (1%) and erythromycin (5 µg/ml) to give approximately 10^7 cfu/ml. The inoculated broths were distributed (150 µl) in 96 wells plate incubated at 30°C with constant shaking in a plate reader and the OD_{600 nm} was recorded every 20 minutes for 12 hours. Blank values (M17-glucose-erythromycin) were subtracted from experimental values to eliminate background readings. ▲, M17-glucose-erythromycin medium without peptide (sterile water was added instead); ■, presence of the mCRAMP drug at 5 (light blue), 10 (violet), 20 (green), and 40 (red) µg/ml, respectively. These results are representative of three independent experiments. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: PT-C SD. Performed the experiments: SP SB M-YM AF SD. Analyzed the data: SP GT PT-C SD. Contributed reagents/materials/analysis tools: RD YS VO. Wrote the paper: PT-C SD.

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