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Abstract

Streptococcus agalactiae (Group B *Streptococcus* or GBS) is a leading cause of invasive infections in neonates whose virulence is dependent on its ability to interact with cells and host components. We here characterized a surface protein with a critical function in GBS pathophysiology. This adhesin, designated PbsP, possesses two Streptococcal Surface Repeat (SSURE) domains, a methionine and lysine-rich region, and a LPXTG cell wall-anchoring motif. PbsP mediates plasminogen (Plg) binding both *in vitro* and *in vivo* and we showed that cell surface-bound Plg can be activated into plasmin by tissue plasminogen activator to increase the bacterial extracellular proteolytic activity. Absence of PbsP results in a decreased bacterial transmigration across brain endothelial cells and impaired virulence in a murine model of infection. PbsP is conserved among the main GBS lineages and is a major plasminogen adhesin in non-CC17 GBS strains. Importantly, immunization of mice with recombinant PbsP confers protective immunity. Our results indicate that GBS have evolved different strategies to recruit Plg which indicates that the ability to acquire cell surface proteolytic activity is essential for the invasiveness of this bacterium.

Introduction

Adhesion of bacterial pathogens to cells and extracellular macromolecules is an essential process in the long-term relationship between microbes and their hosts (Kline *et al.*, 2009, Nobbs *et al.*, 2009). In particular, many microorganisms are able to bind host plasminogen (Plg) on their surface where its activation is often controlled by host factors (Lahteenmaki *et al.*, 2001, Bergmann & Hammerschmidt, 2007, Fulde *et al.*, 2013). Plg is an inactive proenzyme (a zymogen) abundant in the plasma that is activated by proteolysis, a reaction usually catalyzed by the host plasminogen activators, tissue-type (tPA) or urokinase-type (uPA), to produce the active serine protease plasmin (Pln). The Plg/Pln fibrinolytic system controls the degradation of fibrin clots and is important for wound repair and extracellular matrix turnover. By coopting the host Plg/Pln system on their surface, bacteria can increase their adhesive properties and proteolytic activities to promote colonization or invasion of host tissues and evasion from the host immune responses (Lahteenmaki *et al.*, 2001, Bergmann & Hammerschmidt, 2007, Fulde *et al.*, 2013, Foley *et al.*, 2015). Bacteria have evolved independent systems to exploit the host Plg/Pln system at their advantages. Direct binding of host Plg/Pln is mediated by specific surface adhesins or conserved ubiquitous moonlighting proteins (enolase, GAPDH) whereas indirect binding is usually mediated by fibrinogen receptors enabling the formation of fibrinogen-plasminogen complex. Despite this common overlapping function, these receptors could be specifically expressed at different steps of the colonization or invasion processes and thus can be individually and sequentially essential for pathogenicity at specific stages of disease development.

Group B *Streptococcus* (*S. agalactiae* or GBS) is an important cause of invasive infections, including sepsis and meningitis, in neonates and elderly (Le Doare & Heath, 2013, Edmond *et al.*, 2012, Skoff *et al.*, 2009). This extracellular pathogen relies on specific adhesins for harmless colonization and/or threatening invasion. Many GBS adhesins characterized to date

are anchored to the cell wall by a sortase A-dependent mechanism (Nobbs *et al.*, 2009, Mazmanian *et al.*, 1999) and interact with specific host cells or macromolecules during colonization or invasion (Six *et al.*, 2015, Buscetta *et al.*, 2014, Jiang & Wessels, 2014, Wang *et al.*, 2014, Mu *et al.*, 2014, Papasergi *et al.*, 2011, Tazi *et al.*, 2010). During infections, GBS is able to bind host Plg that is activated to Pln by tPA (or uPA) (Magalhaes *et al.*, 2007). Importantly, GBS secrete a protein named Skizzle that increases Plg activation by host factors (Wiles *et al.*, 2010). The resulting surface-bound protease activity promotes disruption of host barriers thereby contributing to bacterial dissemination and invasiveness (Magalhaes *et al.*, 2013, Six *et al.*, 2015). Plg and Pln binding at the GBS surface is mediated by moonlighting proteins, such as α-enolase, glyceraldehyde dehydrogenase and phosphoglycerate kinase, which re-associate with the cell surface following bacterial lysis (Magalhaes *et al.*, 2007, Oliveira *et al.*, 2012, Boone & Tyrrell, 2012). Depending on their phylogenetic lineage, GBS strains also express unrelated fibrinogen receptors (FbsA, FbsB, or FbsC) that catalyse indirect Plg binding (Pietrocola *et al.*, 2005, Gutekunst *et al.*, 2004, Buscetta *et al.*, 2014). In addition, GBS strains belonging to the hypervirulent CC17 clonal complex express a specific multifaceted adhesin, the serine rich repeat Srr2 glycoprotein, with a dual Plg and Fbg binding function (Six *et al.*, 2015, Brochet *et al.*, 2006, Da Cunha *et al.*, 2014).

The pneumococcal protein PfbB (also called PavB) contains a variable number of repetitive sequences (Papasergi *et al.*, 2010, Jensch *et al.*, 2010), referred to as Streptococcal Surface Repeats (SSURE), initially described as a fibronectin (Fnt) binding motif (Bumbaca *et al.*, 2004). However, we showed that this surface protein is a dual Fnt and Plg adhesin (Papasergi *et al.*, 2010). Recently, we identified the GBS SSURE domain-containing protein Gbs0428 by a proteomic analysis of secreted and surface proteins (Papasergi *et al.*, 2013). In this study, we demonstrated that Gbs0428 is a cell wall-anchored surface protein that binds Plg and therefore renamed it PbsP for Plasminogen binding surface Protein. We characterized PbsP as

the major Plg binding protein expressed by our reference strain NEM316 (Glaser *et al.*, 2002) and showed that it is expressed by isolates belonging to the main GBS lineages. In addition, we demonstrate that PbsP is necessary for hematogenous GBS dissemination during invasive diseases and that this conserved adhesin induces immune protection.

Results

PbsP is a GBS cell wall-anchored surface protein

The locus *gbs0428* in the genome of GBS NEM316, a capsular serotype III (CPS III) isolate belonging to the clonal complex 23 (CC23), encodes the SSURE domain-containing protein PbsP (Gbs0428) (Glaser *et al.*, 2002). This 521-aa protein contains an NH2-signal peptide bearing a YSIRK sorting motif (Carlsson *et al.*, 2006, Brega *et al.*, 2013), two 150-aa SSURE domains (Bumbaca *et al.*, 2004) displaying 77% identity, a methionine and lysine-rich (MK-rich) region, and a COOH cell wall-anchoring LPXTG motif (Fig. 1A and B). All sequenced GBS human clinical isolates express a conserved PbsP protein (99.3% of identity) containing two SSURE domains (Da Cunha *et al.*, 2014).

The gene encoding PbsP was deleted in-frame in the NEM316 chromosome. The viability, morphology, and growth in Todd-Hewitt broth of the Δ*pbsP* mutant were similar to those of the WT parental strain (Fig. S1). Western-blot analysis of GBS cell wall extracts using a polyclonal mouse serum (pAb) against a recombinant PbsP revealed an hybridizing band present in the WT strain that was absent in the Δ*pbsP* and *srtA** mutant derivatives (Fig. 2A). The 150 kDa band shown in Fig. 2A is apparently the result of a non-specific reaction, since the band was observed in all GBS cell wall extracts examined and also when using an irrelevant antiserum (anti-GST) instead of anti-PbsP serum (data not shown). Taken together, these results confirm that PbsP is anchored to the cell wall by SrtA (Konto-Ghiorghi *et al.*, 2009, Mazmanian *et al.*, 1999). The apparent molecular weight of this protein is about 40 kDa, a value slightly lower than the predicted 48 kDa of the mature protein. This difference may indicate that PbsP was further processed upon secretion or during its extraction. As expected from previous transcriptomic and proteomic studies (Lamy *et al.*, 2004, Firon *et al.*, 2013, Papasergi *et al.*, 2013), expression analysis reveals that PbsP is overexpressed in the Δ*covRS* mutant (Fig. 2A).

Expression of PbsP at the GBS surface was further analyzed by FACS with the same anti-PbsP serum. PbsP was detected on the surface of the WT NEM316 strain, but not of the Δ*pbsP* or *srtA** mutants, and was highly expressed on the surface of the Δ*covRS* mutant (Fig. 2B). PbsP expression was also detected, although at different levels, on the surface of other WT GBS strains of different serotypes and clonal complexes (Fig. 2B). The surface display of this protein in GBS NEM316, 6313 (CPS III and CC23 as NEM316), and A909 (CPS Ia, CC1 strain) was similar whereas a lower signal intensity was detected with BM110 and COH1 (both CPS III, CC17) and 2603V/R (CPS V, CC19). Of note, no major difference was observed between the *pbsP* genes (>99.5% sequence identity) and corresponding promoter regions and translation signal sequences of these strains (100% sequence identity) (data not shown). On the other hand, we observed that non-capsulated NEM316 gave a similar signal as the WT strain, suggesting that the polysaccharidic capsule did not significantly mask the display of the protein at the bacterial surface (data not shown).

PbsP is a plasminogen binding protein

The PbsP adhesin function(s) were analyzed by ELISA with a purified rPbsP fusion protein against extracellular matrix or blood components including Plg, Fng, Fnt, collagen, C-reactive protein and the complement components factor-H, factor I, factor-B, C1q and C3. This analysis revealed that Plg is the main ligand of PbsP, although low binding levels were observed with other tested components including Fng (Fig. 3A). In the tested conditions, PbsP specifically bound immobilized Plg in a dose-dependent and saturable manner (Fig. 3B). Binding of PbsP to Plg was confirmed by Far Western blotting experiments in which the recombinant protein was used as either bait or prey (Fig 3C). A dissociation constant (Kd) of 617 ± 34 nM was measured by surface plasmon resonance using immobilized rPbsP on the sensor chip and different concentrations of soluble Plg (Fig. 3D).

Binding to fibrin, ECM components and several bacterial proteins is mediated by lysine-

binding motifs in the kringle domains of Plg. To determine whether these lysine binding sites could be involved in interactions with PbsP, increasing concentrations of lysine or lysine analog ε-aminocaproic acid (6-ACA) were included in the ELISA assay. As a negative control, we used L-alanine, which is a non-charged amino acid. PbsP-Plg interactions were inhibited in a dose-dependent manner in the presence of 6-ACA or L-lysine, but not L-alanine, suggesting an involvement of the lysine-binding sites in Plg (Fig. 3E). Lysyl residues are abundant in the SSURE-1, SSURE-2 and MK-rich domains of PbsP, accounting for 11, 14 and 18% of the total residues of the protein, respectively. To determine which domain is involved in Plg binding, we purified GST-tagged recombinant proteins corresponding to full length PbsP, SSURE domains 1 and 2, SSURE domain 2, and the MK-rich domain. ELISA assays showed that all four proteins bind immobilized Plg in a saturable and dose-dependent manner (Fig. 3F). However, the MK-rich domain displayed higher binding to Plg as compared to the SSURE domains (Fig. 3F), in agreement with its higher lysyl content. These data suggest that the ability of PbsP to bind Plg likely reflects a cooperative activity of the SSURE and MK-rich domains.

As opposed to Group A streptococci, GBS does not express a Plg activator and surface bound Plg needs to be activated by host factors (Magalhaes *et al.*, 2007). We therefore tested the ability of Plg bound to PspP to cleave a Pln-specific chromogenic substrate with or without tPA. As expected, the chromogenic substrate S-2251 was cleaved only in the presence of tPA indicating that PspB-bound Plg could be efficiently converted to Pln (Fig. 4A). We next determined if PbsP-bound Pln can degrade its natural substrate Fng following activation by tPA. SDS-PAGE analysis revealed that the alpha and beta chains were almost entirely degraded following 2 and 4 hr of incubation, respectively, while the gamma chain was resistant to cleavage over a 4 hr time period. No detectable degradation of Fng was detected when either Plg or tPA was omitted (Fig. 4B). Collectively these data indicate that PbsP binds

Plg by engaging lysine binding sites on the zymogen molecule and that PbsP-bound Plg can be activated to Pln in the presence of exogenous activators, such as tPA, to degrade ECM components.

PbsP contributes to Plg binding on GBS cell surface

Plg is abundantly present in the host, particularly in the blood, where it reaches concentrations in µM range. We first confirmed recent observations (Magalhaes *et al.*, 2013) that soluble Plg binds on the surface of GBS WT, as revealed with anti-Plg antibodies and FITC-conjugated secondary antibodies (Fig. 5A). Under these conditions, Plg binding on the Δ*pbsP* mutant surface was reduced (2-fold) compared to the WT strain, an effect that was abrogated by genetic complementation of the mutant strain (Fig 5A). We next assessed the contribution of PbsP-mediated Plg binding to acquisition of a cell surface proteolytic activity using the Pln-specific chromogenic substrate. Pln activity was only detected in the presence of tPA and paralleled the level of Plg binding to the bacterial surface (Fig 5A and B). Specifically, plasmin activity was markedly reduced in the absence of PbsP and restored to WT levels by genetic complementation (Fig. 5B).

PbsP is required for adherence to and transmigration across brain endothelial cells

GBS invades brain endothelial cells and causes infection of the central nervous system, a process involving GBS interaction with host extracellular matrix components. We used the brain endothelial cell line hCMEC/D3 as an established model to test GBS adhesion and transmigration (Magalhaes *et al.*, 2013). As compared to the parental NEM316 strain, the Δ*pbsP* mutant was significantly impaired in its ability to adhere to these cells whereas the WT levels of adhesion were restored in the complemented strain (Fig. 6A). However, the levels of adhesion of the Δ*pbsP* mutant were slightly higher than those observed with a *srtA** mutant, which might suggest that other LPXTG adhesins participate to these processes. The levels of

invasion of the *ΔpbsP* mutant were also decreased, as compared to the parental NEM316 strain (Fig. 6B). However this effect was likely secondary to the impaired ability of the mutant strain to adhere to hCMED/D3 cells, since the relative invasion rate (defined as the ratio between invading and adhering bacteria) was similar in the parental strain and the *ΔpbsP* mutant (data not shown).

Plg binding at the surface of GBS was demonstrated to be important for bacterial migration across endothelial barriers after tPA-mediated activation into Pln (Magalhaes *et al.*, 2013). We first confirmed, using a transwell assay, that the ability of NEM316 to migrate through hCMEC/D3 monolayers was greatly enhanced following incubation with Plg and tPA, but was inhibited by the addition of the lysine analogue 6-ACA (Fig. 6C). Remarkably, crossing of the endothelial barrier by the Plg-coated Δ*pbsP* mutant in presence of tPA was almost 2-fold less efficient compared to the WT strain, whereas the complemented mutant displayed a WT phenotype (Fig. 6C).

Immunoprotective PbsP contributes to hematogenous colonization of target organs

To investigate the role of PbsP in the pathogenesis of invasive GBS infection, we intravenously infected CD-1 mice (n = 16 per GBS strain) with 4 x 10^8 CFUs of the WT and the Δ*pbsP* mutant. The mutant strain was less virulent than the WT NEM316 ($p < 0.05$ by log-rank Kaplan-Meyer) as, by nine days post-infection, only 12% of mice infected with the WT strain survived compared to 50% of those infected with Δ*pbsP* (Fig. 7A).

To outline the role of PbsP in GBS virulence, we measured the bacterial load in the blood, kidneys, and brains at early time points, i.e. 48 h post-infection. Similar numbers of WT and Δ*pbsP* mutant bacteria were found in the blood, indicating that PbsP did not play a critical role in the maintenance of bacteriemia (Fig. 7B). In contrast, the absence of PbsP was associated with lower bacterial loads ($p < 0.05$ by Mann-Whitney U test) in the kidneys and brain compared to the WT (Fig. 7C). These data indicate that PbsP plays an important role in

the pathogenesis of invasive GBS disease by favoring systemic spreading of bacteria from the blood to other organs, in particular the brain.

Being critical for GBS virulence and cell surface exposed, we next investigated if PbsP constituted a good candidate to induce active immunization. Adult mice (n = 10 per experiment) were immunized with rPbsP or GST (used as a negative control) and challenged intravenously with 2 x 10^8 CFU of NEM316 WT 3 weeks after the last immunization. Under these conditions, immunization with rPbsP resulted in increased survival (80% *vs* 30% in GST-immunized control animals; $p < 0.05$ by log-rank Kaplan-Meyer; Fig. 8). These data indicate that immunization with PbsP induces efficient protection against GBS infection.

Discussion

The ability of GBS to bind blood Plg is essential for its invasiveness, in particular in promoting invasion of brain endothelial cells *in vitro* and of the central nervous system *in vivo* (Magalhaes *et al.*, 2007, Magalhaes *et al.*, 2013). As most pathogenic streptococci, this bacterium expresses a broad repertoire of unrelated Plg binding proteins whose biological function is, therefore, often difficult to predict. Up to now, two Plg-binding surface proteins have been extensively characterized in GBS, the GAPDH (Seifert *et al.*, 2003, Magalhaes *et al.*, 2007) and the cell wall-anchored LPXTG protein Srr2 (Seo *et al.*, 2013; Six *et al.*, 2015). The GADPH is an abundant cytoplasmic protein, essential for bacterial growth in glucose-containing media (*e.g.* the blood), which is released upon cell lysis and reassociated to the surface of living cells (Oliveira *et al.*, 2012, Madureira *et al.*, 2011). Extracellular GBS GAPDH serves as a receptor for numerous ligands, including Plg and Fg (Seifert *et al.*, 2003, Magalhaes *et al.*, 2013), and exerts immunomodulatory functions (Oliveira *et al.*, 2012, Madureira *et al.*, 2011). This highly conserved protein was detected at the surface of GBS isolates belonging to the main phylogenetic lineages. In contrast, the Srr2 glycoprotein, an LPXTG cell wall protein, is specific for GBS strains that belong to the hypervirulent complex CC17 (Six *et al.*, 2015) and are strongly associated with invasive neonatal meningitis. Moreover, this dual surface adhesin efficiently binds Fng and Plg (Seo *et al.*, 2013; Six *et al.*, 2015). While GAPDH and Srr2 are structurally unrelated, they both interact with Plg in a reaction inhibited by 6-ACA, suggesting an interaction between lysine residues within the adhesins and the lysine binding sites of the Plg Kringle domains.

Thus, the surface protein PbsP is the second LPXTG adhesin mediating Plg binding characterized so far in GBS. As opposed to Srr2, this protein is expressed by human clinical isolates belonging to unrelated CC, including the CC17. PbsP belongs to a family of evolutionarily-related streptococcal surface proteins containing a variable number of an

approximately 150-aa long repeated domain designated "Streptococcal surface repeat domain" (Bumbaca *et al.*, 2004). SSURE-containing proteins were initially characterized in *S. pneumoniae* and shown to mediate binding to both Fnt and Plg (Papasergi *et al.*, 2010). These binding activities are mediated by the SSURE repeats and the binding efficiency apparently reflects the number of repeats (Papasergi *et al.*, 2010, Jensch *et al.*, 2010). In pneumococci, the SSURE-containing protein PfbB (also known as PavB) is involved in bacterial adherence to human epithelial cells, nasopharyngeal colonization, and airways infections (Jensch *et al.*, 2010, Papasergi *et al.*, 2010). The number of SSURE domains within PfbB varies from 2 to 6 depending on the pneumococcal strain considered and sequence analysis revealed that these repeats clustered in two main groups displaying approximately 75% of identity: the conserved first repeats (100% of identity) and the core plus last repeats (>96% of identity) (Jensch *et al.*, 2010) (Fig. S2). All human GBS isolates apparently express a highly conserved 521-aa PbsP adhesin (>98% identity) possessing a similar structure as the pneumococcal PfbB, but containing only two SSURE domains that display 77% of identity and cluster apart from the pneumococcal SSURE sequences (Fig. S2). These structural differences may explain the functional differences observed between the GBS and the pneumococcal adhesins, PbsP mediating binding only to Plg whereas PfbB interacting with both Plg and Fnt.

Although differing in sequence and structure, a constant feature of bacterial and eukaryotic Plg binding motifs is the presence of positively charged amino acids in a hydrophobic surrounding (Fulde et al 2013). The SSURE domains of PbsP and PfbB are lysine-rich ($21 > n > 15$) and the interaction of the two proteins with Plg is inhibited by lysine. This suggests that the SSURE domains of both proteins is involved in Plg binding. However, the GBS PbsP contains a MK-rich domain that is absent in the pneumococcal protein PfbP and *in vitro* binding assays with purified domains demonstrated that this MK-rich domain displays a higher ability to bind Plg compared to the SSURE domains. Thus, it is likely that Plg binding

by PfbB is mediated by the SSURE domains whereas both the SSURE and the additional MK-rich domains of PbsP are involved in this reaction.

Bound Plg is a substrate for activating factors and this activation results in a dramatic increase of the cell surface proteolytic activity, thereby enhancing the ability of the bacteria to degrade host physiological barriers such as the extracellular matrix, the basement membrane, and encapsulating fibrin network. In the case of GBS, this activation is mediated by the host factors tPA and uPA, while this bacterium secretes the protein Skizzle which stabilizes Plg in a cleavable conformation (Wiles *et al.*, 2010). FACS analysis and measurement of tPA-dependent surface proteolytic activity revealed that a GBS NEM316 mutant lacking PbsP binds less Plg and is less proteolytic, compared to the WT strain, although both phenotypes are not abolished. The remaining binding activity is likely due to the Plg-binding properties of the moonlighting GAPDH and phosphoglycerate kinase proteins (Seifert *et al.*, 2003, Magalhaes *et al.*, 2007, Boone *et al.*, 2011). However, our results identify PbsP as a major Plg binding protein in non-CC17 GBS strains (Six *et al.*, 2015).

PbsP expression in GBS is repressed by CovRS (Papasergi *et al.*, 2013; Fig.2), the major global regulator of virulence gene expression (Firon *et al.*, 2013, Lamy *et al.*, 2004, Lembo *et al.*, 2010, Jiang *et al.*, 2008). While the activity of the PbsP promoter (Rosinski-Chupin *et al.*, 2015) is significantly (15-fold) increased in NEM316 Δ*covR* mutant, as determined by using a *lacZ*-fusion, we failed to demonstrate the binding of CovR to the promoter region of *pbsP*, suggesting that this regulation is indirect (data not shown). Inactivation of CovR increases GBS adherence to a variety of cells and matrix components due to the deregulated expression of several adhesins (Lembo *et al.*, 2010, Park *et al.*, 2012, Patras *et al.*, 2013). Our results show that PbsP is required for invasion and transmigration across brain endothelial cells (Fig. 6 and 7). It is therefore probable that PbsP expression is up regulated *in vivo*, together with other adhesins, by a CovR-dependent pathway, to synergistically contribute to the

attachement to endothelial cells and blood brain barrier penetration (Lembo *et al.*, 2010). As a conserved and cell surface localized adhesin, PbsP is an interesting candidate as a component of anti-GBS vaccines. Indeed, PbsP immunization protects mice from invasive infections of the central nervous system by the NEM316 strain.

In conclusion, we reported that PbsP is a conserved GBS cell-wall adhesins with a major role in Plg binding and a possible vaccine candidate against GBS infections. Further detailed studies are necessary to confirm PbsP function during invasive infections by unrelated GBS strains, in particular CC17 strains, and to unravel the coordinated mechanisms used by GBS to successfully hijack the host Plg/Pln fibrinolysis system.

Experimental procedures

Bacterial Strains and Reagents

The following reference GBS strains were used: NEM316 (serotype III, CC23), 6313 (serotype III, CC 23), BM110 (serotype III, CC17), COH1 (serotype III, CC17), A909 (serotype Ia, CC1), and 2603V/R (serotype V, CC19) (Da Cunha *et al.*, 2014, Glaser *et al.*, 2002, Tettelin *et al.*, 2005). The relevant characteristics of the other bacterial strains and plasmids used in this study are summarized in Table 1. GBS were grown at 37 °C in Todd-Hewitt (TH) broth (Difco Laboratories). Antibiotics (Sigma-Aldrich) were used at the following concentrations for *Escherichia coli*: ticarcillin, 100 µg/ml; erythromycin, 150 µg/ml; kanamycin, 25 µg/ml; and for GBS: erythromycin, 10 µg/ml; kanamycin, 500 µg/ml. Human Fnt, Plg and C Reactive Protein (Calbiochem), Factor H, Factor I, Factor B, C1q and C3 (Complement Technologies) and Collagen (Sigma-Aldrich) were purchased and Fng was prepared as previously described (Pietrocola *et al.*, 2005).

DNA manipulation and mutant construction

Purification of GBS genomic DNA and *E. coli* plasmid DNA were performed with the DNeasy Blood and Tissue kit and the Quiaprep Spin Minipreps kit (Qiagen), respectively. Oligonucleotides were provided by Eurofins MWG Operon or Sigma-Aldrich and are listed in Table 2. Analytical PCR used standard Taq polymerase (Invitrogen, Life Technologies). Preparative PCR for cloning and PCR for sequencing (GATC Biotech) were carried out with a high-fidelity polymerase (MyFi or Phusion DNA polymerase, Bioline and ThermoScientific, respectively). The pG1_Δ*pbsP* vector used for *pbsP* deletion was constructed as described (Firon *et al.*, 2013) using a splicing by overlap extension method (Heckman & Pease, 2007) with primers pAF375_EcoRI + pAF376_Δ0428 and pAF377_Δ0428 + pAF378_BamHI. After GBS transformation with pG1_Δ*pbsP* and selection of pG1_Δ*pbsP* integration and de-recombination events, marker-less deletion of *pbsP* was

confirmed on genomic DNA with primers pAF379 + 380 (positive PCR product in case of *pbsP* deletion) and pAF381 + 382 (positive PCR product in case of a WT *pbsP* gene). Primers pAF553_BamHI + pAF554_PstI were used to amplify the full-lenght *pbsP* gene with its predicted Shine-Dalgarno sequence from NEM316 WT genomic DNA. The PCR product was cloned into the pTCV_P_{tet} vector at the corresponding restriction sites to give the constitutive *pbsP* expressing vector pTCV_P_{tet}_ *pbsP*. The full length insert was sequenced to confirm the absence of mutations. The pTCV_P_{tet}_*pbsP* plasmid was introduced in GBS by electroporation and transformants were selected on TH agar supplemented with kanamycin.

Production of recombinant rPbsP and rPbsP domains

Recombinant PbsP (rPbsP) was produced as described (Garibaldi *et al.*, 2010, Papasergi *et al.*, 2010, Buscetta *et al.*, 2014). Briefly, the *pbsP* gene was amplified using primers gbs0428_BamHI and gbs0428_NotI (Table 2) and cloned into the pGEX-SN bacterial expression vector (Cardaci *et al.*, 2012). The corresponding pGEX-SN_PbsP allows the expression of the rPbsP fused to a glutathione S-transferase (GST) tag at its amino-terminal end. To produce recombinant PbsP fragments, the corresponding gene regions were amplified using the following primers: MK-rich$_{423}$_up and MK-rich$_{492}$_low (rMK-rich); SSURE$_{123}$_up and SSURE$_{422}$_low (rSSURE.1+2); SSURE$_{273}$_up and SSURE$_{422}$_low (rSSURE-2) (Table 2). Amplified fragments containing *att* recombination sequences were used to transform the pDEST15 vector by Gateway cloning according to the manufacturer's instructions (Thermo Fisher Scientific). The resulting plasmids pDEST15_MK-rich, pDEST15_SSURE$_{1+2}$, and pDEST15_SSURE$_2$ were used to transform *E. coli* BL21 (DE3). After induction, the recombinant fusion proteins were purified from the cytoplasm of bacterial cells using affinity chromatography (Papasergi *et al.*, 2010). Recombinant GST was produced and purified using the same method and used as a negative control.

Production of Anti-PbsP Antisera

CD1 mice (5 weeks old, Charles River Labs) were injected intraperitoneally with 20 μg of rPbsP or GST (in a total volume of 0.2 ml) in complete (first injection, day 0) or incomplete (second and third injections on days 14 and 28, respectively) Freund's adjuvant emulsions. The use of complete Freund's adjuvant in the first immunization was justified by our previous observations that high-titer sera were more consistently obtained with this adjuvant. However, cares were taken to minimize discomfort to the animals by injecting a low volume (0.1 ml, containing 0.05 mg of mycobacteria) of the oily component of the emulsion. Under these conditions, no significant abdominal distension or other complications at the injection site were observed throughout the experimental period. The mice were bled at 2 weeks after the last immunization, and the sera were tested for reactivity to the purified antigen using ELISA and Western blot assays.

Bacterial extracts and immunoblots

Cell wall extracts were prepared by digestion with mutanolysin (Sigma-Aldrich) of bacterial cells grown to the exponential phase in an osmo-protective buffer, as described (Lalioui *et al.*, 2005, Garibaldi *et al.*, 2010). A total of 30 μg of cell wall proteins (Bradford assay) were run on gels (SDS-PAGE), transferred to nitrocellulose membranes and hybridizations were performed with mouse anti-PbsP serum followed by horseradish peroxidase conjugated goat anti-mouse IgG (R & D Systems). Loading controls consisted of parallel Coomassie-stained gels.

For Far Western analyses, Plg (10 μg) was run on 12% acrylamide gels, transferred on nitrocellulose, and overlaid with 0.15 μM rPbsP or GST in 1% of nonfat dry milk supplemented with 0.05% Tween 20. Complex formation was detected using goat anti-GST IgG (1:4,000: GE Healthcare) followed by alkaline phosphatase-conjugated anti-goat IgG (1:5,000) (Sigma-Aldrich). rPbsP or GST (0.15 μM) were run on 12% acrylamide gels, transferred on nitrocellulose, and overlaid with Plg (1 μg/ml). Complex formation was

detected using anti-Plg rabbit polyclonal antibody (1 :5,000) and horseradish peroxidase-conjugated goat anti-rabbit IgG (1 :10,000; Amersham Biosciences).

ELISA binding assays

Binding of PbsP to host proteins (extracellular matrix or complement components) was determined using ELISA assays. Microplates coating was done with purified proteins (100 µl of 2 µg/ml solutions in 0.1 M carbonate buffer, pH 9.0) overnight at 4°C. Blocking of the wells was done for one hour at 22°C with 200 µl of 2% bovine serum albumin (BSA, Sigma-Aldrich) in PBS, followed by addition of 0.15 µM rPbsP or GST for one hour, and incubation with goat anti-GST (diluted 1:10,000 in PBS 0.1% BSA) for 90 min and the secondary anti-goat IgG (diluted 1:1,000) for one hour. The peroxydase substrate *o*-phenylenediaminedihydrochloride was used to reveal bound rPbsP by measuring absorbance at 490 nm and by substracting the values obtained with the GST control. For competitive ELISA, plates coated as above with Plg were incubated for 1 h at RT with rPbsP (0.15 µM) in the presence of 0 to 300 mM aminocaproic acid (6-ACA), L-Lysine, or L-Alanine (Sigma-Aldrich).

Plg activation assays.

Wells coated with rPbsP or GST (10 µg/ml) were incubated with Plg (2 µg/well) in 100 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA and 1 mg/ml PEG 8000 (Sigma-Aldrich) for 1.5 h at RT. After extensive washing, 5 nM tPA (tissue plasminogen activator) (Sigma-Aldrich) was added and plasmin activity was quantified with the chromogenic substrate S-2251 (H-$_{D}$-Valyl-$_{L}$-leucyl-$_{L}$-lysine-p-nitroaniline di-hydrochloride; Chromogenix). Plates were incubated at 37°C, and absorbance at 415 nm was used to quantify cleavage of the chromogenic substrate.

Fibrinogen degradation assay.

Microtiter plates were coated with 100 µl of rPbsP or GST (0.15 µM) overnight in 0.1 M

carbonate buffer, pH 9.0, at 4°C. Blocking was performed with 200 µl of PBS + 5% of BSA for 1 h at RT. Plasminogen (10 µg/ml) diluted in 100 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mg/ml PEG 8000 was incubated with immobilized rPbsP or GST for 90 min at RT. After washing, fibrinogen (10 µg/well) together with tPA (5 nM) were added to the wells and the mixtures incubated at 37°C for the indicated time points. The reaction was stopped by the addition of reducing SDS-PAGE sample buffer and boiling at 95°C for 5 min. Thereafter, the samples were separated by SDS-PAGE and the degradation of Fng was evaluated by Coomassie blue staining.

Surface plasmon resonance

Surface plasmon resonance (SPR) measurements were carried out on a Biacore X-100 instrument (GE Health-care, Piscataway, NJ, USA). To measure K_D values of plasminogen binding to recombinant rPbsP, goat anti-GST antibody (30 µg/ml), dissolved in 10 mM sodium acetate buffer (pH 5.0), was immobilized onto a carboxy-derivatized sensor chip (CM5). rPbsP (500 nM) was passed over a flow cell, whereas GST alone was passed in a reference cell. Human plasminogen was flowed over the surface of both flow cells at increasing concentrations ranging from 0.4 to 4 µM at a rate of 10 µl/min. Assay channel data was subtracted from reference flow cell data to eliminate the effects of nonspecific interactions. The response units (RU) at steady state were plotted as a function of Plg concentration, and fitted to the Langmuir equation to yield the K_D of the Plg–rPbsP interaction.

Flow Cytometry analysis

Binding of plasminogen or anti-PbsP antibodies to the bacterial cell surface was visualized using flow cytometry immunofluorescence analysis, as previously described (Buscetta *et al.*, 2014). Briefly, GBS strains grown to the log phase in TH were washed in PBS, fixed with 3.7% formaldehyde, and blocked using PBS supplemented with 1% dry milk (mPBS). For

plasminogen-binding studies, bacteria were sequentially incubated with plasminogen (at a final concentration of 0.5 mg/ml in mPBS) and with an anti-plasminogen rabbit polyclonal antibody (diluted 1:1,000 in 1% mPBS). After washing with PBS, bacteria were then treated with FITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich) diluted 1:1,000 in 1% mPBS. To visualize surface-expressed PbsP, bacteria were incubated with anti-rPbsP or anti-GST serum diluted 1:250 followed by FITC- or phycoerythrin-conjugated goat anti-mouse IgG (diluted 1:1,000 or 1:200, respectively; Sigma-Aldrich), as described (Boone *et al.*, 2011, Cardaci *et al.*, 2012). Fluorescent bacteria were analyzed with a MACS Quant VYB, FACSCantoII flow cytometer using the FlowJo software (BD Biosciences).

Plasminogen activation by GBS

The ability of GBS to activate Plg into plasmin was monitored in a quantitative assay using the chromogenic substrate D-Val-Leu-Lys-p-nitroanilide (Sigma-Aldrich). Approximately 5 x 10^8 bacteria were washed with PBS, blocked for 20 min with 1% milk in PBS, mixed with Plg (0.5 mg/ml) in the presence or absence of human tissue-type plasminogen activator (tPA: 20 nM) (Sigma-Aldrich) and incubated for 2h at 37°C. After incubation, the cells were centrifuged, washed, and re-suspended in 400 µl of chromogenic substrate D-Val-Leu-Lys-p-nitroanilide (400 µM final concentration). After 12 h incubation at 37°C, the cells were pelleted and the absorbance of 100 µl of the supernatant fluid was measured at 405 nm in a microplate reader. The 12 h incubation time was chosen, because is representative of the kinetics of this reaction, as determined in previous studies (Megalhaes *et al*. 2007, Megalhaes *et al*. 2013, Six *et al*. 2015).

Adhesion and Invasion

The human brain endothelial cell line hCMEC/D3 was provided by P.O. Couraud (INSERM, Paris, France) and the adherence and invasion assays were performed as described (Buscetta *et al.*, 2014, Weksler *et al.*, 2005). Briefly, bacteria were grown to the mid-log phase and

added to confluent monolayers at a multiplicity of infection (MOI) of 10 bacteria/cell. After one hour incubation, monolayers were washed with PBS to remove non-adherent bacteria, lysed, and plated to enumerate cell-associated bacteria. For the invasion assay, after washing, the monolayers were further incubated for 1 h with medium supplemented with penicillin and streptomycin (200 units/ml and 200 µg/ml, respectively) to kill extracellular bacteria. Percentages of bacterial adhesion and invasion were calculated as recovered cfu/initial inoculum cfu x 100.

GBS migration assay across hBMEC

An endothelial blood-brain barrier *in vitro* model was established by cultivating hCMECs on collagen-coated polycarbonate transwell membrane inserts with a pore size of 3 µm (Corning). This *in vitro* model allows separate access to the upper chamber (blood side) and lower chamber (brain side) and mimics GBS penetration into the brain. The hCMEC monolayer was grown by seeding 500 µl of growth medium containing 1 x 10^6 cells in the upper channel and 1.5 ml growth medium in the bottom chamber of 12 wells tissue culture inserts. The hCMEC were grown for 5 to 7 days at 37 °C in a humidified chamber containing 5% CO_2 to reach confluence. Only monolayers with a trans-endothelial electric resistance (TEER) greater than 200 ohm.cm^{-2} were used, as measured with a Millicell ERS-2 meter (Millipore). Prior to the assay, hCMECs were washed and resuspended in serum-free culture medium without antibiotics. Log-phase GBS cells untreated or treated with a final concentration of human Plg/Pln of 50 µg plus tPA (20 nM) were applied to the apical chamber (total volume of 500 µl with a MOI of 10). At 2 h post-infection, the lower chamber medium was entirely removed and plated onto TH agar to enumerate bacteria crossing the hCMEC monolayer. Simultaneously, the integrity of the hCMEC monolayer was assessed by TEER measurement.

Animals model of GBS infection.

All studies involving mice were performed in strict accordance with the European Union guidelines for the use of laboratory animals. The procedures were approved by the Ethics Committee of the University of Messina (OPBA permit no. 18052010) and by the Ministero della Salute of Italy (permit no. 665/2015).

Virulence of GBS strains were tested with 8 week-old CD1 mice infected intravenously (i.v.) with $5x10^8$ bacteria, as described (Buscetta *et al.*, 2014). Mice were monitored at least once a day for lethality and signs of disease for a total of 14 days after challenge, as described (Garibaldi *et al.*, 2010, Cardaci *et al.*, 2012). Animals with signs of irreversible sepsis were euthanized and GBS invasion of organs confirmed as the cause of disease. In a second set of experiments, GBS-infected mice were sacrificed at 48 h after infection to collect blood, brains and kidneys. The number of CFU was measured in organ homogenates using standard methods (Cardaci *et al.*, 2012). To study the protective effet of PbsP immunization, CD1 mice (5 weeks old, Charles River Labs) were injected intraperitoneally (i.p.) with 20 μg of rPbsP or GST in complete (first injection) or incomplete (second and third injections) Freund's adjuvant emulsions (in a total volume of 0.2 ml) on day 0, 14, and 28. Three weeks after the last immunization, mice were challenged i.v. with 2×10^8 CFUs of the NEM316 WT strain.

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Table 1. GBS strains

I.D.	Strains	Features	Ref.
NEM316	WT	Clinical isolate, serotype III	Glaser *et al.*, 2002
NEM3294	Δ*pbsP*	Deletion of *pbsP* in NEM316	
NEM2511	*SrtA**	NEM316 with an inactive sortase A enzyme	Konto-Ghiorghi *et al.*, 2009
NEM2089	Δ*covSR*	*covSR* deletion in NEM316	Lamy *et al.*, 2004
NEM3745	WT / pTCV_ P_{tet}	NEM316 with the empty vector	
NEM3749	Δ*pbsP* / pTCV_ P_{tet}	NEM3294 with the empty vector	
NEM3751	Δ*pbsP* / pTCV_ P_{tet}_ *pbsP*	NEM3294 with the *pbsP* expression vector	

WT, wild type; pTCV_P_{tet}, empty vector with the P_{tet} constitutive promoter; pTCV_P_{tet}_*pbsP*, vector with the *pbsP* gene under the control of the P_{tet} constitutive promoter.

Table 2. Plasmids and oligonucleotides used in this study

I.D.	Description[a,b,c]	Ref.
Plasmids		
pGEX-SN	*E. coli* expression vector, Amp^R	GE Healthcare Life Sciences
pGEX-SN_*pbsP*	pGEX-SN expression vector carrying *pbsP* (residues 28 to 462)	This study
pG1	Thermosensitive shuttle vector, $oriR^{ts}$ pWV01, Erm^R	Mistou *et al*., 2009
pG1_Δ*pbsP*	pG1 with the *pbsP* deletion cassette	This study
pTCV_P_{tet}	GBS expression vector, constitutive P_{tet} promoter, Erm^R , Km^R	Firon *et al*., 2013
pTCV_P_{tet}_*pbsP*	pTCV_P_{tet} with the full length *pbsP* ORF	This study
pDEST15	*E. coli* expression vector, Amp^R, Cm^R, with *attR* sites for Gateway cloning	Hartley et *al*., 2000
pDEST15_$SSURE_{1+2}$	pDEST15 expression vector with the two PbsP SSURE domains (residues 123 to 422)	This study
pDEST15_$SSURE_2$	pDEST15 expression vector carrying with one PbsP SSURE domain (residues 273 to 422)	This study
pDEST15_MK-rich	pDEST15 expression vector carrying the MK rich sequence (residues 423 to 492)	This study
Oligonucleotides		
gbs0428_BamHI	TTTGGATCCGCATTGTTGCTTTAGGGCAATCT	
gbs0428_NotI	TTTTTTTGCGGCCGCCGGCTTGTGATGACATTTGCATATT	
pAF375_EcoRI	ACATGAATTCGTCCTATGGACAGTCATTCACAT	
pAF376_Δ0428	CTATCCCTATCTTTGTTTAGATTTGTTAATGAGAGTATACCAAC	
pAF377_Δ0428	GTTGGTATACTCTCATTAACAAATCTAAACAAAGATAGGGATAG	
pAF378_BamHI	AGTAGGATCCTTGATGATTCCCATAGATACGTC	
pAF379	TGAAAGTCAAGAAGCAGAGACA	
pAF380	TTTGATAGTTGGGGTATTGTCTG	
pAF381	TACGACAGGTAAAGAGGGACAAG	
pAF382	GGCTGTATTGCCATTAAGAGAGA	
pAF553_BamHI	TGATGGATCCGAAAGAGGAATCCCTTATGAAAATATC	
pAF554_PstI	TGATCTGCAGCTGAATTAAATACTATCCCTATC	

$SSURE_{123}$_up	GGGG**ACAAGTTTGTACAAAAAAGCAGGCTTT**GAGGTAAAGAAATCAACTGATACTG
$SSURE_{273}$_up	GGGG**ACAAGTTTGTACAAAAAAGCAGGCTTT**GAAGTAAAAAATTCTCTAAAGATG
$SSURE_{422}$_low	GGGG**ACCACTTTGTACAAGAAAGCTGGGTT**TTACATATCTTTTTAGCGACATTAAT
MK-$rich_{423}$_up	GGGG**ACAAGTTTGTACAAAAAAGCAGGCTTT**AATAGCACATCAATGATGATG
MK-$rich_{492}$_low	GGGG**ACCACTTTGTACAAGAAAGCTGGGTT**TTACTTTGTTTCACCTGTATTAGGTAA

[a] Em^R, erythromycin resistance; Km^R, kanamycin resistance; Amp^R, ampicillin resistance
[b] Underlined nucleotides represent restriction sites
[c] Recombination sites are indicated by nucleotides in bold

Figure legends

Figure 1. PbsP is a SSURE domain-containing protein.

A. Schematic representation of PbsP. SP, signal peptide; N-terminal, N-terminal domain; SSURE1 and SSURE2, streptococcal surface repeats 1 and 2; MK rich: methionine and lysine-rich region; LPXTG, cell wall anchoring motif.

B. Sequence alignment of the SSURE1 and SSURE2 domains. Asterisk (*), identity; colon (:), residues with strongly similar properties (>0.5 in the Gonnet PAM 250 matrix); period (.) residues with weakly similar properties (<0.5 in the Gonnet PAM 250 matrix).

Figure 2. PbsP is a conserved cell surface protein.

The following GBS strains were compared: WT, wild-type strain NEM316; Δ*pbsP*, deletion mutant lacking PbsP; *srtA**, mutant with an inactive form of sortase A; Δ*covRS* deletion mutant lacking the two-component system CovRS.

A. Western blot analysis of cell wall extracts. Left panel, coomassie staining of cell wall extracts separated by polyacrylamide gel electrophoresis; right panel, corresponding western blot analysis using polyclonal anti-PbsP mouse serum.

B. Expression of PbsP on the GBS surface. Immunofluorescence flow cytometry analysis of PbsP espression on different bacterial strains using mouse polyclonal anti-PbsP serum (blue line) or control anti-GST serum (red line). GBS wild-type strains of different serotypes and clonal complexes are indicated by their common name (6313, A909, COH1, BM110, 2603V/R).

Figure 3. PbsP binds plasminogen *in vitro*

A. Selective binding of PbsP to immobilized plasminogen. Binding of recombinant PbsP to immobilized human plasminogen (Plg), collagen (Coll), fibronectin (Fnt), fibrinogen (Fng), C-reactive protein (CRP) or to the complement components factor H (FH), factor I (FI), factor B (FB), C1q and C3 was quantified by ELISA using anti-GST antibodies. Columns and bars indicate means ± SD from three independent experiments conducted in duplicate.

B. Dose-dependency of PbsP-Plg interactions. ELISA assays were performed with immobilized Plg, to which rPbsP was added at the indicated concentrations. Binding was detected using anti-GST antibodies. Points and bars indicate means ± SD from three independent experiments conducted in duplicate.

C. Far Western blot analysis of PbsP/Plg interactions. Far western blot analyses were done with rPbsP and GST (as negative control) on membranes probed and revealed with Plg and anti-Plg (left panel), and, reciprocally, with Plg on membranes probed and revealed with rPbsP and anti-rPbsP (right panel). Numbers indicate the molecular mass of protein standards in kDa.

D. Binding affinity of PbsP to Plg. Binding is measured as response units (RU) against time by surface plasmon resonance with immobilized rPbsP on a CM5 chip coated with anti-GST and increasing concentrations of Plg ranging from 0.40 (lowermost trace) to 4 μM (uppermost trace). The sensor-graph is representative of three independent experiments conducted in duplicate and the affinity was calculated from curve fitting to a plot of the RU values against Plg concentration (right graph).

E. Selective inhibition of PbsP-Plg interactions. Competitive ELISA assays were done with immobilized Plg to which rPbsP was added in the presence of the indicated concentrations of 6-aminocaproic acid (6-ACA), L-Lysine, or L-Alanine, used as a negative control. Points and bars indicate means ± SD from three independent experiments conducted in duplicate.

F. Binding of PbsP domains to Plg. Schematic representation of recombinant PbsP domains produced as gluthatione-S-transferase (GST) fusion proteins and the corresponding.ELISA assays performed with immobilized Plg, to which the entire protein or its fragments were added at the indicated concentrations. Binding was detected using anti-GST antibodies. Points and bars indicate means ± SD from three independent experiments conducted in duplicate.

Figure 4. PbsP-bound Plg is converted into plasmin by host activators.

A. Activation by tPA of PbsP-bound Plg. Immobilized rPbsP or GST were incubated in microplates with Plg with or without the Plg activator tPA. Activation of rPbsP-bound Plg was quantified with the chromogenic Pln substrate S-2251 (0.6 mM). Points and bars indicate means ± standard deviations from 3 independent experiments performed in triplicate.

B. Degradation of fibrinogen by activated Plg-PbsP complexes. Immobilized rPbsP was incubated with Plg, washed, and incubated with tPA and Fng. At the indicated time points, degradation of the three Fng chains was followed using polyacrylamide gel electrophoresis. A representative Coomassie-stained gel from three experiments is shown.

Figure 5. Plg binding and activation on the GBS surface are dependent on PbsP.

The following GBS strains were compared: WT, NEM316 wild-type strain; Δ*pbsP*, isogenic *pbsP* deletion mutant; Δ*pbsP*+*pbsP*, Δ*pbsP* strain carrying a complementing vector with constitutive *pbsP* expression; WT+P_{tet}, and Δ*pbsP*+ P_{tet}, control strains carrying a vector without inserts; *, $p<0.05$ by one-Way ANOVA and Tukey's multiple comparisons test. Columns and bars indicate the means ± SD of three independent experiments conducted in duplicate.

A. GBS binding to Plg. Bacteria were incubated with Plg followed by fluorescent anti-Plg antibodies and analyzed by FACs. Shown is the increase in mean fluorescent intensity (MFI) measured on 10,000 events per population.

B. Plg activation on the GBS surface. After Plg binding to GBS (as in A), the Plg activator tPA was added and Pln activity was measured optically with the chromogenic Pln substrate D-Val-Leu-Lys-p-nitroanilide.

Figure 6. *In vitro* translocation of GBS through human brain endothelial cells is dependent on Plg-PbsP interactions.

Adhesion (**A**), invasion (**B**) and transmigration (**C**) were assessed using the brain endothelial cell line hCMEC/D3. GBS strains are as in Figs. 2 and 5; * = $p < 0.05$ by one-Way ANOVA and Tukey's multiple comparisons test. Columns and bars represent means + SD of CFU values from three independent experiments conducted in duplicate.

Figure 7. PbsP is required for GBS virulence.

A. Effects of PbsP on survival of GBS-infected mice. Adult CD1 mice (n= 16 per group) were infected i.v. with 5 x 10^8 CFUs of WT NEM 316 or the Δ*pbsP* mutant and survival was monitored. *, $p < 0.05$ by log-rank Kaplan-Meyer analysis.

B-D. Effects of PbsP on organ colonization. Bacterial burden in blood (**B**), kidneys (**C**), and brains (**D**) were quantified 48 hours after i.v. challenge with 5 x 10^8 CFUs of WT or Δ*pbsP* strains. * = $p < 0.05$ by the Mann-Whitney U test.

Figure 8. PbsP-based immunization protects against lethal GBS infection in mice. Animals (n = 10 per group) were immunized with rPbsP protein or with GST as a control and

challenged i.v. with 2 x 10^8 CFU/mouse of wild type strain NEM316. * = $p < 0.05$ by log-rank Kaplan-Meyer analysis.

Figure S1. Effect of deletion of the *pbsP* gene on Gram stain morphology and growth curves of GBS

Gram stain (**A,** 100X magnification) and growth curves in Todd Hewitt broth (**B**). The following GBS strains were compared: WT, NEM316 wild-type strain; Δ*pbsP*, isogenic *pbsP* deletion mutant; Δ*pbsP*+*pbsP*, Δ*pbsP* strain carrying a complementing vector with constitutive *pbsP* expression.

Figure S2. Phylogenetic analysis of streptococcal SSURE domains.

The two SSURE domains (SSURE-1 and -2) of the GBS protein PbsP from strain NEM316, the four and six SSURE domains (SSURE-1 to -6) of the pneumococcal protein PfbB (PavB) from, respectively, strains TIGR4 and R6 were aligned and a phylogenetic tree was computed using the Neighbor Joining method. This analysis defines three clusters supported by significant bootstrap values (100%) calculated from 1,000 replicates. The scale bar (neighbour-joining distance) represents the percentage sequence divergence.

Fig. 1

A

B

```
SSURE1   EVKKSTDTAFKQTVDVPAHYVNAAKGNGPFLAGVNQTIPYEAFGGDGMLTRLILKSSEGA
SSURE2   EVKNSLKMAFKEKVDVPAKYVSAAKAKGPFLAGVNETIPYEAFGGDGMLTRLILKASEGA
         ***:* . ***:.*****:**.***.:********:******************:****
SSURE1   KWSDNGVDKNSPLLPLKGLTKGKYFYQVSLNGNTTGKEGQALLDQIKANDKHSYQATIRV
SSURE2   KWSDNGVDKNSPLLPLKDLTKGKYFYQVSLNGNTAGKKGQALLDQIKANGSHTYQATITI
         ****************.***************:**:***********..*:***** :
SSURE1   YGAKDGKVDLKNMISQKMVTINIPHITTDM
SSURE2   YGTKDGKVDMNTILGQKTVMIHINVAKKDM
         **:******::.::.** * *:*   ..**
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Fig. 2

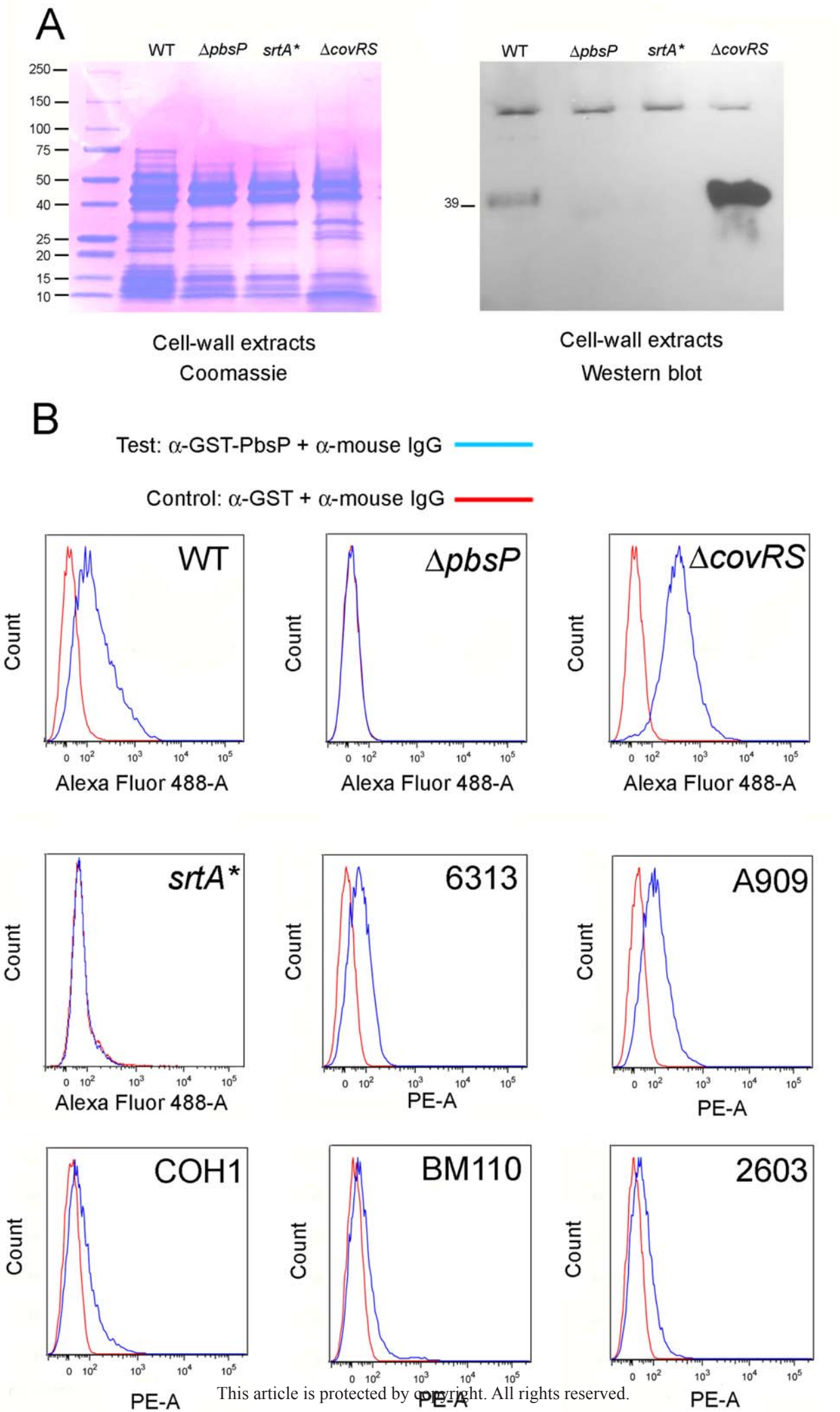
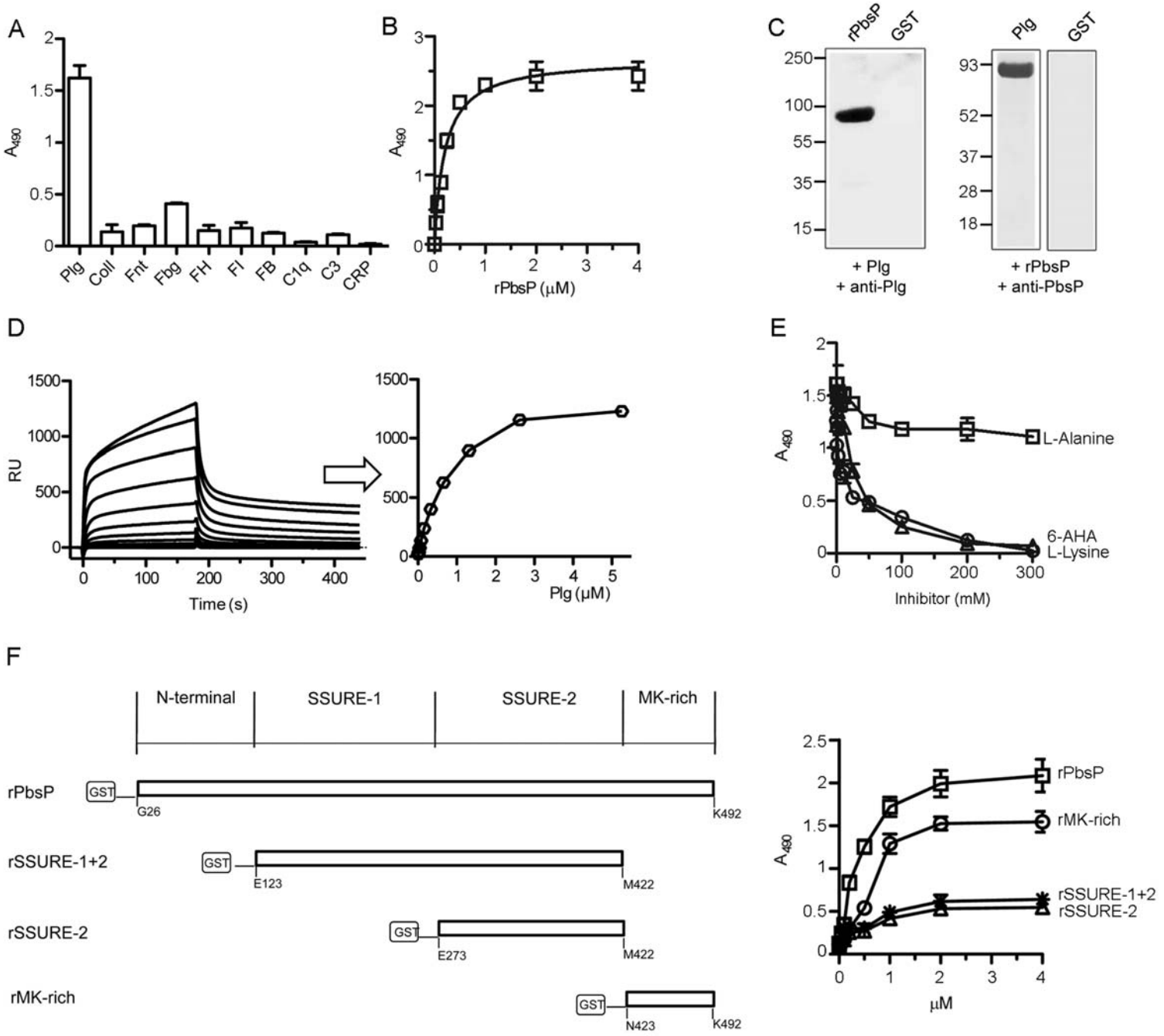


Fig. 3



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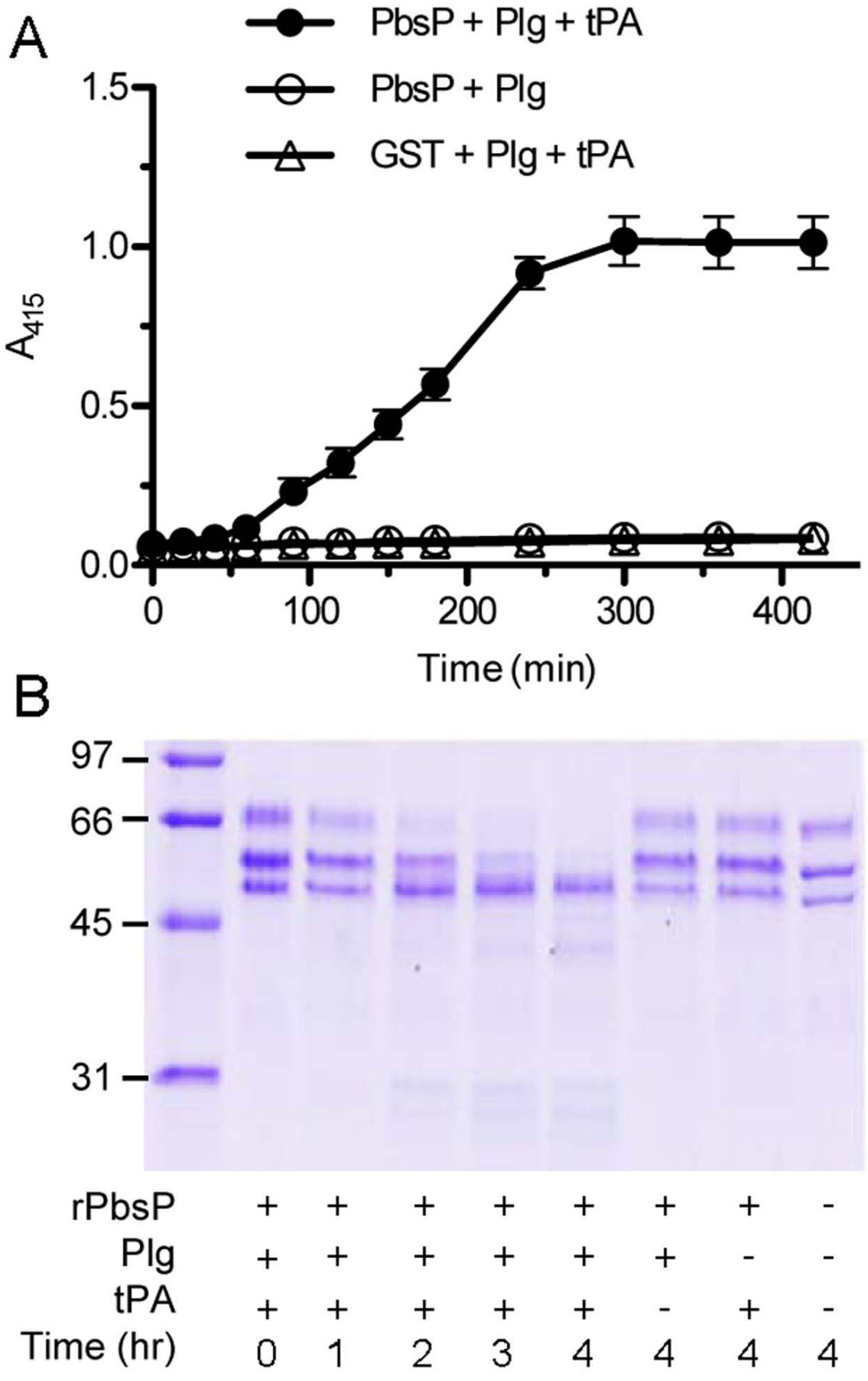


Fig.5

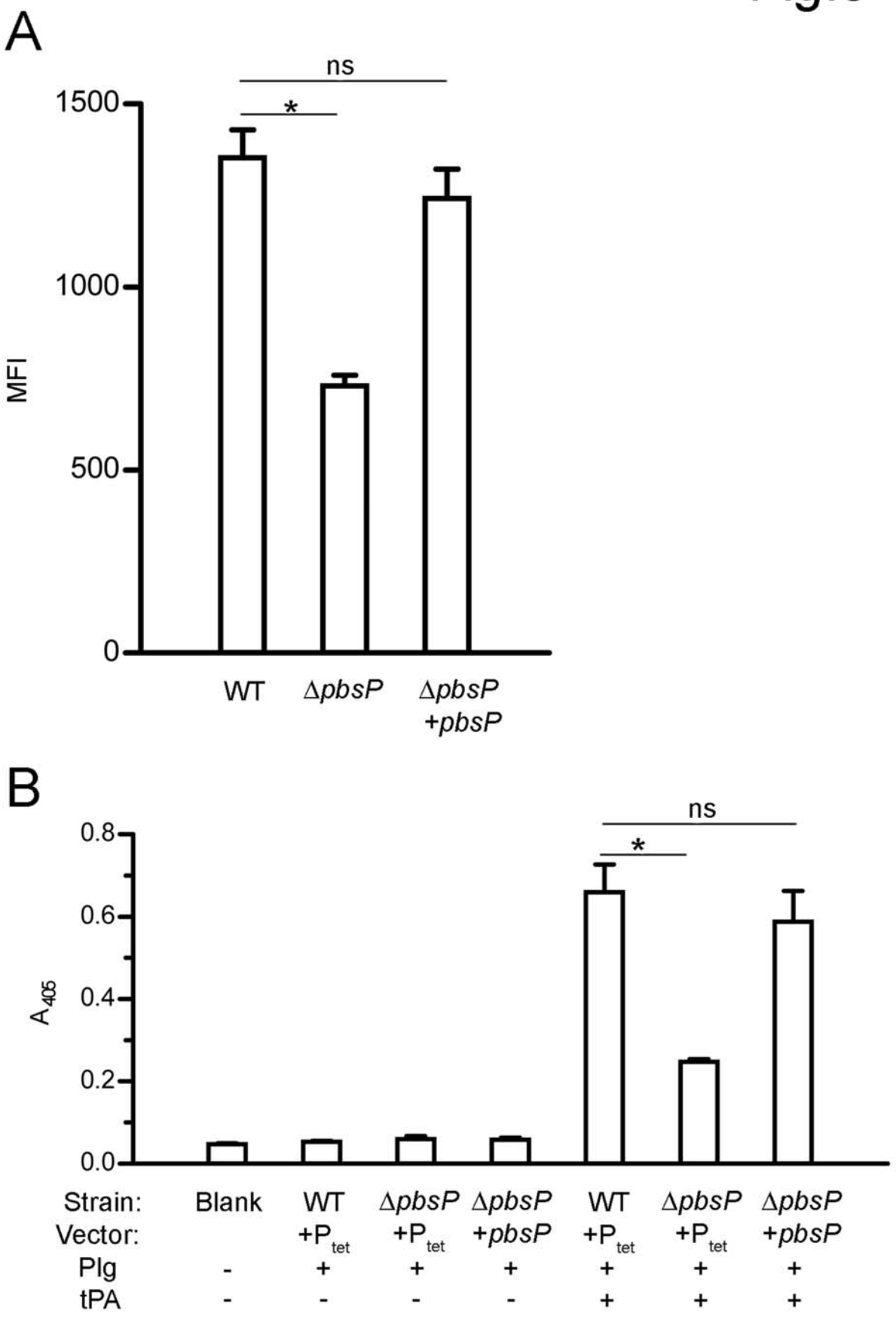


Fig. 6

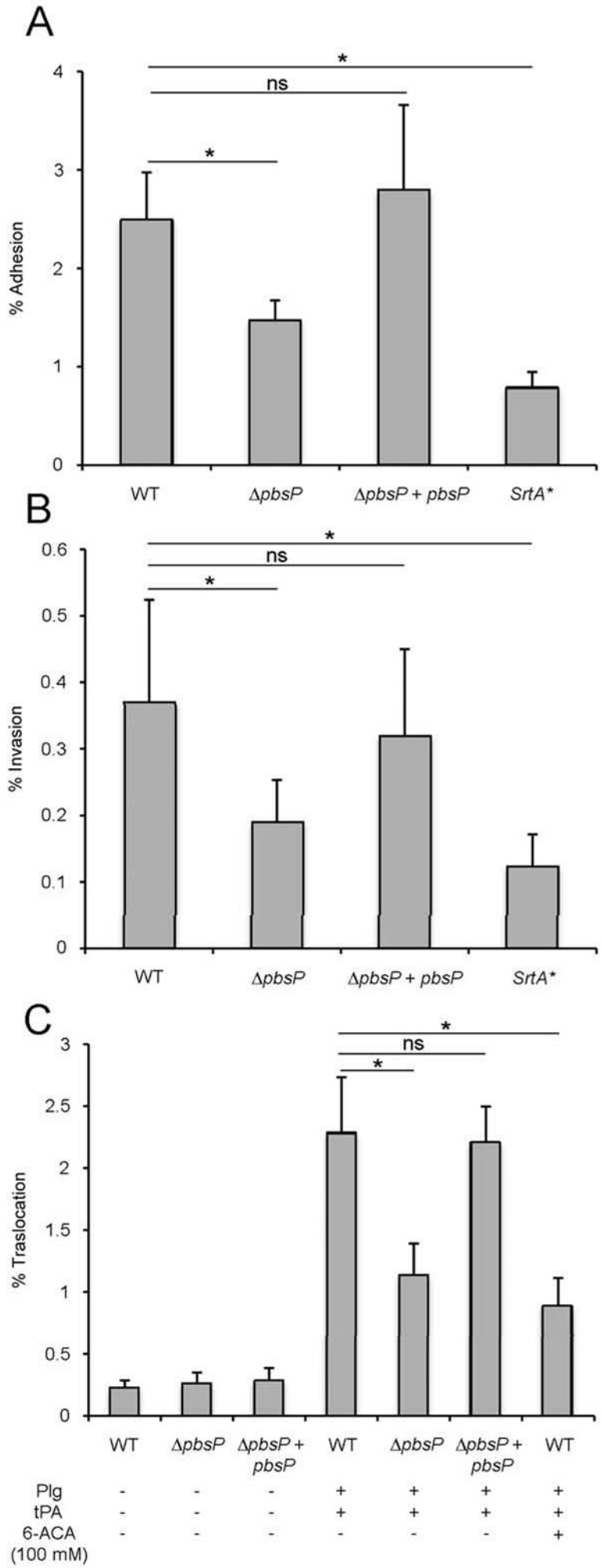


Fig. 7

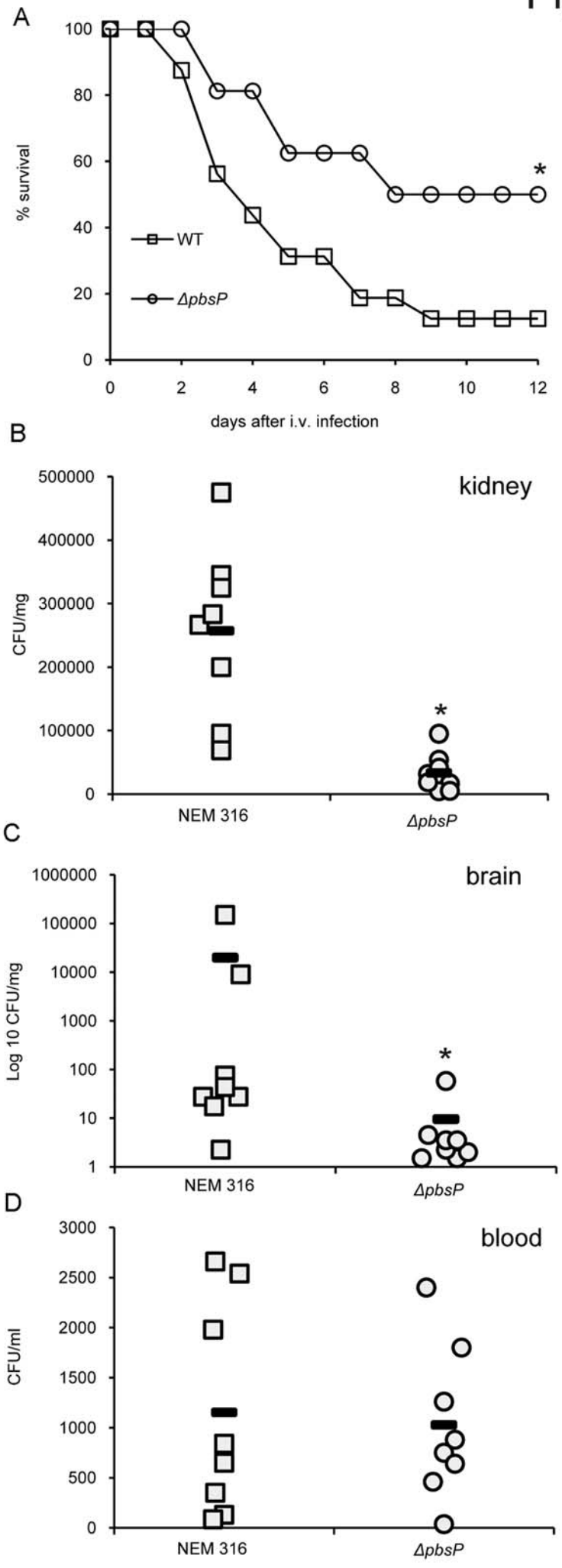
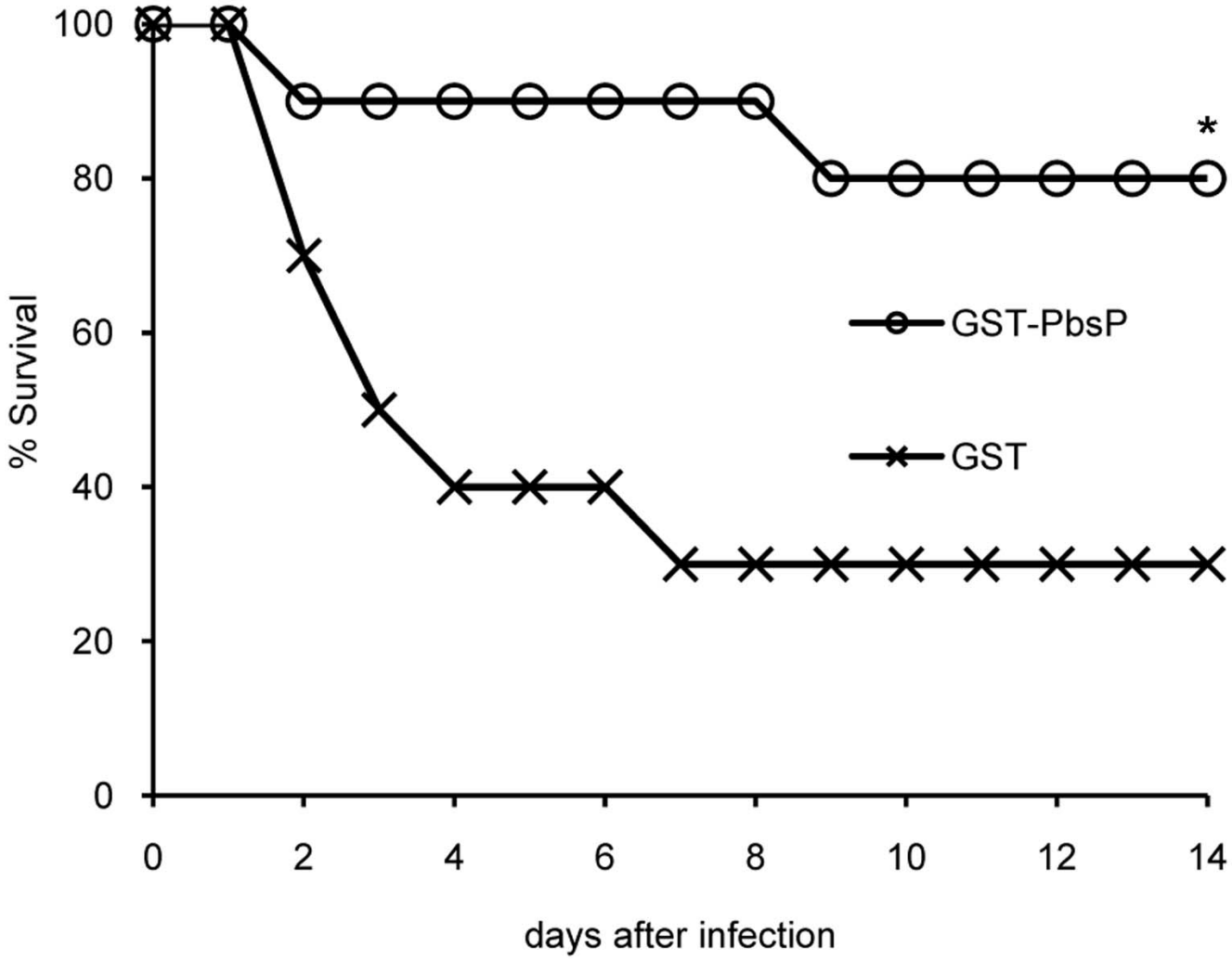
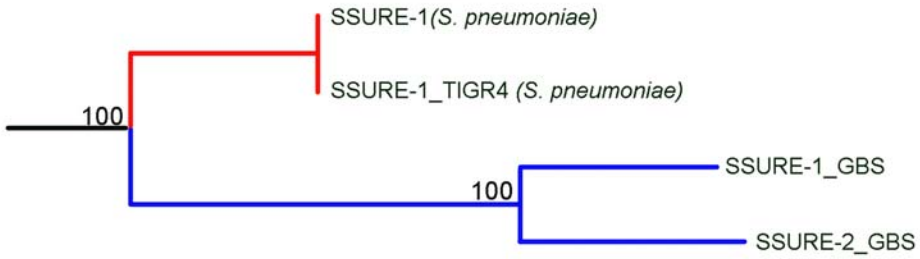


Fig. 8





We describe here Plasminogen binding surface Protein, or PbsP, a highly conserved virulence factor of group B streptococci. This adhesin contains two Streptococcal Surface Repeat domains, a methionine- and lysine-rich region and an LPXTG cell-wall anchoring motif. PbsP largely mediates the ability of group B streptococcal strain NEM316 to bind plasminogen, acquire proteolytic activity and transmigrate through brain endothelial cells, resulting in meningoencephalitis. Moreover, immunization of mice with PbsP confers protective immunity.