University of Messina

PhD School in Surgical and Medical Biotechnologies XXIX Cycle

Coordinator: Prof. G. Raimondo

Functional and molecular characterization of PbsP

(*Plasminogen binding surface Protein*)

of *Streptococcus agalactiae*

PhD student

Romeo Letizia

Supervisor

Prof. Concetta Beninati

Academic year 2016/2017

Introduction

The plasminogen system in the pathogenesis of group B streptococcal infections

Bacterial pathogens have evolved a wide range of strategies to adhere to cells and subsequently invade host tissues (*Kline et al., 2009; Nobbs et al., 2009*). In particular, many microorganisms, including both pathogens and commensals, are able to bind host plasminogen (Plg) on their surface, where its activation can be controlled by host or bacterial factors (*Lahteenmaki et al., 2001; Bergmann and Hammerschmidt, 2007; Fulde et al., 2013*).

Human plasminogen (Plg) is a 92 kDa glycoprotein which is present in high concentrations within human plasma as precursor (a zymogen) of the serine protease plasmin (Pln) (*F. J. Castellino 2005; Bergman et al., 2007; Bhattacharya et al., 2012*). The Plg molecule contains a total of seven structural domains, each with different properties. The N-terminal portion of the molecule consists of an activation peptide (AP) followed by a series of 5 repeating homologous triple-disulfide-linked peptides of approximately 80

amino acids termed kringles (K1–K5) while the C-terminal portion contains

the cleavage (activation) loop (CL) for plasminogen activator (PA) followed by

a serine protease (SP) domain *(F. J. Castellino 2005; Bhattacharya et al., 2012)*

(**Figure 1**). The function of the five kringles is primarily to mediate protein-

protein interactions, such as those involving binding of Plg or Pln to fibrin,

ECM targets, lysine-containing receptors/analogues and bacterial receptors

(*Suenson E et al., 1981; Miles LA et al., 1988; Urano T et al.,1987 M. L. Sanderson-*

Smith et al .2008; S. Bhattacharya et al. 2012).

The activation of inactive Plg into its active form (Pln) is mediated by a

proteolytic mechanism usually catalyzed by the host plasminogen activators

tissue-type (tPA) and urokinase-type (uPA) Plg activators (*Fan ZQ, et al., 1998;*

Violand BNet al., 1976). In order to maintain tissue homeostasis and avoid

random tissue damage, Pln activity must be tightly controlled (*O'Mullane MJet*

al.,1998; O'Mullane MJet al.,1999). This regulation is achieved through the

expression of Plg receptors (PlgRs), Plg activators (PAs) and Pln inhibitors (*E.*

F. Plow et al.,1986; J D Vassalli et al.1991, E. F. Plow et al., 1995).
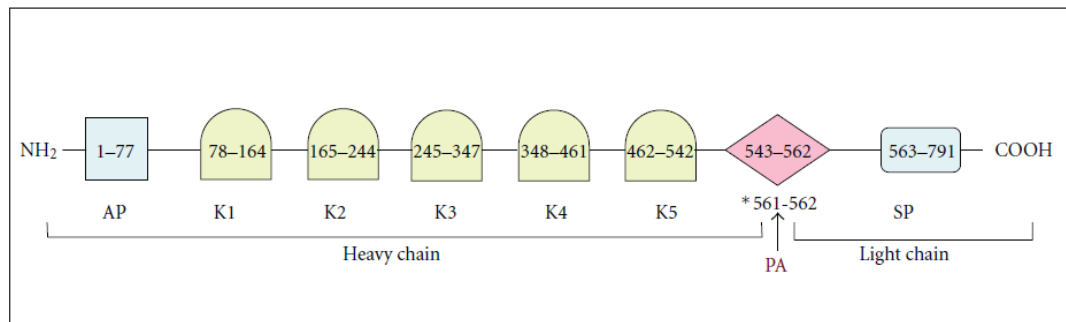
Figure 1. Schematic representation of the human Plg structure. Starting from the N- terminus of the mature protein, the 77-residue activation peptide (AP) is followed by 5 kringle domains (K1–K5) containing lysine binding sites and the catalytic SP domain. The R561-V562 amide bond, which is proteolytically cleaved by activators to generate Pln, is shown with an asterisk and an arrow. The heavy chain and light chains generated after proteolytic cleavage are also shown. *S. Bhattacharya et al., 2012*

Bacteria have independently evolved systems to exploit the host Plg/Pln system to their advantage. They can interact with this system via three distinct mechanisms (*Bergman et al., 2007*): i) bacteria produce PAs, which activate Plg by complex formation or by proteolysis; ii) Plg is recruited to the bacterial cell surface by bacterial PlgRs and then converted into plasmin by host-derived activators; iii) bacteria bind to Plg on the surface of host cells to promote colonization or penetration of body barriers through adhesion to and/or invasion of mucosal or endothelial layers. Bacteria are able to bind to host Plg/Pln directly, through specific surface adhesins or conserved ubiquitous moonlighting proteins such as enolase or GAPDH, or indirectly, through fibrinogen receptors enabling the formation of fibrinogen-plasminogen

complexes (**Figure 2**). Multiple Plg/Pln/Fng receptors can be expressed at different steps of the colonization or invasion processes and can thus be simultaneously or sequentially involved in pathogenesis at specific stages of disease development. For example, Plg expressed on the surface of epithelial cells could be initially be used for mucosal colonization. Then, Pln bound to the bacterial surface could be exploited by bacteria for proteolytic degradation of components of tight junctions or basal membranes, facilitating bacterial translocation across cell barriers and dissemination within tissues. The relevance of Plg binding in streptococcal pathogenesis (particularly in the pathogenesis of infections by group A streptococci and pneumococci) is well documented (*Bergmann et al., 2001, 2005; Sun et al., 2004; Sanderson-Smith et al., 2008;Fulde et al., 2011; Siemens et al., 2011; Teles et al., 2012; Agarwal et al., 2013*). Here, we focus on plasminogen receptors of group B streptococcus.
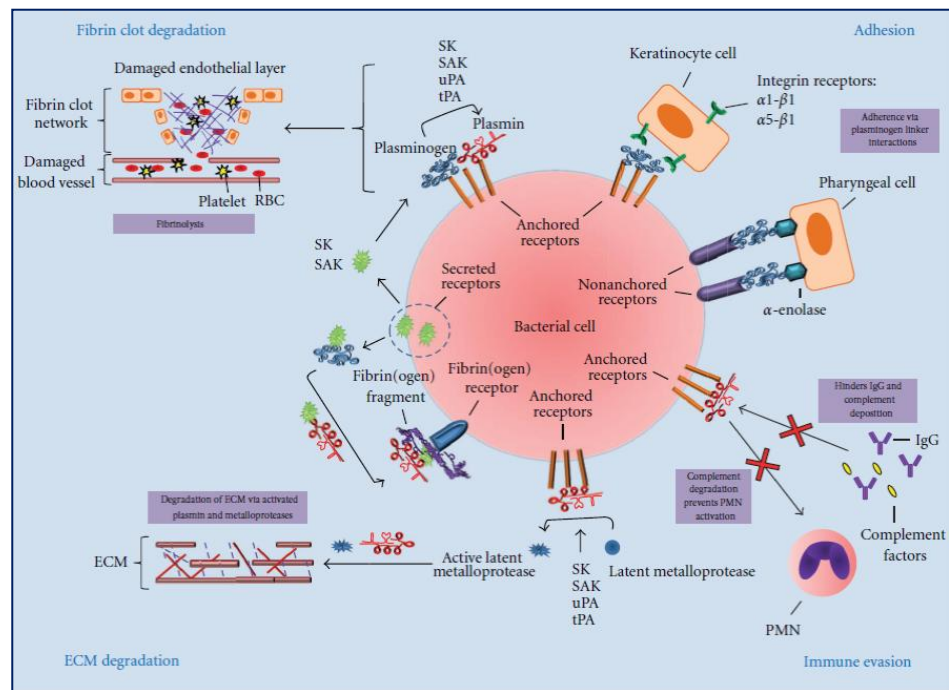
Figure 2. Mechanisms of bacterial cell surface plasmin(ogen) acquisition and its role in bacterial-host interactions. Plasmin(ogen) can be bound directly to the bacterial cell surface via cell-membrane-anchored receptors, nonanchored-cell-surface-associated receptors or indirectly through interactions with fibrinogen and cell surface fibrinogen receptors. Plasmin(ogen) localised on the bacterial cell surface is involved in four main processes; (1) ECM degradation via activated metalloproteases and plasmin; (2) fibrinolysis via plasmin; (3) immune evasion through plasmin-mediated degradation of immune effectors, including complement components and immunoglobulins; (4) adherence to host cells via plasminogen linker interactions with host cell surface receptors. ECM: extracellularmatrix; IgG: immunoglobulin G; RBC: red blood cell; SAK: staphylokinase; SEN: streptococcal α-enolase; Ska: streptokinase; tPA: tissue plasminogen activator; uPA; urokinase plasminogen activator. *M.L. Sanderson-Smith 2012*

Streptococcus agalactiae (also referred to as group B Streptococcus, GBS) is

a Gram-positive encapsulated commensal bacterium of the human

gastrointestinal tract, which is also present in the genital tract of 15–30% of

healthy women (*Campbell et al., 2000; Edwards et al., 2006 Le Doare and Heath,*

2013). In the last two decades it has emerged as one of the leading causes of

neonatal bacterial infections worldwide, commonly resulting in pneumonia,

septicemia or meningitis (*Trivalle et al., 1998; Schuchat et al., 1999; Edwards and Baker, 2005*). GBS is also a significant cause of morbidity and mortality in nonpregnant adults, particularly those with underlying diseases such as diabetes, cirrhosis or malignancy (*Farley et al., 1993*). Neonatal diseases result from transmission of the organism from the pregnant mother to the neonate.

Two distinct GBS-associated clinical syndromes, referred to as early-onset disease (EOD) and late-onset disease (LOD), have been recognized in neonates and characterized by onset of disease during their first 6 days of life or after (from day 7 up to 3 months of age), respectively (*Edwards and Baker, 2005*). For EOD, the mode of transmission in newborns is thought to be vertical, by inhalation of GBS-contaminated amniotic or vaginal fluid during parturition, followed by bacterial translocation across the respiratory epithelium and subsequent systemic infection (*Edwards and Baker, 2005*). In contrast, for LOD, the mode of transmission and the infection route remain elusive, although mother-to-child transmission might also be involved. A plausible scenario would involve early intestinal colonization by GBS with intraluminal multiplication, followed by translocation across the intestinal epithelium and

access to the bloodstream. Indeed, an intestinal portal of entry for LOD is supported by several lines of evidence: (a) 60 and 40% of the neonates asymptomatically colonized with GBS at birth remain positive for bacteria at the rectal level at 4 and 12 wk of life, respectively (*Weindling et al., 1981*); and (b) a longitudinal study of GBS vaginal and rectal colonization in women during and after pregnancy has revealed that carriers are usually colonized for up to 2 yr by a single clone, which is also frequently found in newborn feces for up to 1 yr (*Hansen et al., 2004*). Although intrapartum antibiotic prophylaxis for women at risk for GBS infection has markedly decreased the incidence of EOD, it did not change that of LOD (*Poyart et al., 2008*).

Epidemiological data have reported that serotype III is the most frequently identified GBS serotype causing invasive neonatal disease in different countries (*Poyart et al., 2008; Edmond et al., 2012*). Surprisingly, multi-locus sequence typing (MLST) of GBS isolated from different countries showed that most human carriage and clinical isolates cluster into only five major clonal complexes (CC) (CC1, CC10, CC17, CC19 and CC23) (*Jones N, et al. 2003*) (**Figure3**). Strains of serotype III contain a limited number of clonal

complexes. Among these, a substantial proportion of EOD and the majority of LODs are associated with 'hypervirulent' serotype III strains included in the clonal complex (CC) 17 (CC-17) (*Musser et al., 1989; Lamy et al., 2006; Lin et al., 2006; Phares et al., 2008; Poyart et al., 2008*). These CC17 GBS strains are derived from the same genetic pool (*Teatero et al., 2016*) and are more likely to cause neonatal meningitis than other genotypes (*Jones et al., 2003; Bisharat et al., 2004; Luan et al., 2005; Jones et al., 2006; Lin et al., 2006; Bohnsack et al., 2008; Manning et al., 2009*).

Summary of global multi-locus sequence typing studies[*].

Study[†]	Origin[‡]	Clinical origin[§]	Number of isolates	CC1 (ST1)[‖]	CC10[¶]	CC17	CC19 (ST19, 28)[‖]	CC23	CC26	Other
Jones[5]	World	Ca, Ninv, Ainv	152	16% (14%)	18%	30%	17% (13%–2%)	12%	2%	5%
Luan[55]	Sweden (1988–1997)	Ninv, Ainv	158	15% (9%)	13%	24%	29% (16%–1%)	14%	0	4%
Manning[56]	Canada Alberta (1993–2002)	Ca, Ninv	413	23% (19%)	14%	16%	20% (19%–NA)	22%	NA	5%
Bohnsack[57]	USA (1995–1999)	Ca, Ninv	899	16% (15%)	9%	13%	17% (12%–2%)	40%	0	5%
Sadowi[58]	Poland (1996–2005)	Ca, Ninv, Ainv	114	17% (13%)	18%	14%	12% (9–2%)	37%	1%	1%
Huber[59]	Kenya (2007–2010)	Ca, Ainv	169	12% (9%)	17%	21%	14% (5%–4%)	27%	2%	7%
Brochet[7]	Dakar Bangui (2006–2007)	Ca	163	20% (9%)	6%	12%	28% (4%–15%)	17%	15%	2%

NA, Not available.

[*] As percentage in each clonal complex.

[†] First author of the publication and reference.

[‡] In parenthesis the years of isolation if known.

[§] The epidemiological origin: Ca, carriage, Ninv, neonatal invasive disease, Ainv, adults invasive disease.

[‖] In parenthesis, the percentage corresponding to the indicated sequence types (STs).

[¶] Corresponds to strains from the closely related clonal complexes (CC) CC6, CC8 and CC10.

Figure3: Multi-locus sequence typing (MLST) of GBS isolated from different countries; most human carriage and clinical isolates cluster into only five major clonal complexes (CC) (CC1, CC10, CC17, CC19 and CC23). **Da Cunha et al., 2007**

As mentioned before, GBS normally resides with its human host as an asymptomatic colonizer of the genital and gastrointestinal tracts. However ,in certain circumstances, it can turn into an invasive pathogen causing severe infections. The transition from a commensal to pathogenic lifestyle is related to adaptive regulated changes in expression of specific bacterial genes in response to diverse host environments. This dynamic adaptation of GBS to the human host involves mechanisms for sensing environmental changes. Bacteria respond to changes in environmental stimuli using signal transduction systems (STS), which regulate the expression and transcription of multiple genes whose products are potentially related to virulence or play a role in metabolic pathways. The most common STS in bacteria are the two-component signal transduction systems (TCS). The genome sequence of GBS has revealed the presence of 17–20 TCS that can respond to changes in the external environment (*Poyart et al., 2001; Glaser et al., 2002; Spellerberg et al., 2002; Tettelin et al., 2002; Tettelin et al., 2005; Quach et al., 2008; Faralla et al., 2014*). The most studied TCS used by pathogenic streptococci to adapt to host conditions is the two component regulatory system CovRS (*Lamy et al., 2004;*

Jiang et al., 2005; Jiang et al., 2008). It consists of a membrane-associated histidine kinase sensor (CovS) and a cytoplasmic transcriptional regulator (CovR). The CovRS system act as a global regulator possessing numerous target genes that belong to different functional categories, such as cell envelope, stress response, metabolism and transport proteins, which often function as virulence factors (*Lamy et al., 2004*).

Therefore, the ability to sense the surrounding host microenvironment can result in the expression of specific virulence factors that allow bacterial persistance, dissemination through the blood and colonization of distant host districts. Indeed, GBS relies on specific adhesins for harmless colonization or host invasion. Many of the GBS adhesins characterized thus far are cell-wall proteins, which are anchored to bacterial surface by a sortase A-dependent mechanism (*Mazmanian et al., 1999; Nobbs et al., 2009*) and are therefore accessible to specific receptors present on host cells or in the blood (*Tazi et al., 2010; Papaseri et al., 2011; Buscetta et al., 2014; Jiang and Wessels, 2014; Wang et al., 2014; Mu et al., 2014; Six et al., 2015*). For example, GBS is capable to bind host Plg during infection and, in the presence of host plasminogen activator

tPA (or uPA), Plg is converted to Pln conferring to this pathogen surface proteolytic activity (*Magalhaes et al., 2007*). A recent study indicated that Pg activation on the surface of GBS occurs through cell surface moonlighting proteins, such as α-enolase, glyceraldehyde dehydrogenase and phosphoglycerate kinase that can bind Plg and Pln (*Magalhaes et al., 2007; Boone and Tyrrell, 2012; Oliveira et al., 2012*). Moreover, GBS release extracellularly a protein named Skizzle that can bind Plg and accelerate its conversion to Pln by host activators, although the actual role of this protein in the pathogenesis of GBS disease has not been studied (*Wiles et al., 2010*). In addition, different GBS strains also express unrelated fibrinogen receptors (FbsA, FbsB or FbsC) that might indirectly bind to Plg (*Gutekunst et al., 2004; Pietrocola et al., 2005; Buscetta et al., 2014*). In the context of the hypervirulent CC17 clonal complex, a multifunctional adhesin, the serine rich repeat (Srr2) glycoprotein, that is able to bind both Plg and Fbg can promote GBS invasiveness and the development of meningitis (*Brochet et al., 2006; Da Cunha et al., 2014; Six et al., 2015*).

The aim of the studies presented in this thesis was to identify novel virulence factors of GBS that might be involved in binding to host factors, particularly Plg. First, a novel protein (Gbs0428 or PbsP) was identified by proteomics analysis of the GBS exoproteome and found to display two SSURE domains (*Bumbaca et al., 2004*), homologous with those of the previously characterized P̲lasminogen and f̲ibronectin b̲inding p̲rotein B̲ (PfbB, also called PavB) of *Streptococcus pneumoniae* (*Papasergi et al., 2010*). Next, the role of Gbs0428 in the contest of GBS pathogenesis was investigated. After showing that Gbs0428 is a cell-wall-anchored surface protein that binds Plg, it was found that this adhesin is expressed by isolates belonging to all of the main GBS lineages, including strains of the hypervirulent clonal complex 17 (CC17), which is responsible for a large number of neonatal GBS infections, particularly meningitis cases. In the context of the CC17 prototype BM110 strain, PbsP was found to be markedly upregulated *in vivo*. PbsP was also found to bind Plg largely through the interaction between lysine-binding sites in the kringle 4 domain of Plg with a novel protein domain that was named MK-rich region. Moreover, PbsP-mediated Plg binding conferred proteolytic

activity to GBS surface, which ensured GBS transmigration across endothelial

cells and colonization of the brain. All together these data indicate that PbsP

has an important role in the pathogenesis of GBS infections and might

represent a target in alternative strategies to control GBS infection.

Materials and Methods

Bacterial strains and reagents

In this study we used the following reference GBS strains: 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) (*Glaser et al., 2002; Tettelin et al., 2005; Da Cunha et al., 2014*). 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 mg/ml; erythromycin, 150 mg/ml, kanamycin, 25 mg/ml; and for GBS: erythromycin, 10 mg/ml; kanamycin, 500 mg/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*). Kringle 1-3 and Kringle 1-4 were purchased from Sigma and MyBioSource, respectively. The mini-PLG (residues Val442-Asn790) was obtained by digestion of PLG with porcine

pancreatic elastase (Sigma) as described previously (Christensen, U. and

Mølgaard, L. 1992, Váli, Z. and Patthy, L. 1982). Aminocaproic acid (6-ACA),

L-Lysine or L -Alanine, used as inhibitors, were purchased from Sigma-

Aldrich. N-terminal Mk-rich (residues 423–443), Fr1-Cterminal MK-rich

(residues 444–468), and Fr2-Cterminal MK-rich (residues 469–492) were

purchased as biotinylated peptides from GenScript (**Table4**).

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 3**. Analytical

PCR used standard Taq polymerase (Invitrogen, Life Technologies).

Preparative PCR for cloning and PCR for sequencing (GATC Biotech) were

performed with a high-fidelity polymerase (MyFi or Phusion DNA

polymerase, Bioline and Thermo-Scientific respectively). The pG1_DpbsP

vector used for pbsP deletion was constructed as described (*Firon et al., 2013*)

using a splicing by overlap extension method (Heckman and Pease, 2007) with

primers pAF375_EcoRI1pAF376_D0428 and pAF377_D04281pAF378_BamHI.

After GBS transformation with pG1_DpbsP and selection of pG1_DpbsP

integration and de-recombination events, marker-less deletion of pbsP was

confirmed on genomic DNA with primers pAF3791380 (positive PCR product

in case of pbsP deletion) and pAF3811382 (positive PCR product in case of a

WT pbsP gene). Primers pAF553_BamHI1pAF554_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_Ptet

vector at the corresponding restriction sites to give the constitutive pbsP

expressing vector pTCV_Ptet_pbsP. The full length insert was sequenced to

confirm the absence of mutations. The pTCV_Ptet_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 3**) 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: MKrich423_up and MK-rich492_low (rMK-rich); SSURE123_up and SSURE422_low (rSSURE$_{1-2}$); SSURE273_up and SSURE422_ low (rSSURE$_2$) and Cterminal MK-rich444_up and MK-rich492_low (rCterminal Mk-rich) (**Table 3)**. 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_SSURE112, pDEST15_SSURE2 and pDEST15_Cterminal Mk-rich (**Table2**) 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. With the aim of speeding up the workflow, we purchased biotinylated peptides that

encompassed MK-rich region from GenScript company (Table 4). To have a negative control we used the PinPoint ™ Xa Control Vector (Promega) containing the CAT gene fused to the biotinylated protein tag. After induction, cells containing the PinPoint ™ Xa Control Vector produces a 40kDa protein composed by the CAT peptide (27kDa) and by a 13kDa biotinylated peptide. Biotinylated fusion protein is produced in *E. coli* and are affinity-purified using the SoftLink™ Soft Release Avidin Resin (Promega).

Production of anti-PbsP antisera

CD1 mice (5 weeks old, Charles River Labs) were injected intraperitoneally with 20 ug 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, care was 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 ug 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 antimouse IgG (R&D Systems). Loading controls consisted of parallel Coomassie-stained gels. For Far western analyses, Plg (10 ug) was run on 12% acrylamide gels, transferred on nitrocellulose, and overlaid with 0.15 lM rPbsP or GST in 1% of nonfat dry milk supplemented with 0.05% Tween 20. Complex formation was detected using goat anti-GST IgG (1:4000: GE Healthcare) followed by alkaline phosphatase-conjugated anti-goat IgG

(1:5000) (Sigma-Aldrich). rPbsP or GST (0.15 uM) were run on 12% acrylamide gels, transferred on nitrocellulose and overlaid with Plg (1 ug/ml). Complex formation was detected using anti-Plg rabbit polyclonal antibody (1:5000) and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Amersham Biosciences).

Flow cytometry analysis

Surface-expressed PbsP 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). Then bacteria were incubated with anti-rPbsP or anti-GST serum diluted 1:250 followed by FITC or phycoerythrin-conjugated goat anti-mouse IgG (diluted 1:1000 or 1:200 respectively; Sigma-Aldrich), as described (*Boone et al., 2011; Cardaci et al., 2012*). Fluorescent bacteria were analysed with a MACS Quant VYB, FACS-CantoII flow cytometer using the FlowJo software (BD Biosciences).

Coating of microspheres with recombinant proteins

Fluorescent beads (Fluoresbrite YG 1.00-m microspheres, Polysciences) were conjugated with recombinant PbsP or PbsP fragments (or with GST, as a control) according to the manufacturer's instructions using a protein concentration of 300 ug/ml. The amount of protein coupled on beads was calculated by subtracting from this amount the quantity of protein present in the supernatant after adsorption. Care was taken to use in each experiment beads with similar amounts of coupled proteins. We coupled Fluorescent beads with rPbsP (GST-PbsP), $SSURE_{1-2}$ (GST $SSURE_{1-2}$), $SSURE_2$ (GST $SSURE_2$), rMK-rich (GST-Mk rich), rC-terminal Mk-rich (GST C-ter Mkrich), rGST (as a negative control), N-terminal Mk-rich (Biotin N-ter Mk-rich purchased from GenScript), Fr1C terminal MK-rich (Biotin Fr1Cter Mk-rich purchased from GenScript), Fr2C terminal MK-rich (Biotin Fr2Cter Mk-rich purchased from GenScript) and CAT-Biotin (as a negative control).

Adhesion of PbsP conjugated microspheres to immobilized human proteins

For the microsphere adhesion assay, silane-treated 18-mm^2 glass coverslips were incubated overnight at 4°C with the proteins indicated below (50 ug/ml in carbonate buffer 0.1M,pH9.6), blocked with PBS supplemented with 5% BSA for 2 h at 20 °C, and exposed to 10^8 conjugated- beads in 1 ml of PBS supplemented with 2% BSA. After incubation at 20 °C overnight, the slides were washed and observed under a fluorescent microscope. Results were expressed as number of particles per field of vision at the indicated magnification. At least 20 different fields per slide were counted. The following substrates were used: plasminogen (Plg), collagen (Coll), fibronectin (Fnt), fibrinogen (Fng), C-reactive protein (CRP) or the complement components factor H (FH), factor I (FI), factor B (FB), C1q and C3. For competitive assay, silane-treated 18-mm^2 glass coverslips were incubated overnight at 4 °C with the plasminogen (50 ug/ml in carbonate buffer 0.1M,pH9.6), blocked with PBS supplemented with 5% BSA for 2 h at 20 °C and exposed O.N to 10^8 rPbsP conjugated-beads, in the presence of the

following inhibitors: 0–300 mM aminocaproic acid (6-ACA), L-Lysine or L-Alanine (Sigma-Aldrich). The same procedure was used to test the binding of the whole protein rPbsP and its fragments SSURE1 / 2 SSURE2 and Mkrich to glass coverslips immobilized plasminogen and to identify the minimal region of the MK-rich domain that still maintains the ability to bind Plg. To this end we used N-terminal and C-terminal Mk-rich and Fr1C and Fr2C terminal MK-rich conjugated-beads.

Bacterial attachment to immobilized plasminogen

Microtiter plates were coated overnight at 4 °C with plasminogen at the indicated concentrations in PBS. The wells were washed three times with PBS before the addition of 10^5 CFU of GBS to each well, and the plates were then incubated for 1 h at 37 °C. After extensive washing, the wells were treated with trypsin (2.5 mg/ml, Sigma) for 10 min at 37°C to release the attached bacteria, which were then enumerated by agar plate counts.

ELISA binding assay using whole plasminogen or its fragments

Binding of PbsP to whole plasminogen or to its fragments (kringle 1-3, Kringle 1-4 and Mini-Plg) was determined using ELISA assays. Microplates coating was done with 100 ul of 5ug/ml solutions of whole plasminogen or its fragments in 0.1 M carbonate buffer (pH 9.0) overnight at 4°C. The wells were incubated for 1 h at 22°C with 200 ml of 2% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for blocking, followed by addition of 0.15 uM of rPbsP or rGST for one hour, and incubation with goat anti-GST (diluted 1:10,000 in PBS 0.1% BSA) for 90 min. After adding peroxidase-conjugated secondary anti-goat IgG (diluted 1:1000) for 1 h, 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.

Bacterial adhesion and invasion

Human epithelial (Caco-2 and A549) cell lines were obtained from the American Type Culture Collection. 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 (*Weksler et al.,*

2005; Buscetta et al., 2014, Papasergi et al.,2010). Briefly, bacteria (Nem316 WT,

Nem ΔPbsP, Nem316 ΔPbsP+PbsP) 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 ug/ml respectively) to kill extracellular

bacteria. Percentages of bacterial adhesion and invasion were calculated as

recovered CFU/initial inoculum CFU x 100.

Adhesion of conjugated microspheres to cultured cells

A549 cells were prepared as described above under "bacterial adhesion

and invasion," and protein-coupled beads were added in serum-free DMEM

medium with 2% of BSA at a concentration of 10^8 beads/ml. After 1h of

incubation at 37 °C, monolayers were washed three times, and attached beads

were counted by using a fluorescent microscope. At least 300 cells were

counted. To eliminate GST background the monolayers were pre-incubated with 100ug/ml of rGST in serum-free DMEM and antibiotics O.N. at 37°C ,and extensively washed, and the assay was performed as described above. To discriminate the number of beads associated to cell line, cells were fixed with formaldehyde , and stained with Coumarin- Conjugated Phalloidin (Sigma Aldrich)-to label actin filaments- and DAPI (Sigma Aldrich) for nucleic acid staining according to the manufacturer's instructions.

GBS migration assay across hCMECs

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 um (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 ml of growth medium containing $1x\ 10^6$ 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 5–7 days at 37°C in a humidified chamber containing 5% CO_2 to reach confluence. Only monolayers with a

transendothelial electric resistance (TEER) greater than 200 ohm cm22 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 ug plus tPA (20 nM) were applied to the apical chamber (total volume of 500 ml 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.

In vitro qRT-PCR analysis of PbsP in GBS strains

THB broth was inoculated (1:20) with an overnight culture of GBS strain and incubated at 37°C. Exponentially growing cells (OD560 0.5–0.6) were harvested for 1 min at 13000 rpm at RT, washed twice in PBS and resuspended in 400µl of Tris 10mM pH8 added with 0.4 g of glass beads (425-600 µm diameter, G8772 Sigma). The cells were then mechanically disrupted by vortexing and chilling on ice for several times. After centrifugation at 13 000 g

for 5 min and the supernatants was transferred to a fresh tube.Total RNA was qualitatively analyzed on agarose 1% gel, quantified by nanodrop 1000spectrophotometer (Thermo Scientific) and purified using the "High Pure RNA Isolation Kit"(Roche). cDNA synthesis was performed on purified RNA with random primers using SuperScript II reverse transcription (Invitrogen). For the quantification of PbsP mRNA, qRT-PCR analysis were conducted, in duplicate, with an Applied Biosystems 7500 (Applied Biosystems) using primers and probes listed in **Table 3**. Primers and TaqMan MGB probes were purchased from Applied Biosystems.PCR conditions were as follows: 95°C, 10 min; (95°C, 15 s; 60°C, 1 min) × 40 cycles. Real-time PCR data were normalized in each individual sample by the level of GyrA expression. Gene expression was measured by the comparative CT method ($\Delta\Delta$CT) and was reported as the n-fold difference relative to the normalized expression of BM 110 WT strain.

Animal 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). To investigated about in vivo role of PbsP into the context of the

hypervirulent CC17 lineage, we used BM110, an CC17 prototype strain.

Virulence of GBS strains (BM110 and its ΔpbsP isogenic mutant) were tested

with 8-week-old CD1 mice infected intravenously (i.v.) with 6x 10^7 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 11 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 effect of PbsP immunization, CD1 mice (5 weeks

old, Charles River Labs) were injected intraperitoneally (i.p.) with 50 ug 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 9×10^7 CFUs of the BM110 WT strain. To verify if the PbsP immunization results in a change of bacterial CFU in brains and kidneys, in further experiments GBS infected mice were sacrificed at 48 h after infection to collect organs and to count the number of CFU as indicated above.

Ex vivo qRT-PCR analysis

Mouse samples were obtained from individual animals immediately after the sacrifice. Whole brains and kidneys were collected from mice i.v. infected with 6×10^7 bacteria (BM110 WT) and homogenated in 2ml of DPBS. All samples were put on ice and subjected immediately to a low speed centrifugation (800 g, 5 min, 4°C) to remove the cell debris. The supernatant, containing the bacteria, was transferred into a new tube and centrifuged (10 000 g, 5 min, 4°C). The pellet of all samples was resuspended in 400µl of Tris 10mM pH8 added with 0.4 g of glass beads (425-600 µm diameter, G8772 Sigma). The cells were then mechanically disrupted by vortexing and chilling on ice for several times. After centrifugation at 13 000 g for 5 min and the supernatants was transferred to a fresh tube. Total RNA analysis, purification

and cDNA synthesis were performed as described before. Gene expression

was measured by the comparative CT method ($\Delta\Delta$CT) and was reported as

the n-fold difference relative to the normalized expression of BM 110 WT

strain grown in THB broth.

Table 1. GBS strains

Strains	Features	Ref.
Nem316 WT	Clinical isolate, serotype III (CC23)	Glaser et al. (2002)
Nem ΔpbsP	Deletion of pbsP in NEM316	
NemSrtA*	NEM316 with an inactive sortase A enzyme	Konto-Ghiorghi et al. (2009)
Nem ΔcovSR	covSR deletion in NEM316	Lamy et al. (2004)
Nem ΔpbsP/pTCV_ Ptet_ pbsP	NEM316 with the pbsP expression vector	
BM110 WT	Clinical isolate, serotype III (CC17)	
BM ΔpbsP	Deletion of pbsP in BM110	
BM ΔcovR	covR deletion in BM110	
BM ΔpbsP/pTCV_ Ptet_ pbsP	BM110 with the pbsP expression vector	

WT, wild type; pTCV_Ptet_pbsP, vector with the pbsP gene under the control of the Ptet constitutive promoter.

Tabella 2. Plasmids used in this study

I.D.	Description	Ref.
Plasmids		
pGEX-SN	E. coli expression vector, Amp^R	GE Healthcare Life Sciences
pGEX-SN_pbsP	pGEX-SN expression vector carrying pbsP (residues 28–462)	This study
pG1	Thermosensitive shuttle vector, oriRts pWV01, Erm^R	Mistou et al. (2009)
pG1_DpbsP	pG1 with the pbsP deletion cassette	This study
pTCV_Ptet	GBS expression vector, constitutive Ptet promoter, Erm^R, Km^R	Firon et al. (2013)
pTCV_Ptet_pbsP	pTCV_Ptet 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 123–422)	This study
pDEST15_SSURE$_2$	pDEST15 expression vector carrying with one PbsP SSURE domain (residues 273–422)	This study
pDEST15_MK-rich	pDEST15 expression vector carrying the MK rich sequence (residues 423–492)	This study
pDEST15_Cterminal Mkrich	pDEST15 expression vector carrying the MK rich sequence (residues 444–492)	This study
PinPoint ™ Xa Control Vector	E. coli expression vector, AmpR	Promega

Erm^R, erythromycin resistance; Km^R, kanamycin resistance; Amp^R, ampicillin resistance.

Tabella 3. Oligonucleotides used in this study.

I.D.	Description
gbs0428_BamHI	TTT<u>GGATCC</u>GCATTGTTGCTTTAGGGCAATCT
gbs0428_NotI	TTTTTTT<u>GCGGCCGCC</u>GGCTTGTGATGACATTTGCATATT
pAF375_EcoRI	ACATGAATTCGTCCTATGGACAGTCATTCACAT
pAF376_D0428	CTATCCCTATCTTTGTTTAGATTTGTTAATGAGAGTATACCAAC
pAF377_D0428	GTTGGTATACTCTCATTAACAAATCTAAACAAAGATAGGGATAG
pAF378_BamHI	AGTA<u>GGATCC</u>TTGATGATTCCCATAGATACGTC
pAF379	TGAAAGTCAAGAAGCAGAGACA
pAF380	TTTGATAGTTGGGGTATTGTCTG
pAF381	TACGACAGGTAAAGAGGGACAAG
pAF382	GGCTGTATTGCCATTAAGAGAGA
pAF553_BamHI	TGAT<u>GGATCC</u>GAAAGAGGAATCCCTTATGAAAATATC
pAF554_PstI	TGAT<u>CTGCAG</u>CTGAATTAAATACTATCCCTATC
SSURE$_{123}$_up	GGGG**ACAAGTTTGTACAAAAAAGCAGGCTTT**GAGGTAAAGAAATCAACTGATACTG
SSURE$_{273}$_up	GGGG**ACAAGTTTGTACAAAAAAGCAGGCTTT**GAAGTAAAAAATTCTCTAAAGATG
SSURE$_{422}$_low	GGGG**ACCACTTTGTACAAGAAAGCTGGGTT**TTACATATCTTTTTTAGCGACATTAAT
MK-rich$_{423}$_up	GGGG**ACAAGTTTGTACAAAAAAGCAGGCTTT**AATAGCACATCAATGATGATG
MK-rich$_{492}$_low	GGGG**ACCACTTTGTACAAGAAAGCTGGGTT**TTACTTTGTTTCACCTGTATTAGGTAA
Cterminal MK-rich$_{444}$_up	GGGG**ACAAGTTTGTACAAAAAAGCAGGCTTT**TCATCTAAAATAAATACCGGTATGA
GyrA up	AACTGGGAAGGGATCAATTGTCT
GyrA low	CTTTCCCATTTGAAGTTGTTTCAA
GyrA FAM	ACGTTCACGGACTGAA
PbsP up	TGATGGTGGCTACCTCTGTTAGAA
PbsP low	TGTAGCGATGACCAAAACGACT
PbsP FAM	ATGGCAACGCCGACAC

Underlined nucleotides represent restriction sites. Recombination sites are indicated by nucleotides in bold.

Tabella 4. Biotinylated peptides

I.D.	Description	Ref.
Nterminal Mk-rich (residues 423–443)	NSTSMMMKKDKMTMPMKKEMT	From GenScript
Fr1Cterminal Mkrich (residues 444–468)	SSKINTGMMMSNNKMSANMQMSSQA	From GenScript
Fr2Cterminal Mkrich (residues 469–492)	KSNDKAGKKMSMMSKN	From GenScript

Every peptides were synthesized in fusion with Biotin (N-terminal Biotin-Ahx)

Results

Identification of PbsP by proteomics

In an effort to identify novel virulence factors of *Streptococcus agalactiae*, we first performed a proteomic analysis, which was used to gain a comprehensive view of the complex of extracellular proteins (i.e. the exoproteome) secreted by GBS under varying gene expression conditions. To this end, we compared, by mass spectrometry analysis, the exoproteome of the WT strain NEM316 with that of an isogenic mutant (*ΔcovRS*), bearing a deletion encompassing both the covR and covS genes, which encode for a two-component, master regulator of virulence gene expression in GBS. Whole supernatants of GBS NEM316 WT and its covRS deletion mutant (*ΔcovRS*) grown in CCDM medium were collected during the late exponential phase of growth using a synthetic, entirely dialyzable medium (CCDM ;Carey's chemically defined medium -*Carey et al., 1980*) and directly subjected to LC–MS/MS analysis after precipitation and trypsin treatment. After data acquisition, individual MS/MS spectra were combined, smoothed, and centroided using ProteinLynx version 3.5 (Waters) to obtain the peak list file. Search and identification of peptides were performed in

batch   mode   with   Mascot

(http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF),

using the NCBI database (http://www.ncbi.nlm.nih.gov/). We found, in the

supernatants of the ΔcovRS, but not the WT strain, one peptide

(KTTITAPTANTSTNVESSTDKA) that mapped to the hypothetical protein

gbs0428 in the genome of the NEM 316 stain (*Glaser et al., 2002*).

Bioinformatics analysis of PbsP

Sequence analysis indicated that *gbs0428* is an open reading frame (ORF)

encoding for a hypothetical cell wall-bound protein, based on the presence of a

signal peptide and an LPXTG motif. The protein displays two SSURE domains

homologous with those of the recently characterized *Plasminogen- and*

fibronectin-binding protein B (PfbB) of *Streptococcus pneumoniae* (*S. Papasergi., et al.*

2010). PfbB contains a variable number of repetitive sequences (*Jensch et al.,*

2010; Papasergi et al., 2010), referred to as *Streptococcal SUrface REpeats* (SSURE),

initially described as a fibronectin (Fnt) binding motif (*Bumbaca et al*). These

findings led us to hypothesize that the protein might play a role as a bacterial

adhesin capable of binding to extracellular matrix components. Based on its

homology with PfbB, we tentatively named the protein as plasminongen-binding surface protein (PbsP). The Pfam database (http://pfam.xfam.org/search/sequence) described the SSURE domains of the GBS0428 protein (or PbsP) as being 68 amino acids long, which was in contrast with the original description from Bumbaca et al (*Bumbaca et al., 2004*) of 150 aa-long domains. However, through a manual scrutiny of the sequences of these domains and the use of the RADAR program (http://www.ebi.ac.uk/Tools/pfa/radar/), we determined that the total length of these repeats is actually 150 amino acids, in accordance with the work of Bumbaca et al. (*Bumbaca et al., 2004*). **Fig. 1** summarizes the structural features of PbsP. The protein is 521-aa long and contains an NH2- signal peptide with 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**). Further analysis indicated that the *gbs0428* locus is conserved within the family of *Streptococcaceae*. All sequenced GBS human clinical isolates, including strains belonging to the hypervirulent clonal complex

17 (CC17), display a highly homologous (99.3%) identical *gbs0428* (or *pbsP*)

locus, containing two SSURE domains (*Da Cunha et al., 2014*). Moreover, no

major difference was observed between the *pbsP* promoter regions among GBS.

GBS SSURE domains are closely related to similar domains present in other

streptococcal species, particularly *S. pneumoniae*, as shown in **Fig. 2.** Sequences

displaying significant homology with GBS SSURE domains are found only in

Streptococcaceae, particularly in *S. gordonii*, *S. mitis*, S. *sanguinis*, S. *cristatus*, S.

oralis, S. *anginosus*, S. *intermedius*, in addition to *S. agalactiae* and *S. pneumoniae*.


```
A
NH2 — SP  N-terminal      SSURE1            SSURE2           MK rich LPXTG —COOH

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

Fig1 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)
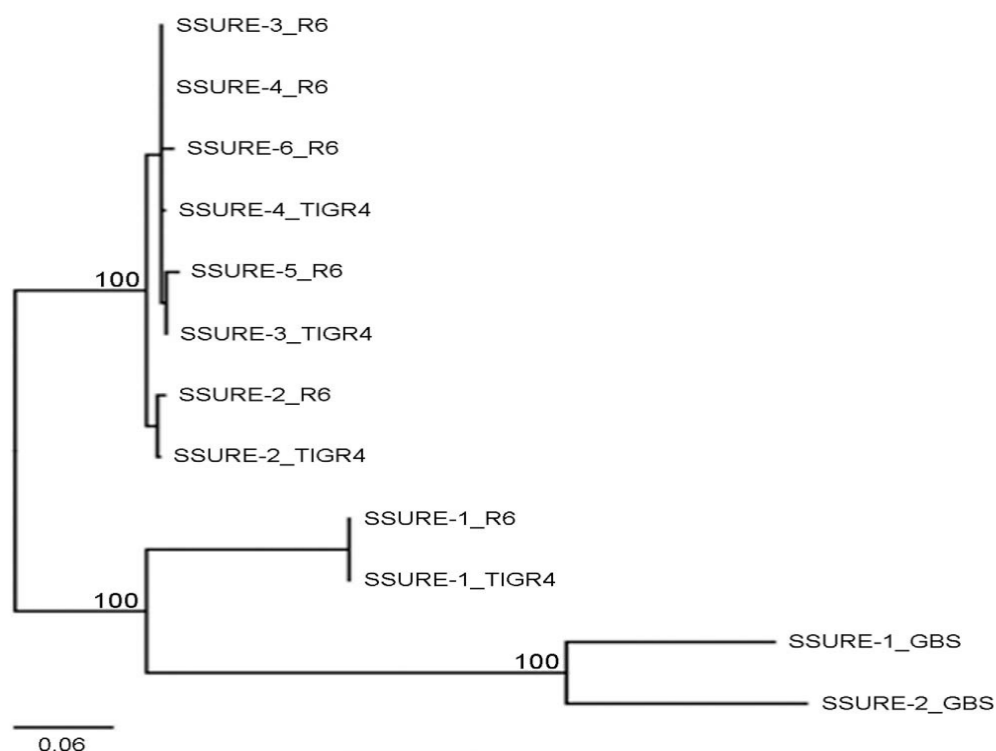
Fig2 Sequence divergence between *S. pneumoniae* **and** *S. agalactiae* **SSURE domains.** The two SSURE domains (SSURE-1 and -2) of the GBS protein PbsP from strain NEM316, the four and six SSURE domains (SSURE-1 to -6) of the pneumococcal protein PfbB (PavB from, respectively, strains TIGR4 and R6 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.

PbsP is expressed in the cell wall and on the surface of GBS

To verify the presence of the PbsP protein on the bacterial surface, we undertook flow cytometry and Western blot analysis of cell-wall-extracts using a polyclonal mouse serum (pAb) raised against a recombinant form of PbsP. Preparation of recombinant PbsP and pAb is reported in the Materials and Methods section. We first compared wild type (WT) NEM 316 with *ΔpbsP* and *ΔcovRS* mutant strains. In the *ΔpbsP* strain, the gene encoding PbsP was deleted in-frame in the NEM316 chromosome, while –as indicated above- the *ΔcovRS* mutant bears a deletion encompassing both the *covR* and *covS* genes.In addition we used a strain (*srtA**) bearing a defective form of sortase A, an enzyme that is

required for linking to the cell wall proteins with an LPXTG motif. All of these mutants showed similar viability, morphology and growth in Todd-Hewitt broth, as compared to the WT parental strain (data not shown).

Western Blot Analysis (**Fig. 3A**) revealed an immunoreactive band in the WT, but not in the *ΔpbsP*, strain, migrating with an apparent molecular weight of 48 KDa, which is slightly higher than the expected mol weight (40 kDa). This discrepancy is likely linked to a relative lack of precision of SDS-PAGE analysis in determining the molecular weight of elongated proteins such as those found on the surface of gram positive bacteria. The PbsP protein was markedly overexpressed in the *ΔcovRS* mutant, in agreement with previous transcriptomics and proteomics studies (*Lamy et al., 2004*; *Firon et al., 2013*). Notably, the protein was not present in cell wall extracts of the *srtA**, strain, indicating a requirement for sortase A for expression in the cell wall of this LPXTG protein. Further evidence for surface expression of PbsP was sought by Facs analysis using pAb. As expected, we detected PbsP on the surface of the WT NEM316 strain, but not on the *ΔpbsP* mutant, while the protein was highly expressed on *ΔcovRS* (**Fig3B**).

To gain a comprehensive view about the expression of this protein in the context of GBS strains belonging to different capsular serotypes (CPS) and clonal complexes (CC), we included in these experiments the representative strains GBS 6313 (CPSIII/CC23, as NEM316), A909 (CPSIa/CC1) BM110 and COH1 (both CPSIII/CC17) and 2603V/R (CPSV/CC19). PbsP expression on the bacterial surface was detected in all of these strains, with the lowest grade of

signal intensity in BM110, COH1 and 2603V/R strains. We also observed that a non-capsulated NEM316 mutant produced a signal of the same intensity as the WT strain, suggesting that the polysaccharide capsule did not significantly mask the display of the protein at the bacterial surface (data not shown).
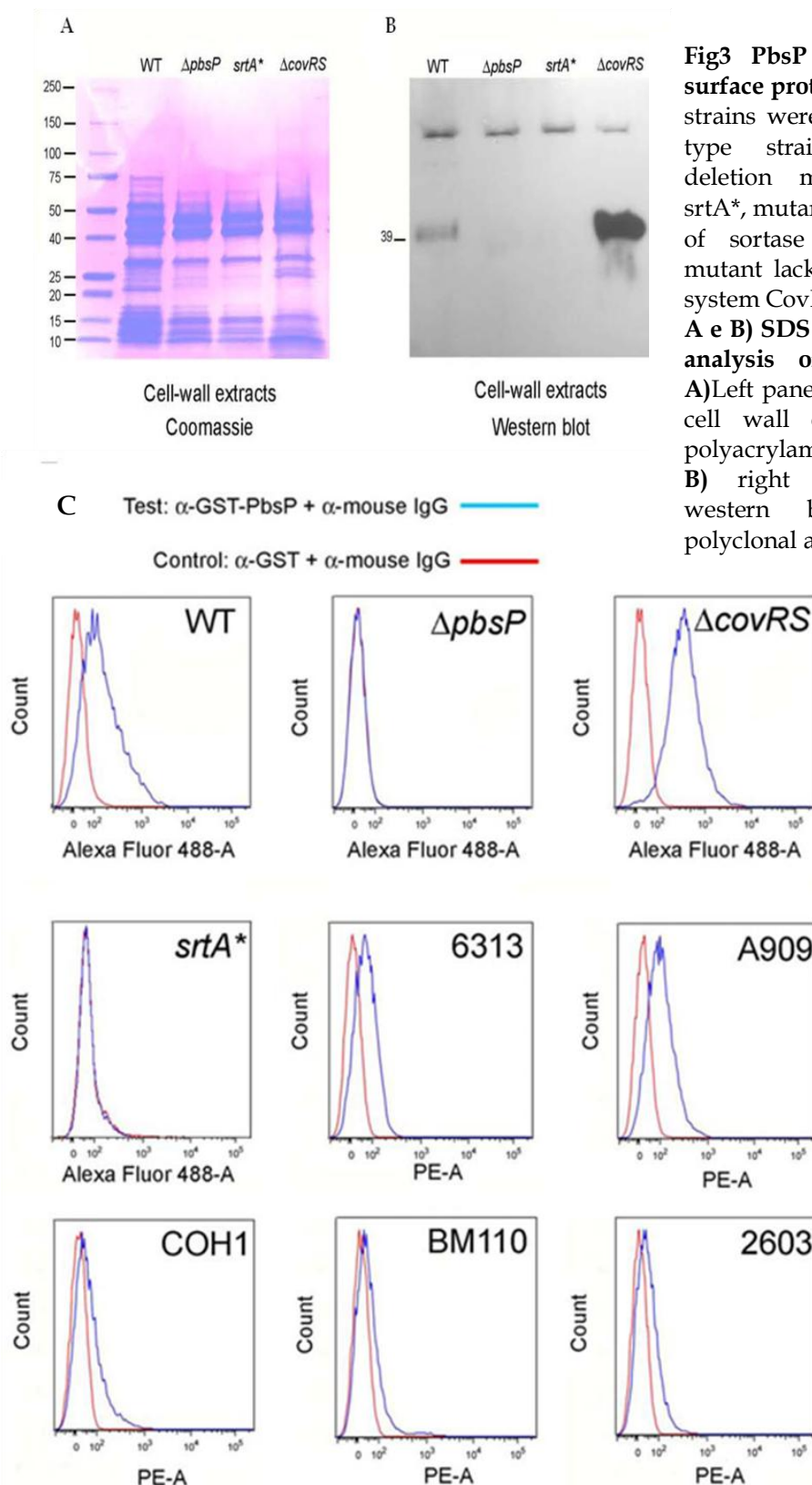
Fig3 PbsP is a conserved cell surface protein. The following GBS strains were compared: WT, wild-type strain NEM316; ΔpbsP, deletion mutant lacking PbsP; srtA*, mutant with an inactive form of sortase A; ΔcovRS deletion mutant lacking the twocomponent system CovRS.

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

C) 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).

PbsP binds to immobilized plasminogen

In order to investigate the functional role of PbsP in bacterial adherence, we measured binding of the recombinant protein to different substrates immobilized on glass coverslips. Recombinant PbsP was absorbed to fluorescent beads with a diameter of 1 micron, to mimic surface exposure of this protein on the bacterial surface. Binding of the beads to extracellular matrix or blood components (such as Plg, Fng, Fnt, collagen, C-reactive protein and the complement components factor-H, factor I, factor-B, C1q and C3) was assessed microscopically. These experiments showed that PbsP-coated beads bound Plg, but not other plasma/matrix components (**Fig4A**). To confirm binding of PbsP to Plg we performed Far western blotting experiments in which the recombinant protein was used as a probe to capture soluble Plg, or vice-versa. As shown in **Fig. 4C**, the ability of PbsP to bind Plg was confirmed using both approaches.

The kringle domains of plasminogen contain lysine-binding motifs that mediate the binding of plasminogen to fibrin, ECM components and several bacterial surface proteins (*Martina L. Sanderson-Smith et al ., 2012*).

To understand whether interactions between plasminogen and PbsP occur through the involvement of lysine binding sites on Plg, increasing concentrations of lysine or of the lysine analog ε-aminocaproic acid (6-ACA) were used to analyze the binding of rPbsP-coated beads to Plg immobilized on glass coverslips. Before adding the rPbsP-beads, slides were pre-incubated with increasing concentration of putative inhibitors. L-alanine, a non-charged amino

acid, was been used as a negative control. In the presence of 6-ACA or lysine , but not L-alanine, PbsP-Plg interaction were inhibited in a dose dependent manner, suggesting an involvment of the lysine-binding motifs in the plasminogen kringle domains (**Fig.4B**).

To test the contribution of PbsP to the overall ability of GBS to bind plasminogen, we compared NEM316 WT and *ΔpbsP* mutant for their capabilities to adhere to plastic plates coated with plasminogen. Under these conditions, adherence of the *ΔpbsP* mutant to plasminogen was almost completely abrogated in comparison with the WT strain (**Fig4D**). Genetic complementation of the *ΔpbsP* mutant restored PbsP expression and its ability to bind immobilized plasminogen, thus confirming that the inability of the mutant strain to bind plasminogen is due to deletion of the *pbsP* gene. All together these data indicate that PbsP is an important adhesin largely mediating binding of GBS to Plg, at least of in the context of CC23 strains such as NEM316.
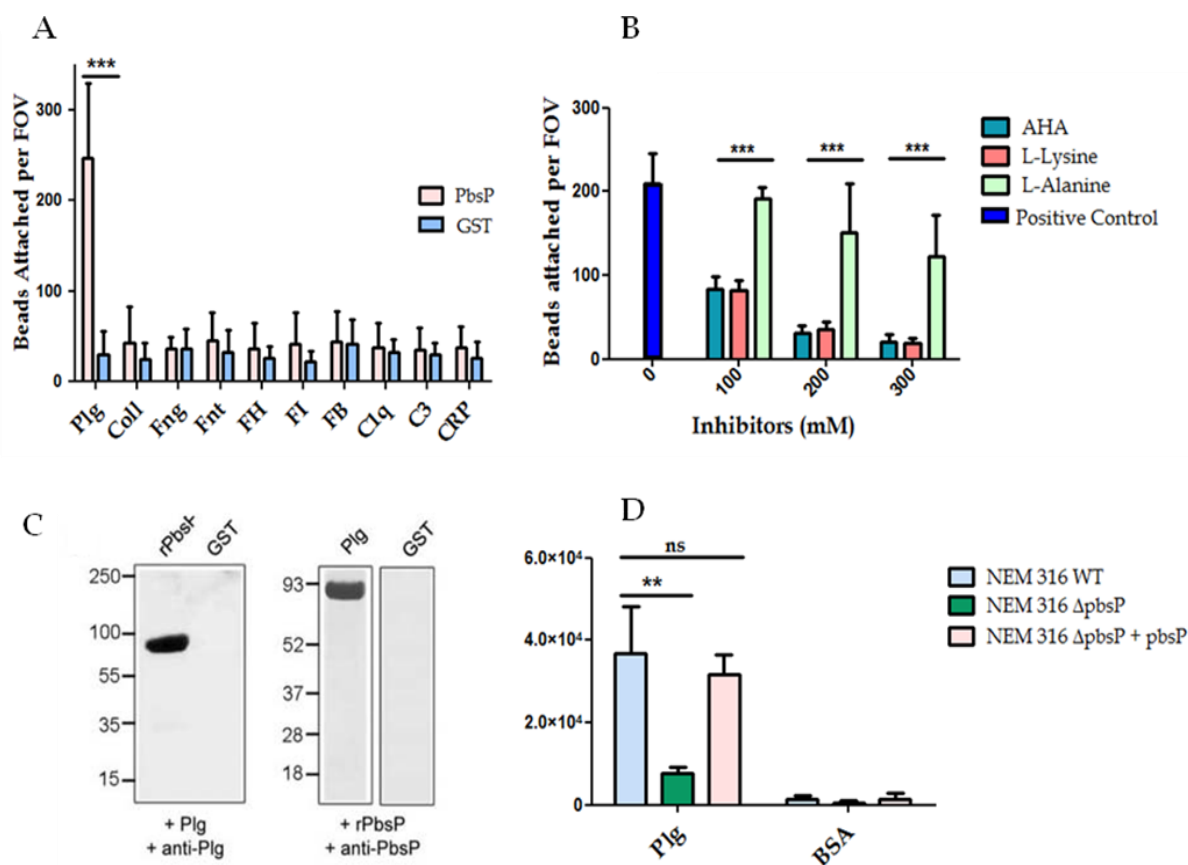
Fig4. PbsP binds human plasminogen in vitro.
A) Selective binding of PbsP to immobilized plasminogen. Binding of the rPbsP-coated beads to immobilized human plasminogen (Plg), collagen (Coll), fibronectin (Fnt), fibrinogen (Fng), C-reactive protein (CRP) or to the complement components factor H (FH), factor I (FI), factor B (FB), C1q and C3 was quantified microscopically counting the number of fluorescent beads atthached on the plasminogen cotated coverslips. Columns and bars indicate means ± SD from three independent experiments.

B) Selective inhibition of PbsP-Plg interactions. Competitive binding assays were done with immobilized Plg to which rPbsP cotated beads were added in the presence of the 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.

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) Contribution of PbsP to the overall ability of GBS to bind plasminogen. Wild-type NEM316 (WT) was compared with its PbsP deletion mutant (ΔPbsP) or ΔpbsP strain carrying a complementing vector with constitutive pbsP expression (ΔpbsP+PbsP). Binding of GBS strains to plasminogen (Plg) immobilized on plastic plates was detected counting the number of CFU after shaving with trypsin. The results shown are mean ± S.D. of CFU counts from three independent experiments. * = p ≤ 0.05 by analysis of variance and Student's Neuman Keuls test.

The plasminogen Kringle 4 domain is involved in PbsP binding

The Plg molecule contains a total of seven structural domains, each with different properties. The N-terminal portion of the molecule consists of an activation peptide (AP) followed by a series of 5 repeating homologous triple-disulfide-linked peptide regions, approximately 80 amino acids in length, termed kringles (K1–K5) containing lysine binding sites and the catalytic SP domain (*Sarbani Bhattacharya et al., 2012*). The function of the five kringles in the heavy chain of Plg is primarily to mediate protein-protein interactions, such as those between Plg or Pm and fibrin, ECM targets, and lysine-containing receptors (*Sarbani Bhattacharya et al 2012*). To identify the plasminogen domain predominantly responsible for interaction with PbsP, we developed an ELISA assay where the whole plasminogen molecule or its fragments (K1-K3, K1-K4 and Mini-Plg a complex of Kringle5 and serine protease domain) are coated on a microplate and binding of PbsP-GST is detected using anti-GST antibodies . This assay showed that Kringle 4, but not the others Kringle domains, was capable to bind PbsP tp the same level as that of the whole plasminogen molecule (**Fig. 5**).
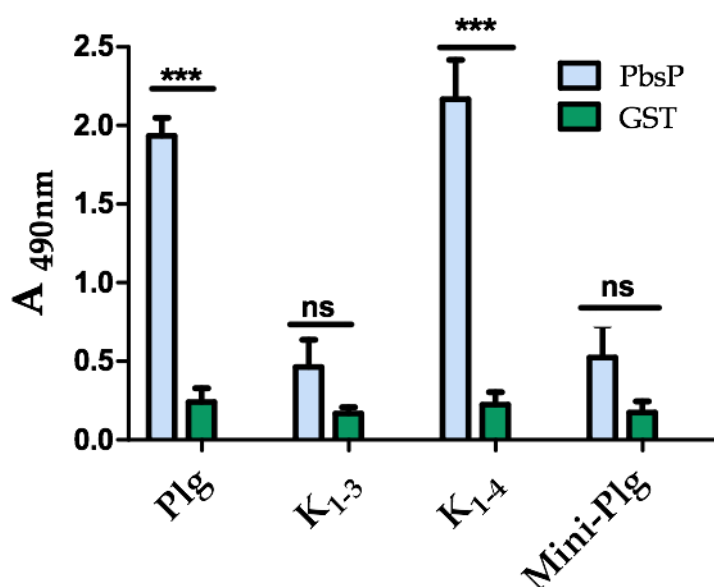
Fig5. Plasminogen Kingle4 is involved in PbsP/Plg interaction Elisa of PbsP/Plg interactions. Elisa were done coating Plasminogen and its fragment on a microplate and the binding with rPbsP and GST (as negative control) was detected using anti-GST antiboby.

The C-terminal region of PbsP is involved in Plasminogen binding

In order to identify the PbsP domain predominantly involved in binding plasminogen, we carried out additional experiments using fluorescent beads coated with different PbsP-fragments. We first obtained recombinant fragments that encompassed different PbsP regions, such as the two SSURE domain (SSURE $_{1-2}$) region, the SSURE 2 domain (SSURE$_2$) and the methionine and lysine-rich region (MK-rich) **(Fig6A).**

This essay revealed that the MK-rich region bound Plg almost as efficiently as the entire protein. Moderate binding was observed with the fragment consisting of the two SSURE domains (SSURE $_{1-2}$) while the single domain fragment (SSURE$_2$) did not display significant interactions with Plg (**Fig6 B).** To identify the minimal region of the MK-rich domain that still maintains the ability to bind Plg, we used two fragments encompassing the Mk-rich region and designated as N-terminalMk-rich and C-terminalMk-rich, respectively **(Fig6A).** N-terminalMk-rich is a biotinylated peptide purchased from GenScript and C-terminalMk-rich is a peptide in fusion with GST cloned in our laboratory.

To analyze the Plg-adhering activities of these fragments, we coupled these fragment with fluorescent beads as described before and we used GST and CAT-Biotin as a negative control. Under these conditions, the C-terminalMk-rich fragment bound Plg as efficiently as the entire MK-rich region and significantly better than N-terminalMK-rich, which showed only slight, but significant, binding (**Fig6 C**).

In order to further narrow down the portion of the Mk-rich domain that is predominantly involved in Plg binding, we obtained an others two biotinylated peptides encompassing C-terminalMk-rich that were designated as Fr1C-terminal MK-rich (Fr1C) and Fr2C-terminalMK-rich (Fr2C) **(Fig6A).** We used these fragments coupled to fluorescent beads, as indicated before. Under these conditions neither Fr1C nor Fr2C efficiently bound Plg, compared to C-terminalMk-rich, indicating that both C- and N-terminal truncation of Mk-rich impaired its capability to bind Plg.
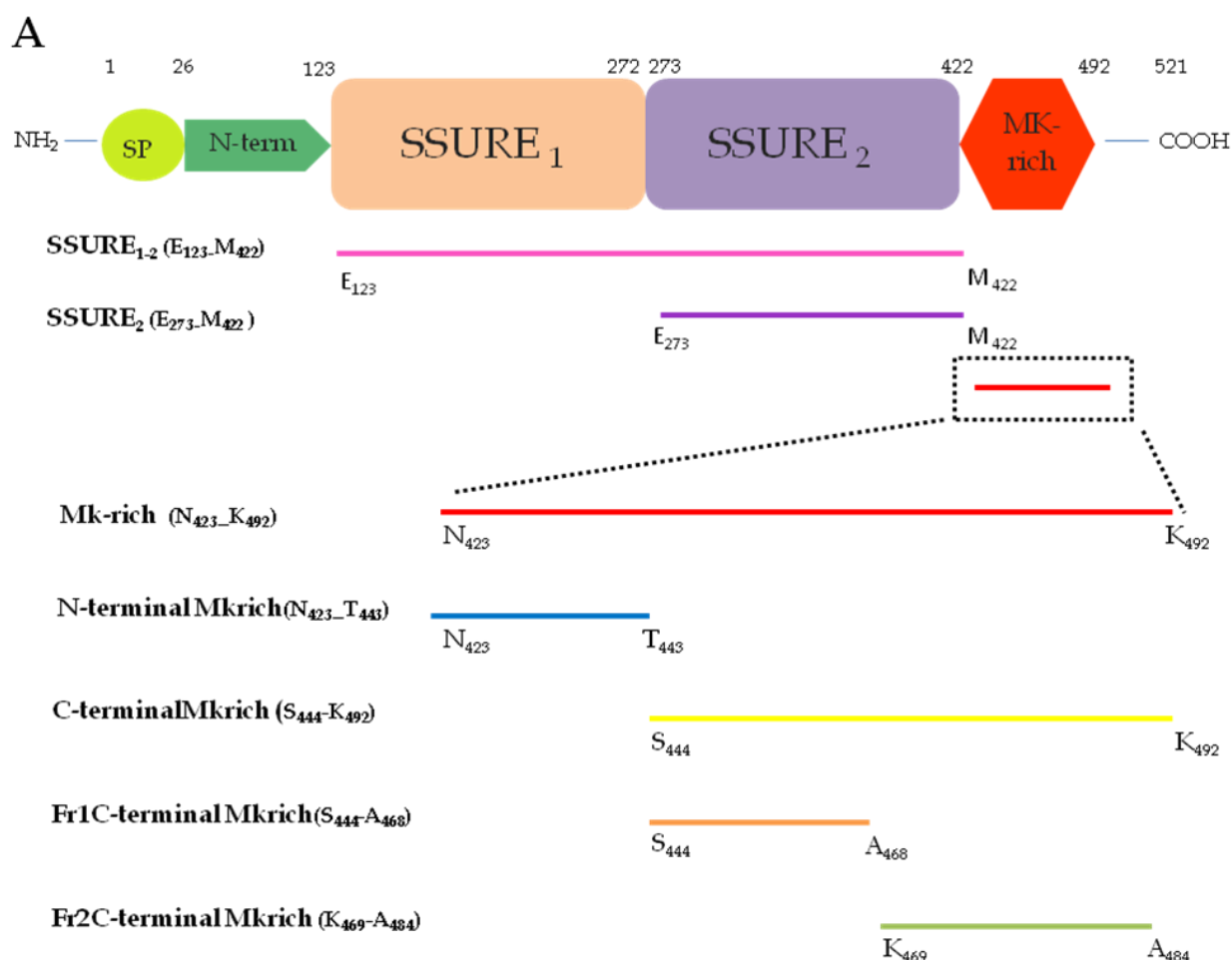
Fig6. A) Schematic representation of PbsP fragments included in these studies.

B
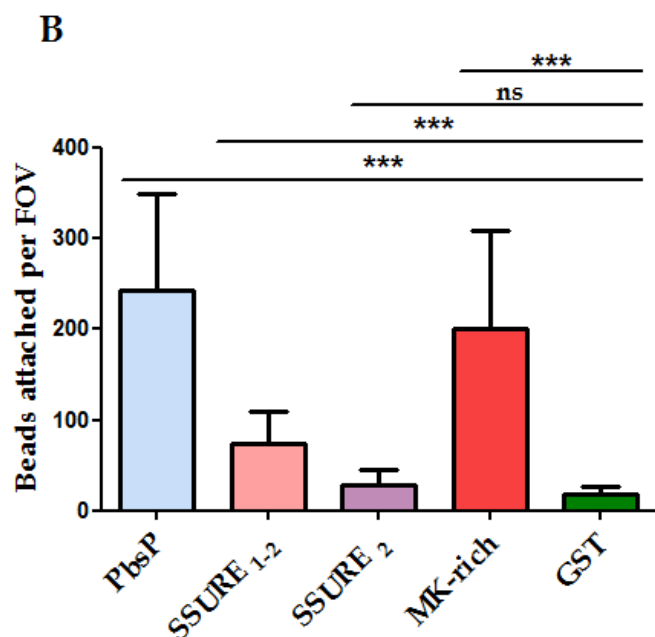
Fig 6. B) fluorescent latex microspheres were coated with the whole PbsP protein, with the SSURE$_{1-2}$, SSURE$_2$ or Mk-rich PbsP fragments expressed as GST fusions (GST-SSURE$_{1-2}$, GST SSURE$_2$, GST-Mk-rich) or with GST. After incubation with plasminogen-sensitized coverslips, adhering particles were counted microscopically.

C

Legend:
- GST-PbsP
- GST-Mk-rich (N$_{423}$-K$_{492}$)
- GST-C-term Mk-rich (S$_{444}$-T$_{492}$)
- GST
- Biotin-N-term Mk-rich (N$_{423}$-T$_{443}$)
- Biotin
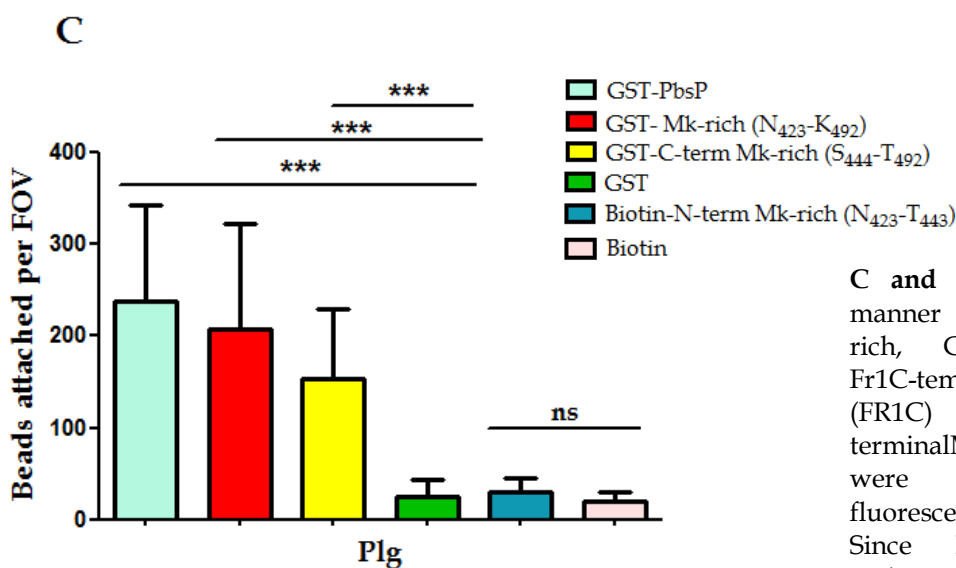
C and D). In the same manner N-terminal-Mk-rich, C-terminal-Mk-rich, Fr1C-teminalMk-rich (FR1C) and Fr2C-terminalMk-rich (FR2C) were coupled with fluorescent latex beads. Since N-terminalMk-rich and FR1C and Fr2C were purchased as biotin fragment , in these tests we used CAT-Biotin and GST coupled beads as a control. Results are expressed as particles per field of vision (*FOV*) at the indicated magnification and represent means ± S.D. of three independent experiments.*, significantly different (*p*<0.05) from GST-coated beads (*A*) as assessed by Student's *t* test.

D

Legend:
- GST-C-term Mk-rich (S$_{444}$-K$_{492}$)
- GST
- Biotin-Fr1C-term Mk-rich (S$_{444}$-A$_{468}$)
- Biotin-Fr2C-term Mk-rich (K$_{469}$-A$_{484}$)
- Biotin
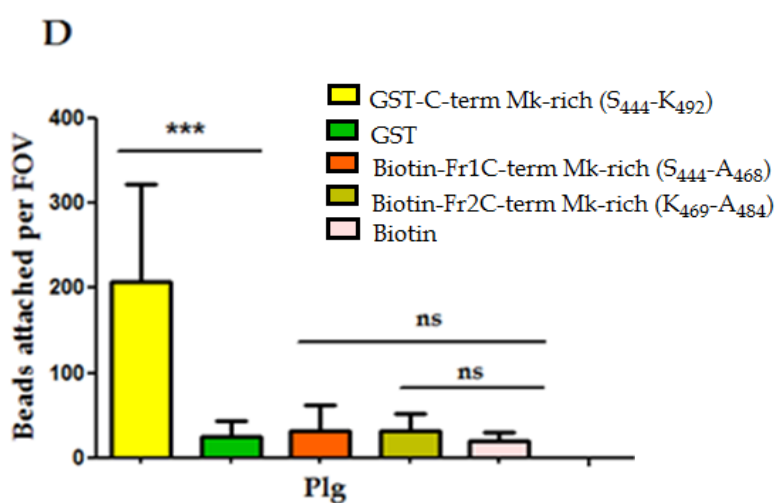
PbsP mediates bacterial adhesion to epithelial cells

Attachment to and invasion of epithelial cells by GBS play a crucial role in the initial stages of infection. Alveolar and intestinal epithelia are considered as likely entry sites in, respectively, early- and late-onset GBS disease (*Hansen Et al., 2004*). In early onset neonatal disease, transmission of GBS generally occurs *via* aspiration of vaginal contents during passage through the birth canal, or of infected amniotic fluid, after the organism has traveled through intact or ruptured amniotic membranes. By this way, infection can remain localized in the lungs, causing pneumonia, or progress to sepsis (in 72% of the cases) and meningitis (2.5%) (*Poyart et al., 2008, Doran and Nizet, 2004*). In late onset disease, the organism is transmitted by the mother or other persons coming into contact with the neonate and is believed to colonize the intestinal mucosa before crossing it (*Tazi, A., et al., 2010*).

Since PbsP is an ortolog of PfbB (plasminogen and fibronectin binding protein B) of *Streptococcus pneumoniae*, which contributes to the ability of this bacterium to adhere to epithelia, we tested whether PbsP contributes to interactions between GBS and epithelial cells. We first compared the ability of NEM316 WT and ΔpbsP to adhere to Caco-2 (intestinal) and A549 (pulmonary) human epithelial cell lines. Binding of the ΔpbsP strain to either CaCo-2 or A549 cells was significantly reduced compared with NEM316, indicating that PbsP is required for optimal bacterial adherence (**Fig. 7A**). Complementation of the *pbsP* mutation with the wild-type allele restored bacterial adherence to A549

and Caco-2 cells. Collectively these results indicate that GBS interactions with epithelial cells are at least partially dependent on surface-expressed PbsP.
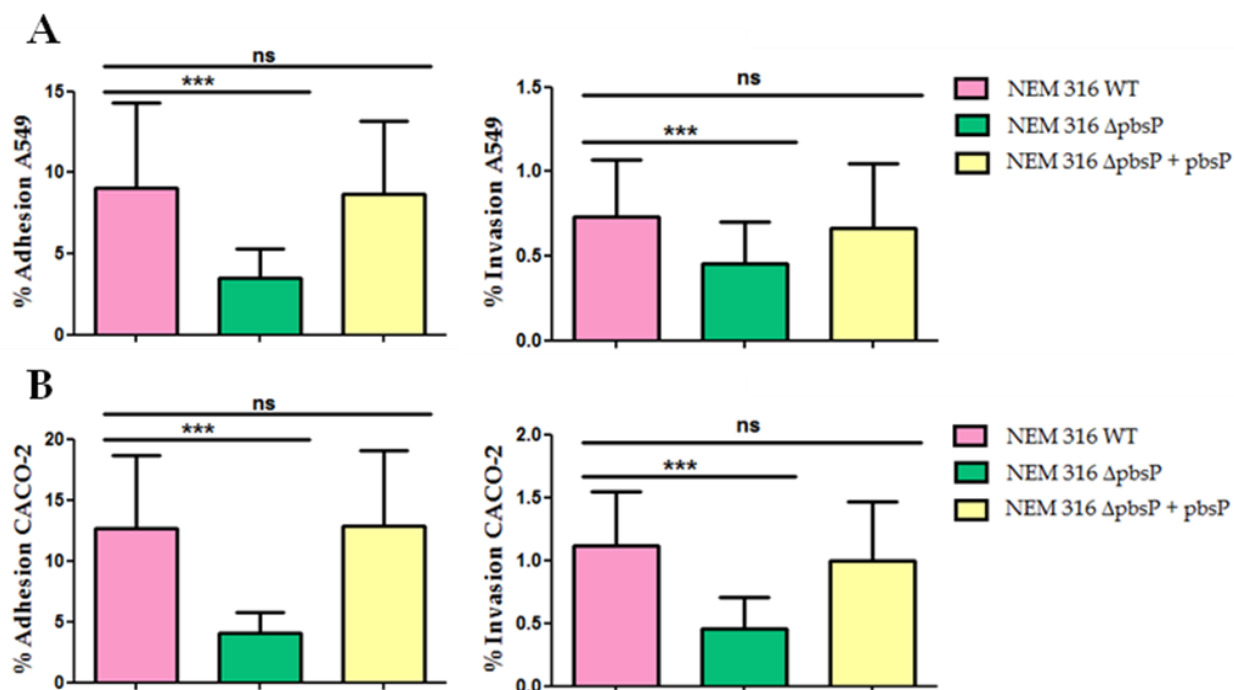
Fig7. PbsP plays a non-reduntant role in the association of GBS NEM316 with epithelial cells. The following GBS strains were compared: WT, NEM316 wild-type strain; ΔpbsP, isogenic pbsP deletion mutant; ΔpbsP+pbsP, ΔpbsP strain carrying a complementing vector with constitutive pbsP expression; Each panel shows the mean ± S.D. of CFU counts from three independent experiments; *, p<0.05 by analysis of variance and Student's Neuman Keuls test. **A**, adhesion and invasion to respiratory epithelial cell lines (A549). **B**, adhesion and invasion to intestinal epithelial cell lines (CACO-2)

SSURE DOMAIN-containing fragments bind efficiently to pulmonary epithelial cells

To determine whether PbsP SSURE domains contribute to bacterial adhesion to epithelial cells, we analyzed the ability of SSURE domains to bind to lung cells in comparison with the C-terminal portion of the protein (MK-rich fragment). To this end, we used fluorescent beads coated with PbsP fragments or with whole PbsP, used as a positive control. The beads were incubated with A549 pulmonary epithelial cells grown on sterile coverslips and, after cell staining, the number of cell-associated beads was counted in 10 different microscopic fields. This experiment revealed that fragments containing SSURE domains bind efficiently to pulmonary epithelial cells. A fragment containing both SSURE domains (SSURE $_{1-2}$, **Fig. 8**) adhered only slightly less efficiently to these cells than the whole protein and this difference was not statistically significant. On the other hand SSURE $_{1-2}$ bound to the cells more efficiently than the fragment encompassing the MK-rich, C-terminal region. The fragment made up by only one SSURE domain (SSURE $_2$ in Fig. 8) adhered as efficiently than the MK-rich fragment. Together, our data indicated that the ranking order of binding to pulmonary epithelial cells of the various fragments (PbsP= SSURE$_{1-2}$ > SSURE$_2$ =MK-rich, **Fig. 8**) differed from the ranking order of binding to plasminogen (PbsP=MK-rich> SSURE$_{1-2}$ = SSURE$_2$, **Fig. 6**). Therefore, binding to Plg might not account, by itself, for the ability of PbsP to bind to epithelials cells, which raises the possibility that PbsP protein is a multifunctional adhesin that can to another receptor on these cells, in addition to Plg. Further studies

will be carried out to verify this hypothesis and to identify the cellular receptors

implicated in binding of PbsP to pulmunary epithelial cells.
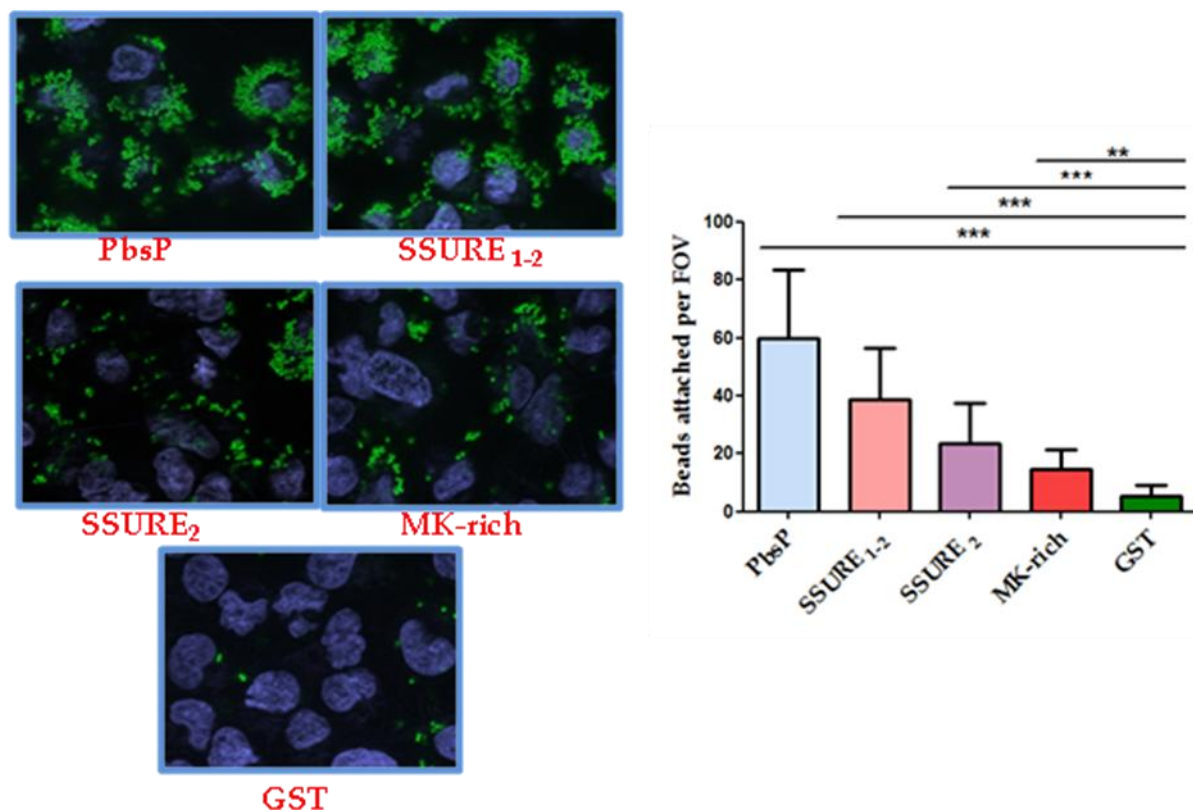
Fig8. Direct binding of PbsP fragments toA549cells. Fluorescent latex microspheres were coated with whole PbsP and PbsP fragments, all expressed as GST fusion proteins (GST SSURE$_{1-2}$, GST SSURE$_2$ and GST Mk-rich). GST was used as a negative control. After incubation with A549 cells, adhering beads were counted microscopically **(A-B)**. A549 cells were labeled with 4',6-diamidino-2-phenylindole for nuclear staining. Columns and bars in panel B represent means ± S.D. of three independent experiments. *, significantly different (p≤0.05) from GST-coated beads, as assessed by Student's t test.

Role of PbsP in the context of the hypervirulent CC17

The experiments reported above indicated that, in the context of the prototype CC23 GBS strain NEM316, PbsP plays a non-redundant role in mediating bacterial Plg binding and adherence to epithelial cells. We were interested to determine whether a similar requirement applies to stains belonging to the hypervirulent CC17 lineage, which is responsible for a large number of neonatal cases of GBS disease, particularly meningitis. Our studies were also prompted by FACS analysis experiments which suggested that PbsP might be less expressed on the surface of GBS CC17 strain COH1 relative to CC23 strains NEM316 and 6313. As a first step, we analyzed the expression of the protein in strain BM110, an additional CC17 prototype strain and whether such expression is under the control of the two component regulatory system CovRS, which, as mentioned above, plays a crucial role as a global regulator of multiple genes involved in host colonization and virulence (*Lamy et al., 2004; Jiang et al., 2008*). To this end, we measured PbsP mRNA levels by real time RT-PCR using strain BM110, and a Δ*covR* deletion mutant of this strain lacking the response regulator (R) component of the signaling system. For comparison, we also tested wild-type (WT) strain NEM316 and its Δ*covR* deletion mutant. The housekeeping gen *gyrA* was used as an internal control. After growth to the exponential phase in Todd-Hewitt broth, the levels of PbsP mRNA were 10-fold lower in wt BM110 relative to wt NEM316. It was found that PbsP expression in BM110 is down regulated by the CovR repressor, since 5- fold higher PbsP mRNA levels were measured in BM110 Δ*covR* bacteria, respectively, as compared with the parental

strain (**Fig. 9A**). These RT-PCR results were confirmed at the protein level by Western-blot analysis of GBS cell wall extracts using an anti-PbsP serum, which produced a fainter PbsP band in wt BM110 in comparison with BM110 Δ*covR* (**Fig.9B**).

Collectively, data from this first set of experiments indicated that PbsP expression is strongly repressed by the CovR regulator in the CC17 prototype strain BM110, at least during *in vitro* growth. This feature may explain low-level expression of the protein in CC17 strains, as observed in the present study.
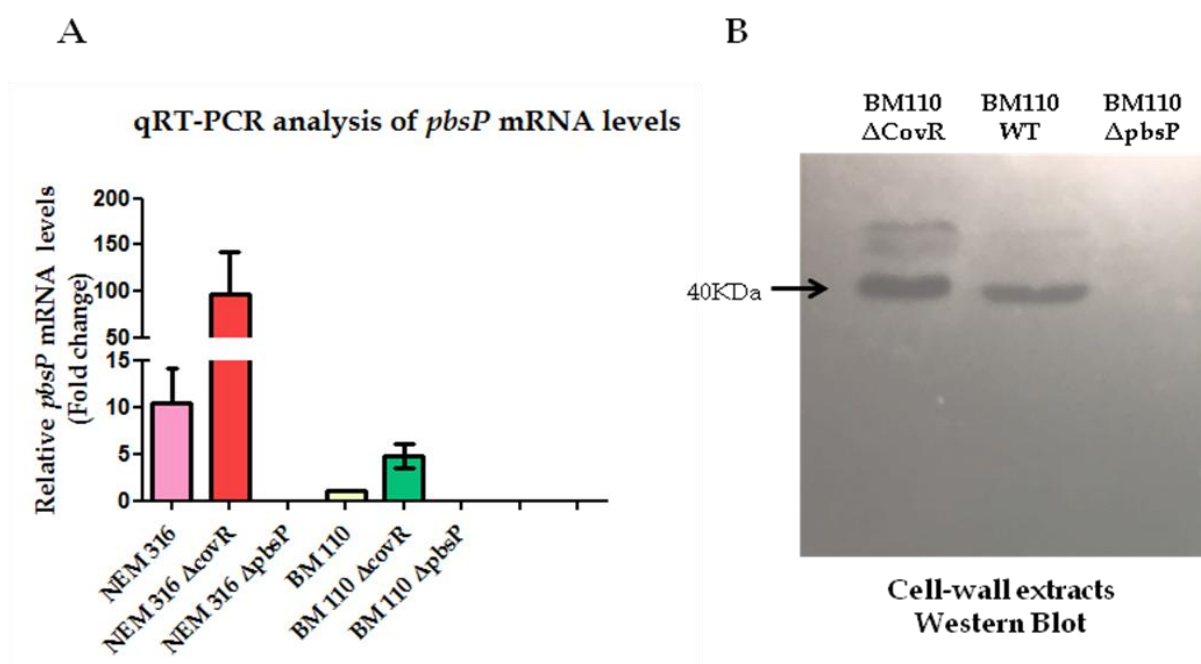
Fig9. qRT-PCR analysis of PbsP in GBS strains(A) and Pbsp expression in cell wall extract of BM110 strain and its mutans (B). A) We used GBS NEM316 and BM110 wild-type strains and ΔpbsP and ΔcovR theirs isogenic deletion mutants. Gene expression was measured by the comparative CT method (ΔΔ CT) and reported as the n fold difference relative to the normalized expression of housekeeping gene (*GyrA*). Results shown are representative of two independent experiments performed in triplicate. **B**) protein level of PbsP was detected by Western Blot analysis using polyclonal anti- PbsP mouse serum. The PbsP level in cell-wall extract of wild-type BM110 is compared with its PbsP deletion mutant (ΔPbsP) or ΔcovR deletion mutant lacking the response regulator (R) component of the signaling system.

PbsP is up-regulated *in vivo* and is required for virulence

As the CsrRS system is involved in the upregulation of virulence genes during GBS infection (*Lamy et al., 2004; Jiang et al., 2005; Jiang et al., 2008*), we hypothesized that PbsP expression, which is tightly controlled by CovR, is upregulated *in vivo*. To investigate this, we performed quantitative RT-PCR (qRT-PCR) analysis of bacterial RNA extracted from kidneys and brains of mice infected i.v. with the BM110 stains. Results were compared with those obtained on *in vitro* grown bacteria using the housekeeping *gyrA* gene as an internal control. As shown in **Fig. 10**, PbsP mRNA levels were approximately 7-fold higher *in vivo* than *in vitro*. To investigate the role of PbsP in the pathogenesis of CC17 GBS infection, the gene encoding PbsP was deleted in-frame in the BM110 chromosome. The viability, morphology and growth in Todd-Hewitt broth of the Δ*pbsP* mutant were similar to those of the WT parental strain (data not shown). We next compared the virulence of BM110 WT with that of a Δ*pbsP* mutant using a mouse model of invasive GBS infection. Under these conditions, only 20% (3 out of 16) of mice infected with the mutant strain died, while 70% of mice infected with the WT strains succumbed to infection by day 4 (**Fig.11**). To further study the role of PbsP in this model, we measured the bacterial load in the blood, brains and kidneys of mice infected with BM110 WT or a Δ*pbsP* mutant as detailed above. At 48h after infection, mice infected with the WT strain had significantly higher bacterial counts than those injected with the Δ*PbsP* mutant both in brains and in kidneys but not in the blood (**Fig. 12 A, B and C**). These data indicate that PbsP may play an important role in the pathogenesis of

invasive GBS disease by favouring hematogenous spreading to target organs, such as the brain. PbsP is highly conserved among clinical isolates of GBS, which could make it as an interesting vaccine candidate. For this reason we next investigated if the protein has the ability to prevent invasive disease after immunization. Adult mice (n 16 per group) were immunized with rPbsP or GST, used as a negative control, and challenged intravenously three weeks later with BM110 WT. Under these conditions, immunization with rPbsP resulted in increased survival (75% or 12/16 versus 37% or 6/16 in control animals; P<0.05 by log-rank (Mantel Cox) test; **Fig. 13**) and in decreased bacterial colony counts in the brains and kidney (**Fig. 14A and B**). These data indicate PbsP immunization can induce significant protection against subsequent systemic infection with GBS.

qRT-PCR analysis of *pbsP* mRNA levels
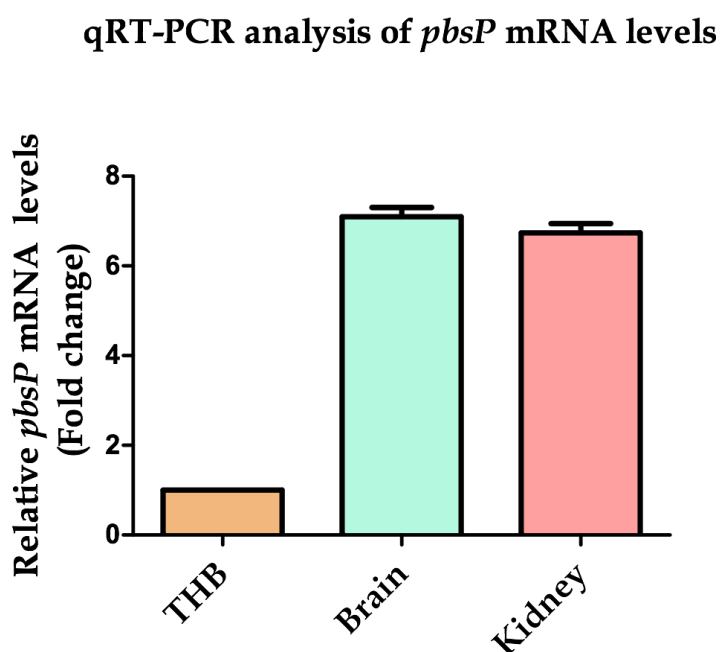
Figure 10. qRT-PCR analysis of PbsP in GBS BM110 in vitro and in vivo. Values are presented as a ratio of expression in brain, and Kidneys of infected CD1 mice relative to expression in TH broth medium. Results shown are representative of two independent experiments performed in triplicate.
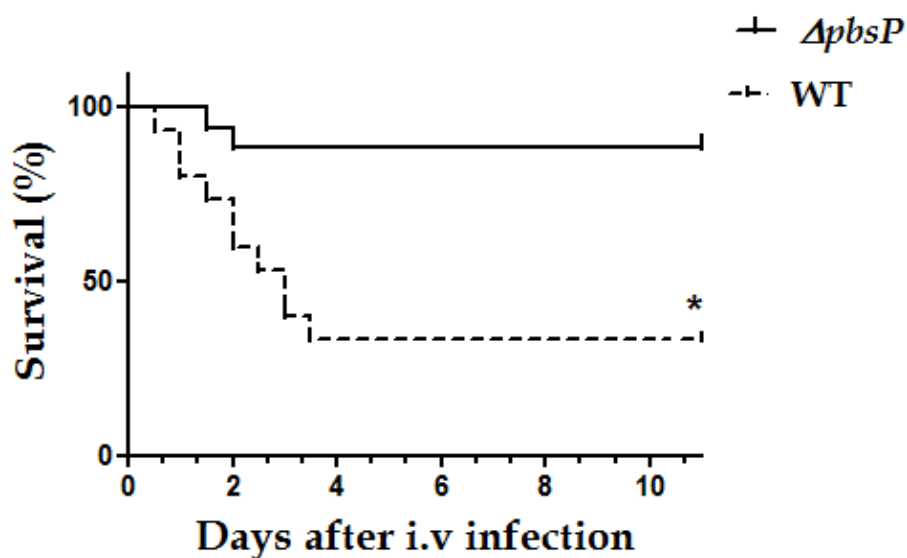
Figure 11. PbsP is a virulence factor of GBS. Comparison of lethality in mice infected with wild-type BM110 (WT) and its *pbsP* deletion (ΔpbsP) mutant. Mice were monitored at least once a day for a total of 11 days after infection with 6x 10^7 bacteria. *, p < 0.05 by log-rank (Mantel Cox) test.
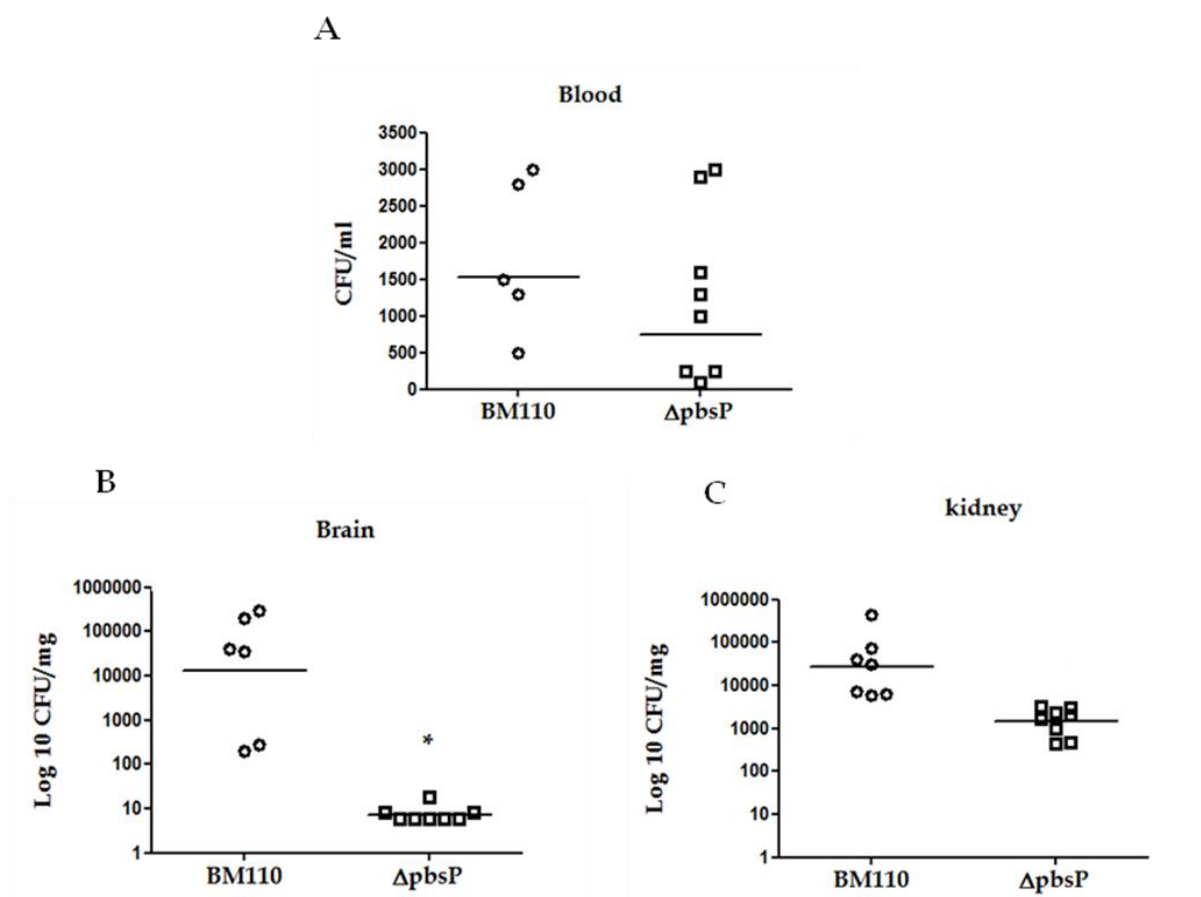
Figure12. Effects of PbsP on organs colonization. Bacterial Burden in bood(**A**) brains (**B**) and kidneys (**C**) were quantified 48hours after i.v. challenge with 6x 10^7 CFUs of WT or ΔpbsP strains. Orizontal Bars indicate the geometrical means. *= p < 0.05 by the Mann Whitney U test.
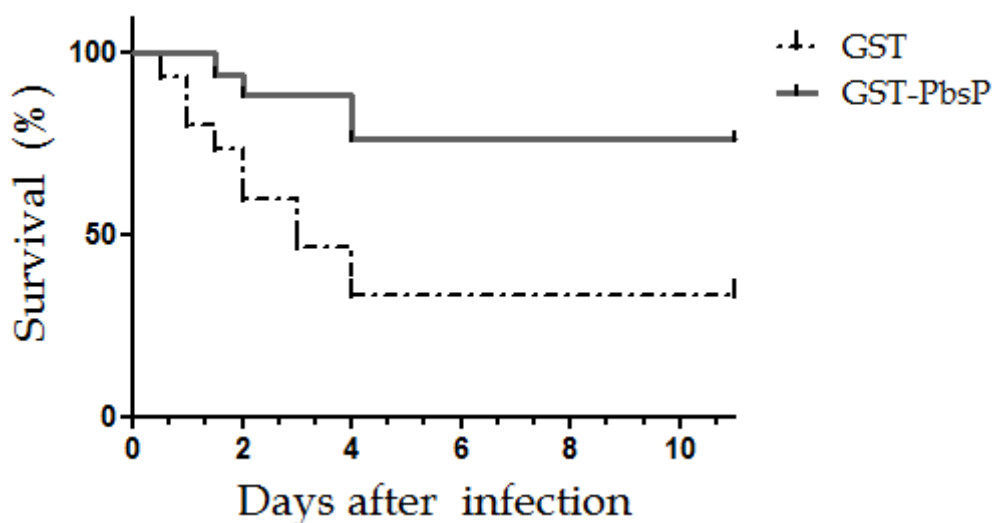
Figure13. Protective effects of GST-PbsP immunization against lethal GBS infection in mice.
Animals were immunized with the recombinant GST-PbsP protein or with the GST as a control and
challenged i.v. with $9x10^7$ CFU/mouse of wild type strain BM110. *, p < 0.05 by log-rank (Mantel Cox)
test.
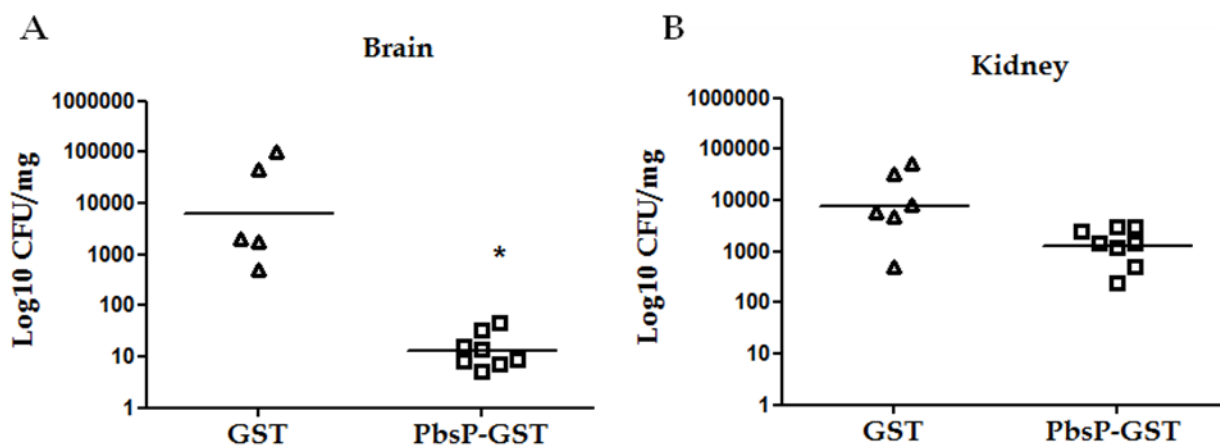
Figure 14. Effects of PbsP immunization on organs colonization. Bacterial Burden in brains (**A**) and
Kidneys (**B**) were quantified 48hours after i.v. challenge with $9x\ 10^7$ CFUs of BM110 strain. Orizontal
Bars indicate the geometrical means. *= p < 0.05 by the Mann Whitney U test.
.

PbsP promotes specific GBS adhesion to and transmigration across brain endothelial cells

Data obtained using either the *pbsP* deletion mutant or active immunization with PbsP suggested that the protein might be involved in translocation of GBS from the blood into the brain, a process in which invasion of the endothelial barrier is a critical step. (*Nizet et al., 1997; Charland et al., 2000; Doran et al., 2005*). To investigate the interaction between GBS and the endothelium, we examined the adhesion and invasion properties of the BM110 strain and its *ΔpbsP* mutant using the brain endothelial cell line hCMEC/D3. The PbsP deletion mutant was significantly impaired in its ability to adhere to and to invade hCMEC/D3, cells as compared with the parental BM 110 strain (**Fig. 15A and 15B**). These results suggest that PbsP is important for *in vitro* GBS adhesion to, and invasion of brain vascular endothelial cells. Previous studies using non-CC17 strains indicated that binding of Plg to L-lysine residues on the bacterial surface and subsequent Plg conversion to plasmin by host tissue plasminogen activator (tPA) are important for migration across brain endothelial cells (*Magaeles et al, 2013*). Therefore we investigated whether PbsP plays a role in Plg-dependent transmigration of BM110 across the endothelium. As expected, the ability of these bacteria to migrate through hCMEC/D3 monolayers was markedly increased in the presence of Plg and tPA **(Fig.15C)** Moreover, this activity was inhibited in a dose- dependent manner in the presence of L-lysine or its analog 6-ACA, but not L-alanine used as a negative control suggesting, an involvement of lysine-binding sites in the interaction

between Plg and bacteria (**Fig.15C**). Notably, the BM110 ΔpbsP mutant

displayed a markedly decreased ability to cross the endothelial barrier in the

presence of Plg and tPA (**Fig. 15C).** Collectively, these data indicate that PbsP

plays a crucial role in plasmin-dependent trasmigration accross brain endotelial
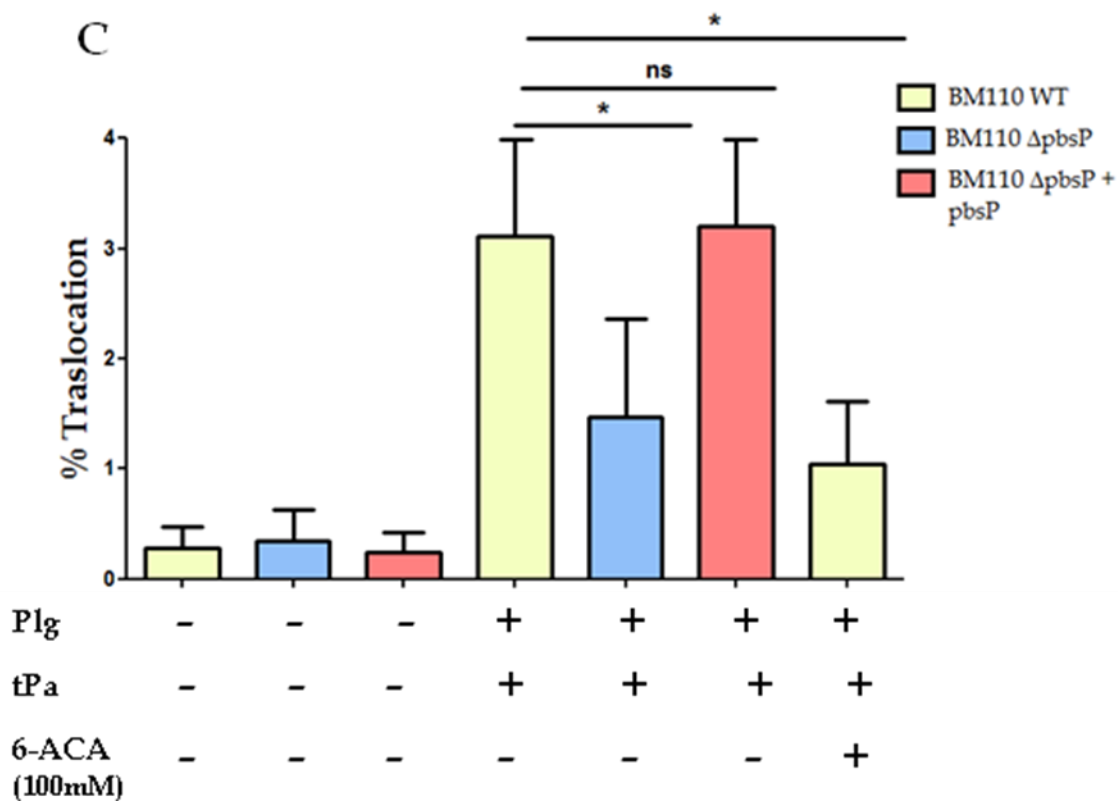
cells in the context of the prototype CC17 strain BM110.
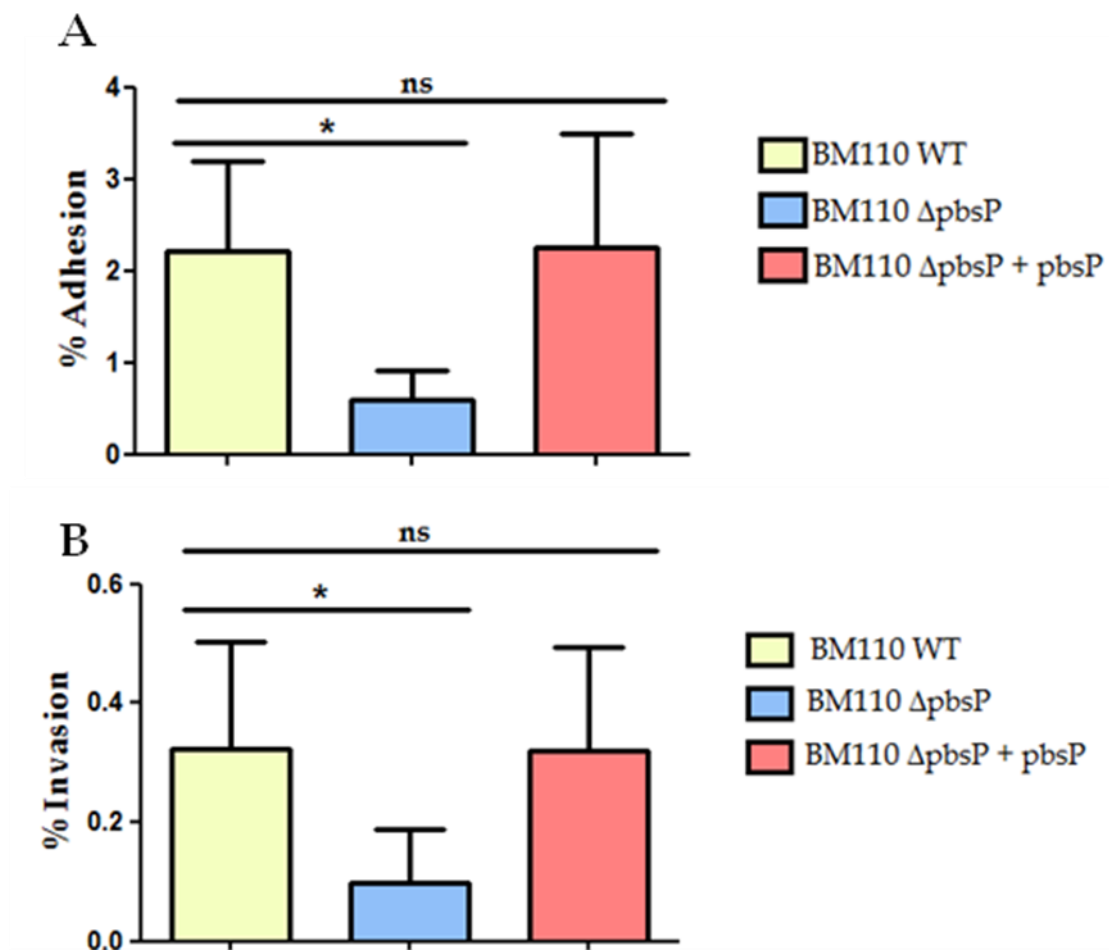
Figure 15. In vitro translocation of GBS BM 110 (WT) and its PbsP defective mutant through human brain endothelial cells is dependent on Plg-PbsP interactions. Adhesion (**A**), invasion (**B**) and transmigration (**C**) were assessed using the brain endothelial cell line hCMEC/D3. *=p< 0.05 by Student t test. Columns and bars represent means + S.D. of CFU values from three independent experiments.

Discussion

In the context of GBS pathogenesis, a crucial role is played by bacterial interaction with Plg. This process confers plasmin-dependent proteolytic activity on cell surface of GBS thereby facilitating bacterial invasiveness through tissue barriers, including the blood-brain barrier, leading to development of meningitis (*Magalhaes et al., 2007; 2013*). GBS is able to express a broad repertoire of unrelated proteins that are potentially capable of binding directly or indirectly to Plg and whose relative importance is difficult to predict. However only two surface proteins of GBS have been shown to directly bind Plg, namely the moonlighting protein GAPDH (*Seifert et al., 2003; Magalhaes et al., 2007*) and Srr2, a cell-wall anchored LPXTG protein (*Seo et al., 2013; Six et al., 2015*). GAPDH is a highly conserved cytosolic enzyme that plays a central role in the glycolytic pathway. It was been demonstrated that GAPDH is released upon cell lysis and reassociated to the surface of living cells (*Madureira et al., 2011; Oliveira et al., 2012*) to bind several ligands, including Plg and Fng (*Seifert et al., 2003; Magalhaes et al., 2013*) and to exert immunomodulatory functions (*Madureira et al., 2011; Oliveira et al., 2012*). This protein is highly conserved and was detected on the surface of GBS isolates belonging to the main phylogenetic lineages. In contrast Srr2 is a LPXTG cell wall anchored protein and is expressed only in GBS strains belonging to the hypervirulent complex CC17 (*Six et al., 2015*). This protein is strongly associated with bacterial dissemination and invasiveness, and with the development of meningitis. Despite GAPDH and Srr2 are structurally unrelated, they are multifunctional adhesins and are

capable to bind not only Plg but also Fng on the bacterial surface (*Seo et al., 2013; Six et al., 2015*). Moreover, for both, Plg-binding is inhibited by 6-ACA, suggesting an interaction between lysine binding sites of the Plg Kringle domains of Plg and lysine residues within the adhesins.

In this study, we identified and characterized a novel Plg-binding protein of GBS, that was named it PbsP, standing for Plasminogen binding surface Protein, since this protein efficiently bound Plg, but not other matrix components. Thus, PbsP is the second Plg-binding cell wall protein characterized so far in GBS. In contrast to Srr2, PbsP is expressed by human clinical isolates belonging to all major lineages, including the CC17. Interestingly, PbsP expression is repressed, in both in Nem316 (CC23) and BM110 (CC17), by CovRS (**Fig. 2**), the major global regulator of virulence gene expression (*Lamy et al., 2004; Jiang et al., 2008; Lembo et al., 2010; Firon et al., 2013*). 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*).

Bioinformatics analysis revealed that PbsP displays two 150-aa long repeated domains designated as 'Streptococcal surface repeat domain' (SSURE) (*Bumbaca et al., 2004*). A protein containing a variable number of SSURE domains (i.e. from 2 to 6 depending on the strain) was initially characterized in *S. pneumoniae* as having the ability to bind to both Fnt and Plg and was therefore named PfbP (*Papasergi et al., 2010*). SSURE domains were shown to be involved in mediating bacterial binding to these matrix components. Moreover

binding efficiency in PfbP was directly related to the number of repeats (*Jensch et al., 2010; Papasergi et al., 2010*). PfbB is involved in bacterial adherence to human epithelial cells, nasopharyngeal colonization and airways infections (*Jensch et al., 2010; Papasergi et al., 2010*). Sequence analysis of this protein showed that SSURE domain repeats clustered in two main groups displaying approximately 75% of identity (**Figure2**). In contrast, PbsP, is has a fixed number of repeats, binds only Plg (but not Fnt) and is expressed in all human GBS isolates with 98% of identity. The two SSURE domains of PbsP cluster apart from the pneumococcal SSURE sequences (**Figure2**), which may explain why the PbsP protein is able to bind only Plg and not Fnt. Bacterial and eukaryotic Plg binding motifs display positively charged amino acids, such as lysine, in a hydrophobic surrounding (*Fulde et al., 2013*). Interestingly, the SSURE domains of PbsP and PfbB are both lysine-rich. Moreover the interaction between PbsP and Plg interaction could be prevented by the addition of lysine or the lysine analog ACA. However, PbsP also contains an additional lysine-rich region, named Mk-rich, that is absent in the pneumococcal PfbB, and appears to also participate in binding to Plg. This was suggested by the greater ability of Mkrich, compared with the SSURE domains, to bind Plg, as observed in the present study.

Therefore, it is likely that binding of PbsP to Plg is due to the cooperative activities of SSURE domains with the Mk-rich region. Moreover, we demonstrated that Kringle4 in Plg mediates binding to PbsP.

Notably, in the present study, a GBS NEM316 mutant lacking PbsP bound less Plg, compared to the WT strain, although this phenotype was not completely 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*). Collectively, our data indicate that PbsP makes a significant contribution to the overall ability of GBS to bind Plg. Moreover, the NEM316 mutant lacking PbsP bound to and invaded pulmonary and intestinal epithelial cells less efficiently than the WT strain. The mutant strain was also impaired in its ability to acquire cell surface proteolytic activity. Accordingly, PbsP was found to play a crucial role in plasmin-dependent trasmigration accross brain endotelial cells. To further study the role of PbsP in this contest, we, next, measured the bacterial load in the brains of mice infected with BM 110 WT or a ΔpbsP mutant and we have found significantly higher bacterial counts in mice infected with the WT strain than those injected with the ΔPbsP mutant, indicating that PbsP play an important role in the pathogenesis of invasive GBS disease by favouring hematogenous spreading to target organs, such as the brain. All together these date suggested that PbsP is an important virulence factor in both clonal complex CC17 and CC23, as confirmed by in vivo experiments that demonstrated the lower gravity of infection in mice infected with mutant lacking PbsP. 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 BM110 strain as evidenced in figure 14 A and B where

immunization with PbsP resulted in increased survival and in decreased bacterial colony counts in the brain. As mentioned above, in the present study, PbsP was found to play a non-redundant role not only in binding of GBS to Plg, but also in the adherence of these bacteria to epithelial and endothelial cells. However, when we compared the ability of various PbsP fragments to adhere to Plg and to epithelial cells, we found that these two activities were not correlated. For example, SSURE domains bound strongly to epithelial cells, but only moderately to Plg. The reverse was true for the MK-rich region, which bound strongly to Plg, but only moderately to epithelial cells. Therefore, it is possible that PbsP is a multifunctional adhesin that can bind both to Plg and to an additional receptor present on the surface of epithelial cells, which is preferentially recognized through the SSURE domains. Further studies will be carried out to verify this hypothesis and to identify cellular receptor/s, different from Plg, which might be implicated in binding of PbsP to epithelial cells.

Since a substantial proportion of EOD and the majority of LODs are associated with the 'hypervirulent' serotype III lineage CC-17 (*Musser et al., 1989; Lamy et al., 2006; Lin et al., 2006; Phares et al., 2008; Poyart et al., 2008*), we focused on the role of PbsP in the contest of this clonal complex. For this purpose we used BM110, a representative strain of the hypervirulent CC17 lineage. We found that PbsP mRNA is upregulated in kidneys and brains of mice infected with the BM110 stain and that a BM110 mutant lacking PbsP was impaired in its ability to colonize the brain. Collectively, these data indicate that PbsP is involved in the pathogenesis of GBS meningitis by virtue of its

ability to recruit Plg on the bacterial surface. This, in turn, allows GBS to acquire Pln-mediated proteolitic activity and to cross the blood-brain barrier. These data indicate that PbsP is a conserved GBS cell-wall adhesin with a major role in Plg binding and a possible vaccine candidate against GBS infections.

References

Agarwal V., Kuchipudi A., Fulde,M., Riesbeck,K., Bergmann,S., and Blom, A. M.(2013). *Streptococcus pneumoniae* EndopeptidaseO (PepO) Isa Multifunctional Plasminogen-and fibronectin-binding protein, facilitating evasion of innate immunity and invasion of host cells. J. Biol.Chem. 288, 6849–6863. doi:10.1074/jbc.M112.405530

Bhattacharya Sarbani et al. (2012) Bacterial Plasminogen Receptors Utilize Host Plasminogen System for Effective Invasion and Dissemination, Journal of Biomedicine and Biotechnology Volume 2012, Article ID 482096, 19 pages doi:10.1155/2012/482096

Bergmann, S., and Hammerschmidt, S. (2007) Fibrinolysis and host response in bacterial infections. Thromb Haemost 98: 512–520.

Bergmann,S., Rohde,M., Chhatwal,G.S., and Hammerschmidt,S. (2001). Alpha-Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding-protein displayed on the bacterial cell surface. Mol. Microbiol. 40, 1273–1287.doi: 10.1046/j.1365-2958.2001.02448

Bergmann S., M. Rohde, K. T. Preissner, and S. Hammerschmidt, "The nine residue plasminogen-binding motif of the pneumococcal enolase is the major cofactor of plasmin mediated degradation of extracellular matrix, dissolution of fibrin and transmigration," Thrombosis and Haemostasis, vol. 94, no. 2, pp. 304–311, 2005.

Bisharat N., Crook DW, Leigh J, Harding RM, Ward PN, Coffey TJ, Maiden MC, Peto T, Jones N. J Clin Microbiol. 2004 May; 42(5):2161-7.Hyperinvasive neonatal group B *streptococcus* has arisen from a bovine ancestor.

Bohnsack, J.F., A. Whiting, M. Gottschalk, D.M. Dunn, R. Weiss, P.H. Azimi, J.B. Philips III, L.E. Weisman, G.G. Rhoads, and F.Y. Lin. 2008. Population structure of invasive and colonizing strains of *Streptococcus agalactiae* from neonates of six U.S. Academic Centers from 1995 to 1999. *J. Clin. Microbiol.* 46:1285–1291. doi:10.1128/ JCM.02105-07

Boone, T.J., Burnham, C.A., and Tyrrell, G.J. (2011) Binding of group B streptococcal phosphoglycerate kinase to plasminogen and actin. Microb Pathog 51: 255–261.

Boone, T.J. and Tyrrell, G.J. (2012) Identification of the actin and plasminogen binding regions of group B streptococcal phosphoglycerate kinase. J Biol Chem 287: 29035–29044.

Brega, S., Caliot, E., Trieu-Cuot, P., and Dramsi, S. (2013) SecA localization and SecA-dependent secretion occurs at new division septa in group B *Streptococcus*. PloSOne 8: e65832.

Brochet, M., E. Couvé, M. Zouine, T. Vallaeys, C. Rusniok, M.C. Lamy, C. Buchrieser, P. Trieu-Cuot, F. Kunst, C. Poyart, and P. Glaser. 2006. Genomic diversity and evolution within the species *Streptococcus agalactiae. Microbes Infect.* 8:1227–1243. doi:10.1016/j.micinf.2005.11.010

Bumbaca, D., Littlejohn, J.E., Nayakanti, H., Rigden, D.J., Galperin, M.Y., and Jedrzejas, M.J. (2004) Sequence analysis and characterization of a novel fibronectin-binding repeat domain from the surface of *Streptococcus pneumonia.* OMICS 8: 341–356.

Buscetta, M., Papasergi, S., Firon, A., Pietrocola, G.,Biondo, C., Mancuso, G., et al. (2014) FbsC, a novel fibrinogen-binding protein, promotes *Streptococcus agalactiae*-host cell interactions. J Biol Chem 289: 21003–21015.

Campbell, J.R., S.L. Hillier, M.A. Krohn, P. Ferrieri, D.F. Zaleznik, and C.J Baker. 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet. Gynecol. 96:498–503

Cardaci, A., Papasergi, S., Midiri, A., Mancuso, G.,Domina, M., Cariccio, V.L., et al. (2012) Protective activity of *Streptococcus pneumoniae* Spr1875 protein fragments identified using a phage displayed genomic library. PloSOne 7: e36588.

Carey RB, Eisenstein TK, Shockman GD, Greber TF, Swenson RM. - *"Soluble group- and type-specific antigens from type III group B Streptococcus"*. Infect Immun 1980;28:195–203.

Carlsson, F., Stalhammar-Carlemalm, M., Flardh, K.,Sandin, C., Carlemalm, E., and Lindahl, G. (2006) Signal sequence directs localized secretion of bacterial surface proteins. Nature 442: 943–946.

Castellino Francis J., Victoria A. Ploplis. Structure and function of the plasminogen/plasmin system. 2005 Apr; Thromb Haemost. 93(4):647-54.

Charland N, Nizet V, Rubens CE, Kim KS, Lacouture S, Gottschalk M. *Streptococcus suis* serotype 2 interactions with human brain microvascular endothelial cells. Infect Immun. 2000 Feb;68(2):637-43.

Christensen, U. and Mølgaard, L. (1992) Positive co-operative binding at two weak lysine binding sites governs the Glu-plasminogen conformational change. Biochem. J. 285, 419-425

Da Cunha, V., Davies, M.R., Douarre, P.E., Rosinski-Chupin, I., Margarit, I., Spinali, S. et al. (2014) *Streptococcus agalactiae* clones infecting humans were selected and fixed through the extensive use of tetracycline. Nat Commun 5: 4544.

Doran KS, Engelson EJ, Khosravi A, Maisey HC, Fedtke I, Equils O, Michelsen KS, Arditi M, Peschel A, Nizet V (2005) Blood-brain barrier invasion by group B *Streptococcus* depends upon proper cell-surface anchoring of lipoteichoic acid. J Clin Investig 115:2499 2507.doi:10.1172/JCI23829

Doran KS, Nizet V. Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. Mol Microbiol. 2004 Oct;54(1):23-31. Review. PMID: 15458402

Edmond, K.M., Kortsalioudaki, C., Scott, S., Schrag, S.J., Zaidi, A.K., Cousens, S., and Heath, P.T. (2012) Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet 379: 547–556.

Edwards, M.S., V. Nizet, and C.J. Baker. 2006. Group B streptococcal infections. In: JS Remington, JO Klein, C Wilson and CJ Baker (eds.), Infectious Diseases of the Fetus and Newborn Infant. Saunders Elsevier, Philadelphia, PA, pp. 403–464

Edwards MS, Baker CJ. Group B streptococcal infections in elderly adults. Clin Infect Dis. 2005 Sep 15;41(6):839-47. Review. PMID: 16107984

Fan ZQ, et al. Tissue factor regulates plasminogen binding and activation. Blood 1998; 91: 1987–98.33.

Faralla C, Metruccio MM, De Chiara M, Mu R, Patras KA, Muzzi A, Grandi G, Margarit I, Doran KS, Janulczyk R. Analysis of two-component systems in group B Streptococcus shows that RgfAC and the novel FspSR modulate virulence and bacterial fitness MBio. 2014 May 20;5(3):e00870-14. doi: 10.1128/mBio.00870-14. PMID: 24846378

Farley MM1, Harvey RC, Stull T, Smith JD, Schuchat A, Wenger JD, Stephens DS. A population-based assessment of invasive disease due to group B *Streptococcus* in nonpregnant adults. N Engl J Med. 1993 Jun 24;328(25):1807-11.

Firon, A., Tazi, A., Da Cunha, V., Brinster, S., Sauvage, E.,Dramsi, S. et al. (2013) The Abi-domain protein Abx1 interacts with the CovS histidine kinase to control virulence gene expression in group B Streptococcus. PLoS Pathog 9: e1003179.

Fulde M., Rohde M., Hitzmann A., Preissner K. T., Nitsche-Schmitz D. P., Nerlich A., et al. (2011). SCM, a novel M-like protein from *Streptococcus canis*, binds (mini)-plasminogen with high affinity and facilitates bacterial transmigration. Biochem. J.434, 523–535 10.1042/BJ20101121

Fulde, M., Steinert, M., and Bergmann, S. (2013) Interaction of streptococcal plasminogen binding proteins with the host fibrinolytic system. Front Cell Infect Microbiol 3: 85.

Garibaldi, M., Rodriguez-Ortega, M.J., Mandanici, F., Cardaci, A., Midiri, A., Papasergi, S. et al. (2010) Immunoprotective activities of a *Streptococcus suis* pilus subunit in murine models of infection. Vaccine 28: 3609–3616.

Glaser, P., Rusniok, C., Buchrieser, C., Chevalier, F., Frangeul, L., Msadek, T. et al. (2002) Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. Mol Microbiol 45: 1499–1513.

Gutekunst, H., Eikmanns, B.J., and Reinscheid, D.J. (2004) The novel fibrinogen-binding protein FbsB promotes *Streptococcus agalactiae* invasion into epithelial cells. Infect Immun 72: 3495–3504.

Hansen, S.M., N. Uldbjerg, M. Kilian, and U.B. Sørensen. 2004. Dynamics of *Streptococcus agalactiae* colonization in women during and after pregnancy and in their infants. J. Clin. Microbiol. 42:83–89. doi:10.1128/JCM.42.1.83-89.2004

Heckman, K.L., and Pease, L.R. (2007) Gene splicing and mutagenesis by PCR-driven overlap extension. Nat Protoc 2: 924–932.

Jensch,I.,Gamez,G.,Rothe,M.,Ebert,S.,Fulde,M.,Somplatzki,D.,etal.(2010). PavB is a surface-exposed adhesion of *Streptococcus pneumoniae* contributing to nasopharyngeal colonization and air ways infections. Mol.Microbiol. 77, 22–43. doi: 10.1111/j.1365-2958.2010.07189.

Jiang J, et al. (2005) Detection of high-affinity and sliding clamp modes for MSH2-MSH6 by single-molecule unzipping force analysis. Mol Cell 20(5):771-81

Jiang, S., and Wessels, M.R. (2014) BsaB, a novel adherence factor of group B Streptococcus. Infect Immun 82: 1007–1016.

Jiang SM, Ishmael N, Dunning Hotopp J, Puliti M, Tissi L, Kumar N, Cieslewicz MJ, Tettelin H, Wessels MR. Variation in the group B Streptococcus CsrRS regulon and effects on pathogenicity. J Bacteriol. 2008 Mar;190(6):1956-65. doi: 10.1128/JB.01677-07. PMID: 18203834

Jones N., J.F. Bohnsack, S. Takahashi, K.A. Oliver, M.S. Chan, F. Kunst, P. Glaser, C. Rusniok, D.W. Crook, R.M. Harding, et al. 2003. Multilocus sequence typing system for group B *streptococcus*. *J. Clin.* Brochet *Microbiol.* 41:2530–2536. doi:10.1128/JCM.41.6.2530-2536.2003

Jones N., Oliver KA, Barry J, Harding RM, Bisharat N, Spratt BG, Peto T, Crook DW; Oxford Group B *Streptococcus* Consortium. Enhanced invasiveness of bovine-derived neonatal sequence type 17 group B streptococcus is independent of capsular serotype. Clin Infect Dis. 2006 Apr 1;42(7):915-24. Epub 2006 Feb 22.

Kline, K.A., Falker, S., Dahlberg, S., Normark, S., and Henriques-Normark, B. (2009) Bacterial adhesins in host microbe interactions. Cell Host Microbe 5: 580–592.

Lahteenmaki, K., Kuusela, P., and Korhonen, T.K. (2001) Bacterial plasminogen activators and receptors. FEMS Microbiol Rev 25: 531–552.

Lalioui, L., Pellegrini, E., Dramsi, S., Baptista, M., Bourgeois, N., Doucet-Populaire, F. et al. (2005) The SrtA Sortase of *Streptococcus agalactiae* is required for cell wall anchoring of proteins containing the LPXTG motif, for adhesion to epithelial cells, and for colonization of the mouse intestine. Infect Immun 73: 3342–3350.

Lamy MC, Zouine M, J Fert, et al. CovS/CovR of Group B *Streptococcus*: a two-component global regulatory system involved in virulence. Mol Microbiol. 2004;54:1250–1268.

Lamy, M.C., S. Dramsi, A. Billoët, H. Réglier-Poupet, A. Tazi, J. Raymond, F. Guérin, E. Couvé, F. Kunst, P. Glaser, et al. 2006. Rapid detection of the "highly virulent" group B *Streptococcus* ST-17 clone. Microbes Infect. 8:1714–1722. doi:10.1016/j.micinf.2006.02.008

Le Doare, K., and Heath, P.T. (2013) An overview of global GBS epidemiology. Vaccine 31 (Suppl. 4): D7–12.

Lembo, A., Gurney, M.A., Burnside, K., Banerjee, A., de los Reyes, M., Connelly, J.E. et al. (2010) Regulation of CovR expression in Group B *Streptococcus* impacts blood-brain barrier penetration. Mol Microbiol 77: 431–443.

Lin FY1, Whiting A, Adderson E, Takahashi S, Dunn DM, Weiss R, Azimi PH, Philips JB 3rd, Weisman LE, Regan J, Clark P, Rhoads GG, Frasch CE, Troendle J, Moyer P, Bohnsack JF.

J Clin Microbiol. 2006 Apr; 44(4):1257-61. Phylogenetic lineages of invasive and colonizing strains of serotype III group B Streptococci from neonates: a multicenter prospective study.

Luan SL, et al. Multilocus sequence typing of Swedish invasive group B *streptococcus* isolates indicates a neonatally associated genetic lineage and capsule switching. J. Clin. Microbiol. 2005; 43:3727–3733. [PubMed: 16081902]

Madureira, P., Andrade, E.B., Gama, B., Oliveira, L.,Moreira, S., Ribeiro, A. et al. (2011) Inhibition of IL-10 production by maternal antibodies against Group B *Streptococcus* GAPDH confers immunity to offspring by favoring neutrophil recruitment. PLoS Pathog 7: e1002363.

Magalhaes, V., Veiga-Malta, I., Almeida, M.R., Baptista, M., Ribeiro, A., Trieu-Cuot, P., and Ferreira, P. (2007) Interaction with human plasminogen system turns on proteolytic activity in

Streptococcus agalactiae and enhances its virulence in a mouse model. Microb Infect 9: 1276–1284.

Magalhaes, V., Andrade, E.B., Alves, J., Ribeiro, A., Kim, K.S., Lima, M., Trieu-Cuot, P., and Ferreira, P. (2013) Group B *Streptococcus* hijacks the host plasminogeno system to promote brain endothelial cell invasion. PloS One 8: e63244.

Manning, S.D., A.C. Springman, E. Lehotzky, M.A. Lewis, T.S. Whittam, and H.D. Davies. 2009. Multilocus sequence types associated with neonatal group B streptococcal sepsis and meningitis in Canada. *J. Clin. Microbiol.* 47:1143–1148. doi:10.1128/JCM.01424-08

Mazmanian, S.K., Liu, G., Ton-That, H., and Schneewind, O. (1999) *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. Science 285: 760–763.

Miles LA, Dahlberg CM, Plow EF. The cell binding domains of plasminogen and their function in plasma. J Biol Chem 1988; 263: 11928–34. 22.

Mu, R., Kim, B.J., Paco, C., Del Rosario, Y., Courtney, H.S., and Doran, K.S. (2014) Identification of a group B streptococcal fibronectin binding protein, SfbA, that contributes to invasion of brain endothelium and development of meningitis. Infect Immun 82: 2276–2286.

Musser, J.M., S.J. Mattingly, R. Quentin, A. Goudeau, and R.K. Selander. 1989. Identification of a high-virulence clone of type III *Streptococcus agalactiae* (group B Streptococcus) causing invasive neonatal disease. *Proc. Nat. Aca. Sci.* 86:4731–4735. doi:10.1073/pnas.86.12.4731

Nizet V, Kim KS, Stins M, Jonas M, Chi EY, Nguyen D, Rubens CE (1997) Invasion of brain microvascular endothelial cells by group B streptococci. Infect Immun 65:5074–5081

Nobbs, A.H., Lamont, R.J., and Jenkinson, H.F. (2009) *Streptococcus* adherence and colonization. Microbiol Mol Biol Rev: MMBR 73: 407–450, Table of Contents.

Oliveira, L., Madureira, P., Andrade, E.B., Bouaboud, A., Morello, E., Ferreira, P. et al. (2012) Group B *streptococcus* GAPDH is released upon cell lysis, associates with bacterial surface, and induces apoptosis in murine macrophages. PloS One 7: e29963.

O'Mullane MJ, Baker MS. Elevated plasminogen receptor expression occurs as a degradative phase event in cellular apoptosis. Immunol Cell Biol 1999; 77: 249–55.).

O'Mullane MJ, Baker MS. Loss of cell viability dramatically elevates cell surface plasminogen binding and activation. Exp Cell Res 1998; 242: 153–64.

Papasergi, S., Garibaldi, M., Tuscano, G., Signorino, G.,Ricci, S., Peppoloni, S. et al. (2010) Plasminogen- and fibronectin-binding protein B is involved in the adherence of *Streptococcus pneumoniae* to human epithelial cells. J Biolog Chem 285: 7517–7524.

Papasergi, S., Brega, S., Mistou, M.Y., Firon, A., Oxaran, V., Dover, R. et al. (2011) The GBS PI-2a pilus is required for virulence in mice neonates. PloS One 6: e18747.

Park, S.E., Jiang, S., and Wessels, M.R. (2012) CsrRS and Environmental pH Regulate Group B Streptococcus Adherence to Human Epithelial Cells and Extracellular Matrix. Infect Immun 80: 3975–3984.

Patras, K.A., Wang, N.Y., Fletcher, E.M., Cavaco, C.K., Jimenez, A., Garg, M. et al. (2013) Group B *Streptococcus* CovR regulation modulates host immune signalling pathways to promote vaginal colonization. Cell Microbiol 15: 1154–1167.

Phares CR1, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ; Active Bacterial Core surveillance/Emerging Infections Program Network. JAMA. 2008 May 7;299(17):2056-65. doi: 10.1001/jama.299.17.2056. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005.

Pietrocola, G., Schubert, A., Visai, L., Torti, M., Fitzgerald, J.R., Foster, T.J., Reinscheid, D.J., and Speziale, P. (2005) FbsA, a fibrinogen-binding protein from *Streptococcus agalactiae*, mediates platelet aggregation. Blood 105: 1052–1059.

Plow EF, Freany F, Plescia J, et al. The plasminogen system and cell surfaces: Evidence for plasminogen and urokinase receptors on the same cell type. J Cell Biol 1986; 103: 2411–30.

Plow EF, T Herren, A Redlitz, L A Miles and J L Hoover-Plow . The cell biology of the plasminogen system. July 1995 FASEB J 9:939-45

Poyart et al., 2001; 40. Poyart C, Lamy MC, Boumaila C, Fiedler F, Trieu-Cuot P. Regulation of D-alanyl-lipotechoic acid biosynthesis in *Streptococcus agalactiae* involves a novel two-component regulatory system. J Bacteriol. 2001;183:6324–6334.

Poyart, C., H. Réglier-Poupet, A. Tazi, A. Billoët, N. Dmytruk, P. Bidet, E. Bingen, J. Raymond, and P. Trieu-Cuot. 2008. Invasive group B streptococcal infections in infants, France. *Emerg. Infect. Dis.* 14:1647–1649. doi:10.3201/eid1410.080185

Quach D, van Sorge NM, Kristian SA, Bryan JD, Shelver DW, Doran KS. The CiaR response regulator in Group B *Streptococcus* promotes intracellular survival and resistance to innate immune defenses. J Bacteriol. 2008 (Epub ahead of print).

Sanderson-Smith ML, K. Dinkla, J. N. Cole et al., "M protein-mediated plasminogen binding is essential for the virulence of an invasive Streptococcus pyogenes isolate," The FASEB Journal, vol. 22, no. 8, pp. 2715–2722, 2008

Sanderson-Smith ML1, De Oliveira DM, Ranson M, McArthur JD. Bacterial plasminogen receptors: mediators of a multifaceted relationship. J Biomed Biotechnol. 2012;2012:272148. doi:10.1155/2012/272148. Epub 2012 Oct 14.

Schuchat A. Group B *streptococcus*. Lancet. 1999 Jan 2;353(9146):51-6. Review. PMID: 10023965

Seifert, K.N., McArthur, W.P., Bleiweis, A.S., and Brady, L.J. (2003) Characterization of group B streptococcal glyceraldehyde-3-phosphate dehydrogenase: surface localization, enzymatic activity, and protein-protein interactions. Can J Microbiol 49: 350–356.

Seo, H.S., Minasov, G., Seepersaud, R., Doran, K.S., Dubrovska, I., Shuvalova, L. et al. (2013) Characterization of fibrinogen binding by glycoproteins Srr1 and Srr2 of *Streptococcus agalactiae*. J Biol Chem 288: 35982–35996.

Siemens N, N. Patenge, J. Otto, T. Fiedler, and B. Kreikemeyer, "*Streptococcus pyogenes* M49 plasminogen/plasmin binding facilitates keratinocyte invasion via integrinintegrin- linked kinase (ILK) pathways and protects from macrophage killing," The Journal of Biological Chemistry, vol. 286, no. 24, pp. 21612–21622, 2011

Six, A., Bellais, S., Bouaboud, A., Fouet, A., Gabriel, C., Tazi, A., Dramsi, S., Trieu-Cuot, P., and Poyart, C. (2015) Srr2, a multifaceted adhesin expressed by ST-17 hypervirulent Group B

Streptococcus involved in binding to both fibrinogen and plasminogen. Mol Microbiol 97: 1209–1222.

Spellerberg B, Rozdzinski E, Martin S, Weber-Heynemann J, Lutticken R. *rgf* encodes a novel two-component signal transduction system of *Streptococcus agalactiae*. Infect Immun. 2002;70:2434–2440.

Suenson E, Thorsen S. Secondary-site binding of glu-plasmin, lys-plasmin and miniplasmin to fibrin. Biochem J 1981; 197: 619–28.

Sun,H, U. Ringdahl, J. W. Momeister et al., "Plasminogen is a critical host pathogenicity factor for group A streptococcal infection," *Science*, vol. 305, no. 5688, pp. 1283–1286, 2004.

Tazi, A., Disson, O., Bellais, S., Bouaboud, A., Dmytruk, N.,Dramsi, S. et al. (2010) The surface protein HvgA mediates group B *streptococcus* hypervirulence and meningeal tropism in neonates. J Exp Med 207: 2313–2322.

Teatero S, Ferrieri P, Martin I, Demczuk W, McGeer A, Fittipaldi N; Toronto Invasive Bacterial Diseases Network.Serotype distribution, population structure and antimicrobial resistance of Group B *Streptococcus* strains recovered from colonized pregnant women. J Clin Microbiol. 2016 Nov 16. pii: JCM.01615-16. [Epub ahead of print] PMID: 27852675

Teles,C.,Smith,A.,andLang,S.(2012).Antibiotic modulation of the plasminogen binding ability of viridians group streptococci. Antimicrobial Agents Chemother. 56, 458463.doi:10.1128/AAC.00452-11

Tettelin H, Masignani V, Cieslewicz MJ, et al. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. Proc Natl Acad Sci USA. 2002;99:12391–12396.

Tettelin, H., Masignani, V., Cieslewicz, M.J., Donati, C.,Medini, D., Ward, N.L. et al. (2005) Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome". Proc Natl Acad Sci USA 102: 13950–13955.

Trivalle C, Martin E, Martel P, Jacque B, Menard JF, Lemeland JF.J Med Microbiol. 1998 Jul;47(7):649-52. PMID: 9839570 Group B streptococcal bacteraemia in the elderly

Urano T, De Serrano VS, Chibber BAK, et al. The control of the urokinase-catalyzed activation of human glutamic acid 1-plasminogen by positive and negative effectors. J Biol Chem 1987; 262: 15959–64.

Váli, Z. and Patthy, L. (1982) Location of the intermediate and high affinity omega-aminocarboxylic-acid-binding sites in human plasminogen. J. Biol. Chem. 257, 2104-2110.

Vassalli J D, A P Sappino, and D Belin The plasminogen activator/plasmin system. J Clin Invest. 1991 Oct; 88(4): 1067–1072. doi: 10.1172/JCI115405.

Violand BN, Castellino FJ. Mechanism of urokinase- catalyzed activation of human plasminogen. J Biol Chem 1976; 251: 3906–12.).

Wang, N.Y., Patras, K.A., Seo, H.S., Cavaco, C.K., Rosler, B., Neely, M.N., Sullam, P.M., and Doran, K.S. (2014) Group B streptococcal serine-rich repeat proteins promote interaction with fibrinogen and vaginal colonization. J Infect Dis 210: 982–991.

Weindling AM, Hawkins JM, Stringer J, Coombes MA. J Clin Pathol. 1981 Dec;34(12):1405. No abstract available. PMID: 7035502 Group B streptococcal serotypes correlated with maternal parity and carrier sites.

Weksler, B.B., Subileau, E.A., Perriere, N., Charneau, P.,Holloway, K., Leveque, M. et al. (2005) Blood-brain barrier-specific properties of a human adult brain endothelial cell line. FASEB J 19: 1872–1874.

Wiles, K.G., Panizzi, P., Kroh, H.K., and Bock, P.E. (2010) Skizzle is a novel plasminogen- and plasmin-binding protein from *Streptococcus agalactiae* that targets proteins of human fibrinolysis to promote plasmin generation.J Biol Chem 285: 21153–21164.

Index