University of Messina

Department of Biomedical Science, Dental Science and of Morphological and Functional Images

PhD course in "Translational Molecular Medicine and Surgery"

XXXVI Cycle

Coordinator: Prof. Antonio Toscano

PbsP, a major virulence factor of *Streptococcus agalactiae*, is regulated by the SaeRS two-component system

PhD student:
Francesco COPPOLINO

Tutor:
Concetta BENINATI

Supervisor:
Arnaud FIRON

Years 2021-2024

Contents

Abstract

Two-component regulatory systems (TCSs) are signal transduction machineries commonly used by prokaryotes to sense and respond to environmental conditions by modifying gene expression. Pathogenic and opportunistic bacteria exploit TCSs to reprogram gene expression during different steps of host infection. In this study, we investigated the role of a two-components regulatory system in *Streptococcus agalactiae*, a leading cause of severe neonatal infections such as sepsis and meningitis, designated SaeRS which was recently discovered to be activated upon contact with vaginal fluids, suggesting its role in host colonization. In this context, SaeRS once activated, is able to up-regulate two important adhesins called PbsP and BvaP, enhancing vaginal mucosal colonization. PbsP (Plasminogen binding surface Protein) is a cell wall-anchored protein, required for binding of surface-associated Plasminogen and Vitronectin. This protein is also a virulence factor involved in bacterial dissemination from the blood to the brain, and a valuable vaccine candidate since immunization with PbsP is protective against infection in murine models. Moreover, *pbsP* gene is present and highly conserved in all sequenced human GBS strains. Here we employed SaeRS loss of function *S. agalactiae* strains to investigate its role *in vivo*, using different murine models of infection. The SaeR-impaired strains showed decreased ability to lead lethality and to disseminate in blood and distant organs. We performed a transcriptomic analysis using a genetic approach to activate signaling pathways of SaeRS. Transcriptome of this constitutionally activated mutant revealed a marked up-regulation of four genes,

comprising *pbsP*, *bvaP*, *saeR*, and *saeS*, which represent the SaeR regulon. Moreover, the genes encoding for the two adhesins PbsP and BvaP was showed to be up-regulated *in vivo* during peritoneal infection. The strong up-regulation of PbsP in constitutionally activated mutant was found to be linked to an hyper-adhesive and hyper-invasive phenotype, using *in vitro* cellular assays. Also the *in vitro* ability to across the microvascular endothelial cells was enhanced in a PbsP-dependent way. These data suggest that SaeRS system is an important regulator of virulence, which is activated during infection and drive host-interaction changes trough the expression of PbsP. Due to its important role, this pathway could be the target of new therapeutic strategies.

Abbreviations

BBB Blood-brain barrier

CC Clonal Complex

CPS Capsular Polysaccharide

CSD Cytoplasmic Sensor Domain

DHp Histidine phospho-transfer

ELISA Enzyme-Linked Immunosorbent Assay

EMC Extracellular Matrix Components

EOD Early-onset disease

GAS Group A *Streptoccoccus*

GBS Group B *Streptococcus*

HK Histidine Kinase

HNP1 Human Neutrophil Peptide 1

IAP Intrapartum Antibiotic Prophylaxis

LBS Lysne-binding site

LOD Late-onset disease

MLST Multilocus Sequence Typing

PCV Polysaccharide conjugate vaccine

PLG Plasminogen

RR Response Regulator

SSURE Streptococcal Surface Repeats

STD Signal Transduction Domain

TCS Two Component System

TM transmembrane

tPA tissue plasminogen activator

uPA urokinase-type plasminogen activator

VN vitronectin

β-H/C β-hemolysin/cytolysin

Introduction

Streptococcus agalactiae

The word Streptococcus comes from the union of the Greek words "*strepto*", chain and "*coccus*", berry. Streptococci were discovered in 1874 by the Austrian surgeon Dr. Theodor Billroth, who reported the presence of chain-forming microorganisms in wound infections (Fig. 1).

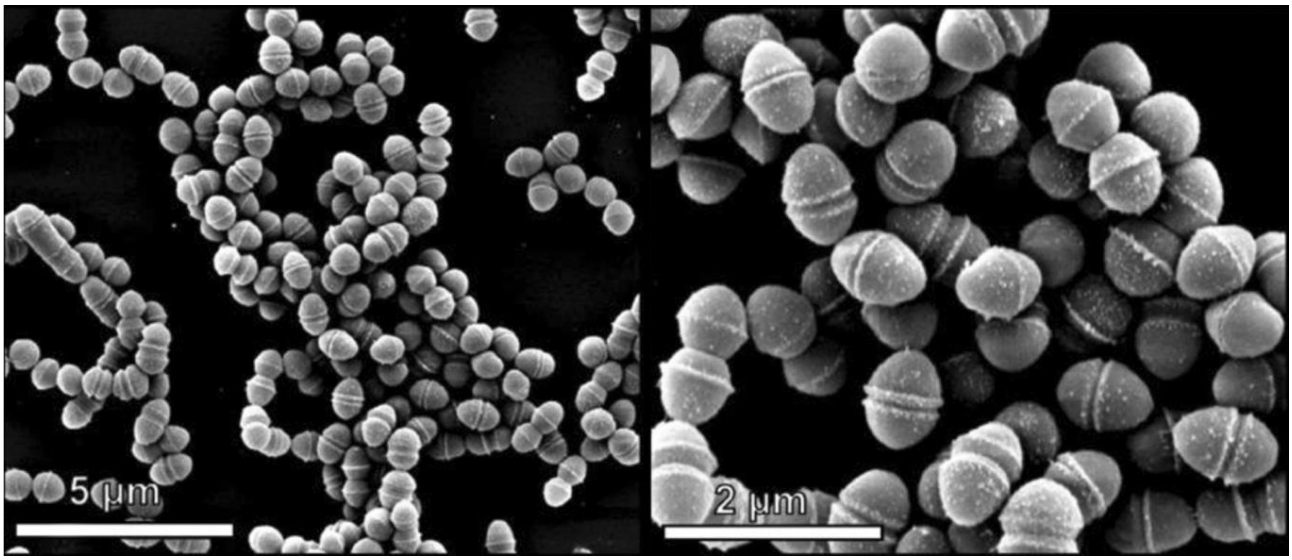


Figure 1. Electron microscopy imaging of Group B *Streptococcus* (1).

In 1879, Luis Pasteur isolated the microorganism from the uterus and the blood of women with puerperal fever, demonstrating that streptococci were responsible for a disease leading, at that time, to high mortality rates. So far, the genus *Streptococcus* includes more than 100 species, that are commonly classified according to two main criteria: the composition and immunological properties of surface antigens and the hemolytic patterns (alpha, beta and gamma) determined using blood agar plates (2).

Streptococcus agalactiae was first described in 1887 as a cause of bovine mastitis (3), which frequently leads to reduced milk production (4, 5), thereby the term *agalactiae* (from the Greek: *a*-, no; "*galactos"*, milk).

The pathobiontic nature of Group B *Streptococcus* (GBS)

S. agalactiae, commonly called group B *Streptococcus* (GBS), is a low-G+C Gram-positive, beta-hemolytic, encapsulated bacterium, belonging to the family of *Streptococcaceae* which includes *Streptococcus* and *Lactococcus* genera. Streptococci are typically commensal bacteria of humans and animals, with variable rates of virulence. In particular, GBS commonly colonizes the gastrointestinal and genital tracts of more than a third of the world's population (6) and is a member of the normal vaginal and intestinal microbiome of healthy adults. However, due to its pathobiontic nature, GBS can shift from commensalism to pathogenicity under specific genetic, immunological or environmental conditions. It is estimated that GBS is present in up to 40% of healthy pregnant women, from whom can be transmitted to newborns at the time of birth (Fig. 2).

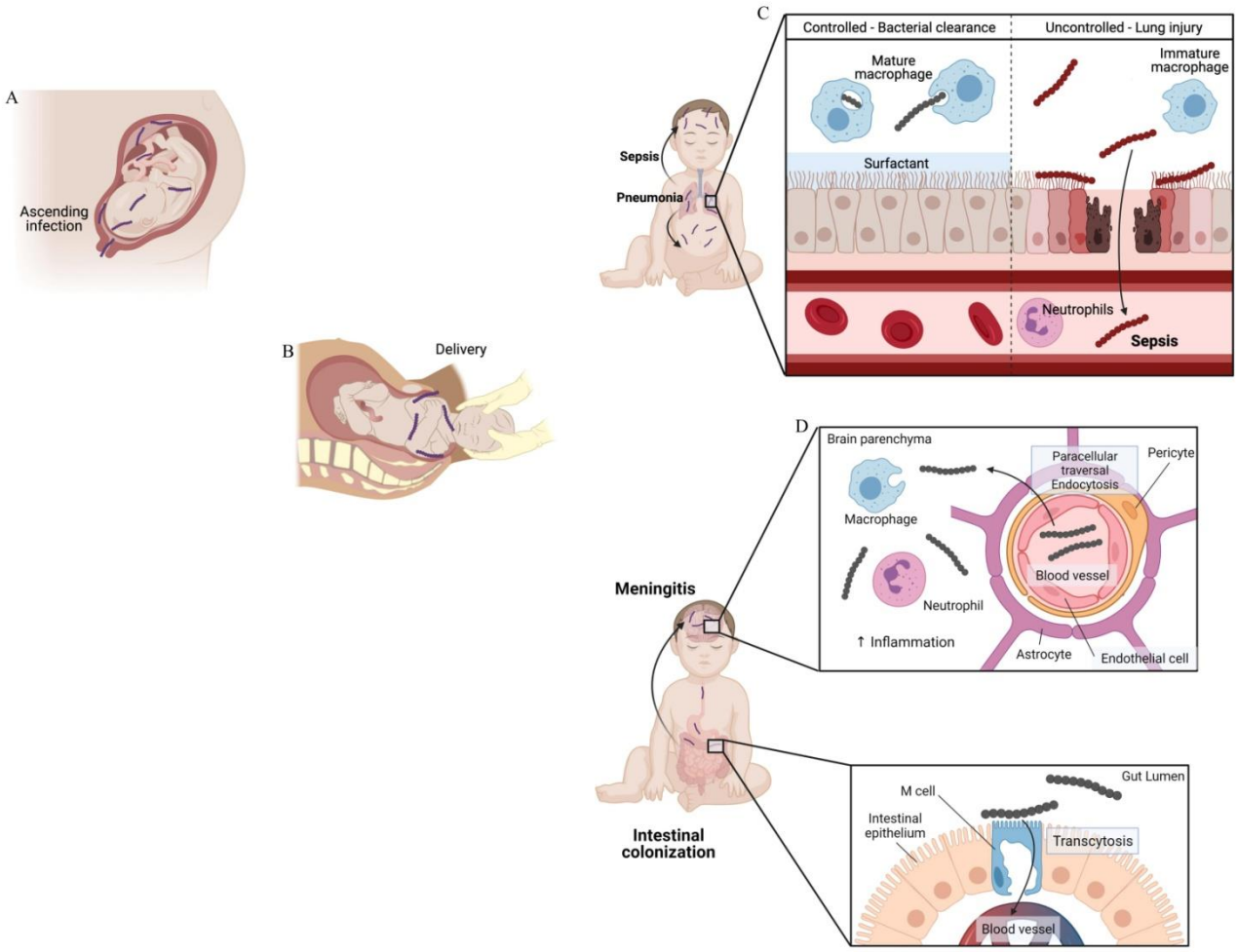


Figure 2. GBS routes of infection in neonates. GBS can be transmitted through ascending infection from the maternal vaginal tract (A) or through aspiration of infected vaginal fluid during delivery (B). In healthy adult lung microenvironment GBS is readily killed by mature macrophages, while developing neonate immune system is more susceptible to GBS invasion (C). Neonates can acquire GBS through vertical transmission or community source resulting in early intestinal colonization (D) (7).

This transmission can result in severe disease. Indeed, neonatal GBS infections are typically classified in two clinical syndromes: early-onset disease (EOD) and the late-onset disease (LOD). While EOD occurs during the first 6 days of life, with symptoms occurring usually within the first 48 h after birth, LOD can occur from 7 days up to three months after birth. Cases of very late-onset disease occur after 3 months of life. EOD

and LOD differ in their pathophysiology and clinical manifestations. EOD usually presents as respiratory distress, followed by septicemia, pneumonia, and, more rarely, meningitis. Instead, LOD commonly manifests as bacteremia and meningitis. EOD generally involves vertical transmission of GBS by inhalation of contaminated amniotic or vaginal fluid during labor, followed by bacterial translocation from the respiratory epithelium to the bloodstream (8) (Fig. 2).

The transmission route of GBS during LOD is poorly understood. Some studies suggest the presence of an early colonization of the neonatal intestinal tract followed by spread of GBS from the intestinal lumen to the bloodstream (9). LOD may develop through transmission from mother, non-maternal caregivers or nosocomial sources. Other risk factors are preterm birth, HIV-infected mothers, and contaminated milk. In addition to newborn infants, GBS can also cause invasive infections in pregnant women and other adults with a compromised immune system, such as oncology, diabetes mellitus or HIV patients (10-12). Moreover, elderly people are frequently affected by invasive GBS infections, and the incidence of these conditions is constantly increasing. Transmission of *S. agalactiae* in adults appears to be dynamic. Pneumonia, endocarditis, osteomyelitis, skin and soft tissue infections and urinary tract infections are the most common clinical features (8, 13). Sexual transmission is an important route for GBS, due to its presence in the urogenital tract as a commensal organism. Furthermore, GBS, albeit at low bacterial load, was also found on the skin and in the respiratory tract (14). It should be noted

that due to incomplete diagnosis, particularly in the case of abortions and preterm delivery, the actual incidence of GBS disease is underestimated.

The first strategy for the prevention of perinatal GBS infections was proposed by the American College of Obstetricians and Gynecologists, the Centers for Disease Control and Prevention and the American Academy of Pediatrics, which published a guideline based on intrapartum antibiotic prophylaxis (IAP) (15). This was followed by revised guidelines for the prevention of GBS disease released in 2002 and the updated guidelines in 2010 (16) which are currently in use. The guideline workflow includes a culture-based screening for GBS vaginal and rectal colonization of all pregnant women between 35 and 37 weeks' gestation. In case of a positive screening, IAP is undertaken through the administration of intravenous penicillin or ampicillin every 4 hours from the start of labor until delivery. In case of allergy to β-lactam antibiotics, cefazolin or clindamycin can be administered. Vancomycin intravenous administration is the only validated alternative in case of penicillin allergy and no bacterial response to the other treatments (17). Despite IAP in colonized women has decreased the incidence of GBS EOD, the incidence of neonatal LOD remained unchanged (18). In addition, GBS morbidity outside of the perinatal risk period has been permanently increasing and today represents the majority of all cases of invasive GBS disease in the United States (Centers for Disease Control and Prevention, 2015). Moreover, IAP negatively influences the microbiota of newborns, and promote the risk of development of *S. agalactiae* resistant strains (19). Thus, GBS disease persists as a major health problem for which additional or alternative

control measures are needed. Vaccination represents the most attractive strategy to reduce global morbidity and mortality associated with GBS. Most cases of invasive infections on newborns occur within the first 24 hours, so maternal immunization rather than direct vaccination of infants is required to prevent neonatal disease. Effective vaccines would stimulate the production of functionally active antibodies, capable of crossing the placenta and protecting fetus against GBS infection. The development of GBS vaccines for maternal immunization has been identified as a priority for World Health Organization (WHO) but, in spite of that, development has been slow and no vaccine has reached a Food and Drug Administration approval.

Was Rebecca Lancefield, in the 1930s, the first to demonstrate that protection against lethal GBS infection in mice could be achieved using capsular polysaccharide (CPS)-specific rabbit sera (20) thus paving the way to the development of a potentially protective vaccines. Among the several GBS virulence factors that have been targeted in vaccine research over the years, CPS is also the most widely studied putative target. Candidate vaccines using CPS alone as immunoprophylactic antigens have shown poor immunogenicity (21). For this reason, the development of GBS polysaccharide conjugate vaccine (PCV) as a stronger inducer of functional CPS-specific IgG responses has been encouraged (22) and so far several formulations of CPS-conjugate have reached clinical trials. In addition, different surface proteins of GBS have been characterized as conserved antigens capable of inducing protective responses in animal studies, such as Rib and alpha proteins (23-27), Sip (28) and C5a peptidase

(29). In the meantime, reverse vaccinology has been successfully applied to identify a conserved sequence encoding components of GBS pili, which induced an immune response directed against different GBS serotypes (30, 31). Despite these prevention strategies the global incidence of invasive GBS disease remains high, confirming this bacterium as the main leading cause of neonatal morbidity and mortality in most industrialized and developing countries.

Streptococcus agalactiae was historically grouped into ten serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX) depending on the antigenic and structural properties of the capsular polysaccharide discovered by Rebecca Lancefield (32) (Fig. 3).

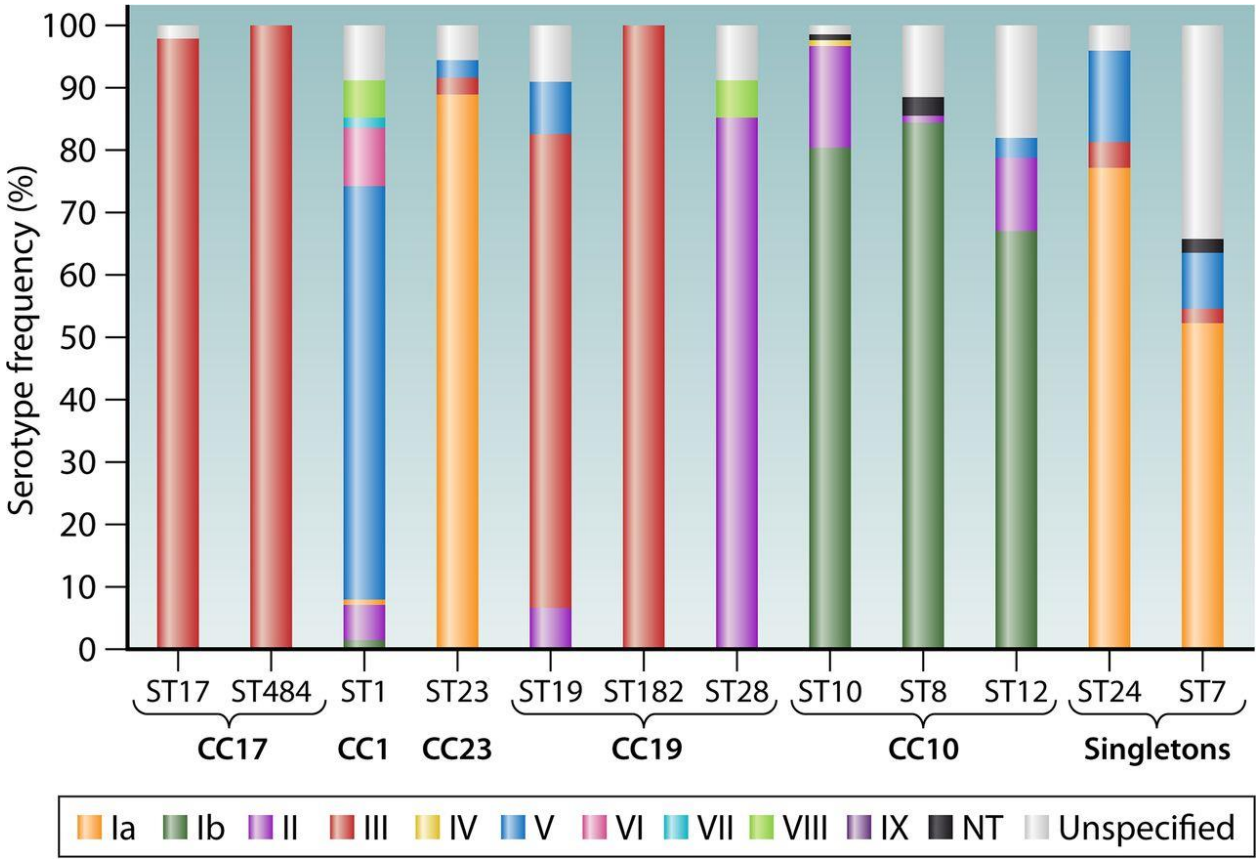


Figure 3. Serotype distribution within each Multilocus Sequence type and their corresponding Clonal Complexes (33).

Epidemiological data collected worldwide have shown that the majority of invasive neonatal diseases are associated with serotype III (34-36). In addition to serotyping, GBS isolates can further classified for their genetic characteristics. In 2003, a multiple gene sequencing scheme (or Multilocus Sequence Typing, ML ST) was proposed, based on sequencing of seven housekeeping genes (*adhP*, *atr*, *glcK*, *glnA*, *pheS*, *sdhA*, and *tkt*) (37). In ML ST, the allelic profile of the strain (i.e. the 'Sequence Type' or ST, followed by a number), is determined by unique sequences of each housekeeping gene. Group of strains sharing at least five of the seven alleles are defined 'clonal complex' or CC, and represent a biologically significant cluster of STs that have diversified very recently from a common founder. Informatics tools based on algorithms, like eBURST, can support the clusterization of ML ST data set into groups of related isolates and clonal complexes, predicting the founding genotype of each clonal complex (38) (Fig. 4).

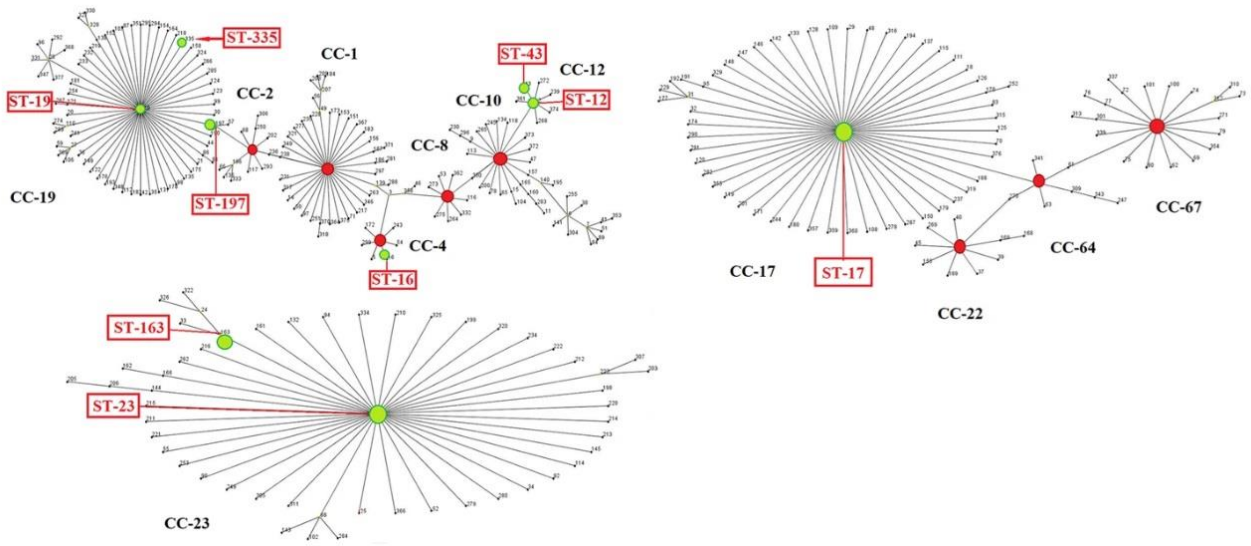


Figure 4. eBURST analysis of GBS strains using STs available in the ML ST database. Central bullet represent the group founder (39).

So far, the predominant CCs worldwide are CC-17, CC-23, CC-19, CC-1 and CC-10. It is also well established that CC-17 represents an "hypervirulent" lineage that is particularly associated with LOD.

GBS virulence factors

The success of GBS as pathogen is linked to several virulence factors that drive the pathogenic process, and typically involve colonization of host surfaces and translocation through host cellular barriers, bacterial evasion from the immune system, activation of inflammatory response and biofilm formation (40). The mechanism underlying the pathogenic activities of the main virulence factors has been extensively studied, but many other factors are poorly understood (Fig. 5).

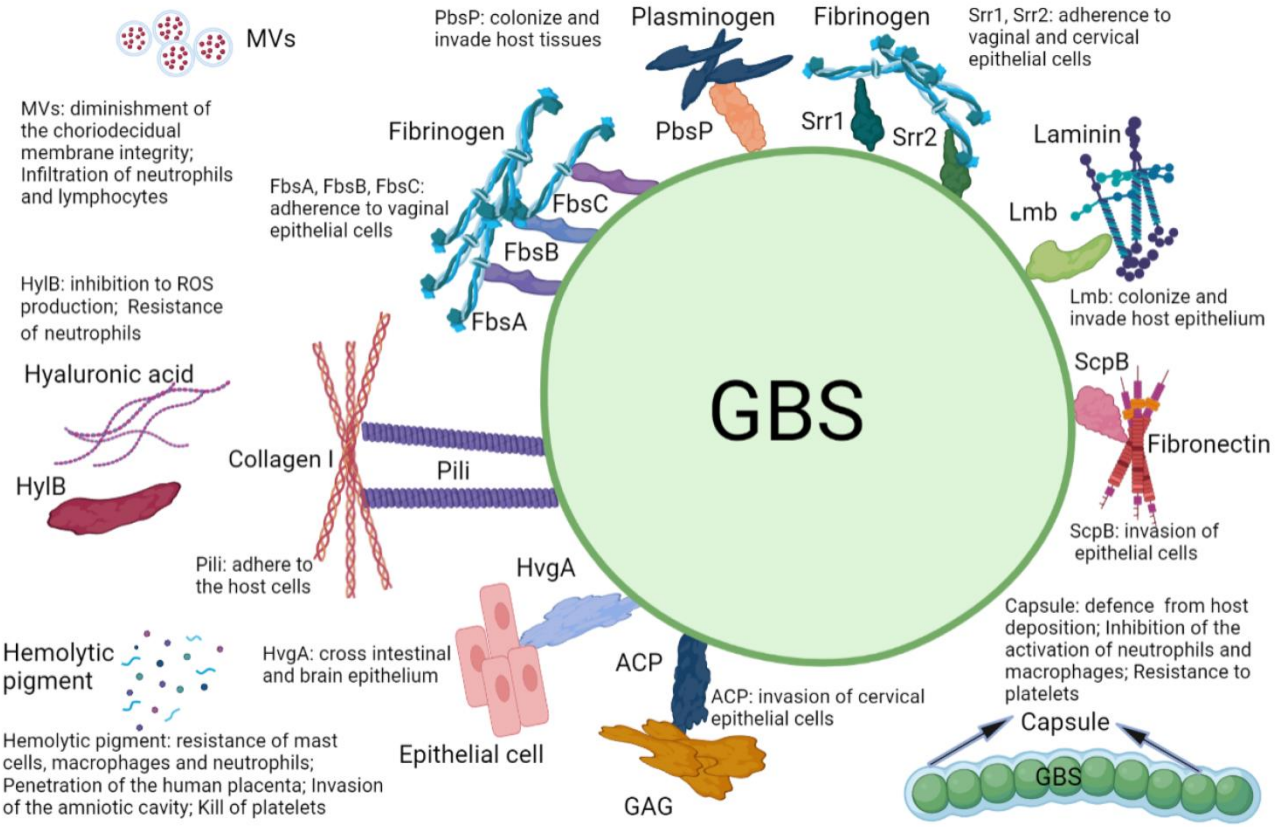


Figure 5. Most well-characterized GBS virulence factors with their specific target and mechanisms (41).

Among the best characterized virulence factors there are the pore-forming toxins, namely, the β-hemolysin/cytolysin (β-H/C) and the Christie Atkins Munch Peterson (CAMP) factor. β-H/C, encoded by *cyl* operon, is a surface-associated ornithine rhamnolipid pigment, which is cytotoxic to red blood cells and many other host cells. This virulence factor is conserved in many clinical isolates and is involved in pathogenesis, promoting GBS invasion of host cell barriers such as the epithelial and endothelial cells of the lung and the blood–brain barrier (BBB). β-H/C is an orange pigment that promotes pathogen resistance to reactive oxygen species (12).

CAMP factor is a secreted protein, identified as a virulence factor because of its ability, when purified, to form pores on membranes and to be lethal in *in vivo* models. CAMP factor is encoded through *cfp* gene, and its expression, as well as that of the *cyl* operon, is regulated by the two-component regulatory system CovRS (cov = control of virulence).

GBS exhibit virulence factors capable to interfere with innate immune clearance mechanisms and triggering a complex immunological response. The capsular polysaccharide (CPS), which represents the type-specific antigen, is the main factor against opsonophagocytic killing. CPS prevents surface deposition of opsonically active complement component C3 on the bacterial surface. Together with the CPS, several other surface components of GBS interfere with opsonophagocytosis mediated by complement cascade. C5a peptidase (also named ScpB), for example, prevents the recruitment of inflammatory cells by destruction of the chemo-attractant C5a (42). The complement-interfering protein (CIP) is a multifunctional secreted protein that mediates binding of factor H, a complement inhibitor, into the surface of the bacterium, thus preventing C3b deposition. CIP can reduce also the classical and lectin complement pathway activation through binding of the C4b factor (43). BibA binds specifically to the human C4-binding protein, a component of the classic complement pathway (44). The β-antigen binds IgA to the bacterial surface, hindering the binding of other opsonizing antibodies. Another efficient mechanism used by GBS to evade the immune system is the degradation of Neutrophil Extracellular Traps (NETs) through nucleases, such as NucA (45). An interesting escape strategy adopted by GBS is to survive inside host-cells.

To date, it is known that GBS can invade and survive within different cell types, however the mechanisms governing intracellular lifestyle of GBS are poorly understood. For instance, the pilus protein PilB promotes intracellular survival within phagocytes (46). Moreover, regulation mediated by two component regulatory systems such as CiaRH and CovRS has been described to be important for GBS survival in brain endothelial cells and macrophages (47, 48). Bacterial adhesion to host cell surfaces constitutes a first important step for GBS colonization and, in turn, host invasion. A large number of cell types have been described as possible targets of GBS. Typically, the first interactions occur with extracellular matrix components (EMCs) or serum proteins, subsequently GBS interacts with host cell surface proteins to invade tissues. Indeed, adhesins represent important virulence factors for GBS. Many of these adhesins are cell-wall anchored proteins, which exhibit modular and repeated domains typically involved in the binding to host factors, and other conserved sequences like the N-terminal signal sequence that drives protein translocation to the membrane, where it is removed after secretion or the C-terminal sorting motif LPxTG (Leu-Pro-x-Thr-Gly) which allows covalent binding to cell wall peptidoglycan (49). The best characterized adhesins are the fibrinogen-binding proteins A, B, and C (FbsA, FbsB, and FbsC) and the serine-rich repeat glycoproteins Srr1 and Srr2, the laminin-binding protein (Lmb), the pilus tip adhesin (PilA), the Streptococcal fibronectin-binding protein A (SfbA), the GBS immunogenic bacterial adhesin (BibA) and the C-17-specific hypervirulent GBS adhesin (HvgA), an allelic variation of *bibA*. FbsA was described to promote adherence (50), while FbsB and FbsC were shown to promote invasion of epithelial

and endothelial barriers (51-54). These proteins indirectly bind also to Plasminogen (Plg). Srr1 and Srr2 were reported to mediate invasion of microvascular endothelial cells (55, 56). Genomic data revealed that Srr2 is a cell wall-anchored protein specific for CC-17 strains, while the non-CC-17 isolates express only Srr1 (57). Both proteins have a key role in the development of GBS disease: Srr1 was demonstrated to promote vaginal colonization and persistence, while Srr2 was shown to increase bacterial survival to phagocytic killing and bacterial persistence in a murine model of meningitis (57, 58). Lmb mediates the binding of GBS to human laminin while ScpB is a fibronectin-binding protein. Another fibronectin-binding protein is SfbA, involved in invasion of brain endothelium and development of meningitis (59). Both proteins are essential for bacterial colonization of damaged epithelium and translocation of bacteria into the bloodstream (42, 60). Pilus adhesin PilA is a multifunctional protein demonstrated to be essential in promoting GBS colonization, persistence, biofilm production and central nervous system invasion (61, 62). BibA is an immunogenic adhesin with anti-phagocytic activity due to its capability to bind human C4-binding protein, a regulator of the classic complement pathway (44). The role of this protein in GBS adhesion was demonstrated by the impaired ability of a *bibA* knockout mutant strain to adhere to both human cervical and lung epithelial cells and to survive in human blood by resisting to opsonophagocytic killing by human neutrophils. HvgA adhesin is an allelic variant of *bibA* present in CC17 strains and confers a higher ability to translocate through the blood-brain barrier, a typical GBS target during LOD.

Plasminogen binding surface Protein (PbsP)

Plasminogen binding surface Protein (PbsP) is a virulence factor that has been discovered through analysis of the NEM316 GBS strain exoproteome in absence of the CovRS TCS. Subsequently, the PbsP structure has been characterized revealing that it is an LPXTG cell wall-anchored surface protein made of a NH_2-signal peptide, bearing a YSIRK sorting motif, two 150-aa repeated sequences (named as Streptococcal Surface Repeats or SSURE domains) (63-65) and a methionine and lysine-rich (MK-rich) region (66) (Fig. 6).

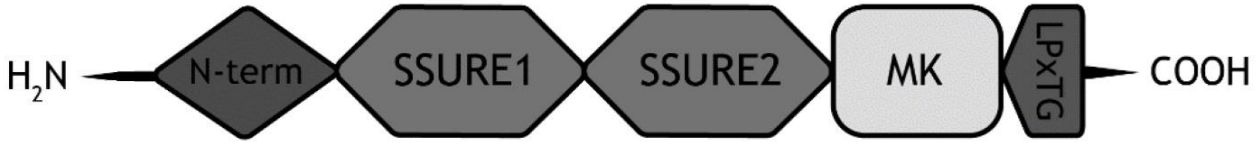


Figure 6. Schematic representation of Plasminogen binding Protein domains (67).

Despite having been described in strain NEM316, the *pbsP* gene is present and highly conserved in all clinical isolates of GBS with 99.3% identity (68), whereas it is not found in non-invasive strains such as *S. dysgalactiae* (69). Results by Buscetta et al. indicate a low-grade presence of PbsP on the bacterial surface, as demonstrated by flowcytometric analysis performed using an anti-rPbsP serum, as well as its increased expression in the Δ*covRS* strain and its abrogation in *srtA*- and *pbsP*-deleted strains. PbsP binding properties were further analysed with purified recombinant PbsP in enzyme-linked immunosorbent assay (ELISA) using extracellular

matrix or blood components as coating proteins. This analysis revealed that Plg is the main ligand of PbsP, and additional data explained how GBS can use this binding to increase their cell surface proteolytic activity, thereby enhancing the ability of bacteria to degrade host physiological barriers such as the extracellular matrix, the basement membrane and encapsulating fibrin networks. In fact, GBS can stabilize the surface-bound Plg in a cleavable conformation through the Skizzle protein and, by this way, host factors, such as tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) can convert it into Plasmin leading to proteolytic activities (70). Moreover, in another paper, PbsP has been shown to also bind to human Vitronectin (Vn) through the SSURE domains, forming a bridge with the α_V integrin subunit, thus highlighting its relevance in mediating streptococcal interaction with different types of epithelial cells (66, 71). These data suggest that PbsP is a multi-ligand protein capable of hijacking, either at the same or at different times during pathogenesis, at least two major components of the host ECM.

PbsP abilities to interacts with host components seems to be required for GBS hematogenous spreading and targeting of different organs during *in vivo* infection assays, as revealed, for instance, by an impairment in the ability of NEM316 Δ*pbsP* to colonize the kidney and the brain (66). Lentini et al. extended the role of PbsP as a virulence factor of CC-17 GBS, by finding that deletion of PbsP selectively impairs bacterial colonization of the brain, despite an unchanged capacity to persist at high levels in the blood and to invade the kidneys, in agreement with the high tropism of CC-17 strains for the central nervous system (72). Expression

of *pbsP* measured by qPCR was also found to be increased in bacteria recovered from the brain or the kidneys. Moreover, immunization with recombinant PbsP prevented brain infection and lethality, both in CC-17 and CC-23 strains.

In a recent published study appended to this thesis, the molecular regions involved in the interactions between Plg and PbsP was identified (67). This study proposes that the MK-rich domain, more specifically the region spanning amino acids S_{444} to N_{484}, is mainly responsible for PbsP binding to plasminogen. On the other hand, the Kringle domain 4 of Plg is the host molecule region involved in the interaction. It is well-known that Kringle domains of Plg are able to interact via their lysine-binding sites (LBS) with multiple ligands, including host Plg receptors/targets, such as fibrin and α2 anti-plasmin, and bacterial proteins (73, 74), but also free L-lysine. The involvement of LBS in Plg-PbsP interaction was confirmed by testing the inhibitory effects of soluble L-lysine. After proving the role of LBS, the role of the abundant lysine residues presents in MK-rich domain using several mutated forms of this domain, in which different lysine residues were replaced by alanine was assessed. Surprisingly, none of the mutated forms showed decreased binding to Plg as compared with the corresponding fragment bearing all lysine residues, indicating that lysine residues in the MK-rich region are not required for interactions between this region and the LBS in the Plg molecule. Methionine is also abundant in the MK-rich domain. Increasing the concentration of free L-methionine partially inhibits binding, suggesting a possible involvement of this amino acid. In conclusion, this study identified the molecular region involved in

Plg-PbsP interaction, which seems to represent a novel bacterial sequence capable of interacting with the LBS of Plg even in the absence of lysine residues.

Two-component regulatory systems: the bacterial sense organs

General structure of two-component systems

In bacterial pathogenesis, two-component systems (TCSs) play a central role among the various signal transduction systems evolved by bacteria. They are involved in sensing various types of stimuli present in the environment such as osmotic pressure, membrane stress, the presence of specific host molecules, pH, antibiotics, and others factors. TCSs are composed of a sensor protein, typically a histidine kinase (HK), and a response regulator (RR) (Fig. 7).

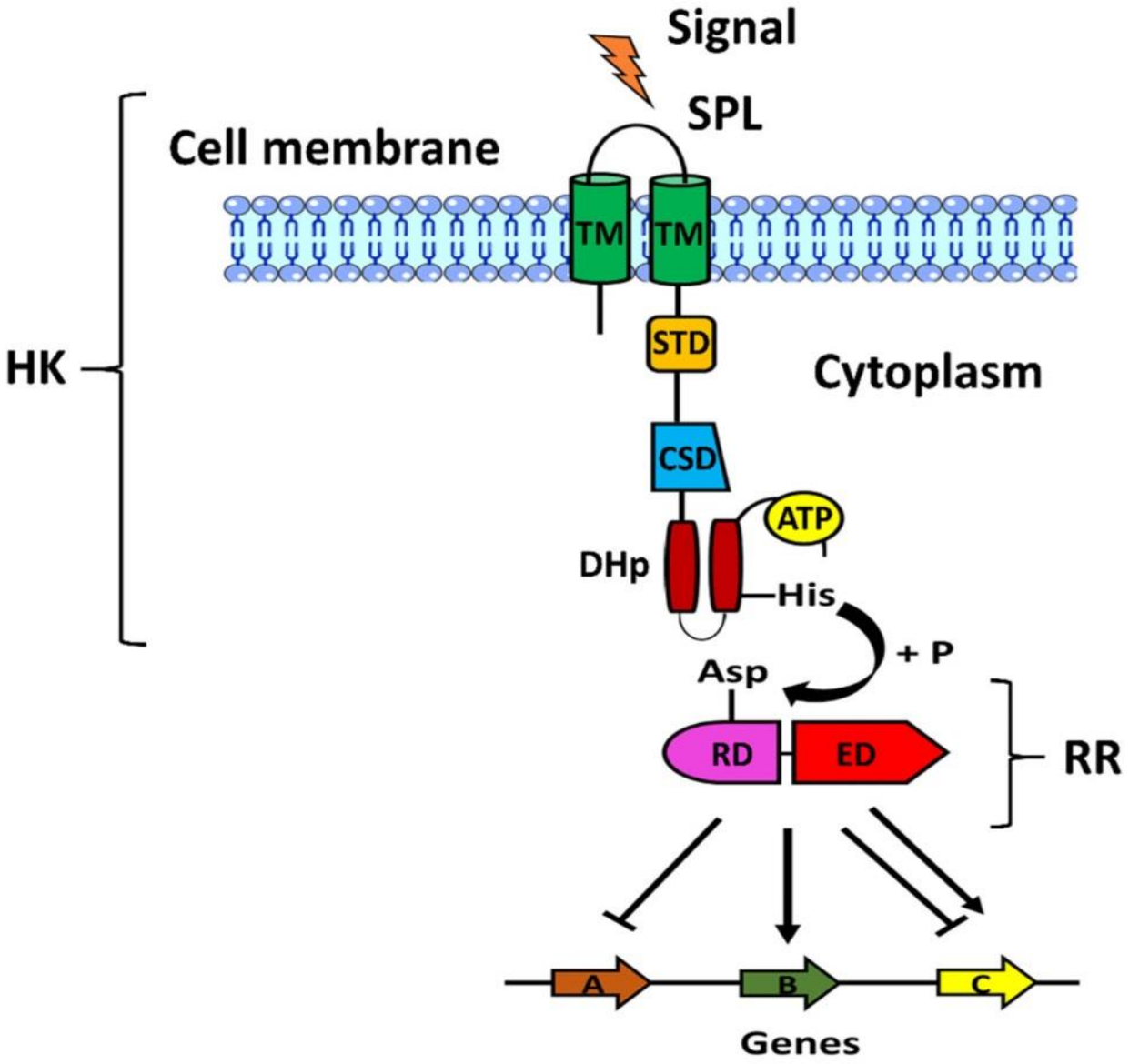


Figure 7. Representation of the main components of a prototypical two-component system (75).

The canonical structure of a sensor protein presents four domains: a sensor domain, bearing two transmembrane (TM) helices, an intracellular signal transduction domain (STD) also defined as HAMP (commonly found in Histidine kinase, Adenyl cyclase, Methyl-accepting proteins, and Phosphatase), a cytoplasmic sensor domain (CSD), and a conserved intracellular kinase domain, also called transmitter module, which

mediates both auto-phosphorylation and the transfer of a phosphoryl-group to the RR. Signal transduction in canonical TCSs occurs between the transmitter module of the sensor protein and the receiver domain of the cognate response regulator (76, 77). The above-mentioned transmitter module is composed of an N-terminal Dimerization and Histidine phospho-transfer (DHp) subdomain and a C-terminal Catalytic and ATP-binding (CA) subdomain. The majority of sensor domains can have three types of structural folding: mixed αβ, all-helical or β-sandwich (78). The response regulators have a simpler structure than sensor proteins. A prototypical RR is made of two domains: a receiver domain and an effector domain. While the first one accepts a phosphoryl group transferred by a cognate sensor, the second one triggers the signaling events. Unlike the effector domains, the receiver domains have a sequence where an aspartate residue is well conserved and is phosphorylated by HK. This event results in conformational changes in the receiver domain with the consequent activation of the effector domain. Structurally, the receiver domain has a conserved (βα)5-fold, in which a five-stranded β-sheet is surrounded by α-helices (79). After the activation of RRs by phosphorylation, several structural changes are induced in some of the α and β elements positioned in the receiver domains, particularly in correspondence of the conserved Thr/Ser and Tyr/Phe key residues (78, 80). Effector domains, also known as output domains, have generally DNA-binding properties and are classified into several subfamilies based on predicted domain structures. The most common effector domains have a winged Helix-Turn-Helix (wHTH) motif or a Helix-Turn-Helix (HTH) motif (81-83). Phosphorylation-induced DNA binding underlies promotion

of gene transcription induced by signal transduction. However, there is a balance between positive and negative controls that determines the net output of the response. Negative control can result directly from the receiver domain of RR, that exhibits a characteristic rate of auto-de-phosphorylation. Additionally, the receiver de-phosphorylation rate can be enhanced by the cognate sensor transmitter module. In fact, several hypotheses were proposed about the mechanism of de-phosphorylation mediated by the sensor protein. For instance, different studies suggest the presence of a conserved DxxxQ motif on the DHp helix α1 (84) in which, substitution of Gln residue with Ala, Glu or His produce a sensor protein defective in phosphatase activity, but still endowed with auto-kinase and phospho-transfer activities (85).

In general, bacteria require numerous TCSs, depending on the specific inputs detected, and therefore by the microenvironment in which they are embedded. Indeed, metabolic adaptations to the host milieu are dependent on the integration of various TCSs-dependent molecular pathways. Gene expression studies are crucial to understand how TCSs act on the genome. The typical approach is to verify gene expression in mutants deleted either in the RR, HK or both, in comparison with the wild-type parental strain (86, 87). Furthermore, mutant strains overexpressing the RR may be suitable to examine target gene expression when the activating stimulus is unknown (88). Various tools are also available to map TCS-targeted sequences and to define signaling networks across different bacterial species or types of infection (89). In this manner, researchers can understand whether RRs may function as activators, repressors, or both.

Two-component systems in GBS

To regulate its marked pathobiontic properties, GBS exploits numerous TCSs that allow it to modulate gene expression depending on the micro-environmental context. Not surprisingly, the number of TCSs identified in GBS is twice as high compared to any other member of the *Streptococcaceae* family. Among the 22 TCSs identified by genomic analysis, only a few have been well investigated, while the mechanisms used by GBS to tightly regulate the expression of virulence factors remain poorly understood. The main GBS TCSs involved in pathogenesis have been classified into three groups (Fig. 8) on the basis of their role in the course of infections:

1) pathogenesis and colonization of the host (CovRS, BgrRS, HssRs, LtdRS);
2) colonization and adhesion to host mucosal tissues (RgfAC, FspSR, SaeRS);
3) resistance to antimicrobial peptides (DltRS, LiaSR, BceRS, CiaRH) (90).

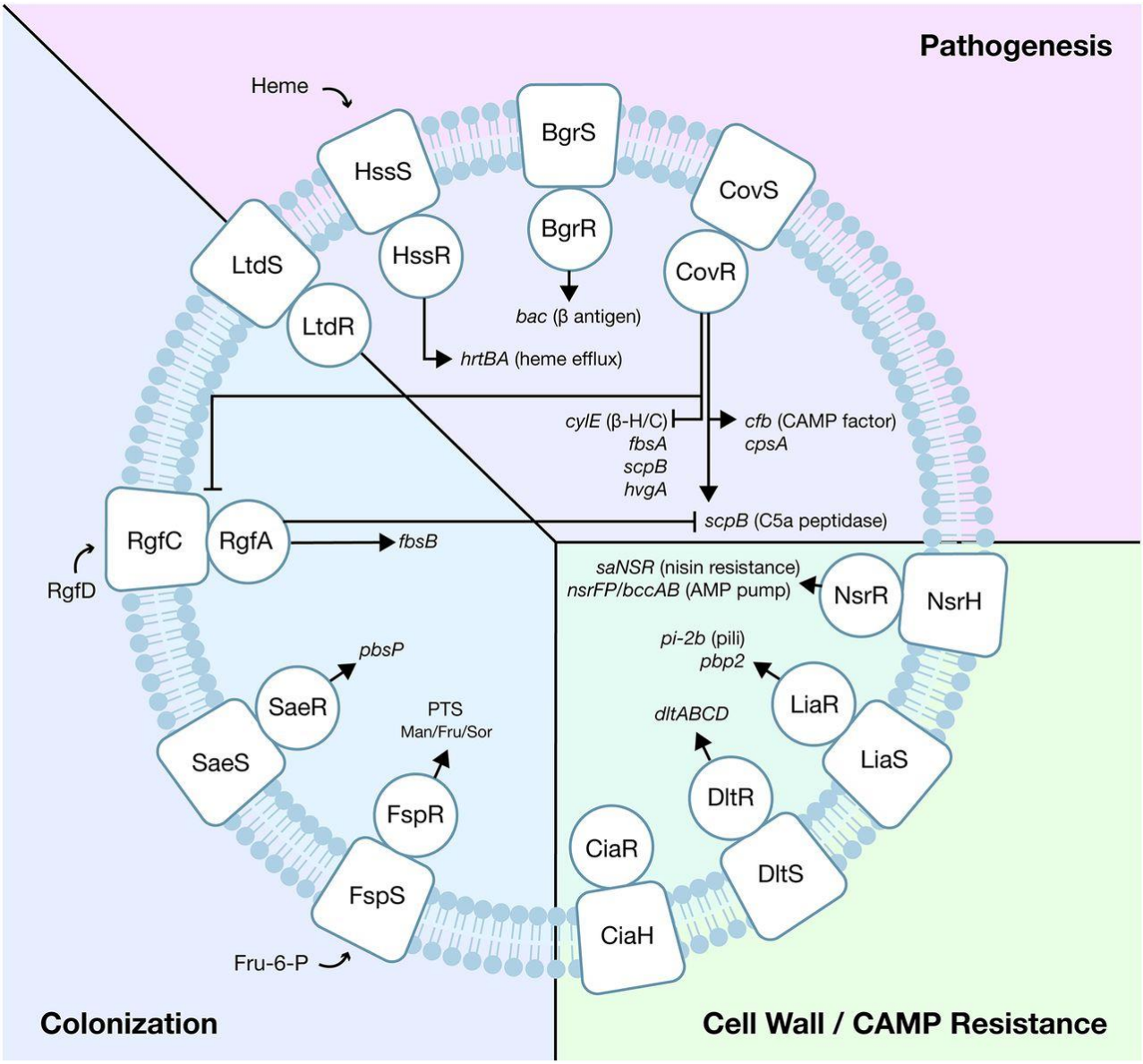


Figure 8. *Streptococcus agalactiae* two-component system and their main target genes (90).

The most-studied TCS in GBS is the CovRS system. This CovRS is an orthologous of the CovRS system of *S. pyogenes* (or Group A *Streptococcus*, GAS). It bears a highly conserved sequence among GBS strains and is able to regulate a large number of virulence-associated genes in different pathogenic species. However, unlike *S. pyogenes*, in which CovR works essentially as a repressor, in GBS CovR modulation of gene

expression is more complex and can result in repression, activation or indirect regulation. When activated by host environment variations such as pH (91) or glucose levels (92), CovS phosphorylates CovR at its conserved aspartic acid residue at position 53 (Asp53). Consequently, CovR changes its affinity for target promoters to directly alter transcription of a large number of GBS genes, depending on the studied clonotype. In fact, behind the virulence variability among different strains of the same species we can discern an important role of differences in regulatory mechanisms. As explained by Mazzuoli et al. in a recent work, CovRS, due to its central and complex role in GBS, is at the center of a fine-tuned regulatory network that is subjected to selective pressures and can generate diversities in streptococcal populations (93). For instance, CovRS can be finely regulated by other proteins. The equilibrium between the phosphatase and kinase activities of CovS depends on its interaction with the membrane protein Abx1.This protein is able to inhibit the CovRS signaling by antagonizing CovS (94). Moreover, the eukaryotic type serine/threonine kinase Stk1 controls virulence indirectly, *via* CovR (95), by direct phosphorylation at its conserved Threonine residue at position 65 (Thr65). The target genes regulated by CovRS in GBS belong to the same functional classes as those regulated by its ortholog in GAS, but aren't identical, despite the close phylogenetical properties of these two species. Mutants with impaired CovRS activity, exhibit a hyper-hemolityc and hyper-pigmented phenotype, linked with transcriptional activation of *cyl* operon (96). In different studies, other virulence factors were demonstrated to be under control of CovR as a repressor, for instance *hvgA/bibA locus* (97), *PI-1 pili* operon, FbsA (50), FbsB (51) and BsaB/FbsC (53, 54) and

PbsP (66). Interestingly, *pbsP* modulation-by-repression mediated by CovRS is not conserved in BM110 (72), supporting a clonotype-specific regulation. Among the genes activated by the presence of phospho-CovR we can mention *cfb* gene, encoding for CAMP factor, as demonstrated by an impaired CAMP activity of Δ*covRS* mutant (96). Moreover, CovRS role was investigated utilizing different cell-types. For instance, GBS A909 Δ*covR* strain showed a reduced capacity to invade human brain microvascular endothelial cells (hBMEC/D3), but increased adherence to these cells. Strikingly, the same mutant displayed an opposite phenotype using human vaginal epithelial cells (HVEC) (98, 99). Finally, CovRS was demonstrated to play a key role *in vivo* murine model, such as urinary tract infection model, vaginal colonization model and sepsis model in neonatal rats (96, 100).

Other TCSs of GBS were described to be involved in pathogenesis. The BgrRS system regulates the expression of *bac* gene, encoding for β-antigen. Interestingly, *bac* gene is located nearby the *brg* operon, responsible for expression of BgrRS. The absence of the sensor BgrS doesn't impair the expression of β-antigen, suggesting a potential crosstalk with other HK (101). The HssRS system modulates the heme transport efflux system HrtBA, regulating the concentration of heme, an important cofactor for bacteria (90).

The DltRS system is encoded by two genes belonging to *dlt* operon, responsible for the D-alanylation of lipoteichoic acid (LTA). Integration of D-Ala in the cell wall of Gram-positive bacteria allows for the maintenance and protection of the cell from turgor pressure and external

stress, such as antibiotics. Data suggest that DltRS help to maintain appropriate levels of D-Ala on LTA based on environmental signals by modulating expression of the *dlt* operon (102).

SaeRS two-component system

SaeRS TCS in *S. aureus*

The *sae* locus (stands for *S. aureus* exoprotein expression) encodes for the SaeRS TCS in *S. aureus*. It was discovered by Giraudo et al. in 1994, in mutants defective in production of different exoproteins (α-hemolysin, β-hemolysin, nuclease, and coagulase) (103). To date, SaeRS is one of the best characterized TCS of *S. aureus* and its roles in virulence factor expression and staphylococcal pathogenesis are well documented and understood. It is a canonical TCS, since the signaling cascade is activated when SaeS, the sensor histidine kinase, detects cognate environmental signals (e.g., human neutrophil peptides, HNPs) (104) and autophosphorylates at the conserved His131 residue. The phosphoryl group is then transferred to Asp51 of SaeR, and the phosphorylated SaeR (SaeR-P) binds to SaeR binding sequence (SBS) andactivates the transcription of the target genes. The *sae* operon includes four genes (saeP, saeQ, saeR, and saeS), and the two promoters P1 and P3 (Fig. 9).

Figure 9. Map of *sae locus* in *Staphilococcus aureus* (105).

The P3 promoter has weak constitutive activation resulting in transcription of a single mRNA bearing *saeR* and *saeS*, located downstream P3. The P1 is located upstream the very first gene saeP and can transcribe for all the four genes with a stronger auto-inducible activation. However, the increase of SaeR and SaeR expression is not expected to further increase the activity of the system because overexpression of *saeRS* does not alter the expression pattern of the Sae-regulon (106). P1 activity is also influenced by growth phase and is maximal in the post-exponential growth phase (107, 108). SaeS is composed of 351 aa and is a bi-functional histidine kinase that present both kinase and phosphatase activities. Structurally consists of a transmembrane domain, a HAMP domain, and a kinase domain. It was demonstrated that the transmembrane domain is necessary and sufficient to recognize and respond to its activation signal, HNP1. Moreover, SaeS seems to be involved in a basal kinase activity, as demonstrated by some induced mutation or SNP variants that affect this property. SaeR belongs to the OmpR family response regulator. It is composed of 228 aa and presents a receiver domain at its N-terminus, with its Asp51 phosphorylation site, and the DNA binding domain ($SaeR^{DB}$) at its C-terminus. It was demonstrated that phosphorylation at Asp51 is

essential for SaeR to bind its target DNA (109). Regarding $SaeR^{DB}$, when isolated it is able to bind DNA, while in the whole SaeR native form seems to be blocked by the unphosphorylated receiver domain. The binding sequence of SaeR-P is $GTTAAN_6GTTAA$, where N = any nucleotide with preference to A/T (109, 110). *saeP* and *saeQ* are auxiliary genes upstream *saeRS*. SaeP is a lipoprotein of 146 aa and SaeQ is a membrane protein of 152 aa. Even if they are not essential for the activation of the SaeRS system, they are required to enhance the SaeS phosphatase activity by forming a SaePQS ternary complex (105). Regarding stimuli recognized by SaeS, both activation and inhibitory signals were discovered so far. For instance, Human neutrophil peptide 1, 2, and 3 (HNP1-3) are antimicrobial peptides that specifically activates SaeRS system. Surprisingly, SaeRS can also be activated by murine neutrophil that don't produce HNP. Moreover, another product of human neutrophils, Calprotectin, is able to activate SaeRS. Instead, among the inhibitory signals, the Silkworm Apolipophorin Protein and the environmental conditions of acid pH (5.5) and 1M NaCl have been reported. SaeR binding sequences were clustered in two different classes of promoters. Class I displays low affinity target promotersand their transcription requires relatively high levels of SaeR-P. Among the factors/genes under the controls of Class I promoters, the main are a coagulase enzyme (*coa*) involved in blood coagulation that interacts with fibrinogen, the fibronectin binding protein A (*fnbA*) involved in invasion of host cells, the extracellular adherence protein (*eap*) that mediates adhesion to host cells and has anti-inflammatory activities mediated through binding to intercellular adhesion molecule type 1 (ICAM-1), *saeP* and others. The class II target promoters seem to have a

high affinity for phospho-SaeR, and the basal level of SaeR phosphorylation is sufficient for their transcription. Two genes were identified under this kind of promoter, *hla* and *hlb*, encoding respectively for α-hemolysin and β-hemolysin. Different studies suggest a possible modulation in expression of *sae* operon by other TCSs.

SaeRS TCS in *S. agalactiae*

A *sae* locus is present also in GBS, indeed SaeRS system genes were identified for the first time by Cook et al. to be highly upregulated *in vivo*, using a murine model of vaginal colonization with GBS A909 strain (111). The *in silico* analysis confirmed a good score of homology between SaeR (48% identical) and SaeS (34% identical) proteins of *S. aureus* and *S. agalactiae* (Fig.10).

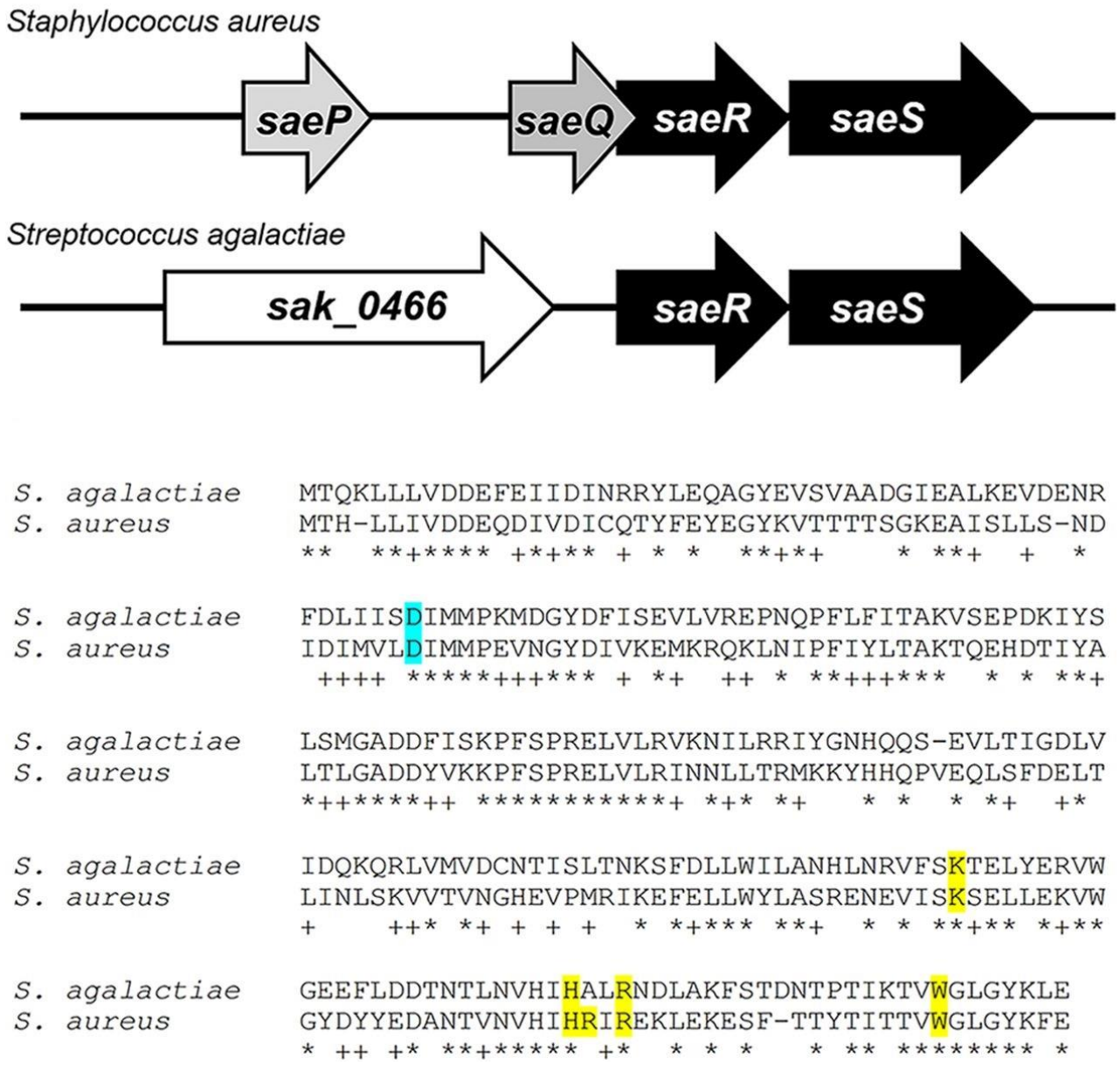


Figure 10. Map of *sae* locus in *Streptococcus agalactiae*. SaeR protein sequence alignment between *S. agalactiae* and *S. aureus*. Residues marked in yellow were shown to be important for DNA binding. Residue marked in blue is the predicted phosphorylatedaspartic acid residue for the SaeR protein in *S. aureus* (111).

The amino acid residues of the $SaeR^{DB}$ (DNA binding domain) are mostly conserved between the two species. However, the SaeR sequences flanking $SaeR^{DB}$ are quite divergent, raising the possibility that the two orthologs recognize different DNA motifs. In the GBS homologous system, *saeP* and

saeQ are replaced by the presents of *pbsP* gene (*sak_0466* in A909 strain). In the same work, *pbsP* gene expression was found to be highly upregulated *in vivo*. Moreover, *pbsP* upregulation after bacterial stimulation with vaginal fluids was demonstrated to be SaeR-dependent. Another gene designated as *sak_1753* (subsequently subsequently called group B streptococcus vaginal adherence protein, BvaP), was found to be upregulated during vaginal colonization and dependent by SaeR, after bacterial stimulation with vaginal fluids. Using murine models,it has been shown that PbsP and BvaP are important for colonization of vaginal tract (111, 112). The activating stimulus of the SaeRS system is still unknown, however, it has been proposed that the signal is likely a small heat-labile peptide of less than 3 kDa (111).

Aim of the thesis

The aim of this thesis was to better investigate about the importance of SaeRS system in modulation of virulence and host-pathogen interactions, focusing on the role of its target gene *pbsP*. The first objective of the work was to consolidate the importance of this system during infection, using *in vivo* murine models. A second objective was to resolve the SaeR regulon, which is the group of genes directly regulated by the SaeRS system, and its genetic modulation. A third objective was to decipher the importance of PbsP modulation during host-cells interactions. A better understanding of this pathogenic mechanism could disclose new therapeutic strategies to fight the rising etiological agent *S. agalactiae*.

Results

Construction and analysis of *S. agalactiae* mutant strains for SaeRS system

In order to decipher the role of SaeRS system in GBS, we produced constitutively deactivated mutants *via* loss of function mutations in SaeR response regulator, and constitutively activated mutants *via* abrogation of the phosphatase activity of the SaeS sensor (Fig. 11).

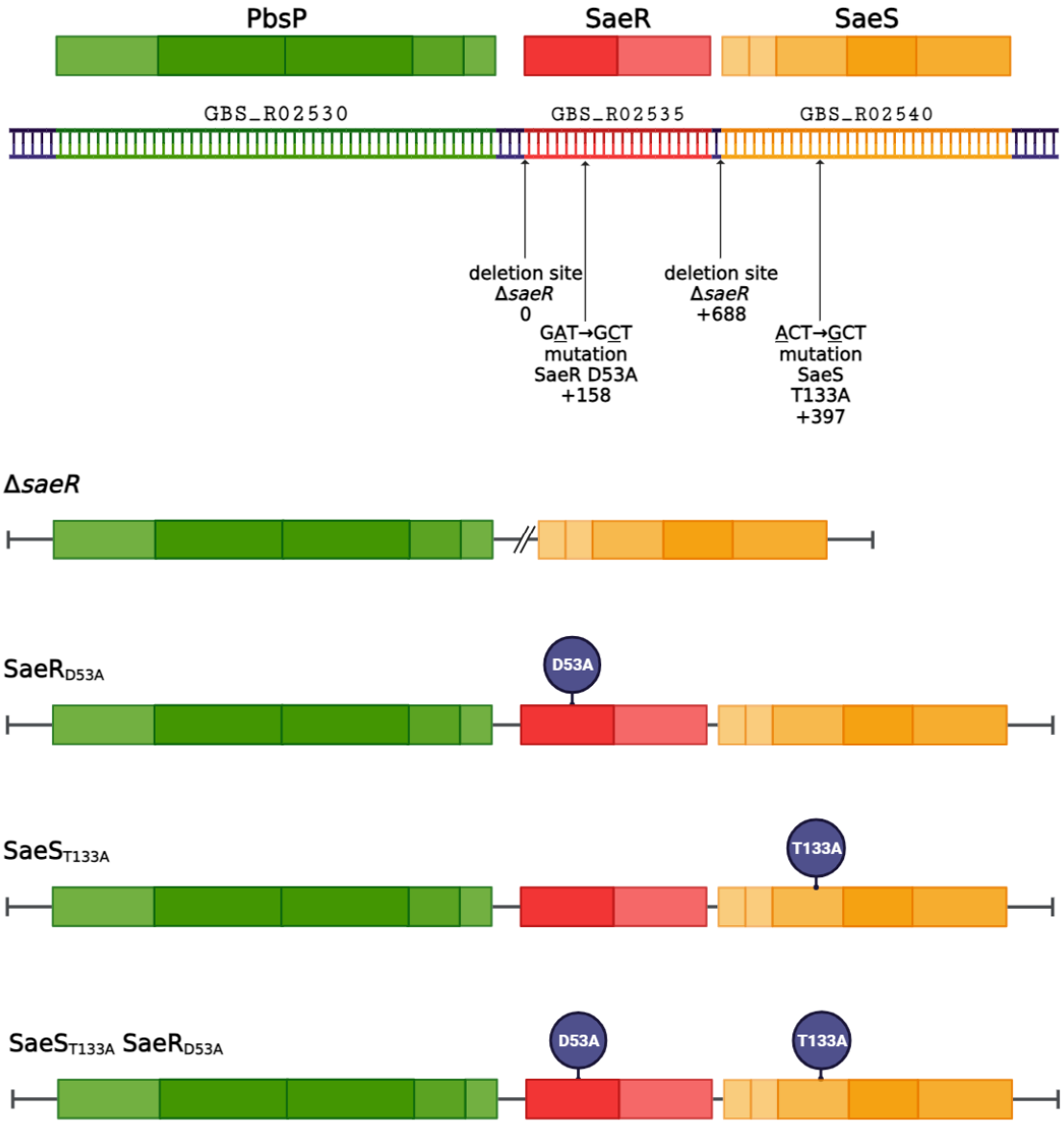


Figure 11. Schematic representation of the *pbsP/saeRS* locus in GBS NEM316 (created with BioRender.com).

In particular, for the SaeR mutants a marker-free, in-frame deletion of the *saeR* gene (Δ*saeR*) and, additionally, a second mutant was obtained with a single nucleotide chromosomal substitution (G<u>A</u>T→G<u>C</u>T) were generated. By this way, the SaeR protein in $SaeR_{D53A}$ strain is non-functional since its Aspartic Acid 53 cannot be phosphorylated by SaeS. Regarding constitutively activated mutants, they were obtained with a single nucleotide chromosomal substitution (<u>A</u>CT→<u>G</u>CT) producing a T133A (Thr→Ala) replacement in SaeS. Since threonyl residue 133 was proposed to be specifically involved in HK phosphatase activity (85), this substitution is expected to result in abrogation of the phosphatase activity of the sensor and in a subsequent accumulation of phospho-activated regulator.

The strains used to prepare the SaeRS system mutants were NEM316, belonging to serogroup III CC-23, which is one of the most studied GBS strains isolated from fatal cases of septicemia in EODs, and BM110 belonging to serogroup III CC-17, known as "hypervirulent" strain and isolated with high frequency in meningitis caused by LODs. The *pbsP-saeRS* locus is highly conserved between the two species, with only few single nucleotide polymorphisms. To obtain maker-free mutations, the upstream and downstream DNA regions flanking the desired mutation sites were amplified by PCR before being fused by overlapping PCR, introducing the mutations, and cloned into the temperature sensitive pG1 vector to produce pG1s bearing the different mutated inserts. GBS cells were electroporated and plasmids integration and excision events were performed as described (see Materials and methods) (113). The

marker-free mutations were confirmed by sequencing. The growth rate of the resulting SaeRS mutants was evaluated in rich THY liquid medium.

Neither SaeR mutant was impaired in its ability to reproduce, as evidenced by similar replication times compared with parental strain, indicating that SaeR mutations did not affect bacterial growth rate in standard laboratory conditions, both in NEM316 a BM110 background strains. On the other hand, evaluating $SaeS_{T133A}$ mutant compared to the parental strain, SaeRS constitutive activation impair the bacterial fitness resulting in a slow-growing mutants (Suppl. 1). The hemolytic phenotype was also evaluated using Blood Agar plates to highlight β-hemolysis of erythrocytes. No differences were observed using NEM316 parental strain and mutants (Suppl. 2). In addition, colony morphology of the mutants was similar to wild type strain. Surprisingly, using the BM110 background, the $SaeS_{T133A}$ mutant revealed a marked hemolytic activity compared to all the other strains.

Taken together, this evidence suggests a clonotype-specific modulation of hemolytic activity mediated by SaeRS system, enhanced in BM110 after activation. Moreover, the constitutive activation of SaeRS slightly impairs the bacterial growth in standard laboratory conditions, both in NEM316 a BM110 background strains.

The SaeR response regulator is required for GBS virulence

In order to assess the role of SaeRS system during pathogenesis, we used a mouse model involving hematogenous dissemination of bacteria to the brain through the bloodstream, which is a crucial step of naturally occurring GBS meningoencephalitis. In this model, standardized groups of mice were intravenously injected with a sub-lethal dose of the different bacterial strains. Animals were observed for health status, appearance, and behavior during the infection, and humanely euthanized upon reaching humane endpoints. Using this *in vivo* model, less than half of the mice infected with Δ*saeR* or $SaeR_{D53A}$ GBS died, while all the animals challenged with wild type NEM316 succumbed to infection. The $SaeS_{T133A}$ mutant was found to be non-virulent in mouse models, probably due to its impaired growth fitness. In light of this, it was not possible to further analyze it in mouse models of infection. To ascertain whether the decreased virulence of the SaeR mutants was linked to impaired *in vivo* growth, we measured bacterial load in the organs of infected mice at 24 and 48 h after challenge with intravenous injection. As shown in Fig. 12, animals infected with either SaeR mutants had significantly lower bacterial counts in the blood, brain, and kidneys than those infected with wild type NEM316.

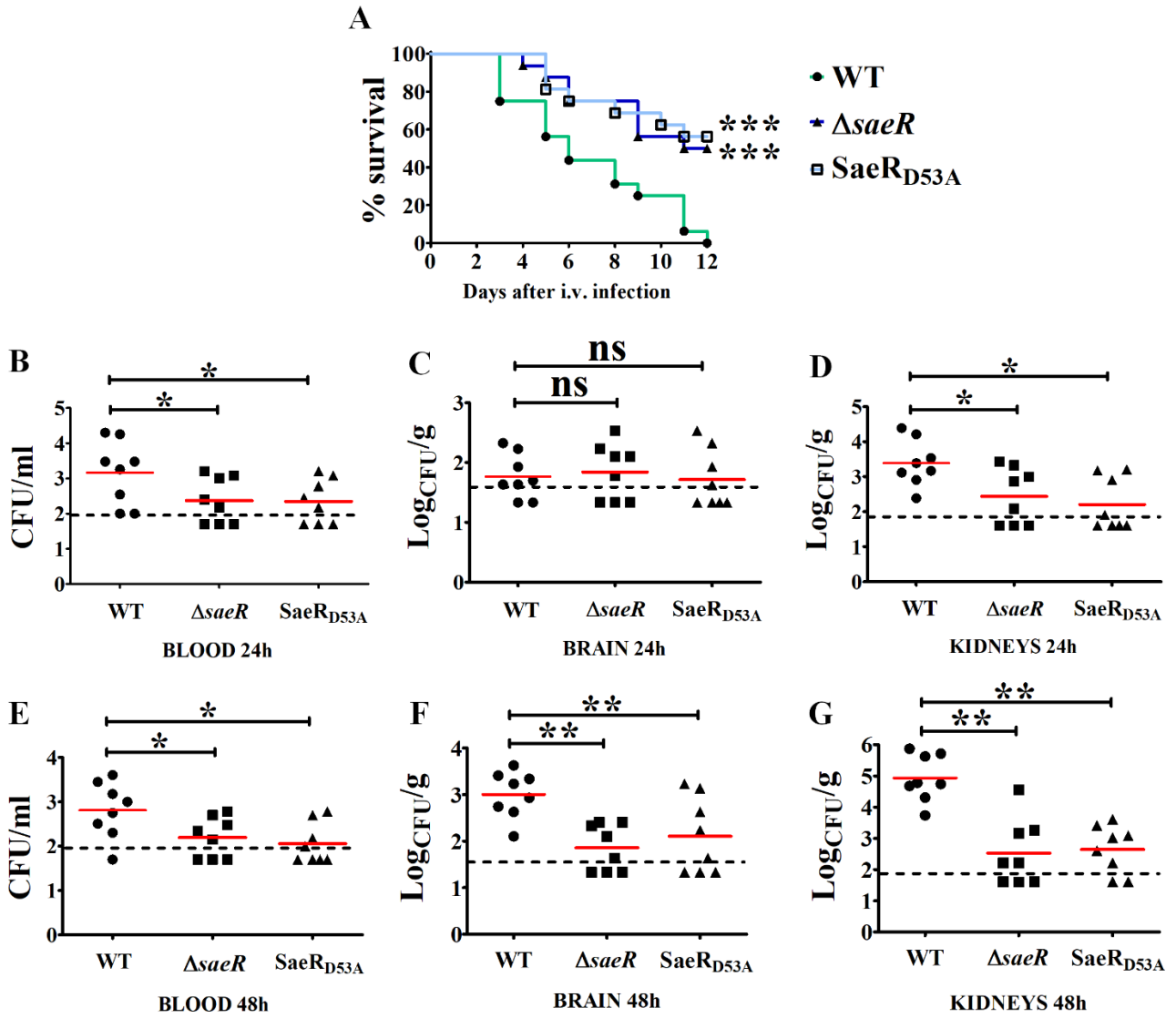


Figure 12. SaeR is required for virulence in a sepsis model. Effect of SaeR mutations on survival of GBS-infected mice (A). Adult CD1 female mice were infected intravenously (i.v.) with 1×10^8 CFU of NEM316 WT or mutant strains. Survival was monitored every 12 h. Cumulative results, n = 2, each involving 4 mice per group. **p<0.01 by Mantel-Cox test. SaeR is required for host invasion. Bacterial burden in the blood (B, E) brains (C, F) and kidneys (D, G) at different time points (24 and 48 h) after i.v. challenge with 1×10^8 CFU of wild-type NEM316) or mutant strains. Cumulative results, n = 2, each involving 4 mice per group. ***p<0.001 by Wilcoxon test; ns, non-signifcant.

According to this data, a similar phenomenon was observed in a peritonitis/sepsis model of GBS, where bacteria injected *via* the intraperitoneal route replicate at the inoculation site and spread systemically to the blood and distant organs. The experimental mice groups were observed for health status, appearance and behavior during the infection, and humanely euthanized upon reaching humane endpoints. Additionally, bacterial load was measured in peritoneal lavage fluid (PLF), blood and kidneys. Using this *in vivo* model, both SaeR mutants showed a reduced ability to persist in the peritoneal cavity, spread systemically and cause death (Fig. 13).

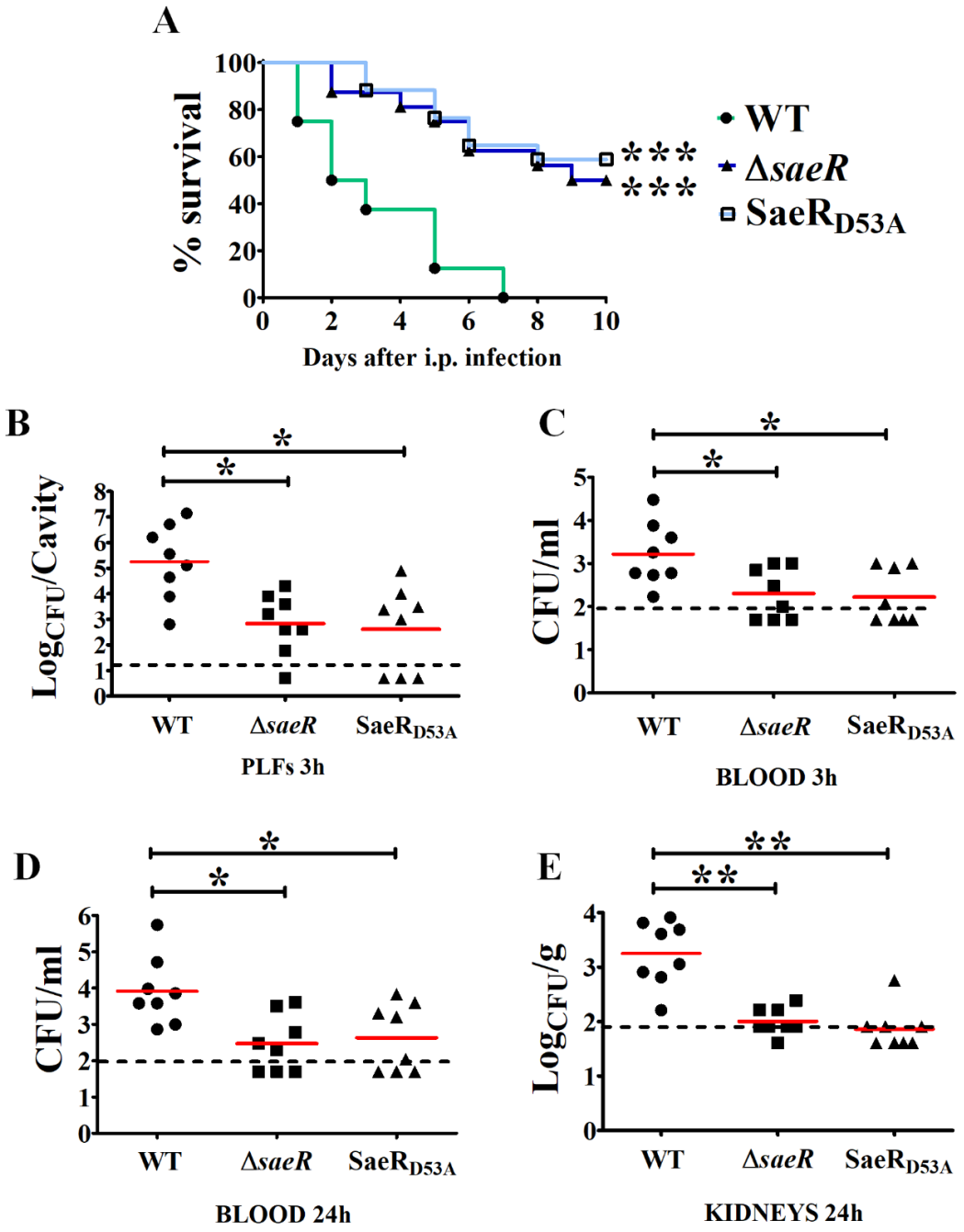


Figure 13. SaeR is required for virulence in a peritonitis model. Effect of SaeR mutations on survival of GBS-infected mice (A). Adult CD1 mice were infected intraperitoneally (i.p.) with 1×10^8 CFU of NEM316 WT or mutant strains. Survival was monitored every 12 h. Cumulative results, n = 2, each involving 4 mice per group. **p<0.01 by Mantel-Cox test. SaeR is required for host invasion. Bacterial persistence in the peritoneum after 3 h of infection (D) and burden in the blood (B, C) and kidneys (E) at different time points (3 and 24 h) after i.p. challenge with 1×108 CFU of wild-type NEM316 GBS or mutant strains. Cumulative results, n = 2, each involving 4 mice per group. ***p<0.001 by Wilcoxon test; ns, non-signifcant.

Collectively, these data indicate that defective SaeR regulator function is associated with a reduced ability of CC-23 GBS to persist *in vivo*, invade vital organs, such as the brain, and cause lethal infection, suggesting that the SaeRS system is required for full expression of virulence in these bacteria.

SaeRS controls a small set of highly upregulated genes

In consideration of the important role of SaeR response regulator in GBS virulence using the above-described *in vivo* models, it was of interest to identify the genes that are transcriptionally controlled by the SaeRS system. To this end, transcriptional response associated with SaeR loss of function conditions (Δ*saeR* and $SaeR_{D53A}$ mutants) and phospho-SaeR accumulation conditions ($SaeS_{T133A}$ mutant) was defined *via* RNA-seq analysis, both in NEM316 and BM110 background. No significant changes in gene expression were detected analyzing non-functional SaeR mutants using stringent criteria compared to the parental strain, after growing in standard laboratory conditions up to the mid-exponential phase. These data, in contrast to the studies reported by L. Cook et al., could theoretically be explained by a lack of SaeRS-activating stimuli under our tested conditions. On the other hand, not surprisingly, the $SaeS_{T133A}$ mutant, in which abrogation of the phosphatase activity of the sensor results in accumulation of phospho-activated regulator, revealed a significant marked up-regulation of four genes (Fig. 14).

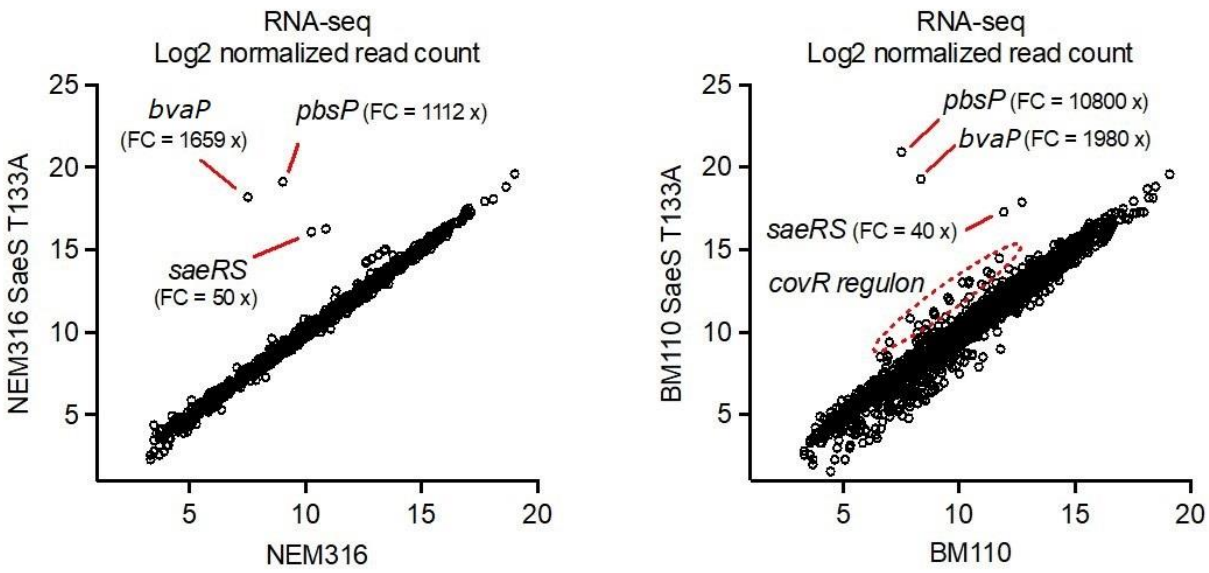


Figure 14. Scatter plot of significant fold changes in the $SaeS_{T133A}$ mutant in NEM316 and BM110 strains.

Among these, two encode for the regulator and sensor components of the SaeRS system itself, while the other two encode for the cell wall proteins PbsP and BvaP. Interestingly, in the BM110 background, also a group of genes belonging to the CovR regulon are slightly, but significantly, up-regulated. In order to confirm the massive up-regulation of the two main target gene of SaeRS, we assess the transcriptional response also by RT-qPCR. Additionally, the increased exposure of the PbsP protein on the bacterial surface was confirmed by Flow cytometry immunofluorescence and ELISA assays, using anti-PbsP antibodies (Suppl. 3). To formally prove that gene up-regulation in the $SaeS_{T133A}$ mutant depends on SaeR phosphorylation, we introduced the $SaeR_{D53A}$ mutation in this strain ($saeS_{T133A}$ $SaeR_{D53A}$). Under these conditions, the upregulation of PbsP was completely abrogated, both at the mRNA and protein level (Suppl. 3), indicating that the $SaeS_{T133A}$ transcriptional changes and phenotype

requires activation of the SaeR response regulator by phosphorylation. Collectively these data indicate that the SaeRS system can trigger, in CC-23 and CC-17 GBS, marked upregulation of a small set of genes representing the SaeR regulon, which includes two previously characterized virulence factors. In order to decipher the genetic regulation of the *pbsP-saeRS locus* related to expression of SaeR regulon, we investigated the activity of the promoters P1 and P2 located inside the *locus* using a beta-galactosidase gene reporter assays. To this end, the promoter regions of *pbsP* and *saeRS* were cloned upstream of the *lacZ* gene (encoding beta-galactosidase enzyme) into the promoter probe vector pTCV-*lacZ*. The resulting plasmids were introduced into BM110 wild type and $SaeS_{T133A}$ strains, and the beta-galactosidase activity of each strain was determined at the stationary growth phase in THY (Fig. 15 A).

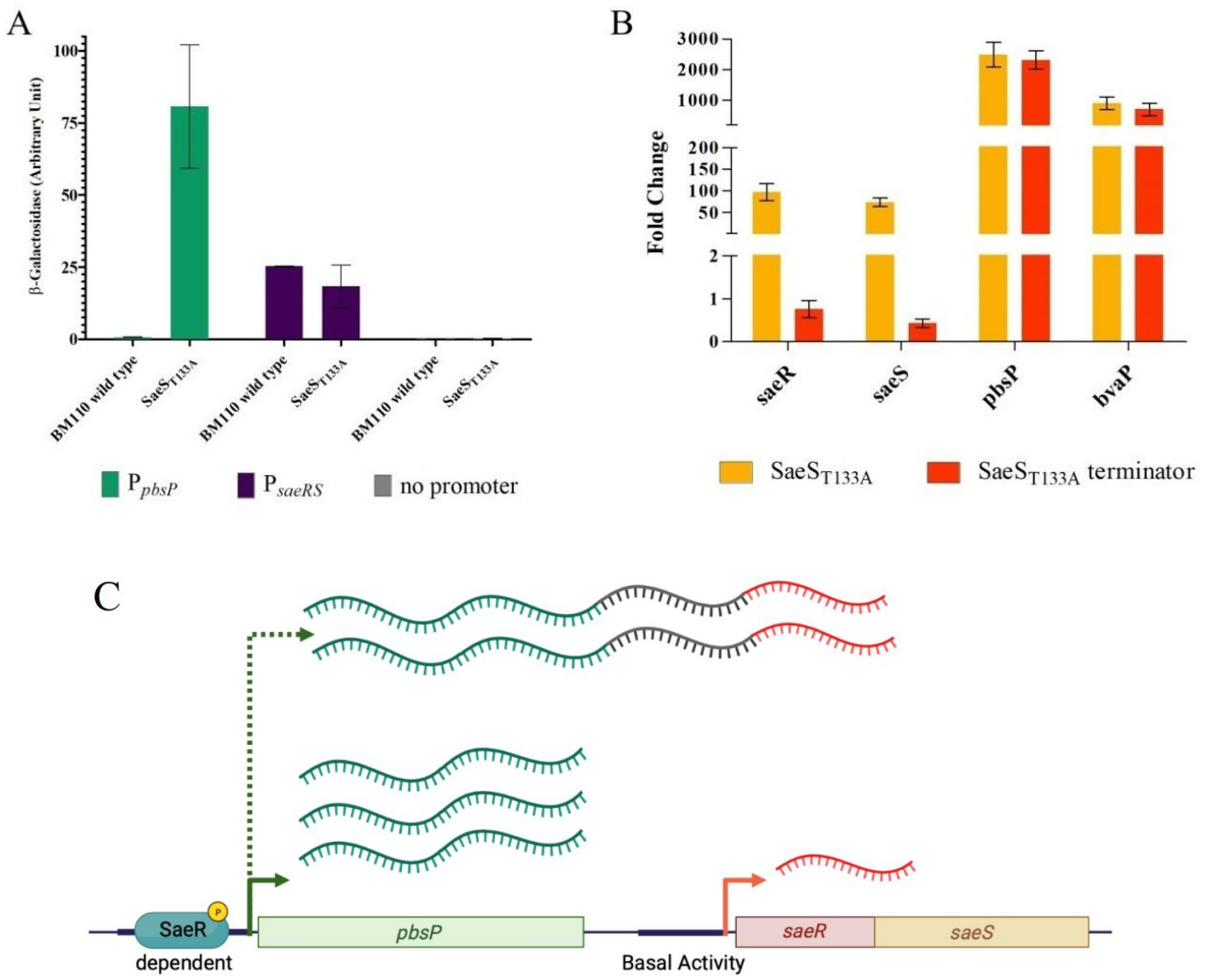


Figura 15. SaeRS genetic model. Activities of the P_{pbsP} and P_{saeR} promoters in the Wild Type and $SaeS_{T133A}$ mutant. Promoters activity was assayed using ß-galactosidase reporter system under the control of the tested promoters in the GBS strains (A). Mean ±SD, n = 2 with technical triplicate. Indirect positive feedback regulation of the *saeRS* locus. The *pbsP* and *saeRS* genes are separated by a 112 bp intergenic region containing a *saeR* putative promoter. Integration of a strong transcriptional terminator downstream *pbsP* interfere with transcriptional activation of *pbsP* and *saeRS* in SaeST133Amutant. Fold change of *saeR*, *saeS*, *pbsP*, and *bvaP* are quantified by RT-qPCR in the $SaeS_{T133A}$and in the $SaeS_{T133A}$ + *pbsP* terminator mutants (B). Values were normalized for those observed in wild type BM110. Mean ±SD, n = 3 with technical triplicate. SaeRS signalling pathway (C). The constitutive promoter P_{saeR} drive the expression of the *saeRS* operon at a basal level. Upon TCS activation, phosphor-SaeR activates the transcription of its target genes *pbsP* and *bvaP*, and indirectly, through a *pbsP* transcriptional-readthrough, up-regulate its own operon (created with BioRender.com).

The P_{pbsP} was strongly active in the $SaeS_{T133A}$ mutant, compared to wild type, which is almost inactive, suggesting that P_{pbsP} activation is mediated by phospho-SaeR, according to transcriptomic data. On the other hand, the P_{saeR} activities revealed no differences between BM110 wild type and $SaeS_{T133A}$ mutant, suggesting a basal-constitutive activation of P_{saeR}, independent from phospho-SaeR, in contrast with transcriptomic data. The up-regulation of *saeRS* genes in $SaeS_{T133A}$ mutant could be theoretically explained by a transcriptional-readthrough phenomenon of P_{pbsP} which, under strong activity conditions, mediate expression of a long mRNA bearing both *pbsP*, *saeR* and *saeS* genes. To prove this hypothesis, we introduced by integration the whole pG1 vector bearing a strong terminator downstream *pbsP* gene in SaeR/S-activated mutant ($SaeS_{T133A}$*terminator*). Expression levels of *saeR, saeS, pbsP* and *bvaP* genes were assess by RT-qPCR (Fig. 15 B). Under these conditions, *saeR* and *saeS* gene expression is abrogated in the presence of the strong terminator, while up-regulation of the target genes *pbsP* and *bvaP* is preserved. Taken together, these data indicate that in the $SaeS_{T133A}$ mutant P_{pbsP} activity drives the expression of the SaeR regulon's four genes, suggesting the occurrence of a transcriptionalread-through phenomenon; moreover, the basal level of SaeR and SaeS are sufficient, upon phosphorylation of SaeR, to determine the massive up-regulation of *pbsP* and *bvaP* target genes in the $SaeS_{T133A}$ strain (Fig. 15 C).

SaeRS-regulated genes are highly expressed *in vivo*

Since the SaeR regulon and its activation under conditions of constitutive activation of SaeRS system was characterized, we investigate if and how the adhesins PbsP and BvaP are modulated *in vivo*. To this end, we developed a model of bacterial stimulation *in vivo* that consist in the recovery of bacteria from the peritoneal exudates of i.p. challenged mice. This model allowed us to look at transcriptional changes in this pathogenic context. We first assessed transcriptional changes in wild type NEM316 bacteria recovered from the peritoneal exudates of i.p. challenged mice. The *pbsP* and *bvaP* transcript levels were measured by RT-qPCR (Fig. 16 A), compared to two well-known virulence factors of GBS, *cylE* (encoding for an enzyme involved in the synthesis of hemolysin) and *bibA* (encoding for the BibA adhesin), which are known to be under control of the CovRS TCS.

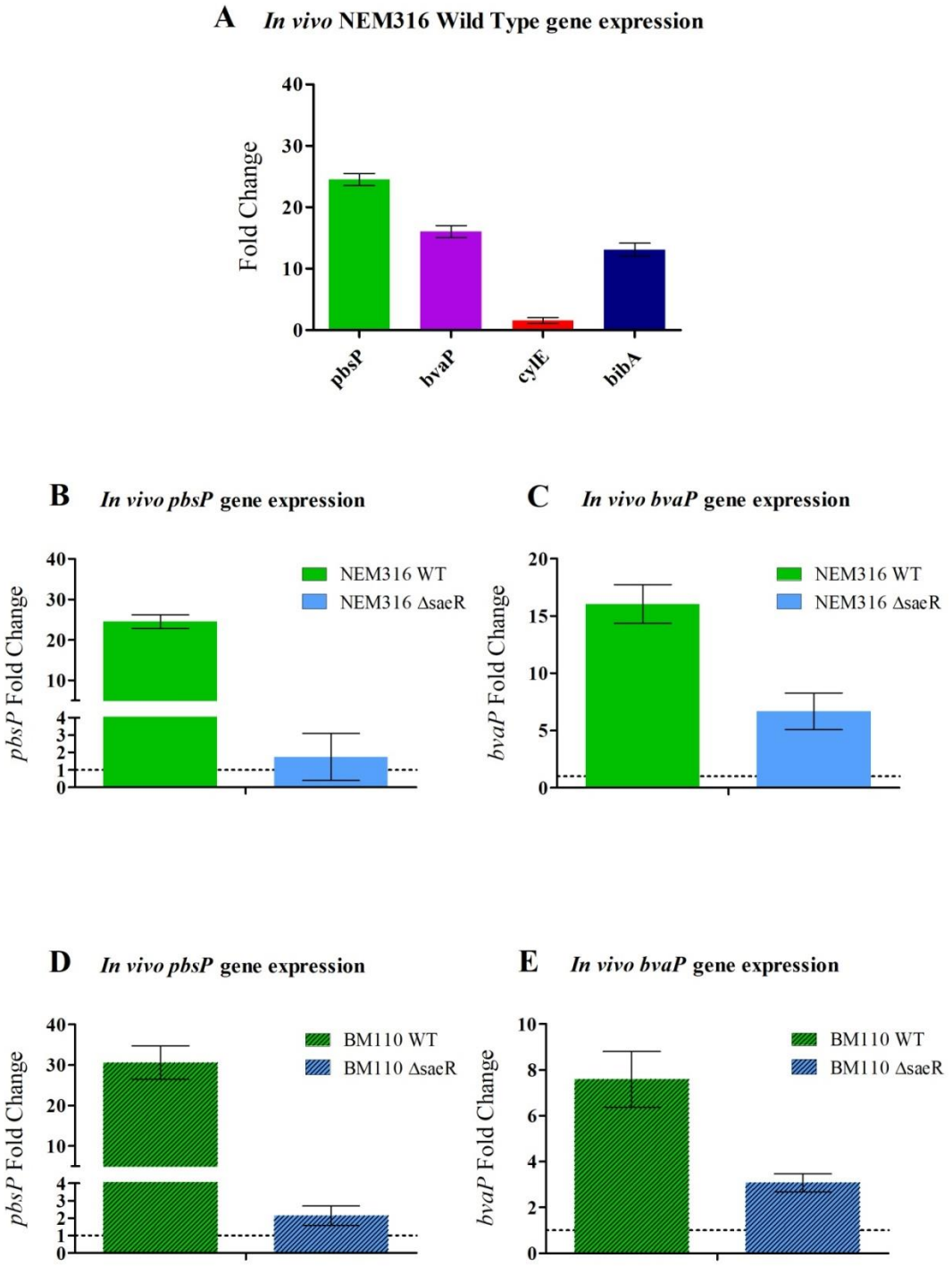


Figura 16. SaeRS regulates expression of PbaP and BvaP *in vivo*. RT-qPCR analysis of selected virulence factors mRNA levels in wild-type NEM316 after inoculation of bacteria in a murine model of peritonitis infection (A). Values were normalized for those observed in wild type NEM316 grown in THY medium. Up-regulation *in vivo* of the SaeRS target virulence factors PbsP and BvaP is markedly reduced in Δ*saeR* mutant strain, both in NEM316 (B, C) and BM110 GBS (D, E) (mean ±SD, n = 2 with technical triplicate).

Under these conditions, *pbsP* and *bvaP* were markedly (20- to 30-fold) upregulated, relative to *in vitro* grown bacteria, with greater fold changes than *cylE* and *bibA*. To ascertain if *in vivo pbsP* and *bvaP* expression is regulated by SaeRS, we next challenged mice with Δ*saeR* bacteria. RT-qPCR analysis of bacterial RNA isolated after peritoneal stimulation revealed that expression of both *pbsP* and *bvaP* was almost totally abrogated using *ΔsaeR* strain compared with wild type bacteria (Fig. 16 B), indicating that SaeR is required for the *in vivo* up-regulation of *pbsP* and *bvaP*. In order to investigate the *in vivo* modulation of virulence factors among different clonotypes, the same experimental model was used also for BM110 wild type and Δ*saeR* strains (Fig. 16 C). Also in this case, *pbsP* and *bvaP* were found to be markedly up-regulated in wild type bacteria after *in vivo* stimulation, with greater fold changes than *cylE* and *hvgA* (an allelic variation of *bibA* in CC-23). Additionally, also in BM110, Δ*saeR* RNA analysis revealed a strong, but non total, decrease in *pbsP* and *bvaP* transcript levels. Taken together, these data indicate that the SaeRS system is mainly responsible for the *in vivo* up-regulation of *pbsP* and *bvaP*.

PbsP upregulation is associated with increased invasion of cell barriers

During GBS pathogenesis, adhesion to host cells and tissue invasion represent two important steps of host colonization. To further investigate the role of the SaeRS system in GBS-host interactions, we assessed the ability of the different SaeRS mutant strains to adhere and invade human cells culture. Using A549 pulmonary alveolar epithelial cells, the $SaeS_{T133A}$ strain exhibited a 5-10-fold increase in ability to adhere to and invade the cell monolayer, compared to wild type NEM316, Δ*saeR* or Δ*pbsP* mutants, the latter bearing an in-frame deletion of *pbsP* gene already characterized in previously works (66). To evaluate if PbsP plays a role in the increased ability of SaeRS activated mutant to interact with host cells, we produced an in-frame deletion of the *pbsP* gene sequence in the $SaeS_{T133A}$ strain ($SaeS_{T133A}$ Δ*pbsP*), always using NEM316 background. Interestingly, when testing this strain for adhesion and invasion, lack of PbsP decreased the ability to adhere to and invade A549 cells to a level that was lower than that of the parental strain (Fig. 17). These data indicate that constitutive activation of the SaeRS system results in a markedly increased ability of CC-23 GBS to interact with and invade lung epithelial cells and that PbsP is the main adhesin involved in this hyper-adhesive and hyper-invasive phenotype.

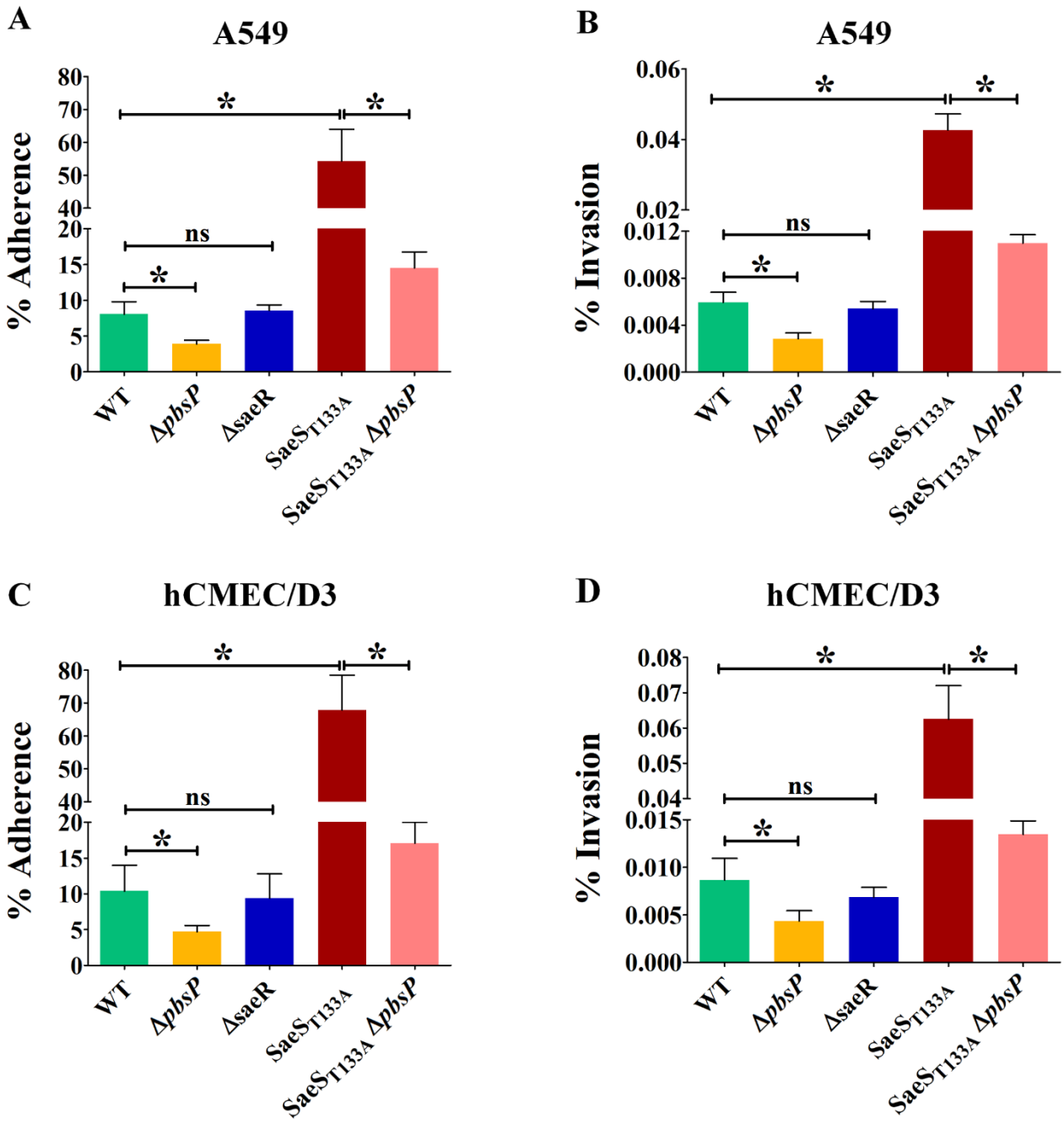


Figura 17. SaeRS activation promotes the ability of GBS to adhere and invade epithelial and endothelial cells in a PbsP-dependent manner. Adhesion and invasion assays were asses for the NEM316 SaeRS and PbsP mutant strains. The $SaeS_{T133A}$ mutant displays markedly increase adherence (A and C) and invasion (B and D) of human lung epithelial cells (A549) and brain microvascular endothelial cells (hCMEC/3), while parental adhesion and invasion levels are restored by PbsP deletion in the $SaeS_{T133A}$ background. PbsP deletion in Wild Type background also displays a decreased adhesion and invasion abilities. Mean ± SD, n = 3 with technical triplicate. *$p<0.05$; by Mann-Whitney test.

In view of the above presented data indicating a role of the SaeRS system in brain invasion (Fig. 12), and of previous studies demonstrating a role of PbsP in this pathogenetic step (72), we further investigated the role of this system in interactions with brain endothelial cell, which are a crucial component of the blood-brain barrier (BBB). According to previously data obtained with A549, $SaeS_{T133A}$ bacteria exhibited increased adherence to and invasion of brain microvascular endothelial cells (hCMEC/3), which was abolished by the absence of PbsP. As shown in previous studies, GBS is able to bind Plasminogen (Plg) through PbsP, enhance the conversion process of Plg to plasmin by tissue Plg activator and exploit this mechanism to migrate across endothelial cells. Thus, in order to investigate the role of SaeRS system in transmigration of GBS across endothelial cells, we used an *in vitro* BBB model involving hCMEC monolayers grown on transwell membrane inserts and bacteria pre-treated with Plg and tissue Plg activator. As shown in Fig. 18, under these conditions, the $SaeS_{T133A}$ mutant strain exhibits a markedly increased ability to transmigrate across monolayers compared to wild type NEM316, which was drastically reduced by *pbsP* deletion.

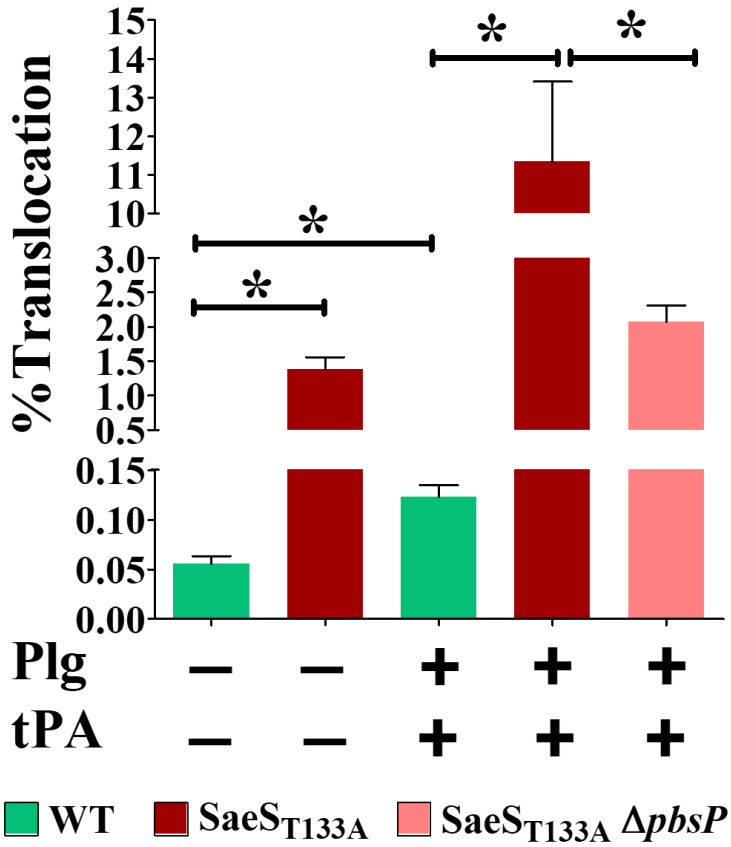


Figura 18. SaeRS activation promotes translocation of GBS through human brain endothelial cells through Plg-PbsP interactions. Translocation was assessed for the NEM316 SaeRS and PbsP mutant using the brain endothelial cell line hCMEC/D3 pre-treated with plasminogen (Plg) and tissue Plg activator (tPA). The $SaeS_{T133A}$ mutant shown a markedly increase traslocation of human endothelial cells, both in presence or absence of activated Plg, while parental translocation levels are restored by PbsP deletion in the $SaeS_{T133A}$ background. Mean ± SD, n = 3 with technical triplicate. *p<0.05; by Mann-Whitney test.

These results suggest that the activation of SaeRS system could modulate the ability of GBS to interacts with host cells and components. As observed in $SaeS_{T133A}$, the constitutive activation of SaeRS system results in a PbsP-dependent increased ability to adhere to and invade both alveolar epithelial cells and microvascular endothelial cells, and to efficiently exploit PbsP overexpression to transmigrate across the endothelial barrier.

Collectively, these data indicate that hyper-expression of PbsP, as seen in the background of constitutive SaeRS activation, results in increased invasion of epithelial and endothelial barriers, with potential implications in virulence.

PbsP upregulation is associated with increased hemolysis in BM110

GBS grown in Blood Agar plates produce a complete lysis of erythrocytes in the media around and under the colonies, called β-hemolysis. GBS hemolytic properties are typically linked to the expression of the *cyl* operon, responsible for β-haemolysin/cytolysin production. CovRS system is the main regulator of *cyl* operon expression and, for instance, Δ*covR* mutants display a hyper-hemolytic phenotype. During construction of SaeRS mutant strains, the hemolytic phenotype was evaluated for each mutant. Among these, BM110 $SaeS_{T133A}$ was the only strain to show hyper-hemolytic activity on Blood Agar plates. To ascertain if this phenotype was linked to target genes of SaeRS regulon, we constructed two double mutants lacking either *pbsP* or *bvaP* genes in the BM110 $SaeS_{T133A}$ background. Surprisingly, the hemolytic activity of BM110 $SaeS_{T133A}$ Δ*pbsP* reverted to wild type levels, while BM110 $SaeS_{T133A}$ Δ*bvaP* did not (Fig. 19).

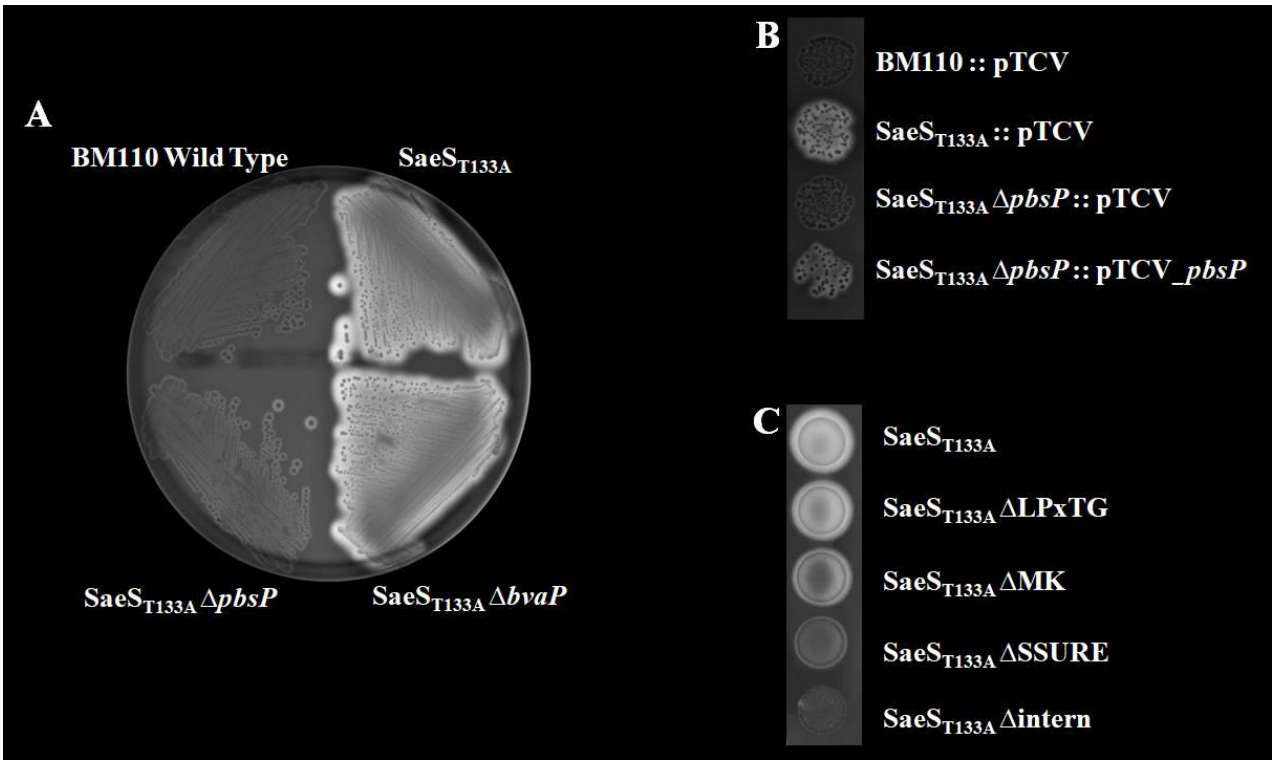


Figura 19. Hyper-haemolytic activity of the SaeS$_{T133A}$ mutant is PbsP-dependent. β-hemolytic activity was tested on Columbia horse blood (5%) agar plates. Hyper-hemL ytic phenotype of the SaeST133Amutant is specifically dependent on PbsP but not on BvaP (A), and is restored in SaeS$_{T133A}$ Δ*pbsP* + *pbsP* strain carrying a complementing vector with constitutive *pbsP* expression (B). The over-expression of the PbsP adhesin domains SSURE + MK (designated Δintern) is required for hyper-hemL ytic activity, while the single domains SSUREs, MK-rich or carboxy-terminal part containing the LPxTG anchoring motif and the hydrophobic C-peptide not (D).

To formally prove a role for PbsP, we complemented the Δ*pbsP* strain in the BM110 SaeS$_{T133A}$with an expression vector containing the *pbsP* gene (pTCV_*pbsP*), and tested for hemolytic activity. As shown in Fig. 19 B, complementation rescued theSaeS$_{T133A}$ hyper-hemolytic phenotype. This formally proves that the hyper-hemolytic phenotype of SaeR/S-activated mutants is specifically linked to PbsP up-regulation. In order to identify the specific domain of PbsP involved in this activity, we produced different mutants bearing deletions of single PbsP domains. As mentioned above,

PbsP is a modular cell-wall-anchored protein of 521-aa structurally composed of an N-terminal domain, two 150-aa SSURE repeat domains (65), a methionine and lysine-rich (MK-rich) region, and a cell wall-anchoring LPxTG motif (66). In the BM110 $SaeS_{T133A}$ background, we produced the domain-specific double mutants $SaeS_{T133A}$ ΔSSURE (in-frame deletion of SSUREs domains), $SaeS_{T133A}$ ΔMK (in-frame deletion of MK-rich domain), $SaeS_{T133A}$ΔLPxTG (in-frame deletion of LPxTG domain), and $SaeS_{T133A}$Δintern (in-frame deletion of the main extracellular part of PbsP, excluding LPxTG domain) (Fig. 20). Interesting, among all, only the $SaeS_{T133A}$Δinternmutant was able to rescue hemolytic activity to parental strain levels, suggesting a role for the main extracellular portion of PbsP in promoting hemolysis upon hyper-expression (Fig. 19 C).

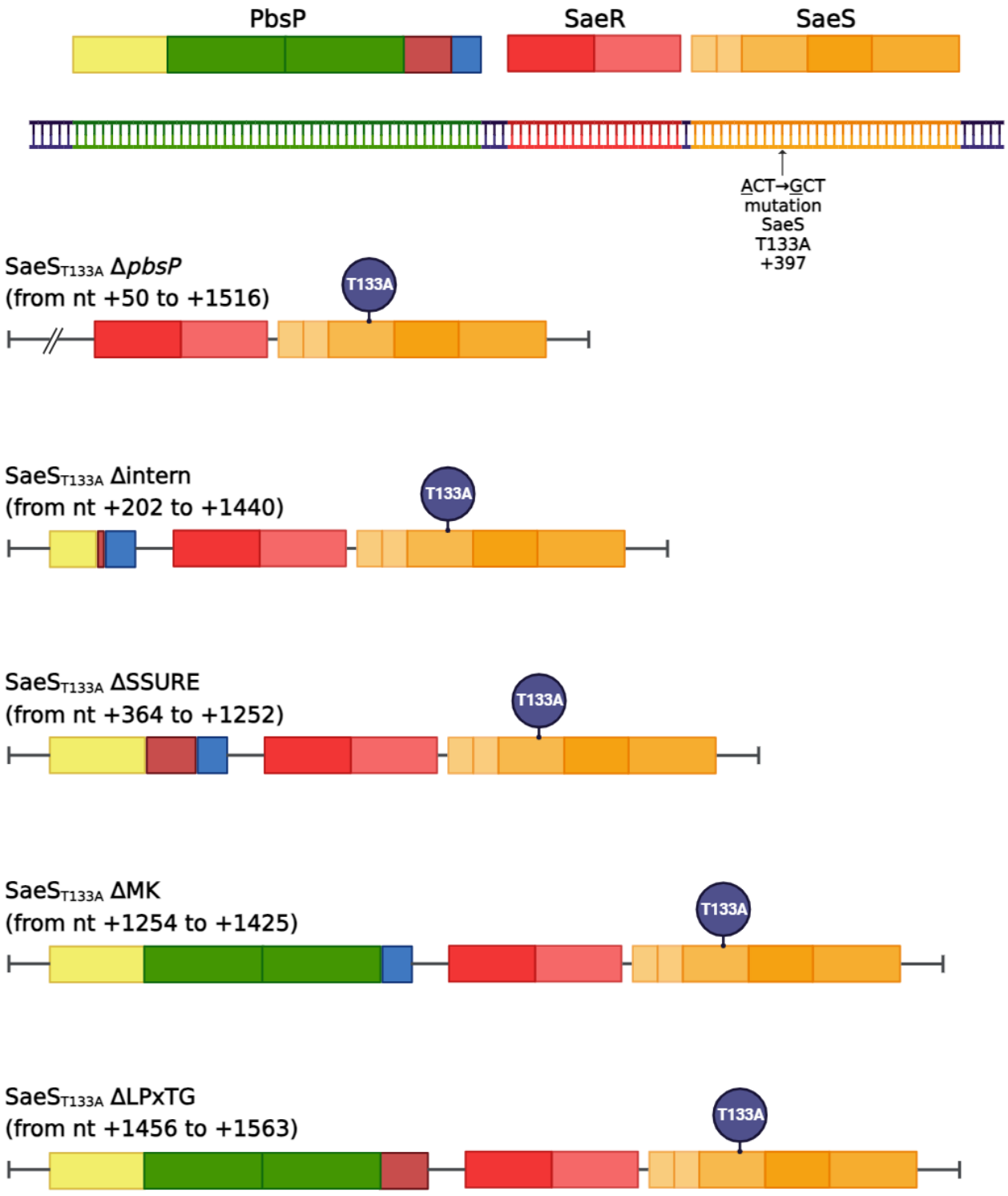


Figura 20. Schematic representation of the structural PbsP domains mutant strains. PbsP domains are differently coloured: LPxTG anchoring motif and the hydrophobic C-peptide (blue), methionine and lysine-rich or MK-rich domain (red), SSURE repeat domains 1+2 (green) and the N-terminal localization domain (yellow).

Discussion

To persist on mucosal surfaces and spread inside the host, bacteria must have mechanisms to adhere to the epithelium, evade the immune system, and reprogram expression of their virulence factors according to changing chemical, physical and metabolic cues in tissue niches. Identifying these genetic adaptation mechanisms would be essential in continuous efforts to develop novel strategies to control difficult to treat infections. The ability of GBS to interact with epithelial cells has been a primary focus of investigation in recent years. The adhesins PbsP and BvaP have emerged as crucial virulence factors involved in interactions between GBS and mucosal surfaces, including vaginal and respiratory epithelia (66, 71, 111, 112). Moreover, PbsP plays an important role in crossing the brain microvascular endothelial cell barrier (72). I provide evidence in this thesis that both PbsP and Bva are regulated in vivo by the SaeRS two component systems during invasive GBS infection, extending previous data obtained in a vaginal colonization model (111). TCSs are composed of a sensor histidine kinase (HK) and a response regulator (RR). Once the HK is activated, a complex intracellular pathway leads to the phosphorylation of a well conserved aspartate residue in the receiver domain of the RR, inducing conformational changes that allow the RR to increase its binding affinities for target sequences in the bacterial genome. The SaeRS TCS is composed of a histidine protein kinase (SaeS) that phosphorylates, and thereby activates, an R protein (SaeR). In the present study, we demonstrated that the response regulator SaeR does not play an essential role in GBS growth *in vitro*, but is required instead *in vivo* to upregulate

expression of the important virulence factor PbsP. To investigate the role of SaeRS TCS in prototypical CC23 NEM316 strain, we first created non-functional SaeR mutants by *saeR* deletion or substitution of a critical amino acid residue (D53) targeted by the kinase activity of SaeS. These mutants were inoculated in mice in order to evaluate their contribution to bacterial virulence in meningoencephalitis and sepsis infection models.

The Δ*saeR* and the $SaeR_{D53A}$ mutants displayed a similar phenotype, since they were both associated with a reduced ability to reproduce locally, invade the bloodstream, persist in the blood and disseminate to distant organs, including the brain and kidneys. These data seem relevant to the pathogenesis of GBS disease, which is characterized by tissue invasion in both localized (e.g. wound) or generalized (e.g. sepsis) infection, as opposed to asymptomatic bacterial colonization. Therefore, data presented here and previous studies collectively point to a crucial role of the SaeRS system in both mucosal colonization (111) and invasive disease. Moreover, previous studies used the A909 GBS, a representative of CC7 GBS, which represents a minor proportion of GBS clinical isolates. In the present study we focused on CC23 strains that were recently associated with an alarming increase in neonatal GBS infection. A further element of novelty of our studies concerns the approach we used to identify the SaeR regulon. We initially observed (using stringent methods to detect differentially regulated genes) that there were minor differences between SaeR mutants and wild-type bacteria in gene expression during *in vitro* growth. Therefore, we hypothesized that this might be linked to a lack of SaeRS-activating stimuli during exposure to laboratory media. To test this

hypothesis, I devised a molecular strategy to induce constitutive activation of the SaeRS system. This was accomplished by abrogating the phosphatase activity of SaeS by a single amino acid substitution in T133. In particular, I focused on generation of $SaeS_{T133A}$ mutants, characterized by non-functional phosphatase activity and constitutively high levels of phospho-activated SaeR. These mutants evidenced the presence of a distinctive SaeR regulon characterized by the marked (200-1,000 fold) upregulation of a few genes only, namely *pbsP*, *bvaP*, *saeR* and *saeS*, as indicated by RNA Seq analysis. These data indicate the presence of a self-regulatory mechanism of the TCS, in addition to the overexpression of two cell wall proteins with marked cell-adhesive activities. Upregulation of these genes was directly linked to SaeR phosphorylation since such upregulation was abrogated by substitution of D53 in the $SaeS_{T133A}$ background. All together, these data emphasize the relevance of SaeRS TCS in the regulation of a much smaller number of genes in GBS compared to *S. aureus*, where the SaeRS TCS controls the expression of many crucial virulence genes both *in vitro* and *in vivo*. These virulence genes include *hla*, *hlb*, and *coa*, and several other genes capable of promoting adhesion and invasion of epithelial and endothelial cells (114, 115).

In the present study I detected a similar pattern of SaeRS-dependent gene regulation in $SaeS_{T133A}$ strain grown *in vitro* and wild type strains obtained *ex vivo*. In particular, I was able to confirm *pbsP* and *bvaP* over-expression in bacteria recovered from peritoneal lavage fluid samples compared with *in vitro* grown GBS. PbsP upregulation in peritoneal exudates was

reminiscent of similar results observed in "hypervirulent" CC17 GBS infecting the brain in a meningo-encephalitis model (66, 67, 71, 72). Here we showed that the recently described BvaP protein is also greatly up-regulated in intraperitoneal streptococci, raising the possibility of a pathogenetic role of this virulence factors in sites other than the vaginal mucosa (112). Moreover, the absence of *saeR* drastically decreased *pbsP* and *bvaP in vivo* expression, formally demonstrating positive regulation of these two virulence factors by the SaeRS TCS in NEM316. This was in contrast with a lack of effect of non-functional SaeR mutations in the expression of CovRS-dependent *cylE* and *bibA* genes in Δ*saeR* streptococci. However, a participation of regulatory systems other than SaeRS in *pbsP* and *bvaP* regulations also suggested by our data, since transcript levels of these genes were higher in Δ*covR* bacteria than in the parental strain. In view of the previously documented role of the CovR/S system in PbsP regulation (72), studies are underway to analyze SaeRS/CovRS interactions in regulation of GBS virulence factors.

The activation of response regulators is the result of the finely regulated phoshorylation-based mechanisms mediated by the kinase and phosphates activities of the sensors, following the recognition of precise environmental stimuli. Our data support the notion that the SaeRS TCS can be activated *in vivo* and induce gene expression mediated by SaeR phosphorylation. The ability of streptococci to adhere to host cells and invade them represent an essential step in GBS pathogenesis. Notably, our data showed that the $SaeS_{T133A}$ strain was extremely proficient in terms of adhesion to and invasion of alveolar epithelial and brain endothelial cells

and that the high levels of GBS-host cell interactions were sustained the over-expression of PbsP on the streptococcal surface. Indeed, hyper-expression of *pbsP* in the $SaeS_{T133A}$ strain could be evidenced not only at the mRNA, but also at the protein level, and greatly increased levels of immune-reactive PbsP were evidenced on the bacterial surface by flow cytometry analysis. Strikingly, the hyper-adhesive hyper-invasive $SaeS_{T133A}$ phenotype was abrogated in *pbsP*- or *saeR*-deleted strains.

The capacity of GBS to use PbsP for blood plasminogen (Plg) binding is essential for invasiveness of brain endothelial cells *in vitro* and of the central nervous system *in vivo* (66, 116, 117). In light of this, we evaluated the role of the SaeRS TCS in streptococcal transmigration across microvascular brain endothelial cell monolayers. Results confirmed the notion that PbsP expression might be important to allow streptococci to bind Plg and acquire plasmin-mediated proteolytic activity to cross the brain endothelial barrier. These results indicate the relevance of SaeRS TCS not only in GBS colonization of the vaginal tract, as previously suggested, but also in bacterial breaching of the blood-brain barrier. Unfortunately, the ability of $SaeS_{T133A}$ strains to cause infection *in vivo* could not be properly evaluated, due the presence of non-specific defects, such as a slight but significant reduction in growth rate.

In conclusion, our studies indicate that the SaeRS TCS plays an important role in GBS virulence and positively regulates the expression of PbsP and BvaP. Particular attention will be devoted in future studies to interactions between SaeRS and CovRS in virulence factor regulation, particularly in view of PbsP regulation by both systems. Moreover, further studies will be needed to investigate the role of SaeRS TCS and other TCSs in regulating PbsP expression in GBS strains belonging to other clonal complexes, particularly in "hypervirulent" CC17 GBS and to clarify the nature of external environmental stimuli capable of triggering SaeRS signaling.

Materials and methods

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this work are listed in Tables 1, Tables 2 and Tables 3.

S. agalactiae			
Strain	**Genotype**	**Plasmid**	**Source or reference**
NEM316	Serotype III, ST-23		
BM110	Serotype III, ST-17		
NEM316 Δ*pbsP*	Δ*pbsP*		Buscetta et al, 2016
NEM316 Δ*saeR*	Δ*saeR*		This study
NEM316 $SaeR_{D53A}$	$SaeR_{D53A}$		This study
NEM316 $SaeS_{T133A}$	$SaeS_{T133A}$		This study
NEM316 $SaeS_{T133A}$Δ*pbsP*	$SaeS_{T133A}$/Δ*pbsP*		This study
NEM316 $SaeS_{T133A}$ $SaeR_{D53A}$	$SaeS_{T133A}$/$SaeR_{D53A}$		This study
BM110 Δ*saeR*	Δ*saeR*		This study
BM110 $SaeR_{D53A}$	$SaeR_{D53A}$		This study
BM110 $SaeS_{T133A}$	$SaeS_{T133A}$		This study
BM110 $SaeS_{T133A}$*terminator*	$SaeS_{T133A}$/*terminator*		This study
BM110 $SaeS_{T133A}$Δ*pbsP*	$SaeS_{T133A}$/Δ*pbsP*		This study
BM110 $SaeS_{T133A}$ΔLPxTG	$SaeS_{T133A}$/ΔLPxTG		This study
BM110 $SaeS_{T133A}$ ΔMK	$SaeS_{T133A}$/ΔMK		This study
BM110 $SaeS_{T133A}$ ΔSSURE	$SaeS_{T133A}$/ΔSSURE		This study
BM110 $SaeS_{T133A}$ Δintern	$SaeS_{T133A}$/Δintern		This study
BM110 $SaeS_{T133A}$Δ*bvaP*	$SaeS_{T133A}$/Δ*bvaP*		This study
BM110		pTCV	This study
BM110 $SaeS_{T133A}$	$SaeS_{T133A}$	pTCV	This study
BM110 $SaeS_{T133A}$Δ*pbsP*	$SaeS_{T133A}$/Δ*pbsP*	pTCV	This study
BM110 $SaeS_{T133A}$Δ*pbsP*	$SaeS_{T133A}$/Δ*pbsP*	pTCV_*pbsP*	This study
BM110		pTCV-*lacZ*	This study
BM110		pTCV-*lacZ*_P*pbsP*	This study

BM110		pTCV-*lacZ*_P*saeRS*	This study
BM110 $SaeS_{T133A}$	$SaeS_{T133A}$	pTCV-*lacZ*	This study
BM110 $SaeS_{T133A}$	$SaeS_{T133A}$	pTCV-*lacZ*_P*pbsP*	This study
BM110 $SaeS_{T133A}$	$SaeS_{T133A}$	pTCV-*lacZ*_P*saeRS*	This study

E. coli			
Strain	**Genotype**	**Plasmid**	**Source or reference**
XL-1 Blue	*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lac [F′proABlacIq ZΔM15 Tn10 (Tetr)]*		Stratagene
TOP10	*F⁻mcrAΔ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galUgalKλ–rpsL(Str^R) endA1 nupG*		Invitrogen

Table 1. Bacterial strains used in this study.

S. agalactiae was cultured in Todd Hewitt supplemented with 5 g/liter of yeast extract (THY) at 37°C under steady state conditions. *E. coli* XL-1 Blue and *E. coli* TOP10 were used for cloning experiments. *E. coli* strains were cultured in Luria Bertani broth at 37°C with vigorous shaking (200 rpm). Media were solidified with 1.6 % agar. Antibiotics were used at the following concentrations: for *E. coli*, erythromycin 150 µg/mL; for *S. agalactiae*: kanamycin 500 µg /mL ; erythromycin 10 µg/mL.

Plasmid	**Resistence**	**Properties**	**Source or reference**
pG1	Erythromycin		Devaux et al., 2018
pG1_Δ*pbsP*	Erythromycin		Courtesy of Arnaud Firon
pG1_Δ*saeR*	Erythromycin		Courtesy of Arnaud Firon
pG1_SaeR$_{D53A}$	Erythromycin		Courtesy of Arnaud Firon
pG1_SaeS$_{T133A}$	Erythromycin		Courtesy of Arnaud Firon
pG1_*pbsP_terminator*	Erythromycin		Courtesy of Arnaud Firon
pG1_ΔLPxTG	Erythromycin		Courtesy of Arnaud Firon
pG1_ΔMK	Erythromycin		Courtesy of Arnaud Firon
pG1_ΔSSURE	Erythromycin		Courtesy of Arnaud Firon
pG1_Δintern	Erythromycin		Courtesy of Arnaud Firon
pG1_Δ*bvaP*	Erythromycin		Courtesy of Arnaud Firon
pTCV-*erm*	Kanamycin/Erythromycin		Courtesy of Arnaud Firon
pTCV_*pbsP*	Kanamycin/Erythromycin		(Poyart et al., 2001)
pTCV-*lacZ*	Kanamycin/Erythromycin		(Poyart and Trieu-Cuot, 1997)
pTCV-*lacZ*_P*pbsP*	Kanamycin/Erythromycin		Courtesy of Arnaud Firon
pTCV-*lacZ*_P*saeRS*	Kanamycin/Erythromycin		Courtesy of Arnaud Firon

Table 2. Plasmids used in this study.

Primers for qPCR	
gyrA_FW	ACC TGG GAA GGG ATC AAT TGT CT
gyrA_RV	CTT TCC CAT TTG AAG TTG TTT CAA
pbsP_FW	TGA TGG TGG CTA CCT CTG TTA GAA
pbsP_RV	AGT CGT TTT GGT CAT CGC TAC A
saeR_FW	TTG ACG ATT GGT GAT TTG GTT ATC
saeR_RV	GGA AAT TGT ATT GCA GTC CAC CAT
bvaP_FW	GCT TGC GGT GCT GTG AAA G
bvaP_RV	GGA GGA GCA TTC GTA GCT CTT G
cylE_FW	ATC GGA GGA GGC GTT AAT CA
cylE_RV	TGC TGA CGC TTG GTA GTT GCT
bibA_FW	ACC AGT CCA AAC ACT CCT ATT TC
bibA_RV	TTT GCC TAC ACC TGG ATA TTA TGC
MGB hydrolysis probes	
gyrA-VIC	ACG TTC ACG GAC TGA A
pbsP-6-FAM	ATG GCA ACG CCG ACA C
saeR-6-FAM	ACC AAA AAC AAC GTT TGG T
bvaP-Texas Red	AGC TGA AAC ACC TTG TG
cylE-6-FAM	CTA AAA AAA CAG CCA ATA TG
bibA-Cy5	TCA AAT AAG TTC CCA CAG AGT

Table 3. Primers and oligonucleotides used in this study.

Strains constructions

S. agalactiae genomic DNA was purified using DNeasy Blood and Tissue kit (Qiagen) and used as template in PCR reactions. Oligonucleotides used in this study are listed in Table 3. Preparative PCRs for cloning were performed using a high-fidelity polymerase (Phusion™ Plus DNA Polymerase, ThermoFisher Scientific). To construct the different mutants of *S. agalactiae*, two chromosomal regions upstream and downstream of the targeted mutation sites were amplified and fused by overlap extension PCR. The ends of the resulting fragments bear the specific sequences for *Eco*RI and *Bam*HI restriction enzymes. The temperature-sensitive pG1 plasmid (118) was amplified with divergent oligonucleotides. The resulting fragments, displaying overlapping regions, were joined by Gibson assembly reaction. The reaction final mixture was used to transform *E. coli* XL1 by Heat Shock transformation. Recombinant clones were screened by colony PCR using primers pEX-Rev and M13 (uni-43). The obtained pG1 mutational plasmid were verified by sequencing and used for electroporation of *S. agalactiae* (Eporator, Eppendorf). Transformants were selected by growing on THY agar plates containing erythromycin at 30°C. Plasmid integration into the bacterial genome was mediated by isolating transformants on THY agar plates containing erythromycin 10 µg/mL (Ery) at 37°C. Integrants were further isolated to single colonies, on the same selective medium, and incubated at 37°C. Plasmid excision was favoured by growing cells 30°C in THY broth without erythromycin. After several passages of culture in the same conditions, bacteria were plated on THY agar plates and Ery sensitive clones were selected to be

analyzed by colony PCR (MyTaq™ HS DNA Polymerase, Meridian Bioscience). Mutations were confirmed by sequencing of chromosomal DNA.

Mouse infection models

Virulence of GBS strains was tested *via* sepsis and peritonitis models. For sepsis model, 8-week-old CD1 mice were infected intravenously (i.v.) with 5 x 10^8 bacteria, as described (53). Mice were monitored for lethality and signs of disease for a total of 14 days after challenge. 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 24 and 48 h after infection to collect blood, brains and kidneys. The number of CFU was measured in organ homogenates using standard methods of bacterial count. For peritonitis model, 8-week-old CD1 mice were infected intraperitoneally (i.p.) with 5 x 10^8 bacteria, as described. Mice were monitored for lethality and signs of disease for a total of 14 days after challenge. 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 3 h and 24 h after infection to collect peritoneal lavage fluids (PLFs), blood, and kidneys. The number of CFU was measured in organ homogenates using standard methods. For GBS *in vivo* stimulation, 7-week-old CD1 mice were infected intraperitoneally (i.p.) with 1 x 10^9 bacteria. GBS-infected mice were sacrificed at 1 h and PLFs from 5 mice were collected and pooled. Infected PLFs were subjected to differential

lysis and differential centrifugation steps in order to remove eukaryotic cellular cells from the samples. The bacterial pellet was finally re-suspended in extraction buffer and processed for RNA extraction.

Extraction and purification of bacterial nucleic acid

Bacterial pellets (GBS grown to mid-log phase in THY, OD_{600} 0,5) were suspended in 350 µl of Tris-HCl (pH 8, 10 mM) in 1.5 mL microcentrifuge tubes, to which 34 mg of glass beads (106 µm, Sigma-Aldrich G-8893) were added. The tubes were placed in a RETSH MM30 homogenizer and shaken at 30 Hz for 20 minutes. To isolate total RNA in the homogenized samples, RNA purification columns (RNeasy minikit, Qiagen) were used, according to the manufacturer's protocols and stored at −80 °C after quantification by Nanodrop 2000 (ThermoFisher Scientific) readings and gel electrophoresis analysis.

RNA-sequencing and analysis

RNAs purification, sample processing and data analysis for RNA-seq were done as described by Mazzuoli et al (93). Briefly, RNAs purification were done from three independent cultures, with replica done in different days, in 10 mL of THY, 50 mM HEPES pH 7.4. RNA stabilization reagents (RNAprotect, Qiagen) were added at mid-exponential phase (OD_{600} 0,5–0.6) for 5 min at ambient temperature. Cells were harvested at 4˚C, washed with 1 mL cold PBS, and the bacterial pellets stored at minus 80˚C. Cells were mechanically lysed and total RNA extracted following manufacturer

instructions (FastPreps and FastRNA ProBlue, MP Biomedicals). Residual DNA were digested (TURBO DNase, Ambion) and samples qualities were validated (Agilent Bioanalyzer 2100, Qubit 3.0, Life Technologies) before rRNA depletion, libraries construction and sequencing (Ribozero rRNA, TruSeq Stranded mRNA, Hiseq2500, Illumina).

For RNA-seq, single-end strand-specific 65 bp reads were cleaned (cutadapt version 1.11) and only sequences at least 25 nt in length were considered for further analysis. Alignment on the corresponding reference genomes (Bowtie v1.2.2 with BM110 RefSeq NZ_LT714196 and NEM136 RefSeq NC_004368) and gene counts data (featureCounts, v1.4.6-p3, Subreads package; parameters: -t gene -g Name -s 2) were analysed with R (v3.6.1) and the Bioconductor package DESeq2 (v1.26.0). Normalization and dispersion were estimated and statistical tests for differential expression were performed applying the independent filtering algorithm. A generalized linear model including the replicate effect as blocking factor was set in order to test for the differential expression between the mutant and the WT strains. For each comparison, raw p-values were adjusted for multiple testing according to the Benjamini and Hochberg procedure and genes with an adjusted p-value lower than 0.005 were considered differentially expressed. The coverage profiles were obtained for each strand using bedtools (v2.25.0), normalized using the DESeq2 size factors and then averaged across the biological replicates

RT-qPCR

To measure target genes mRNA, reverse transcriptions of total RNA were performed using the M-MLV reverse transcriptase (Invitrogen, Cat. No. 28025013) and Random Primers (Promega, Cat. No. A3500), using 1 µg of DNase treated total RNA in 20 µL of reaction according to the manufacturer's protocol. All reactions on cDNA were carried out on three replicates using specific primer pairs and MGB hydrolysis probes. Quantitative PCR (qPCR) was carried out with the Taqman Gene Expression Master MIX (Applied Biosystem, Cat. No. 4369016) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Relative gene expression levels were calculated with the ΔΔCT method where expression values were normalized with the expression of the housekeeping *gyrA* gene.

B-galactosidase assay

To investigate the *pbsP* and *saeRS* promoters' activity, amplified fragments corresponding to the two gene regulatory regions were cloned by Gibson assembly between the *Eco*RI and *Bam*HI restriction sites of pTCV-lacZ plasmid. For β-galactosidase assays, cells were harvested after o/n culture in THY kan 500, washed twice with cold Z-buffer (60 mM $Na_2HPO_4xH_2O$, 40 mM $NaH_2PO_4xH_2O$, 10 mM KCl, 1 mM $MgSO_4x7H_2O$, pH=7) and resuspended in cold Z-buffer supplemented with 50 mM 2-mercaptoethanol. Subsequently, bacteria were permeabilized with 0.5% (v/v) toluene and 4.5% (v/v) ethanol. β-galactosidase activity was assessed

at 28°C using o-nitrophenyl-beta-Dgalactopyranoside (ONPG) 4 mg/mL as substrate. Reaction kinetics at 28 °C were followed by recording the OD_{420} every 4 min for 60 min in a microplate reader.

PbsP exposition on bacterial surface

Surface exposed PbsP was visualized using flow cytometry immunofluorescence analysis and enzyme-linked immunosorbent assay (ELISA) through anti-rPbsP antibodies. Using flow cytometry immunofluorescence, GBS strains grown to the Log phase in THY were washed in PBS, fixed with 3.7% formaldehyde, and blocked with 1% non-fat dry milk (Santa Cruz Biotechnology Inc. sc-2324) in PBS solution. To visualize surface-expressed PbsP, bacteria were incubated with mouse anti-rPbsP or anti-GST murine serum diluted 1:25 followed by phycoerythrin-conjugated goat anti-mouse IgG diluted 1:50 (ThermoFisher, Cat. No. 12-i10-82). Fluorescent bacteria were analyzed with a MACS Quant VYB, FACS CantoII flow cytometer using the FlowJo software (BD Biosciences). Using ELISA, GBS strains were grown to the Log phase in THY were washed in PBS, normalized to OD_{600} 1 and coated o/n at 4°C. To block free protein binding sites, the wells were treated for 1 h with BSA (2%, v/v) in PBS. Surface exposed PbsP was detected with anti-rPbsP murine serum diluted 1:2000, followed by rabbit Horseradish Peroxidase (HRP)-conjugated anti-rabbit IgG diluted 1:1000. After washing, o-phenylenediamine dihydrochloride was added, and the absorbance at 490 nm was determined using an Infinite 200 PRO plate reader (Tecan).

Adhesion, invasion and translocation assays

Human epithelial cell lines A549 (ATCC CCL-185; lung carcinoma) and human brain endothelial cell line hCMEC/D3 were used in this study. Cells were cultured in 24-well plates at a density of 1×10^5 cells/well in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of fetal bovine serum (FBS). At 24 h before the adherence or invasion assays, the medium was removed and replaced with serum-free medium. Bacteria were grown to the mid-log phase and added to sub-confluent monolayers at a multiplicity of infection (MOI) of 30 bacteria/cell. After a one-hour incubation, monolayers were washed with PBS to remove non-adherent bacteria, lysed with sterile dH2O, and plated to enumerate cell-associated bacteria. For the invasion assay, after washing, the monolayers were further incubated for 1h with medium supplemented with penicillin and streptomycin (200 units/mL, 100 µg/mL, respectively) to kill extracellular bacteria. Percentages of bacterial adhesion and invasion were calculated as 100× (recovered CFU/initial inoculum CFU). To test the translocation ability of GBS, an endothelial blood-brain barrier *in vitro* model was used by cultivating hCMEC/D3 cells in collagen coated-polycarbonate trans-well membrane inserts with a pore size of 3 µm, as previously described (53). This model allows access to the upper ("blood side") and lower ("brain side") chambers and mimics GBS penetration into the brain. The hCMEC/D3 cells were grown for 5–7 days at 37°C in a humidified chamber containing 5% CO_2 to reach confluence. Prior to the assay, the integrity of the monolayer was verified by adding the Evans blue stain to the upper chamber. The hCMEC/D3 cells were then washed and

resuspended in serum-free culture medium without antibiotics. Log-phase GBS were added to the upper chamber together with Plg and/or tPA. At 2 h post-infection, the lower chamber medium was entirely removed and plated onto THY agar to measure translocation.

Acknowledgments

The studies reported in this thesis were carried out at the Metchnikoff Laboratory, University of Messina. Parts of the research presented was conducted at the Institut Pasteur of Paris and at the University of Pavia.

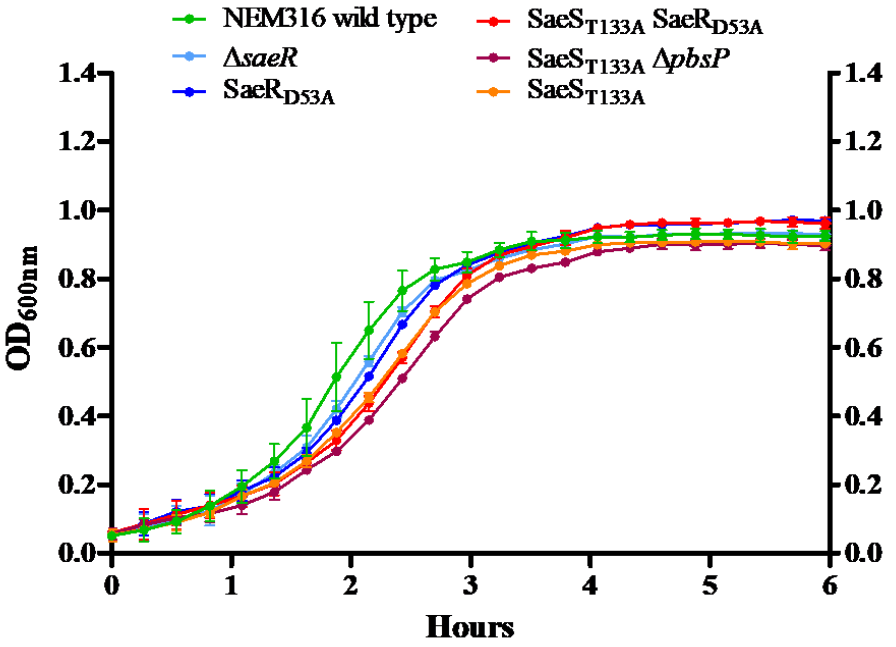
Ringrazio in primis la mia Tutor, la Prof.ssa Concetta Beninati, per avermi supportato enormemente in questo percorso con immensa concretezza. I suoi consigli mi hanno fatto crescere professionalmente e non solo. Grazie anche all'intero gruppo da Lei guidato, i colleghi/amici dei Laboratori Metchnikoff che mi sono sempre stati affianco, nei momenti belli e in quelli meno belli.

J'aimerais remercier Patrick de m'avoir chaleureusement accueilli au sein de l'unité Biologie des Bactéries Pathogènes à Gram-positif. J'aimerais aussi remercier toutes les personnes de l'unité pour leur aide précieuse et en particulier Arnaud qui m'a aidé à devenir un meilleur chercheur et qui m'a transmis sa passion pour la génétique bactérienne.

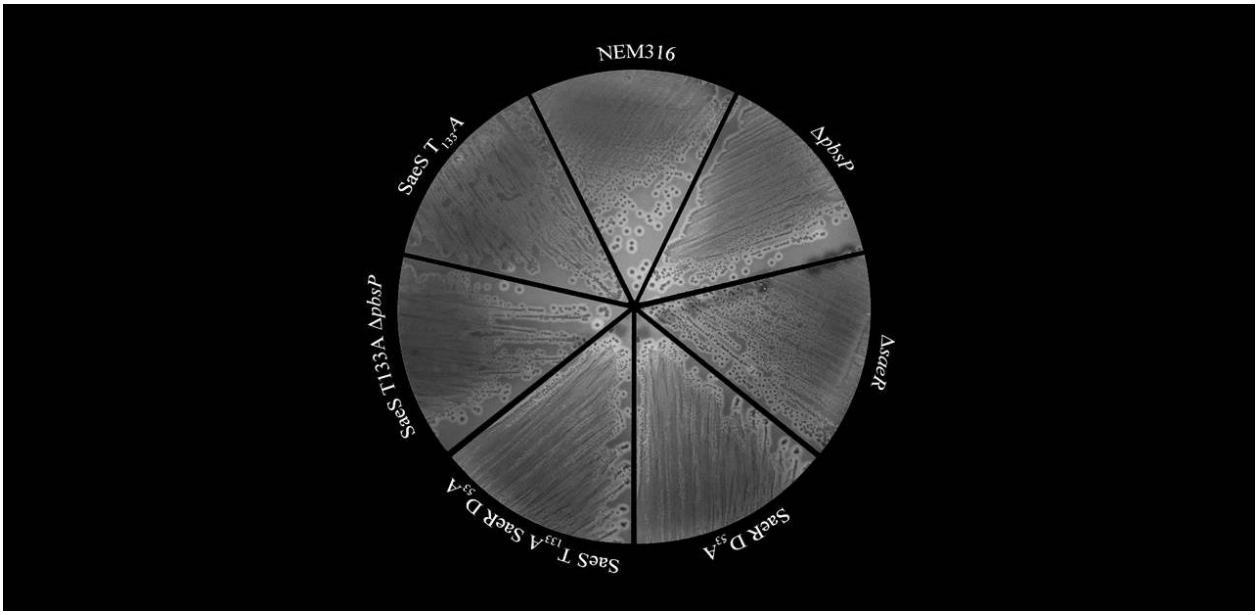
Un ringraziamento anche al gruppo dell'Università di Pavia, in particolar modo al Prof. Giampiero Pietrocola che mi ha accolto, aiutato e consigliato, alla Prof. Giulia Barbieri e tutto il gruppo di colleghi che hanno accompagnato questa esperienza.

Infine grazie alla mia famiglia, Mamma, Papà e Sorella, per avermi sempre supportato e senza i quali non sarei qui.

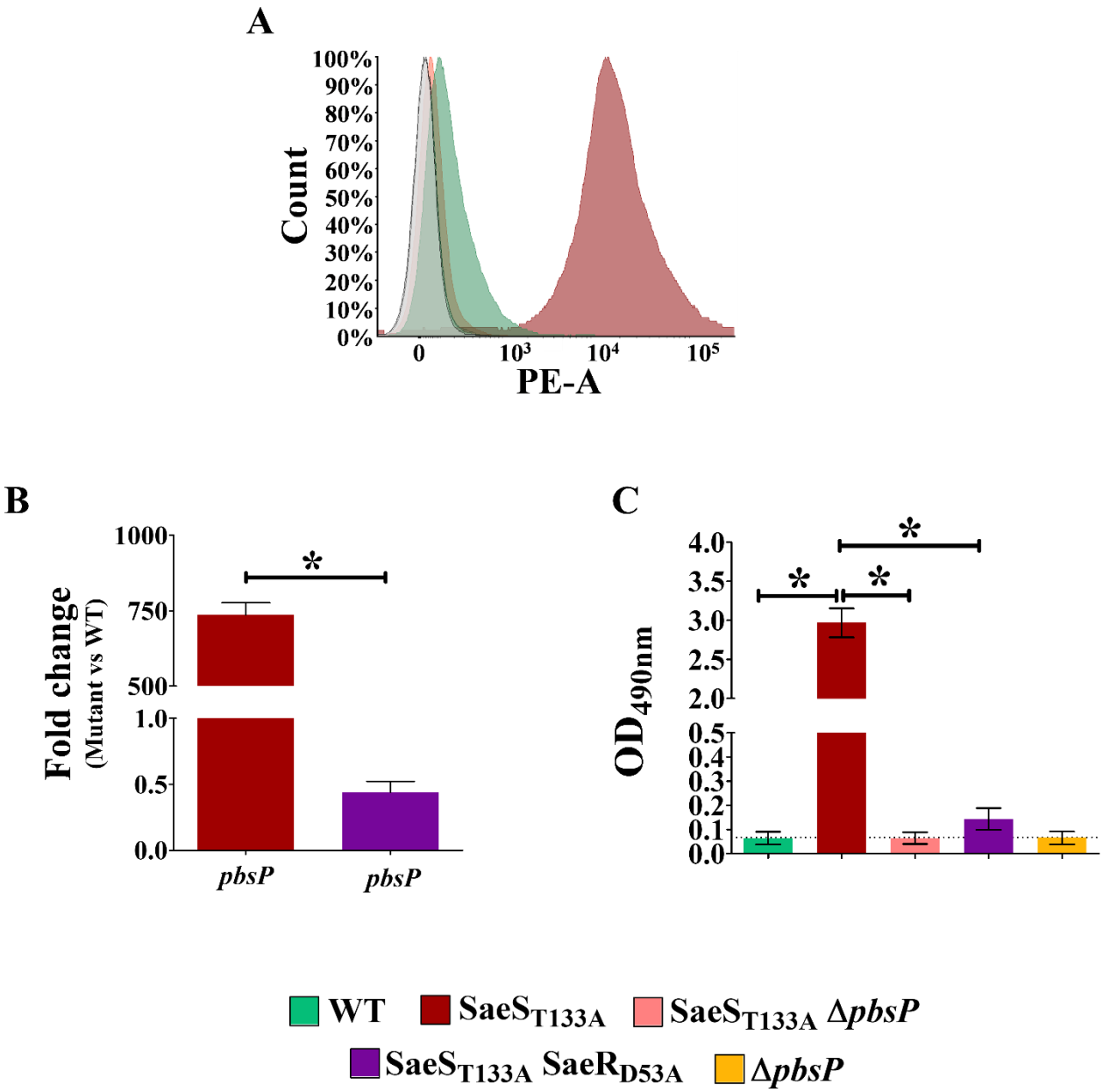
Supplementary Information



Supplementary figure 1. Growth curves of the NEM316 strain and of the SaeRS two-component system mutant strains in THY medium at 37°C in 96-well microplates, evaluated as optical density (OD_{600}) measured over time. Mean ± SD, n = 2 with technical triplicate.



Supplementary figure 2. β-hemolytic phenotype of the NEM316 strain and of the mutant strains used in this study. Hemolytic activities were assayed on Columbia horse blood (5%) agar plates.



Supplementary figure 3. SaeRS regulates PbsP. RT-qPCR analysis of *pbsP* mRNA levels in NEM316 SaeRS system mutant strains. Values were normalized for those observed in wild type (B). Mean ±SD, n = 2 with technical triplicate. *p<0.05; ***p<0.001 by Student's t test; ns, non-signifcant. Expression of PbsP on the GBS surface was confirmed by Immunofluorescence flow cytometry analysis (A) and enzyme-linked immunosorbent assay (C) using mouse polyclonal anti-PbsP serum. Mean ±SD, n = 2 with technical triplicate. *p<0.05; ***p<0.001 by Student's t test; ns, non-signifcant.

References

1. Chambers SA, Gaddy JA, Townsend SD. Synthetic Ellagic Acid Glycosides Inhibit Early Stage Adhesion of Streptococcus agalactiae Biofilms as Observed by Scanning Electron Microscopy. Chemistry. 2020 Aug 06;26(44):9923-8. PubMed PMID: 32084298. Pubmed Central PMCID: PMC7442748. Epub 20200421. eng.
2. Ferretti J, Kohler W. History of Streptococcal Research. In: Ferretti JJ, Stevens DL, Fischetti VA, editors. Streptococcus pyogenes: Basic Biology to Clinical Manifestations. Oklahoma City (OK)2016.
3. Nocard M, editor Mollereau. Sur une mammite contagieuse des vaches latieres. Annales Institute Pasteur; 1887.
4. Keefe GP. Streptococcus agalactiae mastitis: a review. The Canadian veterinary journal = La revue veterinaire canadienne. 1997 Jul;38(7):429-37. PubMed PMID: 9220132. Pubmed Central PMCID: 1576741.
5. Ruegg PL. A 100-Year Review: Mastitis detection, management, and prevention. Journal of dairy science. 2017 Dec;100(12):10381-97. PubMed PMID: 29153171.
6. Tavares T, Pinho L, Bonifácio Andrade E. Group B Streptococcal Neonatal Meningitis. Clin Microbiol Rev. 2022 Apr 20;35(2):e0007921. PubMed PMID: 35170986. Pubmed Central PMCID: PMC8849199. Epub 20220216. eng.
7. Furuta A, Brokaw A, Manuel G, Dacanay M, Marcell L, Seepersaud R, et al. Bacterial and Host Determinants of Group B Streptococcal Infection of the Neonate and Infant. Front Microbiol. 2022;13:820365. PubMed PMID: 35265059. Pubmed Central PMCID: PMC8899651. Epub 20220221. eng.
8. Edwards MS, Baker CJ. Group B streptococcal infections in elderly adults. Clin Infect Dis. 2005 Sep 15;41(6):839-47. PubMed PMID: 16107984. Epub 20050816. eng.
9. Hansen SM, Uldbjerg N, Kilian M, Sørensen UB. Dynamics of Streptococcus agalactiae colonization in women during and after pregnancy and in their infants. J Clin Microbiol. 2004 Jan;42(1):83-9. PubMed PMID: 14715736. Pubmed Central PMCID: PMC321715. eng.
10. Farley MM. Group B streptococcal disease in nonpregnant adults. Clin Infect Dis. 2001 Aug 15;33(4):556-61. PubMed PMID: 11462195. Epub 20010720. eng.
11. Sendi P, Johansson L, Norrby-Teglund A. Invasive group B Streptococcal disease in non-pregnant adults : a review with emphasis on skin and soft-tissue infections. Infection. 2008 Mar;36(2):100-11. PubMed PMID: 18193384. Epub 20080112. eng.
12. Hanna M NASGB. StatPearls [Internet]. 2023;Treasure Island (FL): StatPearls Publishing.
13. Tazi A, Morand PC, Réglier-Poupet H, Dmytruk N, Billoët A, Antona D, et al. Invasive group B streptococcal infections in adults, France (2007-2010). Clin Microbiol Infect. 2011 Oct;17(10):1587-9. PubMed PMID: 21883671. Epub 20110829. eng.
14. van der Mee-Marquet N, Fourny L, Arnault L, Domelier AS, Salloum M, Lartigue MF, et al. Molecular characterization of human-colonizing Streptococcus agalactiae strains isolated from throat, skin, anal margin, and genital body sites. J Clin Microbiol. 2008 Sep;46(9):2906-11. PubMed PMID: 18632904. Pubmed Central PMCID: PMC2546740. Epub 20080716. eng.
15. Group B streptococcal infections in pregnancy. ACOG Technical Bulletin Number 170--July 1992. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics. 1993 Jul;42(1):55-9. PubMed PMID: 8103484.
16. Verani JR, Schrag SJ. Group B streptococcal disease in infants: progress in prevention and continued challenges. Clinics in perinatology. 2010 Jun;37(2):375-92. PubMed PMID: 20569813.
17. Prevention of Group B Streptococcal Early-Onset Disease in Newborns: ACOG Committee Opinion, Number 797. Obstetrics and gynecology. 2020 Feb;135(2):e51-e72. PubMed PMID: 31977795.

18. Van Dyke MK, Phares CR, Lynfield R, Thomas AR, Arnold KE, Craig AS, et al. Evaluation of universal antenatal screening for group B streptococcus. The New England journal of medicine. 2009 Jun 18;360(25):2626-36. PubMed PMID: 19535801.
19. Creti L, Libman E, Rizzo D, Fichten CS, Bailes S, Tran DL, et al. Sleep in the Postpartum: Characteristics of First-Time, Healthy Mothers. Sleep disorders. 2017;2017:8520358. PubMed PMID: 29181201. Pubmed Central PMCID: 5664311.
20. Lancefield RC. Two Serological Types of Group B Hemolytic Streptococci with Related, but Not Identical, Type-Specific Substances. J Exp Med. 1938 Jan 1;67(1):25-40. PubMed PMID: 19870707. Pubmed Central PMCID: 2133550.
21. Baker CJ, Edwards MS. Group B streptococcal conjugate vaccines. Archives of disease in childhood. 2003 May;88(5):375-8. PubMed PMID: 12716700. Pubmed Central PMCID: 1719562.
22. Leroux-Roels G, Maes C, Willekens J, De Boever F, de Rooij R, Martell L, et al. A randomized, observer-blind Phase Ib study to identify formulations and vaccine schedules of a trivalent Group B Streptococcus vaccine for use in non-pregnant and pregnant women. Vaccine. 2016 Apr 4;34(15):1786-91. PubMed PMID: 26928074.
23. Stalhammar-Carlemalm M, Stenberg L, Lindahl G. Protein rib: a novel group B streptococcal cell surface protein that confers protective immunity and is expressed by most strains causing invasive infections. J Exp Med. 1993 Jun 1;177(6):1593-603. PubMed PMID: 8496678. Pubmed Central PMCID: 2191029.
24. Larsson C, Stalhammar-Carlemalm M, Lindahl G. Experimental vaccination against group B streptococcus, an encapsulated bacterium, with highly purified preparations of cell surface proteins Rib and alpha. Infect Immun. 1996 Sep;64(9):3518-23. PubMed PMID: 8751893. Pubmed Central PMCID: 174257.
25. Larsson C, Stalhammar-Carlemalm M, Lindahl G. Protection against experimental infection with group B streptococcus by immunization with a bivalent protein vaccine. Vaccine. 1999 Feb 5;17(5):454-8. PubMed PMID: 10073723.
26. Larsson C, Lindroth M, Nordin P, Stalhammar-Carlemalm M, Lindahl G, Krantz I. Association between low concentrations of antibodies to protein alpha and Rib and invasive neonatal group B streptococcal infection. Archives of disease in childhood Fetal and neonatal edition. 2006 Nov;91(6):F403-8. PubMed PMID: 17056838. Pubmed Central PMCID: 2672751.
27. Kobayashi M, Schrag SJ, Alderson MR, Madhi SA, Baker CJ, Sobanjo-Ter Meulen A, et al. WHO consultation on group B Streptococcus vaccine development: Report from a meeting held on 27-28 April 2016. Vaccine. 2019 Nov 28;37(50):7307-14. PubMed PMID: 28017431. Pubmed Central PMCID: 6892266.
28. Brodeur BR, Boyer M, Charlebois I, Hamel J, Couture F, Rioux CR, et al. Identification of group B streptococcal Sip protein, which elicits cross-protective immunity. Infect Immun. 2000 Oct;68(10):5610-8. PubMed PMID: 10992461. Pubmed Central PMCID: 101513.
29. Santillan DA, Rai KK, Santillan MK, Krishnamachari Y, Salem AK, Hunter SK. Efficacy of polymeric encapsulated C5a peptidase-based group B streptococcus vaccines in a murine model. American journal of obstetrics and gynecology. 2011 Sep;205(3):249 e1-8. PubMed PMID: 21802065. Pubmed Central PMCID: 3213321.
30. Nuccitelli A, Cozzi R, Gourlay LJ, Donnarumma D, Necchi F, Norais N, et al. Structure-based approach to rationally design a chimeric protein for an effective vaccine against Group B Streptococcus infections. Proc Natl Acad Sci U S A. 2011 Jun 21;108(25):10278-83. PubMed PMID: 21593422. Pubmed Central PMCID: 3121859.
31. Nuccitelli A, Rinaudo CD, Maione D. Group B Streptococcus vaccine: state of the art. Therapeutic advances in vaccines. 2015 May;3(3):76-90. PubMed PMID: 26288735. Pubmed Central PMCID: 4530403.
32. Caliot É, Dramsi S, Chapot-Chartier MP, Courtin P, Kulakauskas S, Péchoux C, et al. Role of the Group B antigen of Streptococcus agalactiae: a peptidoglycan-anchored polysaccharide

involved in cell wall biogenesis. PLoS Pathog. 2012;8(6):e1002756. PubMed PMID: 22719253. Pubmed Central PMCID: PMC3375309. Epub 20120614. eng.
33. Furfaro LL, Chang BJ, Payne MS. Perinatal Streptococcus agalactiae Epidemiology and Surveillance Targets. Clin Microbiol Rev. 2018 Oct;31(4). PubMed PMID: 30111577. Pubmed Central PMCID: PMC6148196. Epub 20180815. eng.
34. Musser JM, Mattingly SJ, Quentin R, Goudeau A, Selander RK. Identification of a high-virulence clone of type III Streptococcus agalactiae (group B Streptococcus) causing invasive neonatal disease. Proc Natl Acad Sci U S A. 1989 Jun;86(12):4731-5. PubMed PMID: 2660146. Pubmed Central PMCID: PMC287347. eng.
35. Poyart C, Réglier-Poupet H, Tazi A, Billoët A, Dmytruk N, Bidet P, et al. Invasive group B streptococcal infections in infants, France. Emerg Infect Dis. 2008 Oct;14(10):1647-9. PubMed PMID: 18826837. Pubmed Central PMCID: PMC2609873. eng.
36. Edmond KM, Kortsalioudaki C, Scott S, Schrag SJ, Zaidi AK, Cousens S, et al. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet. 2012 Feb 11;379(9815):547-56. PubMed PMID: 22226047. Epub 20120104. eng.
37. Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan MS, Kunst F, et al. Multilocus sequence typing system for group B streptococcus. J Clin Microbiol. 2003 Jun;41(6):2530-6. PubMed PMID: 12791877. Pubmed Central PMCID: PMC156480. eng.
38. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 2004 Mar;186(5):1518-30. PubMed PMID: 14973027. Pubmed Central PMCID: PMC344416. eng.
39. Motallebirad T, Fazeli H, Ghahiri A, Shokri D, Jalalifar S, Moghim S, et al. Prevalence, population structure, distribution of serotypes, pilus islands and resistance genes among erythromycin-resistant colonizing and invasive Streptococcus agalactiae isolates recovered from pregnant and non-pregnant women in Isfahan, Iran. BMC Microbiol. 2021 May 04;21(1):139. PubMed PMID: 33947330. Pubmed Central PMCID: PMC8096152. Epub 20210504. eng.
40. Spellerberg B. Pathogenesis of neonatal Streptococcus agalactiae infections. Microbes Infect. 2000 Nov;2(14):1733-42. PubMed PMID: 11137046. eng.
41. Liu Y, Liu J. Group B Streptococcus: Virulence Factors and Pathogenic Mechanism. Microorganisms. 2022 Dec 15;10(12). PubMed PMID: 36557736. Pubmed Central PMCID: PMC9784991. Epub 20221215. eng.
42. Cheng Q, Stafslien D, Purushothaman SS, Cleary P. The group B streptococcal C5a peptidase is both a specific protease and an invasin. Infect Immun. 2002 May;70(5):2408-13. PubMed PMID: 11953377. Pubmed Central PMCID: PMC127948. eng.
43. Pietrocola G, Rindi S, Rosini R, Buccato S, Speziale P, Margarit I. The Group B Streptococcus-Secreted Protein CIP Interacts with C4, Preventing C3b Deposition via the Lectin and Classical Complement Pathways. Journal of immunology. 2016 Jan 1;196(1):385-94. PubMed PMID: 26608922. Pubmed Central PMCID: 4683360.
44. Santi I, Scarselli M, Mariani M, Pezzicoli A, Masignani V, Taddei A, et al. BibA: a novel immunogenic bacterial adhesin contributing to group B Streptococcus survival in human blood. Mol Microbiol. 2007 Feb;63(3):754-67. PubMed PMID: 17212592. Epub 20070108. eng.
45. Derre-Bobillot A, Cortes-Perez NG, Yamamoto Y, Kharrat P, Couve E, Da Cunha V, et al. Nuclease A (Gbs0661), an extracellular nuclease of Streptococcus agalactiae, attacks the neutrophil extracellular traps and is needed for full virulence. Mol Microbiol. 2013 Aug;89(3):518-31. PubMed PMID: 23772975.
46. Maisey HC, Quach D, Hensler ME, Liu GY, Gallo RL, Nizet V, et al. A group B streptococcal pilus protein promotes phagocyte resistance and systemic virulence. FASEB J. 2008 Jun;22(6):1715-24. PubMed PMID: 18198218. Pubmed Central PMCID: PMC2721339. Epub 20080115. eng.

47. CumL ey NJ, Smith LM, Anthony M, May RC. The CovS/CovR acid response regulator is required for intracellular survival of group B Streptococcus in macrophages. Infect Immun. 2012 May;80(5):1650-61. PubMed PMID: 22331428. Pubmed Central PMCID: PMC3347446. Epub 20120213. eng.
48. 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. 2009 Apr;191(7):2023-32. PubMed PMID: 19114476. Pubmed Central PMCID: PMC2655536. Epub 20081229. eng.
49. Bhat AH, Nguyen MT, Das A, Ton-That H. Anchoring surface proteins to the bacterial cell wall by sortase enzymes: how it started and what we know now. Curr Opin Microbiol. 2021 Apr;60:73-9. PubMed PMID: 33611145. Pubmed Central PMCID: PMC7990056. Epub 20210218. eng.
50. Schubert A, Zakikhany K, Pietrocola G, Meinke A, Speziale P, Eikmanns BJ, et al. The fibrinogen receptor FbsA promotes adherence of Streptococcus agalactiae to human epithelial cells. Infect Immun. 2004 Nov;72(11):6197-205. PubMed PMID: 15501744. Pubmed Central PMCID: PMC523014. eng.
51. Gutekunst H, Eikmanns BJ, Reinscheid DJ. The novel fibrinogen-binding protein FbsB promotes Streptococcus agalactiae invasion into epithelial cells. Infect Immun. 2004 Jun;72(6):3495-504. PubMed PMID: 15155657. Pubmed Central PMCID: PMC415667. eng.
52. Pietrocola G, Schubert A, Visai L, Torti M, Fitzgerald JR, Foster TJ, et al. FbsA, a fibrinogen-binding protein from Streptococcus agalactiae, mediates platelet aggregation. Blood. 2005 Feb 01;105(3):1052-9. PubMed PMID: 15383464. Epub 20040921. eng.
53. Buscetta M, Papasergi S, Firon A, Pietrocola G, Biondo C, Mancuso G, et al. FbsC, a novel fibrinogen-binding protein, promotes Streptococcus agalactiae-host cell interactions. J Biol Chem. 2014 Jul 25;289(30):21003-15. PubMed PMID: 24904056. Pubmed Central PMCID: PMC4110306. eng.
54. Jiang S, Wessels MR. BsaB, a novel adherence factor of group B Streptococcus. Infect Immun. 2014 Mar;82(3):1007-16. PubMed PMID: 24343649. Pubmed Central PMCID: PMC3957996. Epub 20131216. eng.
55. Seo HS, Mu R, Kim BJ, Doran KS, Sullam PM. Binding of glycoprotein Srr1 of Streptococcus agalactiae to fibrinogen promotes attachment to brain endothelium and the development of meningitis. PLoS Pathog. 2012;8(10):e1002947. PubMed PMID: 23055927. Pubmed Central PMCID: PMC3464228. Epub 20121004. eng.
56. Seo HS, Minasov G, Seepersaud R, Doran KS, Dubrovska I, Shuvalova L, et al. Characterization of fibrinogen binding by glycoproteins Srr1 and Srr2 of Streptococcus agalactiae. J Biol Chem. 2013 Dec 13;288(50):35982-96. PubMed PMID: 24165132. Pubmed Central PMCID: PMC3861647. Epub 20131028. eng.
57. Six A, Bellais S, Bouaboud A, Fouet A, Gabriel C, Tazi A, et al. Srr2, a multifaceted adhesin expressed by ST-17 hypervirulent Group B Streptococcus involved in binding to both fibrinogen and plasminogen. Mol Microbiol. 2015 Sep;97(6):1209-22. PubMed PMID: 26094503. Epub 20150717. eng.
58. Sheen TR, Jimenez A, Wang NY, Banerjee A, van Sorge NM, Doran KS. Serine-rich repeat proteins and pili promote Streptococcus agalactiae colonization of the vaginal tract. J Bacteriol. 2011 Dec;193(24):6834-42. PubMed PMID: 21984789. Pubmed Central PMCID: PMC3232834. Epub 20111007. eng.
59. Mu R, Kim BJ, Paco C, Del Rosario Y, Courtney HS, Doran KS. Identification of a group B streptococcal fibronectin binding protein, SfbA, that contributes to invasion of brain endothelium and development of meningitis. Infect Immun. 2014 Jun;82(6):2276-86. PubMed PMID: 24643538. Pubmed Central PMCID: PMC4019170. Epub 20140318. eng.
60. Spellerberg B, Pohl B, Haase G, Martin S, Weber-Heynemann J, Lütticken R. Identification of genetic determinants for the hemolytic activity of Streptococcus agalactiae by ISS1 transposition.

J Bacteriol. 1999 May;181(10):3212-9. PubMed PMID: 10322024. Pubmed Central PMCID: PMC93778. eng.
61. Maisey HC, Hensler M, Nizet V, Doran KS. Group B streptococcal pilus proteins contribute to adherence to and invasion of brain microvascular endothelial cells. J Bacteriol. 2007 Feb;189(4):1464-7. PubMed PMID: 17041051. Pubmed Central PMCID: PMC1797338. Epub 20061013. eng.
62. Banerjee A, Kim BJ, Carmona EM, Cutting AS, Gurney MA, Carlos C, et al. Bacterial Pili exploit integrin machinery to promote immune activation and efficient blood-brain barrier penetration. Nat Commun. 2011 Sep 06;2:462. PubMed PMID: 21897373. Pubmed Central PMCID: PMC3195231. Epub 20110906. eng.
63. Carlsson F, Stalhammar-Carlemalm M, Flardh K, Sandin C, Carlemalm E, Lindahl G. Signal sequence directs localized secretion of bacterial surface proteins. Nature. 2006 Aug 24;442(7105):943-6. PubMed PMID: 16929299.
64. Brega S, Caliot E, Trieu-Cuot P, Dramsi S. SecA localization and SecA-dependent secretion occurs at new division septa in group B Streptococcus. PLoS One. 2013;8(6):e65832. PubMed PMID: 23762438. Pubmed Central PMCID: 3676364.
65. Bumbaca D, Littlejohn JE, Nayakanti H, Rigden DJ, Galperin MY, Jedrzejas MJ. Sequence analysis and characterization of a novel fibronectin-binding repeat domain from the surface of Streptococcus pneumoniae. Omics : a journal of integrative biology. 2004 Winter;8(4):341-56. PubMed PMID: 15703481.
66. Buscetta M, Firon A, Pietrocola G, Biondo C, Mancuso G, Midiri A, et al. PbsP, a cell wall-anchored protein that binds plasminogen to promote hematogenous dissemination of group B Streptococcus. Mol Microbiol. 2016 Jul;101(1):27-41. PubMed PMID: 26888569. Epub 20160601. eng.
67. Coppolino F, Romeo L, Pietrocola G, Lentini G, De Gaetano GV, Teti G, et al. Lysine Residues in the MK-Rich Region Are Not Required for Binding of the PbsP Protein From Group B Streptococci to Plasminogen. Front Cell Infect Microbiol. 2021;11:679792. PubMed PMID: 34568085. Pubmed Central PMCID: PMC8455988. Epub 20210908. eng.
68. Da Cunha V, Davies MR, Douarre PE, Rosinski-Chupin I, Margarit I, Spinali S, et al. Streptococcus agalactiae clones infecting humans were selected and fixed through the extensive use of tetracycline. Nat Commun. 2014 Aug 4;5:4544. PubMed PMID: 25088811. Pubmed Central PMCID: 4538795.
69. Arya J, Sharma D, Kumar D, Jakhar R, Khichi A, Dangi M, et al. Comparative genome analysis of Streptococcus strains to identify virulent genes causing neonatal meningitis. Infect Genet Evol. 2023 Jan;107:105398. PubMed PMID: 36572056. Epub 20221224. eng.
70. Wiles KG, Panizzi P, Kroh HK, Bock PE. 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. 2010 Jul 2;285(27):21153-64. PubMed PMID: 20435890. Pubmed Central PMCID: 2898333.
71. De Gaetano GV, Pietrocola G, Romeo L, Galbo R, Lentini G, Giardina M, et al. The Streptococcus agalactiae cell wall-anchored protein PbsP mediates adhesion to and invasion of epithelial cells by exploiting the host vitronectin/α. Mol Microbiol. 2018 Oct;110(1):82-94. PubMed PMID: 30030946. Epub 20181004. eng.
72. Lentini G, Midiri A, Firon A, Galbo R, Mancuso G, Biondo C, et al. The plasminogen binding protein PbsP is required for brain invasion by hypervirulent CC17 Group B streptococci. Sci Rep. 2018 Sep 25;8(1):14322. PubMed PMID: 30254272. Pubmed Central PMCID: PMC6156580. Epub 20180925. eng.
73. Castellino FJ, McCance SG. The kringle domains of human plasminogen. Ciba Foundation symposium. 1997;212:46-60; discussion -5. PubMed PMID: 9524763.

74. Bhattacharya S, Ploplis VA, Castellino FJ. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. Journal of biomedicine & biotechnology. 2012;2012:482096. PubMed PMID: 23118509. Pubmed Central PMCID: 3477821.
75. De Gaetano GV, Lentini G, Fama A, Coppolino F, Beninati C. In Vivo Role of Two-Component Regulatory Systems in Models of Urinary Tract Infections. Pathogens. 2023 Jan 10;12(1). PubMed PMID: 36678467. Pubmed Central PMCID: 9861413.
76. Stock JB, Ninfa AJ, Stock AM. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol Rev. 1989 Dec;53(4):450-90. PubMed PMID: 2556636. Pubmed Central PMCID: PMC372749. eng.
77. Parkinson JS, Kofoid EC. Communication modules in bacterial signaling proteins. Annu Rev Genet. 1992;26:71-112. PubMed PMID: 1482126. eng.
78. Wang S. Bacterial Two-Component Systems: Structures and Signaling Mechanisms2012 06 September 2012.
79. Casino P, Rubio V, Marina A. Structural insight into partner specificity and phosphoryl transfer in two-component signal transduction. Cell. 2009 Oct 16;139(2):325-36. PubMed PMID: 19800110. Epub 20091001. eng.
80. Bachhawat P, Stock AM. Crystal structures of the receiver domain of the response regulator PhoP from Escherichia coli in the absence and presence of the phosphoryl analog beryllofluoride. J Bacteriol. 2007 Aug;189(16):5987-95. PubMed PMID: 17545283. Pubmed Central PMCID: PMC1952025. Epub 20070601. eng.
81. Galperin MY. Diversity of structure and function of response regulator output domains. Curr Opin Microbiol. 2010 Apr;13(2):150-9. PubMed PMID: 20226724. Pubmed Central PMCID: PMC3086695. Epub 20100311. eng.
82. Sidote DJ, Barbieri CM, Wu T, Stock AM. Structure of the Staphylococcus aureus AgrA LytTR domain bound to DNA reveals a beta fold with an unusual mode of binding. Structure. 2008 May;16(5):727-35. PubMed PMID: 18462677. Pubmed Central PMCID: PMC2430735. eng.
83. Maris AE, Sawaya MR, Kaczor-Grzeskowiak M, Jarvis MR, Bearson SM, Kopka ML , et al. Dimerization allows DNA target site recognition by the NarL response regulator. Nat Struct Biol. 2002 Oct;9(10):771-8. PubMed PMID: 12352954. eng.
84. Zhao R, Collins EJ, Bourret RB, Silversmith RE. Structure and catalytic mechanism of the E. coli chemotaxis phosphatase CheZ. Nat Struct Biol. 2002 Aug;9(8):570-5. PubMed PMID: 12080332. eng.
85. Huynh TN, Noriega CE, Stewart V. Conserved mechanism for sensor phosphatase control of two-component signaling revealed in the nitrate sensor NarX. Proc Natl Acad Sci U S A. 2010 Dec 07;107(49):21140-5. PubMed PMID: 21078995. Pubmed Central PMCID: PMC3000247. Epub 20101115. eng.
86. Liu W, Dong H, Li J, Ou Q, Lv Y, Wang X, et al. RNA-seq reveals the critical role of OtpR in regulating Brucella melitensis metabolism and virulence under acidic stress. Sci Rep. 2015 Aug 05;5:10864. PubMed PMID: 26242322. Pubmed Central PMCID: PMC4542472. Epub 20150805. eng.
87. Low LY, Harrison PF, Lin YH, Boyce JD, Rood JI, Cheung JK. RNA-seq analysis of virR and revR mutants of Clostridium perfringens. BMC Genomics. 2016 May 23;17:391. PubMed PMID: 27216822. Pubmed Central PMCID: PMC4877802. Epub 20160523. eng.
88. Kaihami GH, Breda LCD, de Almeida JRF, de Oliveira Pereira T, Nicastro GG, Boechat AL, et al. The Atypical Response Regulator AtvR Is a New Player in Pseudomonas aeruginosa Response to Hypoxia and Virulence. Infect Immun. 2017 Aug;85(8). PubMed PMID: 28533471. Pubmed Central PMCID: PMC5520423. Epub 20170719. eng.
89. Rajeev L, Garber ME, Mukhopadhyay A. Tools to map target genes of bacterial two-component system response regulators. Environ Microbiol Rep. 2020 Jun;12(3):267-76. PubMed PMID: 32212247. Pubmed Central PMCID: PMC7318608. Epub 20200405. eng.

90. Thomas L, Cook L. Two-Component Signal Transduction Systems in the Human Pathogen Streptococcus agalactiae. Infect Immun. 2020 Jun 22;88(7). PubMed PMID: 31988177. Pubmed Central PMCID: 7309623.
91. Santi I, Grifantini R, Jiang SM, Brettoni C, Grandi G, Wessels MR, et al. CsrRS regulates group B Streptococcus virulence gene expression in response to environmental pH: a new perspective on vaccine development. J Bacteriol. 2009 Sep;191(17):5387-97. PubMed PMID: 19542277. Pubmed Central PMCID: PMC2725634. Epub 20090619. eng.
92. Di Palo B, Rippa V, Santi I, Brettoni C, Muzzi A, Metruccio MM, et al. Adaptive response of Group B streptococcus to high glucose conditions: new insights on the CovRS regulation network. PLoS One. 2013;8(4):e61294. PubMed PMID: 23585887. Pubmed Central PMCID: PMC3621830. Epub 20130409. eng.
93. Mazzuoli MV, Daunesse M, Varet H, Rosinski-Chupin I, Legendre R, Sismeiro O, et al. The CovR regulatory network drives the evolution of Group B Streptococcus virulence. PLoS Genet. 2021 Sep;17(9):e1009761. PubMed PMID: 34491998. Pubmed Central PMCID: PMC8448333. Epub 20210907. eng.
94. Firon A, Tazi A, Da Cunha V, Brinster S, Sauvage E, Dramsi S, et al. The Abi-domain protein Abx1 interacts with the CovS histidine kinase to control virulence gene expression in group B Streptococcus. PLoS Pathog. 2013 Feb;9(2):e1003179. PubMed PMID: 23436996. Pubmed Central PMCID: PMC3578759. Epub 20130221. eng.
95. Lin WJ, Walthers D, Connelly JE, Burnside K, Jewell KA, Kenney LJ, et al. Threonine phosphorylation prevents promoter DNA binding of the Group B Streptococcus response regulator CovR. Mol Microbiol. 2009 Mar;71(6):1477-95. PubMed PMID: 19170889. Pubmed Central PMCID: PMC3133594. Epub 20090123. eng.
96. Lamy MC, Zouine M, Fert J, Vergassola M, Couve E, Pellegrini E, et al. CovS/CovR of group B streptococcus: a two-component global regulatory system involved in virulence. Mol Microbiol. 2004 Dec;54(5):1250-68. PubMed PMID: 15554966. eng.
97. Tazi A, Disson O, Bellais S, Bouaboud A, Dmytruk N, Dramsi S, et al. The surface protein HvgA mediates group B streptococcus hypervirulence and meningeal tropism in neonates. J Exp Med. 2010 Oct 25;207(11):2313-22. PubMed PMID: 20956545. Pubmed Central PMCID: PMC2964583. Epub 20101018. eng.
98. Lembo A, Gurney MA, Burnside K, Banerjee A, de los Reyes M, Connelly JE, et al. Regulation of CovR expression in Group B Streptococcus impacts blood-brain barrier penetration. Mol Microbiol. 2010 Jul;77(2):431-43. PubMed PMID: 20497331. Pubmed Central PMCID: PMC2909351. Epub 20100519. eng.
99. Patras KA, Wang NY, Fletcher EM, Cavaco CK, Jimenez A, Garg M, et al. Group B Streptococcus CovR regulation modulates host immune signalling pathways to promote vaginal colonization. Cell Microbiol. 2013 Jul;15(7):1154-67. PubMed PMID: 23298320. Pubmed Central PMCID: PMC3657335. Epub 20130130. eng.
100. Sullivan MJ, Leclercq SY, Ipe DS, Carey AJ, Smith JP, Voller N, et al. Effect of the Streptococcus agalactiae Virulence Regulator CovR on the Pathogenesis of Urinary Tract Infection. J Infect Dis. 2017 Feb 01;215(3):475-83. PubMed PMID: 28011914. Pubmed Central PMCID: PMC6455028. eng.
101. Rozhdestvenskaya AS, Totolian AA, Dmitriev AV. Inactivation of DNA-binding response regulator Sak189 abrogates beta-antigen expression and affects virulence of Streptococcus agalactiae. PLoS One. 2010 Apr 19;5(4):e10212. PubMed PMID: 20419089. Pubmed Central PMCID: PMC2856668. Epub 20100419. eng.
102. Poyart C, Lamy MC, Boumaila C, Fiedler F, Trieu-Cuot P. Regulation of D-alanyl-lipoteichoic acid biosynthesis in Streptococcus agalactiae involves a novel two-component regulatory system. J Bacteriol. 2001 Nov;183(21):6324-34. PubMed PMID: 11591677. Pubmed Central PMCID: PMC100127. eng.

103. Giraudo AT, Calzolari A, Cataldi AA, Bogni C, Nagel R. The sae locus of Staphylococcus aureus encodes a two-component regulatory system. FEMS Microbiol Lett. 1999 Aug 01;177(1):15-22. PubMed PMID: 10436918. eng.
104. Geiger T, Goerke C, Mainiero M, Kraus D, Wolz C. The virulence regulator Sae of Staphylococcus aureus: promoter activities and response to phagocytosis-related signals. J Bacteriol. 2008 May;190(10):3419-28. PubMed PMID: 18344360. Pubmed Central PMCID: PMC2395011. Epub 20080314. eng.
105. Jeong DW, Cho H, Jones MB, Shatzkes K, Sun F, Ji Q, et al. The auxiliary protein complex SaePQ activates the phosphatase activity of sensor kinase SaeS in the SaeRS two-component system of Staphylococcus aureus. Mol Microbiol. 2012 Oct;86(2):331-48. PubMed PMID: 22882143. Pubmed Central PMCID: PMC3468659. Epub 20120827. eng.
106. Gordon RJ, Lowy FD. Pathogenesis of methicillin-resistant Staphylococcus aureus infection. Clin Infect Dis. 2008 Jun 01;46 Suppl 5(Suppl 5):S350-9. PubMed PMID: 18462090. Pubmed Central PMCID: PMC2474459. eng.
107. Novick RP, Jiang D. The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing. Microbiology (Reading). 2003 Oct;149(Pt 10):2709-17. PubMed PMID: 14523104. eng.
108. Steinhuber A, Goerke C, Bayer MG, Döring G, Wolz C. Molecular architecture of the regulatory Locus sae of Staphylococcus aureus and its impact on expression of virulence factors. J Bacteriol. 2003 Nov;185(21):6278-86. PubMed PMID: 14563862. Pubmed Central PMCID: PMC219404. eng.
109. Sun F, Li C, Jeong D, Sohn C, He C, Bae T. In the Staphylococcus aureus two-component system sae, the response regulator SaeR binds to a direct repeat sequence and DNA binding requires phosphorylation by the sensor kinase SaeS. J Bacteriol. 2010 Apr;192(8):2111-27. PubMed PMID: 20172998. Pubmed Central PMCID: PMC2849438. Epub 20100219. eng.
110. Nygaard TK, Pallister KB, Ruzevich P, Griffith S, Vuong C, Voyich JM. SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. J Infect Dis. 2010 Jan 15;201(2):241-54. PubMed PMID: 20001858. Pubmed Central PMCID: PMC2798008. eng.
111. Cook LCC, Hu H, Maienschein-Cline M, Federle MJ. A Vaginal Tract Signal Detected by the Group B Streptococcus SaeRS System Elicits Transcriptomic Changes and Enhances Murine Colonization. Infect Immun. 2018 Apr;86(4). PubMed PMID: 29378799. Pubmed Central PMCID: PMC5865029. Epub 20180322. eng.
112. Thomas LS, Cook LC. A Novel Conserved Protein in Streptococcus agalactiae, BvaP, Is Important for Vaginal Colonization and Biofilm Formation. mSphere. 2022 Dec 21;7(6):e0042122. PubMed PMID: 36218343. Pubmed Central PMCID: PMC9769775. Epub 20221011. eng.
113. Biswas I, Gruss A, Ehrlich SD, Maguin E. High-efficiency gene inactivation and replacement system for gram-positive bacteria. J Bacteriol. 1993 Jun;175(11):3628-35. PubMed PMID: 8501066. Pubmed Central PMCID: 204764.
114. Kuroda H, Kuroda M, Cui L, Hiramatsu K. Subinhibitory concentrations of beta-lactam induce haemolytic activity in Staphylococcus aureus through the SaeRS two-component system. FEMS Microbiol Lett. 2007 Mar;268(1):98-105. PubMed PMID: 17263851.
115. Zurek OW, Nygaard TK, Watkins RL, Pallister KB, Torres VJ, Horswill AR, et al. The role of innate immunity in promoting SaeR/S-mediated virulence in Staphylococcus aureus. Journal of innate immunity. 2014;6(1):21-30. PubMed PMID: 23816635. Pubmed Central PMCID: 4435966.
116. Magalhães V, Veiga-Malta I, Almeida MR, Baptista M, Ribeiro A, Trieu-Cuot P, et al. Interaction with human plasminogen system turns on proteolytic activity in Streptococcus agalactiae and enhances its virulence in a mouse model. Microbes Infect. 2007 Sep;9(11):1276-84. PubMed PMID: 17890121. Epub 20070609. eng.
117. Magalhães V, Andrade EB, Alves J, Ribeiro A, Kim KS, Lima M, et al. Group B Streptococcus hijacks the host plasminogen system to promote brain endothelial cell invasion. PLoS

One. 2013;8(5):e63244. PubMed PMID: 23658816. Pubmed Central PMCID: PMC3642152. Epub 20130502. eng.
118. Devaux L, Sleiman D, Mazzuoli MV, Gominet M, Lanotte P, Trieu-Cuot P, et al. Cyclic di-AMP regulation of osmotic homeostasis is essential in Group B Streptococcus. PLoS Genet. 2018 Apr;14(4):e1007342. PubMed PMID: 29659565. Pubmed Central PMCID: 5919688.

Additional papers

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 08 September 2021
doi: 10.3389/fcimb.2021.679792

Lysine Residues in the MK-Rich Region Are Not Required for Binding of the PbsP Protein From Group B Streptococci to Plasminogen

Francesco Coppolino[1†], Letizia Romeo[2†], Giampiero Pietrocola[3], Germana Lentini[2], Giuseppe Valerio De Gaetano[2], Giuseppe Teti[4], Roberta Galbo[5] and Concetta Beninati[2,6]*

[1] Department of Biomedical, Dental and Imaging Sciences, University of Messina, Messina, Italy, [2] Department of Human Pathology and Medicine, University of Messina, Messina, Italy, [3] Department Molecular Medicine, Biochemistry Section, University of Pavia, Pavia, Italy, [4] Charybdis Vaccines Srl, Messina, Italy, [5] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy, [6] Scylla Biotech Srl, Messina, Italy

OPEN ACCESS

Edited by:
Maurizio Fraziano, University of Rome Tor Vergata, Italy

Reviewed by:
Angela Silva Barbosa, Butantan Institute, Brazil
Barbara Spellerberg, Ulm University Medical Center, Germany

***Correspondence:**
Roberta Galbo
rgalbo@unime.it

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *16 March 2021*
Accepted: *23 August 2021*
Published: *08 September 2021*

Citation:
Coppolino F, Romeo L, Pietrocola G, Lentini G, De Gaetano GV, Teti G, Galbo R and Beninati C (2021) Lysine Residues in the MK-Rich Region Are Not Required for Binding of the PbsP Protein From Group B Streptococci to Plasminogen. Front. Cell. Infect. Microbiol. 11:679792. doi: 10.3389/fcimb.2021.679792

Binding to plasminogen (Plg) enables bacteria to associate with and invade host tissues. The cell wall protein PbsP significantly contributes to the ability of group B streptococci, a frequent cause of invasive infection, to bind Plg. Here we sought to identify the molecular regions involved in the interactions between Plg and PbsP. The K4 Kringle domain of the Plg molecule was required for binding of Plg to whole PbsP and to a PbsP fragment encompassing a region rich in methionine and lysine (MK-rich domain). These interactions were inhibited by free L-lysine, indicating the involvement of lysine binding sites in the Plg molecule. However, mutation to alanine of all lysine residues in the MK-rich domain did not decrease its ability to bind Plg. Collectively, our data identify a novel bacterial sequence that can interact with lysine binding sites in the Plg molecule. Notably, such binding did not require the presence of lysine or other positively charged amino acids in the bacterial receptor. These data may be useful for developing alternative therapeutic strategies aimed at blocking interactions between group B streptococci and Plg.

Keywords: ***Streptococcus agalactiae*, MK-rich domain, plasminogen, cell wall-proteins, adhesion molecules**

INTRODUCTION

A wide variety of bacterial species are capable of interacting with plasminogen (Plg), a process that is thought to enhance their ability to colonize and invade host tissues (Lottenberg et al., 1994; Boyle and Lottenberg, 1997; Lahteenmaki et al., 2001; Walker et al., 2005; Sanderson-Smith et al., 2012; Fulde et al., 2013). Plg is produced in the liver and is released in plasma, where it reaches elevated concentrations (around 200 mg/l or 2 μM). Lower amounts of this protein are associated with various types of tissues, in which Plg is found predominantly on the cell surface and in the extracellular matrix. The presence of Plg on the surface of host cells can enhance microbial adherence, as shown using epithelial cells (Papasergi et al., 2010; Bhattacharya et al., 2012; Agarwal et al., 2013). Moreover, soluble Plg can be recruited from plasma or exudates to microbial surfaces to be converted to plasmin (Pl), an active form of the molecule endowed with potent protease activity (Castellino and Ploplis, 2005). By this mechanism, surface-associated plasmin can contribute to

degradation of the extracellular matrix and fibrin barriers, leading to tissue disruption and hematogenous dissemination to distant organs, such as the brain (Bhattacharya et al., 2012; Peetermans et al., 2016).

The mature circulating form of Plg (791 amino acids, 93 kDa) bears an Activation Peptide (AP) domain followed by 5 Kringle domains (K1–K5, each displaying three loops and three intra-domain disulfide bridges) and by the S1 Peptidase Domain (SPD) at the C-terminal region (Claeys et al., 1976; Castellino and Powell, 1981). The Kringle domains of Plg are able to interact *via* their lysine-binding sites (LBS) with multiple ligands, including host Plg receptors/targets, such as fibrin and α2 anti-plasmin, and bacterial proteins (Castellino and McCance, 1997; Bhattacharya et al., 2012). The Kringle LBS interact not only with lysine residues present in Plg-binding proteins, but also with free L-lysine and with the analogous zwitterionic ligand ϵ-aminocaproic acid (EACA). EACA inhibits fibrinolysis by competing with fibrin for binding to LBS in the Pl molecule and is used to treat acute bleeding disorders. Structural analysis of Kringle domains indicates that LBS consist of shallow surfaces bearing a dipole in which the opposite charges are separated by a hydrophobic region of highly conserved aromatic residues (Tulinsky et al., 1988). LBS in Kringle domains bind free lysine and EACA in the following order of affinity: K1 > K4 > K5 > K2 (Lin et al., 2000; Sun et al., 2002) while the K3 domain displays only slight lysine-binding activity. Streptokinase and staphylokinase are extracellularly secreted products of, respectively, *Streptoccoccus pyogenes* and *Staphylococcus aureus* that directly bind and activate Plg (Verhamme and Bock, 2014; Verhamme et al., 2015; Nguyen and Vogel, 2016; Rafipour et al., 2019). Other extracellular proteins, such as Skizzle from GBS, and surface-associated Plg-binding bacterial products (often referred to as bacterial Plg receptors), rely instead on external activators, such as host-derived uPA or tPA, for conversion of bound Plg into Pl (Wiles et al., 2010; Peetermans et al., 2016). Bacterial Plg receptors include "moonlighting" cytoplasmic enzymes, such as α-enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Winram and Lottenberg, 1996; Pancholi and Fischetti, 1998; Bergmann et al., 2003; Seifert et al., 2003; Boël et al., 2005) as well as specialized lipoproteins or cell wall proteins, such as Plg-binding streptococcal M- and M-like proteins (Berge and Sjobring, 1993; Wistedt et al., 1995; Ringdahl and Sjobring, 2000; Bhattacharya et al., 2012). A common feature of bacterial surface Plg receptors is their ability to interact with LBS, as indicated by inhibition of these interactions using free lysine or EACA.

Streptococcus agalactiae (also named group B *Streptococcus* or GBS) is a gram-positive encapsulated bacterium that can behave as a commensal of the human gastrointestinal and genital tracts or as an agent of invasive infections. Clinical manifestations of GBS disease include sepsis and meningitis in neonates and an increasing variety of conditions in adults with predisposing factors, in pregnant women and in the elderly (Skoff et al., 2009; Edmond et al., 2012; Le Doare and Heath, 2013). Plg binding and acquisition of plasmin-mediated proteolytic activity by GBS have a crucial role in its ability to spread hematogenously to the brain and other organs (Magalhaes et al., 2013). Plg binding by GBS is at least partially mediated by PbsP (standing for Plasminogen binding surface Protein), a cell-wall-anchored protein of 521-aa. PbsP contains an N-terminal domain, two 150-aa SSURE repeat domains (Bumbaca et al., 2004), a methionine and lysine-rich (MK-rich) region, and a cell wall-anchoring LPxTG motif (Buscetta et al., 2016). PbsP is involved in hematogenous dissemination and blood brain barrier invasion by GBS (Buscetta et al., 2016; Lentini et al., 2018) and is an interesting vaccine candidate, because of its high degree of conservation among GBS clinical isolates and its strong upregulation *in vivo* (Cook et al., 2018; Lentini et al., 2018). Moreover, PbsP is a multifunctional adhesin capable of also binding vitronectin, which might contribute to its ability to promote GBS adherence to epithelial cells (De Gaetano et al., 2018). In view of its importance in pathogenesis, we sought in the present study to obtain insights into the molecular regions responsible for PbsP binding to Plg. It was found that such interactions require a specific sequence in the MK-rich domain of PbsP and the Kringle 4 LBS of Plg. However, the presence of lysine or other positively charged amino acid residues in the MK-rich region was not required for binding of this region to Plg. These data may be useful to develop therapeutic strategies aimed at preventing GBS interactions with Plg and to better understand binding of bacterial receptors to Plg.

MATERIALS AND METHODS

Peptides, Recombinant Fragments and Antibodies

The recombinant PbsP (rPbsP), SSURE domains (rSSURE-1+2 and rSSURE-2) and rMK-rich domain used in this study were produced as described (Garibaldi et al., 2010; Papasergi et al., 2010; Buscetta et al., 2014; Buscetta et al., 2016). The peptides indicated as Fr1, Fr2, Fr3 and Fr4, encompassing the MK-rich domain, were purchased from GenScript Itd (Hong Kong).

To obtain the M12K fragment and its mutated forms designated Mut1, Mut2, Mut3 and Mut4 (**Supplementary Table 1**), the corresponding oligonucleotides were obtained by ATG:biosynthetics GmbH (Germany). After amplification with the primers described in **Supplementary Table 2**, PCR products were cloned into a Gateway pDONR221 vector (Thermo Fisher Scientific, Waltham, Massachusetts) according to the manufacturer's instructions. Next, the recombinant purified plasmids were used to obtain expression vectors pDEST15_M12K, pDEST15_Mut1, pDEST15_Mut2, pDEST15_Mut3, and pDEST15_Mut4. Transformed *E. coli* BL21 (DE3) strains were grown to purify the peptides as fusions to Gluthatione S-Transferase, as previously described (Buscetta et al., 2016). Polyclonal antiserum against purified Plg was raised in rabbits using purified human Plg as an antigen. Purification of rabbit antibodies from sera was performed by affinity chromatography using protein G-Sepharose columns (GE Healthcare, Buckinghamshire, UK). Goat anti-rabbit IgG horseradish

peroxidase (HRP)-conjugated secondary antibody was purchased from Dako Cytomation (Glostrup, Denmark). Unless stated otherwise, all other reagents were purchased from Sigma-Aldrich (St. Louis, Missouri).

Plasminogen and Kringle Domains

Human plasma was obtained from healthy volunteers with informed consent and permission of the ethical board of the University of Pavia (permit no. 19092013). After centrifugation, the plasma fraction was frozen in aliquots and stored at −20°C (Pietrocola et al., 2016). Plasminogen was purified from plasma by affinity chromatography on a Lys-Sepharose column (Deutsch and Mertz, 1970). Kringle 1–3 (P1667) and Kringle 1–4 (MBS634949) were purchased from Sigma-Aldrich and MyBiosource (San Diego, California), respectively. Mini-Plg (residues Val_{442}-Asn_{790}) was obtained by digestion of Plg with porcine pancreatic elastase (Sigma-Aldrich), as previously described (Váli and Patthy, 1982; Christensen and Mølgaard, 1992).

ELISA, Dot and Western Blot Assays

Binding of Plg or its fragments to recombinant proteins (rPbsP, rSSURE-1+2, rSSURE-2, rMK-rich, M12K, Mut1, Mut2, Mut3 and Mut4) was determined by ELISA, dot and Western blot assays as described (Pietrocola et al., 2016). For ELISA assays, microtiter wells were coated a 4°C with equimolar amounts of rPbsP or PbsP fragments. Blocking of the wells was done for 1h at room temperature (RT) with 200 µl of 4% bovine serum albumin (BSA, Sigma-Aldrich) in PBS, followed by the addition of Plg, K 1-3, K 1-4 or Mini-Plg. The plates were then incubated with rabbit polyclonal anti-Plg (1:2000 in PBS 0,1% BSA) and anti-rabbit horseradish peroxidase-conjugated IgG (diluted 1:10000 in PBS 0,1% BSA). Plates were developed with o-phenylenediamine dihydrochloride (OPD) and the absorbance (490 nm) was determined using an ELISA plate reader. For competitive ELISA, plates were coated and blocked as above and incubated for 1h at RT with rPbsP or PbsP fragments in the presence of the indicated inhibitors. In selected experiments, plates were sensitized with rPbsP or its fragments, probed with Plg, K 1-3, K 1-4 or Mini-Plg and developed, with rabbit polyclonal anti-Plg, followed by anti-rabbit horseradish peroxidase-conjugated IgG.

RESULTS

The Kringle 4 Domain of Plg Is Required for Binding to PbsP

In order to identify the molecular regions involved in interactions between PbsP and Plg, we analyzed the binding of isolated recombinant PbsP domains to different Plg fragments by ELISA. In these assays we used the whole PbsP protein, the MK-rich domain and 2 different SSURE domain fragments (rSSURE-1+2 and rSSURE-2; **Figure 1A**). These products were immobilized on plates and probed with the whole Plg protein or three different Plg fragments encompassing Kringle 1 to 3, Kringle 1 to 4 and Mini-Plg (**Figure 1B**). Under these conditions, the MK-rich domain bound Plg almost as efficiently as the whole PbsP protein (**Figure 1C**). In contrast, the SSURE domains showed only moderate, albeit significant, binding to Plg. Notably, both the MK-rich domain and whole PbsP bound the Plg fragment encompassing Kringle 1 to 4, but not other fragments, indicating that the Kringle 4 domain is required for PbsP-Plg interactions. To further study the role of the MK-rich domain, we tested the ability of the isolated MK-rich fragments to compete with whole PbsP for binding to Plg. The whole protein was adsorbed onto microtiter plates and Plg binding was assessed in the presence of inhibitors. As shown in **Figure 1D**, the addition of the MK-rich domain produced a dose-dependent inhibition in the binding of Plg to PbsP and could almost completely abrogate such binding at the highest doses, while rSSURE-1+2 was only partially effective as an inhibitor. Collectively these data indicate that the Plg-binding ability of PbsP predominantly resides in its MK-rich domain and requires the Kringle 4 domain.

Free L-Lysine Inhibits Interactions Between Plg and the MK-Rich Domain

In order to identify the molecular regions involved in PbsP-Plg interactions, we next investigated the involvement of LBS sites in binding of the MK-rich fragment to Plg by testing the inhibitory effects of soluble L-lysine. To this end, Plg binding to immobilized PbsP or its fragments was tested in the presence of soluble L-lysine or its analog EACA, which is frequently used to probe the interactions between LBS and Plg-binding proteins (Lin et al., 2000). Under these conditions, both free lysine and EACA produced a dose-dependent inhibition of Plg binding to the MK-rich fragment (**Figure 2A**). In contrast, alanine (a non charged amino acid) and arginine (a charged amino acid), used as controls, were totally ineffective. As expected, similar results were obtained when assessing the ability of lysine and EACA to inhibit Plg binding of whole PbsP (**Figure 2B**). Collectively these data suggest that LBS are involved in interactions between Plg and the MK-rich domain of PbsP.

A C-Terminal Fragment of the MK-Rich Domain Is Sufficient for Binding to Plg

In view of the significant Plg-binding activities of the MK-rich domain of PbsP, further studies focused on the mechanisms of such interactions. In order to identify the minimal region of the MK-rich domain that still maintains reactivity with Plg, we produced truncated forms of this domain. First we produced an N-terminally truncated form (designated Fr1) spanning amino acids S_{444} to N_{484}, and a shorter fragment (designated Fr2) spanning the remaining part of the domain (N_{423} to T_{443}; **Figure 3A**). When tested by ELISA and dot blot analysis, Fr1 fully retained the ability of the entire MK-rich domain to bind Plg, while Fr2 was inactive (**Figures 3B, D**). Next, we produced a truncated form of Fr1, designated Fr4, (K_{469}-N_{484}) lacking the 25 N-terminal amino acids and Fr3, the complementary fragment, spanning the 25 N-terminal amino acids (S_{444}-A_{468}). As shown in **Figures 3C, D**, neither of these fragments bound Plg to any extent. In addition, assays using Fr2, Fr3 and Fr4 as

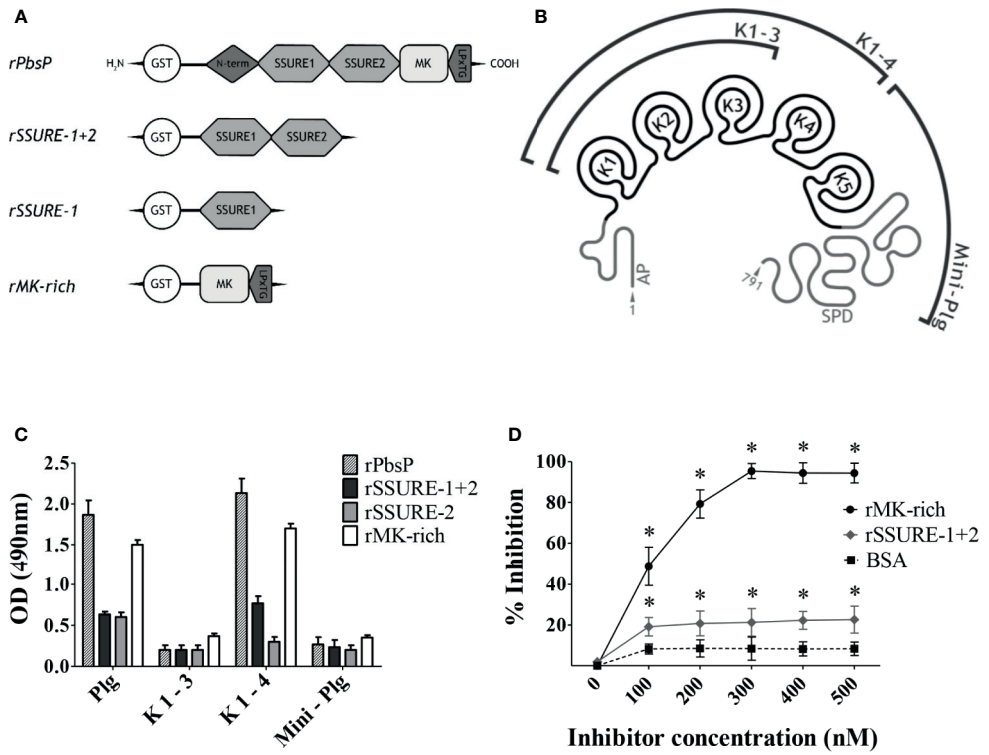


FIGURE 1 | The Kringle 4 domain of Plg is required for binding to PbsP. **(A)**, Schematic representation of recombinant Plasminogen binding surface Protein (PbsP) and its derived recombinant fragments. **(B)**, Structure of human Glu-Plasminogen and its fragments: AP domain, K 1-3 encompassing Kringle 1 to 3, K 1-4 encompassing Kringle 1 to 4 and Mini-Plg encompassing Kringle 5 and S1 Peptidase Domain (SPD). **(C)**, ELISA for selective binding of PbsP and its fragments to Plg and its derivatives. An equimolar amount of rPbsP and its derived fragments (250 nM) was immobilized on the surface of microtiter wells, and their binding was tested with Plg, K1-3, K1-4, or Mini-Plg (100 nM). Complexes were detected with polyclonal anti-Plg antibodies followed by HRP-conjugated goat anti-rabbit IgG. **(D)**, Selective inhibition of PbsP-Plg interactions. Competitive ELISA assays were done with immobilized rPbsP (250 nM) to which Plg (100 nM) was added in the presence of increasing concentrations of rSSURE-1+2 or rMK-rich fragments. BSA (bovine serum albumin) was used as a control inhibitor. Inhibition ability is shown as percentage. Data are means ± SD from three independent experiments conducted in duplicate. *p <0.05 as determined by Wilcoxon rank sum test analysis.

inhibitors indicated that none of these fragments inhibited binding of Plg to the MK-rich domain (**Figure 3E**). In contrast, as expected, Fr1 showed strong inhibitory activities under the same conditions (**Figure 3E**). These results indicate that the 21 N-terminal amino acids (N_{423} to T_{443}) of the MK-rich region are not required for Plg binding and that Fr1, but not shorter fragments, recapitulates the ability of the MK-rich domain to bind Plg.

Lysine Residues in the MK-Rich Domain Are Not Involved in Plg Binding

Previous studies have demonstrated that terminal or internal lysine residues in a number of bacterial Plg receptors are required for optimal interactions with Plg (Bergmann et al., 2001; Derbise et al., 2004; Wiles et al., 2010; Moreau et al., 2017; Nagarajan et al., 2019). In view of the ability of L-lysine to inhibit interactions between the MK-rich domain and Plg, as shown above, we hypothesized that LBS in the Plg molecule interact with one or more of the lysine residues that are abundantly present in the MK-rich domain. To verify this hypothesis, we produced several mutated forms of the MK-rich domain in which different lysine residues were replaced by alanine (**Supplementary Table 1**). Next, we used these mutated forms to assess their binding to Plg by ELISA and Western blot analysis. Surprisingly, none of the mutated forms showed decreased binding to Plg as compared with the corresponding fragment bearing all lysine residues and designated as M12K (**Figures 4A, C**). In particular, an MK-rich fragment in which all lysine residues were replaced by alanine (rMut4) bound Plg as efficiently as the wild-type form of the molecule (**Figures 4A, C**). Next, it was of interest to ascertain whether the rMut4 fragment in which all lysine residues were replaced by alanine could still be inhibited by free lysine in its interactions with Plg. **Figure 4B** shows that this was indeed the case, since both L-lysine and EACA inhibited Plg binding by the rMut4 fragment. These data indicate that lysine residues in the MK-rich region are not required for interactions between this region and the LBS in the Plg molecule.

A remarkable feature of the MK-rich region is the abundant presence of methionine, which accounts for 26% of all amino acid residues (**Figure 3A**). Since methionine is a nonpolar amino acid, we hypothesized that hydrophobic interactions are involved

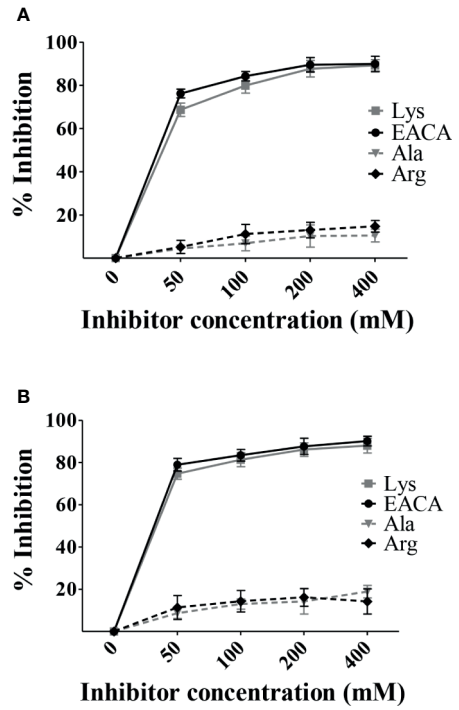


FIGURE 2 | Free L-lysine inhibits interactions between Plg and the MK-rich domain. **(A, B)**, Selective inhibition of rMK-rich/rPbsP-Plg interactions. Competitive ELISA assays were done with immobilized rMK-rich or rPbsP (250 nM) to which Plg (100 nM) was added in the presence of increasing concentrations of ε-aminocaproic acid (EACA) or L-lysine (Lys). L-alanine (Ala) and L-arginine (Arg) were used as negative controls. Inhibition ability is shown as percentage. Data are means ± SD from three independent experiments conducted in duplicate.

in binding of the MK-rich region to Plg LBS, which contain pockets of hydrophobic residues. To explore the possible involvement of methionine residues in binding of the MK-rich region to Plg, we tested the effects of increasing concentrations of free L-methionine on this interaction. The presence of methionine at 50 mM or higher concentrations significantly, albeit partially, inhibited binding of the rMut4 fragment to Plg (**Figure 5**), suggesting the possible involvement of methionine residues in such binding.

DISCUSSION

Our previous studies have indicated that the cell wall protein PbsP significantly contributes to the ability of GBS to bind plasminogen and acquire plasmin-dependent protease activity (Buscetta et al., 2016). *In vivo*, PbsP plays a major role in brain invasion by GBS, a process that is at least partially mediated by surface plasmin activity (Buscetta et al., 2016; Lentini et al., 2018). Therefore, better understanding of the molecular mechanisms by which PbsP binds plasminogen may lead to the development of adjunctive methods to control GBS disease.

In the first part of our present study we sought to identify the molecular region of Plg involved in binding to PbsP. It was found that such binding was inhibited by free L-lysine suggesting the involvement of LBS in one or more Kringle domains of Plg. In this respect, our data resemble observations conducted with a wide variety of other bacterial Plg receptors, whose interactions with Plg are lysine- or EACA-inhibitable (Lahteenmaki et al., 2001; Bergmann et al., 2011; Bhattacharya et al., 2012; Sanderson-Smith et al., 2012; Peetermans et al., 2016). However, relatively few studies have investigated the specific type of LBS or Kringle domain involved. Plg-binding M proteins from *Streptococcus pyogenes* selectively interact with K2, which contains a low-affinity LBS (Wistedt et al., 1998; Fu et al., 2008), while SCM, an M-like protein from *Streptococcus canis*, binds the K5 of Plg and Mini-Plg (Fulde et al., 2011). The LenA protein from *Leptospira interrogans* was found to bind to a Plg fragment spanning K1 to K3 (Verma et al., 2010). To our knowledge, specific binding of bacterial receptors to the K4 domain of Plg has not been previously reported.

We found here that PbsP and its MK-rich region bound to a polypeptide encompassing the K1-4 Plg domains but not to a shorter fragment encompassing K1-3, indicating that the K4 domain is required for binding. It is likely that this requirement reflects direct binding of the MK-rich region to the K4 domain rather than indirect effects of the K4 domain in changing the conformation of K1-3 in a way that promotes binding of these domains to Plg. In fact, Kringles are considered functionally and structurally independent domains that are highly stabilized by internal bonds, including disulfide bridges (Mulichak et al., 1991; De Vos et al., 1992). However, further studies involving isolated Kringle domains will be needed to formally demonstrate binding of the MK-rich region to K4. Collectively our data and those of previous investigations suggest a high degree of specificity in the binding to distinct LBS of the various bacterial proteins studied thus far, likely reflecting considerable differences in the Plg-binding motifs of the various receptors.

In the second part of our study we sought to identify the molecular region(s) of PbsP involved in binding to Plg. In agreement with previous studies (Buscetta et al., 2016), we found that both the SSURE domains and the MK-rich domain (i.e. a PbsP region rich in methionine and lysine) were capable of binding Plg and that the latter domain was highly efficient in this activity. By testing various fragments of the MK-rich domain, we identified a 49 amino-acid-long sequence, designated Fr1, that could largely recapitulate the Plg-binding ability of whole PbsP. Fr1 constitutes the major portion of the MK-rich domain and spans a region in which methionine and lysine residues make up 21 and 17%, respectively, of the entire amino acid sequence. The presence of positively charged amino acids, such as lysine, in the context of hydrophobic residues, such as methionine, is a common feature of Plg binding motifs (Peetermans et al., 2016). Moreover, lysine residues in these motifs are often crucially required for the ability of either host- or pathogen-derived proteins to bind Plg (Miles et al., 1991; Ponting et al., 1992; Bergmann et al., 2001; Derbise et al., 2004; Wiles et al., 2010; Bhattacharya et al., 2012; Sanderson-Smith et al., 2012;

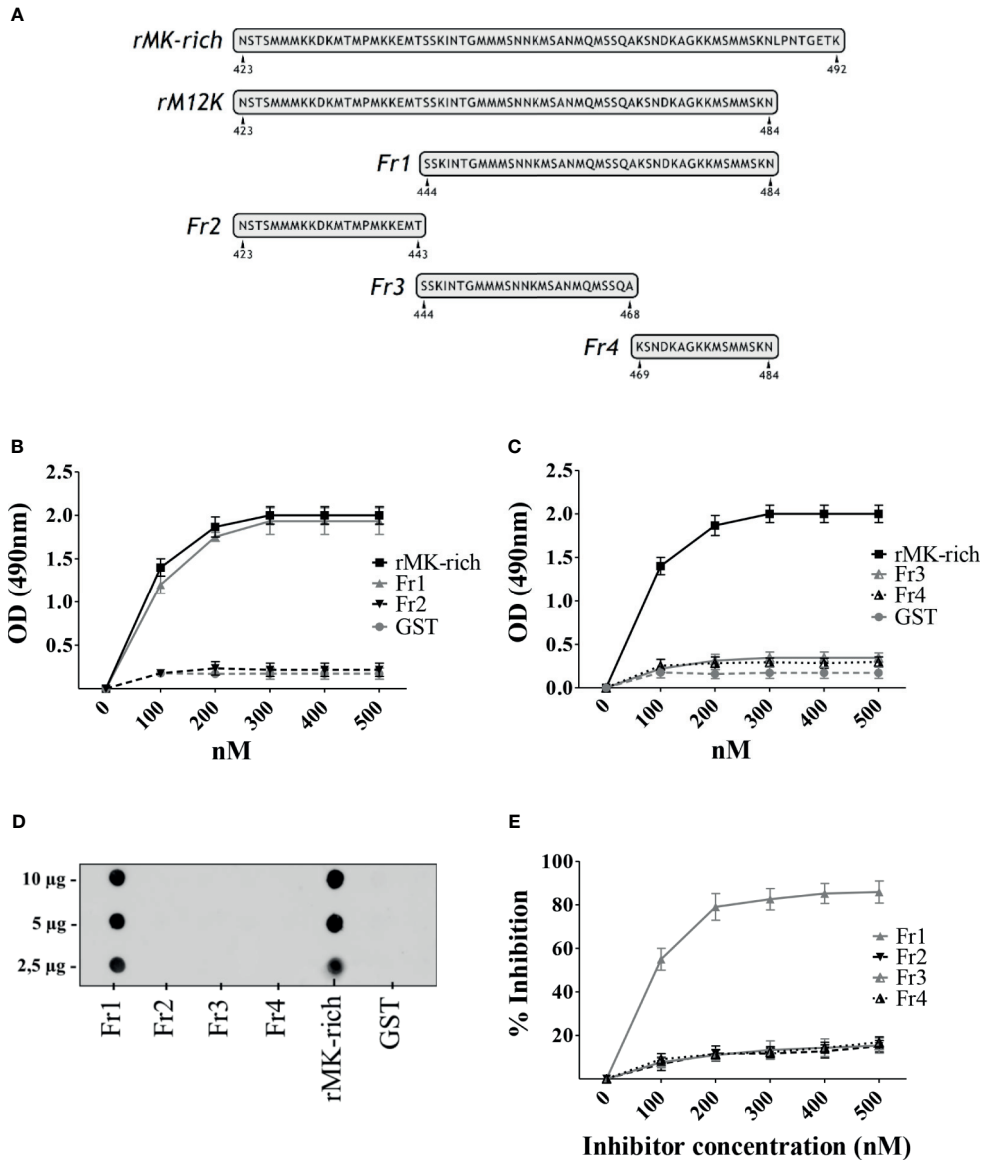


FIGURE 3 | A C-terminal fragment of the MK-rich domain is sufficient for binding to Plg. **(A)**, Schematic representation and sequence of truncated forms of MK-rich domain. **(B, C)**, Binding of MK-rich and its truncated forms to Plg. Recombinant MK-rich and its derived fragments (250 nM) were immobilized on the surface of microtiter wells, and their binding was tested with increasing concentrations of Plg. Bound Plg was detected with rabbit antibodies to human Plg followed by HRP-conjugated goat anti-rabbit IgG. **(D)**, Dot blot analysis of MK-rich/fragments-Plg interactions. Increasing concentrations of rMK-rich or its fragments were spotted onto the nitrocellulose membranes and probed using 1µg of Plg, which were detected using anti-Plg antibodies. GST was used as negative control. Shown are data from one representative experiment of two producing similar results. **(E)**, Selective inhibition of MK-rich-Plg interactions. Competitive ELISA assays were done with immobilized rMK-rich (250 nM) to which Plg (100 nM) was added in the presence of increasing concentrations of Fr1, Fr2, Fr3 and Fr4. Inhibition ability is shown as percentage. Data are means ± SD from three independent experiments conducted in duplicate.

Moreau et al., 2017; Nagarajan et al., 2019). For these reasons we hypothesized that one or more of the lysine residues of this domain mediated such interactions. Surprisingly, however, this was not the case, since mutant forms of the MK-rich domain lacking one or all lysine residues fully retained the ability to bind Plg, as shown here by direct binding or inhibition studies. These data are reminiscent of recent studies in which mutagenesis of lysine residues to alanine resulted in minimal or no inhibition in Plg binding activity. For example, loss of lysine residues in the internal nonapeptide of a1 and a2 repeats of the M-like proteins of *S. pyogenes* did not decrease its Plg binding affinity. However, Plg binding was abolished by mutagenesis of arginine and histidine residues, despite the presence of lysine residues (Sanderson-Smith et al., 2006; Sanderson-Smith et al., 2007;

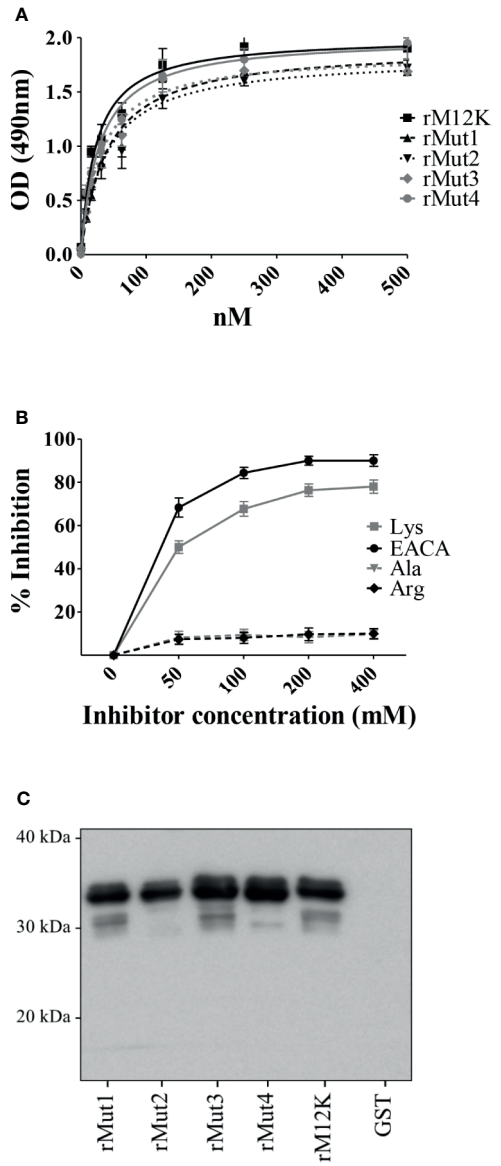


FIGURE 4 | Lysine residues in the MK-rich domain are not involved in Plg binding. **(A)**, Binding of M12K and its mutated forms to Plg. Recombinant M12K and its mutated forms (250 nM) were immobilized on the surface of microtiter wells, and their binding was tested with increasing concentrations of Plg. Bound Plg was detected with rabbit anti-Plg antibodies and HRP-conjugated goat anti-rabbit IgG. **(B)**, Selective inhibition of Mut4-Plg interactions. Competitive ELISA assays were done with immobilized rMut4 (250 nM) to which Plg (100 nM) was added in the presence of the indicated concentrations of (EACA) or L-lysine (Lys). L-alanine (Ala) and L-arginine (Arg) were used as negative controls. Inhibition ability is shown as percentage. Data are means ± SD from three independent experiments conducted in duplicate. **(C)**, Western blot analysis of binding to Plg of M12K and its mutated forms. Recombinant M12K or its mutated forms were loaded onto SDS-PAGE (5µg/lane), transferred to nitrocellulose membrane and probed with Plg (1µg/ml). Bound Plg was detected using a primary polyclonal anti-Plg antibody followed by secondary HRP-conjugated goat anti-rabbit IgG. GST was used as negative control. Numbers indicate the molecular mass of protein standards in kDa. Shown are data from one representative experiment of two producing similar results.

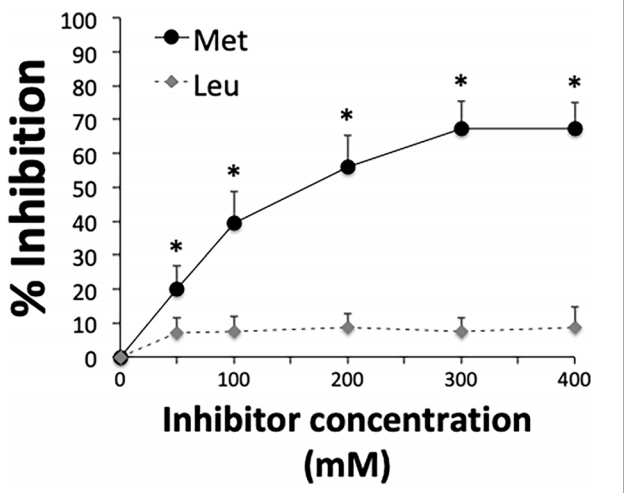


FIGURE 5 | Free L-methionine inhibits interactions between Plg and the MK-rich domain. Selective inhibition of the interactions between Plg and the rMut4 fragment that lacks all of the lysine residues present in the MK-rich fragment. Competitive ELISA assays were done with immobilized rMut4 fragment (250 nM) to which Plg (100 nM) was added in the presence of increasing concentrations of L-methionine (Met). L-leucine (Leu) was used as a negative control. Inhibition ability is shown as percentage. Data are means ± SD from three independent experiments conducted in duplicate. *$p < 0.05$ as determined by Wilcoxon rank sum test analysis.

Sanderson-Smith et al., 2012). Therefore it appears that the absence of lysine residues can be compensated for by the presence of other positively charged amino acids such as arginine or histidine within Plg binding motifs in these bacterial Plg receptors. It should be noted, however, that the MK-rich region of PbsP does not contain positively charged amino acids other than lysine. Therefore our data showing robust, lysine-inhibitable interactions between Plg and a mutated MK-rich fragment lacking all lysine residues provide an unusual example of an LBS ligand devoid of positively charged amino acid residues. We speculate that methionine residues in the MK-rich region of PbsP engage in hydrophobic interactions with aromatic residues in the in the LBS pocket of the K4 domain. Our data showing partial inhibition of these interactions in the presence of free L-methionine are compatible with this possibility.

In conclusion, we identified here a novel bacterial sequence that can interact with the LBS of Plg even in the absence of lysine or other positively charged amino acids. These data may be useful to devise alternative therapeutic strategies to prevent Plg-mediated invasion of host tissues by GBS and other bacterial pathogens.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

FC, LR and GP conducted most of the experiments and analyzed data. Other experiments were conducted by GL, GG and RG. CB and GT contributed to the study design, wrote the paper and revised the final version of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The work was funded in part by the PRIN (Programma di Ricerca Scientifica di Rilevante Interesse Nazionale) grant 2017M8R7N9_002 from the Ministero dell'Università and Ricerca Scientifica (MIUR) of Italy and by FFABR (Fund For Basic Research Activities) 2017 and 2019 from the University of Messina. Additional funding was provided by a grant for PhD student (PON n° DR_36_TRAN_PON_IND_1) at Doctoral School in Translational Molecular Medicine and Surgery, Department of Biomedical, Dental and Imaging Sciences, University of Messina, Messina, Italy.

ACKNOWLEDGMENTS

We thank Dr. Patrick Trieu-Cuot from Institute Pasteur, Paris, France, for helpful discussion and suggestions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.679792/full#supplementary-material

REFERENCES

Agarwal, V., Kuchipudi, A., Fulde, M., Riesbeck, K., Bergmann, S., and Blom, A. M. (2013). *Streptococcus Pneumoniae* Endopeptidase O (PepO) Is a Multifunctional Plasminogen- and Fibronectin-Binding Protein, Facilitating Evasion of Innate Immunity and Invasion of Host Cells. *J. Biol. Chem.* 288 (10), 6849–6863. doi: 10.1074/jbc.M112.405530

Berge, A., and Sjobring, U. (1993). PAM, A Novel Plasminogen-Binding Protein From Streptococcus Pyogenes. *J. Biol. Chem.* 268, 25417–25424. doi: 10.1016/S0021-9258(19)74408-1

Bergmann, R., Dinkla, K., Nitsche-Schmitz, D. P., Graham, R. M. A., Luttge, M., Sanderson-Smith, M. L., et al. (2011). Biological Functions of GCS3, A Novel Plasminogen-Binding Protein of Streptococcus Dysgalactiae Ssp. Equisimilis. *Int. J. Med. Microbiol.* 301, 157–164. doi: 10.1016/j.ijmm.2010.06.007

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.x

Bergmann, S., Wild, D., Diekmann, O., Frank, R., Bracht, D., Chhatwal, G. S., et al. (2003). Identification of a Novel Plasmin(Ogen)-Binding Motif in Surface Displayed Alpha-Enolase of Streptococcus Pneumoniae. *Mol. Microbiol.* 49, 411–423. doi: 10.1046/j.1365-2958.2003.03557.x

Bhattacharya, S., Ploplis, V. A., and Castellino, F. J. (2012). Bacterial Plasminogen Receptors Utilize Host Plasminogen System for Effective Invasion and Dissemination. *J. BioMed. Biotechnol.* 2012, 482096. doi: 10.1155/2012/482096

Boël, G., Jin, H., and Pancholi, V. (2005). Inhibition of Cell Surface Export of Group A Streptococcal Anchorless Surface Dehydrogenase Affects Bacterial Adherence and Antiphagocytic Properties. *Infect. Immun* 73 (10), 6237–6248. doi: 10.1128/IAI.73.10.6237-6248.2005

Boyle, M. D., and Lottenberg, R. (1997). Plasminogen Activation by Invasive Human Pathogens. *Thromb. Haemostasis* 77 (1), 1–10. doi: 10.1055/s-0038-1655727

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 Pneumoniae. *Omics* 8, 341–356. doi: 10.1089/omi.2004.8.341

Buscetta, M., Firon, A., Pietrocola, G., Biondo, C., Mancuso, G., Midiri, A., et al. (2016). PbsP, A Cell Wall-Anchored Protein That Binds Plasminogen to Promote Hematogenous Dissemination of Group B Streptococcus. *Mol. Microbiol.* 101, 27–41. doi: 10.1111/mmi.13357

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. doi: 10.1074/jbc.M114.553073

Castellino, F. J., and McCance, S. G. (1997). The Kringle Domains of Human Plasminogen. *Ciba Found Symp*. 212, 46–60. doi: 10.1002/9780470515457.ch4

Castellino, F. J., and Ploplis, V. A. (2005). Structure and Function of the Plasminogen/Plasmin System. *Thromb. Haemostasis* 93, 647–654. doi: 10.1160/TH04-12-0842

Castellino, F. J., and Powell, J. R. (1981). Human Plasminogen. *Methods Enzimol* 80 Pt C, 365–378. doi: 10.1016/S0076-6879(81)80031-6

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 (Pt 2), 419–425. doi: 10.1042/bj2850419

Claeys, H., Sottrup-Jensen, L., Zajdel, M., Petersen, T. E., and Magnusson, S. (1976). Multiple Gene Duplication in the Evolution of Plasminogen. Five Regions of Sequence Homology With the Two Internally Homologous Structures in Prothrombin. *FEBS Lett.* 61 (1), 20–24. doi: 10.1016/0014-5793(76)80161-5

Cook, L. C. C., Hu, H., Maienschein-Cline, M., and Federle, M. J. (2018). A Vaginal Tract Signal Detected by the Group B Streptococcus SaeRS System Elicits Transcriptomic Changes and Enhances Murine Colonization. *Infect. Immun.* 86, e00762–17. doi: 10.1128/IAI.00762-17

De Gaetano, G. V., Pietrocola, G., Romeo, L., Galbo, R., Lentini, G., Giardina, M., et al. (2018). The Streptococcus Agalactiae Cell Wall-Anchored Protein PbsP Mediates Adhesion to and Invasion of Epithelial Cells by Exploiting the Host Vitronectin/Alpha(V) Integrin Axis. *Mol. Microbiol.* 110, 82–94. doi: 10.1111/mmi.14084

Derbise, A., Song, Y. P., Parikh, S., Fischetti, V. A., and Win, V. (2004). Role of the C-Terminal Lysine Residues of Streptococcal Surface Enolase in Glu- and Lys-Plasminogen-Binding Activities of Group A Streptococci. *Infect. Immun.* 72, 94–105. doi: 10.1128/iai.72.1.94-105.2004

Deutsch, D. G., and Mertz, E. T. (1970). Plasminogen: Purification From Human Plasma by Affinity Chromatography. *Science* 170 (3962), 1095–1096. doi: 10.1126/science.170.3962.1095

De Vos, A. M., Ultsch, M. H., Kelley, R. F., Padmanabhan, K., Tulinsky, A., Westbrook, M. L., et al. (1992). Crystal Structure of the Kringle 2 Domain of Tissue Plasminogen Activator at 2.4-A Resolution. *Biochemistry* 31 (1), 270–279. doi: 10.1021/bi00116a037

Edmond, K. M., Kortsalioudaki, C., Scott, S., Schrag, S. J., Zaidi, A. K., Cousens, S., et al. (2012). Group B Streptococcal Disease in Infants Aged Younger Than 3 Months: Systematic Review and Meta-Analysis. *Lancet* 379, 547–556. doi: 10.1016/S0140-6736(11)61651-6

Fu, Q., Figuera-Losada, M., Ploplis, V. A., Cnudde, S., Geiger, J. H., Prorok, M., et al. (2008). The Lack of Binding of VEK-30, an Internal Peptide From the Group A Streptococcal M-Like Protein, PAM, to Murine Plasminogen Is Due to Two Amino Acid Replacements in the Plasminogen Kringle-2 Domain. *J. Biol. Chem.* 283 (3), 1580–1587. doi: 10.1074/jbc.M705063200

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. doi: 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. doi: 10.3389/fcimb.2013.00085

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. doi: 10.1016/j.vaccine.2010.01.009

Lahteenmaki, K., Kuusela, P., and Korhonen, T. K. (2001). Bacterial Plasminogen Activators and Receptors. *FEMS Microbiol. Rev.* 25, 531–552. doi: 10.1111/j.1574-6976.2001.tb00590.x

Le Doare, K., and Heath, P. T. (2013). An Overview of Global GBS Epidemiology. *Vaccine* Suppl 4, D7–12. doi: 10.1016/j.vaccine.2013.01.009

Lentini, G., Midiri, A., Firon, A., Galbo, R., Mancuso, G., Biondo, C., et al. (2018). The Plasminogen Binding Protein PbsP Is Required for Brain Invasion by Hypervirulent CC17 Group B Streptococci. *Sci. Rep.* 8, 14322. doi: 10.1038/s41598-018-32774-8

Lin, L. F., Houng, A., and Reed, G. L. (2000). Epsilon Amino Caproic Acid Inhibits Streptokinase-Plasminogen Activator Complex Formation and Substrate Binding Through Kringle-Dependent Mechanisms. *Biochemistry* 39 (16), 4740–4745. doi: 10.1021/bi992028x

Lottenberg, R., Minning-Wenz, D., and Boyle, M. D. (1994). Capturing Host Plasmin(Ogen): A Common Mechanism for Invasive Pathogens? *Trends Microbiol.* 2 (1), 20–24. doi: 10.1016/0966-842x(94)90340-9

Magalhaes, V., Andrade, E. B., Alves, J., Ribeiro, A., Kim, K. S., Lima, M., et al. (2013). Group B Streptococcus Hijacks the Host Plasminogen System to Promote Brain Endothelial Cell Invasion. *PloS One* 8 (5), e63244. doi: 10.1371/journal.pone.0063244

Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K., and Plow, E. F. (1991). Role of Cell-Surface Lysines in Plasminogen Binding to Cells: Identification of Alpha-Enolase as a Candidate Plasminogen Receptor. *Biochemistry* 30, 1682–1691. doi: 10.1021/bi00220a034

Moreau, C., Terrasse, R., Thielens, N. M., Vernet, T., Gaboriaud, C., and Di Guilmi, A. M. (2017). Deciphering Key Residues Involved in the Virulence-Promoting Interactions Between Streptococcus Pneumoniae and Human Plasminogen. *J. Biol. Chem.* 292, 2217–2225. doi: 10.1074/jbc.M116.764209

Mulichak, A. M., Tulinsky, A., and Ravichandran, K. G. (1991). Crystal and Molecular Structure of Human Plasminogen Kringle 4 Refined at 1.9-A Resolution. *Biochemistry* 30 (43), 10576–10588. doi: 10.1021/bi00107a029

Nagarajan, R., Sankar, S., and Ponnuraj, K. (2019). Crystal Structure of GAPDH of Streptococcus Agalactiae and Characterization of Its Interaction With Extracellular Matrix Molecules. *Microb. Pathogen.* 127, 359–367. doi: 10.1016/j.micpath.2018.12.020

Nguyen, L. T., and Vogel, H. J. (2016). Staphylokinase has Distinct Modes of Interaction With Antimicrobial Peptides, Modulating Its Plasminogen-Activation Properties. *Sci. Rep.* 6, 31817. doi: 10.1038/srep31817

Pancholi, V., and Fischetti, V. A. (1998). α-Enolase, A Novel Strong Plasmin (ogen) Binding Protein on the Surface of Pathogenic Streptococci. *Protein Chem. Struct.* 273 (23), 14503–14515. doi: 10.1074/jbc.273.23.14503

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. Biol. Chem.* 285, 7517–7524. doi: 10.1074/jbc.M109.062075

Peetermans, M., Vanassche, T., Liesenborghs, L., Lijnen, R. H., and Verhamme, P. (2016). Bacterial Pathogens Activate Plasminogen to Breach Tissue Barriers and Escape From Innate Immunity. *Crit. Rev. Microbiol.* 42, 866–882. doi: 10.3109/1040841X.2015.1080214

Pietrocola, G., Nobile, G., Gianotti, V., Zapotoczna, M., Foster, T. J., Geoghegan, J. A., et al. (2016). Molecular Interactions of Human Plasminogen With Fibronectin-Binding Protein B (FnBPB), a Fibrinogen/Fibronectin-Binding Protein From *Staphylococcus Aureus*. *J. Biol. Chem.* 291 (35), 18148–18162. doi: 10.1074/jbc.M116.731125

Ponting, C. P., Marshall, J. M., and Cederholm-williams, S. A. (1992). Plasminogen: A Structural Review. *Blood Coagul Fibrinol.* 3 (5), 605–614. doi: 10.1097/00001721-199210000-00012

Rafipour, M., Keramati, M., Aslani, M. M., Arashkia, A., and Roohvand, F. (2019). The Beta-Domain of Streptokinase Affects Several Functionalities, Including Specific/Proteolytic Activity Kinetics. *FEBS Open Bio.* 9, 1259–1269. doi: 10.1002/2211-5463.12657

Ringdahl, U., and Sjobring, U. (2000). Analysis of Plasminogen-Binding M Proteins of *Streptococcus Pyogenes*. *Methods* 21, 143–150. doi: 10.1006/meth.2000.0985

Sanderson-Smith, M. L., De Oliveira, D. M. P., Ranson, M., and McArthur, J. D. (2012). Bacterial Plasminogen Receptors: Mediators of a Multifaceted Relationship. *J. BioMed. Biotechnol.* 2012, 272148. doi: 10.1155/2012/272148

Sanderson-Smith, M. L., Dowton, M., Ranson, M., and Walker, M. J. (2007). The Plasminogen-Binding Group A Streptococcal M Protein-Related Protein Prp Binds Plasminogen via Arginine and Histidine Residues. *J. Bacteriol.* 189, 1435–1440. doi: 10.1128/JB.01218-06

Sanderson-Smith, M. L., Walker, M. J., and Ranson, M. (2006). The Maintenance of High Affinity Plasminogen Binding by Group A Streptococcal Plasminogen-Binding M-Like Protein Is Mediated by Arginine and Histidine Residues Within the A1 and A2 Repeat Domains. *J. Biol. Chem.* 281, 25965–25971. doi: 10.1074/jbc.M603846200

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 (5), 350–356. doi: 10.1139/w03-042

Skoff, T. H., Farley, M. M., Petit, S., Craig, A. S., Schaffner, W., Gershman, K., et al. (2009). Increasing Burden of Invasive Group B Streptococcal Disease in Nonpregnant Adult-2007. *Clin. Infect. Dis.* 49, 85–92. doi: 10.1086/599369

Sun, Z., Chen, Y. H., Wang, P., Zhang, J., Gurewich, V., Zhang, P., et al. (2002). The Blockage of the High-Affinity Lysine Binding Sites of Plasminogen by EACA Significantly Inhibits Prourokinase-Induced Plasminogen Activation. *Biochim. Biophys. Acta* 1596 (2), 182–192. doi: 10.1016/s0167-4838(02)00233-9

Tulinsky, A., Park, C. H., Mao, B., and Llinàs, M. (1988). Lysine/fibrin Binding Sites of Kringles Modeled After the Structure of Kringle 1 of Prothrombin. *Proteins* 3 (2), 85–96. doi: 10.1002/prot.340030203

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 (4), 2104–2110. doi: 10.1016/S0021-9258(19)68152-4

Verhamme, I. M., and Bock, P. E. (2014). Rapid Binding of Plasminogen to Streptokinase in a Catalytic Complex Reveals a Three-Step Mechanism. *J. Biol. Chem.* 289, 28006–28018. doi: 10.1111/jth.12939

Verhamme, I. M., Panizzi, P. R., and Bock, P. E. (2015). Pathogen Activators of Plasminogen. *J. Thromb. Haemost.* 13, S106–S114. doi: 10.1111/jth.12939

Verma, A., Brissette, C. A., Bowman, A. A., Shah, S. T., Zipfel, P. F., and Stevenson, B. (2010). Leptospiral Endostatin-Like Protein A Is a Bacterial Cell Surface Receptor for Human Plasminogen. *Infect. Immun.* 78 (5), 2053–2059. doi: 10.1128/IAI.01282-09

Walker, M. J., McArthur, J. D., McKay, F., and Ranson, M. (2005). Is Plasminogen Deployed as a Virulence Factor? *Trends Microbiol.* 13 (7), 308–313. doi: 10.1016/j.tim.2005.05.006

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. doi: 10.1074/jbc.M110.107730

Winram, S. B., and Lottenberg, R. (1996). The Plasmin-Binding Protein Plr of Group A Streptococci Is Identified as Glyceraldehyde-3-Phosphate Dehydrogenase. *Microbiology* 142, 2311–2320. doi: 10.1099/13500872-142-8-2311

Wistedt, A. C., Kotarsky, H., Marti, D., Ringdahl, U., Castellino, F. J., Schaller, J., et al. (1998). Kringle 2 Mediates High Affinity Binding of Plasminogen to an Internal Sequence in Streptococcal Surface Protein PAM. *J. Biol. Chem.* 273, 24420–24424. doi: 10.1074/jbc.273.38.24420

Wistedt, A. C., Ringdahl, U., Mulleresterl, W., and Sjobring, U. (1995). Identification of a Plasminogen-Binding Motif in Pam, A Bacterial Surface Protein. *Mol. Microbiol.* 18, 569–578. doi: 10.1111/j.1365-2958

Conflict of Interest: CB and GT act as scientific advisors for, respectively, Scylla Biotech Srl. and Charybdis Vaccines Srl. without receiving any compensation for these activities. Charybdis Vaccines S.r.l. and Scylla Biotech S.r.l. did not provide funding for this study and had no role in its conduction.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in

this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.