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The role of CpsABCD in *Streptococcus agalactiae* capsule biosynthesis

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SUMMARY

Streptococcus agalactiae or group B Streptococcus (GBS) is a Gram-positive bacterium asymptomatically colonizing 15-35% of women in the gastrointestinal and urogenital tracts. During delivery, neonates born to mothers who carry GBS can be infected themselves and develop severe diseases such as sepsis, pneumonia and meningitis. Pre-partum screenings and prophylactic treatment with antibiotics have reduced the incidence of neonatal GBS disease to 0.04% in USA. But still, in the western world, *S. agalactiae* represents the major cause of bacterial meningitis in newborns and half of the infected suffer long-term neurodevelopmental defects. Moreover, GBS has also emerged as a pathogen in other patient populations such as the elderly, pregnant women, diabetics and individuals who are immunocompromised. Vaccines based on the capsule polysaccharide (CPS) of this pathogen are currently under development.

The CPS is the main virulence factor of GBS, preventing complement deposition and opsonophagocytosis. The production of a CPS is ubiquitous in bacteria, and the Wzy pathway constitutes one of the prototypical mechanisms to produce these structures. This pathway has been characterized in detail in *S. pneumoniae*. Briefly, the repeating units of sugars composing the CPS are synthesized inside the cell by a group of glycosyltransferases. The repeating units are then flipped outside the membrane and incorporated into the growing polysaccharide chain by a polymerase. Lastly, the polysaccharide is attached to the cell wall peptidoglycan to create the CPS layer surrounding the bacterium. All the enzymes involved in this process are encoded in a single operon.

The aim of this work is to investigate the role of the CpsABCD proteins encoded in the *cps* operon of GBS. These proteins are highly conserved in all GBS serotypes, as well as in some other related bacteria, but they are not involved in the synthesis of the basic repeating units of sugars. CpsA is reported to be a transcriptional regulator and/or an enzyme attaching the CPS to the cell wall. CpsBCD homologous proteins in *S. pneumoniae* constitute a putative phosphoregulatory system, but their role in GBS capsule biosynthesis is unclear. To investigate the role of these proteins we developed twelve knockout and

functional GBS mutant strains and we examined them for CPS quantity, size, and attachment to the cell surface, as well as CpsD phosphorylation. Moreover, we used a bacterial two hybrid assay to investigate interdependencies between these proteins.

We observed that in GBS CpsB, C and D constitute a phosphoregulatory system where the CpsD autokinase phosphorylates its C-terminal tyrosines in a CpsC-dependent manner. These Tyr residues are also the target of the cognate CpsB phosphatase. Analysis of *cps* operon transcription by qRT-PCR on the mutant strains suggested that CpsABCD are not involved in transcriptional regulation of this operon. Furthermore, all the mutant strains retained the capability to produce a CPS, confirming that these proteins are not involved in the synthesis of polysaccharides, however, differences in CPS length and attachment to the cell wall were observed. In particular, we observed that the CpsC extracellular domain appeared necessary for the production of high molecular weight polysaccharides and that the LytR domain of CpsA is required for the attachment of the CPS to the bacterial cell surface. Protein-protein interactions between CpsD and CpsC and between CpsA and CpsC were observed.

These results allowed us to propose tentative roles for the proteins and their interdependencies. We propose a model where these proteins are fine-tuning the steps terminating the CPS biosynthesis, i.e. the balance between polymerization and attachment to the cell wall. In said model, CpsA competes with the CPS polymerase and attaches the CPS to the cell wall. This interplay depends on the cyclic phosphorylation of the CpsCD complex which modulates the activity of CpsA balancing the two competing activities.

Ultimately, to investigate how differences in CPS length, amount and localization impact on *S. agalactiae* ability to interact with cells, an *in vitro* adhesion-invasion assay, using lung epithelial cells have been tested. Our results showed that strains with CPS length different from the wild type were defective in associations to cells. Moreover, strains lacking the capsule or producing very little CPS were more efficient in invading cells irrespective of the CPS length.

RIASSUNTO

Streptococcus agalactiae, anche detto streptococco gruppo B (GBS), è un batterio Gram-positivo comunemente identificato come colonizzatore asintomatico del tratto gastrointestinale e urogenitale nel 15-35% delle donne. Durante il parto, GBS può essere trasmesso dalla madre colonizzata al neonato, il quale può sviluppare sepsi, polmoniti o meningiti. La diffusione di screening e trattamenti profilattici pre-parto ha significativamente ridotto l'incidenza delle malattie neonatali causate da GBS. Negli Stati Uniti, ad esempio, l'incidenza media di queste infezioni è dello 0.04%. Nel mondo occidentale, tuttavia, *S. agalactiae* rappresenta ancora la prima causa di meningiti batteriche nei neonati e la metà degli infetti soffre di difetti nello sviluppo neurologico a lungo termine. Patologie causate da GBS sono riscontrate anche in altri tipi di pazienti, quali gli anziani, le donne gravide, i diabetici e gli immunodepressi. Alcuni vaccini contro GBS sono attualmente in fase di sviluppo e sono basati sul polisaccaride capsulare (CPS) di *S. agalactiae*.

Il CPS è il maggior fattore di virulenza di GBS ed è in grado di inibire la deposizione del complemento sulla superficie del patogeno e l'opsonofagocitosi. La presenza di una capsula polisaccaridica che riveste la superficie batterica è una caratteristica comune a molti batteri e il pathway Wzy e uno dei tipici meccanismi usati per produrre i polisaccaridi che compongono questa struttura. Questo processo è stato descritto in dettaglio per *S. pneumoniae*. La produzione del CPS inizia all'interno della cellula con la sintesi delle unità saccaridiche da parte di una serie di glicosiltrasferasi. Successivamente le unità sono trasferite sul lato esterno della membrana batterica dove una polimerasi incorpora le unità ripetute al polisaccaride nascente. Il polisaccaride viene infine attaccato al peptidoglicano della parete cellulare e crea lo strato della capsula. Tutti i geni che codificano gli enzimi responsabili di questo processo si trovano in un unico operone.

Lo scopo di questa tesi è di investigare il ruolo delle proteine CpsABCD codificate dall'operone *cps*. Queste proteine sono conservate in tutti i sierotipi di GBS e in altri batteri, ma non sono direttamente coinvolte nella biosintesi delle unità saccaridiche che compongono il CPS. In letteratura, CpsA è descritto come

un regolatore trascrizionale o un enzima che attacca il CPS alla parete cellulare. Le proteine omologhe di CpsBCD di *S. pneumoniae* compongono un sistema di fosforegolazione la cui funzione nell'ambito della biosintesi del CPS non è stata chiarita. Per studiare il ruolo di CpsABCD in GBS abbiamo sviluppato dodici mutanti in cui i geni *cpsABCD* sono stati deleti o mutati. Per ognuno di questi mutanti sono stati caratterizzati la quantità, la dimensione e la localizzazione cellulare del CPS prodotto, e lo stato di fosforilazione di CpsD. Inoltre mediante l'uso del bacterial two hybrid assay sono state analizzate le interazioni tra alcune di queste proteine.

I risultati ottenuti hanno dimostrato che, anche in GBS, CpsB, C e D compongono un sistema di fosforegolazione in cui CpsD è l'autochinasi e CpsB è la fosfatasi. CpsD fosforila le tirosine che si trovano al suo C-terminale e la sua attività è dipendente dalla presenza della coda C-terminale di CpsC. Le tirosine di CpsD sono a loro volta defosforilate da CpsB. La trascrizione dell'operone *cps* è stata analizzata mediante qRT-PCR in tutti i mutanti e i risultati hanno mostrato che le proteine CpsABCD non sono coinvolte nella regolazione trascrizionale dell'operone. Inoltre, l'osservazione che i mutanti mantengono la capacità di produrre il CPS, conferma che queste proteine non partecipano alla sintesi del polisaccaride. Tuttavia, per alcuni mutanti sono state osservate differenze nella lunghezza del CPS e nella sua localizzazione. I dati ottenuti suggeriscono che il dominio extracellulare di CpsC è necessario per la produzione di polisaccaridi ad alto peso molecolare e il dominio LytR di CpsA è responsabile del trasferimento del CPS alla parete cellulare. Infine, lo studio delle interazioni tra proteine ha dimostrato che CpsC interagisce con CpsD e CpsA.

Queste evidenze sperimentali hanno permesso di suggerire delle possibili funzioni per CpsABCD. Queste proteine sono principalmente coinvolte nella regolazione dei due processi che terminano la biosintesi del CPS: la polimerizzazione e il trasferimento del polisaccaride alla parete cellulare. Nel modello che proponiamo, l'elongazione del CPS da parte della polimerasi viene interrotta dall'azione di CpsA che trasferisce il polisaccaride neosintetizzato alla parete cellulare. L'azione di CpsA viene modulata dallo stato di fosforilazione del complesso CpsCD che è quindi responsabile del bilanciamento dei due processi di

elongazione e trasferimento del CPS alla parete cellulare.

Infine, abbiamo studiato l'impatto delle differenze fenotipiche del CPS sulla capacità di GBS di interagire con le cellule umane. A questo scopo è stato impiegato un saggio di adesione-invasione *in vitro* usando cellule epiteliali polmonari e alcuni dei mutanti sviluppati. I risultati ottenuti hanno mostrato che i mutanti aventi un CPS di lunghezza diversa dal ceppo wild type presentano difetti di adesione alle cellule. Inoltre i ceppi privi di CPS o aventi una bassa quantità di CPS sono in grado di invadere le cellule più efficacemente, indipendentemente dalla lunghezza del polisaccaride prodotto.

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INTRODUCTION

Brief history of *Streptococcus agalactiae*

Streptococcus agalactiae is a Gram-positive bacterium also known as group B streptococcus (GBS) on the basis of the classification done by R. Lancefield in 1933 (Lancefield, 1933). *S. agalactiae* is a coccus with spherical shape and less than 2 μm in diameter. It is usually observed in pairs or short chains of cocci. It is catalase-negative, facultative anaerobic and β-hemolytic. It does not form spores and is not motile (Scanziani *et al.*, 1999).

In the early 1930s, *S. agalactiae* was mainly recognized as a pathogen of dairy cattle, causing acute and chronic mastitis leading to reduced milk production (Keefe, 1997). Moreover, it was commonly isolated from the vagina of healthy women, but was considered a human commensal of the gastrointestinal and urogenital tract. By the end of the decade, some cases of fatal puerperal sepsis associated with GBS were reported (Fry, 1938). During the 1970s, interest in group B streptococci increased dramatically when this bacterium emerged as the leading cause of neonatal sepsis in nurseries throughout the U.S. (Franciosi *et al.*, 1973). Approximately 10%–35% of women are asymptomatic carriers of GBS (Barton *et al.*, 1973). During delivery, newborns may become infected and develop severe diseases such as sepsis, pneumonia and meningitis (Schuchat, 1998, Thigpen *et al.*, 2011). In the 80s, the incidence of GBS infection was 1.7/1000 newborns and the mortality was close to 50% (Boyer *et al.*, 1983). In this decade, clinical trials demonstrated that the administration of intravenous antibiotics during labor, to women at risk of transmitting GBS to their newborns, could prevent early-onset disease (infection occurring in the first week of life) (Lim *et al.*, 1986).

The guidelines for prevention of perinatal GBS disease are based on pre-partum screening and prophylactic treatment with antibiotics (penicillin or erythromycin), and have been disseminated in different western countries since

the 90s. These measures have gradually reduced the incidence of early-onset disease to approximately 0.04% in USA. However, they are not sufficient to prevent late-onset disease (infection occurring in the period between the first week after birth and first 3 months of life) (Fig. 1) and invasive diseases in adult and elder people (Verani *et al.*, 2010, Edwards & Baker, 2005). Overall, the case fatality rates from early-onset GBS disease have declined to 4–6% in recent years, as a consequence of improvements in therapy and management (Rodriguez-Granger *et al.*, 2012). And yet, in the western world, *S. agalactiae* represents the major cause of bacterial meningitis in newborns (Thigpen *et al.*, 2011) (Fig. 2) and half of the infected suffer long-term neurodevelopmental defects (Rodriguez-Granger *et al.*, 2012).

No penicillin-resistant isolates of GBS have been observed so far, however, resistance to erythromycin and clindamycin has become relatively common in both genital tract isolates and invasive strains (Phares *et al.*, 2008). Vaccination of adolescent or pregnant women is considered an attractive solution.

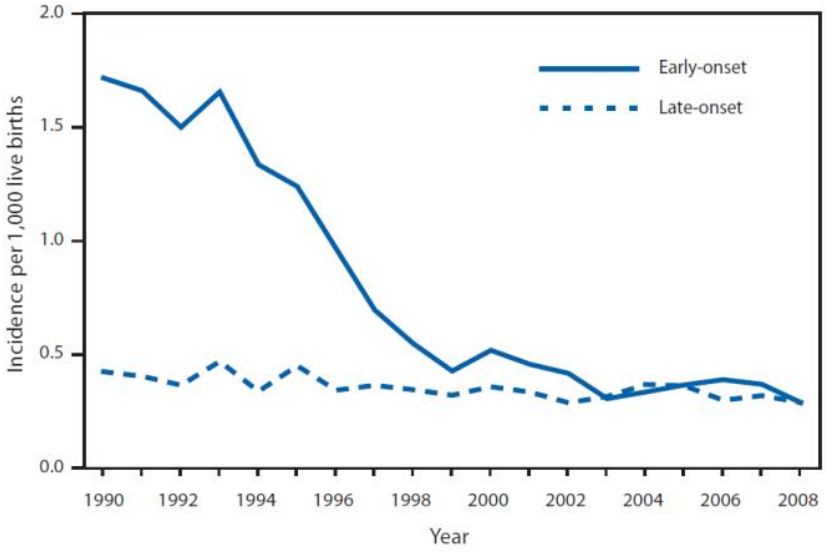

FIGURE 1. **Incidence of early- and late-onset invasive GBS disease in USA from 1990 to 2008.** Adapted from (Jordan *et al.*, 2008).

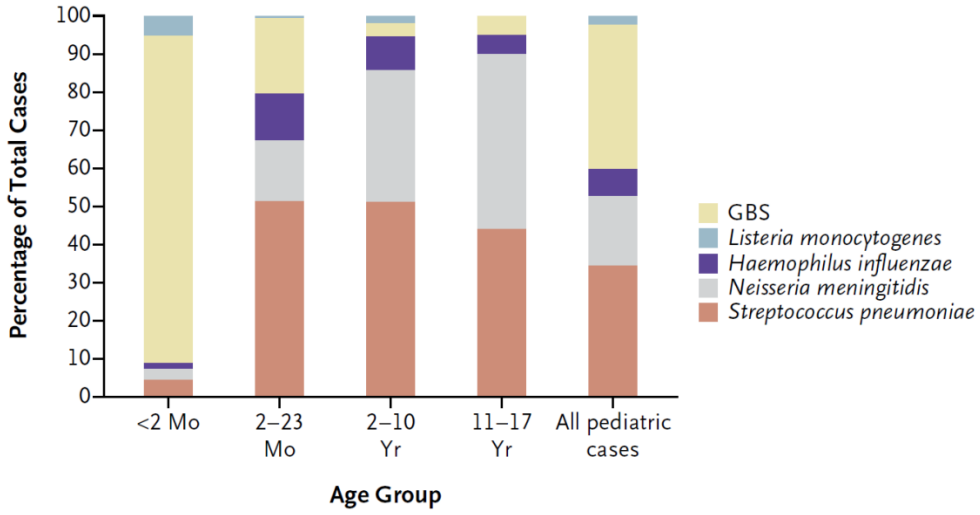

FIGURE 2. **Proportions of cases of bacterial meningitis reported in 2003–2007 caused by each pathogen, according to age group**. Mo, months; Yr, years. Adapted from (Thigpen *et al.*, 2011). Copyright Massachusetts Medical Society.

S. agalactiae **pathogenesis**

The pathogenesis of neonatal GBS infection begins with the asymptomatic colonization of the female genital tract. During labor the pathogen can be transmitted to the newborn (Schuchat, 1998), moreover, Bennett and coworkers reported that GBS can traverse placenta and weaken its membranes (Bennett *et al.*, 1987) causing an increased risk of rupture of the placental membranes and preterm delivery or death of the fetus (Regan *et al.*, 1996, Chen *et al.*, 2013).

In newborns, early-onset infections occur in the first week after birth with symptoms typically starting in the first hours. These cases result from the ascension of GBS from the vagina to the amniotic fluid or from the contact with infected vaginal fluids during labor (Verani *et al.*, 2010). During these processes GBS can be aspirated into the fetal lungs, potentially causing pneumonia and respiratory failure. From there, the organism can gain access to the bloodstream by passing through the alveolar epithelium (Fig. 3). Once in the bloodstream, GBS may cause septicemia and reaches the other organs and tissues. The lack of a completely functional immune system makes the neonates particularly prone to GBS invasive disease (Doran & Nizet, 2004).

Late-onset infection occurs in infants up to 3 months of age, and is generally characterized by symptoms related to bacteremia and a high incidence of meningitis (Doran & Nizet, 2004). GBS causes meningitis thanks to its ability to traverse the human blood-brain barrier (Fig. 3) (Nizet *et al.*, 1997). Prophylactic treatments have not reduced the incidence of these diseases (Verani *et al.*, 2010) and the prevention of these infections is difficult. Children born from mothers without GBS-specific antibodies are more prone to acquire late-onset infection, both during delivery and hospitalization. Moreover, GBS strains isolated from infected neonates have been found in the mother's breast milk suggesting this route as a possible mechanism of colonization (Le Doare & Kampmann, 2014, Olver *et al.*, 2000).

Finally, GBS has emerged as an important pathogen also in other patient populations such as the elderly, pregnant women, diabetics and the immunocompromised (Doran & Nizet, 2004). Clinical manifestations of GBS infection in adults include skin, soft tissue and urinary tract infections, bacteremia, pneumonia, arthritis and endocarditis (Edwards & Baker, 2005).

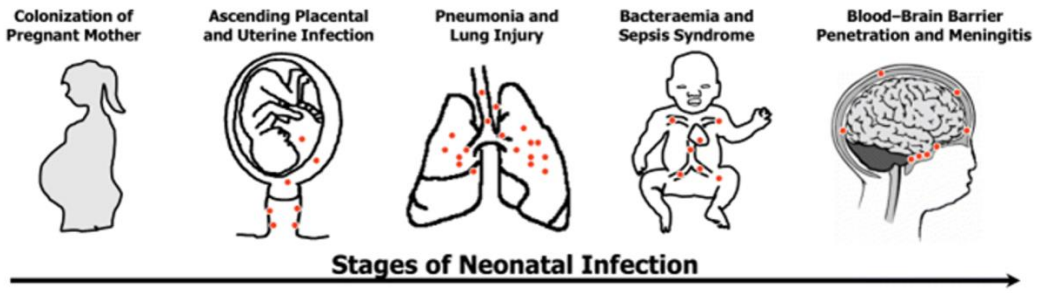

FIGURE 3. **Stages in the molecular and cellular pathogenesis of neonatal GBS infection**. Adapted from (Doran & Nizet, 2004).

S. agalactiae's virulence factors

S. agalactiae is normally found in the gastrointestinal and urogenital tract, but during infection it colonizes other compartments such as the placental membranes, lung epithelium, the bloodstream and the brain (Doran & Nizet, 2004, Nizet *et al.*, 1997). Moreover, despite that GBS is an extracellular pathogen, it has been shown that invasion of human cells play an important role in pathogenesis (Lindahl *et al.*, 2005). To survive and cause disease GBS has evolved a plethora of virulence factors which are involved in different critical points of the infectious process such as the adherence to and the penetration of the epithelial and endothelial cellular barriers, the avoidance of immunologic clearance mechanisms and the proinflammatory activity (Lindahl *et al.*, 2005). GBS virulence factors are typically integral components of the bacterial surface or secreted extracellular components (Fig. 4).

A critical virulence factor involved in adhesion and invasion of cellular barriers is the β-hemolysin/cytolysin (CylE). This pore-forming toxin is responsible for the lysis of red blood cells giving the typical clearer zone surrounding the GBS colonies when grown on blood agar plates. Moreover, this toxin lyses also other human cells such as lung epithelium and endothelial cells in the the blood-brain barrier (Nizet *et al.*, 1997). The β-hemolysin/cytolysin also has a role in inflammation, in fact, pore formation in macrophages can induce apoptosis and generation of ROS activating the sepsis cascade (Ring *et al.*, 2000). Another pore-forming toxin of GBS is the CAMP factor. This secreted protein oligomerizes in the target membrane to form discrete pores and trigger cell lysis (Lang & Palmer, 2003). The hyaluronate lyase instead is a virulence factor that facilitate spread of bacteria by breaking down the hyaluronic acid polymers present in the extracellular matrices of the placenta and lung (Liu & Nizet, 2004).

Proteins important for GBS adhesion to epithelia include the Lmb adhesin which binds to laminin (Spellerberg *et al.*, 1999), the surface anchored proteins FbsA and PavA mediating the binding to fibrinogen (Schubert *et al.*, 2002, Mitchell, 2003) and the Rib protein (Stalhammar-Carlemalm *et al.*, 1999). The C5a peptidase ScpB is a surface-localized serine protease involved both in adhesion to cells and in the avoidance of immunologic clearance. This protein

binds to fibronectin promoting bacterial adhesion and invasion of epithelial cells (Beckmann *et al.*, 2002). Moreover, because of its protease activity, it inactivates human C5a, leading to attenuation of neutrophil chemotaxis (Hill *et al.*, 1988). Another factor involved in avoidance of immunologic clearance is the streptococcal β-protein that captures to complement inhibitor protein factor H (Mitchell, 2003).

A non-proteinaceous virulence factor of GBS is the capsular polysaccharide (CPS). The CPS surrounds the external surface of the bacterium and is characterized by the presence of a terminal sialic acid identical to a sugar epitope widely displayed on the surface of mammalian cells. This epitope has evolved in GBS to resemble host 'self' and avoid immune recognition (Doran & Nizet, 2004). Moreover the CPS masks other antigenic determinants on the surface of the bacterium. Through this virulence factor, GBS is capable of preventing complement deposition and opsonophagocytosis (Hill *et al.*, 1988).

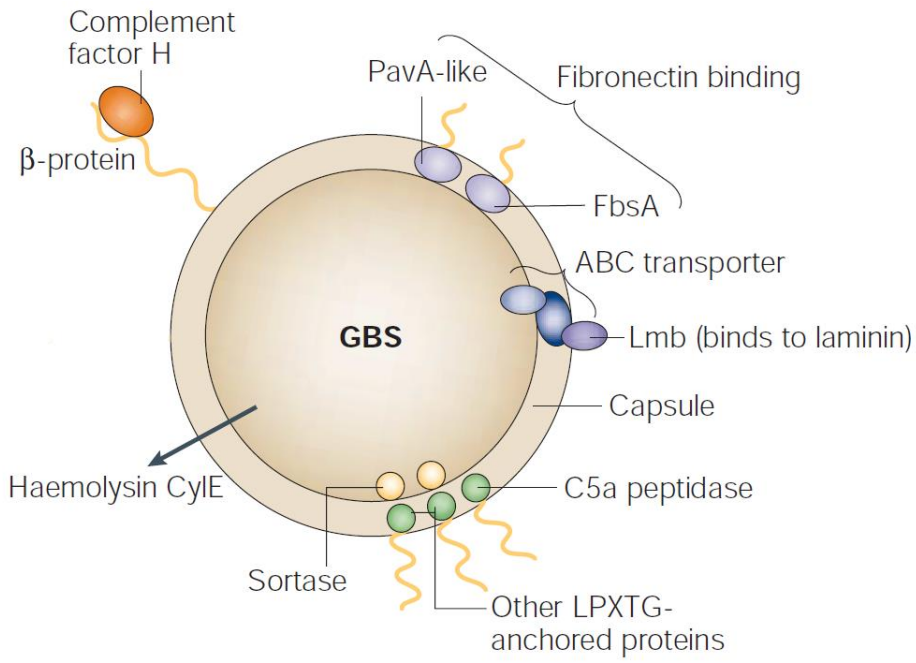

FIGURE 4. **Summary of the main virulence factors of GBS.** Adapted from (Mitchell, 2003).

Prevention of GBS infection

In most of the western countries pregnant women at 35-37 weeks of gestation are routinely screened for GBS carriage by vaginal and rectal swabs. Colonized women are treated before labor with oral erythromycin or intravenous penicillin to decrease the risk of vertical transmission. Other countries such as UK and Finland offer a less effective risk-based strategy at time of delivery (Colbourn *et al.*, 2007, Homer *et al.*, 2014). An interesting epidemiological study was conducted in 2007 by Colbourn and coworkers to determine the cost-effectiveness of prenatal strategies for preventing GBS in the UK (Colbourn *et al.*, 2007). Based on the incidence of GBS infection in newborns from mothers with different risk factors, healthcare costs were calculated for several prevention strategies, including different prenatal screening and treatment regimens, and vaccination alone or in combination with the other measures. Results suggested that treatment with antibiotic for all the women and the screening of women with very low or absent risk factors would be the most cost-effective strategy. However, if a vaccine for GBS was available, vaccination for all and treatment of women with high risk factors would be the best choice. Vaccination would in fact reduce the antibiotic exposition and the consequent allergic reactions and antibiotic resistance (Colbourn *et al.*, 2007). Moreover it may reduce the incidence of late-onset GBS disease and infections of adults or the elderly (Edwards & Baker, 2005).

Currently, vaccination against GBS is in the early phases of development and various vaccines have been tested in women in Phase I and II trials (Baker *et al.*, 1999, Paoletti & Madoff, 2002). Studies done in the 1970s demonstrated an association between low levels of maternal antibody to GBS and susceptibility of the newborn to GBS disease and showed that antibodies are transferred from mother to newborn (Baker & Barrett, 1973). Prenatal immunization is a strategy which has been shown to be safe and protective for both the mother and her fetus/infant (Steinhoff, 2013). For these reason the vaccination strategy proposed for GBS is to immunize mothers between 24 and 28 weeks of gestation. Vaccination is expected to induce mucosal immunity in the mother reducing maternal GBS colonization and transmission to the newborn. Moreover,

protective vaccine-induced antibodies crossing the placenta would protect the baby and are expected to persist in the infant for about 3 months after birth reducing late-onset infection (Colbourn *et al.*, 2007, Chen *et al.*, 2013).

Unfortunately, design of a Phase III efficacy trial to evaluate the safety and efficacy of maternal immunization to prevent GBS disease in infants presents some critical points that need to be considered. The low incidence of GBS newborn infections requires that the trial is conducted on a high number of mothers to obtain statistically significant results (Madhi *et al.*, 2013). Moreover, given the current use of antibiotics for prenatal prophylaxis, it is unethical to design a placebo-controlled randomized clinical trial to determine the efficacy of the vaccine (Johri *et al.*, 2006). Serological protection correlates could represent an answer to this problem, and efforts are ongoing to establishing an antibody threshold correlating with protection from GBS infections in newborn (Dangor *et al.*, 2015).

GBS vaccine candidates and molecular epidemiology

Initial attempts to develop a GBS vaccine focused on the use of the capsular polysaccharide as antigen. In the 1930s experiments demonstrated that polysaccharide-specific rabbit antibodies were protective against GBS infections when injected in mice (Lancefield, 1938).

Ten different variants of the capsular polysaccharide have been characterized so far (Ia, Ib, II-IX) (Cieslewicz *et al.*, 2005, Berti *et al.*, 2014) and these variants define the GBS serotypes. A recent epidemiological study on the distribution of GBS serotypes in developed countries showed that serotype III was the most frequently identified, followed by serotypes Ia, V, Ib and II (Fig. 5) and that the distribution of the serotypes changed very little in the last 30 years (Edmond *et al.*, 2012, Rodriguez-Granger *et al.*, 2012). In developing countries the distribution of the serotypes is slightly different, with prevalence of the serotypes Ia, II and III and a higher proportion of non-typeable isolates (approx. 20%) (Johri *et al.*, 2013). Serotype III is the most prevalent in newborn disease, accounting for half of the infections (Phares *et al.*, 2008). A specific clone of this

serotype in particular, the clonal complex CC17, is strongly associated with neonatal meningitis (Bellais *et al.*, 2012). For pediatric and adult cases, serotype V predominated (31% of cases), followed by serotypes Ia (Phares *et al.*, 2008).

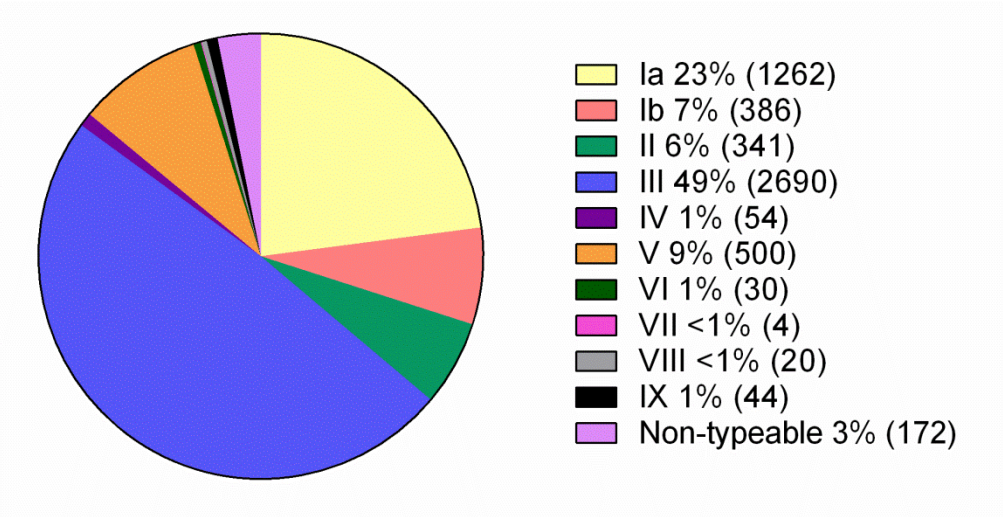

FIGURE 5. D**istribution of GBS serotypes in developed countries, 1980–2011**. Adapted from (Edmond *et al.*, 2012).

In the 1980s the first human clinical trials were conducted using the purified serotype III CPS from GBS as antigen. GBS CPS was demonstrated to be safe and immunogenic, but the immunological response was variable between subjects and the vaccine was not able to elicit B-cell memory (Baker *et al.*, 1988). To overcome these problems, polysaccharides were conjugated to proteins. The first GBS glycoconjugate vaccine trial conducted in humans involved a serotype III CPS conjugated to tetanus toxoid (III–TT) (Kasper *et al.*, 1996). Conjugate vaccines based on nine GBS serotypes have been prepared and tested pre-clinically, although there is no cross protection between serotypes. For this reason capsular conjugate vaccines will need to be multivalent in order to provide sufficient coverage against the prevalent serotypes (Johri *et al.*, 2006). Bivalent (II-TT, III-TT) and tetravalent (Ia-TT, Ib-TT, II-TT, III-TT) vaccines have been tested in humans and mouse and showed promising results (Baker *et al.*, 2003, Paoletti *et al.*, 1994). In order to achieve a 95% population coverage in Europe or

North America, five serotypes would need to be included (Ia, Ib, II, III and V) in a multivalent vaccine (Johri *et al.*, 2006). However, there are regions such as Japan where this combination would not be appropriate due to the different distribution of serotypes (Lachenauer *et al.*, 1999).

GBS proteins were also investigated as potential vaccine candidates to overcome the serotype specificity of the CPS antigen. The surface proteins tested included the C5a peptidase, Lmb, Sip, and LrrG which are present and highly conserved in all the serotypes. Promising results were obtained but further development of these vaccine candidates is uncertain (Johri *et al.*, 2006). Reverse vaccinology strategies have also been used to identify immunogenic surface proteins that could confer protection from different serotypes showing that pilus components could represent good candidates (Maione *et al.*, 2005).

Biological relevance of bacterial capsules

The production of a capsular polysaccharide is a common feature of several pathogenic Gram-positive and -negative bacteria. As mentioned above, the primary function of these molecules is to shield the bacterial surface from interactions with the host immune system and prevent opsonophagocytosis (Hill *et al.*, 1988). The importance of this virulence factor makes the CPS a very good vaccine candidate. *Haemophilus influenza* type b, *Neisseria meningitidis*, *Streptococcus pneumoniae* represent a few examples of encapsulated bacteria for which licensed vaccines have been developed using their capsular polysaccharides as antigens (Pace, 2013).

Isogenic GBS mutants lacking the capsule, or without the terminal sialic acid, were shown to bind greater amounts of C3b, to be more susceptible to killing by human neutrophils and to be less virulent in a neonatal rat model (Wessels *et al.*, 1989, Marques *et al.*, 1992). Despite the importance of this virulence factor, little is known about if and how GBS regulates CPS during the different steps of the pathogenesis. Studies have shown that the presence of the CPS is important for biofilm formation in an *in vitro* model (Xia *et al.*, 2014), however, it is also reported that the CPS interferes with adhesion and invasion of cultured epithelial

and endothelial cells (Hulse *et al.*, 1993, Tamura *et al.*, 1994). These observations suggest that GBS may regulate the expression of the CPS in response to the host environment. The transcriptional regulators RogB and CovR/S of *S. agalactiae* were shown to regulate the transcription of the genes encoding the enzymes responsible for the CPS biosynthesis (Gutekunst *et al.*, 2003, Lamy *et al.*, 2004). Interestingly these two transcription factors regulate other virulence factors, however, the regulation of the CPS by them appears to be strain-specific and it is not clear whether differences in the transcription of the *cps* genes correlate with differences in CPS amount (Rajagopal, 2009). Analysis of GBS transcriptome in different growth conditions has only shown that the transcription of the *cps* genes is reduced when GBS is grown in human blood instead of laboratory medium (Mereghetti *et al.*, 2008). Moreover, differences in doubling time during GBS growth have been observed to correlate with the amount of CPS produced, or more specifically, when GBS grows fast it appears to produce more CPS than when the growth is slower (Ross *et al.*, 1999). It was also observed that when GBS is grown with a short doubling time it invades epithelial cells more efficiently (Malin & Paoletti, 2001).

Similarly to GBS, also other bacteria such as *S. pneumoniae* and *N. meningitidis* present this dual role of the CPS (Yother, 2011, Kugelberg *et al.*, 2008). In *S. pneumoniae* CcpA have been suggested as possible transcriptional regulator coordinating the CPS expression with the bacterial metabolism (Giammarinaro & Paton, 2002), and the function of some proteins of the CPS biosynthesis pathway have been shown to be affected by oxygen levels (Geno *et al.*, 2014). Also in *N. meningitidis* some transcriptional regulators involved in CPS regulation are described (Kugelberg *et al.*, 2008) and other mechanisms such as phase variation and transposon insertion events have been shown to alter the expression of the genes encoding the enzymes for the CPS biosynthesis (Uria *et al.*, 2008). However, the detailed mechanisms used to regulate the expression of this important virulence determinant have yet to be established.

The biosynthesis of microbial polysaccharides

Similar mechanisms are involved in the synthesis of capsular polysaccharides between different bacteria. In particular, the Wzy-, synthase-, and ABC transporter-dependent mechanisms occur in gram-negative bacteria (Whitfield, 2006), whereas only the Wzy- and synthase-dependent mechanisms are described in gram-positive bacteria (Yother, 2011). All these mechanisms are described in detail in two excellent reviews (Whitfield, 2006, Yother, 2011), but in this work we will focus only on the Wzy mechanisms which is the one used by GBS.

The Wzy pathway is typical of polysaccharides with multiple different sugars and glycosidic linkages. This mechanism has been characterized in detail and described both for the CPS of *S. pneumoniae* (Yother, 2011) and for group 1 and 4 capsules of *E. coli* (Whitfield, 2006). Figure 6 illustrates the Wzy pathway of *S. pneumoniae* which represents the prototype for Gram-positive bacteria. Briefly, the portal enzyme CpsE (WchA homology group) catalyzes the transfer of a sugar-1-P from a UDP-sugar to an undecaprenyl-phosphate lipid in the membrane. Then the basic repeating units of the CPS are synthesized at the cytosolic side of the membrane by processing other nucleotide diphospho-sugars and attaching them to the lipid anchored moiety. This is accomplished by the sequential action of glycosyltransferases. The units anchored to the membrane lipids are transferred to the outer side of the membrane by the flippase (Wzx homolog), where the polymerase (Wzy homolog) is responsible for the polymerization of the units into the full-length polysaccharide. The polysaccharide is then released from the undecaprenyl-phosphate and shed or covalently attached to the cell wall peptidoglycan by an unknown enzyme. The free undecaprenyl-phosphate is flipped back to the inside of the membrane and reused to synthesize another repeating unit (Yother, 2011). In Gram-negative bacteria a complex spanning the periplasmic space and the outer membrane is responsible for the transfer of the synthesized polysaccharide to the external surface of the bacteria (Cuthbertson *et al.*, 2009).

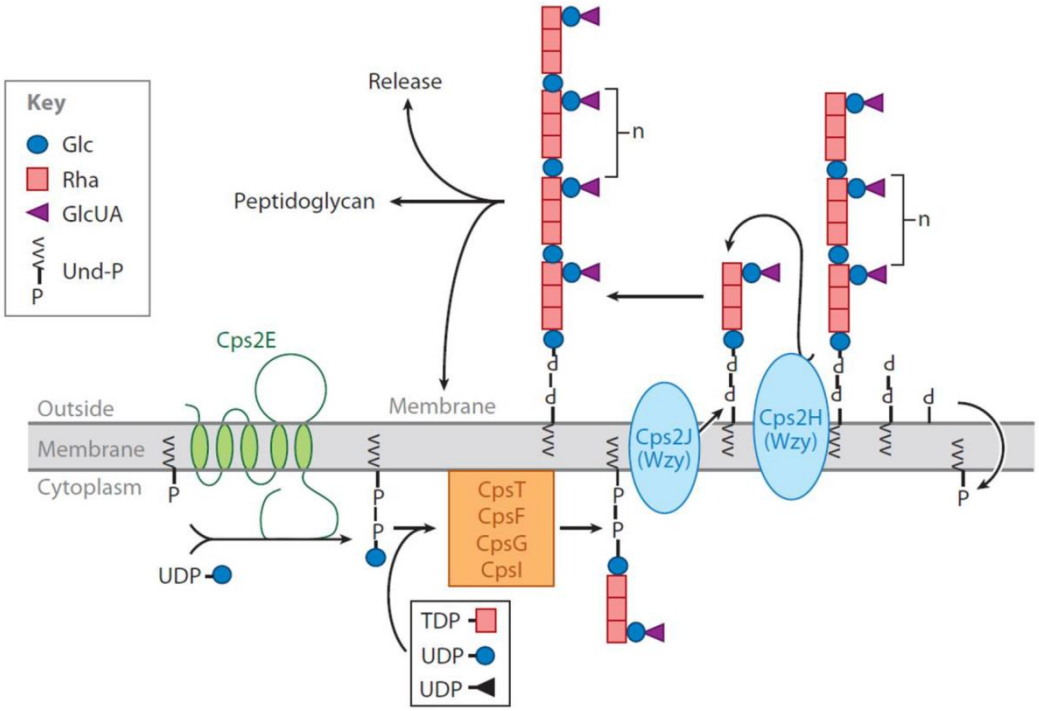

FIGURE 6. **The Wzy CPS biosynthesis pathway of *S. pneumoniae*.** Glc, glucose; Rha, rhamnose; GlcUA, glucuronic acid; Und-P, undecaprenyl-phosphate; NDP-, nucleotide diphospho-. Adapted from (Yother, 2011).

The *cps* operon

The genetic loci for the Wzy-dependent polysaccharide biosynthesis are similar in all the bacteria. The Wzy polymerase and Wzx flippase are the defining enzymes of this pathway and their presence in genetic loci is generally predicted by their putative membrane topologies (Yother, 2011). These two genes are usually quite conserved and are generally flanked by genes encoding enzymes unique to specific capsular serotypes in the different bacteria (Cieslewicz *et al.*, 2005, Yother, 2011). In *S. pneumonia*, for example, the enzymes of the Wzy-dependent polysaccharide biosynthesis pathway are found in the *cps* operon. This operon presents a 5' region encoding for proteins conserved among serotypes, a central region containing the genes for the polymerase, the flippase and the other serotype specific glycosyltransferases, and a 3' variable region encoding enzymes responsible for the biosynthesis of specific sugar moieties or for their chemical modifications (Bentley *et al.*, 2006, Yother, 2011).

The *cps* locus of GBS was initially identified by Rubens and coworkers in a serotype III GBS strain by the use of a transposon mutant library (Rubens *et al.*, 1993). This locus has a general organization similar to the *cps* locus of *S. pneumoniae.* It is approximately 18 kb long and it is composed of 16-18 genes depending on the serotype (Cieslewicz *et al.*, 2005). The locus is an operon transcribed in a single transcript starting from upstream the first gene (Yamamoto *et al.*, 1999). The *cps* operon can be divided into three main regions (Cieslewicz *et al.*, 2005). The central part of the operon (*cpsE-L*) determines the capsule serotype and comprises genes encoding for the glycosyltransferases, the polymerase (*cpsH*) and the flippase (*cpsL*). The last four genes of the operon (*neuA-neuD*) encode enzymes that synthesize the sialic acid. Finally, the first genes of the operon (*cpsA-D*) are not directly involved in the biosynthesis of the CPS repeating units (Cieslewicz *et al.*, 2001) (Fig. 7).

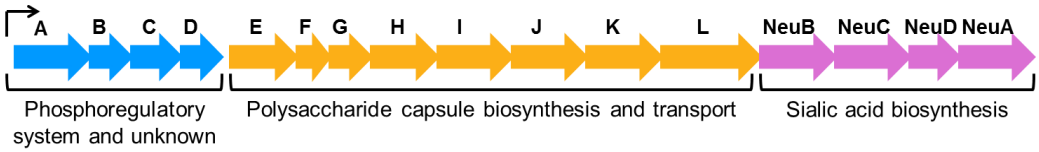

FIGURE 7. **GBS *cps* operon.** General organization of the cps operon of GBS. The example is from serotype Ia GBS.

CpsABCD of *S. agalactiae*

The functions of the enzymes encoded by the central and the last portions of the *cps* operon have been identified experimentally or by homology (Yamamoto *et al.*, 1999, Cieslewicz *et al.*, 2005). They are all involved in the biosynthesis of the sialic acid, in the assembly and transport of the repeating units of the CPS and in their polymerization into the full-length polysaccharide. However, the function of the proteins encoded by the first four genes of the *cps* operon is still not clear. The genes *cpsA-D* are conserved among all the GBS capsule serotypes (>97% aa identity) and have orthologues in other encapsulated streptococci such as *S. pneumoniae* (Yamamoto *et al.*, 1999). Their function in

GBS has been previously investigated by Cieslewicz and coworkers through the construction and characterization of knockout mutants. All the mutants retained the ability to produce the CPS although a clear reduction in the total amount of polysaccharide was observed for all the strains. A reduction in *cps* operon transcription was shown in the Δ*cpsA* mutant suggesting that CpsA may be required for transcription of the *cps* operon. Whereas, for CpsC and CpsD a more undefined role in polymerization/export of CPS have been was suggested (Cieslewicz *et al.*, 2001). Lately, Hanson and coworkers showed that the recombinant CpsA bound the *cps* operon promoter *in vitro*, suggesting that this protein may be a transcription regulator (Hanson *et al.*, 2012).

CpsABCD homologues in other bacteria

Orthologues of the *cpsABCD* genes are found in several Gram-positive bacteria utilizing the Wzy pathway. In Gram-negative bacteria *cpsA* and *cpsB* orthologues are not present, while orthologues to CpsC and CpsD are readily identified. Interestingly, in Gram-negative bacteria CpsC and CpsD are found as the single multi-domain protein Wzc (Olivares-Illana *et al.*, 2008, Whitfield & Paiment, 2003).

CpsA is a 485 aa membrane protein with a major extracellular portion (Hanson *et al.*, 2011). This protein belongs to the LytR-CpsA-Psr (LCP) protein family, together with another two paralogues commonly found in Gram-positive bacteria. This family of proteins is suggested to be involved in the final steps of cell wall assembly (Hubscher *et al.*, 2008) and the function of these proteins seems to partially overlap (Eberhardt *et al.*, 2012). The extracellular domains of the CpsA homologues of *S. pneumoniae* and *B. subtilis* have recently been crystallized and were proposed to be responsible for hydrolysis of the pyrophosphate linkage between the CPS and the undecaprenyl-phosphate anchor (Kawai *et al.*, 2011), and subsequent attachment of CPS to the peptidoglycan (Eberhardt *et al.*, 2012). Interestingly, these functions appear different from those suggested for CpsA in GBS.

Concerning CpsBCD, orthologous proteins in *S. pneumoniae* (46-64% aa identity) were described to constitute a phosphoregulatory system which has been studied in some detail. The CpsD orthologue was shown to be a cytoplasmic autokinase that is trans- and cis-phosphorylating four tyrosines found in its C-terminal tail (Bender & Yother, 2001). The CpsC orthologue is a membrane protein with an uncharacterized

extracellular domain and an intracellular tail responsible for interaction with CpsD and consequent retention of the protein close to the membrane (Bender & Yother, 2001). Finally, the CpsB orthologue is the phosphatase of the system, and responsible for CpsD dephosphorylation (Bender & Yother, 2001, Hagelueken *et al.*, 2009). Similarly, the Wzc protein of *E. coli* is composed of a membrane domain and a cytoplasmic autokinase domain (Whitfield & Paiment, 2003). Also in *E. coli* a phosphatase Wzb responsible for Wzc dephosporylation was found to be encoded in the *wzy* locus (Hagelueken *et al.*, 2009).

Despite that functional roles for these proteins have already been described, it has not been elucidated if/how this system is involved in CPS biosynthesis. *S. pneumoniae* strains with deletions in *cpsB* suggested ambiguous consequences of CpsD phosphorylation, with some studies reporting a reduced CPS production correlated with the increased phosphorylation (Morona *et al.*, 2000) and others instead showing an increase in the amount of CPS produced (Bender *et al.*, 2003). In *E. coli* Wzc oligomerizes in the inner membrane and interacts with the Wza oligomer spanning the outer membrane. In this complex, the cyclical phosphorylation of Wzc is suggested to regulate the processes of CPS polymerization and transport through the periplasmic space and outer membrane (Collins *et al.*, 2007). In Gram-positive bacteria, CpsD cyclical phosphorylation is instead suggested to be responsible for regulating the two processes of CPS synthesis and attachment to the cell wall peptidoglycan (Kadioglu *et al*., 2008) but the mechanism of action has not been clearly established so far.

AIM OF THE THESIS

The capsular polysaccharide is the main virulence factor of GBS and is a promising antigen selected for the development of a vaccine to fight this pathogen. The chemical structure and the biological function of the CPS have been investigated in some detail, however very little is known about the biosynthesis of this molecule and about the regulation of this process.

The aim of this work is to investigate the role of the CpsABCD proteins encoded by the first four genes of the *cps* operon. These proteins are conserved between the different GBS serotypes and among bacteria synthesizing the CPS using the Wzy-pathway, but they are not predicted to be involved in the biosynthesis of the repeating units of sugars and in their polymerization.

Experimental studies investigating the role of CpsABCD in GBS are limited, and present potential discrepancies compared to *S. pneumoniae* and other related species. In GBS CpsA is suggested to be a transcriptional regulator of the *cps* operon, and CpsBCD are suggested to have a role in determining the characteristics of the CPS polymer. By homology, these three proteins are suggested to be members of a phosphoregulatory system but this putative function was never directly investigated in GBS. Moreover the specific role of this system in CPS biosynthesis has not been elucidated in any Gram-positive bacteria.

To investigate the role of these proteins we developed a panel of knockout and functional mutant strains, and analyzed the effects on *cps* operon transcription, CPS quantity, size, and attachment to the cell surface, as well as CpsD phosphorylation. *In vivo* molecular interactions between the CpsABCD proteins were also studied. The resulting data provided novel insights on the role of each individual protein, as well as their interdependencies, and showed that these proteins are responsible for balancing the processes of polymerization and attachment to the cell wall of the CPS.

Moreover we took advantage of the differences in CPS phenotypes of selected mutant strains to investigate the biological impact of the CPS in the interaction with epithelial cells.

EXPERIMENTAL PROCEDURES

Bioinformatic analysis

The aminoacid sequences of CpsA, CpsB, CpsC and CpsD of GBS 515 (serotype Ia) were downloaded from the GeneBank Database (accession numbers EAO72243, EAO72192, EAO72213 and EAO72226). The protein sequences were analyzed using the online tool Pfam (http://pfam.xfam.org/ (Finn *et al.*, 2014)) to identify conserved domains. The predicted subcellular localization of the proteins was predicted using the online tool PSORTb 3.0.2 (http://www.psort.org/psortb/ (Yu *et al.*, 2010)). Subsequently the online tool Octopus (http://octopus.cbr.su.se/ (Viklund & Elofsson, 2008)) was used to predict the membrane topology of the predicted membrane proteins. Alignments with homologous proteins of *S. pneumoniae* and *S. aureus* were performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/ (Sievers *et al.*, 2011)).

Bacterial strains and growth conditions.

GBS strains 515 and 515Δ*cpsE* were provided from Dr. Dennis Kasper (Harvard Medical School, Boston, MA, USA) and Dr Michael Cieslewicz (Cieslewicz *et al.*, 2001) respectively. GBS strains were grown in Todd-Hewitt broth (THB) at 37°C, 5% CO_2. Tryptic soy broth, 15 g/L agar (TSA) was used as solid medium. Strains were stored at -80°C in THB medium, 15% glycerol. MAX Efficiency® DH5α™ Competent Cells (Life Technologies) and chemically competent HK100 *E.coli* cells were prepared in-house, and used for transformation, propagation, and preparation of plasmids. Chemically competent BL21 and BTH101 *E.coli* cells were prepared in-house, and used for transformation, protein expression and for the Bacterial two Hybrid assay. *E. coli* was grown at 37°C with agitation (180 rpm) in Luria-Bertani broth (LB), or on 15 g/L agar plates (LBA). Erythromycin (Erm) was used for selection of GBS (1 µg/ml) or *E. coli* (100 µg/ml) containing the pJRS233-derived plasmids (Perez-Casal *et al.*, 1993) used for mutagenesis. Kanamycin (Kan) was used for selection of *E. coli* (50 µg/ml) containing the pET24b-derived plasmids (Novagen, South Africa) and the pKT25-derived plasmids (Euromedex, France). Ampicillin (Amp)

was used for selection of *E. coli* (100 µg/ml) containing the pUT18C-derived plasmids (Euromedex, France) and the pET15-derived plasmids (Novagen, Germany).

Construction of GBS mutant strains

To prepare each mutant strain, the shuttle vector pJRS233 (Perez-Casal *et al.*, 1993) containing the gene locus with an in-frame deletion or a codon substitution was constructed. Mutant strains obtained are described in Table 2, and primers used for the development of constructs are listed in Table 1. Constructs for genes with codon substitutions were prepared using a splicing by overlap extension PCR (SOEing-PCR) strategy (Horton *et al.*, 1989). Briefly, amplicons up- and downstream of the codon substitution were amplified from GBS 515 gDNA using the PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies). Internal primers used to amplify the two parts of the genes have 15 bp overlapping tails and introduce the codon substitution, and amplicons are then joined together by SOEing-PCR. The resulting fragment was ligated into pJRS233 using BamHI and XhoI restriction sites.

Constructs for genes with in-frame deletions were prepared using the Polymerase Incomplete Primer Extension (PIPE) method (Olsen & Eckstein, 1989). Briefly, the gene and 900-1,000 bp up- and downstream of the coding sequence were amplified from GBS 515 gDNA and cloned into pET24b using NotI and XhoI (*cpsA* inserts) or BamHI and XhoI (*cpsB-C-D* inserts) restriction sites. In-frame deletions were developed by amplifying the plasmid using primers with 15 bp overlapping tails annealing at the two sides of the region to delete. Linear plasmids were transformed into HK100 competent cells able to re-circularize the plasmid. Following propagation and purification of the plasmid, the inserts containing the in-frame deletions were transferred into pJRS233 plasmid by restriction digestion, ligation, and transformation of *E. coli* DH5α (Life Technology).

Constructs for chromosomal complementation were prepared by cloning the respective wt loci into pJRS233.The various pJRS233 constructs were used for insertion/duplication and excision mutagenesis (Fig. 8) (Perez-Casal *et al.*, 1993,

Cieslewicz *et al.*, 2001). Briefly, pJRS233-derived plasmids purified from *E. coli* were used to transform electrocompetent GBS 515 cells by electroporation (Framson *et al.*, 1997). Transformants were selected by growth on TSA + Erm at 30°C for 48 h. Integration was performed by growth of transformants at 37°C (non-permissive temperature for the suicide shuttle vector) with Erm selection. Excision of the integrated plasmid was performed by serial passages in THB at 30°C, and parallel screening for Erm-sensitive colonies on plate. Mutants were verified by PCR sequencing of the loci.

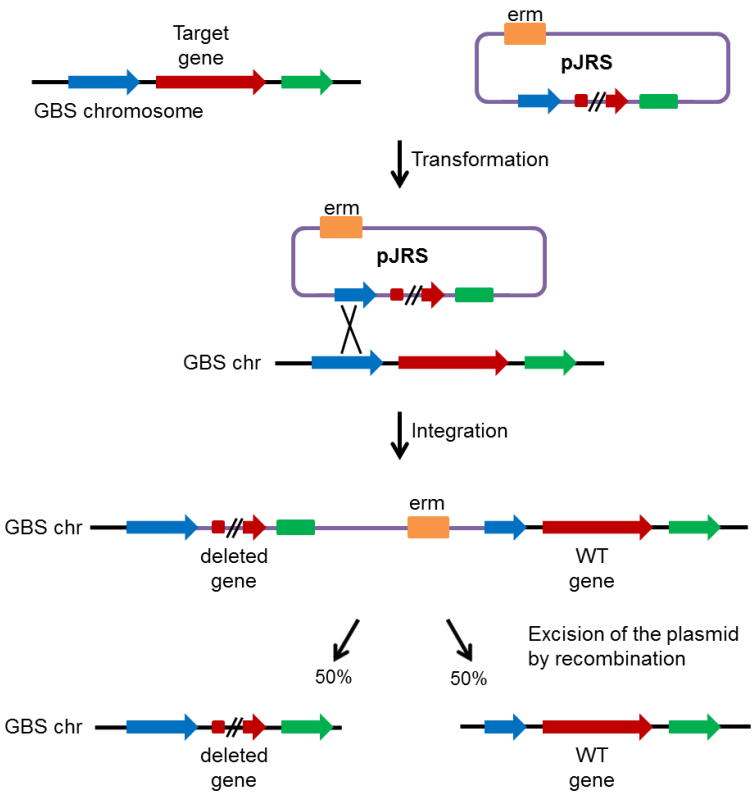

FIGURE 8. **Mutagenesis strategy used to develop GBS mutant strains.** Cartoon representing the insertion/duplication and excision mutagenesis strategy commonly used to develop GBS mutant strains.

Growth curves

Bacteria were inoculated 1:50 from O/N cultures into 200 µl of fresh THB and grown on plate at 37°C. Each strain was inoculated into five independent

wells. OD_{600} was monitored every 20 min for 400 min using a plate reader (TECAN, Switzerland). Growth curves have been designed by plotting the mean of the ODs measured at each time point for the five biological replicates.

qRT-PCR analysis

RNA extracts were prepared as described (Faralla *et al.*, 2014). Briefly, bacteria were harvested at two time points, at OD_{600}=0.4 (log phase) and OD_{600}=1.7 (early stationary phase). To rapidly arrest transcription, 10 ml of bacteria were cooled on ice and added to 10 ml of frozen THB medium in a 50 ml conical tube. GBS cells were then collected by centrifugation for 15 min at 3,220 *g*, 4°C, and resuspended in 800 µl of TRIzol (Life Technologies). Bacteria were disrupted mechanically by agitation with Lysing matrix B in 2 ml tubes (MP Biomedicals, Santa Ana, CA) using a Fastprep-24 homogenizer (MP Biomedicals, Santa Ana, CA) for 60 s at 6.5 m/s for two cycles, and kept on ice for 2 min between the cycles. Samples were then centrifuged for 5 min at 8,000 *g*, 4°C and RNA was extracted with Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. RNA samples were treated with DNase (Roche) for 2 h at 37°C and further purified using the RNA MiniPrep kit (Qiagen), including a second DNase treatment on the column for 30 min at room temperature (RT), according to the manufacturer's instructions. cDNA was prepared using the Reverse Transcription System (Promega) by using 500 ng of RNA per reaction. Real time quantitative PCR (qRT-PCR) was performed on 50 ng of cDNA that was amplified using LightCycler® 480 DNA SYBR Green I Master (Roche). Reactions were monitored using a LightCycler® 480 instrument and software (Roche). Three technical replicates were monitored for each strain/condition analyzed. To quantify *cps* operon transcription level, primers annealing on *cpsA* and *cpsE* were used for all the strains with the exception of *cpsA* mutants where primers for *cpsD* and *cpsE* were used. The transcript amounts in each condition were standardized to an internal control gene (*gyrA*) and compared with standardized expression in the wild-type (wt) strain ($\Delta\Delta C_T$ method). The primers used are listed in Table 1.

Production of α-CpsA, α-CpsB and α-CpsD mouse sera

The *cpsB* and the *cpsD* genes were amplified from GBS 515 gDNA. For *cpsA* only the portion of the gene codifying for the extracellular part of CpsA was amplified. Primers used to amplify the genes are listed in the Table 1. The *cpsA* and *cpsD* inserts were cloned by PIPE method (Olsen & Eckstein, 1989) into a modified pET-15 vector (Novagen), enabling the expression of the protein with an N-terminal 6xHis-tag followed by a cleavage site for the TEV (tobacco etch virus) protease. The *cpsB* insert was cloned by restriction enzymes digestion (NdeI and XhoI) into the pET24b vector (Novagen), enabling the expression of the protein with a C-terminal 6xHis-tag. Plasmids were propagated in *E. coli* DH5α. Subsequently, the plasmids were transformed into *E. coli* BL21(DE3) cells (Novagen) where the expression of the 6xHis-tagged fusion proteins were induced according to the manufacturer's instructions. The bacterial pellet was resuspended in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 10 mM imidazole, and was lysed by sonication. Extracts were pelleted and the supernatant was purified using a FF-Crude His-Trap HP nickel chelating column (GE Healthcare, Little Chalfont, United Kingdom). The recombinant proteins were eluted with 300 mM imidazole, and the buffer was exchanged to PBS using an Amicon Ultra 3K centrifugal filter (Millipore, Cork, Ireland). Antisera specific for CpsA, CpsB and CpsD were produced by immunizing (prime and two boost) 8 CD1 mice with 20 µg of purified recombinant protein formulated with 400 µg of Alum.

Protein extracts

Bacteria were grown in 30 ml THB at 37°C until exponential growth phase was reached (OD_{600} = 0.4). Cells were pelleted, washed in PBS and resuspended in 800 µl of Tris-HCl 50 mM pH 7.5 with cOmplete Protease Inhibitor and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche), transferred into Lysing Matrix B 2 ml Tubes (MP Biomedicals, Santa Ana, CA) and lysed using the FastPrep-24™ Automated Homogenizer (6 cycles at 6.5 m/s for 30 s). Tubes were centrifuged 500 *g* for 5 s, the supernatant was collected and further centrifuged at max speed for 15 min at 4°C to separate the soluble fraction (supernatant) from the total fraction (pellet).

Flow cytometry

Flow cytometry using α-CPSIa mAb was performed as described elsewhere (Berti *et al.*, 2014) with minor differences. Briefly, bacteria were grown overnight on TSA plates at 37°C, harvested using a sterile loop and diluted in PBS to OD_{600} = 0.3. The bacterial suspension (400 µL) was centrifuged at 8,000 *g*, resuspended in 200 µl of heat-inactivated fetal calf serum and incubated for 20 min at RT with shaking. Bacteria were diluted 1:10 in PBST, 0.1% BSA with 1:10,000 diluted α-CPSIa and incubated for 1 h at 4°C. Samples were washed twice in PBST, resuspended in goat anti-mouse allophycocyanin (APC)-conjugated $F(ab')_2$ fragment IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:200 in PBST, and incubated for 30 min at 4°C. Bacteria were washed twice in PBS, fixed in PBS, 2% paraformaldehyde for 20 min at RT, centrifuged and resuspended in 150 µl PBS. All data were collected using a FACS CANTO II (BD) by acquiring 10,000 events, and data analysis was performed with Flow-Jo software (v.8.6, TreeStar Inc., Ashland, OR).

Quantification of the capsular polysaccharide attached to the cell surface

Alkaline extraction of CPS from GBS bacteria was performed as previously described (Wessels *et al.*, 1990). Briefly, bacteria were grown in 50 ml THB at 37°C for 8 h (stationary phase). Viable counts were performed and confirmed that CFU numbers between the strains were comparable. GBS cells were collected by centrifugation for 15 min at 3,220 *g* at 4°C, resuspended in 1.1 ml of PBS, 0.8 N NaOH and incubated at 37°C for 36 h. Samples were neutralized by addition of HCl and pelleted by centrifugation for 10 min at 10,000 *g*, 4°C. 850 µl of the supernatant were diluted in 7.15 ml of water, and centrifuged for 10 min at 3,220 *g* at 4°C. 7.2 ml of the supernatant were loaded on a Vivaspin 10 tube (Sigma) and centrifuged at 3,220 *g* until most of the solution passed through the membrane. After two washes with 1 ml dH_2O, the CPS extract was recovered from the membrane by resuspension in 1.6 ml of water. The amount of CPS present in the extract was estimated by measuring the sialic acid content using the colorimetric resorcinol-hydrochloric acid method (Svennerholm, 1957). Briefly, 120 µl of extract were mixed with 380 µl of water and 500 µl of resorcinol

solution (0.2% resorcinol, 0.3 mM copper sulfate, 30% (v/v) HCl). Samples were boiled for 20 min, cooled to room temperature and absorbance was measured at 564 nm. The sialic acid content of the samples was then determined by comparison with a concomitantly prepared standard curve using serial dilutions of purified sialic acid.

Quantification of CPS in the growth medium

Bacteria were grown in 10 ml THB at 37°C for 8 h. GBS cells were pelleted by centrifugation for 15 min at 3,220 *g* at 4°C, and the growth medium was collected and filtered using a 0.22 µm Nalgene Syringe Filter (Thermo Scientific). The amount of capsular polysaccharide released in the growth medium was estimated by dot blot. Serial dilutions (1:2) were prepared in PBS. Two µl of each serial dilution were spotted onto a nitrocellulose membrane. The membrane was dried for 20 min and blocked by soaking in 5% skim milk in PBS, Tween-20 0.05% (PBST). Detection by immunoblotting was performed as described below (immunoblotting experiments).

Cell wall extracts

Bacteria were grown in 10 ml THB at 37°C for 8 h. GBS cells were pelleted by centrifugation for 15 min at 3,220 *g* at 4°C and washed in PBS. Extracts of CPS attached to the peptidoglycan were prepared by incubating the bacterial pellet with 200 U of mutanolysin (Sigma) diluted in 50 µl of protoplasting buffer (0.1 M potassium phosphate, 40% sucrose, 10 mM $MgCl_2$) for 1 h at 37°C. 20 µl of Proteinase K solution (Life Technologies) were added, and samples were incubated at 56°C for 30 min. After centrifugation for 5 min at 10,000 *g* at 4°C the supernatant was collected.

Immunoblotting experiments

CPS or protein extracts were separated by electrophoresis on NuPage 4-12% Bis-Tris gels (Life Technologies) according to the manufacturer's instructions. Western blot was performed using the iBlot® Blotting System (Life Technologies) according to the manufacturer's instructions. Nitrocellulose

membranes were blocked by soaking in 5% (w/v) skim milk in PBST with the exception of membranes probed with the α-P-Tyr mAb, which were blocked in 3% (w/v) BSA in PBST. Primary mouse α-CPSIa mAb (30E9/B11), were obtained by immunization with Ia glycoconjugate. Mouse α-P-Tyr mAb (Sigma, clone PT-66), mouse α-RNA polymerase β mAb (Thermo Scientific, clone 8RB13) and mouse α-CpsA, α-CpsB and α-CpsD polyclonal sera were also used as primary antibodies. All the primary antibodies and sera were diluted 1:2,000 in 1% (w/v) BSA in PBST and membranes were incubated for 1 h at RT. After three 5 min washes in PBST, membranes were incubated in 1:15000 of secondary goat anti-mouse antibody conjugated to horseradish peroxidase. Detection was performed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer's instructions.

Immunogold labelling and electron microscopy

Immunogold electron microscopy of the GBS CPS in wt and mutant strains was performed as previously described (Barocchi *et al.*, 2006). Briefly, bacteria were grown in THB until exponential growth phase was reached (OD_{600} = 0.4). Cells were pelleted, washed in PBS and fixed in PBS, 2% paraformaldehyde for 20 min at RT. Twenty µl of sample were added to 200-square mesh formvar copper grids coated with a thin carbon film (Ted Pella, Redding, CA) and incubated at RT for 5 min. The excess of solution was blotted by Whatman filter paper. The grids were than incubated for 1 hin blocking buffer (1% normal rabbit serum, 1% BSA, 1× PBS), and subsequently incubated with 1:1,000 α-CPSIa mAb in blocking buffer. Samples were washed five times for 5 min in blocking buffer and incubated with secondary gold-conjugated antibodies at 1:40 (goat anti-mouse IgG, 10 nm (Agar Scientific, UK)). Samples were washed in distilled water five times for 5 min each and blotted. Grids were stained for 45 sec with aqueous 1% uranyl acetate pH 4.5, blotted, air dried at RT, and finally observed in a FEI Tecnai G2 spirit operating at voltage of 80 kV and at a magnification of 87,000. Images were collected with a CCD Olympus.SIS Morada 2K*4K*.

Purification of capsular polysaccharide from bacterial pellets and spent media

The 515 strain and the Δ*cpsA*, CpsC(Δext) and CpsD(K49A) mutant strains were grown in 1 liter THB at 37°C for 8 h. The pellet and the medium obtained from the cultures were separated by centrifugation for 30 min at 8,600 g at 4°C. The purification process was based on previously described procedures (Wessels *et al.*, 1990). Briefly, pellets were washed in PBS and successively inactivated by incubation with PBS, 0.8 N NaOH at 37°C for 36 h. After centrifugation at 4,000 rpm for 20 min, 1 M Tris buffer (1:9, v/v) was added to the supernatant and diluted with 1:1 (v/v) HCl to reach a neutral pH. Spent growth media were inactivated by filtration 0.22 µm. For both bacterial pellets and media the same purification process was applied. Briefly, 2 M $CaCl_2$ (0.1 M final concentration) and ethanol (30% v/v final concentration) were added to the solution. After centrifugation at 4,000 *g* for 20 min, the supernatants were subjected to a tangential flow filtration on a 30,000-molecular weight cutoff (Hydrosart Sartorius, 50 cm^2 surface) against 16 volumes of 50 mM TRIS, 500 mM NaCl, pH 8.8, and 8 volumes of 10 mM sodium phosphate, pH 7.2. Then, the samples were loaded in a preparative size exclusion column (Sephacryl S500 column, GE Healthcare) by using 10 mM sodium phosphate, 500 mM NaCl pH 7.2 as eluent buffer. The CPS samples were subjected to full N-acetylation. After complete drying, samples were solubilized in 300 mM Na_2CO_3/300 mM NaCl pH 8.8. A 1:1 diluted solution of 4.15 µL/mL acetic anhydride in ethanol was added, and the reaction was incubated at room temperature for 2 h. Samples were then purified in a preparative size exclusion column (Sephadex G15 column, GE Healthcare) by using MilliQ water. The polysaccharide content was determined using the colorimetric resorcinol-hydrochloric acid assay (Svennerholm, 1957). The purity of the polysaccharide preparation was assessed by colorimetric assays, which indicated a content of residual proteins below 3% (w/w) and nucleic acids below 1% (w/w). Endotoxin content was <30 endotoxin units/µg of saccharide, measured by the *Limulus* amebocyte lysate (LAL) test.

NMR Spectroscopy

^{1}H NMR experiments were recorded by a Bruker Avance III 400 spectrometer, equipped with a high precision temperature controller, and using 5-mm broadband probe (Bruker). TopSpin software (v.3.2, Bruker) was used for data acquisition and processing. ^{1}H NMR spectra were collected at 25 +/- 0.1°C with 32,000 data points over a 10 ppm spectral width, accumulating an appropriate number of scans for high signal/noise ratio. The spectra were weighted with 0.2 Hz line broadening and Fourier-transformed. The transmitter was set at the water frequency which was used as the reference signal (4.79 ppm). All monodimensional proton NMR spectra were obtained in a quantitative manner using a total recycle time to ensure a full recovery of each signal (5 x Longitudinal Relaxation Time T_1).

HPLC-SEC

CPS samples were eluted on a TSK gel 6000PW (30 cm × 7.5 mm) column (particle size, 17 μm; Sigma 8-05765) with TSK gel PWH guard column (7.5 mm ID × 7.5 cm L; particle size, 13 μm; Sigma 8-06732) (Tosoh Bioscience) and calibrated with a series of defined pullulan standards (Polymer) of average molecular weights ranging from 20,000 to 1,330,000 Da. Void and bed volume calibration was performed with λ-DNA (λ-DNA Molecular Weight Marker III, 0.12–21.2 kbp; Roche) and sodium azide (NaN_3) (Merck), respectively. The mobile phase was 10 mM sodium phosphate pH 7.2, at a flow rate of 0.5 mL/min (isocratic method for 50 min). The polysaccharide samples were analyzed at a concentration of 0.2-0.4 mg/mL, using 10 mM sodium phosphate buffer pH 7.2 as mobile phase, at a flow rate of 0.5 mL/min.

Bacterial two-hybrid

A bacterial two-hybrid assay (BACTH) was employed to test potential interactions between CpsA, CpsC and CpsD (Karimova *et al.*, 1998). CpsC, CpsD and CpsA coding sequences were amplified using the primers described in Table 1 and cloned into pUT18C and pKT25 plasmids (Euromedex, France) at the C-terminal of the domains T18 and T25 of the adenylate cyclase of *Bordetella*

pertussis. CpsC was cloned both in a full length version and with the C-terminal 33-aa tail deleted. The nucleotide sequences of the modified regions of the plasmids were confirmed by sequencing. Interactions between proteins were tested by introducing the plasmids into the adenylate cyclase-deficient *Escherichia coli* strain BTH101 (Euromedex, France). Empty plasmids were tested together with all the fusion proteins as negative control. Positive control plasmids pKT25-zip and pUT18C-zip were provided by the manufacturer (Karimova *et al.*, 1998). Colonies containing both plasmids were selected by plating on LB + Kan 50 µg/ml + Amp 100 µg/ml agar plates and growing them overnight (O/N) at 37°C. Four colonies were selected for each transformation and independently inoculated into 1 ml of LB, 50 µg/ml Kan, 100 µg/ml Amp, 1 mM IPTG and grown O/N at 30°C. Two µl from each culture spotted onto LB agar plates with additives as seen above and 80 µg/ml X-gal. After incubation O/N at 30°C, the plates were examined for the formation of blue colonies, indicative of a protein-protein interaction.

Biofilm formation assay on polystyrene plates

The biofilm formation assay was performed as described (Rinaudo *et al.*, 2010). GBS strains grown to stationary phase in THB, 1% glucose were diluted 1:50 in fresh medium and 100 µl cultures were inoculated into a polystyrene flat-bottom 96-well plate (Costar). Plates were incubated without shaking at 37°C for 18 h aerobically in 5% CO_2. Media, including any unattached bacteria, were decanted from the wells. Wells were washed with PBS and subsequently air-dried. Adherent bacteria were stained for 10 min with a 0.5% (w/vol) solution of Crystal Violet. After rinsing three times with PBS, bound dye was released from stained cells using 30% glacial acetic acid. This allowed indirect measurement of biofilms formed on both the bottom and sides of the well. Biofilm formation was quantified by measuring absorbance of the solution at 545 nm with a microplate reader (Tecan, Switzerland). The assay was run in five replicates for three times.

***In vitro* adhesion/invasion assay**

An *in vitro* adhesion/invasion assay was performed as described (Korir *et*

al., 2014). The A549 cell line (ATCC CCL-185), a human alveolar epithelial carcinoma cell line, was maintained by incubation at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) and 0,2% Primocin (InvivoGen, France). Passages from 12-18 were used for the assay. The day before the experiment cells were trypsinized, resuspended in infection medium (DMEM, 10% FBS and no antibiotics), plated on a 24-well plate ($2x10^5$ cells/well) and incubated for 24 h at 37°C with 5% CO_2. GBS strains were grown in THB to exponential phase, washed once with PBS and resuspended in infection medium. Prior to infection, host cells were washed with PBS. Then, they were infected with bacteria using a multiplicity of infection (MOI) of one bacterial cell per host cell. After 2 h of incubation at 37°C with 5% CO_2, wells were washed five times with PBS to remove non-adherent bacteria. To determine the number of associated bacteria (attached and invaded), host cells were lysed with cold PBS, 1% saponin (Sigma) for 10 min. Lysates were subjected to vortex mixing and plated on TSA plates after serial dilutions in PBS. Plates have been incubated overnight at 37°C, and CFU were counted. To test invasion, infection medium containing 100 µg/ml of gentamicin (Gibco) and 5 µg/ml of penicillin G (Sigma) was added to each well and incubated at 37°C for another 2 h to kill extracellular bacteria. Wells were then washed five times with PBS, and intracellular bacteria were enumerated as described above. All data were expressed as percentages of the total number of bacteria per well after the 2-h infection. The ratio between invaded and associated bacteria was also calculated. Assays were run in triplicate at least three times.

TABLE 1. **Oligonucleotides.** Restriction sites are marked in bold, overlapping regions used for mutagenesis are underlined, nucleotidesubstitutions resulting in amino acid substitutions are marked in bold and underlined. For, forward; Rev, reverse; Ampl., amplification.

Name	Sequence	Description
NotA5F	TAAA**GCGGCCGC**CTCTATCACTGACAACAATGG	Ampl. of *cpsA* + flanking regions, For, 894 bp upstream *cpsA* start, NotI
XhA3R	TATC**CTCGAG**GAAGAAGTATATTGTGGCGTA	Ampl. of *cpsA* + flanking regions, Rev, 916 bp downstream *cpsA* end, XhoI
KOA3F	TCGCGCCGTCAACAAAAGAACACAATGGAGGAATAAC	*ΔcpsA* mutagenesis, For, overlap KOA5R
KOA5R	TTGTTGACGGCGCGAATGATTAGACATTGTAA	*ΔcpsA* mutagenesis, Rev, overlap KOA3F
M1A3F	ACTACTTTATATGGATAACAAGAATGATTGATATTCATTC	CpsA(Δext) mutagenesis, For, overlap M1A5R
M1A5R	TCCATATAAAGTAGTAGCAACGAAAATAGAAGC	CpsA(Δext) mutagenesis, Rev, overlap M1A3F
M2A3F	TCTATATTAGCGGTTAACAAGAATGATTGATATTCATTCTC	CpsA(ΔLyt-R) mutagenesis, For, overlap M2A5R
M2A5R	ACCGCTAATATAGATATTAAATACCCCTTCTTTATG	CpsA(ΔLyt-R) mutagenesis, Rev, overlap M2A3F
BaB5F	TAAA**GGATCC**TTATGTTAGCTTAATTGAACTTAGCA	Ampl. of *cpsB* + flanking regions, For, 904 bp upstream *cpsB* start, BamHI
XhB3R	AAAG**CTCGAG**GACATAACAGAGTTCCTAGTA	Ampl. of *cpsB* + flanking regions, Rev, 960 bp downstream *cpsB* end, XhoI
KOB3F	ATTCATTCTCATATCCATTACATTTAGGAGATTTCATGAA	*ΔcpsB* mutagenesis, For, overlap KOB5R
KOB5R	GATATGAGAATGAATATCAATCATTCTTGTTATTCCTC	*ΔcpsB* mutagenesis, Rev, overlap KOB3F
M1BF	GTTGCGCATATAGAG**GC**GTATAACGCTTTAGA	CpsB(R139A) mutagenesis, For, overlap M1BR
M1BR	TCTAAAGCGTTATAC**GC**CTCTATATGCGCAAC	CpsB(R139A) mutagenesis, Rev, overlap M1BF
M2BF	CATAACCTTGATGTT**GC**ACCGCCATTTTTAGC	CpsB(R206A) mutagenesis, For, overlap M2BR
M2BR	GCTAAAAATGGCGGT**GC**AACATCAAGGTTATG	CpsB(R206A) mutagenesis, Rev, overlap M2BF
BaC5F	ACAA**GGATCC**ACTGTCGAGTCACAAGCATTA	Ampl. of *cpsC* + flanking regions, For, 905 bp upstream *cpsC* start, BamHI
XhC3R	CAAT**CTCGAG**TTAAACTCTTCAAGATAGCCACG	Ampl. of *cpsC* + flanking regions, Rev, 943 bp downstream *cpsC* end, XhoI
KOC3F	ATGAATAAAATAGCTATAGTACCAGATTTGAATAAACTT	*ΔcpsC* mutagenesis, For, overlap KOC5R
KOC5R	AGCTATTTTATTCATGAAATCTCCTAAATGTAATGGT	*ΔcpsC* mutagenesis, Rev, overlap KOC3F
M1C3F	ATTATGGGTATTTTGTAAGGAGAATATAATGACTCGTT	CpsC(ΔC-term) mutagenesis, For, overlap M1C5R
M1C5R	CAAAATACCCATAATAACTAAAACAATAGTTGATAATCC	CpsC(ΔC-term) mutagenesis, Rev, overlap M1C3F
M2C3F	TCAACAAGGATATATGTTACTCAAGTAGAGGATATC	CpsC(Δext) mutagenesis, For, overlap M2C5R
M2C5R	ATATATCCTTGTTGAAGAAGTATATTGTGGCGTAA	CpsC(Δext) mutagenesis, Rev, overlap M2C3F

Name	Sequence	Description
BaD5F	TTTA**GGATCC**CAAAAAGAACGGGTGAAGGAA	Ampl. of *cpsD* + flanking regions, For, 1018 bp upstream *cpsD* start, BamHI
XhD3R	TCTA**CTCGAG**CTACCATTACGACCTACTCTA	Ampl. of *cpsD* + flanking regions, Rev, 966 bp downstream *cpsD* end, XhoI
KOD3F	GAAATAGTTGATAGCAAAAGGGATAGAAAAAGGAAGTAA	*ΔcpsD* mutagenesis, For, overlap KOD5R
KOD5R	GCTATCAACTATTTCTAAACGAGTCATTATATTCTC	*ΔcpsD* mutagenesis, Rev, overlap KOD3F
M1DF	GGAAGGGGAAGGA**GC**ATCCACTACTTCA	CpsD(K49A) mutagenesis, Rev, overlap M1DR
M1DR	TGAAGTAGTGGAT**GC**TCCTTCCCCTTCC	CpsD(K49A) mutagenesis, For, overlap M1DF
M2D3F	GTTAGTGAATCTGTTGGAAAAAGGGATAGAAAAAGG	CpsD(ΔP-Tyr) mutagenesis, For, overlap M2D5R
M2D5R	AACAGATTCACTAACTTTATTAAGAATAATACCTAAGAAC	CpsD(ΔP-Tyr) mutagenesis, Rev, overlap M2D3F
1015F	AGGTTTACTTGTGGCGCTTG	qRT-PCR, For, annealing to *gyrA*
1015R	TCTGCTTGAGCAATGGTGTC	qRT-PCR, Rev, annealing to *gyrA*
1292F	TCAACTGGACAACGCTTCAC	qRT-PCR, For, annealing to *cpsA*
1292R	AAGTTGAGCTCCTGGCATTG	qRT-PCR, Rev, annealing to *cpsA*
1288F	TGCTCATATGTGGCATTGTG	qRT-PCR, For, annealing to *cpsE*
1288R	AGAAAAGATAGCCGGTCCAC	qRT-PCR, Rev, annealing to *cpsE*
1289F	TCAATGCGATCCGTACAAAC	qRT-PCR, For, annealing to *cpsD*
1289R	GTGGATTTTCCTTCCCCTTC	qRT-PCR, Rev, annealing to *cpsD*
CF	TAGG**GGATCC**CATGAATAAAATAGCTAATACAG	BACTH, For, ampl. of *cpsC*, BamHI
CR	CATTA**GAATTC**GATTAAAGTTTATTCAAATCTGG	BACTH, Rev, ampl. of *cpsC*, EcoRI
CMutR	CATTA**GAATTC**GATTACAAAATACCCATAATAAC	BACTH, Rev, ampl. of CpsC(ΔC-term), EcoRI
DF	AGGA**GGATCC**CATGACTCGTTTAGAAATAG	BACTH, For, ampl. of *cpsD*, BamHI
DR	TACA**GAATTC**GATTACTTCCTTTTTCTATC	BACTH, Rev, ampl. of *cpsD*, EcoRI
AF	GGAG**GGATCC**AATGTCTAATCATTCGCGCCG	BACTH, For, ampl. of *cpsA*, BamHI
AR	ATCA**GGTACC**CTTGTTATTCCTCCATTGTGTTC	BACTH, Rev, ampl. of *cpsA*, KpnI
AextF	**CTGTACTTCCAGGGC**TCAACCATTGATTTGACAAATAATC	Recombinant extracellular domain of CpsA, For, ampl. of *cpsA*, 15pb overlap with pET-TEV
AextR	**AATTAAGTCGCG**TTATTCCTCCATTGTGTTCTT	Recombinant extracellular domain of CpsA, Rev, ampl. of *cpsA*, 15pb overlap with pET-TEV
1291f	AACA**CATATG**ATTGATATTCATTCTCAT	Recombinant CpsB, For, ampl. of *cpsB*, NdeI
1291r	AAT**CTCGAG**AATGTAATGGTTTTTTAATATAG	Recombinant CpsB, Rev, ampl. of *cpsB*, XhoI
1289f	**CTGTACTTCCAGGGC**ATGACTCGTTTAGAAATAGTTGATAGC	Recombinant CpsD, For, ampl. of *cpsD*, 15pb overlap with pET-TEV
1289r	**AATTAAGTCGCGTTA**CTTCCTTTTTCTATCCCTTTTTCCGTAA	Recombinant CpsD, Rev, ampl. of *cpsD*, 15pb overlap with pET-TEV

RESULTS

Bioinformatic analysis of CpsABCD proteins of *S. agalactiae*

The amino acid sequences of CpsABCD of GBS 515 (serotype Ia) were analyzed using Pfam to identify conserved domains. The online tool PSORTb was used to predict the subcellular localization and subsequently Octopus was used to predict the membrane topology of the membrane proteins. Results from these analyses are summarized in the cartoon in figure 9 and were consistent with previous literature on *S. agalactiae* and *S. pneumonia* (Hanson *et al.*, 2012, Byrne *et al.*, 2011).

CpsA is predicted to be a membrane protein with intracellular N-terminus and extracellular C-terminus. Three putative transmembrane helices are found among the first 96 aa of the protein, and the latter 389 aa are predicted to be extracellular. In the extracellular portion of the protein two conserved domains are identified, the proximal DNA polymerase processivity factor domain (DNA_PPF, Pfam accession no. PF02916) and the distal LytR_cpsA_psr domain (Pfam accession no. PF03816). As reported by Hanson and coworkers (Hanson *et al.*, 2012), the identification of the DNA_PPF domain is curious because the sequence of CpsA is very divergent from traditional DNA_PPF sliding clamp, moreover proteins belonging to this family bind directly to DNA, and in CpsA this domain is extracellular. Therefore, the function of this domain in CpsA is unknown. As for the LytR_cpsA_psr domain, it is found in the extracellular domain of a number of putative membrane-bound proteins related to the cell envelope, but its function is annotated as unknown.

CpsB is predicted to be localized in the cytoplasm and to possess a Polymerase and Histidinol Phosphatase domain (PHP, Pfam accession no. PF02811). The orthologous gene in *S. pneumoniae* (64% aa sequence identity) is described to be a phosphatase, and two amino acids in particular are reported to be important for phosphatase activity (Hagelueken *et al.*, 2009). These two amino acids are among the conserved residues found also in CpsB (R139 and R206).

CpsC is predicted to be a membrane protein with intracellular N- and C-termini. Two transmembrane helices are predicted after 25 aa from the beginning

of the protein and 33 aa before its end. The major central portion of the protein is extracellular and a Wzz superfamily domain (Pfam accession no. PF02706) is identified in this region. This domain is found in a number of related proteins involved in the synthesis of lipopolysaccharide, O-antigen polysaccharide, capsule polysaccharide and exopolysaccharides.

Finally, CpsD is predicted to be found in the cytoplasm, and possesses an AAA (ATPases Associated with diverse cellular Activities) domain (Pfam accession no. PF13614). CpsD is potentially a kinase, also by virtue of comparison with the orthologous genes CapB (40% aa sequence identity) in *S. aureus* (Olivares-Illana *et al.*, 2008) and Wzd (59% aa sequence identity) in *S. pneumoniae* (Henriques *et al.*, 2011). The conserved catalytic lysine residue described in the homologous proteins was identified also in CpsD(K49). Moreover we identified a repeated motif YGX in the C-terminal tail of CpsD, potentially constituting a phosphoacceptor region (Morona *et al.*, 2003).

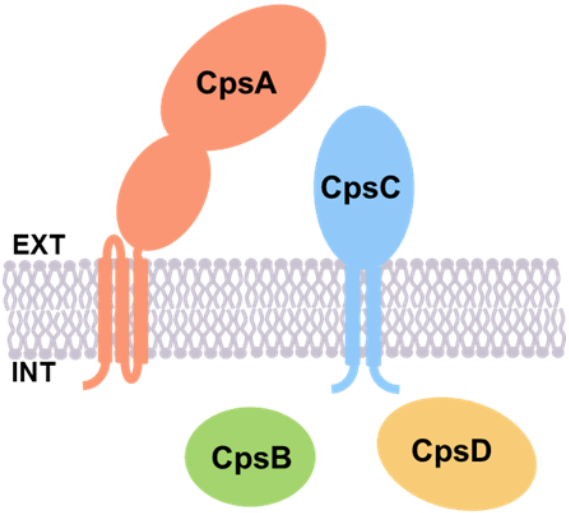

FIGURE 9. **Predicted subcellular localization and membrane topology of CpsABCD.** Cartoon representing the CpsABCD proteins based on the information obtained by analyzing the protein sequences with PSORTb and Octopus.

Generation of isogenic CpsABCD mutant strains in GBS

To investigate the function of *cpsABCD*, twelve isogenic mutant strains were obtained in the GBS 515 (serotype Ia) genetic background (Table 2). The knock-out (KO) mutants Δ*cpsA*, Δ*cpsB*, Δ*cpsC* and Δ*cpsD* contained in-frame deletions where a large part of the gene sequence was removed. Furthermore, we designed *ad hoc* functional mutant strains to investigate the role of specific domains and/or enzymatic activities of the proteins. Thus, the entire extracellular portion of CpsA, or the LytR domain only, were deleted, generating strains CpsA(Δext) and CpsA(ΔLytR). We generated the two mutant strains CpsB(R139A) and CpsB(R206A), containing alanine substitutions in those conserved aminoacids reported to be important for phosphatase activity (Hagelueken *et al.*, 2009). Little is known about CpsC and its orthologues, although the predicted C-terminal intracellular tail was suggested to be essential for CpsD activity in *S. aureus* and *S. pneumoniae* (Soulat *et al.*, 2006, Byrne *et al.*, 2011). We created the mutant strains CpsC(ΔC-term) and CpsC(Δext) where the predicted intracellular tail (33 aa), and extracellular domain (101 aa) of CpsC were deleted. As for CpsD, the putative kinase, the conserved catalytic residue Lys49 was mutated into alanine, generating strain CpsD(K49A). In parallel, the repeated motif YGX in the C-terminal tail of CpsD was truncated (12 aa), generating strain CpsD(ΔP-Tyr). All the mutants were viable and none of them showed significant growth defects as observed from their growth kinetics (Fig. 10).

TABLE 2. **GBS strains used in this work**

Strain name	Description	Mutated protein [full length aa]
GBS 515	Wild type strain	-
Δ*cpsE*	*cpsE* deletion	Details in (Cieslewicz *et al.*, 2001)
Δ*cpsA*	*cpsA* deletion	Deletion of aa 11-452 [458]
CpsA(Δext)	Deletion of the CpsA extracellular domain	Deletion of aa 96-458 [458]
CpsA(ΔLytR)	Deletion of the CpsA LytR domain	Deletion of aa 236-458 [458]
Δ*cpsB*	*cpsB* deletion	Deletion of aa 4-240 [243]
CpsB(R139A)	Point mutation in the phosphatase active site	Arginine to alanine in position 139 [243]
CpsB(R206A)	Point mutation in the phosphatase active site	Arginine to alanine in position 206 [243]
Δ*cpsC*	*cpsC* deletion	Deletion of aa 1-222 [230]
CpsC(ΔC-term)	Deletion of the CpsC intracellular C-terminal portion	Deletion of aa 198-230 [230]
CpsC(Δext)	Deletion of the CpsC extracellular domain	Deletion of aa 53-153 [230]
Δ*cpsD*	*cpsD* deletion	Deletion of aa 11-225 [232]
CpsD(K49A)	Point mutation in the autokinase active site	Lysine to alanine in position 49 [232]
CpsD(ΔP-Tyr)	Phosphoacceptor site C-terminal deletion	Deletion of aa 213-224 [232]

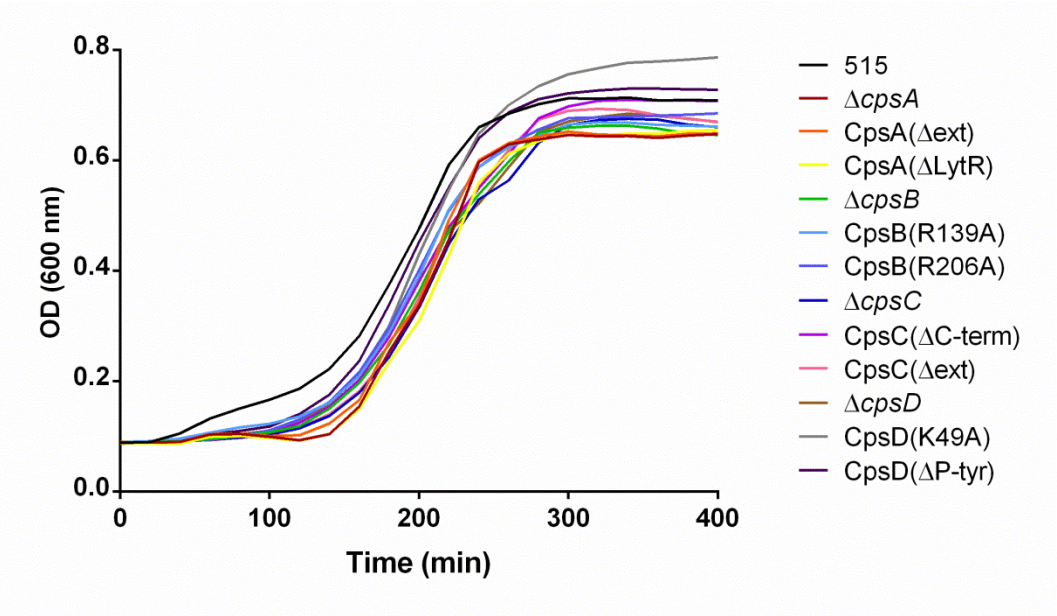

FIGURE 10. **Growth curves of the wild type and mutant strains.** Optical density (nm=600) of the wild type strain 515 and of the *cps* mutant strains in THB medium was monitored for 400 min. Growth curves are the mean of five different biological replicates.

Analysis of the cps operon transcription in CpsABCD mutant strains

CpsA has previously been reported to be involved in *cps* operon transcription (Cieslewicz *et al.*, 2001, Hanson *et al.*, 2012). We used qRT-PCR to analyze the transcription of the operon in all the mutant strains. Bacteria were harvested in logarithmic and early stationary phase and the transcription was measured using primers annealing to *cpsA* (with the exception of CpsA mutants, where we used primers annealing to *cpsD*) (Fig. 11). Compared to the wild type strain 515, mutant strains showed no prominent differences in relative expression of the *cps* operon, suggesting that none of the *cpsABCD* genes is involved in transcriptional regulation of the *cps* operon in the conditions tested. We also noted that the transcription of the *cps* operon was reduced in early stationary phase compared to exponential phase of growth.

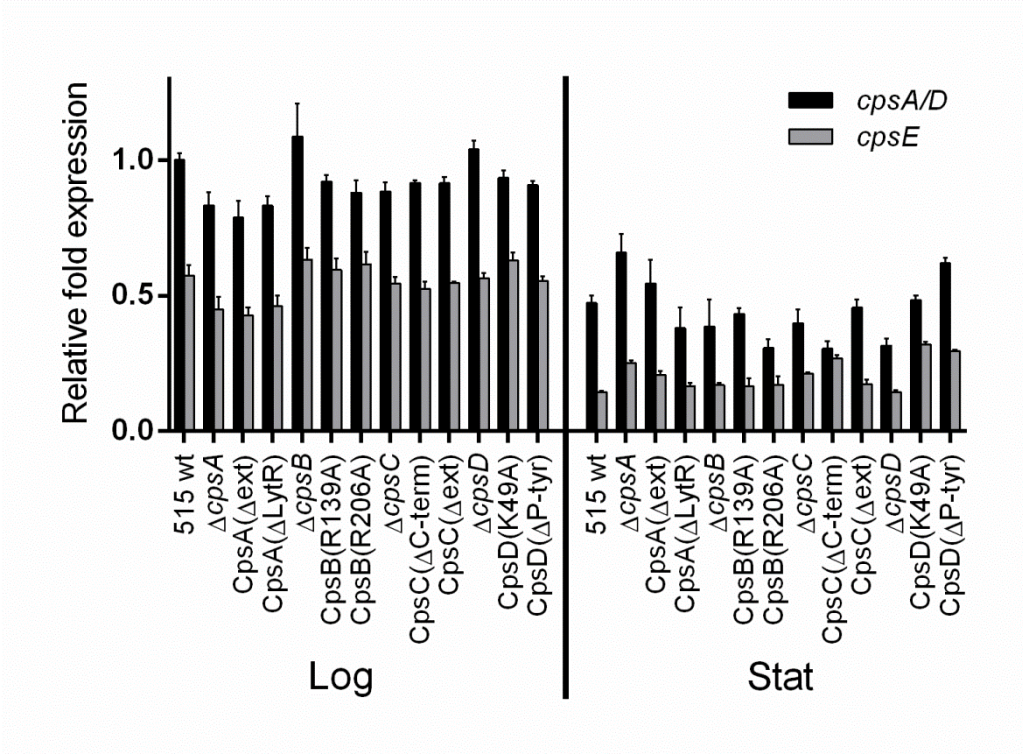

FIGURE 11. Q**uantification of the *cps* operon transcription.** *Cps* operon transcription of the wt strain 515 and of its derivative *cps* mutant strains was measured by qRT-PCR using primers annealing to c*psA/D* (black bars) and to *cpsE* (grey bars). Bacteria were harvested in logarithmic (Log) and early stationary phase (Stat). The relative fold expression for each strain was calculated in comparison with the wt strain 515 in log phase. Columns represent means of three independent experiments performed with triplicate samples. Error bars represent standard deviation.

The *cps* operon transcription was also quantified using primers annealing to *cpsE*, the first gene downstream *cpsABCD*. For all the strains, transcription measured with these primers was reduced in comparison to the transcript quantified on *cpsA*. This effect suggests possible RNA degradation of the long transcript starting from the 3' end. Primers annealing to the last gene of the operon (*neuA*) were tested on the wild type strain and gave a signal even lower than the one measured with primers on *cpsE* thus confirming this hypothesis (data not shown). Using primers annealing to *cpsE* we could also exclude the presence of polar effects on the transcription of the *cps* operon due to the mutations introduced (Fig. 11).

CpsBCD forms an interdependent kinase/phosphatase system

The presence of CpsABD in the wild type and mutant strains was verified by Western Blot on total protein extracts (Fig. 12). In all *cpsA* mutants anti-sera failed to detect the presence of CpsA, possibly because antibodies are binding to the LytR domain of the protein which is not present in any of these strains. All the other mutants produced an amount of CpsA comparable to the wild type. CpsB and CpsD are present in all the protein extracts with minor expression differences, with exception for the respective knockout mutant strains. A reduced expression is observed for CpsB in the CpsA(Δext) and CpsA(ΔLytR) strains, possibly because the 3' end of the *cpsA* gene is absent in these strains and harbors the ribosome binding site (RBS) of *cpsB*.

In *S. pneumoniae* the orthologous CpsBCD have been shown to constitute a phosphoregulatory system. The autokinase CpsD phosphorylates its C-terminally located tyrosines (Morona *et al.*, 2003). CpsB is the cognate phosphatase and CpsC is a membrane protein required for CpsD autokinase activity (Byrne *et al.*, 2011, Bender & Yother, 2001). We hypothesized that these proteins have similar functions in *S. agalactiae*. The presence of putative phosphoproteins in total bacterial extracts was examined using an α-P-Tyr mAb (Fig. 12). CpsD appeared phosphorylated in the wild type strain, albeit showing

only a faint band. The three *cpsB* mutants showed an increased level of phosphorylation of CpsD, consistent with absent/reduced phosphatase activity of CpsB, and confirming that the amino acids R139 and R206 are necessary for phosphatase activity. A slight increase in CpsD phosphorylation is observed also in the CpsA(Δext) and CpsA(ΔLytR) strains, consistent with the reduced translation of the CpsB protein (see above). All the mutations in CpsD resulted in undetectable phosphorylation of this protein. We conclude that CpsD is an autokinase and when non-functional, it cannot be phosphorylated by other bacterial kinases. Moreover, we showed that one or more of the four tyrosines at the C-terminal of CpsD constitute the phosphoacceptor site, and that the lysine in position 49 is necessary for autokinase activity. In addition, if CpsC is absent or lacks the C-terminal intracellular tail, then CpsD is not phosphorylated. While, if only the extracellular portion of the protein is deleted, autophosphorylation activity of CpsD is preserved. Thus, the CpsC intracellular 33 aa tail is necessary for CpsD phosphorylation while the CpsC extracellular domain is dispensable.

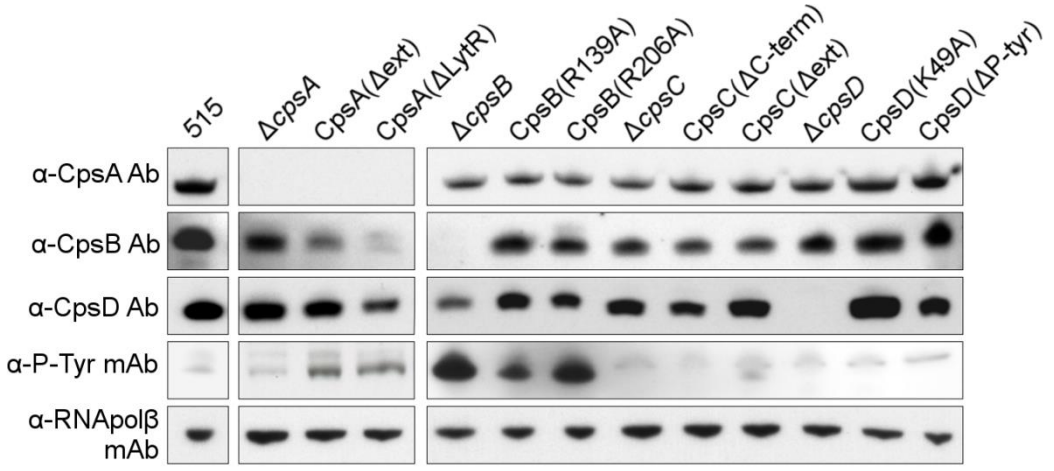

FIGURE 12. **CpsABD proteins in wt and mutant strains.** Western blots showing CpsA (α-CpsA), CpsB (α-CpsB), CpsD (α-CpsD), tyrosine phosphorylation of CpsD (α-P-Tyr mAb) and the loading control RNA polymerase subunit β (α-RNApolβ mAb) in total protein extracts from 515 wt and the *cps* mutant strains.

Aberrant CPS production and localization in CpsABCD mutant strains

CPS production in wt and *cps* mutant strains was verified by flow cytometry using an α-CPSIa mAb. All the mutant strains exhibited a clear shift in mean fluorescence compared to the negative control (Fig. 13*A*), confirming that the strains produce a CPS possessing epitopes recognized by the monoclonal antibodies raised against the wild type CPS. The amount of CPS present on the surface of the different strains was quantified in bacterial extracts obtained by alkaline treatment (Wessels *et al.*, 1990). We detected lower amounts of surface-associated CPS in all the KO mutant strains compared to the wild type (Fig. 13*B*), consistent with previously published data (Cieslewicz *et al.*, 2001). Most of the functional mutants also showed a significant capsule reduction, with the exception of mutants with point mutations in CpsB (CpsB(R139A) and CpsB(R206A)) and the CpsD(ΔP-Tyr) strain which lacks the putative tyrosine phosphoacceptor tail (Fig. 13*B*).

In *S. agalactiae* the CPS is covalently attached to the cell wall peptidoglycan (Deng *et al.*, 2000). Certain mutant strains had little CPS attached to the bacterial cell surface (Fig. 13*B*) despite having normal *cps* operon transcription. We hypothesized that some of the CPS produced may be shed. To examine this possibility, serial dilutions of spent growth media were spotted on a nitrocellulose membrane and probed with an α-CPSIa mAb. All the *cpsA* mutants showed an increased amount of CPS in the medium compared to the wt (Fig. 14*A*). The same phenotype was observed for the mutant strain with impaired autokinase activity (CpsD(K49A)) but not for the other *cpsD* mutants. The mutants Δ*cpsB*, Δ*cpsC* and CpsC(Δext) showed no detectable CPS in the culture supernatant, comparable to the negative control Δ*cpsE* which does not produce any CPS whatsoever. The Δ*cpsA* and CpsD(K49A) strains were chromosomally complemented and the amount of CPS released in the media by the complemented strains was restored to wild type levels (Fig. 14*B*). The mutant strains with increased CPS in the medium concomitantly showed a significant reduction in the amount of CPS attached to the bacterial surface (Fig. 13*B*), suggesting a defective attachment of CPS to the cell wall. These data suggest that CpsA could be the enzyme responsible for attachment of CPS to the cell wall, and that CpsD

autokinase activity is required. Specifically, the LytR domain of CpsA seems to be necessary for CPS attachment to the surface, since CPS is shed when this domain is removed.

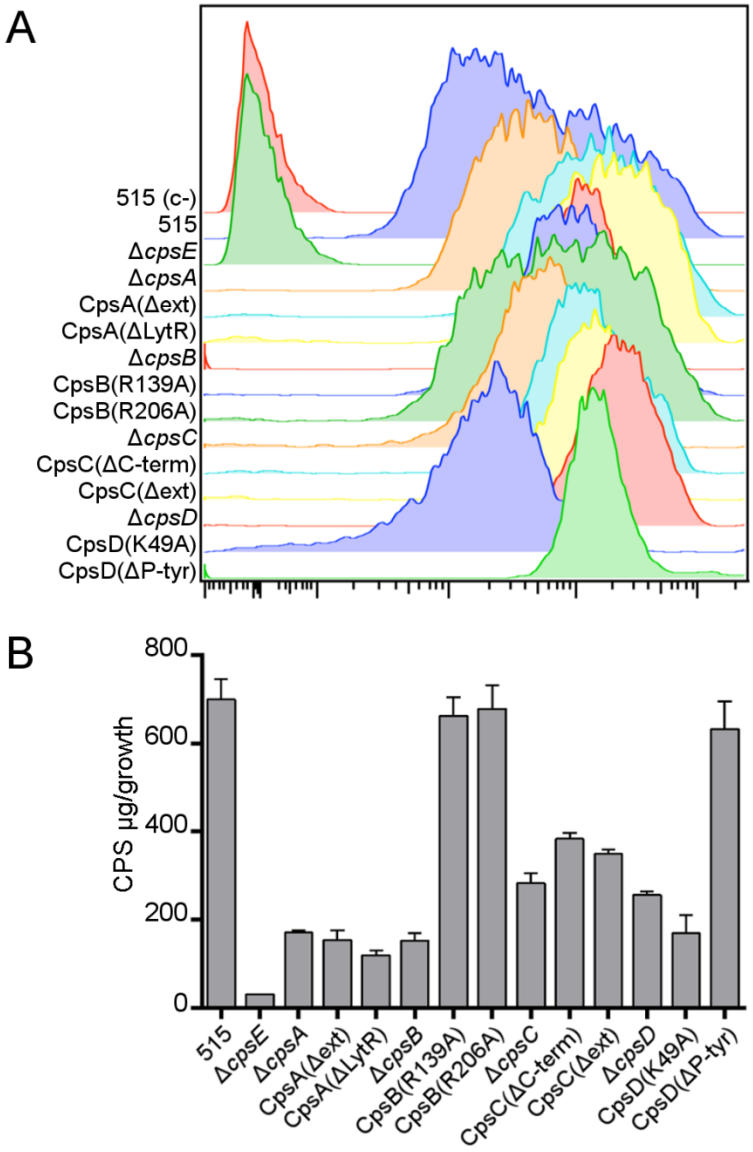

FIGURE 13. **CPS production in the *cps* mutant strains.** *A*, WT and *cps* mutant strains were incubated with a primary α-CPSIa mAb followed by a secondary goat α-mouse antibody conjugated to allophycocyanin (APC), and analyzed by flow cytometry. The unencapsulated Δ*cpsE* strain was included as a negative control. *B*, CPS from bacterial pellets was measured by a resorcinol assay. Columns represent means of three independent experiments performed with triplicate samples. Error bars represent standard deviation.

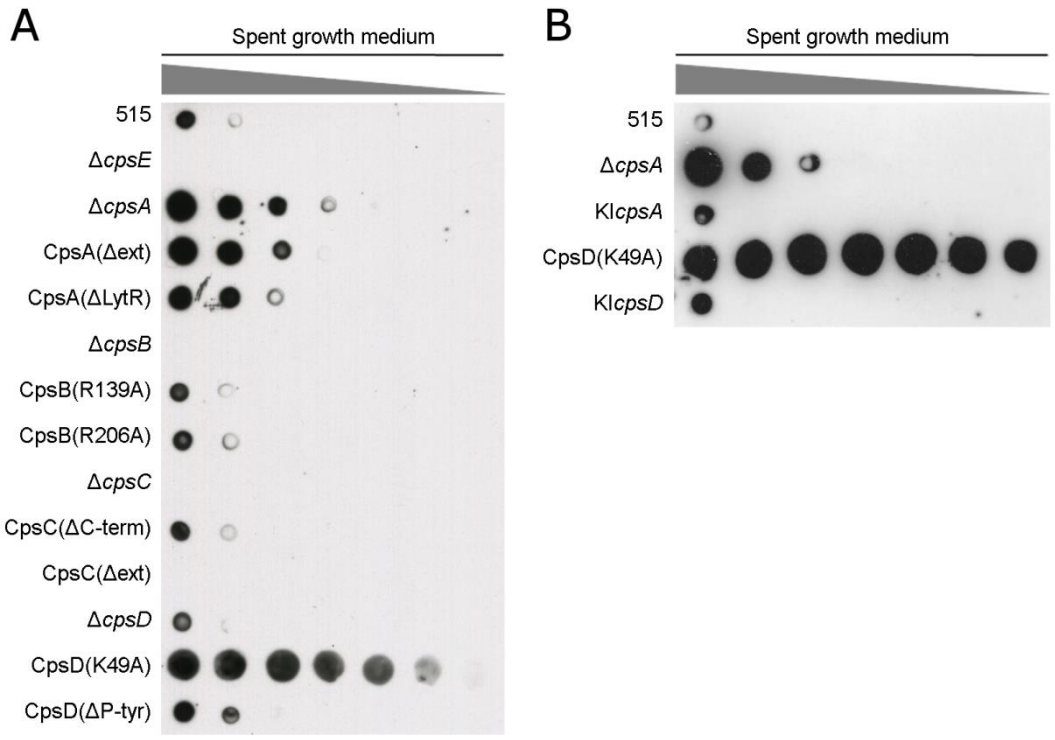

FIGURE 14. **Aberrant CPS localization in mutant strains**. Dot blots showing serial dilutions (1:2) of spent growth media spotted on a nitrocellulose membrane and probed with an α-CPSIa mAb. *A*, dot blot on the wild type and on all the *cps* mutants. *B*, dot blot on selected mutant strains and on their respective complemented strains.

CPS length anomalies are observed in selected mutant strains

CPS from bacteria was extracted by mutanolysin treatment, separated on a polyacrylamide gel and examined by immunoblot with an α-CPSIa mAb (Fig. 15*A*). Aberrant CPS length was observed in some mutant strains when compared to CPS extracted from the wt. We observed that strains where CpsD is absent/non-functional (the three *cpsD* mutant strains and the CpsC(ΔC-term) mutant) displayed CPS with an unusually high molecular weight. Interestingly, these mutant strains are those where phosphorylation of CpsD was absent, suggesting that phosphorylation of CpsD may influence CPS chain length. In contrast, a very short CPS was produced by the strains lacking the extracellular domain of CpsC. Surprisingly, we could not detect any CPS in samples from Δ*cpsB* mutant by Western blot, despite previously having confirmed CPS production by FACS

analysis and CPS quantification (Fig. 13*AB*). Mutant strains that exhibited aberrant CPS phenotypes (i.e. Δ*cpsB* and all the *cpsC* and *cpsD* mutant strains) were chromosomally complemented. CPS extracts were prepared and analyzed by Western blot (Fig. 15*B*) and all the complemented strains appeared indistinguishable from the wild type, confirming that the phenotypes observed are solely due to the specific mutations introduced.

Immunogold transmission electron microscopy was performed in an attempt to visualize the CPS at the bacterial surface (Fig. 16). Wild type bacteria were observed both as electron dense diplococci or chains of cocci. Gold beads linked to α-CPSIa were uniformly distributed in a thin layer peripheral to the bacterial surface. The negative control (Δ*cpsE*), devoid of CPS, had very few if any beads associated with the bacteria. In the CpsD(ΔP-tyr) mutant, beads formed a wider layer around the bacterial periphery with very few beads in close proximity to the bacteria. In addition, scattered beads were also observed at a large distance from the bacterial surface, suggesting a more extended CPS. The Δ*cpsB* mutant, in comparison, showed a bead distribution similar to the wt but with much fewer gold beads. This is consistent with results shown in Fig. 13*B* indicating a lower CPS amount in this mutant.

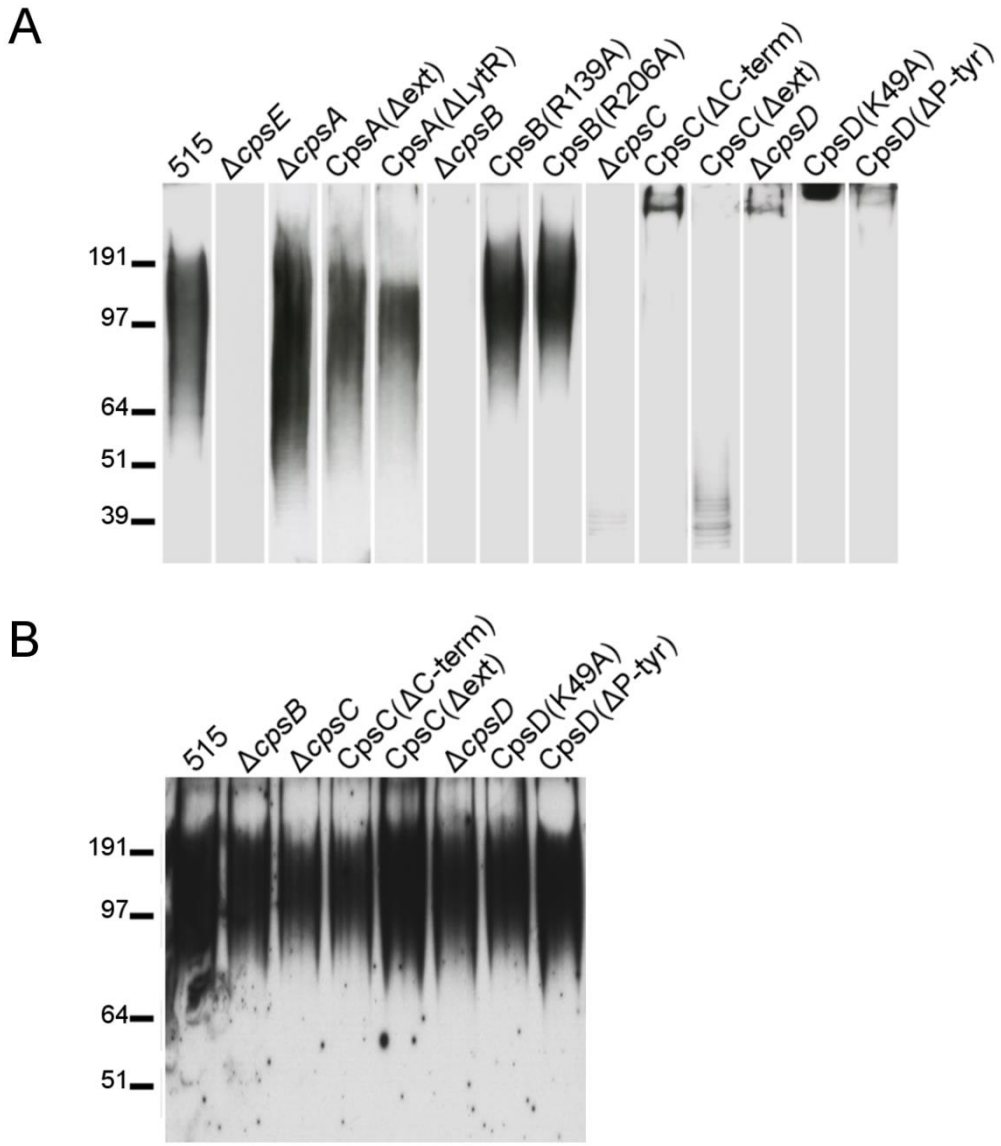

FIGURE 15. **CPS length differences in the wt and *cps* mutant strains.** *A*, Western blot of CPS bacterial surface extracts from the wt and all the *cps* mutant strains. Lanes were exposed for different times, in order to permit visualization of CPS from all strains. *B*, Western blot of CPS bacterial surface extracts from the wt strain and from chromosomally complemented strains. CPS was detected with an α-CPSIa mAb. A protein molecular weight marker is included for approximate comparison.

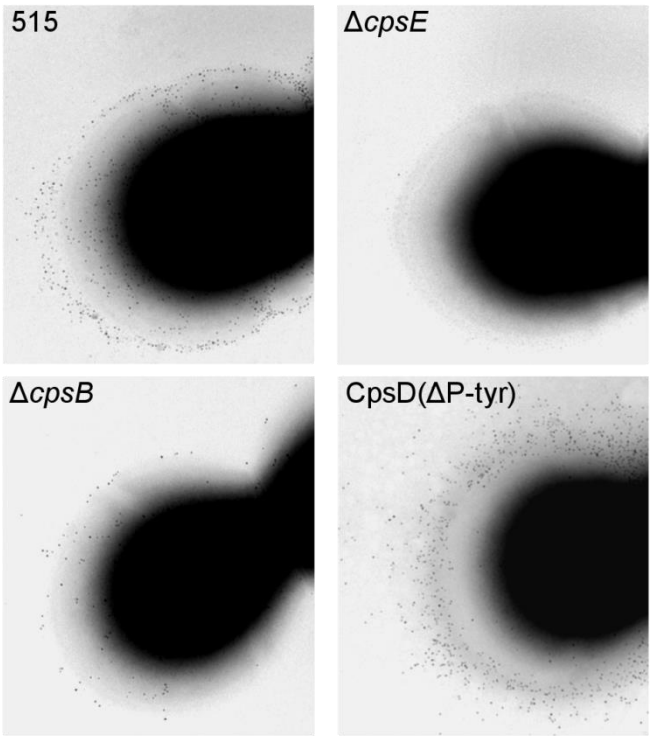

FIGURE 16. Immunogold TEM on whole bacteria using an α-CPSIa mAb as primary antibody, and a secondary gold-beads conjugated antibody. Bacterial strains are indicated.

Biochemical characterization of the CPS in selected mutant strains

On the basis of the Western Blot data, we selected a panel of strains exhibiting different CPS properties for further analytical characterization of the capsular polysaccharides. The Δ*cpsA* and CpsD(K49A) strains were selected because they partially release the CPS in the growth medium. CpsC(Δext) was chosen because it produces a very short polysaccharide. One liter cultures were used to obtain bacterial pellets that underwent alkaline treatment, and the CPS was subsequently purified by ethanol precipitation and diafiltration. A simplified procedure was set up to purify the CPS from the growth media. The NMR analysis confirmed the saccharide structural identity of purified CPS obtained from wild type and mutant strains, irrespective of whether they were derived from media or bacteria (Fig. 17).

In the Δ*cpsA* and CpsD(K49A) strains, the amount of CPS collected from the media represented the majority of the total CPS produced (70% and 77%,

respectively). In comparison, very little CPS was purified from the medium of the wild type strain 515. The relative molecular weight distribution of purified CPS was determined by HPLC using a pullulan reference standard to build a calibration curve. The average chain length of the CPS purified from the media of the two mutant strains was significantly higher than the CPS of the wild type strain (Tab. 3, Fig. 18). The Δ*cpsA* mutant released a CPS approximately 5 to 10 times longer than the wild type. Interestingly, the CPS purified from the bacterial surface of Δ*cpsA* strain was instead comparable to that of the wild type.
Using the CpsD(K49A) mutant we observed that the purified CPS from both fractions exhibit an extremely high molecular weight distribution (more than 10 times longer than the wt), to the point where further size estimation was impossible.

We also investigated the CPS purified from the bacterial surface of the mutant strain CpsC(Δext), which showed shorter surface CPS in the immunoblot experiments (Fig. 15*A*). The amount of CPS extracted from the bacterial pellet of this mutant was higher than in the other mutant strains, and the molecular weight distribution of the purified CPS determined by HPLC indicated a 6-fold smaller CPS compared to the wt (Tab. 3, Fig. 18). The growth medium of this strain was not analyzed since previous experiments suggested a minimal release of CPS (Fig. 14*A*). In conclusion, if CpsA is disrupted the amount of shed CPS increases dramatically and that CPS is very long, while retained CPS is shorter than that of the wild type. Concomitantly, if CpsD is unable to autophosphorylate, then attached and released CPS are both longer than the wild type, and increased shedding is again observed. Finally, it is noteworthy that shed CPS purified from medium was consistently much longer than wt CPS from the bacterial surface.

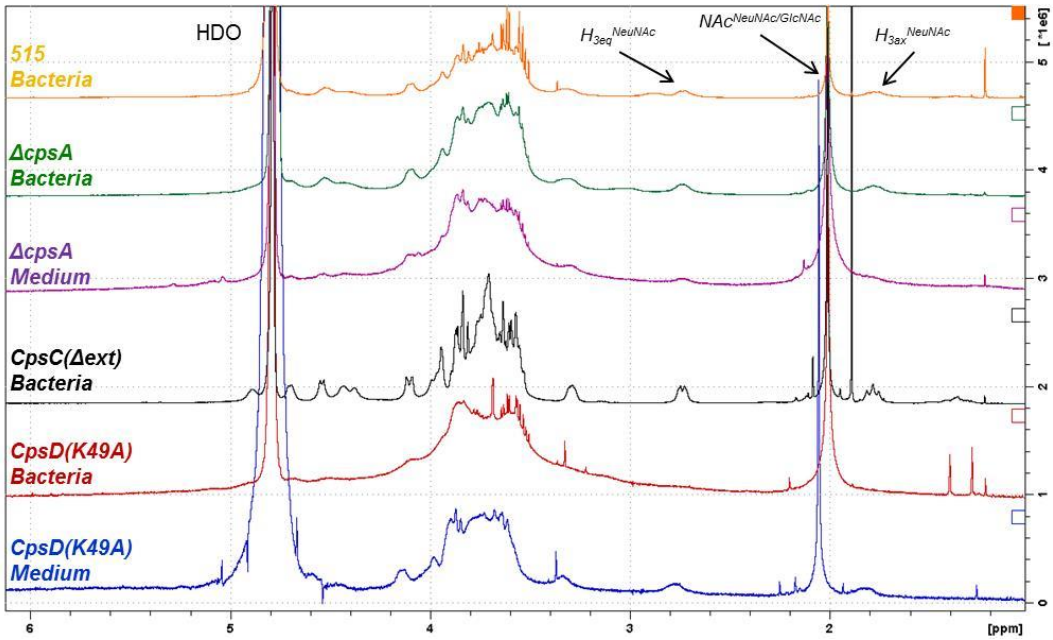

FIGURE 17. **Comparison of 1H NMR spectra obtained for CPS extracts purified from bacterial pellets and from spent growth media.**

TABLE 3. **Biochemical characterization of the purified CPS from selected mutant strains.** Quantification of the CPS purified from bacterial pellets and from spent growth media from 1 liter cultures. The mean size of the CPS was estimated by HPLC-SEC. The size range represents 95% of the area of the molecular size distribution.

Strain	Fraction	CPS (mg)	Size (kDa)	Range (kDa)
515	Bacteria	4.1	167	49 - 616
	Medium	0.5	NA	NA
Δ*cpsA*	Bacteria	2.8	237	62 - 1075
	Medium	6.7	>1330	115 - >1330
CpsC(Δext)	Bacteria	3.3	55	23 - 136
CpsD(K49A)	Bacteria	1.7	>1330	1013 - >1330
	Medium	5.6	>1330	>1330

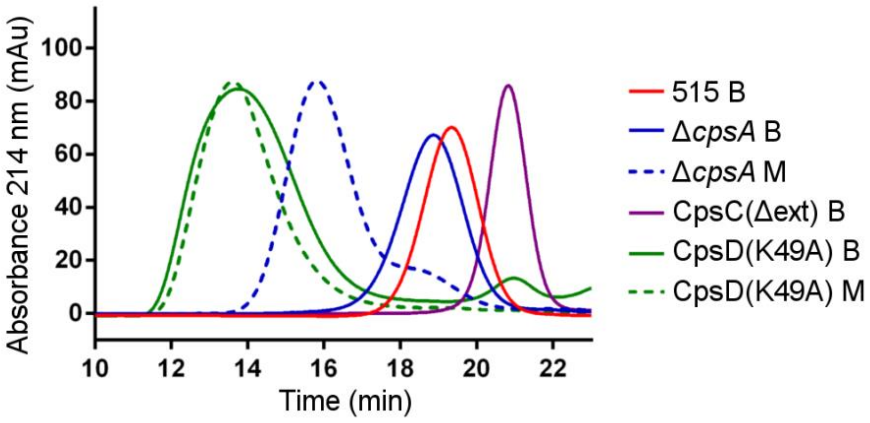

FIGURE 18. **Molecular size analysis of CPS from selected strains.** Profiles for CPS extracts purified from bacterial pellets (solid lines) and from spent growth media (dotted lines) analyzed by SEC-HPLC. The molecular size of the polysaccharide was calculated by comparison with a calibration curve generated using pullulan standards.

CpsC interacts with CpsA and CpsD

As seen in Western Blot experiments (Fig. 15*A*), mutant strains lacking CpsC or the extracellular domain of CpsC produced a short CPS, suggesting that the extracellular part of CpsC is in some way assisting polymerization. We hypothesized that CpsC interferes with CpsA termination of CPS polymerization. This implied a possible interaction between CpsC and CpsA, which was investigated using a Bacterial Two Hybrid (BACTH) system (Karimova *et al.*, 1998) (see Experimental Procedures for details). We observed a CpsA self-interaction, suggesting a possible oligomerization of CpsA. Interestingly, we also observed an association between heterologously expressed CpsC and CpsA (Fig. 19*A*). Such a direct interaction has not been previously reported, and suggests that CpsC may be forming a transient or stable complex with CpsA, thereby modulating the attachment of CPS to the cell wall.

A protein-protein interaction between the two homologous protein Wzd (CpsC) and Wze (CpsD) in *S. pneumoniae* has previously been shown (Henriques *et al.*, 2011). Having observed that CpsD autokinase activity required the presence of CpsC (Fig. 12) we investigated a putative interaction between CpsC and CpsD of GBS by BACTH system. Indeed, we observed that heterologously expressed CpsC and CpsD interacted (Fig. 19*B*). Interestingly, we observed that this

interaction was abrogated when the CpsC C-terminal 33 aa tail was removed, suggesting that the tail directly interacts with CpsD.

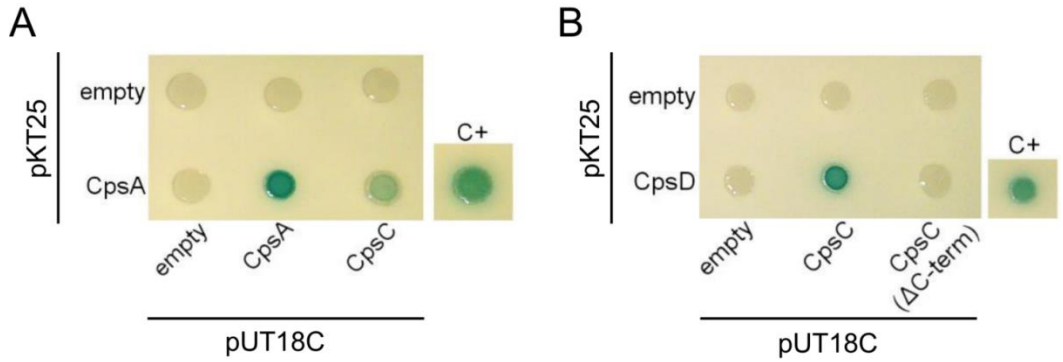

FIGURE 19. **Analysis of protein interactions between CpsACD using a bacterial two hybrid system.** A, Bacterial two hybrid (BACTH) analysis of CpsA and CpsD. T25-CpsA was tested for interaction with T18-CpsA and T18-CpsC. B. BACTH analysis of CpsC and CpsD. T25-CpsD was tested for interaction with T18-CpsC and T18-CpsC(ΔC-Term). Empty plasmids were tested together with fusion proteins as negative controls. The positive control used was the leucine zipper GCN4 fused to the T25 and T18 fragments (Karimova *et al.*, 1998). The formation of blue colonies indicates a protein–protein interaction. Experiments were performed in triplicates and the same results were obtained for each replicate.

CPS defects in mutant strains are associated with reduced adhesion to plates

The wild type strain 515 is a known biofilm-forming strain (Rinaudo *et al.*, 2010). The crystal violet assay on polystyrene plates is an initial screening for the adhesive properties of bacteria (O'Toole *et al.*, 2000). We used this assay to investigate whether the different CPS phenotypes observed in the *cps* mutants may have an impact on the biofilm properties of GBS. GBS 515 wt gave a positive signal in the crystal violet assay (Fig. 20). In comparison, the unencapsulated isogenic mutant Δ*cpsE* showed a weak signal. Similar phenotypes have already been observed and suggest that the presence of the CPS is required for bacterial aggregation and adhesion to plates (Xia *et al.*, 2014). Such adhesion does not seem to directly correlate with CPS amounts, in fact strains producing as much CPS as the wt strain (i.e. the functional CpsB mutants and the CpsD(ΔP-tyr)

strain) (Fig. 13*B*) present opposite adhesion phenotypes (Fig. 20). All the strains showing reduced adhesion to plate possess very long attached polysaccharides (CpsC(ΔC-term) and all the *cpsD* mutants) (Fig. 15*A*) or shed the CPS in the growth medium (CpsD(K49A) and the *cpsA* mutants) (Fig. 14*A*). In conclusions our data suggest that strains with reduced CPS amounts or shorter CPS length are able to adhere to plates. On the contrary, absence of CPS, increase in CPS length and shed CPS are negative factors for biofilm formation.

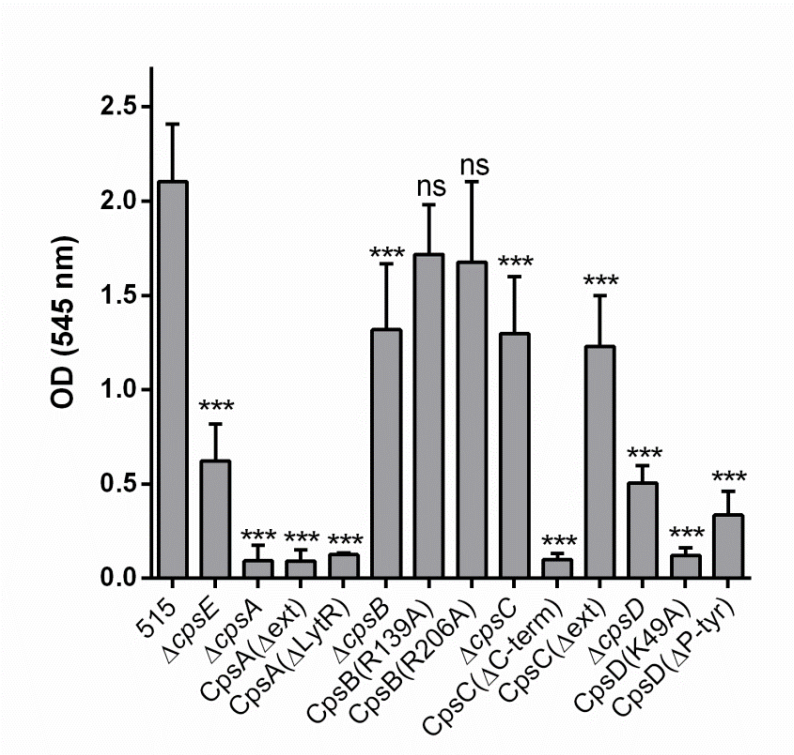

FIGURE 20. **Biofilm formation assay on polystyrene plates.** Adhesion to 96-well polysturene plate is measured by crystal violet assay for GBS 515 and all the *cps* mutant strains after growth in THB supplemented with 1% glucose for 18 hours. Solubilized crystal violet is quantified measuring the absorbance at 540 nm and is used as measure of the number of bacteria adhering to the well. Columns represent means of five replicates. Error bars represent standard deviation. Results were analyzed by one-way ANOVA comparing the mean of each column to the mean of the wild type strain 515. ns, not significant; ***, $p<0.001$.

GBS strains with CPS defects have different adhesion/invasion properties

During the initial stages of the infection GBS needs to attach to and invade epithelial cells. Alveolar epithelia are described as an entry site in early-onset disease. We tried to test whether aberrant CPS length, localization and amount may have an impact on the ability of GBS to associate with epithelial cells. An *in vitro* adhesion-invasion assay was used to test interactions between selected *cps* mutant strains and the lung epithelial cell line A549.

By comparing the wild type and the unencapsulated strain Δ*cpsE* we observed that GBS required the presence of the CPS to associate with cells (Fig. 21*A*). In contrast, the presence of the CPS interfered with the invasion process (Fig. 21*B*) as previously observed (Hulse *et al.*, 1993, Alkuwaity *et al.*, 2012). Moreover, our results showed that strains producing very little CPS, such as the Δ*cpsB* and the Δ*cpsD* mutants (Fig. 13*B*), are less prone to associate with pulmonary cells, but are more capable invaders (Fig. 21*AB*). The same phenotypes were observed for the CpsC(Δext) mutant which produces a short capsule. In contrast, strains presenting little CPS attached to the bacterial surface, but increased shed CPS (the Δ*cpsA* and the CpsD(K49A) mutants) were both defective in the process of cell invasion. The same phenotype was observed also for the CpsD(ΔP-tyr) strain which produces an amount of CPS comparable to the wild type but with increased polymer length, and for the CpsC(Δext) mutant characterized by a short CPS (Fig. 21*B*). Furthermore, all the *cpsD* mutants which present long CPS on the bacterial surface, were deficient in the adhesion process (Fig. 21*A*). It is noteworthy that the invasion-adhesion properties of these strains are not correlated to the ability to adhere to the plates, as previously observed in the crystal violet biofilm assay (Fig. 20). This suggests that the phenotypes observed go beyond mere physicochemical properties of the bacterial surface (e.g. charge). Moreover, the reduced invasive capability of most of the strains is not dependent on the reduced association with cells. In conclusion, our results suggest that strains with reduced or increased CPS length are defective in adhesion to lung epithelial cells *in vitro*. In fact, the only mutant with phenotypes similar to the wild type was the Δ*cpsA* which produces an attached CPS with the same size of the wt. On the other hand, we observed that the unencapsulated strain and the

mutants producing very little CPS are more efficient in invading these cells irrespective of the CPS length.

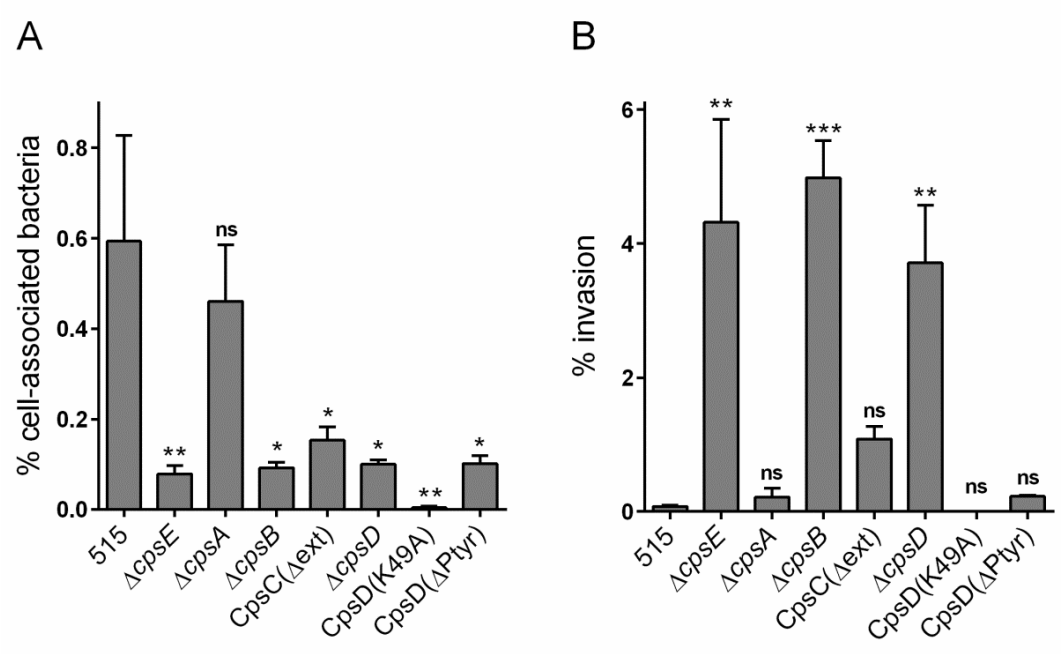

FIGURE 21. **Association to the A549 lung epithelial cell line of the *cps* mutant strains.** *A,* bacteria associated to the A549 lung epithelial cells after 2 h of infection. The number of bacteria is expressed relative to the total number of bacteria in the well after the infection. *B,* percentage of invasion was calculated as percentage of bacteria associated to the A549 cells which are found inside the cells. Each panel shows the mean values from three independent experiments each performed in triplicates. Error bars represent SEM. Results were analyzed by one-way ANOVA comparing the mean of each column to the mean of the wild type strain 515. ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

DISCUSSION

The *cpsABCD* genes are relatively well-conserved intra- and interspecies (Yamamoto *et al.*, 1999, Cieslewicz *et al.*, 2005). In this work we focused on these four conserved genes and their role in *S. agalactiae* CPS biosynthesis. Previous studies on homologous proteins from *S. pneumoniae* have provided molecular details on the phosphorylation and dephosphorylation involving CpsB, C, and D (Morona *et al.*, 2000, Bender & Yother, 2001, Byrne *et al.*, 2011), but the role of this phosphoregulatory system in the context of the CPS biosynthesis is not completely understood. Moreover, the notion that similar events may be occurring in *S. agalactiae* is merely an argument by analogy. We attempted mutational studies to experimentally elucidate the role of CpsABCD in *S. agalactiae* CPS biosynthesis, including mutations that could shed light on the potential interdependencies between these proteins. Our data suggest that CpsA, B, C and D proteins are not essential for the biosynthesis of the capsular polysaccharide repeating units, since all the mutant strains retained the ability to produce a CPS recognizable by monoclonal antibodies against the wild type CPS. However, we observed differences in CPS length and localization in our mutant strains, suggesting that these proteins are involved in controlling CPS elongation and attachment to the cell wall. Following is a step-by-step discussion of the working model we propose for CpsABCD (Fig. 22).

In the final steps of CPS biosynthesis, the newly synthesized repeating unit (RU) anchored to a polyisoprenoid phosphate lipid is flipped to the outer side of the bacterial membrane, where CpsH is presumably responsible for the polymerization of the repeating units (Fig. 22*A*). By analogy with other Wzy-dependent systems, such polymerization occurs bottom-up. The nascent CPS is removed from the lipid through a phosphotransferase reaction, and subsequently linked to a single membrane-anchored RU (Yother, 2011). The final product is a CPS that is removed from the membrane lipid and covalently attached to GlcNAc in the peptidoglycan backbone (CPS-PG) (Deng *et al.*, 2000). This linkage effectively renders further polymerization impossible. Our results suggest that the LytR domain of CpsA is necessary for this activity. In fact, we observed that

when this domain was removed, mutants showed defects in CPS attachment to the bacterial surface and, as a corollary, increased amounts of CPS was shed into the growth medium. CpsA belongs to the LytR-CpsA-Psr (LCP) protein family, together with two paralogues, and it was suggested that these enzymes are involved in the final steps of cell wall assembly (Hubscher *et al.*, 2008). A study of the homologous Cps2A protein in *S. pneumoniae* proposed that it may be responsible for transfer of CPS from the membrane lipid to the cell wall peptidoglycan (Kawai *et al.*, 2011). However, the deletion of *cps2A* in *S. pneumoniae* was not sufficient to obtain a clear CPS release phenotype, possibly due to redundancy of LCP protein activities (Eberhardt *et al.*, 2012). In comparison, we observed increased CPS release for all the *cpsA* mutant strains, even though attachment of CPS to the bacterial surface was not abolished completely. Thus, a possible redundancy between LCP proteins remains a possibility. It is noteworthy that we find no evidence for CpsA involvement in the transcriptional regulation of the *cps* operon, in contrast to previous literature (Cieslewicz *et al.*, 2001, Hanson *et al.*, 2012).

We speculate that the CpsH polymerase and CpsA compete for the same substrate (the nascent CPS). While the enzymatic reaction involving CpsH results in elongation of the CPS by one RU at a time, the CpsA reaction instead terminates elongation by securing CPS to the cell wall. We suggest that the activity of CpsA is moderated by CpsC (Fig. 22*B*). This notion is supported by the direct interaction of CpsC and CpsA in a bacterial two-hybrid system (Fig. 19*A*). Moreover, deletion of the extracellular part of CpsC results in abnormally short CPS, suggesting a premature termination of CPS synthesis by CpsA (Fig. 18).

An interaction between CpsC and CpsD was also observed, strictly dependent on the presence of the short 33 aa intracellular C-terminal tail of CpsC (Fig. 19*B*). Homologues of CpsC and CpsD in Gram-negative bacteria are found as a single multi-domain protein (Olivares-Illana *et al.*, 2008, Whitfield & Paiment, 2003). Taken together, this suggests that CpsC and CpsD form a heterodimer or more complex multimers and act in concert. We show that CpsD is an autokinase and phosphorylates tyrosines in its C-terminus. In our model we propose that the phosphorylation state of CpsD directs the conformation of the

CpsC extracellular domain through interaction with the C-terminal tail (Fig. 22*B*). The notion of CpsCD acting in concert is supported by a model of the homologous proteins CapAB in *S. aureus*, suggesting an octamer complex with conformational changes induced in response to the phosphorylation state of CapB (Olivares-Illana *et al.*, 2008).

Among our mutant strains we observed both very long and very short CPS. We believe that these phenotypes are a result of CpsCD exerting control over the action of CpsA. I.e., a very short CPS suggests unchecked CpsA action, and premature termination of CPS biosynthesis. CpsCD can be considered to have entered a 'permissive' state, which coincides with CpsD being hyperphosphorylated. In contrast, when CpsD is absent or non-phosphorylated (CpsC(ΔC-term) and all the *cpsD* mutants) long CPS are produced, implying that the termination of CPS biosynthesis is impeded. We also observed that when the CpsD protein is in full-length form but non-functional (CpsD(K49A) mutant) the CPS is not only longer than in the wild type, but it is also released into the medium. This phenotype is similar to those observed for the *cpsA* mutants, and further supports the notion that CpsD dephosphorylation is directly or indirectly inhibiting CpsA activity. Admittedly, the data on mutants Δ*cps*D, Cps(ΔP-tyr) and CpsC(ΔC-term) are not immediately consistent with this model, as the CPS is still attached to the bacterial surface despite having an increased size. However, these mutants all have in common structural truncations that may affect the integrity of the CpsCD complex, resulting in an anomalous configuration with unpredictable consequences. Theoretically possible alternatives to our model include a direct inhibition of polymerization by CpsA, or that CpsC facilitates the polymerization process. In our view, both possibilities are less consistent with the data presented here, compared to the model we propose.

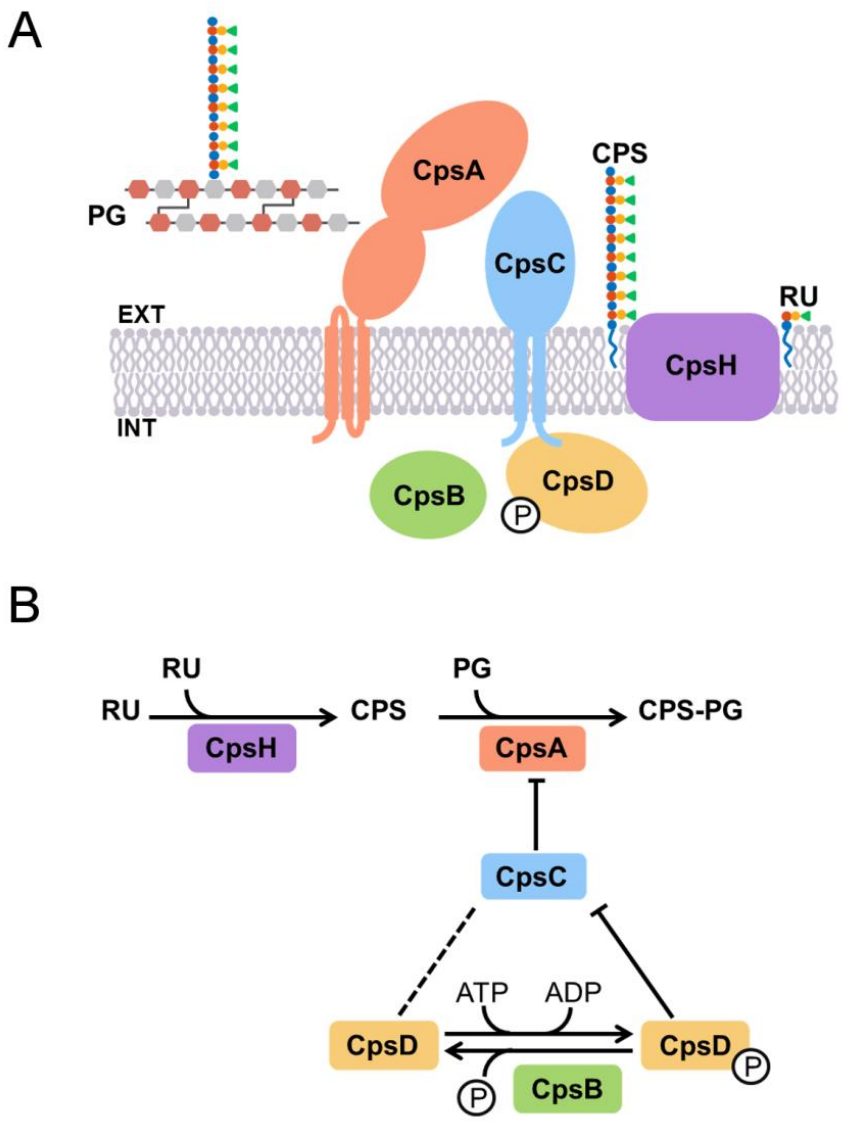

FIGURE 22. **Model of CpsABCD involvement in CPS biosynthesis**. *A*, Topology and subcellular localization of the CpsABCD proteins and of the CpsH polymerase based on computer predictions and/or literature. Repeating unit (RU), the capsular polysaccharide (CPS) and the cell wall peptidoglycan (PG) are also represented in the panel. *B*, Schematic representation of the working model proposed for the CpsABCD proteins. Arrows represent enzymatic reactions, the bar-headed line is an inhibitory effect, and the dotted line represents interdependency.

A comparison between the phenotypes of mutant strains Δ*cps*A and CpsD(K49A) presents an interesting enigma. In both cases, a majority of the CPS is shed into the medium and is also unusually long. Prior to PG attachment by CpsA, the CPS is tethered to the membrane through the lipid moiety. If the CPS is not transferred from the lipid moiety to the PG, polymerization continues unhindered and results in CPS polymers that are 10-fold longer or more compared to the wild type size. We speculate that shedding occurs because the lipid moiety alone is insufficient to keep such a large molecule tethered in the membrane through hydrophobic interaction. In the Δ*cpsA* strain only normal-sized CPS is found attached, while in the CpsD(K49A) mutant the attached CPS is as long as the shed CPS. A difference between the mutants is that the CpsD(K49A) has a fully functional CpsA, although the CpsCD is in a permanent 'non-permissive' state in relation to CpsA. On the other hand, in the case of the Δ*cpsA* mutant, CpsCD undergoes normal phosphorylation cycling, and will thus periodically enter a 'permissive' state where the CPS is subjective to hydrolysis, and may become anchored to PG by other LCP proteins, as previously suggested for *S. pneumoniae* (Eberhardt *et al.*, 2012). Presently, we do not have a clear understanding of how these mutations result in two somewhat different phenotypes.

In summary, this work examines the concerted action of CpsABCD in the Gram-positive bacterium *S. agalactiae*. Through the use of multiple functional and structural mutations, the resulting phenotypes allowed us to approach the proteins as a system, and define interdependencies. CpsABCD sit at the finishing line of CPS biosynthesis, and the cyclic phosphorylation of CpsD is a main switch that ensures secure attachment to the cell wall, indirectly determining the average length of CPS. A steady-state is obtained through autophosphorylation of CpsD and dephosphorylation by CpsB. Perturbances in this system lead to distinct anomalies in capsular localization and polymer length. Apparently, the bacteria are employing a sweet spot in the CpsABCD system, where the current equilibrium results in CPS of a 'suitable' length and that is securely anchored to the cell surface.

The reason why GBS produces a CPS of a particular design represents a

fascinating subject. In an attempt to understand the biological consequences of CPS deviations, we performed *in vitro* biofilm and cell infection assays. By comparing the wild type and the Δ*cpsE* mutant, we observed that the unencapsulated strain is less efficiently adhering both to polystyrene plates and to the A549 pulmonary cells layer. In contrast, the mutant strain showed an increased percentage of invading bacteria in comparison with the wild type. These results suggest that invasion is attenuated by the CPS and that on the other hand the CPS is necessary to promote association to cells. Inhibition of cell invasion by the CPS has already been reported using GBS and different cell types (Gibson *et al.*, 1995, Hulse *et al.*, 1993). However, our results are partially in contrast with previously published data showing that strains devoid of CPS were more efficient both in cells adhesion and invasion (Soriani *et al.*, 2006). With regard to the *in vitro* biofilm assay, our findings are similar to those reported by Xia and coworkers (Xia *et al.*, 2014).

To our knowledge the impact of the differences in CPS amount and length on the adhesion-invasion process has not been investigated in GBS or other related bacteria. To this aim, we selected a panel of mutant strains with different capsule phenotypes and we analyzed them using the assays described. We observed that increased CPS lengths caused defects in adhesion both to polystyrene plates and to the A549 pulmonary cells layer. These findings suggest that long CPS may mask specific components important for adherence or may nonspecifically attenuate this process by steric hindrance or surface charge (Absolom, 1988). Interestingly, also the strains that were completely devoid of CPS or producing very little CPS were poorly associated to cells, thus suggesting that the presence of the polysaccharide is a prerequisite for adhesion to this cell line. From the invasion assay we observed that the unencapsulated strain and the mutants producing very little CPS were more efficient in invading cells irrespective of the CPS length. This result confirms the theory proposed for different encapsulated bacteria that the CPS attenuate the invasion process (Soriani *et al.*, 2006, Malin & Paoletti, 2001). In strains that were shedding the CPS, we observed that the presence of the CPS in the medium does not have an apparent impact on GBS association to A549 cells. Analyzing strains with very

little but long CPS attached to the bacterial surface (the Δ*cpsD* and the CpsD(K49A) mutants) we saw that the strain releasing the CPS in the medium was significantly less efficient in cell invasion compared to the other strain. Experiments showed that preincubation of the cell monolayer with purified capsule is not inhibiting the invasion of the cells by GBS (Hulse *et al.*, 1993) so the reduced invasion that we observed could be an effect of the reduced association.

In conclusion our results showed that strains with CPS length different from the wild type were defective in associations to lung epithelial cells *in vitro*. Moreover, we observed that the unencapsulated strain and the mutants producing very little CPS were more efficient in invading these cells, irrespective of the CPS length. However, strains without CPS are known to be more susceptible to killing by human neutrophils and less virulent (Wessels *et al.*, 1989, Marques *et al.*, 1992). These findings suggest two possible scenarios, the first is that during evolution GBS may have found an equilibrium resulting in a CPS with specific features that ensure the best trade-off to adapt to the different environment encountered during pathogenesis. The second is that the length of the CPS or its amount may be regulated in response to external factors, i.e. reducing CPS production to promote intracellular invasion and increasing capsule expression to evade host defenses. A recent paper showed that the phosphatase activity of the CpsB homologue in *S. pneumoniae* increases with growth in high-oxygen conditions causing a reduction in the total amount of CPS produced (Geno *et al.*, 2014). Chemical compounds able to inhibit the activity of this protein have been described and were shown to reduce CPS production in *S. pneumoniae* (Standish *et al.*, 2012). However, taken together, these two works underline that there is not a clear correlation between the activity of the CpsB phosphatase and the amount of CPS produced by *S. pneumoniae.*

The CPS of GBS and of other bacteria represents an important virulence factor involved in several aspects of bacterial pathogenesis. With this work we have shed light on the mechanism used by GBS to ensure the attachment of the CPS to the cell wall and to indirectly determine the length of this molecule. We also observed that differences is CPS size, amount and localization are correlated

to different adhesion-invasion properties. However, the understanding of whether CPS in GBS and other bacteria adapts to the external environment remains a fascinating subject for future studies. We believe that a detailed comprehension of the CPS biosynthesis pathway would not only be scientifically exciting, but would also permit to identify target mechanisms for drug design and biotechnological applications.

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PUBLICATIONS

The results of the scientific work presented in this thesis led to the production of a manuscript and to the filing of two patent applications:

- C. Toniolo, E. Balducci, M. R. Romano, D. Proietti, I. Ferlenghi, G. Grandi, F. Berti, I. Margarit Y Ros, and R. Janulczyk. *Streptococcus agalactiae* capsule polymer length and attachment is determined by the proteins CpsABCD. J. Biol. Chem. - under revision.

- E. Balducci, F. Berti, I. Margarit Y Ros, R. Janulczyk, C. Toniolo. European patent application, "Purification of secreted polysaccharides from S. agalactiae". Filed May 2014.

- E. Balducci, F. Berti, R. Janulczyk, C. Toniolo. European patent application, "Polysaccharides produced by CpsC mutants". Filed May 2014.

Other contributions during the Ph.D. studies:

- Berti, F., E. Campisi, C. Toniolo, L. Morelli, S. Crotti, R. Rosini, M.R. Romano, V. Pinto, B. Brogioni, G. Torricelli, R. Janulczyk, G. Grandi & I. Margarit, (2014) Structure of the type IX group B Streptococcus capsular polysaccharide and its evolutionary relationship with types V and VII. J. Biol. Chem. 289: 23437-23448.

- R. Rosini, E. Campisi, M. De Chiara, H. Tettelin, D. Rinaudo, C. Toniolo, M. Metruccio, S. Guidotti, U. B. S. Sørensen, M. Kilian, M. Ramirez, R. Janulczyk, C. Donati, G. Grandi, I. Margarit Y Ros. Genomic analysis reveals the molecular basis for capsule loss in the Group B Streptococcus population. PlosONE - submitted.

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