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Streptococcus agalactiae adapts to glucose stress conditions by modulating gene expression profile

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“Success is not final, failure is not fatal: it is the courage to continue that counts”

Winston Churchill

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1. ABSTRACT

Diabetes mellitus is considered a risk factor for Group B *Streptococcus* (GBS) infections. Typically, this pathology is associated to high glucose levels in the bloodstream. Although clinical evidences support this notion, the physiological mechanisms underlying GBS adaptation to such conditions are not yet defined. In the attempt to address this issue, we performed comparative global gene expression analysis of GBS grown under glucose-stress conditions and observed that a number of metabolic and virulence genes was differentially regulated. Of importance, we also demonstrated that by knocking-out the *csrRS* locus the transcription profile of GBS grown in high-glucose conditions was profoundly affected, with more than a third of glucose-dependent genes, including the virulence factor *bibA,* found to be controlled by this two-component system. Furthermore, *in vitro* molecular analysis showed that CsrR specifically binds to the *bibA* promoter and the phosphorilation increases the affinity of the regulator to this promoter region. Moreover, we demonstrated that CsrR acts as a repressor of *bibA* expression by binding to its promoter *in vivo*. In conclusion, this work by elucidating both the response of GBS to pathological glucose conditions and the underlined molecular mechanisms will set the basis for a better understanding of GBS pathogenesis.

2. INTRODUCTION

2.1 Group B Streptococcus

Streptococcus agalactiae, also named Group B Streptococcus or GBS, is an encapsulated Gram positive coccus. In 1933, Rebecca Lancefield identified the group B antigen, a cell wall-associated carbohydrate that distinguishes GBS from other streptococcal species (Lancefield, 1934). It forms small 3 to 4 mm, grey-white colonies that have a narrow zone of beta hemolysis on blood agar plate.

GBS strains are classified into ten serotypes according to immunogenic characteristics of the capsule polysaccharides (Ia, Ib, II, III, IV, V, VI,VII, VIII and IX). Approximately 10% of serotypes are non-typeable (Kong *et al*., 2002; Bisharat *et al*., 2005; Gherardi *et al*., 2007; Skoff *et al*., 2009)

GBS is principally a microbe of bovine and human origin although strains have been isolated from fish, dogs, piglets and occasionally from other animal species. There is no definitive evidence that infected cattle serve as a reservoir for transfer of the Group B Streptococcus to human. Indeed different studies have demonstrated biochemical, biological and serological differences between bovine and human strains.

Group B organisms were only rarely considered as agents of human infections while recognized as commensals among the normal flora of human upper respiratory tract and the female genitourinary tract. More than 30 years ago, the attention to Group B Streptococcus as a major cause of neonatal sepsis dramatically increased. In that period, in fact, half of the patients with GBS

infection died. Up to now, remain unclear the reason for the emergence of Group B Streptococci as etiological agent of neonatal disease.

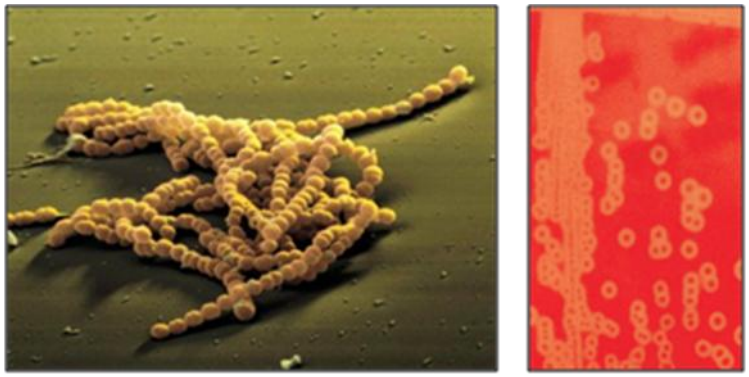

Figure 2.1 *Streptococcus agalactiae*. (A) Scanning Electron Microscopy (SEM) of *Streptococcus agalactiae.* (B) Colonies *Streptococcus agalactiae* on a blood agar plate. Note the zone of clear haemolysis.

2.2 Epidemiology of GBS

Group B Streptococcus colonizes the urogenital tract of more 30% of the healthy population and in particular it colonizes the vagina of 25-40% of healthy women (Dillon *et al*., 1982; Schuchat, 1998; Hansen *et al*., 2004). It has been found in the urethra in both men and women without causing infections and in the upper respiratory tract. Colonization also is observed in wound and soft tissue cultures in the absence of obvious infection. Determining the acquisition and transmission of *S. agalactiae* can be puzzling, as it is very invasive but produces little inflammation at the entry site.

This bacterium is an important cause of infection in three populations:

- Pregnant women

- Neonates
- Nonpregnant adults

GBS causes a variety of perinatal infections in pregnant women, including both symptomatic and asymptomatic bacteriuria, endometritis, amnionitis, meningitis, pyelonephritis, and post partum wound infections (Pass *et al*., 1982). It also has been suggested that GBS urinary tract infections or urinary tract, rectal, or genital colonization in pregnant woman may lead to late term abortions and preterm and low-birth-weight infants. Pregnant women are colonized at multiple sites, including rectum, vagina, cervix and throat, but many of them carry GBS in asymptomatically way (Regan *et al*., 1991). However, GBS colonization in pregnant women is important because of the risk for transmission to their newborns. Most infections and colonization of newborns are due to aspiration of contaminated vaginal and amniotic fluid before or during parturition (Doran & Nizet, 2004). This pathogen is the leading cause of neonatal bacterial diseases in the United States of America; infection in newborns has been divided in early-onset disease (EOD) and late-onset disease (LOD) depending on the infants' age and disease manifestations. Infants with EOD most commonly have sepsis or pneumonia while meningitis and bone and soft tissue infections can also occur (Edwards, 2001; Puopolo *et al*., 2005). LOD is less frequent than EOD and the mortality rate is lower. In contrast morbidity is high, as around 50% of neonates that survive to GBS infection suffer complication, including mental retardation, hearing loss and speech and language delay (Schuchat, 1998; Schrag *et al*., 2000; Edwards, 2001).

Although GBS is commonly associated with neonatal diseases (Johri *et al*., 2006) and thought of as causes of disease in pregnant women, it causes substantial

morbidity and mortality among non pregnant adults (Fig. 2.2) and appears to be increasing in incidence in that population.

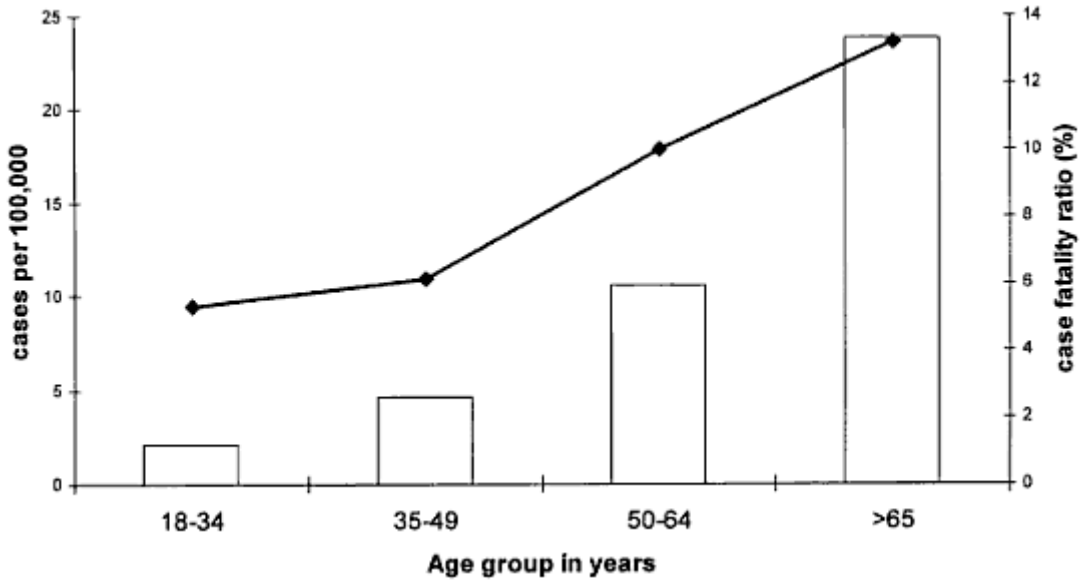

Figure 2.2 Incidence of invasive Group B streptococcal disease by age (case per 100000) (open bars) and case fatality ratio (%) (Black line) in person > 18 years of age (Gram-positive pathogens - Vincent A. Fischetti ,American Society for Microbiology, 2006).

The reported annual incidence of GBS infection in nonpregnant adults in the general population is between 4 and 7 per 100,000 (Schrag *et al*., 2000; Zangwill *et al*., 1992 Farley *et al*., 1993; Blumberg *et al*., 1996; Phares *et al*., 2008; Skoff *et al*., 2009). However, the risk is as high as 26 per 100,000 in patients ≥65 years of age (Schrag *et al*., 2000; Phares *et al*., 2008). This risk reflects an increase in incidence between 1999 and 2005 in a population-based surveillance study in ten states in the United States (Phares *et al*., 2008). In view of the reductions in GBS infection in neonates and pregnant women, GBS infection in adults is now estimated to account for over three-fourths of invasive GBS disease in the United States and for 90 percent of the mortality. At least 1300 GBS-related deaths

occurred among elderly persons in 2003; the case fatality rate for elderly adults is estimated at 15 percent (Edwards and Baker, 2005).

Of ten known GBS serotypes, the most commons reported among adults are types Ia, III and V. A population- based study found serotype V to be the most common one causing disease in non pregnant adults and the second most common serotype in pregnant women (Harrison *et al*., 1998); in fact more than 25% of the subjects had invasive GBS disease caused by type V strains (Table 2.1).

Table 2.1 Serotype distribution of Group B streptococcal isolates from non-pregnant adults with invasive GBS infection, 1992-1999 (Edward and Baker, 2005).

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GBS serotype	N° (%) of subjects (n=589)
Ia	143 (24.3)
Ib	72 (12.2)
II	70 (11.9)
III	97 (16.5)
IV	2 (0.3)
V	162 (27.5)
VI	0
VII	0
VIII	1 (0.2)
Non-typeable	42 (7.1)

Numerous studies have allowed description of the clinical spectrum of disease, including clinical features, risk factors, therapy, and outcomes. The most common syndromes caused by GBS in adults are skin, soft tissue, and bone infections. These infections are often complications of chronic diabetes or decubitus ulcer. Cellulitis, foot ulcers, and abscesses are the most common manifestation, but also necrotizing fasciitis have occasionally been reported (Edwards and Baker, 2005). Patients with indwelling catheters are at higher risk for GBS bacterimia; polymicrobial bacteremia, often with *Staphylococcus aureus* is identified in 26 to 30% of patients with GBS colonization (Jackson *et al*., 1995). GBS can also cause pneumonia, increasing the mortality rate of patients. Less common GBS infections such as arthritis, urinary infection, meningitis, and peritonitis can also occur, especially in patients with common predisposing factors, such as diabetes, osteoarthritis, and underlying joint disease. *S agalactiae* infection is extremely rare in healthy individuals and is almost always associated with underlying abnormalities. Risk factors that promote GBS infections include diabetes mellitus, elderly, malignancy, liver disease, neurological deficits, renal failure, other forms of immune impairment such as human immunodeficiency virus infection, cancer and venous insufficiency (Jenkins *et al*., 2010).

Elderly patients with diabetes mellitus have displayed peripheral neuropathy or peripheral vascular diseases following trauma, particularly to the lower extremities. GBS takes advantage of this condition by crossing the endothelial barrier and promoting bacterial invasion of the foot (Edwards and Baker, 2005). Indeed, GBS is also found in biopsy of patients with foot infections, a common status of patients suffering from diabetes (Urban *et al*., 2011). Group B streptococcal infection in elderly people (≥70 years) is strongly linked to

congestive heart failure and being bedridden, with urinary tract infection, pneumonia, and soft-tissue infection as the most common manifestations of infection. Neurologic illness is associated with pneumonia in elderly people, possibly due to aspiration of Group B *Streptococci* from the upper respiratory tract.

2.3 Molecular pathogenesis of GBS

Group B Streptococcus infection in human is a complex and multifactorial process which involves several virulence determinants that contribute to neonatal disease. An important role is mediated by the capsule, which remains together to the β-haemolytic activity the main virulence factor for Group B Streptococcus. In addition, a number of molecules both surface-exposed or secreted are necessary for GBS infection process (Table 2.2).

The GBS pathogenic process can be described in four main steps:

A. Colonization of mucosal surfaces;

B. Translocation through host cellular barriers;

C. Evasion of immunological clearance;

D. Activation of inflammatory response.

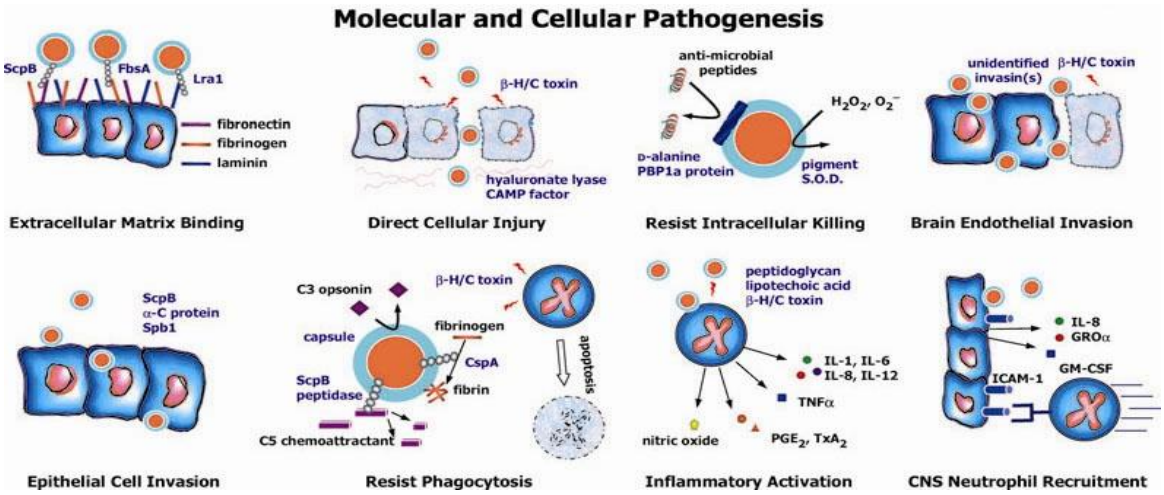

Figure 2.3 Schematic representation of the molecular and cellular pathogenesis of GBS (Doran and Nizet, 2004).

A. Colonization of mucosal surfaces

GBS adheres to a variety of human cells including vaginal, intestinal and respiratory tract epithelium, blood–brain barrier endothelium and placental membranes. In particular GBS behaves as a commensal organism that colonizes the lower gastrointestinal and genital tracts of healthy adults occupying a niche that places infants at risk of vertical transmission. The interaction between GBS and epithelial cells is mediated by several bacterial molecules that interact with both low and high affinity with different host cell components (Wibawan *et al*., 1992).

Several studies have demonstrated that GBS is able to interact with the extracellular matrix (ECM) components like fibronectin, fibrinogen and laminin. Indeed, GBS binds to immobilized fibronectin (Tamura and Rubens, 1995) but not to the soluble form (Butler *et al*., 1987). The GBS fibronectin-binding property

seems to be associated with the surface-anchored C5a peptidase, ScpB (Beckmann *et al*., 2002). The binding to fibronectin facilitates mucosal colonization and mediates GBS internalization into host cells (Cheng *et al*., 2002a). On the other hand, the adherence of GBS to laminin involves the laminin binding protein, Lmb (Spellerberg *et al*., 1999), while attachment of GBS to fibrinogen is mediated by two unrelated surface fibrinogen binding protein, FbsA and FbsB (Gutekunst *et al*., 2004; Schubert *et al*., 2002). GBS binding to fibrinogen and fibronectin is positively regulated by the transcriptional regulator RogB, which modulates the expression of a number of genes coding ECM binding proteins (Gutekunst *et al*., 2003). The recently described pilus-like structures present on the surface of GBS (Lauer *et al*., 2005) also seems to be involved in the adherence of bacteria to lung epithelial cells. In particular the knock-out mutant strain for the ancillary protein shows an impaired ability to adhere to epithelial cells (Maisey *et al*., 2006).

B. Translocation through host cellular barriers

The strategy used by GBS to traverse defined anatomic epithelial barriers, such as the cervical or vaginal epithelium, entering bloodstream is still poorly understood. However, the GBS ability to penetrate host cellular barriers is the first distinguishing feature of its pathogenicity.

GBS has the ability to invade chorionic but not amniotic epithelial cells (Winram *et al*., 1998). Nevertheless, GBS can traverse placental membranes, weak their tensile strength and access to the fetus within the amniotic cavity. This process induces placental membrane rupture or trigger premature delivery. After

aspiration of infected amniotic or vaginal fluid, the initial focus of GBS infection takes place in the newborn lung. From there, the organism rapidly gains access to the bloodstream and is circulated through other organs and tissues.

GBS has the ability to invade both alveolar epithelial and pulmonary endothelial as initially noted in newborn macaques (Rubens *et al*., 1991), and later confirmed in human tissue culture lines derived from both cellular barriers (Gibson *et al*., 1993; Rubens *et al*., 1992). Cellular invasion by GBS occurs when the organism triggers its own endocytotic uptake and enters the cell within a membrane-bound vacuole. In the host, this process requires rearrangement of microfilament components of the cytoskeleton and the signalling pathways mediated by PI 3-kinase, seem to be involved (Tyrrell *et al*., 2002).

There is a close correlation between cellular invasion and GBS virulence potentiality because clinical isolates from infants with bloodstream infections invade epithelial cells better than strains from the vaginal mucosa of asymptomatic women (Valentin-Weigand and Chhatwal, 1995). Other proteins like ScpB or the alpha C surface protein are involved in GBS epithelial cell invasion (Bolduc *et al*., 2002; Cheng *et al*., 2002b).

Early-onset GBS pneumonia is characterized by widespread damage to lung epithelium and endothelium, with haemorrhage and neutrophils entering the alveolar airspaces. GBS enters the bloodstream as a consequence of the loss of barrier integrity and the β-haemolysin/cytolysin (β-H/C) appears the protein largely involved in this process. *cylE* is the gene necessary and sufficient for GBS β-H/C expression (Pritzlaff *et al*., 2001). This pore-forming toxin lyses lung epithelial and endothelial cells and compromises their barrier function (Gibson *et al*., 1999; Nizet *et al*., 1996). Besides, the GBS β-H/C promotes GBS intracellular

invasion and triggers the release of interleukin-8 (IL-8) that is the principal chemoattractant for human neutrophils (Doran *et al*., 2002).

GBS is also the leading cause of bacterial meningitis in human newborns and the bacterium has the propensity to breech the specialized endothelium comprising the human blood–brain barrier. In fact, it has been reported that COH1, a highly encapsulated GBS strain, was able to invade and translocate across a polarized brain microvascular epithelial cell monolayer without marked changes in transendothelial electrical resistance (Nizet *et al*., 1997). As for the epithelial cell barriers, the GBS β-H/C is directly cytolytic for human brain endothelial cells (Doran *et al*., 2003).

GBS virulence factors play a pivotal role in penetration of host cellular barriers. It is reported that bloodstream isolates of GBS secrete high levels of an enzyme that degrades hyaluronic acid, the main polysaccharide component of host connective tissue (Kjems *et al*., 1980; Pritchard and Cleary, 1996). Another important extracellular protein involved in invasion is CAMP factor; this protein oligomerizes in the target membrane to form discrete pores and triggers cell lysis (Lang and Palmer, 2003) and it is toxic when injected intravenously in rabbits (Skalka and Smola, 1981).

Recently, it has been proposed a novel paracellular route used by GBS to traverse epithelial cells monolayer. In this new model, the crossing of bacteria across the cell is not associated with the loss of monolayer's integrity suggesting that paracellular translocation is likely to be an active but transient phenomenon used by GBS to translocate from the site of colonization to target organs (Soriani *et al*., 2006).

C. Evasion of immunological clearance

Phagocytic cells including polymorphonucleates (PMNs) and macrophages are the first line of defence against GBS and their early action determines to outcome of the infection. However, the effective uptake and killing of GBS by these cells requires opsonization of the bacterium and deposition of complement components (Jarva *et al*., 2003). Neonates that are deficient in a) phagocytic cell function, b) specific anti-GBS immunoglobuling and c) classic and alternate complement components, are also particularly prone to GBS invasive disease.

Polysaccharide capsule is fundamental to the avoidance of immune response. Indeed, sialylated GBS capsule protects the bacterium by PMNs mediated opsonophagocytic killing, preventing C3 deposition on the bacterial surface. Isogenic GBS mutant defected for capsule synthesis is more susceptible to killing by PMNs. As a consequence, lethal doses of capsule-deficient strains are in mice 100-fold greater than the one of the parental wild-type strain (Marques *et al*., 1992; Wessels *et al*., 1989).

Other multifunctional GBS determinants that contribute to bacterial resistance to the host clearance mechanisms have been identified (Jarva *et al*., 2003). Among them, FbsA seems to be important for the survival of bacteria in human blood (Schubert *et al*., 2002). The beta-C protein binds the Fc domain of human IgA, potentially sequestering this important host mucosal defence molecule (Jerlstrom *et al*., 1996), while GBS strains expressing the alpha-C protein appear more resistant to phagocytic killing (Madoff *et al*., 1991). Besides, the presence of tandem repeats within the alpha C sequence is correlated to antigenic variability that allows the bacterium to avoid opsonophagocytic killing triggered by specific

antibody (Madoff *et al*., 1996). The C5a-ase possesses a domain that specifically cleaves human complement component C5a, a chemoattractant for human PMNs, reducing the acute neutrophils response to sites of infection (Bohnsack *et al*., 1997). A recently identified novel cell surface protease named CspA targets host fibrinogen, producing adherent fibrin-like cleavage products that coat the bacterial surface and interfere with opsonophagocytic clearance (Harris *et al*., 2003).

The application of signature-tagged mutagenesis for *in vivo* screening in a GBS neonatal rat sepsis model has identify unexpected virulence genes encoding factors that are crucial for the bacterium survival to immune clearance. For example, *ponA*, which codes for an extracytoplasmic penicillin-binding protein (PBP1a), promotes resistance to phagocytic killing independent of capsule (Jones *et al*., 2003) and protects form cationic anti-microbial peptides (defensins, cathelicidins) produced by host epithelial cells and phagocytes (Hamilton *et al*., 2006). An analogue function is associated with the D-alanylation of lipotechoic acid in the bacterial cell wall (Poyart *et al*., 2003).

The GBS β-H/C triggers cytolytic events in macrophages and neutrophils (Liu *et al*., 2004) and can also induce macrophage apoptosis (Buratta *et al*., 2002; Ulett *et al*., 2003) Curiously, GBS has been shown to survive for prolonged periods within the phagolysosome of macrophages and to be >10-fold more resistant to hydrogen peroxide killing than catalase-positive *Staphylococcus aureus* (Wilson and Weaver, 1985). Superoxide dismutase (SodA) (Poyart *et al*., 2001) and the orange carotenoid pigment (Liu *et al*., 2004) are the main defences of GBS against oxidative stress. Indeed, the pigmentation is a property unique among haemolytic streptococci and genetically linked to the *cylE* gene. Carotenoids

neutralize hydrogen peroxide and singlet oxygen, therefore providing a shield against the key elements of phagocyte oxidative burst killing (Liu *et al*., 2004).

D. Activation of inflammatory responses

The host inflammatory response to GBS invasive infections, is associated to the sepsis syndrome and multiorgan dysfunction. Peptidoglycan and other GBS components associated with the cell wall, not including the surface polysaccharide capsule, appear to be the most provocative agents in triggering host cytokine cascades, in particular the proximal mediators tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1). GBS induction of NF-kB signalling and TNF-α release from human monocytes *in vitro* requires CD14 and the receptors for complement components 3 and 4 (Medvedev *et al*., 1998). Recently, the importance of complement components in amplifying GBS TNF-α induction was corroborated when reduced levels of the cytokine were observed in the blood of C3 or C3 receptor-deficient mice stimulated with GBS (Levy *et al*., 2003). Knockout mouse studies indicate GBS cell wall peptidoglycan-induced activation of p38 and NF-kB, depends on the cytoplasmic TLR adaptor protein MyD88, but does not proceed via the well studied TLR2 and/or TLR4 (Henneke *et al*., 2002). Of interest, GBS β-H/C and cell wall components act synergistically to induce macrophage production of inducible nitric oxide synthase (iNOS) and generation of nitric oxide (NO) (Ring *et al*., 2002), a potent factor in the sepsis cascade. LOD is characterized by meningitis with or without accompanying sepsis Localization of GBS in the brain and Central Nervous System (CNS) during LOD, triggers a strong host inflammatory response. Indeed, in the infant rat model,

early GBS meningitis is characterized by acute neutrophilic inflammation in the subarachnoid space and ventricles, vasculopathy and neuronal injury. The initiation of the CNS inflammatory response is triggered by the blood–brain barrier endothelium, which activates a specific pattern of gene transcription for neutrophil recruitment, including production of chemokines, endothelial receptors and neutrophil activators (Doran *et al*., 2003). The principal provocative factor for the blood–brain barrier inflammatory gene response is the GBS β-H/C, and this toxin has also been shown to contribute to the development of meningitis (Doran *et al*., 2003).

Table 2.2 Key virulent factors of Group B Streptococcus (Doran and Nizet, 2004)**.**

Virulence factor	Genetic basis	Chemical nature	Molecular or cellular action(s)	Proposed contribution(s) to disease pathogenesis
Exopolysaccharide surface capsule	*cps*A-L, *neu*A-D	High-molecular-weight polymer with terminal sialic acid residues	Impairs complement C3 deposition and activation Decreases immune recognition, perhaps through molecular mimicry of host sialic acid epitopes	Blocks opsonophagocytic clearance Delays neutrophil recruitment
β-Haemolysin/cytolysin	*cyl*E	CylE protein (79 kD)	Forms pores in cell membranes Induces apoptosis Promotes cellular invasion Triggers iNOS, cytokine release	Direct tissue injury Penetration of epithelial barriers Induction of sepsis syndrome Phagocytic resistance
+ linked pigment	*cyl* locus	Carotenoid	Antioxidant effect blocks H_2O_2, singlet oxygen	Impairment of oxidative burst killing
Hyaluronate lyase	*hyl*B	HylB enzyme (110 kD)	Cleaves hyaluronan and chondroitin sulphate	Spread through host tissues Impairment of leukocyte trafficking
C5a peptidase	*scp*B	ScpB protein (120 kD)	Cleaves human C5a Binds fibronectin	Inhibit PMN recruitments Extracelluar matrix attachment Epithelial adherence and invasion
CAMP factor	*cfb*	CAMP protein (24 kD)	CAMP reaction (co-haemolysin) Binds to Fc portion of IgG, IgM	Direct tissue injury Impairment of antibody function
Lipotechoic acid	Complex	Amphiphilic glycerol phosphate polymer of complex lipids and short-chain fatty acids	Binds host cell surfaces Binds host pattern recognition receptors (TLRs) Alanylation inhibits host anti-microbial peptides	Epithelial cell attachment Activation of the sepsis syndrome Resistance to neutrophil killing
C protein (alpha and beta components)	*bca* (alpha) *cba* (beta)	Alpha: protein with mutiple identical tandem repeats (14–145 kD); beta: 84–94 kD variants	Binds cervical epithelial cells Blocks intracellular killing by neutrophils non-immune binding of IgA	Epithelial cell adherence Epithelial cell invasion Resistance to phagocytic clearance
Serine protease	*csp*A	CspA protein (142 kD)	Cleaves fibrinogen to fibrin-like fragments	Resistance to phagocytic clearance? Promotes tissue spread
Fibrinogen receptor	*fbs*A	FbsA protein (44.2 kD)	Binds fibrinogen through repetitive structure motifs	Extracellular matrix attachment Epithelial adherence Resistance to opsonophagocytic killing

2.4 Regulation of gene expression

The GBS pathogenesis implies that this bacterium can survive in a large number of human body compartments, encountering different environmental conditions, such as different pH, availability of carbon source and temperature. The modulation of gene expression allows the adaptation through different mechanisms of regulation that control the production of proteins involved in adhesion, nutrient acquisition, survival to host immune system (Sitkiewicz *et al*., 2009; Mereghetti *et al*., 2008; Mereghetti *et al*., 2009).

A. Two component regulatory system

One common mechanism used by bacteria to regulate gene expression is the alteration of sigma factors associated with RNA polymerase. In light of paucity of sigma factors, GBS may rely more on other mechanisms for regulation on gene expression. GBS strains generally possess approximately 17-20 predicted two component regulatory systems (TCS) (Glaser *et al*., 2002; Tettelin *et al*., 2002; Tettelin *et al*., 2005), a number significantly grater than has been reported for closely related species such as GAS (13) or *Lactococcus lactis* (8) (Bolotin *et al*., 2001; Ferretti *et al*., 2001). TCSs allow for the sensing of specific environmental stimuli or conditions followed by transduction of the signal to a response regulator. In the basic model, a membrane-bound histidine protein kinase (sensor) is autophosphorilated when the signal is detected. The phosphoryl group is then transferred to the cytoplasmatic response regulator. Phosphorilation of the regulator alters its binding affinity for the promoters of target genes, thereby affecting their transcription (Stoch *et al*., 2000). The best characterized TCS in

GBS is the CsrS histidine kinase and CsrR response regulator pair, also known CsrRS (for capsule synthesis regulator, regulator and sensor components; also called CovRS). This TCS was originally identified by two separate groups, both of which demonstrated that it has global effects on gene expression that impact the virulence of GBS (Lamy *et al*., 2004; Jiang *et al*., 2005). The GBS CsrRS regulatory system is an ortholog of the GAS CovRS system that is known to be important for GAS virulence (Levin and Wessels, 1998; Federle *et al*., 1999). In GBS, *csrS* (*sag1624*) and *csrR* (*sag1625*) are components of a seven-gene operon that is not regulated by CsrR (Lamy *et al*., 2004). The function of other genes in the operon has not been fully elucidated. In a comprehensive analysis of this TCS, Lamy and colleagues reported that a *csrRS* deletion mutant displayed multiple phenotypic changes compared to the wild type strain, including increased hemolytic activity, reduced CAMP factor activity and increased adherence. The mutant was also unable to grow normally in human serum. Not surprisingly, the mutant was significantly attenuated for virulence in the neonatal rat sepsis infection model (Lamy *et al*., 2004). The phenotypic changes that occurred in the *csrRS* mutant have been confirmed using microarrays to assess changes in gene expression. In GBS, the CsrRS TCS was shown to affect 140 genes during grown *in vitro*, with en equal numbers up-regulated and down-regulated. Many of affected genes encode proteins predicted to be secreted or localized to the cell surface. It is interesting that the target genes regulated by the CsrRS TCS in GBS and in GAS are not identical, despite the fact that the two systems are orthologous. The differences in target genes may be a reflection of the different host compartments encountered by these two pathogens.

Whereas the *S*. *pyogenes* system represses expression of all the major virulence factors it is known to control, Jiang and colleagues indicate evidence of both up- and down-regulation of different virulence determinants by GBS CsrRS. The GBS CsrRS system has divergent effects on different target genes or that it has regulatory activity on another regulator that, in turn, acts on one or more regulated structural genes. Interaction of the CsrRS system with other regulators could be part of a regulatory cascade or network in which multiple elements link an environmental stimulus with a series of downstream responses. The possibility of one or more intermediate regulators could explain the observed up (CAMP factor) and down (beta-hemolysin and C5a peptidase)-regulatory effects on different target genes (Jiang *et al*., 2005).

CsrRS has a pivotal role in GBS phatogenesis of GBS infection; in fact transcriptional analysis showed that up to 7% of genes are under the control of this TCS (Jiang *et al*., 2008; Lamy *et al*., 2004). The genes regulated by CsrRS belong to differential functional categories, as cell envelope, cellular processes, metabolism, and virulence factor. Recently, it has been reported that CsrRS has an important role in the translocation of GBS from the acid pH of the vagina to the neutral pH of the newborn tissues, promoting a changing from a colonizing to an invasive phenotype (Santi *et al*., 2009).

B. Carbon catabolite repression

Carbon metabolism and its regulation are central to prokaryotic life. Sugars serve as the most facile source of carbon and energy, both of which are needed to replenish essential nucleotide cofactors and other metabolites in the cell. When

faced with a wide variety of carbon and energy sources, a bacterium has to make metabolic decisions, opting for preferential use of one source over another in order to maintain optimal growth (Deutscher, 2008; Stulke and Hillen, 1998; Titgemeyer and Hillen, 2002). Simultaneous utilization of all available sugars would be metabolically inefficient and would lead to slower growth. The ability to utilize preferred sugars depends on a regulatory process called carbon catabolite repression (CCR) (Stulke and Hillen, 1999; Titgemeyer and Hillen, 2002; Warner and Lolkema, 2003). CCR causes silencing of genes specific for the utilization of nonpreferred sugars until the bacterium has consumed the preferred sugar(s).

CCR has been studied in considerable detail in the model free-living, Gram-positive bacterium *Bacillus subtilis* (Stulke and Hillen, 2000; Titgemeyer and Hillen, 2002; Warner and Lolkema, 2003). The main global regulator of CCR in this organism is catabolite control protein A (CcpA) (Chauvaux, 1996; Henkin *et al*., 1991). CcpA belongs to the LacI/GalR family of activator-repressor transcription factors and influences the expression of a wide range of catabolic operons in *B. subtilis* (Belitsky *et al*., 2004; Grundy *et al*., 1994; Grundy *et al*., 1993; Henkin, 1996; Hueck and Hillen 1995; Kim *et al*., 2002; Simpson and Russell, 1998; Stulke and Hillen, 2000; Warner *et al*., 2000; Warner and Lolkema, 2003). CcpA has also been identified to function in the regulation of catabolic operons and catabolite repression in many streptococcal species (Iyer *et al*., 2005; Asanuma *et al*., 2004, Dong *et al*., 2004, Rogers and Scannapieco, 2001, van den Bogaard *et al*., 2000). Candidate genes or operons that are subject to CcpA-dependent CCR are often identifiable by the presence of an operator sequence, called the catabolite-repressible element (*cre*), to which CcpA binds

(Asanuma *et al*., 2004; Kim and Chambliss, 1997; Miwa *et al*., 1997; Ramseier *et al*, 1995). In streptococcal species, several *in vivo* and *in vitro* studies have shown that the *cre* consensus sequence is WTGNAANCGNWNNCW (N is any base and W is A or T), where the underlined bases are involved in CcpA binding (Tomoyasu *et al*., 2010; Kim and Chambliss, 1997; Miwa *et al*., 2000; Schumacher *et al*., 2004; Warner and Lolkema. 2003).

The affinity of CcpA for *cre* sequences is enhanced by binding to another protein, the histidine phosphoprotein (HPr). HPr is an integral component of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), where it normally functions in the transfer of high-energy phosphate from phosphoenolpyruvate to the enzyme II complex during sugar uptake (Deutscher *et al*., 2006; Postma and Lengeler, 1985; Reizer *et al*., 1999). The presence of a preferred sugar, such as glucose, in the medium activates phosphorylation of HPr on a conserved serine residue at position 46 by the Hpr kinase, which itself is activated by metabolites such as the high-energy glycolytic intermediate fructose-1,6-bisphosphate (Brochu and Vadeboncoeur, 1999; Frey *et al*., 2003; Poncet *et al*., 2004; Reizer *et al*., 1998; Thevenot, 1995). CcpA interacts with the phosphoserine form of HPr, P∼Ser-HPr, to form a dimeric complex. This interaction increases the affinity of CcpA for the *cre* box. Binding of this dimeric complex typically causes repression of promoters, facilitating CCR (Asanuma *et al*., 2004; Deutscher *et al*., 1995; Deutscher *et al*., 1994). CcpA residues involved in binding of P∼Ser-HPr (Kraus *et al*., 1998) and those involved in binding of *cre* (Kim and Chambliss, 1997) have been characterized, and the crystal structure of the CcpA-P∼Ser-HPr complex has been recently solved (Schumacher *et al*., 2004). However, several reports have shown that although there is slightly low

affinity, CcpA can recognize and bind to the cre without HPr-(Ser- 46-P) and fructose-1,6-bisphosphate *in vitro* (Tomoyasu *et al*., 2010).

2.5 Involvement of BibA and pullulanase in GBS pathogenesis

Studies of the role of carbohydrate metabolism in streptococcal pathogenesis have focused on two mechanisms: adherence to eukaryotic cells and acquisition of crucial nutrients. Regulation of gene encoding for proteins involved in these pathways could be an important basis for understanding the molecular mechanisms underlying GBS pathogenesis.

A. BibA: Group B Streptococcus immunogenic bacterial adhesin

Recently, Santi and colleagues (Santi *et al*., 2007) have reported the presence of BibA, an immunogenic surface-associated antigen expressed by GBS that is involved in virulence. The protein product of the *bibA* (*sag2063*) gene in the GBS strain 2603 V/R (Tettelin *et al*., 2002) is a polypeptide of 630 amino acids containing a leader peptide (residues 1–27), a N-terminal domain (residues 28–400), a proline rich region (residues 401–568) that consists of 42 copies of a PEAK/PDVK motif, and a canonical cell wall anchoring domain (residues 596–630). The anchoring domain is formed by the consensus LPXTG sequence, followed by a hydrophobic transmembrane segment and a charged C-terminal tail. As shown in figure 2.4, four allelic variants of this protein have been identified: variant I, found in strains 2603 V/R (V) and 18RS21 (II); variant II, in strains NEM316 (III) and 515 (Ia); variant III, in strains CJB111 (V), H36B (Ib), and A909 (Ia); and variant IV, in the COH1 (III) strain.

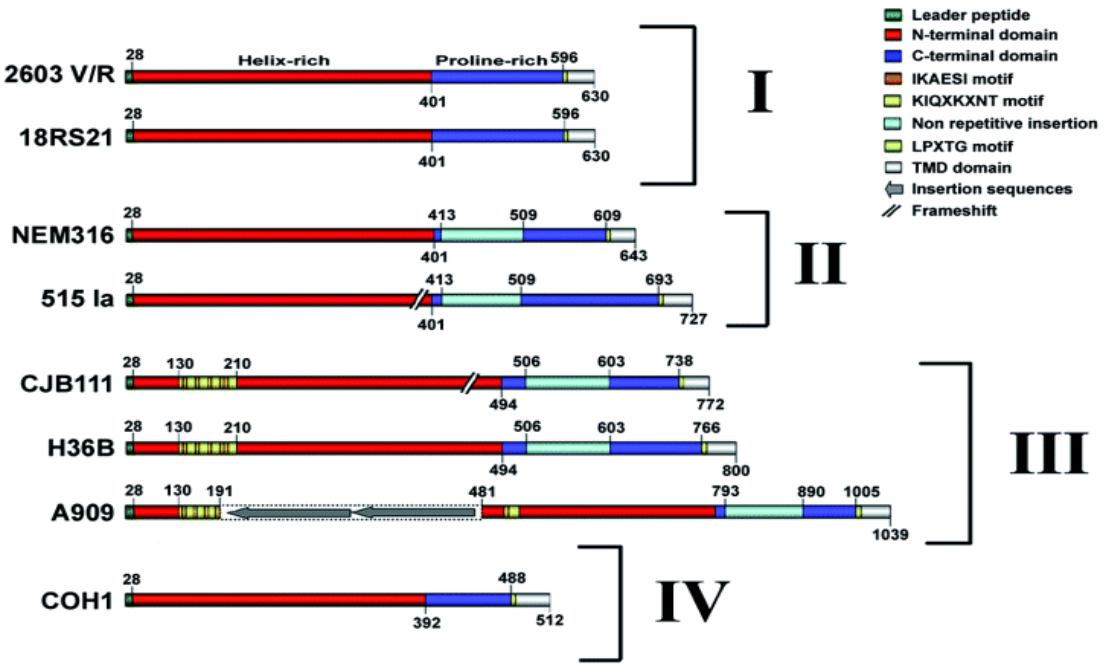

Figure 2.4 Overview of sequence organization in BibA proteins.

BibA is expressed on the surface of several GBS strains, but is also recovered in GBS culture supernatants. The two forms seem to have an identical MW and this suggests that secretion of BibA might be due to a proteolytic cleavage of the cell wall-anchoring domain (Santi *et al*., 2007). Exposure of BibA on the bacterial surface is correlated with protection, because mice immunized with BibA are protected against challenge by a GBS strain with high levels of surface exposure of the antigen. Furthermore, serum samples from mice immunized with recombinant BibA induced neutrophil-mediated *in vitro* opsonophagocytic killing of GBS (Santi *et al*., 2009b).

Deletion of the *bibA* gene severely reduced the capability of GBS to survive in human blood and to resist opsonophagocytic killing by human neutrophils. *bibA* knockout mutant strain of GBS shows an impaired capability to adhere to both

human cervical and lung epithelial cells, demonstrating the effective role of BibA as adhesin (Santi *et al*., 2007).

The presence of this protein on GBS surface is influenced by various environmental factors (Mereghetti *et al*., 2008; Santi *et al*., 2009a). GBS adapts to different environmental conditions by modulating the transcription of genes involved in pathogen-host interaction (Boskey *et al*., 1999). An important condition that affects the gene expression is the pH; in the human host, GBS encounters pH conditions that vary from the acidic pH of the vagina or intracellular endocytic compartments to the near-neutral pH of amniotic fluid or the fetal. Moreover, a comparative global gene expression analysis of GBS grown at acidic and neutral pHs has shown a down regulation of *bibA* expression when GBS is grown at acidic pH compare to a grown at pH 7 (Santi *et al*., 2009a). Furthermore, the regulation of the gene is under the control of two component system CsrRS, in fact the expression level of *bibA* doesn't change in the *csrRS* deletion mutant strain grown in both conditions, meaning that the signal is transducted by these regulatory components.

B. Pullulanase

The use of carbon sources is essential to the ability of bacteria to colonize the host and potentially cause disease in humans. In particular, highly polymerized α-glucan polysaccharides, such as starch and glycogen, are most likely to be found in environmental niches. Indeed, it is known that dietary-derived starches are very abundant in the human colon (Anderson *et al*., 1981; Levitt *et al*., 1987), while glycogen is deposited in large amount in the vaginal ephitelium during times of

high estrogen availability. Because of the complex structures of highly polymerized α-glucans, bacteria require an appropriate combination of enzymes for de-polymerization to oligo- and monosaccharides. Among these enzymes are ascribed pullulanases. Pullulanases have a glycosidic hydrolase activity towards α-glucan polysaccharides and are considered key extracellular components in bacterial metabolism.

Recently it is identified in GBS a novel surface-exposed α-glucan-degrading enzyme, named SAP (*Streptococcus agalactiae* pullulanase), belonging to the streptococcal family of pullulanases (Santi *et al*., 2008). The *sap* gene (*sag1216*) is highly conserved among Group B streptococcus (GBS) strains; homologous genes, such as those for *pulA* and *spuA*, are present in other pathogenic streptococci. SAP is a member of the class 13 glycoside hydrolase (GH13; α-amylase) family and is a type I pullulanase; *in vitro* studies have shown that recombinant SAP can degrade α-glucans such as pullulan, glycogen, and starch (Santi *et al*., 2008). Furthermore, fluorescence-activated scanning analysis and confocal imaging studies performed on whole bacteria indicate that the presence of α-glucan polysaccharides in culture medium upregulates the expression of SAP on the bacterial surface (Santi *et al*., 2008). As reported for other streptococcal pullulanases, specific anti-SAP antibodies are found in human sera from healthy volunteers. Investigation of the functional role of anti-SAP antibodies revealed that incubation of GBS in the presence of sera from animals immunized with SAP reduced the ability of the bacterium to degrade pullulan.

3. Material and methods

3.1 Bacterial strains and growth conditions

GBS type V strain 2603V/R and isogenic mutant strain 2603Δ*csrRS* have been described previously (Tettelin *et al*., 2002; Jiang *et al*., 2005). *E. coli* DH10BT1 and HK100 strains were used for cloning purposes. *E. coli* BL21 (DE3) strain was used for protein production. Unless otherwise specified, for experiments testing the effects of glucose, GBS was cultured at 37°C in Todd-Hewitt broth (Difco) and in a sugar-free complex medium (CM: 10 g/l proteose peptone, 5 g/l trypticase peptone, 5 g/l yeast extract, 2.5 g/l KCl, 1 mM urea, 1 mM arginine). *E. coli* was grown in Luria–Bertani broth and ampicillin was used at a final concentration of 100 µg/ml for recombinant strains.

3.2 Microarray analysis of gene expression

Microarray comparison was performed on the wild-type strain 2603 V/R and the isogenic mutant strain 2603Δ*csrRS*.

The two strains were grown in THB until late exponential phase, washed in PBS and resuspended in CM until OD_{600} of 0.5. The bacteria were washed in PBS and resuspendend in CM in the absence or presence of 1% glucose at 37°C for 30 minutes of incubation. Total RNA was extracted with RNeasy Mini Kit (Qiagen) and treated with RNase-free DNase (Qiagen) according to the manufacturer's instructions. The concentration of total RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA integrity was

verified using a Bioanalyzer 1000 (Agilent). For each strain, total bacterial RNA was isolated from four independent culture pools and the samples were sent to Roche NimbleGen Systems, where cDNA synthesis and labeling were performed. Changes in gene expression levels were evaluated using the NimbleGen GBS DNA microarray (17 probes for each gene, 3 replicates for probe consisting of 60-mer synthetic oligonucleotides for each gene). All hybridizations, staining, and processing were performed by personnel at Roche NimbleGen, Inc. (Madison, WI, USA).

Briefly, to synthesize double-stranded cDNA, 10 ug of RNA were retrotranscripted using the Invitrogen SuperScript Double-Stranded cDNA Synthesis Kit. To eliminate RNA contaminations, a step with RNase was performed. The cDNA was precipitated with phenol:chloroform:isoamyl alcohol and quality control was analyzed using a spectrophotometer. The cDNA was labeled using the NimbleGen One-Color DNA Labeling Kits (labeling with Cy3).

Equal amounts of cDNA was then hybridized onto the Slide 12x135K (Design Name: 100920_TI208435_60mer_HX12) for *Streptococcus agalactiae*, using the NimbleGen Hybridization System and according to protocol "NimbleGen Arrays User's Guide, Gene Expression Arrays, Version 5.1" (NimbleGen Roche). cDNA were hybridized onto microarray at +42°C for 16 hours. After hybridization, microarray was disassembled, washed following the NimbleGen's protocol. The slides were dried in NimbleGen Microarray Dryer (NimbleGen Roche) for 2 minutes and immediately scanned with the MS 200 Microarray Scanner (NimbleGen Roche) following the protocol. Scanned image and data were extracted and analyzed using NimbleScan software. NimbleScan software normalizes expression data using quantile normalization as described by Bolstad,

(Bolstad *et al*., 2003). Gene calls are generated using the Robust Multichip Average (RMA) algorithm as described by Irizarry (Irizarry *et al*., 2003a; Irizarry *et al*., 2003b).

The raw data were analyzed using the DNASTAR software.

The microarray experiment has been submitted to the Array Express database of the European Bioinformatic Institute (http://www.ebi.ac.uk/microarray-as/ae/) with accession number A-MEXP-2195 (Chip design).

3.3 Quantitative reverse transcriptase PCR

Quantitative real-time PCR (qRT-PCR) was used to validate microarray experiments. One microgram of RNA previously extracted for microarray was incubated with random primers and used for cDNA synthesis (at 42°C, one hour incubation) using ImProm-II Reverse Transcriptase (Promega). Fifteen microliters of cDNA were used as template for PCR amplification using gene specific primers as listed in Table 3.1. The amplification was performed using FastStart Universal SYBR Green Master (Rox) (Roche), employing Light Cycler 480 System (Roche). The expression levels of all the genes tested by quantitative RT-PCR were normalized using the *gyrA* expression as an internal standard. Each sample was tested in triplicate during a trial, and three independent experiments were performed.

3.4 Flow cytometry analysis

To verify the exposure of BibA on bacterial surface, GBS was incubated for 30 minutes in CM in the absence or presence of 1% glucose employing the same

conditions used for microarray experiments. After the grown, the bacteria were washed twice with PBS, suspended in newborn calf serum (Sigma), incubated for 20 min at room temperature, and dispensed in a 96-well plate (20 µl per well). The bacteria were fixed in 1% paraformaldehyde for 15 minutes at room temperature. Eighty microliters of anti-BibA immune rabbit serum diluted in PBS/0.1% BSA was added to the bacterial suspension to a final dilution of 1:200. Incubation was performed at 4°C for 1 hour. After washing, bacteria were labeled with R-Phycoerythrin (PE)-conjugated secondary antibodies (final dilution 1:100) (Jackson Immuno Research, PA, USA) at 4°C. Pre immune serum from rabbit was used as a negative control. Bacterial staining was analyzed by using a FACS CANTO II Flow cytometer equipped with three laser system (405, 488, 633 nm), eight Color Configuration and BD FACSDiva^{TM} v6.1.3 software (BD Bioscience, SANJOSE, CA) and the data were analyzed with FlowJo 7.2.2 program.

3.5 SDS-PAGE and Immunoblot analysis

In order to prepare GBS extracts relative to the secreted protein fraction, supernatant of bacteria cultures grown in the same conditions used for microarray were collected. Proteins in 1 ml of supernatant were precipitated with 10% of trichloroaceticacid (TCA) for 1 hr at 4°C. Protein were then pelletted, washed with cold acetone and resuspended in Tris-HCl pH 6,8. Bacterial proteins were separated by 4-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with milk (5% w/v) and, then, incubated with anti-BibA immune rabbit serum at a 1:1000 dilution, secondary antibody (ECL, horseradish peroxidase-

linked anti-mouse IgG, GE Healthcare) at a 1:1000 dilution and developed with ECL enhanced chemiluminescence detection substrate (SuperSignal West Pico, Pierce).

3.6 Cloning, production and purification of recombinant proteins CsrR

The *csrR* gene (*sag1625*) was amplified by PCR from GBS genome using the primers (Fw *csrR*, Rv *csrR*) listed in Table 3.1, carrying the cleavage sites of the restriction enzyme *Nde*I and *Xho*I at 5' end. The PCR product was digested with the specific restriction enzymes and cloned into pET 21b (Novagen) previously linearized with the same restriction enzymes. The construct was introduced into BL21 (DE3) by transformation.

The recombinant bacteria were grown at 37°C to an optical density at 600 nm of 0.5 (mid exponential phase), at which time 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added. After 3 hours, the cells were harvested by centrifugation, resuspended in buffer A (50 mM Na_2HPO_4 [pH 8], 0.3 M NaCl) and distrupted by sonication (10 cycles, 30 sec ON 30 sec OFF). The purification on the soluble fraction was performed with His Gravi Trap columns (GE Healthcare): the lysate was loaded onto the columns and after several washing in buffer B (50 mM Na_2HPO_4 [pH 8], 0.3 M NaCl, 20 mM imidazole) the recombinant proteins were eluted with high concentration imidazole buffer (50 mM Na_2HPO_4 [pH 8], 0.3 M NaCl, 250 mM imidazole). Protein concentration was estimated using the Bradford assay (Bradford, M. M. 1976), and protein content was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE). After the purification, the protein was dialyzed against buffer A to remove the imidazole content.

3.7 Electrophoretic mobility shift assays on *bibA* promoter

Elecrophoretic mobility shift assays were performed in order to verify the binding of CsrR to the *bibA* promoter. Biotin-labeled primers (Fw *bibA*, Rv *bibA*, Table 3.1) were used to amplify DNA fragments corresponding to the promoter region of *bibA* (*sag2063*). As unrelated sequence, *sag0017* promoter was amplified by PCR using the biotin-labeled primers (Fw *sag0017*, Rv *sag0017*, Table 3.1). Various amounts of purified recombinant CsrRS (phosphorylated and not phosphorylated) were incubated with 1 ng of labeled probes in 20 µl of buffer Z (25 mM HEPES, pH 7.6, 50 mM KCl, 12.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 20% glycerol, 0.1% triton) for 20 minutes at room temperature. The reactions were stopped with 2 µl of 50% glycerol and the protein-DNA complexes were separated on native 6% polyacrylamide gels in 0.5X TBE (45 mM Tris, pH 8.0, 45 mM boric acid, 1 mM EDTA) at 100 V (20 V/cm) at room temperature. Afterwards, electrophoretic transfer to a nylon membrane (GE Healthcare) was performed in 0.5X TBE at 380 mA for 45 minutes, and the transferred DNA was cross-linked to the membrane with UV light. After incubation in blocking buffer (2% milk in PBS with 0.5% Triton [PBS-T]) for 1 hour at room temperature, the membrane was incubated with streptavidin-horseradish peroxidase (HRP) conjugate (Pierce) for 1 hour at room temperature at a final dilution 1:1000. The membrane was washed and visualized with SuperSignal chemiluminescence reagent (Pierce). EMSAs were also performed using phosphorylated His-tagged CsrRS: the *in vitro*

modification was obtained incubating 10 µg of protein with 32 mM acetyl phosphate in freshly made phosphorylation buffer (20 mM NaH_2PO_4, [pH 8.0], 10 mM $MgCl_2$, 1 mM DTT) in a total volume of 100 µl for 90 minutes at room temperature (Jiang *et al*., 2004).

The specificity of CsrR binding to the *bibA* promoter was tested by competition EMSA performed using increasing quantities (250x-500x) of either unlabelled *bibA* promoter (used a specific competitor) or *sag0017* promoter (used as a non specific competitor).

3.8 Chromatin immunoprecipitation

The transcriptional role of CsrR in *bibA* gene regulation was investigated *in vivo* by ChIP. GBS wild type strain and isogenic mutant strain 2603Δ*csrS* grown in CM in presence or not of glucose were fixed with 1% formaldehyde at room temperature for 15 minutes under gentle agitation and cross linking reaction was stopped by the addition of glicyne (0.125 M) for 10 minutes. Bacteria were harvested by centrifugation, washed twice in 1 volume of cold phosphate-buffered saline, washed once in 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), and 0.25% Triton X-100, and resuspended in 2 ml TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The samples were sonicated in ice and the average size of sheared DNA was determined to be ~0.5 kb. Cell debris was removed by centrifugation and the supernatant was used as the chromatin input for the immunoprecipitation reactions, after an initial stage of pre-clearing with 100 µl 50% protein A-Sepharose slurry (Pharmacia) for 45 minutes at 4°C. Precleared cell extracts (0.9 ml) were incubated overnight with 10 µl CsrR antiserum in 1x

radioimmunoprecipitation assay (RIPA) buffer (140 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% Na deoxycholate) at 4°C, and CsrR-DNA complexes were immunoprecipitated with 50 µl 50% protein A slurry (preequilibrated in 1x RIPA buffer) for 3 hours at 4°C in sterile disposable minicolumns (Bio-Rad). After four washing steps in RIPA buffer, one in LiCl buffer (250 mM LiCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5% NP-40 [Igepal], 0.5% Na deoxycholate), and twice in TE, the immuno-complex was resuspended in 100 µl TE. Treatment for 30 minutes at 37°C with RNase A (20 µg/ml) and overnight digestion with 50 µg/ml proteinase K in 0.5% SDS at 37°C were performed to recover DNA. Cross-linking was reversed for 6 hours at 65 °C and DNA was extract with organic solvents and resuspended in 100 µl of distillated water. The presence of the target promoter sequences in the chromatin immunoprecipitates was detected by DotBlot analysis (Danielli *et al*., 2006)

3.9 Cloning, expression and purification of recombinant proteins CcpA

The *ccpA* gene (sag*0707*) was cloned into the pET-15 vector (Novagen) using the Polymerase Incomplete Primer Extension (PIPE) methodology described by Klock and Lesley (2009). The primers Fw *ccpA*, Rv *ccpA*, Fw pET-15, Rv pET-15 are listened in Table 3.1. PCR product and vector were directly transformed into *E.coli* HK100 recipient cells. Single ampicillin resistant colonies were selected and checked for the presence of recombinant plasmid by colony PCR. Competent *E.coli* BL21 (DE3) cells were transformed with the plasmids purified from positive clones.

The recombinant bacteria were grown at 37°C to an optical density at 600 nm of 0.5 (mid exponential phase), at which time 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added. After 3 hours, the cells were harvested by centrifugation, resuspended in buffer A (50 mM Na_2HPO_4 [pH 8], 0.3 M NaCl) and distrupted by sonication (10 cycles, 30 sec ON 30 sec OFF). The purification on the soluble fraction was performed with His Gravi Trap columns (GE Healthcare): the lysate was loaded onto the columns and after several washing in buffer B (50 mM Na_2HPO_4 [pH 8], 0.3 M NaCl, 20 mM imidazole) the recombinant proteins were eluted with high concentration imidazole buffer (50 mM Na_2HPO_4 [pH 8], 0.3 M NaCl, 250 mM imidazole). Protein concentration was estimated using the Bradford assay (Bradford, M. M. 1976), and protein content was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After the purification, the protein was dialyzed against buffer A to remove the imidazole content.

3.10 Electrophoretic mobility shift assays on *sap* promoter

Elecrophoretic mobility shift assays were performed in order to verify the interaction of CcpA with the *sap* promoter. A biotin labeled Fw *sap* and Rv *sap* primers were used to amplify the promoter by PCR; as negative control of the experiment, *bibA* promoter used in previous EMSA was employed. 25 ng of both DNA were incubated with the recombinant protein in 20 µl of buffer Z (25 mM HEPES, pH 7.6, 50 mM KCl, 12.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 20% glycerol, 0.1% triton) for 20 minutes at room temperature. The DNA-protein

complexes were separated on 1% agarose gel, run in 1X TAE (40 mM Tris-acetate, 1mM EDTA) at 100 V (20 V/cm) at room temperature.

To better define the binding site for CcpA on *sap* promoter, EMSA probes were prepared by annealing biotin-labeled forward and reverse oligonucleotides (Fw *cre* box, Rv *cre* box, Fw mutated *cre*, Rv mutated *cre*, Table 3.1) by incubation at 95°C for 5 min and successive gradual cooling to room temperature. Various amounts of purified recombinant CcpA were incubated with 1 ng of labeled probes in 20 µl of buffer Z for 20 minutes at room temperature. The reactions were stopped with 2 µl of 50% glycerol and the protein-DNA complexes were separated on native 6% polyacrylamide gels in 0.5X TBE (45 mM Tris, pH 8.0, 45 mM boric acid, 1 mM EDTA) at 100 V (20 V/cm) at room temperature. Afterwards, electrophoretic transfer to a nylon membrane (GE Healthcare) was performed in 0.5X TBE at 380 mA for 45 minutes, and the transferred DNA was cross-linked to the membrane with UV light. After incubation in blocking buffer (2% milk in PBS with 0.5% Triton [PBS-T]) for 1 hour at room temperature, the membrane was incubated with streptavidin-horseradish peroxidase (HRP) conjugate (Pierce) for 1 hour at room temperature at a final dilution 1:1000. The membrane was washed and visualized with SuperSignal chemiluminescence reagent (Pierce). The specificity of CcpA binding to the *cre* sequence was tested by competition EMSA performed using increasing quantities (100x-500x) of either unlabelled *cre* box (used a specific competitor) or mutated *cre* (used as a non specific competitor).

Table 3.1 Primers used for this work

Primer	Sequence (5'-3')
Fw RT *gyrA*	CTTGACGAAGGTGAGACAATTC
Rv RT *gyrA*	TTGAAGCGAACAGAGTAGCC
Fw RT *cfb*	TTAAGGCTTCTACACGACTACC
Rv RT *cfb*	CAAGTGACAACTCCACAAGTG
Fw RT *bibA*	TGCCTACACCTGGATATTATGC
Rv RT *bibA*	GGCTTAGCTTCTGGTTTAACG
Fw RT *cylX*	CTGAGTTTCTTACGGAAGGTGGTG
Rv RT *cylX*	ATCAACGACACTGCCATCAGCACA
Fw RT *potB*	ACACCTGCTCTTACACCATTC
Rv RT *potB*	GGGAATTAACGCCTTCTTAACC
Fw RT *sag1333*	AGGCGTCAATGACTTTCATGGTGC
Rv RT *sag1333*	ACCATATCGCCTGCTTGAACCCTA
Fw RT *sag2021*	CCACATGGTTCTAGTGGAAGCGTT
Rv RT *sag2021*	TTTCTGTTGACGGTGGTGTTGGCT
Fw RT *sag0677*	GTTTGCAGTTGCTGGACCACAAGA
Rv RT *sag0677*	GAGTTGCCTCACTAGCAGTTTCCA
Fw RT *sap*	TGATGCGGCTGCGATTGAATTAGC
Rv RT *sap*	ATGTTCTCCAGCCCTCACCAATCA
Fw *csrR*	GGAATTCCATATGGGTAAAAAGATCTTAATAATCGAAG
Rv *csrR*	CCCGCTCGAGTTTTTCACGAATCACATAGCCCATT
Fw *bibA*	ATAATAGATTATTTTAGATAGAAACAACCC
Rv *bibA*	CATATTCGCTCCTTTATATAGTTAGTTG
Fw s*ag0017*	GAGGACGGTTTGCTAAATCGTTAGG
Rv *sag0017*	TTCATTTTTATTAAACTACTCCTTTACGAT
Fw *ccpA*	CTGTACTTCCAGGGCATGAATACAGATGATACGATTACGATTTA
Rv *ccpA*	AATTAAGTCGCGTTACTAATTATTTGTTGTGCCACGTTTAACAA
Fw pET-15	TAACGGACTTAATTAACGGTCTCCAGCTTGGCTGTTTTGGC

Rv pET-15	GCCCTGGAAGTACAGGTTTTCGTGATGATGATGATGATG
Fw *sap*	AGGAAATTTTTGATAAAAAAGCTAGGCAATATT
Rv *sap*	TATCATTCTCCTTTTTTAATGAATTGTTACC
Fw *cre* box	TTACTTGTTGCAAGCGCTTGCGTAAATTG
Rv *cre* box	CAATTTACGCAAGCGCTTGCAACAAGTAA
Fw mutated *cre*	TTACTTGTATCGTTAAAGCTAGTAAATTG
Rv mutated *cre*	CAATTTACTAGCTTTAACGATACAAGTAA

4. RESULTS

4.1 Regulation of GBS gene expression by glucose

To elucidate the response of GBS to glucose stress conditions, we performed a comparative global gene expression analysis of the 2603 V/R GBS strain grown at mid-exponential phase in a pepton-based complex medium (CM) devoid of sugars versus bacteria incubated for 30 min in CM containing 1% glucose. We found that, in such conditions, 27.5% of the genes were differentially expressed, with 353 of them up-regulated and 225 down-regulated. As expected, among the most regulated functional families, we found genes related to energy metabolism. Genes encoding transport and binding proteins were also highly regulated (Fig. 4.1). Of importance, a number of virulence genes were modulates by glucose, indicating a role in the adaptation of GBS to stress conditions. The microarray data were validated by real-time RT-PCR on eight genes, using total RNA isolated from wild-type and *csrRS* knockout strains grown in the presence or absence of glucose (see Table 4.1). The changes in response to glucose stress conditions observed in wild-type GBS and in the mutant strain were very similar to those measured by global gene expression analysis.

A list of the most regulated genes at high glucose conditions is reported in Table 4.2.

4.2 Functional categories

A. Stress response of GBS in high glucose condition

Growth in high glucose medium involves a considerable change in expression of genes involved in adaptation and stress response. Among stress response genes, a dramatic change was observed in the transcript for *sag1677*, encoding a universal stress protein, whose expression was highly down-regulated (27-fold) following exposure to glucose. Several genes, including *sag1135*, *sag1136*, *sag1137*, encoding stress proteins were down-regulated, too. Interestingly, these stress proteins are homologs of Gls24, a general stress protein of *Enterococcus faecalis* which has been reported to have a crucial role in stress response as well as in virulence (Teng *et al*., 2004).

B. Transcriptional regulators

After 30 minutes of incubation with 1% glucose several transcriptional regulators were modulated. In particular, *sag1128*, *sag2017*, *sag0554* belonging to the putative Cro/CI family and *sag1749*, *sag1655*, *sag0427*, being part of the putative Mer family of regulators, were 2-5 fold up-regulated. Of interest, the latter family has been reported to act as a key activator in nitric oxide defense system in pneumococci, thus ensuring both survival and systemic infection (Stroeher *et al*., 2007; Brown *et al*., 2003)

In response to the availability of glucose sources, bacteria inhibit multiple enzymes responsible for alternative sugar metabolism pathways through the

carbon catabolite repression system (CCR), mediated by the catabolite control protein A (CcpA, *sag0707*). Bioinformatic analysis revealed that a number of transcriptional regulators, that were highly down-regulated under glucose conditions (up to 20-fold), has in their promoter region a consensus sequence for the binding of CcpA (http://regprecise.lbl.gov/RegPrecise/gmregulon.jsp?gmproject_id=6875). They included: *sag0277*, encoding a Mga-like protein, a positive regulator of virulence in GAS (Almengor *et al*., 2007; *sag1348*, *fruR*, lactose phosphotrasferase system repressor; *sag0119*, *rbsR*, ribose operon repressor; *sag0042*, phosphosugar-binding transcriptional regulator belonging to RpiR family; *sag2073*, a transcriptional regulator belonging to GntR family; *sag2161*, encoding for a transcriptional regulator of the Crp/Fnr family and CcpA itself.

C. Transport genes

Several genes encoding transport proteins were found to be up-regulated in high glucose conditions. These included *sag0745*, coding for a putative transporter of the NRAMP family, involved both in Mn^{2+} and Fe^{2+} uptake (Janulczyk *et al*., 2003; Papp-Wallace and Maguire, 2006) and genes encoding for proteins involved in potassium uptake, such as *sag1590*, *sag1591*, *sag1631* (belonging to the trk family) and *sag1090*. We also observed a five fold upregulation of the *sag1711* gene, coding for a putative CorA protein involved in magnesium transport (Warren *et al*., 2004). A number of genes belonging to the transport family and found to be up-regulated by glucose are interestingly involved in peptide uptake, a process essential to satisfy GBS growth requirement, given that this bacterium

has a limited capacity to synthesize amino acid. Among up-regulated genes, although regulated at different extent, we found the *dps* gene (*sag1444)*, coding for a putative peptide/proton symporter (Samen *et al*., 2004); a putative histidine ABC transporter (*sag0947* to *sag0949*); a spermidine-putrescine transporter, *potABCD* (*sag1108* to *sag1111*), implicated in the pathogenesis of *Streptococcus pneumoniae* in various infection models (Shah *et al*., 2011); the *sag0290* to *sag0292* operon (regulated up to 8-fold), encoding a putative polar amino acid ABC transporter; the *sag1145* gene, encoding for the sodium:alanin symporter protein; the *sag0715* to *sag0718* and *sag0947* to *sag0949* genes, all encoding for amino acid ABC transporters. Furthermore, we found up-regulated the region comprising the *sag0241 to sag0244* genes, encoding an ABC transporter for glycine betaine, whose accumulation in *B.subtilis* and *L.lactis* confers protection against osmotic and cold stress to the bacterium (Hoffmann *et al*., 2002; Hoffmann and Bremer, 2011; Obis *et al*., 1999).

As expected, in high glucose condition genes involved in the transport of complex carbohydrates were also down-regulated, including the region from *sag1441* to *sag1443*, encoding for the maltose-maltodextrin transport system (*malE-F-G)*; *sag0955* and *sag1925* (*msmK*) genes, encoding a sugar-ABC transporter and a sugar transport ATP-binding protein, respectively; the ribose ABC transporter region (from *sag0114* to *sag0117*, namely *rbsA-B-C-D*); the cellobiose ABC transporter (*sag0328* to *sag0330*). Furthermore several multiple transport systems (PTS), which allow uptake of various carboydrate sources, such as *sag1805*, *sag1813*, *sag1814*, *sag1948-1951*, *sag1898-1902* and *sag0192*, showing a specificity for different sugars, were found to be highly down-regulated (up to 120-fold).

D. Wide-ranging changes in GBS adaptive metabolism

The expression of several genes involved in a wide range of metabolic pathway was dramatically modulated by a 30 minutes incubation in 1% glucose, mirroring a rapid adaptation of GBS cellular process metabolism to conditions and nutrients in the new environment. We observed that bacteria grown in such a rich medium were growing more rapidly, thus, as expected, gene encoding for DNA replication, recombination and repair and gene encoding for membrane biosynthesis were found to be highly up-regulated, while genes encoding for enzymes involved in substrate degradation were found to be down-regulated. Transcript changes were observed in aminoacid classes, in particular the *sag2165* and *sag2167*, which encode for a carbamate kinase and an ornithine carbamoyltransferase, respectively, were highly down-regulated (up to 20-fold). Both enzymes are components of the arginine deaminase system, which has been suggested to aid bacterial survival in acidic environments by catalyzing the release of ammonia from arginine (Gruening *et al*., 2006). The *sag1907* gene (*eda-2*), encoding for keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase, was found to be highly down-regulated, too. Conversely few genes were slightly up-regulated, such as genes belonging to the aspartate and serine families.

We found a drastic down-regulation (up to 130-fold) of genes implicated in energy metabolism of sugars, including the *sag0033* to *sag0042* locus, encoding for the sialic acid operon. The list also comprises a carbohydrate kinase (*sag1906*) belonging to PfkB family; a putative hexulose-6-phosphate synthase (*sag1812*); L-a ribulose-5-phosphate 4-epimerase (*sag1810*); a putative hexulose-6-phosphate isomerase (*sag1811*); and the *sag0118* gene encoding for a

ribokinase. Also genes encoding for proteins required for fermentation processes were found to be down-regulated, including *sag1637*, *sag0053* and *sag0054*, annotated as alcohol dehydrogenases (*adh*, *adhP*, *adhE)*. The repression of genes codifying for proteins implicated in biosynthesis and degradation of polysaccharides such as *sag1901*, glucuronyl hydrolase; *sag0041*, acetyl xylan esterase; *sag1216*, pullulanase; *sag0856*, glycogen synthase (*glgA)*; *sag0854*, glucose-1-phosphate adenylyltransferase (*glgC)*; *sag0853* glycogen branching enzyme (*glgB)* was also observed.

E. Virulence and host-pathogen interaction genes

Pathogenic bacteria by modulating the expression of surface-associated or secreted virulence factors can adapt to host conditions and improve their capacity to persist in specific niches. Although pathologic high glucose conditions are often associated to an increased risk for GBS infections, we found that transcription of known or putative virulence factors was down-regulated in response to glucose stress conditions, indicating that in this particular scenario such determinants may be dispensable to GBS invasiveness. In particular the expression of pore-forming toxins including a) the *cyl* gene cluster (*sag0662* to *sag0673*), required for GBS hemolysin production, which is responsible for promoting invasion of host cells and triggering cell lysis (Pritzlaff *et al*., 2001) ,and b) the *cfb* gene (*sag2043*), encoding the CAMP factor (Lang and Palmer, 2003), was down-regulated in a range between 6 to12 fold.

A similar pattern of expression was observed for several surface-expressed proteins containing the LPXTG cell wall-sorting motif, such as *bibA* (*sag2063*).

Among virulence factors involved in host cell adherence and invasion, the expression of the *sag1234* gene encoding for the laminin binding protein, Lmb, which promotes adherence of GBS to host cells by binding to ECM laminin (Spellerberg *et al*., 1999), was repressed by glucose. Furthermore, we found that also the expression of *sodA* (*sag0788*), a gene which plays a crucial role against oxidative stress (Poyart *et al*., 2001), and of the hyaluronate lysase (*sag1197*), which cleaves hyaluronic acid, a major component of the connective tissue, thus promoting GBS spreading during infection, was significantly reduced.

On the contrary only few genes were found to be up-regulated, such as *sag0677*, encoding an unknown function LPXTG protein, and *sag2021*, encoding for a protein binding to human glycoprotein GP-340 thus preventing bacterial colonization (Brady *et al*., 2010). The same activation trend was observed for the *sag1739* to *sag1744* operon, encoding proteins which although not officially classified among virulence factors, have been reported to be involved in the respiration metabolism and to play a role in virulence and GBS growth *in vivo* (Yamamoto *et al*., 2005).

4.3 The response to glucose involves the two component system CsrRS

By comparing previously reported information on genes controlled by the CsrRS two-component system (Lamy *et al*., 2004) and the array of genes modulated by glucose, we found a number of common genes, including *sag0662* to *sag0673* (*cyl* operon), *sag2043* (*cfb*) and *sag2063* (*bibA*). Therefore to postulate whether glucose-dependent regulation of gene expression was under the control of CsrRS

system, we carried out transcriptome analysis of an isogenic *ΔcsrRS* mutant strain grown under glucose rich conditions and compared it to an isogenic wild type strain grown at the same conditions. Of importance, a large number of glucose-dependent genes (~36%), mainly factors implicated in GBS transposon function and virulence, resulted to be under CsrRS regulation (Fig. 4.2).

Table 4.1 Real time PCR confirmed the results of microarray experiments.

	2603 V/R		Δ*csrRS*	
	microarray	real-time	microarray	real-time
potB	2,0up	2,1 up	1,8 up	3,3 up
bibA	2,4 down	2,1 down	1,5 down	1,4 down
cfb	5,8 down	7,8 down	3,7 down	3,3 down
cylX	9,0 down	11,8 down	2,4 down	4,6 down
sap	20,6 down	39,3 down	74,7 down	129,2 down
sag1333	3,3 down	3,9 down	1,6 down	1,5 down
sag2021	3,8 up	2,1 up	1,9 up	1,2 up
sag0677	2,9 up	1,3 up	1,1 down	1,2 up

Table 4.2 List of genes highly regulated in GBS strain 2603V/R after incubation in high glucose medium.

TIGR locus	Annotation	Family	Fold change	Regulation
sag0008	Transcription-repair coupling factor (*mfd)*	DNA metabolism	6.1	Up
sag0185	Hypothetical protein	Cell envelope	10.24	Down
sag0281	Hypothetical protein	Cell envelope	7.04	Down
sag2169	Hypothetical protein	Cell envelope	8.35	Up
sag0040	ROK family protein	Unknown function	48.35	Down
sag1643	Glutamine amidotransferase, class I	Unknown function	19.91	Up
sag0034	Sugar ABC transporter	Transport and binding proteins	128.03	Down
sag1949	PTS system	Transport and binding proteins	84.29	Down
sag1950	PTS system	Transport and binding proteins	119.63	Down
sag1951	PTS system	Transport and binding proteins	92.69	Down
sag1642	ABC transporter	Transport and binding proteins	19.12	Up
sag0664	CylG protein (*cylG*)	Cellular processes	12.25	Down
sag0663	CylD protein (*cylD*)	Cellular processes	11.63	Down
sag0667	CylA protein (*cylA*)	Cellular processes	10.16	Down
sag0666	CylZ protein (*cylZ*)	Cellular processes	9.92	Down
sag0662	CylX protein (*cylX*)	Cellular processes	8.99	Down
sag0668	CylB protein (*cylB*)	Cellular processes	8.82	Down
sag0669	CylE protein (*cylE*)	Cellular processes	8.79	Down
sag0670	CylF protein (*cylF*)	Cellular processes	8.63	Down
sag0671	CylJ protein (*cylJ*)	Cellular processes	7.01	Down
sag0672	CylI protein (*cylI*)	Cellular processes	6.76	Down
sag0673	CylK protein (*cylK*)	Cellular processes	5.98	Down
sag2043	cAMP factor (*cfb*)	Cellular processes	5.76	Down
sag1733	Universal stress protein family	Cellular processes	96.38	Up
sag0777	ATP- dependent RNA helicase	Transcription	21.94	Up
sag1216	Pullulanase (*sap*)	Energy metabolism	20.61	Down
sag0856	Glycogen synthase (*glgA*)	Energy metabolism	20.31	Down
sag1907	Keto-hydroxyglutarate-aldolase (*eda-2)*	Amino acids biosynthesis	48.04	Down
sag2165	Ornithine carbamoyltransferase (*argF-2)*	Amino acids biosynthesis	10.84	Down

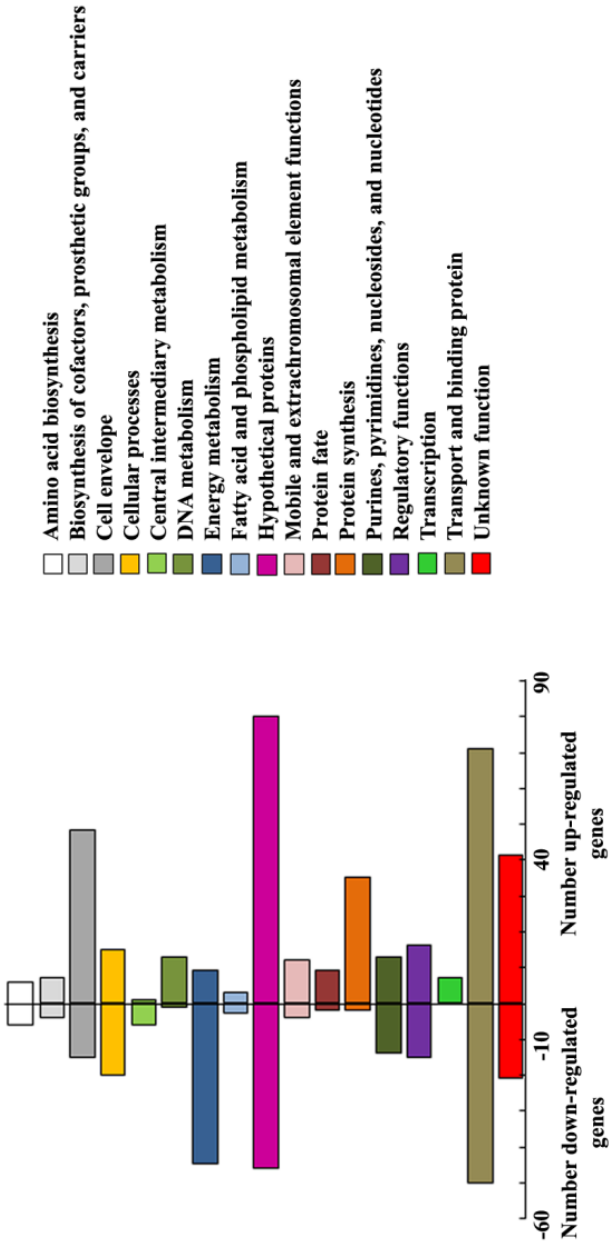

Figure 4.1 Differential regulation of gene expression in GBS strain 2603V/R after exposure to absence or presence of 1% glucose. Genes were classified into 17 functional categories. Bars indicate the number of genes differentially regulated in the absence versus presence of glucose.

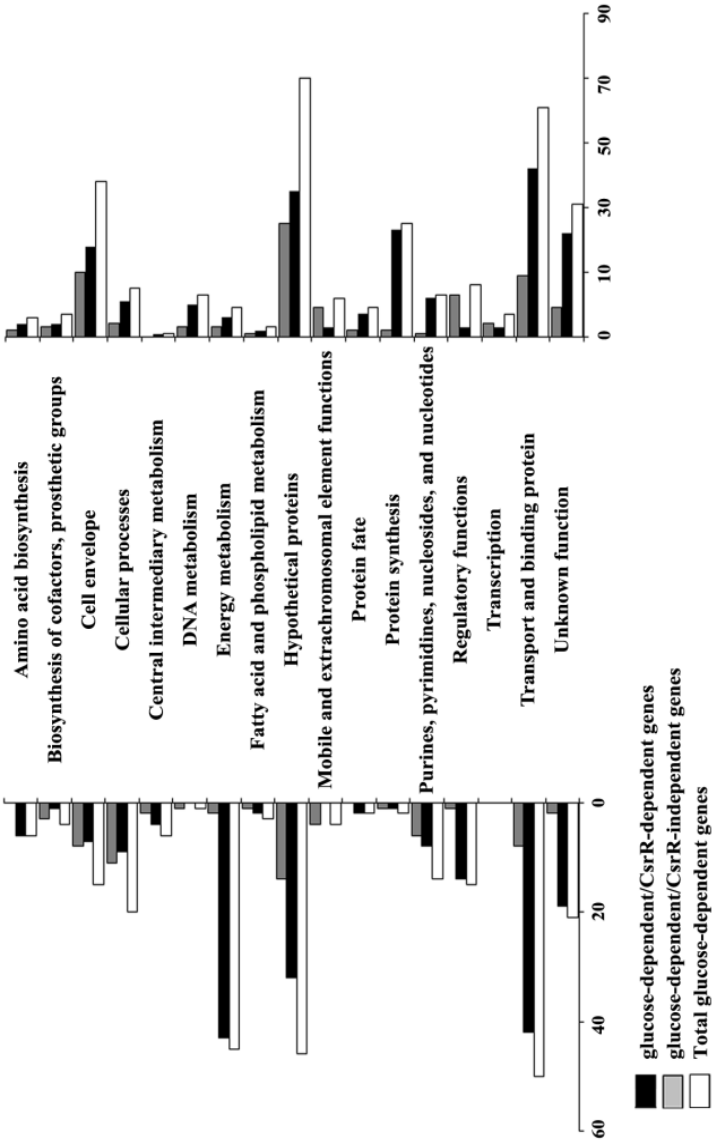

Figure 4.2 Differential regulation of gene expression in GBS strain 2603 V/R versus the isogenic Δ*csrRS* mutant strain after incubation in medium with 1% glucose versus a sugars-free complex medium. White bars indicate the number of glucose-regulated genes in the wild-type strain; black bars indicate the number of genes that are glucose- dependent and CsrR- dependent; grey bars indicate the number of genes that are glucose-dependent and CsrRS-independent.

4.4 Glucose influences the BibA exposure on cell wall surface

Recently in our group, a well-conserved, cell wall–anchored protein that we refer to as GBS immunogenic bacterial adhesin, or BibA, was identified (Santi *et al*., 2007). BibA is immunogenic in humans and confers resistance to phagocytic killing, thus contributing to GBS survival in human blood (Santi *et al*., 2007). *bibA* expression is modulated by the CsrRS two-component regulatory system, which regulates the expression of genes encoding known virulence factors, such as β-hemolysin (Lamy *et al*., 2004; Jiang *et al*., 2008). Lamy *et al.* have recently shown that *bibA* is up-regulated in a Δ*csrRS* mutant strain (Lamy *et al*., 2004). Furthermore, Santi *et al*. reported that *bibA* is down-regulated when GBS is grown at acidic pH and they confirmed a CsrRS dependent regulation in response to such environmental factor (Santi *et al*., 2009a).

To investigate the role of glucose in *bibA* regulation, we performed a comparative gene expression analysis growing the bacteria in presence of this sugar and we found a down regulation of gene expression (2.4 fold). Further analysis of the transcript by real time PCR confirmed the data observed in microarray experiment (2.1 fold).

To further confirm that glucose has a repressor effect on BibA protein expression we performed a western blot analysis. For this reason we analyzed the TCA precipitated surnatants from bacterial growth after exposure to glucose. Western blot analysis clearly showed that BibA expression was highly reduced in high glucose conditions (data not shown). The bacteria were also analyzed by FACS analysis using an anti-BibA antiserum to detect the protein presence on bacterial

surface. As reported in figure 4.3, BibA surface expression was down-regulated following bacteria transition from a medium without glucose (blue line) to a medium with high glucose content (green line).

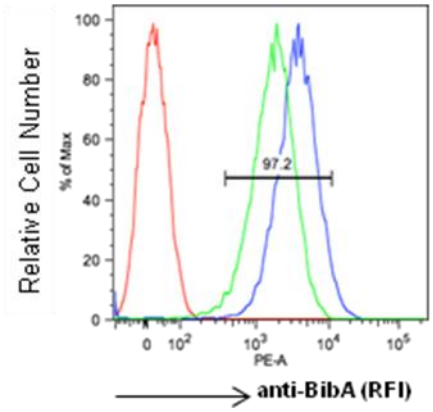

Figure 4.3 Flow cytometry analysis of 2603V/R strain grow in CM in the absence (blue line histogram) and presence of glucose (green line histogram) incubated with a polyclonal mouse anti-BibA antibody and stained with PE-conjugated anti-rabbit IgG antibody. The red histogram indicates bacteria treated only with secondary antibody.

4.5 CsrR specifically binds to the *bibA* promoter

Lamy and colleagues (Lamy *et al*., 2004) identified a potential CsrR recognition sequence, through DNase I footprinting and sequence comparisons of upstream regions from three genes, including *bibA* gene from NEM 316 strain (*gbs2018*). A comparison of the DNA regions protected by CsrR from DNase I cleavage allowed to identify a highly conserved nonanucleotide motif 5'-TATTTTAAT-3'. This sequence is 100% conserved in the CsrR binding regions of the *cylX* and *bibA* promoters. For *bibA* gene, two binding sites for CsrR were identified at -60 bp and -144 bp from translational starting point.

Based on these evidences, we analyzed the *bibA* promoter in 2603 V/R strain and found a putative CsrR binding site at -146 bp from translational starting point (Fig. 4.4).

To better define CsrRS-mediated gene regulation, we deeper investigated its mechanism of action on *bibA* expression. To determine whether CsrR interacts directly with the *bibA* promoter region, we performed electrophoresis mobility shift assay (EMSA) using recombinant CsrR protein. A biotin-DNA probe, which corresponded to the promoter region of *bibA*, was incubated with increasing concentrations of CsrR. As shown in figure 4.5A, CsrR induced a shift in the mobility of the fragment at a concentration of 0.8 µg, postulating that *bibA* expression could be controlled by this two component system.

As already described, CsrS is the sensor kinase protein of the CsrRS TCS responsible for the phosphorylation of the regulator component (CsrR) at a conserved aspartate residue. Aspartate phosphorylation usually alters the effector

activity by modulating DNA binding affinity and as a consequence, controlling of gene expression.

Recently, Jiang and colleagues (Jiang *et al*., 2008) demonstrated that phosphorylation of CsrR on aspartate, increases its affinity to promoters of different genes. To understand the relevance of CsrR phosphorylation in *bibA* regulation, we performed *in vitro* EMSA using a chemically phosphorylated CsrR through acetyl-phosphate. This modification leads to an increased affinity of CsrR for the *bibA* promoter, as testified by the observation of a shift of *PbibA* band already when 0.6 µg of recombinant CsrR were added (Fig. 4.5A).

To verify the specificity of CsrR binding to the *bibA* promoter, we performed a competitive EMSA by adding a 250-fold or 500-fold excess of unlabelled probes corresponding to the *bibA* promoter or a non-correlated promoter (*sag0017*). As displayed in figure 4.5B, the addition of the unlabelled *bibA* promoter (lanes 3-4) impaired the binding of recombinant CsrR to the *PbibA* biotin probe, while the presence of a non specific competitor did not have any effect (lanes 5-6). These data confirm the capability of CsrR to specifically bind the *bibA* promoter and that CsrR phosphorilation increases the affinity for the DNA region corresponding to the *bibA* promoter.

5'ATAATAGATTATTTTAGATAGAAACAACCCAAACCCTTAGATTTTACTAAGGGTTTTTTGTGT
GGAAAATAACATATATATCCGATTAAATTATAAAAAAATAATCTTTTGATTATTTTTACTAGATT
GTTGAGCAAAAAAACATTAAAAAAAAGATGTT**TATTTTAAT**AAAATGTAAATAAATTAAAGAAAA
GTGTTGAAATAAAAAACTCCAAGTTATATAATTAGTTAAGGAACTCAAATTAAATTAAAATAAAC
GTGGTCCTATCCTAATAAATTAGGATTTTCAACTAACTATATAAAGGAGCGAAT<u>ATG</u>3

Figure 4.4 Intragenic region between nucleotides 2045046 - 2045361 (Reverse Strand) The potential CsrR binding site is highlighted in red. The underline region is the first translated codon.

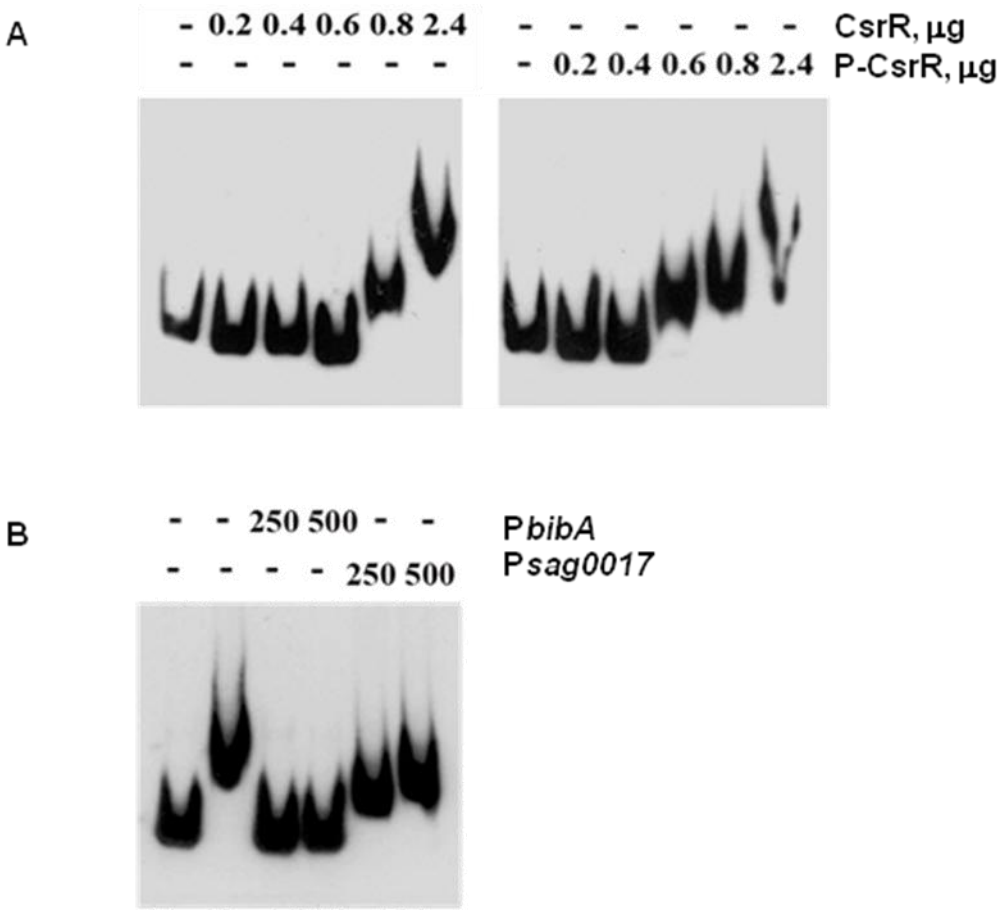

Figure 4.5 A)The *bibA* promoter (1 ng) was incubated with different amounts of purified CsrR and phosphorylated CsrR. B) Unlabeled *bibA* and *sag0017* promoters were tested for their ability to sequester CsrR from biotin-labeled *bibA* promoter (1 ng). Lane1: labelled P*bibA*. Lane 2**:** CsrR (0.8 µg) incubated with labelled P*bibA*. Lane 3-4**:** Competition assay with P*bibA* (250x-500x) as specific competitor. Lane 5-6**:** Competition assay with P*sag0017* as non specific competitor (250x-500x). In three experiments the protein-DNA complexes were separated on a native 6% polyacrylamide gel.

4.6 CsrR acts as repressor of *bibA* expression

These data by postulating a direct interaction between CsrR and the *bibA* promoter suggest that CsrR controls the expression of this gene *in vivo*.

To investigate how the presence of glucose can influence the role of CsrR in *bibA* expression we performed chromatin immuno-precipitation (ChIP) analysis. Bacterial cultures grown to an OD_{600} of 0.5 were exposed for 30' to glucose and *in vivo* cross-linked with formaldehyde. After sonication, protein-DNA complexes were immunoprecipitated with a nonsaturating amount of polyclonal CsrR antiserum. The immunoprecipitated DNA (IP) was analyzed by dot blot analysis, spotting on membrane the IP and flowthrough (FT) and using ^{32}P-labelled *bibA* promoter as probe. FT samples indicated that the DNA amount loaded in each spot was comparable (data do not shown). As shown in figure 4.6, quantification of dot blot intensity relative to DNA immunoprecipitated with CsrR antiserum revealed a 3 fold increase in the amount of ^{32}P-labelled *bibA* promoter in GBS cultures grown in high glucose (1%) medium compared to sugar-free conditions. The same experiment was performed using an isogenic Δ*csrRS* deletion mutant and no differences in the magnitude of immunoprecipated DNA were observed (data not shown).

These data strongly postulate that CsrR acts as a repressor of *bibA* expression *in vivo*.

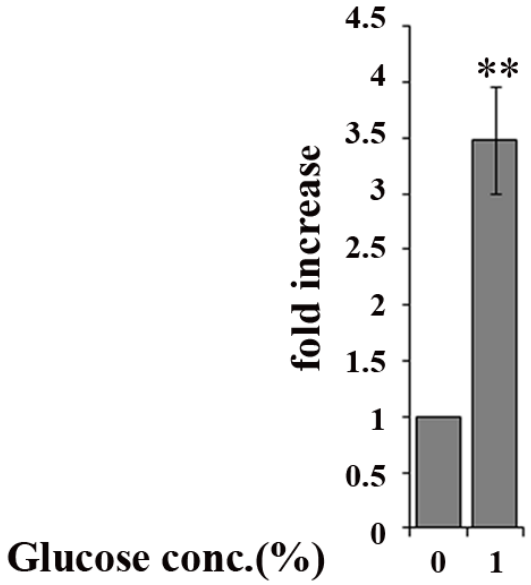

Figure 4.6 Comparison of ^{32}P-labelled *bibA* promoter levels immunoprecipitated with a CsrR antiserum in 2603 V/R wild type strain grown in medium devoid of glucose (0) or in the presence of 1% glucose (1) (*n*=3). **, P ≤ 0.01. Error bars represent the SD. *n* indicates the number of independent experiments.

4.7 Promoter region of *sap* gene shows the CcpA binding site

In addition to genes that appeared to be glucose and CsrRS dependent, we found a large group of genes (63%), involved in GBS metabolism, transport and protein synthesis, that was modulated by glucose both in the wild-type strain and in the *csrRS* mutant strain, indicating that their expression was glucose dependent but CsrRS independent.

GBS, as other streptococcal species (Tomoyasu *et al*., 2010; Abranches *et al*., 2008), has a complex metabolic system that controls carbohydrate catabolism, named CcpA regulatory system, in which enzymes necessary for the metabolism of alternative sugar are inhibited in the presence of glucose. In particular, CcpA, under the stimuli of environmental sugars, regulates the expression of metabolic genes and virulence determinants (Tomoyasu *et al*., 2010; Abranches *et al*., 2008; Almengor *et al*., 2007). Genes subjected to CcpA regulation are identifiable by the presence of a target DNA sequence, called the catabolite-responsive element (*cre*) site, to which CcpA binds.

To investigate the presence of the *cre* box in the promoter region of GBS genes, we performed a bioinformatic analysis of the 2603 V/R genome using the program Reg Precise (http://regprecise.lbl.gov/RegPrecise/gmregulon.jsp?gmproject_id=6875). By this comparative analysis we found that in 2603 V/R strain, 60 operons, conteining 139 genes showed an identical sequence as the one recognized by CcpA. In particular, we found that 77% of them were differentially regulated in the presence

of glucose, strengthening the hypothesis that CcpA may have a role in their regulation. Streptococcal pullulanases are metabolic enzymes with a glycosidic hydrolase activity towards α-glucan polysaccharides and are considered key extracellular components in bacterial metabolism. One of the most regulated gene under high glucose experimental conditions is *sap*, recently demonstrated to encode for a GBS pullulanase enzyme capable to catabolize complex sugars and to induce functional antibodies (Santi *et al*., 2008). In particular, SAP is affected by presence of glucose and resulted to be highly down regulated (20 fold) when bacteria were grown with this sugar. Of importance, microarray analysis comparing 2603 V/R WT strain and an isogenic Δ*csrRS* mutant strain, revealed that *sap* was still highly down-regulated, indicating that this modulation was independent from this two component system.

Analysis of *sap* promoter (Nucleotides 1226300 - 1226469 Reverse Strand) with the program DBTBS (http://dbtbs.hgc.jp/) revealed that this region carries a putative *cre* box, placed between -65 bp and -52 bp from the starting +1bp and displaying a 5' TGCAAGCGCTTGC 3' sequence relative to the *cre* consensus sequence (Fig. 4.7) as described in the literature (Tomoyasu *et al*., 2010).

5'AGGAAATTTTTGATAAAAAAGCTAGGCAATATTGCTTAGCTTTTTTGTAATGCTATTGATAGT
TTTAGTGAAAATTTCAAAAAAATAAAGAAATCATTTACTTGT**TGCAAGCGCTTGC**GTAAATTGT
TATGATTTTATTGGTAACAATTCATTAAAAAAGGAGAATGATA 3'

Figure 4.7 Intragenic region between nucleotides 1226300 - 1226469 (Reverse Strand). The potential CcpA binding site is highlighted in red.

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4.8 CcpA is able to recognize the sap promoter

The test the ability of recombinant CcpA to bind the *sap* promoter, we performed an EMSA. For this analysis two PCR-generated DNA fragments were synthesized: one relative to the promoter of the *sap* gene, containing a *cre* site and a second one, the *bibA* promoter, which does not display a canonical binding motif for CcpA. Both DNA fragments were separately incubated in buffer Z with 4 different amounts of CcpA and loaded on 1% agarose gel. As shown in Fig. 4.8, after electrophoresis of the reaction mixtures, we observed that both regions, (*bibA* promoter, lanes 1-5; *sap* promoter, lanes 6-10), were shifted by the presence of recombinant CcpA protein. However, for the promoter containing the *cre* box, a bandshift was observed already at 2 µg of recombinant CcpA (lane 8), while a retardation band for *bibA* was observed only at 4 µg (lane 5). These data suggest that CcpA has a general affinity for DNA sequences, but it binds the *cre* motif with a higher affinity: in fact a lower amount of protein is required for gel retardation with *sap*.

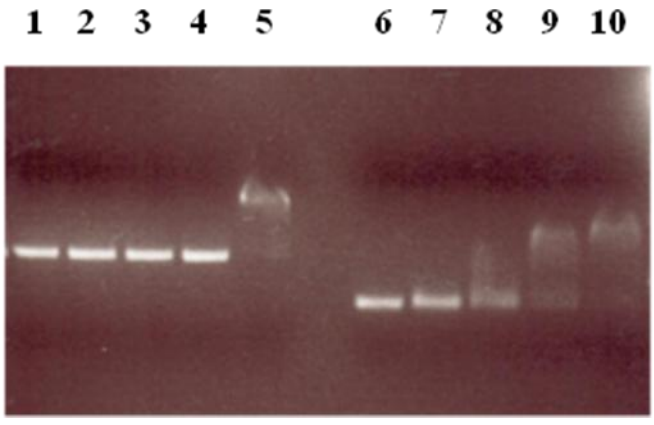

Figure 4.8 The *bibA* promoter (25 ng, Lanes 1 to 5) and the *sap* promoter (25 ng, Lanes 6 to 10), were incubated with two different amounts of purified CcpA (1 µg, Lanes 2 and 7; 2 µg, Lanes 3 and 8; 3 µg, Lanes 4 and 9; 4 µg, Lanes 5 and 10).

4.9 CcpA binds specifically the promoter of pullulanase

To assess whether the recombinant protein CcpA could bind the *cre* box in pullulanase promoter, EMSA was performed with CcpA using, as probe, biotin-labeled DNA fragment, corresponding to the *cre* site from the *sap* promoter (*cre box*, Table 3.1). The probe was incubated with different concentration of recombinant protein and the complex was resolved on native polyacrylamide gel. The lower amount of CcpA able to induce the mobility retardation of the probe was 0.6 µg (data do not shown).

After establishing the minimum concentration at which the shift was observed, we performed EMSA using biotin-labeled DNA fragments, carrying both a wild type and a mutated *cre* sequence (mutated *cre*, Table 3.1). Figure 4.9A shows that recombinant CcpA was able to induce a shift in mobility of the *cre box* probe, while it was not able to bind the fragment carrying a mutated *cre* sequence.

CcpA specific binding to the *cre* sequence was also tested by competition EMSA. As competitors, unlabeled wild type and mutated *cre* sequences were used. As shown in Fig. 4.9B CcpA binding to labelled *cre box* sequence was reversed when either a 100-fold or 500-fold excess of unlabelled *sap cre* sequence was added, while it was not affected by competition with identical concentrations of a mutated *cre* sequence.

In conclusion, we postulate that recombinant CcpA is able to bind the *cre* box present in the promoter of pullulanase in a specific manner. Indeed, CcpA does not recognize a mutated *cre* sequence. The competition assay has also demonstrated that an unlabeled wild type *cre box* probe, but not the mutated one,

competes for the binding to the protein, enforcing the specific affinity of CcpA for this sequence. These results suggest that the *sap* gene could be directly regulated by CcpA, and its repression observed under high glucose conditions depending on this regulator.

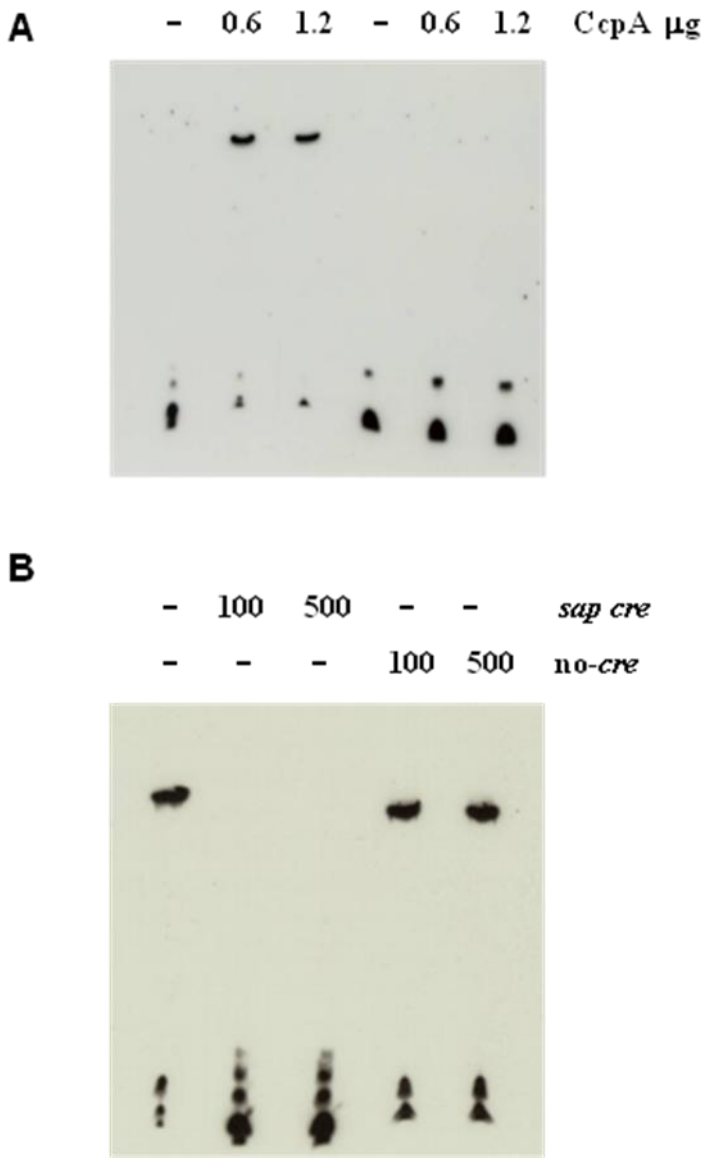

Figure 4.9 A) The *sap cre* (1 ng, Lanes 1 to 3) and the mutated *cre* probes (1 ng, Lanes 4 to 6), were incubated with two different amounts of purified CcpA.

B) Unlabeled *sap cre* and mutated *cre* fragment were tested for their ability to sequester CcpA from biotin-labeled *sap cre* probe (1 ng). Lane 1**:** CcpA (1.5 µg) incubated with labelled *sap cre*. Lane 2-3**:** Competition assay with *sap cre* (100x-500x) as specific competitor. Lane 4-5**:** Competition assay with mutated *cre* as non specific competitor (100x-500x). In three experiments the protein-DNA complexes were separated on a native 6% polyacrylamide gel.

5. Discussion

Diabetes mellitus is characterized by inappropriately high concentrations of glucose in plasma as a result of a relative or absolute lack of insulin. Patients with uncontrolled diabetes mellitus can be more susceptible to bacterial infections, which can lead to higher morbidity and mortality than in healthy, non-diabetic patients because of dysfunction in polymorphonuclear neutrophil bactericidal function, cellular immunity, and complement activation. For this reason, diabetic patients tend to have a higher incidence and increased severity of infections than non-diabetic patients.

Short and transient periods of hyperglycaemia result in abnormalitiesin granulocyte adherence, chemotaxis, phagocytosis, and bactericidal function. These impairments in immune function have been associated with an increased risk of infections. Systemic hyperglycaemia results in derangement of the immune system including neutrophil function, cellular immunity, and complement function.

As previous studies have shown, *Klebsiella pneumoniae*, followed by Streptococcus spp. were the most commonly isolated organisms among diabetic patients, whereas in the non-diabetic group Streptococcus spp., followed by Staphylococcus spp., were most common (Zheng *et al*., 2012).

Although GBS is commonly associated with neonatal diseases and postpartum infections, it is also an important cause of morbidity and mortality among adults. GBS infection has been reported to occur in adults with serious underlying conditions and, in particular, diabetes has been reported to be one of the most important risk factor promoting GBS infections. Patients with diabetes display

peripheral neuropathy or peripheral vascular diseases following trauma, particularly to the lower extremities. GBS takes advantage of this condition by crossing the endothelial barrier and promoting bacterial invasion of the foot. Indeed, GBS is also found in biopsy of patients with foot infections, a common status of patients suffering from diabetes. The impact of hyperglycemia upon susceptibility to GBS infection has not been fully elucidated, but, at least in part, this effect seems to be due to impairment of neutrophil effector functions. Indeed, Mazade *et al*. have reported that high glucose levels impair neutrophil-mediated phagocytosis and killing of GBS and reduce superoxide production thus ensuring spreading of infection (Mazade and Edwards, 2001).

Although clinical evidences indicate a strong correlation between individuals with high blood glucose levels and the propensity to acquire GBS systemic infections, the signaling events triggered in this bacterium by high glucose conditions and the relative modulation of the expression of virulence determinants are still unknown.

Glucose concentration in blood varies since 80 mg/dL in healthy people until 600 mg/dL in diabetic patients with severe complications. A clinical study has reported a patient admitted in hospital with blood glucose concentration of 1288 mg/dL (Yanai *et al.,* 2012) that corresponds to ~1% of sugar. To investigate the adaptation of GBS to high glucose presence, we performed a gene expression analysis growing bacteria in the absence and presence of this carbon source. One of the limitation for setting *in vitro* experiments looking at the effect of carbon sources on bacteria stress response is the fact that metabolites generated during the incubation of bacteria with sugars can alter the pH of the milieu and, differently from what happens *in vivo*, where the environment (i.e. blood) is rapidly

buffered, may dramatically affect the activation of intracellular pathways. In order to precisely delineate the impact of glucose, we defined that the duration of the experiments was limited to 30 minutes, a time point at which pH was still constant as at the initial conditions (data not shown).

We found that glucose affected transcription of ~30% of genes; in particular the most highly regulated genes belong to transport and binding proteins and metabolism families.

Recent reports have clearly elucidated the molecular mechanisms by which GBS adapts to different environmental conditions, including pH, carbon source availability, biological fluids and temperature (Mereghetti *et al*., 2009; Sitkiewicz *et al*., 2009; Santi *et al*., 2009). During these events a wide range of genes involved in adhesion to the host, nutrient acquisition and survival to the immune system are differently modulated. For example, global gene expression analysis of GBS grown in amniotic fluid, blood and pH stress conditions has revealed a number of mechanisms used by this bacterium to adapt to the host. Our study proposes that a general trend of adaptive regulation is triggered during the growth of GBS in the hyperglycemic milieu of the blood, as for diabetic patients. In particular, we observed that, at a gene expression level, GBS quickly adapts to environmental carbon sources changes by modulating both transcription and translation of genes involved in transport, metabolism and virulence.

One intriguing finding of our analysis is that the exposure of GBS to glucose stress conditions appears to down-regulate several virulence factors, suggesting that in such a scenario these determinants may be dispensable to bacterial invasiveness. In this context, genes encoding for pore-forming toxins, such as hemolysin β and CAMP factor, both known to be crucial in promoting cell invasion

and lysis (Pritzlaff *et al*., 2001; Lang *et al*., 2003), were found to be highly down-regulated. Furthermore, the presence of glucose reduced the transcription of genes involved in host cell adherence, such as *Lmb* (Spellerberg *et al*., 1999) and serum resistance, such as *bibA* (Santi *et al*., 2009).

The down-regulation of virulence determinants in a high glucose milieu clearly point out the reliance of GBS fitness on the access to the metabolites derived by the catabolism of carbon sources. Indeed, over the past few years it has been reported that catabolism of carbohydrates plays a key role in the pathogenesis of invasive streptoccocci, including *Streptococccus pneumoniae*, *Streptococcus pyogenes* and GBS (Almengor *et al*., 2007; Shelburne *et al*., 2008a; iyer and Camilli, 2007; Jones *et al*., 2000). Johns and colleagues have recently applied signature-tagged transposon mutagenesis to a neonatal sepsis model and showed the importance of carbohydrate catabolism for GBS infectivity (Jones *et al*., 2000). In particular, by knocking out genes involved in complex carbohydrate metabolism, including a maltose-binding protein (*mal*), a phosphotransferase (PTS) and a sucrose hydrolase (*scrB*), they observed an attenuated virulence (Jones *et al*., 2000). Furthermore these genes, which clearly impact GBS virulence, were also found to be down-regulated in our study, suggesting a direct link between the modulation of genes coding for virulence factors and the one responsible for carbohydrate utilization, leading to an attenuated status of bacteria. Indeed, virulence is an energy consuming condition and bacteria occupying carbohydrate-rich niches, such as the blood of hyperglycemic individuals, may avoid the activation of virulence-associated pathways.

Response of GBS to variations in environment relies on two major types of transcriptional regulators, the two-component gene regulatory systems (TCS) and

stand-alone regulators (such as CcpA). The best characterized TCS in streptococci is the CsrR/S system, which plays a key role in connecting the expression of complex carbohydrate metabolism genes together with that of virulence factors, thereby contributing pathogenesis (Lamy *et al*., 2004; Churchward, 2007; Mascher *et al*., 2003). In our study a wide range of glucose-dependent genes, in particular factors implicated in GBS transposon function and virulence, resulted to be under CsrRS regulation, strongly suggesting that effectors of this TCS are involved in the response and adaptation of GBS to high glucose condition. As previously reported for the response of GBS to pH stress conditions (Santi *et al*., 2009), also in the case of high glucose conditions the contribution of CsrRS to the transcription of glucose-dependent genes was found to be independent on CsrR abundance, as its expression remained constant along the time span of the experiment. This is in agreement with our findings showing that CsrR enhances its affinity for the *bibA* promoter in the presence of glucose. Based on these evidences, we speculate that as for the pH-dependent activation of CsrR (Santi *et al*., 2009), the transmission of a signal through the CsrS sensor protein during glucose-stress conditions, may be responsible for the increased affinity for regulated promoters (Jiang *et al*., 2008).

The clearest link between complex carbohydrate utilization and virulence factor production is represented by the transcriptional regulator CcpA, exhaustively studied in GAS (Almengor *et al*., 2007; Shelburne *et al*., 2008b), but also present in other streptococcal species (Tomoyasu *et al*., 2010; Abranches *et al*., 2008). This complex metabolic system controls carbohydrate catabolism by inhibiting enzymes necessary for the metabolism of alternative sugar in the presence of glucose. In particular, CcpA, under the stimuli of environmental sugars,

modulates both metabolic and virulence genes by binding to the *cre* site and repressing their expression (Tomoyasu *et al*., 2010; Abranches *et al*., 2008; Almengor *et al*., 2007). In our study, we demonstrated that the *cre* box-containing promoter of *sap*, one of the most regulated genes in the presence of glucose, is a target of CcpA *in vitro*, suggesting that this protein may contribute to the regulation of metabolic genes under glucose stress conditions. Indeed, we found that numerous genes reported to be potentially controlled by CcpA were among the most down-regulated under high glucose conditions. These data might a starting point for future investigations to better define the role of carbon catabolism in GBS virulence.

The need for an increased knowledge of bacterial pathogenesis to better design curative strategies is among the most challenging objective of modern society. In this context, the data reported in this paper by highlighting mechanisms used by GBS to adapt to pathological conditions, may contribute to the understanding of GBS infectivity and put the basis for the development of next generation therapeutic and preventive agents.

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