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Regulation of CovR expression in Group B *streptococcus* impacts blood-brain barrier penetration

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Summary

Group B *Streptococcus* (GBS) is an important cause of invasive infections in humans. The pathogen encodes a number of virulence factors including the pluripotent β -hemolysin/cytolysin (β -H/C). As GBS has the disposition of both a commensal organism and an invasive pathogen, it is important for the organism to appropriately regulate β -H/C and other virulence factors in response to the environment. GBS can repress transcription of β -H/C using the two component system, CovR/CovS. Recently, we described that the serine/threonine kinase Stk1 can phosphorylate CovR at threonine 65 to relieve repression of β -H/C. In this study, we show that infection with CovR deficient GBS strains resulted in increased sepsis. Although CovR deficient GBS showed decreased ability to invade the brain endothelium *in vitro*, they were more proficient in induction of permeability and proinflammatory signaling pathways in brain endothelium and penetration of the blood brain barrier (BBB) *in vivo*. Microarray analysis revealed that CovR positively regulates its own expression and regulates the expression of 153 genes. Collectively, our results suggest that the positive feedback loop which regulates CovR transcription modulates host cell interaction and immune defense and may facilitate the transition of GBS from a commensal organism to a virulent meningial pathogen.

Keywords

Streptococcus agalactiae; transcription; regulation; two component system; sepsis; meningitis; blood brain barrier

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Introduction

Group B Streptococci (GBS, *Streptococcus agalactiae*) are β -hemolytic, Gram-positive cocci that are the most common cause of bacterial infections in human neonates, and are emerging pathogens of certain adult populations (Baker & Edwards, 1995). GBS reside as commensal organisms in the lower gastro-intestinal and genital tracts of healthy women. Transmission of GBS to the newborn can occur *in utero* due to ascending infection or from aspiration of contaminated vaginal fluids during birth. GBS disease in human newborns can manifest as pneumonia, sepsis and meningitis (Baker & Edwards, 1995, Rajagopal, 2009).

GBS encode a number of factors that are important for its disease pathogenesis (Maisey *et al.*, 2008, Rajagopal, 2009). This includes the cell surface associated toxin known as β -hemolysin/cytolysin (β -H/C). GBS strains lacking β -H/C are severely attenuated for virulence in pneumonia, sepsis, meningitis and arthritis models of infection (Doran *et al.*, 2003, Doran *et al.*, 2002, Ring *et al.*, 2002, Hensler *et al.*, 2005, Puliti *et al.*, 2000). This is due to the fact that β -H/C promotes activation of inflammatory responses and invasion of host cell barriers that include the lung epithelium and the endothelial cells which constitute the blood-brain barrier (BBB) (Nizet *et al.*, 1996, Nizet *et al.*, 1997a, Gibson *et al.*, 1999, Doran *et al.*, 2002, Doran *et al.*, 2003, Nizet, 2002). β -H/C has also been described to promote liver injury and cardiac failure in animal models of infection (Hensler *et al.*, 2008). Because of the ability of GBS to efficiently transition from commensal to invasive niches in the human host, appropriate expression of β -H/C and other virulence factors in response to the external environment is critical for the pathogen's life cycle.

Regulation of virulence factor expression in bacteria is primarily accomplished by two component signaling systems (TCS, (Hoch & Silhavy, 1995, Beier & Gross, 2006)). A typical TCS consists of a membrane-associated sensor histidine kinase and its cognate response regulator. Environmental signals are detected by the sensor histidine kinase, which then phosphorylates its cognate response regulator at a conserved aspartate residue. Aspartate phosphorylation often alters the DNA binding affinity of the response regulator and thus changes gene expression for successful environmental adaptation (Hoch & Silhavy, 1995, Stock *et al.*, 2000).

In GBS, the TCS comprising the sensor histidine kinase CovS (or CsrS) and the response regulator CovR (or CsrR) repress the transcription of the *cylE* gene encoding β -H/C (Jiang *et al.*, 2005, Lamy *et al.*, 2004). CovR/S (Cov, control of virulence) or CsrR/S (Csr, capsule synthesis regulator) was identified in GBS based on its sequence homology to the CovR/S or CsrR/S TCS of Group A Streptococcus (GAS, (Jiang *et al.*, 2005, Lamy *et al.*, 2004, Federle *et al.*, 1999, Levin & Wessels, 1998). Homology modeling suggest that GBS CovR is similar to response regulators of the OmpR family and has an N terminal receiver domain that is linked to the C terminal, DNA binding effector domain by the flexible linker domain (see homology model in (Lin *et al.*, 2009)). CovS was described to enhance CovR repression of *cylE* and other CovR regulated GBS genes (Jiang *et al.*, 2005, Jiang *et al.*, 2008). Although not shown directly, this is most likely due to increased CovR phosphorylation at the conserved aspartate residue at position 53 (D53, (Jiang *et al.*, 2005, Lin *et al.*, 2009)). Recently, we described that a serine/threonine kinase Stk1 can also phosphorylate CovR at a threonine residue in position 65 which decreases aspartate (D53) phosphorylation and promoter binding (Lin *et al.*, 2009). Consequently, we observed that 1) alanine substitution of the conserved aspartate at position 53 (D53A CovR) abolished CovR function, 2) glutamate substitution of threonine at position 65 (T65E CovR, mimics constitutive Stk1 phosphorylation) decreased aspartate phosphorylation, promoter binding & CovR function and 3) alanine substitution of threonine at position 65 (T65A CovR) modestly increased CovR regulation in GBS (Lin *et al.*, 2009). The purpose of this study was to evaluate

whether post-translational modifications of CovR were important for GBS virulence. Our studies indicate that GBS strains with decreased CovR function (T65E CovR, D53A CovR and $\Delta covR$) show a dramatic increase in their ability to cause bloodstream infections, activate innate immune signaling pathways and penetrate the blood brain barrier. We also show that CovR positively regulates its own expression in GBS. Taken together, these results suggest that a positive feedback loop enables GBS to regulate CovR function for virulence factor expression during disease pathogenesis.

Results

Role of CovR in GBS virulence

To determine if CovR regulation is important during GBS infection, we used the murine sepsis model of infection to compare virulence potential of strains with decreased CovR function (T65E CovR, D53A CovR and $\Delta covR$) to those proficient for CovR function (WT, T65A CovR). Interestingly, we observed that the mice infected with the $\Delta covR$ strain or those encoding T65E or D53A CovR succumbed to the infection within 2 days after injection, in contrast to infection with WT or T65A CovR (Fig. 1). Survival of mice infected with the CovR deficient strains was statistically significant compared to WT or T65A CovR (P value <0.0001). The accelerated morbidity and mortality observed during infection with CovR deficient GBS was not observed in mice infected with a double $\Delta covR\Delta cylE$ mutant (Fig. S1).

We have previously demonstrated that β -H/C contributes to the development of GBS meningitis and blood brain barrier (BBB) immune activation (Doran *et al.*, 2003). To investigate the role of CovR in BBB penetration and central nervous system (CNS) infection, we used a murine model of hematogenous GBS meningitis (Doran *et al.*, 2005, Doran *et al.*, 2003). As shown in Fig. 2A the number of CFU recovered from the brains of mice infected with the CovR deficient strains (T65E, D53A, and $\Delta covR$) were significantly higher than the CovR proficient (WT, T65A CovR) strains. However, the average CFU of CovR proficient and deficient strains isolated from the blood and spleens of infected mice at this time was not significantly different (Fig. 2B and C). We also observed a 2-8 fold increase in chemokine KC (the murine functional homologue of IL-8) levels in the brains of mice infected with CovR deficient GBS compared to WT or T65A CovR (Fig. 2D). Taken together, these data indicate that regulation of CovR expression contributes to GBS virulence, BBB penetration and chemokine expression.

Contribution of CovR to GBS invasion and blood brain barrier penetration

The increase in bacterial CFU and KC expression observed in the brains of mice infected with CovR deficient GBS ($\Delta covR$, D53A or T65E) prompted us to examine their ability to invade brain endothelium *in vitro*. Studies have indicated that GBS adherence and invasion of the BBB are essential steps in the pathogenesis of meningitis (Doran *et al.*, 2005, Doran *et al.*, 2003). The tissue culture model of the BBB consisting of immortalized human brain microvascular endothelial cells (hBMEC, (Stins *et al.*, 1997)) has been extensively used to compare GBS adherence and invasion of the BBB (Nizet *et al.*, 1997b, Doran *et al.*, 2005, Doran *et al.*, 2003). Our studies indicate that while adherence of $\Delta covR$ or GBS encoding D53A or T65E CovR to hBMEC was not significantly different from the WT (Fig. 3A), these strains showed a dramatic decrease in their ability to invade hBMEC (Fig. 3B). In contrast, invasion of hBMEC by GBS encoding T65A CovR was not significantly different from the WT (Fig. 3B). Notably, the $\Delta covR$ strain also showed a significant decrease in invasion of A549 lung epithelial cells ($<1\%$, see Fig. S2). This decrease in invasion of host cells was not due to the inability of $\Delta covR$ to adhere to A549 or to grow in media such as RPMI (Figs. S2 and S3) as indicated in NEM316 (Lamy *et al.*, 2004). Trypan blue staining

of hBMEC and A549 cells following infection with either WT or $\Delta covR$ did not indicate any significant differences in cell viability (data not shown), suggesting the absence of cytotoxic effects. Taken together, our results suggest that CovR regulation may be important for GBS host cell invasion.

Despite the fact that CovR deficient GBS exhibit decreased ability to invade brain endothelium *in vitro* (Fig. 3), CovR inactivation led to increased BBB penetration *in vivo* (Fig. 2). Therefore, we hypothesized that infection with CovR deficient GBS alters the integrity of the BBB endothelium, which can facilitate bacterial access to the CNS. To test this hypothesis, two different methods were used to assess hBMEC integrity and barrier function. First, we measured hBMEC permeability to Evans Blue dye following GBS infection and second, we monitored changes in trans-endothelial electrical resistance (TEER) across hBMEC monolayers in real time using an ECIS instrument (Applied BioPhysics, Troy, NY). These studies showed that infection with the CovR deficient strain ($\Delta covR$) resulted in increased hBMEC permeability to Evans Blue (Fig. 4A) and a time dependent decrease in the integrity of the BBB endothelium, when compared to the WT (Fig. 4B). A significant and marked delay in the decrease in hBMEC resistance was observed following infection with the control β -H/C deficient strain ($\Delta cylE$, Fig. 4B), suggesting that this CovR regulated toxin contributes to BBB disruption.

CovR mediated activation of brain endothelium

Studies have demonstrated that BBB endothelium responds to the β -H/C toxin with functional gene expression to promote the characteristic neutrophilic inflammatory response of acute bacterial meningitis (Doran *et al.*, 2003). We sought to determine the hBMEC global gene activation program during infection with GBS CovR substitutions that exhibit increased β -H/C expression (Lin *et al.*, 2009). The transcriptional response of hBMEC to infection with either WT or GBS encoding D53A or T65E CovR strains were performed as described (Doran *et al.*, 2003, van Sorge *et al.*, 2008). Consistent with previous studies (Doran *et al.*, 2003), the most highly induced hBMEC genes following WT GBS infection were CXC subfamily chemokines IL-8, CXCL1 and CXCL2 which act mainly on cells of neutrophil lineage. Notably, the CovR deficient strains stimulated a 3-20 fold increase in transcription of twenty nine hBMEC genes including those encoding proinflammatory cytokines/chemokines compared to WT (IL-8, CXCL2, CCL20, IL-6 and IL-11, Table S2). As observed in the microarray analysis, quantitative RT-PCR confirmed that transcript levels of IL-6, IL-8, CXCL2, and CCL20 were significantly upregulated in cells infected with CovR deficient GBS compared to uninfected or hBMEC infected with WT GBS (Fig. 5). These data indicate that CovR regulation plays a significantly role in the modulation of the BBB endothelial response during GBS infection.

CovR regulation of gene expression in GBS serotype Ia strain A909

Previous reports have indicated that although CovR/S can regulate the expression of 150 genes, regulation of only 39 genes was conserved among the GBS strains analyzed to date (Jiang *et al.*, 2008). Our studies indicated that CovR-deficient GBS, despite their hypo-invasive phenotype *in vitro*, exhibit increased virulence potential, BBB penetration and ability to provoke proinflammatory signaling pathways. Transcriptional profiling analysis indicated that, in A909, CovR repressed and activated the expression of 89 and 64 genes, respectively (Table S3), and also confirmed that expression of *covR* (SAK_1639) was decreased in the $\Delta covR$ strain (456.13-fold less than WT). A comparison of CovR regulated genes in A909 to those determined in 2603v/r, NEM316 and 515 indicates that only 27 CovR repressed and 3 CovR activated genes are conserved between these GBS strains (see underlined SAK loci in Table S3). As expected (Lamy *et al.*, 2004, Jiang *et al.*, 2008, Lin *et al.*, 2009, Rajagopal *et al.*, 2006), a significant increase in *cylE* (21 fold, β -H/C) and

decrease in *cfb* (7.1 fold, CAMP factor) transcription was observed in the $\Delta covR$ strain compared to WT (Table S3). Twenty five genes were uniquely repressed by CovR in A909 (see * in Table S3, A). This includes repression of the TCS *rgfC/rgfA* (Spellerberg *et al.*, 2002, Rajagopal, 2009), the iron siderophore *fhu* transport system (Clancy *et al.*, 2006) and the fibrinogen binding protein FbsB (SAK_0955 (Gutekunst *et al.*, 2004, Rosenau *et al.*, 2007)). A comparison of genes activated by CovR in A909 to those in NEM316, 515 and 2603v/r indicated that 51 genes were uniquely up regulated (see * in Table S3, B). These include a moderate increase in transcription of hyaluronate lyase (SAK_1284), the histidine triad protein (SAK_1318) and components of the λ prophage Sa03 (SAK_0607 to SAK_0654, Tettelin *et al.*, 2005).

A positive feed back loop regulates CovR expression

Interestingly, the microarray analysis indicated that transcription of *covS* was lower in the $\Delta covR$ strain (≥ 2.8 -fold, Table S3, B); similar results were seen in the microarray analysis of GBS 515 (Jiang *et al.*, 2008). These observations prompted us to investigate whether CovR can activate its own expression. Transcription of *covR* and *covS* is controlled from two promoters ((Lamy *et al.*, 2004), P_{SAK_1640} and P_{covR} , Fig. 7A). We recently described that the substitutions D53A or T65E prevents CovR promoter binding in contrast to the T65A substitution (Lin *et al.*, 2009). Therefore, we hypothesized that if CovR activates its expression by binding to P_{SAK_1640} and/or P_{covR} promoters, then transcription and expression of *covR* and *covS* should be decreased in GBS encoding D53A and T65E and not in T65A CovR. To test this hypothesis, qRT-PCR was performed to evaluate the expression of *covR* and *covS* in WT, $\Delta covR$ and GBS encoding D53A, T65E and T65A CovR. Consistent with our hypothesis, transcription of *covS* and *covR* were significantly lower (≥ 5 fold, see Table 1) in the D53A and T65E CovR strains unlike the T65A strain that showed a moderate increase in *covR/S* transcription (≥ 2.4 -fold, Table 1).

To further confirm that the decrease in *covR* transcription correlated to a decrease in CovR protein levels, antibodies were raised to purified CovR protein and quantitative westerns were performed. Since the microarray analysis indicated that CovR does not regulate σA (*rpoD*, SAK_1461) in A909 (GSE9370), CovR expression was normalized to σA as described (Seepersaud *et al.*, 2006). As expected, a band corresponding to CovR protein was not observed in the $\Delta covR$ strain (Fig. 6). Consistent with the dramatic decrease in *covR* transcription (Table 1), CovR protein levels were lower in GBS encoding either D53A (0.01 ± 0.05) or T65E (0.08 ± 0.01) CovR compared to WT (Fig. 6).

CovR directly binds to the P_{covR} promoter

Given the dramatic decrease in CovR transcription and expression in GBS encoding T65E CovR (Table 1, Fig. 6) and our findings that T65E CovR does not bind promoter DNA (Lin *et al.*, 2009), we hypothesized that CovR may bind to its own promoters P_{SAK_1640} and/or P_{covR} to activate its expression. Examination of the CovR promoter sequence in P_{SAK_1640} and P_{covR} indicated the presence of only one CovR binding site TATAAAT (Lamy *et al.*, 2004) in P_{covR} (Fig 7A). Thus, we hypothesized that CovR may bind to and activate transcription of *covR/S* from the P_{covR} promoter and not from the P_{SAK_1640} promoter. To test this hypothesis, we examined the interaction of recombinant CovR protein with the promoters, P_{covR} and P_{SAK_1640} , using electrophoretic mobility shift assay (EMSA). Using methods described for OmpR (Kenney *et al.*, 1995), we confirmed that greater than 90% of the CovR protein was phosphorylated when treated with acetyl phosphate or phosphoramidate (data not shown). Our results indicated that aspartate phosphorylated CovR (CovR~P) binds to the P_{covR} promoter (Fig. 7B) and binding is competitively inhibited by unlabeled P_{covR} (Fig. S4). The decrease in probe (P_{covR}) mobility and competitive inhibition by unlabeled P_{covR} is similar to that observed with P_{cylX} (Lin *et al.*,

2009). Incubation of CovR with the phosphodonor was required to observe DNA binding to P_{covR} similar to P_{cylX} (Lin *et al.*, 2009). The decrease in P_{covR} mobility (Fig. 7B) was also observed with the T65A CovR~P protein but not with D53A or T65E CovR~P (data not shown). We also observed that WT CovR~P did not bind to the P_{SAK_1640} promoter (Fig. 7C). These results suggest that CovR binds to the P_{covR} promoter to positively regulate its expression. We predict that the decrease in CovR transcription and protein levels in GBS encoding D53A or T65E CovR may be due to their inability to activate transcription from the P_{covR} promoter. Although previous studies have suggested that transcription of *covR/S* is independent of CovR in NEM316 (Lamy *et al.*, 2004), we confirmed that CovR~P binds to P_{covR} of NEM316 (Fig. S5). Similar results were observed with P_{covR} from GBS NCTC10/84 and COH1 (data not shown). Collectively, our results suggest that the positive feedback loop which regulates CovR expression enables GBS to appropriately express virulence factors for disease pathogenesis.

Discussion

Signaling systems are essential for all living organisms to respond to their dynamic external environment. The importance of TCS in adaptive responses and regulation of virulence factor expression has been described for many pathogenic organisms (Calva & Oropeza, 2006, Beier & Gross, 2006, Laub & Goulian, 2007, Bourret, 2008). This study reports our investigation of CovR regulation of gene expression and virulence of the GBS serotype Ia strain A909. Our studies indicate that CovR deficient GBS promote inflammatory activation, BBB penetration and enhanced mortality. The dramatic inflammatory response and accelerated morbidity and mortality observed in the complete absence of CovR suggests that during infection, CovR mediated repression of virulence factors may blunt BBB cytokine secretion and enable GBS to avoid innate immune mechanisms for its persistence in the host. Therefore, fine-tuned regulation of virulence factors in response to the external environment is critical for GBS pathogenesis.

Our studies also indicate that CovR activates the expression of 64 and represses the expression of 89 genes in A909 including the cytotoxins β -H/C and CAMP factor. As only 30 CovR regulated genes of A909 are represented in the previously identified CovR core-regulon (Jiang *et al.*, 2008), these data further confirm the diversity of CovR regulation in GBS. Despite the dramatic increase in expression of factors important for GBS adherence and invasion, we observed that CovR deficient strains showed decreased invasion of host cells such as hBMEC and A549 lung epithelial cells. We cannot rule out the possibility that this decrease in recovered intracellular bacteria may in part be due to decreased intracellular survival of these strains. The ability of GBS to leave the bloodstream and penetrate the BBB is thought to be achieved by a combination of events that includes direct invasion and transcytosis of brain endothelium, break down of BBB due to bacterial toxins (e.g. β -H/C) and the induction of a host inflammatory response which itself may act to compromise barrier function. Here we show that CovR mutants increased BBB permeability and provoked a significant increase in gene expression to promote inflammatory cytokine and chemokine release specific to neutrophil signaling and activation. Neutrophil recruitment is thought to be part of the very first line of CNS defense against bacterial infection as many Gram-positive and Gram-negative meningeal pathogens induce expression of these genes in hBMEC (Doran *et al.*, 2003, van Sorge *et al.*, 2008, Sokolova *et al.*, 2004). We predict that the marked increase in expression of β -H/C in CovR deficient GBS facilitates the induction of a robust inflammatory response, and subsequent neutrophil recruitment compromises the integrity of the BBB leading to disease progression and eventual mortality. Our results further suggest that the inflammatory response plays a significant role in the pathogenesis of GBS CNS infection and that the invasive capability of the organism is not required for BBB activation or disruption.

Paxadoxically, previous studies have indicated that CovR mutants derived from GBS NEM316, 2603v/r or 515 that exhibit significantly increased β -H/C expression were attenuated for sepsis (differences ranged from 10-100 fold compared to the isogenic WT (Jiang *et al.*, 2005, Lamy *et al.*, 2004)). We predict that the differences in virulence of CovR mutants can be attributed to the route and models of infection used in the respective studies. The intraperitoneal route of infection used by Jiang *et al* (Jiang *et al.*, 2005) requires GBS to invade the epithelium to gain access into the bloodstream to cause sepsis, in contrast to the direct intravenous inoculation used in this study. As CovR deficient GBS demonstrate decreased epithelial cell invasion, this might account for the previously observed virulence attenuation. Notably, the CovS mutant of 2603v/r showed increased virulence in the murine septic arthritis model of infection (Jiang *et al.*, 2008), indicating the differential role of CovR/CovS in this strain.

Consistent with our observations, recent reports have indicated that GBS isolates obtained from patients with severe sepsis or necrotizing fasciitis and toxic shock include hyper-hemolytic variants (Sigge *et al.*, 2008, Sendi *et al.*, 2009). A 3bp deletion in *covR* that eliminated valine in position 31 was associated with the serotype Ib hyper-hemolytic variant from the patient with necrotizing fasciitis (Sendi *et al.*, 2009). Whether this deletion decreased or abolished CovR phosphorylation at D53 and/or its DNA binding ability in GBS is not known. It is likely that in some GBS strains, CovR mutants are under selective pressure during invasive disease similar to that observed with CovR/S in GAS (Sumby *et al.*, 2006, Walker *et al.*, 2007). Consequently, strategies employing CovR/S as a model system for attenuated virulence or as target in future antimicrobial therapies will not be valid for all GBS strains. The strain specific role of TCS such as that of CovR/S in GBS has been previously reported for other TCS in bacterial pathogens such as *Streptococcus pneumoniae* (Hendriksen *et al.*, 2007, Paterson *et al.*, 2006) and *Staphylococcus aureus* (Cassat *et al.*, 2006).

Our findings that A909 CovR impacts bloodstream and CNS infection, and that a positive feedback loop enables CovR to activate its expression provides insight into GBS environmental adaptation. Because the dramatic increase in inflammatory response observed in the complete absence of CovR can be detrimental to the host and consequently to the pathogen, regulation of CovR function (rather than its presence or absence) may play a crucial role during the course of GBS infection. Based on the findings of this study, we predict that during its lifestyle as a commensal organism, CovS may sense environmental signals to phosphorylate CovR at the conserved aspartate D53 residue located in the receiver domain. Aspartate phosphorylation induces CovR promoter binding that increases CovR expression from P_{covR} and also enhances CovR mediated repression of GBS virulence factors such as β -H/C, FbsA, FbsB and iron acquisition systems (Fig. 8). Conversely, during invasive infection, we predict that decreased phosphorylation of the conserved aspartate (due to other sensor kinases such as Stk1 (Lin *et al.*, 2009) or the lack of CovS activation signals) decreases CovR expression from P_{covR} and alleviates CovR repression of GBS virulence factors, and iron acquisition systems (Fig. 8). Support for this hypothesis is provided by recent observations that a number of virulence factors that showed increased expression at pH 7 (invasive state) compared to pH 5 (acidic environment of the vagina, commensal state) are repressed by CovR/S in GBS 2603v/r (Santi *et al.*, 2009).

Notably, we observed that CovR positively regulates its own expression in GBS. These observations are in contrast to previous observations in GAS and *Streptococcus mutans*, where CovR homologues have been shown negatively regulate their own expression (Gusa *et al.*, 2006, Gusa & Scott, 2005, Chong *et al.*, 2008). In GAS, CovR regulates the expression of ~ 15% of the genome and is primarily a repressor of virulence gene expression (Graham *et al.*, 2002). Extensive studies have described that the GAS CovR recognizes the

ATTARA consensus sequence and directly represses its own promoter, P_{cov} (Gusa *et al.*, 2006, Gusa & Scott, 2005). Up to 7 CovR consensus binding sites are located in the GAS P_{cov} promoter spanning the region from -58 upstream to the transcription start site, to + 346 within the CovR open reading frame (Gusa & Scott, 2005). In contrast to GAS, the GBS CovR 1) recognizes the TATTTAAT consensus sequence and only one binding site is located upstream to the transcriptional start site in the CovR promoter (Fig. 7A and (Lamy *et al.*, 2004)), 2) activates and represses approximately similar numbers of genes (Lamy *et al.*, 2004, Jiang *et al.*, 2005, Jiang *et al.*, 2008) and this study) and 3) is also regulated by Stk1 (Lin *et al.*, 2009). Although further characterization is necessary to demonstrate that CovR directly activates its own expression in GBS, our collective results on *covR* transcription and expression (protein levels) in the substitution mutants and P_{covR} DNA binding suggests that CovR positively regulates its own expression.

The importance of positive feedback loops of TCS to bacterial disease pathogenesis has been previously described for a number of pathogens including *Salmonella*, *Bordetella pertussis*, and *S. pneumoniae* (Scarlato *et al.*, 1990, Shin *et al.*, 2006, Alloing *et al.*, 1998). As most two component regulators require external signals for activation at the conserved aspartate, it is beneficial for organisms to regulate their expression (Mitrophanov & Groisman, 2008). This mode of regulation enables organisms to conserve their energy resources and minimize non-essential cross talk between signaling systems. Given that CovR represses the expression of > 70 genes which include a number of virulence factors, it is conceivable that the presence of a positive feed back loop would enable GBS to regulate CovR and virulence factor expression in response to the host niches and environmental conditions encountered during its disease cycle. Identification of the external signals/cues that are sensed by the sensor kinases such as CovS and Stk1 for regulation of CovR function will provide a deeper understanding of the role of this important regulator in GBS virulence.

Experimental Procedures

General growth

The strains, plasmids and primers used in this study are listed in Table S1. The wild type (WT) GBS strain A909 is a serotype 1a capsular polysaccharide clinical isolate (Madoff *et al.*, 1991). The $\Delta covR$ strain is isogenic to A909 and was constructed as described (Rajagopal *et al.*, 2006). GBS strains encoding the chromosomal, site directed substitutions in CovR such as D53A, T65A and T65E are isogenic to A909 and were derived previously (Lin *et al.*, 2009). Routine cultures of GBS were performed in Tryptic Soy Broth (TSB, Difco Laboratories) in 5% CO₂ at 37°C. The *Escherichia coli* strain BL21DE3 and plasmid pLR136 were used to produce CovR-His₆ fusion protein as described (Rajagopal *et al.*, 2006). Routine cultures of *E. coli* were performed in Luria-Bertani broth (LB, Difco Laboratories) at 37°C. All chemicals were purchased from Sigma-Aldrich, unless mentioned otherwise. GBS cell growth was monitored at 600 nm after incubation in 5% CO₂ at 37°C unless mentioned otherwise. Antibiotics were added at the following concentrations when necessary: For GBS, erythromycin 1 µg ml⁻¹; spectinomycin 300 µg ml⁻¹; For *E. coli*, erythromycin 300 µg ml⁻¹ spectinomycin 50 µg ml⁻¹; ampicillin 50 µg ml⁻¹.

Mouse infection studies

All animal experiments were approved by the Institutional Animal Care and Use Committee, Seattle Childrens Research Institute, and performed using accepted veterinary standards. Six week old male CD-1 mice obtained from Charles River Laboratories (MA, USA) were injected via the tail vein with 1×10^9 CFU of either WT A909 or isogenic $\Delta covR$, T65A, T65E and D53A strains ($n = 8$ per group). Survival of the mice was monitored up to 20 days after infection. To examine BBB penetration and chemokine expression CD-1 mice were

infected via tail vein with 3×10^6 - 10^7 WT, $\Delta covR$, T65A, T65E and D53A CovR ($n = 5$ per group); blood, brain and spleens from infected mice were collected aseptically 24-48 hr after infection. Note, at this time the mice had not succumbed to the infection. Bacterial counts in brain and spleen homogenates were determined by plating serial 10-fold dilutions on TSB agar. Chemokine KC (IL-8 homologue) ELISA assays were performed on supernatants of brain homogenates from above using the DuoSet® kit as described by R&D Systems, USA.

Adherence and Invasion of hBMEC and A549

The hBMEC line, immortalized by transfection with the SV40 large T antigen (Stins *et al.*, 1997) was used in these studies. Propagation of hBMEC and GBS adherence and invasion assays was performed as described previously (Doran *et al.*, 2005). Parallel invasion experiments with A549 lung epithelial cells were performed as described (Doran *et al.*, 2002). A 2 fold increase or decrease in adherence or invasion compared to the isogenic WT was considered significant as described (Doran *et al.*, 2005).

Assessment of hBMEC permeability and barrier integrity

The barrier function and permeability of hBMEC monolayers during GBS infection was assessed using two methods. First, hBMEC were established on collagen-coated Transwell plates, pore size 3 μ m (Transwell-COL; Corning-Costar Corp., Acton MA) as described previously (Doran *et al.*, 2003). Monolayers were incubated with the WT or the $\Delta covR$ mutant (MOI = 1) for 6 hours at 37°C in 5% CO₂. Transwells inserts were then transferred to a fresh plate containing HBSS (Hanks Balanced Salt Solution) in the bottom chamber and 25 μ l of 0.4% Evans Blue solution in PBS was added to the upper chamber. Following a 40 min incubation hBMEC permeability was assessed by colorimetric quantification of Evans Blue present in the bottom chamber (OD=600 nm). Uninfected wells served as a control for background levels of dye migration. Second, changes in transendothelial electrical resistance (TEER) were monitored by electric cell-substrate impedance sensing (Giaever & Keese, 1984, Giaever & Keese, 1991, Giaever & Keese, 1993, Lo *et al.*, 1995) using an ECIS ZTheta Instrument and 8W10E+ arrays (Applied BioPhysics, Troy, NY). HBMEC monolayers were established on gold plated electrodes in 8 well array slides attached to a computer operated sensing apparatus to allow measurements in real time. At confluence hBMEC reached a TEER of ~1200-1400 ohms, which is similar to that reported previously for hBMEC using this method (Tripathi *et al.*, 2007). Monolayers were then infected with WT, $\Delta covR$ or $\Delta cylE$ strains (1×10^5 CFU/well) and the system measured the cell membrane capacitance (Cm), the resistance from the cell-electrode interaction (α), and the barrier function properties of the cell monolayer (Rb). Deconvolution of the overall ECIS signal into these parameters is performed by the ECIS software by fitting the mathematical model derived by (Giaever & Keese, 1991) to the experimental data by least-square optimization procedures. Data is shown as a decrease in resistance as a proportion of control plotted versus time.

hBMEC microarray analysis

Briefly, hBMEC monolayers were cultured, washed and infected with GBS as described (Doran *et al.*, 2003). Subsequently, total RNA was isolated from hBMEC monolayers using the RNeasy miniprep kit (Qiagen Inc., USA) according to the manufacturer's protocol. Microarray analysis using Human WG-6 vS arrays from Illumina Inc. (USA) was performed as described (van Sorge *et al.*, 2008). A statistical algorithm developed for high-density oligonucleotide arrays was used for data analysis and a three fold increase or decrease in gene expression was considered significant (Doran *et al.*, 2003). hBMEC microarrays were performed with two independent biological replicates of each strain (WT A909, D53A CovR, T65E CovR) and media only controls. cDNA synthesis and quantitative PCR (qPCR)

for IL-6, IL-8, CXCL1, CXCL2, CCL20 and GAPDH was performed using primers and methods described previously (van Sorge *et al.*, 2008).

GBS microarrays and qRT-PCR

Total RNA from GBS was isolated using the RNeasy Mini kit (QIAGEN, Inc. USA) as described (Lin *et al.*, 2009) RNA integrity and concentration was determined using an Agilent 2100 Bioanalyzer (Agilent, USA). Purified RNA from three independent biological replicates for each strain was sent to NimbleGen Systems, Inc. for full expression services using the *Streptococcus agalactiae* A909 chip (cat. no. A4327-00-01; NimbleGen). Microarray data was interpreted and analyzed using the program GeneSpring GX (GeneSpring GX 7.3.1; Agilent Technologies, CA). Genes with statistically significant differences among groups were calculated using the Welch t-test (parametric, with variances not assumed equal) with a P-value cutoff of 0.05 and an associated Benjamini and Hochberg False Discovery Rate multiple testing correction (Benjamini & Hochberg, 1995). All fold changes were defined as relative to A909 WT. The microarray data is deposited at Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo>), accession number GSE9370. qRT-PCR was performed using a one-step QuantiTect SYBR Green RT-PCR kit (QIAGEN, Inc., USA) as described (Lin *et al.*, 2009). The reference gene used for all runs was the housekeeping ribosomal protein S12 gene *rpsL* (Jiang *et al.*, 2005).

Electrophoretic mobility shift assay (EMSA)

Purification of CovR-His₆ fusion protein was performed as described previously (Rajagopal *et al.*, 2006). Primers to amplify promoter regions P_{cov} and P_{SAK_1649} are listed in Table S1. Promoter regions were PCR amplified using Pfu DNA Polymerase (Stratagene, La Jolla, CA) and A909 genomic DNA was used as the template. PCR fragments were purified with the QIAquick PCR Purification Kit (QIAGEN, Inc., Valencia, CA). End labeling of the PCR fragments and binding reactions were carried out as described recently (Lin *et al.*, 2009).

Generation of CovR antibody

His-tagged CovR protein was purified as described previously (Rajagopal *et al.*, 2006). Antisera against the CovR protein was generated in female New Zealand white rabbits as a service from Lampire Biological Laboratories (Ottsville, PA). In brief, rabbits were immunized subcutaneously with 0.25 mg of CovR conjugated to the highly immunogenic protein keyhole limpet hemocyanin (KLH) that was emulsified in complete Freund's adjuvant for the first dose, and in incomplete Freund's adjuvant for subsequent doses (days 21 and 42 after initial immunization). Serum was prepared from blood collected approximately 2 weeks after the last dose was given. Pre-absorbed antisera was obtained by incubating the serum with the $\Delta covR$ mutant overnight at 4°C in 0.02% sodium azide followed by centrifugation.

Quantitative Western blot analysis

GBS strains were grown to an OD₆₀₀ of 0.6 and cytoplasmic protein fractions were isolated and quantified as described (Seepersaud *et al.*, 2006). Equal amount of cytoplasmic protein from each strain (15µg) was subjected to 12% SDS-PAGE followed by western blotting as described (Seepersaud *et al.*, 2006). In brief, the membrane containing cytoplasmic proteins was blocked in 1:1 Odyssey blocking buffer (LI COR Biosciences, USA) in PBS, and incubated at room temperature for 2 hrs with a 1:7500 dilution of primary CovR antibody, respectively. Subsequently, secondary antibody was added at 1:10,000 and washes were performed following the Odyssey Li-Cor infrared imager instructions. Band intensity was normalized to the constitutively expressed sigma factor σ^A (RpoD) as described (Seepersaud *et al.*, 2006).

Statistical analysis

The Mann-Whitney test was used to evaluate differences between cytokine levels and CFU/organ between GBS strains. Survival analyses were performed using a log rank test. ANOVA was used to evaluate differences in hBMEC permeability as measured by Evans Blue migration after GBS infection. In all cases, p value of <0.05 was considered statistically significant. These tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CovR mutants show increased virulence in the murine model of GBS sepsis

Eight, six week old male CD-1 mice were intravenously injected with 1×10^9 CFU of WT, $\Delta covR$ or isogenic strains encoding D53A, T65E or T65A CovR. Kaplan Meier survival curve shows percent survival of mice after the infection. Note that 100% of mice infected with the CovR deficient strains (T65E CovR, D53A CovR and $\Delta covR$) succumbed to the infection within 2 days post inoculation in contrast to the WT or T65A CovR. Survival of mice infected with the CovR deficient strains was statistically significant compared to WT or T65A CovR (P value <0.0001).

Figure 2. Increased infection in the brain of mice infected with CovR deficient GBS

Five, six week old male CD-1 mice were intravenously injected with $3 \times (10^6 - 10^7)$ CFU of WT, $\Delta covR$ or isogenic strains encoding D53A, T65E or T65A CovR. At approximately 48 hrs post infection, blood, spleens and brains were harvested from the infected mice and CFU were enumerated and cytokine KC (IL8 equivalent) levels were measured. Note the increase in CFU of CovR deficient GBS (T65E CovR, D53A CovR and $\Delta covR$) in the brain (see **A**, $P < 0.05$) and not in the spleen (see **B**) or blood (see **C**), when compared to WT or T65A CovR. An increase in cytokine KC was observed in the brains of mice infected with CovR deficient GBS (see **D**, $P = 0.007$).

Figure 3. The CovR deficient GBS demonstrate decreased invasion of human brain microvascular endothelial cells (hMBEC)

Adherence and invasion of HBMEC by WT GBS A909, $\Delta covR$ and strains encoding the site directed CovR substitutions D53A, T65E and T65A were performed at an MOI (multiplicity of infection) of 1 as described previously (Doran *et al.*, 2003). Percent adherence and invasion is normalized to that of the WT as described (Doran *et al.*, 2005). Note that while adherence to HBMEC by CovR deficient GBS (T65E CovR, D53A CovR and $\Delta covR$) is similar to WT or T65A CovR (see A), these strains demonstrate a severe decrease in invasion (see B). * indicates significantly different from WT ($P = <0.001$).

Figure 4. CovR contributes to hBMEC permeability and decreased barrier function

(A) hBMEC permeability was assessed by Evans Blue dye migration following infection with WT or the $\Delta covR$ mutant (MOI = 1). Following a 6 hr infection, Evans Blue dye was added to the upper chamber and quantified colorimetrically in the lower chamber (OD = 600nm). An increase in absorbance is indicative of hBMEC permeability to Evans Blue; the uninfected or media only control demonstrated a low level of background dye migration. All experiments were repeated at least twice in triplicate; data from a representative experiment is shown. The error bars indicate 95% confidence intervals of the mean of three wells, ** $p < 0.01$, ns = not significant.

(B) shows a time dependent decrease in hBMEC resistance due to infection with the GBS $\Delta covR$ strain compared to WT or the control $\Delta cylE$ mutant. Resistance was assessed by electrical cell substrate sensing and is normalized to the uninfected or media only control.

Figure 5. Increased expression of genes important for the immune response in HBMEC
qRT-PCR was performed on RNA isolated from HBMEC cells infected with either WT or the CovR substitutions D53A and T65E. Increased transcription of IL-6, IL-8, CXCL1, CXCL2 and CCL-20 were observed in HBMEC that are infected with D53A or T65E compared to the WT.

Figure 6. CovR expression is decreased in GBS strains encoding D53A and T65E CovR
 Quantitative western analysis was performed on equal protein concentrations isolated from WT A909, $\Delta covR$, and GBS encoding D53A, T65E and T65A CovR. The blot shown represents one of five independent experiments. Band intensities were normalized to the non-CovR regulated housekeeping gene *rpoD* encoding σA as described ((Seepersaud *et al.*, 2006), see lower panel). CovR-His₆ denotes the lane that contained 150 ng of purified full length His-Tagged CovR protein. Note that expression of CovR is decreased in GBS encoding D53A and T65E CovR when compared to WT or T65A CovR.

Figure 7. CovR binds to the *P_{covR}* promoter

The structure for the *CovR/S* operon and the promoters *P_{SAK_1640}* and *P_{covR}* that regulate *CovR* expression are shown (see A and (Lamy *et al.*, 2004)). The *CovR* binding site is underlined and the translational start codon ATG and subsequent coding sequences are indicated in italics in the *P_{covR}* sequence. Electrophoretic Mobility Shift Assay (EMSA) was performed using purified WT *CovR* protein and the promoters *P_{covR}* promoter (**B**) and *P_{SAK_1640}* (**C**), respectively. Promoters *P_{covR}* and *P_{SAK_1640}* were incubated with equimolar concentrations of acetyl phosphate treated WT *CovR* (*CovR*~P). In all cases, Lane 1 represents probe-only control and lanes 2-7 represent increasing amounts of *CovR* from 0.9 to 21.9 μ M.

Figure 8. Role of the positive feed back loop of CovR in GBS virulence

We propose that during its lifestyle as a commensal, CovS senses external signals and autophosphorylates at a conserved histidine residue (H278). Subsequently, CovS phosphorylates CovR at the conserved aspartate residue (D53) located in the receiver domain (represented as an unshaded circle in CovR). Aspartate phosphorylation at the receiver domain induces promoter DNA binding via the effector domain (represented as a patterned circle in CovR) resulting in increased CovR transcription from the P_{covR} promoter and CovR repression of virulence factors (β -H/C, Fibrinogen binding proteins FbsA, FbsB and Iron transport proteins, FhuCDBG). Conversely, during invasion of host tissues, sensor kinases such as Stk1 may sense external signals and autophosphorylate at the threonine residue (T305, (Silvestroni *et al.*, 2009)). Stk1 then phosphorylates CovR at threonine in position 65 to alleviate aspartate phosphorylation and promoter binding (Lin *et al.*, 2009). In addition, the absence of CovS activation signals can also decrease aspartate phosphorylation and CovR promoter binding. Together, these lead to decreased CovR expression from the P_{covR} promoter and also alleviate CovR repression of GBS virulence factors (β -H/C, FbsA, FbsB and Iron transport proteins FhuCDBG).

Table 1Transcription of *covR* and *covS* in $\Delta covR$ and GBS encoding D53A, T65A and T65E CovR

Gene Expression				
Locus	qRT-PCR*			
	$\Delta covR$	D53A	T65E	T65A
CovR autoregulation				
<i>covR</i>	N/A	0.1 ± 0.1	0.15 ± 0.1	3.4 ± 0.8
<i>covS</i>	0.3 ± 0.05	0.1 ± 0.15	0.1 ± 0.1	2.4 ± 0.1

* qRT-PCR was performed as described in Experimental Procedures. Gene expression is denoted as fold difference relative to the WT GBS strain A909. Standard error is indicated.