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# RovS and Its Associated Signaling Peptide Form a Cell-To-Cell Communication System Required for *Streptococcus agalactiae* Pathogenesis

David Pérez-Pascual,<sup>a,b</sup> Philippe Gaudu,<sup>a,b</sup> Betty Fleuchot,<sup>a,b</sup> Colette Besset,<sup>a,b</sup> Isabelle Rosinski-Chupin,<sup>c</sup> Alain Guillot,<sup>a,b</sup> Véronique Monnet,<sup>a,b</sup> Rozenn Gardan<sup>a,b</sup>

INRA, UMR1319 MICALIS, Jouy-en-Josas, France<sup>a</sup>; AgroParistech, UMR MICALIS, Jouy-en-Josas, France<sup>b</sup>; Institut Pasteur, Unité de Biologie des Bactéries Pathogènes à Gram Positif, Paris, France<sup>c</sup>

**ABSTRACT** Bacteria can communicate with each other to coordinate their biological functions at the population level. In a previous study, we described a cell-to-cell communication system in streptococci that involves a transcriptional regulator belonging to the Rgg family and short hydrophobic peptides (SHPs) that act as signaling molecules. *Streptococcus agalactiae*, an opportunistic pathogenic bacterium responsible for fatal infections in neonates and immunocompromised adults, has one copy of the *shp/rgg* locus. The SHP-associated Rgg is called RovS in *S. agalactiae*. In this study, we found that the SHP/RovS cell-to-cell communication system is active in the strain NEM316 of *S. agalactiae*, and we identified different partners that are involved in this system, such as the Eep peptidase, the PptAB, and the OppA1-F oligopeptide transporters. We also identified a new target gene controlled by this system and reexamined the regulation of a previously proposed target gene, *fbxA*, in the context of the SHP-associated RovS system. Furthermore, our results are the first to indicate the SHP/RovS system specificity to host liver and spleen using a murine model, which demonstrates its implication in streptococci virulence. Finally, we observed that SHP/RovS regulation influences *S. agalactiae*'s ability to adhere to and invade HepG2 hepatic cells. Hence, the SHP/RovS cell-to-cell communication system appears to be an essential mechanism that regulates pathogenicity in *S. agalactiae* and represents an attractive target for the development of new therapeutic strategies.

**IMPORTANCE** Rgg regulators and their cognate pheromones, called small hydrophobic peptides (SHPs), are present in nearly all streptococcal species. The general pathways of the cell-to-cell communication system in which Rgg and SHP take part are well understood. However, many other players remain unidentified, and the direct targets of the system, as well as its link to virulence, remain unclear. Here, we identified the different players involved in the SHP/Rgg system in *S. agalactiae*, which is the leading agent of severe infections in human newborns. We have identified a direct target of the Rgg regulator in *S. agalactiae* (called RovS) and examined a previously proposed target, all in the context of associated SHP. For the first time, we have also demonstrated the implication of the SHP/RovS mechanism in virulence, as well as its host organ specificity. Thus, this cell-to-cell communication system may represent a future target for *S. agalactiae* disease treatment.

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Address correspondence to David Pérez-Pascual, david.perez@jouy.inra.fr.

Communication among bacteria allows them to synchronize their behavior at the population level and thus regulate various biological processes, including biofilm formation, natural competence, or bioluminescence production (1, 2). In pathogenic microorganisms, many cell-to-cell communication systems control the expression of virulence factors, such as the LasI/LasR and RhII/RhIR systems in *Pseudomonas aeruginosa* (3), the Agr system in *Staphylococcus aureus* (4), the Fsr system in *Enterococcus faecalis* (5), and the PapR/PlcR system in *Bacillus cereus* (6). In these cell-to-cell communication processes, bacteria produce, release, detect, and respond to signaling molecules (7, 8). Whereas the signaling molecules of Gram-negative bacteria belong mainly to the acyl-homoserine lactone family, those of Gram-positive bacteria consist mainly of peptides (9).

In a previous study, we described a new cell-to-cell communication system in streptococci that is formed by a transcriptional regulator belonging to the Rgg family and a short hydrophobic peptide (SHP) that acts as a signaling molecule (10, 11). This system has been studied in detail in *Streptococcus thermophilus* strain LMD-9 at locus 1358 (*shp1358/rgg1358* system), at which both genes are divergently transcribed. First, SHP1358 is produced as a propeptide; it is then likely processed by the membrane-associated Eep peptidase, secreted into the extracellular medium, and finally imported into the cell by the oligopeptide transporter Ami. Once inside the cell, the mature form of SHP1358 interacts with the transcriptional regulator Rgg1358 to control expression of the *shp1358* gene and the *ster\_1357* gene; the last is located downstream of *rgg1358* and encodes a cyclic peptide

with an unknown function (12). This system has also been found in the pathogenic bacterium *Streptococcus pyogenes*, which has a complex regulatory network involving two copies of very similar *shp/rgg* loci (13, 14). Furthermore, genomic analysis has shown that SHP-associated Rgg transcriptional regulators are specific to the streptococcus family; they are present in almost all species of this genus (12). The widespread conservation of the SHP/Rgg cell-to-cell communication system in *Streptococcus* suggests that it plays an important role in the genus.

*Streptococcus agalactiae*, also known as Group B *Streptococcus* (GBS), is a commensal bacterium that may be present, asymptotically, in the gastrointestinal and genitourinary tracts of up to 30% of healthy adults (15). GBS is also the leading agent of severe and invasive bacterial infections which can provoke neonatal pneumonia, sepsis, and meningitis (of early or late onset) in human newborns. GBS transmission from colonized mothers to their newborns can occur *in utero* when infections ascend or, more frequently, when the neonate aspirates contaminated amniotic/vaginal fluids during delivery (16). In addition, *S. agalactiae* has been recognized as an ever-growing cause of severe invasive infections in older or immunocompromised adults (17). Although pre-natal detection and, consequently, intrapartum antibiotic prophylaxis have greatly reduced GBS incidence in neonates and in pregnant women, the incidence of GBS-caused disease is still significant, mainly in infants, where it manifests itself as a late-onset disease, and in elderly or immunocompromised adults, where it occurs as a chronic disease (18). Several GBS virulence factors, including capsule, adhesins, hemolysin, proteases, pili, pigment, and extracellular nuclease, have been identified (19–21). When these virulence factors are adequately expressed as a consequence of favorable host conditions, GBS survival may be enhanced, and for this reason, regulatory and signaling molecules are attractive targets when it comes to developing new prophylactic strategies or therapeutic agents aimed at fighting bacterial infections (19).

Sequencing of the GBS genome has revealed that it contains genes encoding around 20 two-component systems (TCS) and more than 90 putative stand-alone regulators (22). In the latter group, there are three genes that code for putative Rgg-like transcriptional regulators, including a single copy of the SHP-associated Rgg transcriptional regulator, known as RovS (11, 23). Samen et al. showed that RovS controls the expression of some genes that code for virulence factors in *S. agalactiae* strain 6313, including *fbsA*, which encodes a fibrinogen-binding protein; *sodA*, which encodes a superoxide dismutase; and the *cyl* operon, which encodes proteins responsible for hemolysin production (23). Recently, Cook et al. reexamined the activity of this RovS transcriptional regulator in strain A909, taking into account the peptide encoded by the adjacent *shp*<sub>1520</sub> gene. They demonstrated that, as proposed for other streptococci, RovS is not a stand-alone regulator but rather a peptide-associated transcriptional regulator that is part of a cell-to-cell communication system that controls the expression of *shp*<sub>1520</sub> (24). However, their study did not provide information about other putative target genes or the relevance of this cell-to-cell communication system for *S. agalactiae* pathogenesis.

In this study, we describe the SHP/RovS cell-to-cell communication system in *S. agalactiae* strain NEM316 and identify the different molecular players involved. A new additional target gene regulated by this system is identified, and a previously proposed target gene, *fbsA*, is reexamined, taking into account the role of

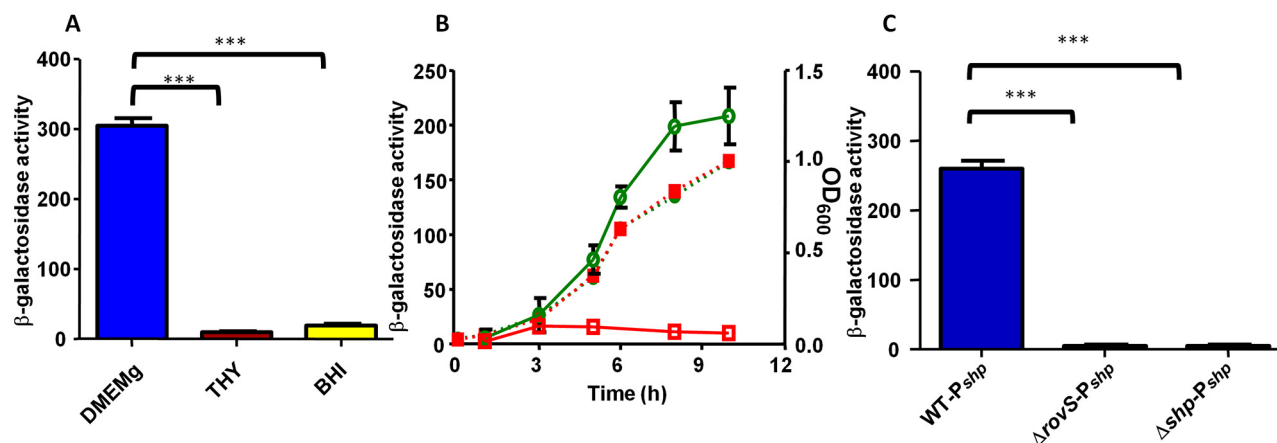
SHP-associated RovS (23). Finally, our results are the first to demonstrate that the SHP/Rgg system mediates streptococcal virulence, based on infection patterns in a murine model, including the bacterium's organ specificity in the host.

## RESULTS

**SHP/RovS forms a cell-to-cell communication system in *S. agalactiae* strain NEM316.** In a previous study, we identified a short gene that encodes a short hydrophobic peptide (SHP); this gene is located upstream of the *rovS* gene in GBS (11). Thus, it seemed highly probable that SHP and RovS act together as part of a cell-to-cell communication system, as has already been seen in other streptococci (12, 13). Notably, the *shp/rovS* locus is present in all 285 sequenced genomes of GBS deposited in GenBank, and both *shp* and *rovS* display more than 99% identity at the DNA level among the strains. As the *shp/rovS* locus sequence is highly conserved across all GBS strains, we chose to use strain NEM316 as a representative with which to decipher the role of the SHP/RovS system, as this strain was responsible for a fatal case of septicemia in an infected infant (22).

First, we confirmed the functionality of the SHP/RovS system in GBS strain NEM316. In the previously described SHP/Rgg systems, transcription of the *shp* gene is positively controlled by the SHP-Rgg complex (12, 13). We therefore constructed a plasmid (*P*<sub>shp</sub>) with a transcriptional fusion between the *lacZ* reporter gene and the promoter of the *shp* gene, which was subsequently transferred into the wild-type (WT) strain, resulting in the WT-*P*<sub>shp</sub> strain (Table S1). We observed that *shp* expression was reduced 15- to 30-fold in cultures grown in the rich media Todd-Hewitt broth supplemented with yeast extract (THY) and brain heart infusion (BHI) medium compared to those grown in the chemically defined medium Dulbecco's modified Eagle's medium with some modifications adapted to GBS growth (DMEMg) (see Materials and Methods), which was peptide free (Fig. 1A). Expression kinetics during growth in DMEMg revealed that *shp* induction was highest at the end of the exponential phase (Fig. 1B). A plasmid-borne transcriptional fusion between *lacZ* and the promoter region of the *rovS* gene showed that *rovS* was constitutively expressed at low levels ( $15 \pm 2$  MU) in DMEMg (Fig. 1B). To elucidate the roles of RovS and SHP in *shp* gene expression, particularly with regard to SHP's function as a signaling molecule, we constructed two mutant strains in which either the *rovS* gene or the *shp* gene was deleted. The plasmid *P*<sub>shp</sub> was used to transform both mutants, yielding the  $\Delta$ *rovS*-*P*<sub>shp</sub> and  $\Delta$ *shp*-*P*<sub>shp</sub> strains (see Table S1 in the supplemental material). As shown in Fig. 1C, the *shp* gene was not significantly expressed in either of the mutant strains when grown in DMEMg. Similar results were obtained in BHI medium (data not shown). We were able to restore the expression of *shp* to WT levels in the  $\Delta$ *shp*-*P*<sub>shp</sub> strain by coculturing it with strain WT-pTCVlac, which is an SHP-producing strain (Fig. S1), or by adding a 1  $\mu$ M concentration of a synthetic peptide (sSHP) that corresponded to the mature form (DILIVGG) of SHP found in the growth medium (25) (Fig. S2). From this, we concluded that *shp* expression is dependent on both the RovS transcriptional regulator and the extracellular SHP signaling molecule.

In the SHP/Rgg system in *S. thermophilus* and *S. pyogenes*, the membrane protease Eep is responsible for SHP maturation (12, 13). To check if this is also the case in *S. agalactiae*, we deleted the Eep-encoding gene *gbs1901* (which encodes a protein sharing 69%



**FIG 1** The SHP/RovS cell-to-cell communication system is active in strain NEM316. (A) Effects of culture media on *shp* expression.  $\beta$ -Galactosidase activity was measured in the WT-*P<sub>shp</sub>* strain grown in DMEMg, THY, or BHI medium at 37°C until it reached the early stationary phase ( $OD_{600}$ , ~0.8). (B) Expression kinetics of the *shp* and *rovS* genes.  $\beta$ -Galactosidase activity was measured during the growth of the WT-*P<sub>shp</sub>* (○) and WT-*P<sub>rovS</sub>* (□) strains. Dotted lines indicate the growth of each strain as measured at an  $OD_{600}$ , and solid lines indicate  $\beta$ -galactosidase activity. (C) *shp* expression depends on RovS and SHP.  $\beta$ -Galactosidase activity was measured in the WT-*P<sub>shp</sub>*,  $\Delta$ *rovS*-*P<sub>shp</sub>*, and  $\Delta$ *shp*-*P<sub>shp</sub>* strains grown in DMEMg at 37°C until they reached the early stationary phase ( $OD_{600}$ , ~0.8). The data presented are the means  $\pm$  standard deviations (SD) of results from three independent experiments. Differences were considered to be extremely significant if *P* values were  $<0.001$  (\*\*\*).

identity with *S. thermophilus* Eep peptidase) and transferred the plasmid *P<sub>shp</sub>* into the  $\Delta$ *eep* strain, yielding the  $\Delta$ *eep*-*P<sub>shp</sub>* strain. In the  $\Delta$ *eep* genetic background, expression of the *shp* gene decreased significantly but not totally (by 70% of WT levels) (Fig. 2A). Moreover, when sSHP was added to the  $\Delta$ *eep*-*P<sub>shp</sub>* culture, a partial complementation of *shp* expression was observed (Fig. 2A). These results demonstrate that Eep peptidase is involved in the SHP/RovS cell-to-cell communication system. However, its role is complex because it is, most probably, involved in different steps of the SHP/RovS mechanism. To focus our study on the role of Eep in the maturation of SHP, we performed an additional experiment in which the production of mature SHP was independent from the positive-feedback loop necessary for the expression of *shp* and disconnected from the detection of mature SHP. Supernatant of a  $\Delta$ *eep* strain in which a *shp* plasmid-borne gene was under the control of a strong constitutive promoter ( $\Delta$ *eep*-*P23shp* strain) was mixed for 2 h with a  $\Delta$ *shp* reporter strain ( $\Delta$ *shp*-*P<sub>shp</sub>*) unable to produce any SHP but with a functional *eep* gene. Under this condition, we measured an intermediate level of expression for the *shp* gene ( $96 \pm 5$  MU). As expected, with the same reporter strain ( $\Delta$ *shp*-*P<sub>shp</sub>* strain), no  $\beta$ -galactosidase activity was detected with its own supernatant, whereas a high level ( $336 \pm 25$  MU) was measured with supernatant of the  $\Delta$ *shp*-*P23shp* strain (Fig. 2B). This indicates that the Eep peptidase is an important component of the maturation of SHP.

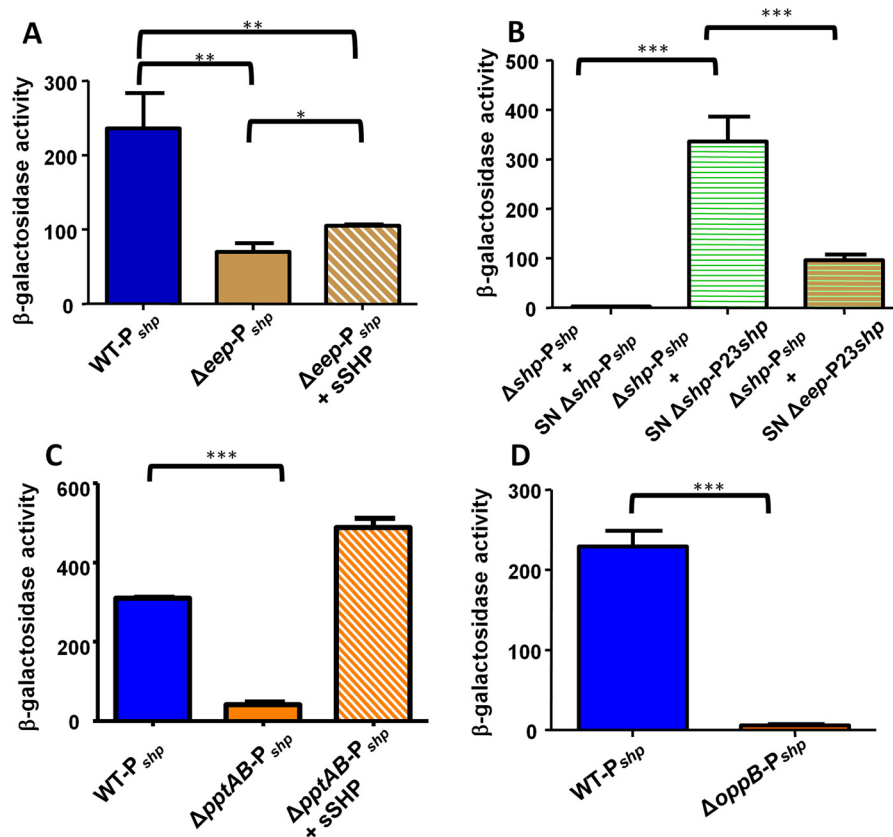
It is known that SHP pheromones are secreted (12, 25, 26). Based on the recently published work in which the pheromone peptide transporter PptAB was identified for *E. faecalis* (27), we identified the orthologous genes *gbs0359* to *-0360* in strain NEM316 of GBS by BLAST. The corresponding proteins share an identity of 67% for PptA and 35% for PptB. We constructed a  $\Delta$ *pptAB* deletion mutant and introduced the plasmid *P<sub>shp</sub>*, yielding the  $\Delta$ *pptAB*-*P<sub>shp</sub>* strain (Table S1). In the  $\Delta$ *pptAB* genetic background, *shp* gene expression was diminished to 13% of the WT level (Fig. 2C). Furthermore, the expression of the *shp* gene was restored when 1  $\mu$ M of sSHP was added to the culture me-

dium (Fig. 2C). These results show that the PptAB transporter system is responsible for the secretion of SHP.

In both *S. thermophilus* and *S. pyogenes*, the mature SHP is reimported by an oligopeptide transporter (12, 13). In *S. agalactiae*, oligopeptide uptake is undertaken by a single oligopeptide ABC transporter system: *oppA1-F* (encoded by *gbs0144* to *gbs0148* in strain NEM316) (22, 28). We inactivated the Opp system by deleting the *oppB* gene and then transferred the plasmid *P<sub>shp</sub>* into this strain, thus producing strain  $\Delta$ *oppB*-*P<sub>shp</sub>*. The *shp* gene was not expressed in the  $\Delta$ *oppB* genetic background in DMEMg (Fig. 2D). These results demonstrate that the OppA1-F ABC transporter system is involved in the SHP/RovS system, most probably because it imports the mature form of SHP into the cell.

***gbs1556* is a newly identified target of the SHP/RovS system.** The *gbs1556* gene is located downstream of the *shp* gene and is transcribed in the same orientation (Fig. 3A) (22). They are separated by only 60 nucleotides (nt), which suggests that they may be cotranscribed. To test this hypothesis, reverse transcription (RT)-PCR assays were performed; *rovS* and *gbs1556* as well as the intergenic region between *shp* and *gbs1556* were amplified using mRNA from the WT strain as the template, whereas no amplification was obtained for the intergenic region between *rovS* and *shp* (Fig. 3B). This observation confirms that *shp* and *gbs1556* form an operon that is controlled by a promoter region located upstream of the *shp* gene.

Analysis of the sequence of Gbs1556 revealed the presence of a peptide signal signature in its N-terminal domain (predicted by SignalP software v.4.1), suggesting that Gbs1556 is a secreted protein. We therefore used a label-free proteomic approach that combined SDS-PAGE and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to compare the secretomes of NEM316 and the  $\Delta$ *rovS* mutant grown in DMEMg. As expected, Gbs1556 was much more abundant in the supernatant of the NEM316 strain (an average of 93 mass spectra were detected) than in that of the  $\Delta$ *rovS* mutant strain (an average of 1 mass spectrum was detected) ( $P < 0.01$ ). No significant differences between the



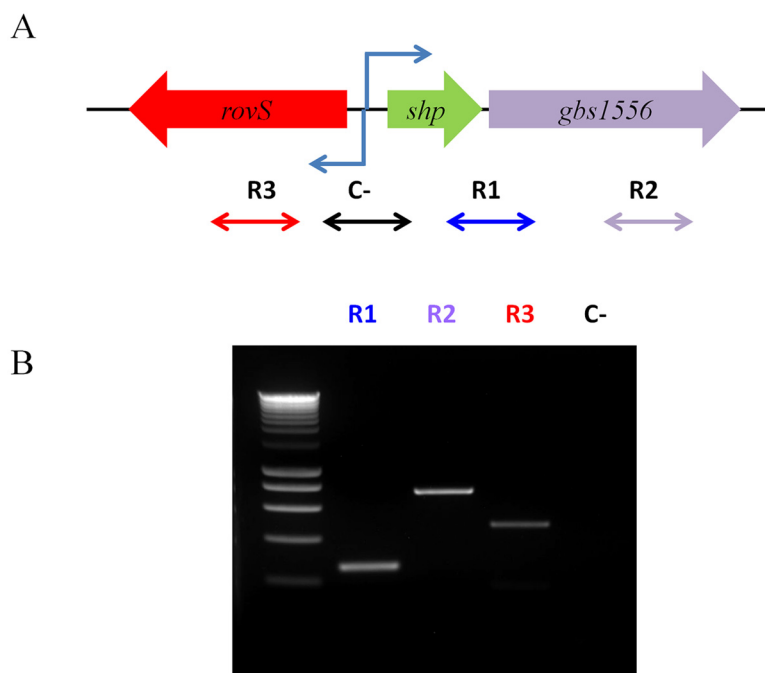
**FIG 2** The membrane peptidase Eep (A and B), the PptAB exporter system (C), and the oligopeptide transporter system OppA1-F (D) are involved in the SHP/RovS cell-to-cell communication system.  $\beta$ -Galactosidase activity was measured in the WT-*P<sub>shp</sub>*,  $\Delta$ *oppB*-*P<sub>shp</sub>*,  $\Delta$ *pptAB*-*P<sub>shp</sub>*, and  $\Delta$ *eep*-*P<sub>shp</sub>* strains grown in DMEMg at 37°C until they reached the early stationary phase ( $OD_{600}$ , ~0.8). An external complementation assay was performed by adding a 1  $\mu$ M concentration of sSHP to the cultures of  $\Delta$ *pptAB*-*P<sub>shp</sub>* and  $\Delta$ *eep*-*P<sub>shp</sub>* (A and C) cells. Supernatants (SN) of  $\Delta$ *shp* and  $\Delta$ *eep* strains in which a *shp* plasmid-borne gene was under the control of a strong constitutive promoter (*P23shp*) were mixed for 2 h with the  $\Delta$ *shp*-*P<sub>shp</sub>* reporter strain, and the  $\beta$ -galactosidase activity was measured (B). The data presented are the means  $\pm$  SD of results from three independent experiments. Differences were considered to be significant if *P* values were  $<0.05$  (\*), highly significant if *P* values were  $<0.01$  (\*\*), and extremely significant if *P* values were  $<0.001$  (\*\*\*).

abundances of the 70 other identified proteins in the supernatants of the two strains were observed (data not shown). This difference detected by the proteomic approach was confirmed at the RNA level by quantitative real-time PCR. The mRNA level of *gbs1556* was 22-fold lower in the  $\Delta$ *rovS* mutant than that observed in the WT strain.

**The SHP/RovS cell-to-cell communication system negatively regulates *fbsA* expression.** To better understand the role of the RovS transcriptional regulator and SHP in the regulation of the *fbsA* (*gbs1087*) gene, which has been described as being negatively regulated by RovS (23), we studied the transcription of this gene in strains grown in DMEMg and rich media. After creating a plasmid-borne transcriptional fusion that contained the *lacZ* reporter gene under the control of the promoter region of the *fbsA* gene (*P<sub>fbsA</sub>*), we transferred this fusion into the NEM316,  $\Delta$ *rovS*, and  $\Delta$ *shp* strains (Table S1). In DMEMg, the *P<sub>fbsA</sub>* fusion was expressed in the WT-*P<sub>fbsA</sub>* strain and highly expressed in both the  $\Delta$ *rovS*-*P<sub>fbsA</sub>* and  $\Delta$ *shp*-*P<sub>fbsA</sub>* strains (Fig. 4A). The differences observed using the transcriptional fusions were confirmed by quantitative real-time PCR; the mRNA levels of *fbsA* were 2.7- and 1.7-fold higher in the  $\Delta$ *rovS* and  $\Delta$ *shp* genetic backgrounds, respectively, than in NEM316. The overproduction of the FbsA protein in mutant strains was confirmed by testing adhesion to hu-

man fibrinogen; we observed higher adhesion for both the  $\Delta$ *rovS* and  $\Delta$ *shp* strains than for the NEM316 strain when all strains were grown in DMEMg (2.5- and 2-fold higher, respectively) (Fig. 4C). In BHI medium, there was no significant difference in levels of *fbsA* expression among the WT,  $\Delta$ *rovS*, and  $\Delta$ *shp* backgrounds (Fig. 4B). These results were also confirmed by the human fibrinogen adhesion test; when the  $\Delta$ *rovS* and  $\Delta$ *shp* strains were grown in BHI medium, no significant differences were observed between their degrees of binding to immobilized fibrinogen and that of strain NEM316 (Fig. 4D). Taken together, these results demonstrate that in DMEMg, both RovS and SHP negatively regulate the expression of the *fbsA* gene.

**Identification of the DNA-binding motif recognized by RovS.** The transcriptional start sites of the *shp* and *rovS* genes of strain NEM316 were identified by RNA-seq experiments (see Fig. S3 in the supplemental material), allowing the identification of  $\sim 10$  boxes of the promoters of both genes (Fig. 5A). The *rovS* gene is a paralog of the *rgg2* gene in *S. pyogenes*. The *shp*/*rgg* loci are highly conserved at the nucleotide level with transcriptional start sites and  $\sim 10$  sequences of the *shp* and *rovS* genes, which are identical in the two species. The DNA motif in the promoter region of the *shp* gene that is recognized by Rgg2 has been experimentally identified using DNA protection assays (14). This motif



**FIG 3** *gbs1556* and *shp* are cotranscribed. (A) Schematic representation of the *shp/rovS* locus. The broken arrows represent the  $-10$  region of the putative promoters. The double arrows associated with the names R1, R2, R3, and C- represent the potential fragments that were tested in the RT-PCR assay. (B) RT-PCR analysis of the *shp-gbs1556* locus in strain NEM316. Each reaction was performed with 10 ng *S. agalactiae* RNA. Each line contains the product derived from the PCR highlighted in the drawing.

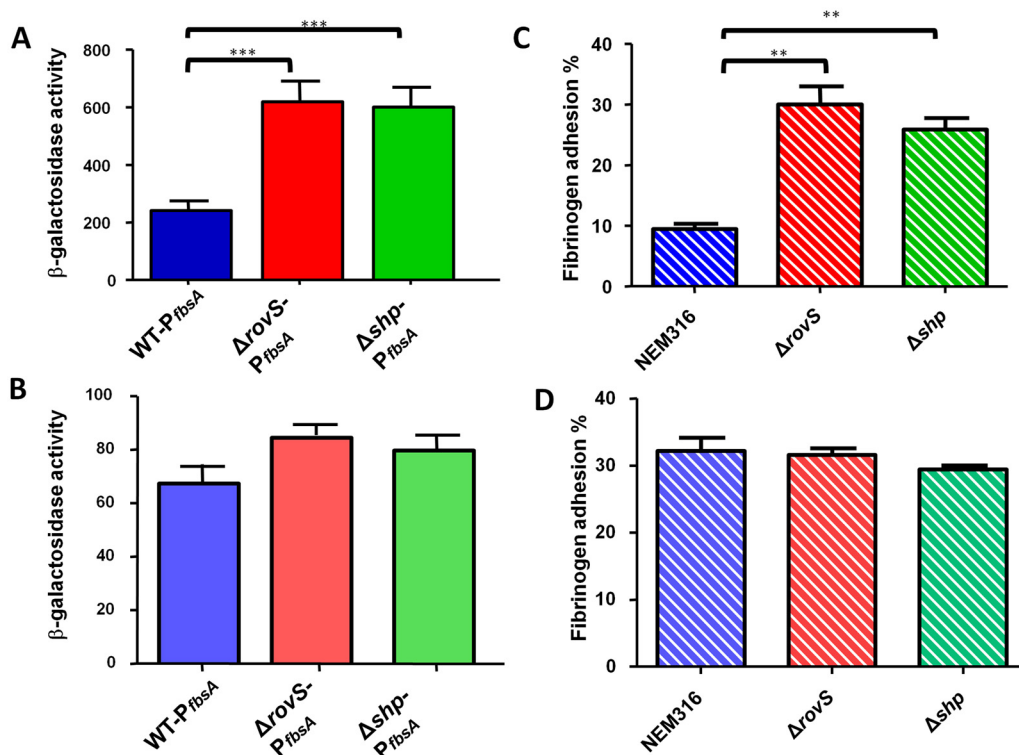
is also present in the promoter region of the *shp* gene of *S. agalactiae*, but the three promoter-proximal adenosines in the *S. pyogenes* motif are replaced by three thymidines in the *S. agalactiae* motif (Fig. 5A and B). Thus, we performed electrophoretic mobility shift assays (EMSAs) to quantify interactions between RovS and the promoter region of the *shp* gene in GBS. A 134-bp DNA probe ( $P_{shp}$  probe) centered on the conserved motif was amplified using PCR. We then mixed  $5 \times 10^{-4}$  pmol of the labeled probe with increasing concentrations of His-tagged RovS, which ranged from 0 to 250 nM, and analyzed DNA binding by EMSA. As expected, we observed increasing binding of RovS to the DNA probe with increasing concentrations of RovS (Fig. 5C). RovS did not bind to a probe containing the promoter region of the *ldh* gene ( $P_{ldhL}$  probe), and the specificity of binding was also confirmed with competition assays. The shift of the labeled  $P_{shp}$  probe was drastically decreased when the unlabeled  $P_{shp}$  probe was added, a sign that there was competition between the two types of probes for binding sites; however, it was not affected by the addition of the unlabeled  $P_{ldhL}$  probe (Fig. 5C). When a  $P_{shp}$  probe that contained a partially deleted DNA binding motif was used, no RovS binding was detected, even at a RovS concentration of 750 nM (Fig. 5B and C). The binding of RovS to the  $P_{shp}$  probe was also evaluated in the presence of 100  $\mu$ M sSHP, which resulted in a slight decrease in binding when RovS was present at 10 and 50 mM (Fig. S4). Of note, similar observations were made for two other SHP/Rgg systems, the SHP/Rgg1358 mechanism in *S. thermophilus* (12) and the SHP2/Rgg2 system in *S. pyogenes* (14).

In order to investigate the interaction between RovS and the promoter region of the *fbxA* gene ( $P_{fbxA}$  probe), we tested RovS binding to DNA fragments that were located upstream of this gene and that contained a previously identified RovS DNA binding

motif (23). Notably, this motif shares no similarities with the motif identified in the promoter region of the *shp* gene. No shift was detected even when RovS was added at 750 nM (Fig. 5C), indicating that RovS does not bind to the promoter region of this gene under our assay conditions.

**SHP/RovS is necessary for GBS strain NEM316's successful persistence in host liver and spleen.** To determine the role of SHP/RovS in GBS virulence, we investigated the persistence of the NEM316,  $\Delta rovS$ , and  $\Delta shp$  strains in different organs of mice that had been infected with a sublethal dose of bacteria ( $10^6$  CFU). The brain, liver, spleen, and lungs were analyzed. Two days postinfection, bacteria were found in these organs, but no differences were observed in the amounts of bacteria present in the lungs and brain (data not shown). However, bacterial counts were significantly lower in the spleens and livers of mice infected with the  $\Delta rovS$  mutant (1.4 and 1.5 log) (Fig. 6A and B) and the  $\Delta shp$  mutant (1.3 and 1 log) (Fig. 6C and D) than in those infected with the WT strain NEM316. In conclusion, SHP/RovS seems to be a determining factor in the ability of GBS to persist in infected liver and spleen tissues.

**SHP/RovS is needed for GBS adhesion to and invasion of the HepG2 hepatic cell line.** Since the SHP/RovS system was shown to be involved in GBS persistence in the host's liver and seems to help control the expression of some virulence factors, we studied the ability of the  $\Delta rovS$  and  $\Delta shp$  mutant strains to adhere to and invade the human HepG2 hepatic cell line. When cells were infected at a multiplicity of infection (MOI) of 10, the  $\Delta rovS$  and  $\Delta shp$  strains were present in smaller amounts in hepatic cells 1 h postinfection than was strain NEM316 (Fig. 7A). Adhesion ability was also diminished in both mutant strains: it decreased 50% compared to WT levels (Fig. 7B). These results confirm that the



**FIG 4** Expression of the *fbsA* gene (A and B) and adhesion to immobilized human fibrinogen (C and D).  $\beta$ -Galactosidase activity was measured in the WT-*P<sub>fbsA</sub>*,  $\Delta$ *rovS*-*P<sub>fbsA</sub>*, and  $\Delta$ *shp*-*P<sub>fbsA</sub>* strains for *fbsA* expression; each strain was grown in DMEMg (A) or BHI medium (B) at 37°C until it reached the early stationary phase ( $OD_{600}$  ~0.8). The adhesion assay was performed in microtiter wells previously coated with a fixed amount of human fibrinogen. A total of  $10^6$  CFU of culture grown in DMEMg (C) or BHI medium (D) was placed in each well, and the plates were then incubated for 30 min at 37°C. After the wells were washed to eliminate unbound bacteria, adhered cells were recovered and quantified in terms of CFU/ml. The data presented are the means  $\pm$  SD of results from three independent experiments. Differences were considered to be highly significant if *P* values were <0.01 (\*\*) and extremely significant if *P* values were <0.001 (\*\*\*).

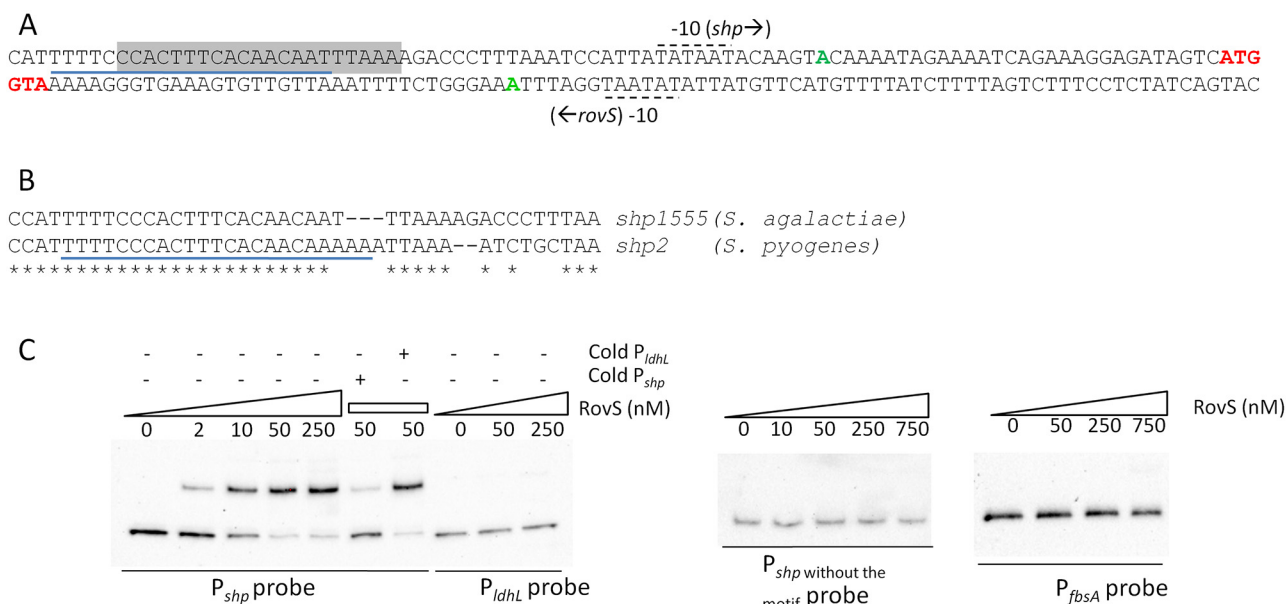
SHP/RovS cell-to-cell communication system plays an important role in the bacterial colonization of host liver tissues.

## DISCUSSION

The increasing incidence of invasive GBS disease in adults, together with the emergence of antibiotic-resistant strains (15), has underscored the fact that there is a current need to develop new or complementary strategies against GBS infection. Cell-to-cell communication systems have become an attractive target when exploring new prophylactic or treatment strategies against several pathogens, including *S. aureus* and *P. aeruginosa* (29, 30). For this reason, using *S. agalactiae* strain NEM316, we reexamined the activity of the RovS transcriptional regulator, which had previously been described as a stand-alone regulator (23), together with that of its genetically associated SHP signaling molecule. Based on our results, we propose the following model for SHP/RovS cell-to-cell communication (Fig. 8). The SHP pheromone is matured by the Eep protease and at least another unknown protease. Then, SHP is secreted into the extracellular medium via the PptAB exporter system. Finally, mature SHP is imported into the cell by the oligopeptide transporter OppA1-F, where it interacts with RovS to positively regulate the transcription of the *shp* and *gbs1556* genes, which are cotranscribed. While *rovS* expression is constitutive at a low level throughout growth under our conditions, we observed a triggering of *shp* gene expression at the beginning of growth and an exponential increase in its expression with increasing biomass yield.

Our results demonstrate that Eep peptidase is an important direct actor in the maturation of SHP but not the only one. Indeed, the expression of the *shp* gene was not abolished in a  $\Delta$ *eep* mutant. This indicates that, most probably, another protease is involved in the maturation of SHP, at least when the Eep protease is not present in the cell. A similar hypothesis was suggested for the production of the pheromone cCF10 in *E. faecalis* (27). Our results also highlight the fact that the maturation of SHP is not the only role of the Eep protease in the SHP/RovS mechanism. Indeed, the partial complementation provided by sSHP in the  $\Delta$ *eep* mutant suggests that Eep is involved in a step after the secretion and maturation of SHP, and that remained to be identified.

Very recently, an ABC transporter, PptAB, required for secretion of at least three different peptide sex pheromones was identified in *E. faecalis* (27). The Eep protease is involved in the production of most of these sex pheromones which need to be imported by an oligopeptide transporter to be functional. These similarities with the SHP/RovS mechanism led us to test the role of the orthologue of *pptAB* of GBS in the export of SHP. The significant decrease in the expression of *shp* in the  $\Delta$ *pptAB* mutant and the strong functional complementation with the addition of sSHP show that the PptAB transporter is responsible of the secretion of the mature SHP into the extracellular medium. The way that SHP is exported has been puzzling for a long time. It seems that PptAB is in charge of the export of different kinds of signaling peptides in different Gram-positive organisms, as the Eep peptidase is in-



**FIG 5** Analysis of RovS binding to DNA. (A) DNA region (double-stranded) upstream from the *shp* gene in *S. agalactiae* NEM316. The ATGs of the *shp* and *rovS* genes are in red. The transcriptional start sites of the *shp* and *rovS* genes, as determined by RNA-seq experiments (Fig. S3), are in green. The -10 region of *shp* and *rovS* are underlined with dashed black lines. The DNA binding motif recognized by *S. pyogenes* Rgg2 is underlined in blue. The fragment of the motif that was removed from the probe called  $P_{shp}$  without the motif probe in gray shading. (B) DNA regions located upstream from the gene *shp1555* in *S. agalactiae* NEM316 versus those located upstream from *shp2* in *S. pyogenes* strain nz131. The DNA binding motif recognized by *S. pyogenes* Rgg2 and identified in the DNase protection assay is underlined in blue (26). The 3' ends of the DNA fragments are located 51 nt upstream from the ATG of the *shp* genes. (C) DNA binding of the His-tagged RovS protein to the promoter region of *fbsA* ( $P_{fbsA}$  probe) and to the promoter region of *shp* with ( $P_{shp}$  probe) and without ( $P_{shp}$  without the motif probe) the putative binding motif. The *Idh* probe ( $P_{IdhL}$  probe) is an *idhL* promoter fragment that was used as a negative control.

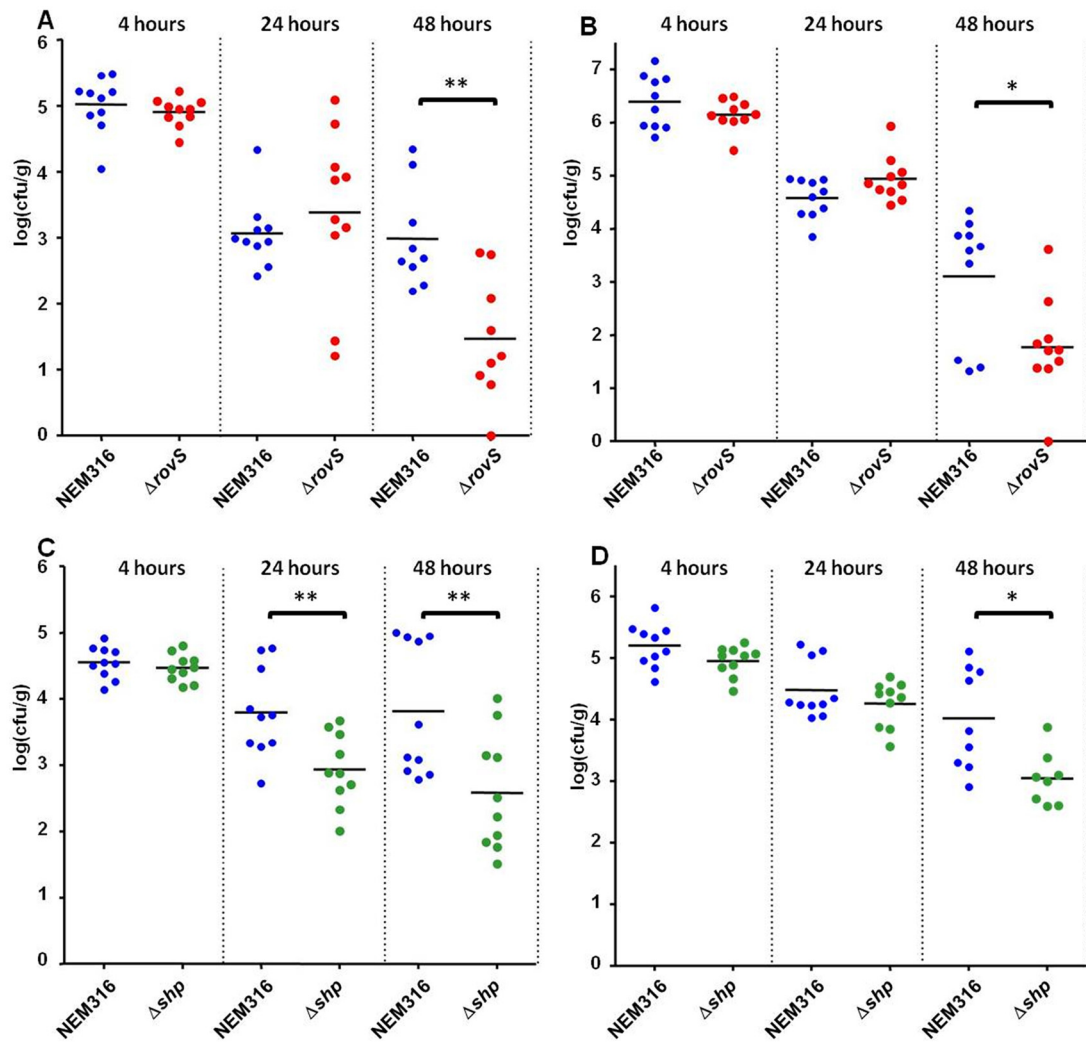
involved in the production of these pheromones. It will be challenging to understand how and where PptAB and Eep work together to produce and secrete these signaling peptides in streptococci.

In the present study, it was also found that the nutritional composition of the culture medium is a determining factor for SHP/RovS cell-to-cell communication activity; the system's positively regulated target was highly expressed in a chemically defined medium that was free of peptides and relatively underexpressed in rich culture media (BHI medium and THY). It is known that the OppA1-F operon is expressed at similar levels in THY and chemically defined medium (CDM) (28). Thus, a potential explanation of the inhibitory effect of the rich medium on *shp* expression is that free nutritional oligopeptides present in the rich medium compete with the SHP pheromone for OppA1-F transport, thus reducing subsequent *shp* expression. Consistent with this idea is the observation that, when 0.05% tryptone was added to DMEMg, expression of the *shp* gene was repressed (data not shown). A similar phenomenon has been observed in other Rgg-like regulatory systems. For example, in *S. thermophilus*, medium composition affects the expression of the target gene *ster\_1357* (which is controlled by the SHP1358/Rgg1358 system) (10) and of the *comX* gene (controlled by another Rgg-like protein, ComR), which triggers the competence state (31). It also affects expression of the *agr* system regulon in *S. aureus* (32, 33). GBS is able to colonize a diverse array of host niches; it alternates between living as a commensal in the maternal genitourinary tract and living as an opportunistic pathogen in different host organs, such as the lungs or placenta. Although it is difficult to assess the peptide compositions of these different niches, we can speculate that the SHP/RovS cell-to-cell communication system is a sophisticated mechanism that

modulates the expression of target genes by taking into account such environmental characteristics as nutrient availability.

A combination of genetic and proteomic approaches allowed us to identify *gbs1556* as a new target gene positively regulated by the SHP/RovS system. The *gbs1556* gene occurs, with 100% identity, in the 285 GBS sequences available in the NCBI database. It encodes a 530-amino-acid secreted protein of unknown function that has a conserved domain (amino acids 380 to 488) characteristic of a transglutaminase/protease enzyme. Transcriptome analyses revealed that the *gbs1556* gene was significantly overexpressed when bacteria were grown in human amniotic fluid or blood (34, 35), suggesting that Gbs1556 plays a role in GBS infection. A recent study has shown that the *gbs1556* gene is also upregulated during GBS infection of macrophages, which raises the question of whether it interacts with the host immune system (36).

The *fbsA* gene had previously been identified as a target of RovS. When a *rovS* mutant and its parental strain were grown in THY, *fbsA* expression was 1.8-fold higher in the mutant (23). We confirmed that RovS affects *fbsA* expression. When a *rovS* mutant and its parental strain were grown in DMEMg, there was a 2.7-fold increase in *fbsA* expression in the mutant. The fact that no significant differences were observed for *fbsA* in BHI medium may have resulted from the different methodologies and different rich media used in the two studies (23). It is obvious from these results that the absence of peptides in the medium boosted the activity of the SHP/RovS system. Furthermore, although we could detect a specific binding of purified RovS to a DNA fragment containing the *shp* promoter region, we did not see its binding to a DNA fragment containing the *fbsA* promoter region, which contrasts with the results of Samen et al. This discrepancy might be ex-



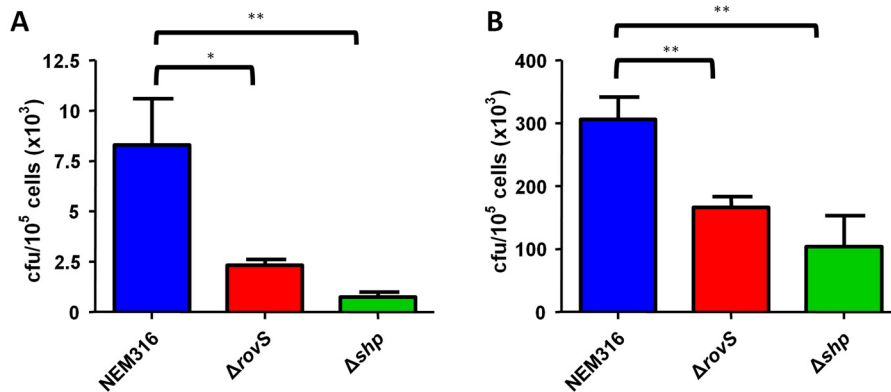
**FIG 6** GBS persistence in the livers (A and C) and spleens (B and D) of mice infected with NEM316. Numbers of CFU per gram of tissue are shown for each strain. To infect mice with the NEM316 (blue),  $\Delta\text{rovS}$  (red), and  $\Delta\text{shp}$  (green) strains,  $10^6$  CFU of the cultures was injected intravenously. Subsequently, groups of 10 mice infected with each strain were sacrificed 4, 24, and 48 h postinfection, and their organs were removed and immediately processed to quantify bacterial loads. Differences were considered to be significant if  $P$  values were  $<0.05$  (\*) and highly significant if  $P$  values were  $<0.01$  (\*\*).

plained by the fact that Samen's team used very high concentrations of DNA and RovS in their EMSAs to detect shifts on the agarose gels; in contrast, we used chemiluminescent detection. In conclusion, based on these results, we hypothesize that *shp* and *gbs1556* are direct targets of the SHP/RovS system but that the *fbsA* gene is probably an indirect target.

The results of this experimental study are the first to strongly demonstrate that the SHP/RovS cell-to-cell communication system plays a role in the bacterial pathogenesis of GBS *in vivo*. Nearly 100% of mice infected with the NEM316,  $\Delta\text{rovS}$ , or  $\Delta\text{shp}$  strain developed septicemia 4 h postinfection. The absence of RovS or SHP led to a large decrease in the bacterial burden in their livers and spleens after 48 h, which highlights that the SHP/RovS system may demonstrate organ specificity. A similar organ tropism has been hypothesized for the Agr system in *Listeria monocytogenes*, in which the absence of *agrD* decreases the bacterial load in the livers and spleens of infected mice (37), and for the LuxI/LuxR-like quorum-sensing system in the Gram-negative human pathogen *Burkholderia pseudomallei* (38).

In order to colonize the organs of their hosts, pathogenic bacteria must have the ability to adhere to and invade epithelial cells (39). The disruption of the *shp* and *rovS* genes resulted in a moderate but significant decrease in GBS' ability to adhere to and invade human HepG2 hepatic cells, which concurred with our results obtained *in vivo*. Although the disruption of the SHP/RovS system increased the expression of the *fbsA* gene and enhanced bacterial binding to immobilized fibrinogen, the results obtained from the cell culture experiments suggest that FbsA is not the major adhesin involved in the binding of GBS to hepatocytes. It should be noted that the liver is one of the targets of GBS sepsis, with infection leading to significant adverse effects, such as hepatocyte necrosis, before it provokes organ failure (40). Hence, GBS with a defective SHP/RovS cell-to-cell communication system may have trouble infecting these organs because there is faulty coordination of the bacterial population; consequently, its systemic spread and infection capacity may be limited.

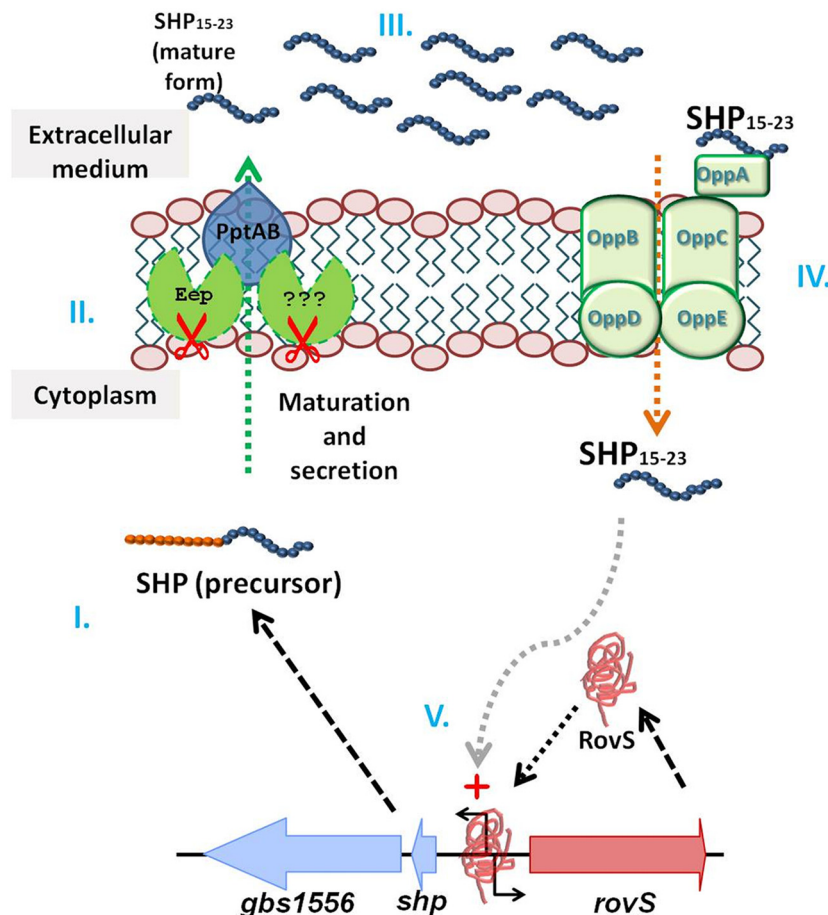
In summary, the present study shows that GBS has a cell-to-cell communication system that is directly involved in its pathogenesis



**FIG 7** SHP/RovS promotes invasion of (A) and adhesion to (B) HepG2 hepatic epithelial cells. The figures show the bacterial loads of the *S. agalactiae* NEM316,  $\Delta$ *rovS*, and  $\Delta$ *shp* strains recovered from the intracellular compartment (A) or the cellular surface (B) of infected HepG2 hepatic epithelial cells 1 h postinfection. The data presented are the means  $\pm$  SD of results from three independent experiments. Differences were considered to be significant if *P* values were  $<0.05$  (\*) and highly significant if *P* values were  $<0.01$  (\*\*).

controlling the expression of the *fbxA* gene, besides a newly identified target gene. Until now, SHP-associated Rgg regulators have been described as either activators or repressors, with their activity

being entirely dependent on the presence of their cognate pheromone. This study highlights the complexity of the SHP-associated RovS transcriptional regulator, an unusual member of the Rgg



**FIG 8** Schematic representation of the SHP/RovS cell-to-cell communication mechanism. The SHP pheromone is produced as the precursor (I), matured mainly by the Eep membrane-associated peptidase, and secreted into the extracellular medium via the PptAB transporter (II). Mature SHP from nt 15 to 23 (SHP<sub>15-23</sub>) is accumulated outside the cell (III) to be imported back into the cell by the oligopeptide transporter OppA1-F (IV). Finally, SHP<sub>15-23</sub> interacts with RovS to positively regulate the transcription of the *shp* and *gbs1556* genes (V), which are cotranscribed.

family that has both repressing and activating functions. Furthermore, RovS can control the expression of its targets directly or indirectly, although the mechanisms involved in indirect regulation remain unclear. Overall, our research indicates that the SHP/RovS system is an important regulator of GBS virulence and therefore constitutes a novel target in efforts to control or moderate its pathogenesis.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The strains and plasmids used in this study are described in Table S1 in the supplemental material. *S. agalactiae* NEM316, a serotype III strain (ATCC 12403), was used in this study. *Escherichia coli* strain TG1-dev was used for cloning experiments (41). *S. agalactiae* strains were grown at 37°C in brain heart infusion (BHI) medium, Todd-Hewitt broth supplemented with yeast extract (THY), or Dulbecco's modified Eagle's medium (DMEM); the latter is a chemically defined medium (Sigma-Aldrich) with some modifications adapted to GBS growth as follows. DMEM was supplemented with 50 mM Tris-HCl (pH 8), 50 mM  $\beta$ -glycerophosphate (pH 7.5), a vitamin cocktail (see Table S2 in the supplemental material), and 0.3% (wt/vol) glucose; the resulting medium was designated DMEMg. All experiments were performed, without agitation, in Erlenmeyer flasks filled to less than 1/10 of their volume. *E. coli* was cultured in Luria-Bertani broth at 37°C with aeration by shaking. In all cases, 1.5% (wt/vol) agar was added to the media as necessary. Antibiotics were used as needed at the following concentrations: for *S. agalactiae*, 5  $\mu$ g  $\cdot$  ml<sup>-1</sup> erythromycin or 2  $\mu$ g  $\cdot$  ml<sup>-1</sup> tetracycline; and for *E. coli*, 100  $\mu$ g  $\cdot$  ml<sup>-1</sup> ampicillin or 50  $\mu$ g  $\cdot$  ml<sup>-1</sup> kanamycin.

**DNA manipulation techniques.** Standard DNA recombination procedures were used. The oligonucleotides used for PCR are listed in Table S3. *S. agalactiae* was transformed using electrocompetent cells (42).

**Construction of deletion mutants.** The genes *rovS*, *shp*, *oppB*, *pptAB*, and *eep* were deleted from *S. agalactiae* as described previously (43). Briefly, the two DNA fragments that flanked the target gene were amplified by PCR and ligated with a second PCR amplification, using the overlapping-PCR method. The resulting PCR product and the plasmid pBR322-pG<sup>+</sup> host8 were digested with BamHI and XbaI restriction enzymes, ligated, and transformed into the *E. coli* TG1-dev strain. The resulting plasmids were designated pBR322-pG<sup>+</sup> host8::updown, followed by the name of the corresponding gene. Proper plasmid construction was verified by sequencing, and the plasmids were then transformed into *S. agalactiae* strain NEM316 with a subsequent selection step at 30°C on BHI agar plates. Cells in which the plasmid was successfully integrated into the chromosome were selected via tetracycline selection at 37°C. A few integrant strains were isolated, and serial dilutions were performed in BHI medium at 30°C; they were conducted without antibiotics in order to allow vector excision. Cells were then plated on BHI agar, and isolated colonies were tested for tetracycline sensitivity to detect pG<sup>+</sup> host8 excised clones. The successful deletion of the *rovS*, *shp*, *oppB*, *pptAB*, and *eep* genes was confirmed by sequencing.

**Construction of lacZ transcriptional fusions.** The pTCV-lac plasmid (44) was used to examine the expression of the *shp*, *rovS*, and *fbxA* genes in *S. agalactiae* strain NEM316. The putative promoter region of each gene was PCR amplified and fused to the vector in front of the *lacZ* reporter gene. The specific primers used for each transcriptional fusion are listed in Table S3. Following digestion with BamHI and EcoRI restriction enzymes, each PCR product was ligated into pTCV-lac, which had been digested with the same restriction enzymes, resulting in the creation of the plasmids P<sub>shp</sub>, P<sub>rovS</sub>, and P<sub>fbxA</sub>. The insertion was confirmed by PCR and sequencing. These plasmids were transferred into the NEM316,  $\Delta$ rovS,  $\Delta$ shp,  $\Delta$ oppB,  $\Delta$ pptAB, and  $\Delta$ eep strains, and recombinant clones were selected with erythromycin, resulting in the strains listed in Table S1.

**SHP overexpression plasmid construction.** A DNA fragment which contained the coding region of *shp* was amplified using DP58 and DP59 primers (Table S3). To ensure the constitutive expression of the *shp* gene,

the P23 promoter region was amplified using the LJ14 and LJ15 primers (Table S3) from the pBS23 vector. The P23 promoter region was cloned upstream of *shp* gene by ligation of BamHI sites, and the obtained product was introduced into the pTCV-lac plasmid by ligation of EcoRI sites, resulting in the P23shp vector. This plasmid was introduced in the NEM316,  $\Delta$ shp, and  $\Delta$ eep strains (Table S1).

**Determination of  $\beta$ -galactosidase activity.** Overnight cultures of various strains of *S. agalactiae* (BHI medium at 37°C) were diluted 100-fold in fresh DMEMg, THY, or BHI medium and cultivated at 37°C in a 6% CO<sub>2</sub> environment. When needed, the sSHP DILIIIVGG synthetic (purchased from Genepep) were added to DMEMg cultures at 1  $\mu$ M at an optical density at 600 nm (OD<sub>600</sub>) of 0.05. At different points during growth, 2-ml samples were taken to determine the OD<sub>600</sub> and  $\beta$ -galactosidase activity of the culture using the method previously described (44). Enzymatic activity was expressed in Miller units and calculated using the following formula:  $(10^3 \times \text{OD}_{420})/(\text{reaction time [minutes]} \times \text{OD}_{600} \times \text{cell volume used in the assay})$ .

For coculture assays, the WT-pTCVlac and  $\Delta$ shp-pTCVlac strains as SHP potential donors and the  $\Delta$ shp-P<sub>shp</sub> strain as an SHP recipient were used. Strains were first grown separately in DMEMg until they reached an OD<sub>600</sub> of 0.5. Then, both cultures were diluted in DMEMg to reach an initial OD<sub>600</sub> of 0.05, and equal volumes of the WT-pTCVlac and  $\Delta$ shp-P<sub>shp</sub> strains were mixed together. At an OD<sub>600</sub> of ~0.8,  $\beta$ -galactosidase activity was measured as described above.

**Quantitative real-time PCR.** The expression of *gbs1556* and *fbxA* was analyzed by quantitative real-time PCR in the NEM316,  $\Delta$ rovS, and  $\Delta$ shp genetics backgrounds. Total RNA extractions were performed from different strains grown in DMEMg and harvested at an OD<sub>600</sub> of 0.5, using the TRIzol reagent (Invitrogen) method as previously described (11). Three extractions were independently performed for each strain. cDNA synthesis was done with 500 ng DNase I-treated RNA by using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The quantitative real-time PCR was carried out using the SYBR Green PCR master mix (Eurogentec) as recommended. PCRs were performed in triplicate and run on a Mastercycler Ep realplex detector (Eppendorf). The critical threshold cycle was defined for each sample. The expression levels of the studied genes were normalized using the NEM316 *rpoB* gene, whose transcription level is constant under our experimental conditions (45). For each gene, an analysis of variance was performed on the cycle threshold (*C<sub>T</sub>*) corrected by the *C<sub>T</sub>* of *rpoB* in order to determine whether the difference in expression levels of the strains was significant ( $P < 0.05$ ). Data were computed using the comparative critical-threshold method ( $2^{-\Delta\Delta C_T}$ ) (46).

**RT-PCR.** RT-PCR experiments were performed with cDNA synthesized with total RNA extracted from strain NEM316 grown in DMEMg and harvested at an OD<sub>600</sub> of 0.5, as described above. We designed primers (Table S2) specific to amplified intra- or intergenic regions. PCR products were separated by electrophoresis on a 1% agarose gel.

**Adhesion to immobilized fibrinogen.** Purified human fibrinogen was fixed in 96-well microtiter plates (20  $\mu$ g/well) by overnight incubation at 4°C. The wells were washed twice with phosphate-buffered saline (PBS). The NEM316,  $\Delta$ rovS, and  $\Delta$ shp GBS strains were grown in DMEMg until an OD<sub>600</sub> of 0.8 was reached; they were then adjusted to a concentration of 10<sup>6</sup> CFU/ml in PBS. Subsequently, 100  $\mu$ l of GBS suspension was added per well in triplicate and incubated for 30 min at 37°C. The wells were then washed with PBS to remove unbound bacteria and treated with 100  $\mu$ l of 0.02% Triton X-100 and repeated pipetting to free the attached bacteria. The number of bound bacteria was determined by plating serial dilutions of the recovered bacteria on BHI agar plates.

**Purification of His-tagged RovS.** The plasmid pET28a::RovS was constructed to allow the expression and purification of the N-terminally His-tagged RovS protein in *E. coli*. The *rovS* gene was amplified by PCR using the oligonucleotides RovS-NdeI and RovS-XhoI. The resulting fragment was digested with the restriction enzymes NdeI/XhoI and ligated into the expression vector pET28a, which had previously been digested

with the same restriction enzymes. The resulting plasmid was used to transform *E. coli* strain Rosetta in order to obtain TIL 1387 (*E. coli* Rosetta/pET28a::rovS). The production and purification of RovS were performed as previously described (12).

**EMSA.** DNA probes of the *shp*, *ldhL*, and *fbxA*, promoter regions were amplified by PCR using the oligonucleotides described in Table S3. DNA probes were 3'-end labeled, and gel shift reactions were carried out using the 2nd-generation digoxigenin (DIG) gel shift kit (Roche) in accordance with the manufacturer's instructions. The 10- $\mu$ l DNA binding reaction mixtures, which coupled probes to the RovS protein, involved incubation for 30 min at 30°C. Samples were then loaded onto 4 to 16% native polyacrylamide gels (native PAGE, 4 to 16% Bis-Tris gel; Invitrogen) and exposed to 70 V for approximately 2 h in 1 $\times$  Tris-borate-EDTA (TBE) buffer. The labeled probe/protein complexes were transferred to a positively charged nylon membrane (GE Healthcare, Amersham; Hybond-N<sup>+</sup>) by electroblotting for 1 h in a mini trans-blot cell (Bio-Rad) in 0.5 $\times$  TBE buffer. DNA complexes were visualized using a Bio-Rad ChemiDoc system in accordance with the manufacturer's instructions.

**Extracellular protein analysis by LC-MS/MS.** Cultures of the *S. agalactiae* NEM316 and  $\Delta$ rovS strains were grown in 100 ml of DMEMg until they reached the late exponential phase. For secretome analysis, the supernatants were recovered by centrifugation at 6,000  $\times$  g for 10 min at 4°C and concentrated by ultrafiltration using 0.22- $\mu$ m-pore-size filters (Millipore). Then, 10  $\mu$ g of each protein suspension was separated using one-dimensional short-migration electrophoresis (10). In-gel digestion of the proteins was performed using the ProGest system (Genomic Solutions) as previously described (47). The LC-MS/MS procedures, as well as the statistical analyses, are described in the supplemental material and herein.

**Internalization and adherence assays.** Confluent human HepG2 hepatocellular carcinoma cells were infected with the *S. agalactiae* NEM316,  $\Delta$ rovS, and  $\Delta$ shp strains at a multiplicity of infection (MOI) of 10 bacteria per eukaryotic cell for 1 h in 6% CO<sub>2</sub> at 37°C. Cell monolayers were then washed three times with Dulbecco's PBS, and cells were lysed by adding 500  $\mu$ l of a cold 0.02% Triton X-100 solution and pipetting it repeatedly. Total (intracellular plus surface-adherent) GBS association was estimated by plating serial dilutions of epithelial cell lysates on BHI agar. To estimate internalization, an experiment as described above was performed. Following the period of infection, cell monolayers were then incubated for an additional hour in fresh DMEM that contained gentamicin (100  $\mu$ g  $\cdot$  ml<sup>-1</sup>) and vancomycin (150  $\mu$ g  $\cdot$  ml<sup>-1</sup>) to kill extracellular bacteria. The amount of intracellular bacteria was quantified by removing the supernatants, disrupting the cells, and plating them on BHI agar in order to count viable bacterial colonies. The adherence was calculated as follows: total CFU minus internalized CFU. The assays were performed in triplicate and were repeated at least three times.

**Organ persistence in a mouse model.** Six-week-old pathogen-free Swiss CD1 mice (Charles River Laboratories, France) were used for infection assays. The NEM316,  $\Delta$ rovS, and  $\Delta$ shp GBS strains were grown in DMEMg at 37°C in 6% CO<sub>2</sub>, harvested in the late exponential phase (OD<sub>600</sub> ~0.8), and washed twice in 0.9% NaCl before being used for animal infection. Groups of 10 mice were first anesthetized with ketamine (100  $\mu$ g  $\cdot$  g<sup>-1</sup>; Merial) and xylazine (12  $\mu$ g  $\cdot$  g<sup>-1</sup>; Bayer) prior to bacterial administration. Each animal received an intravenous injection of 100  $\mu$ l of cell suspension, containing 10<sup>6</sup> CFU. At 4, 24, and 48 h postinfection, 10 mice of each group were sacrificed; aseptic spleen, liver, and brain extractions were performed. Organs were homogenized with an Ultra-Turrax T 25 basic (IKA Works, Inc.) in preparation for bacterial quantification. Serial dilutions of the homogenized solution were plated on BHI medium, and 20 colonies per sample were further analyzed for pigment production and hemolytic activity on Granada agar plates and Columbia agar supplemented with 5% sheep's blood (bioMérieux), respectively.

For the lung persistence analysis, 50  $\mu$ l of a 10<sup>6</sup>-CFU bacterial suspension was administered intranasally to groups of 10 mice. The amount of bacteria in the lungs was quantified in the same way as described above for the other organs.

**Ethics statement.** Animal experiments were performed in accordance with European Directive 2010/2063/UE and were approved by the institutional review ethics committee, COMETHEA, of the INRA Center in Jouy-en-Josas, France.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02306-14/-/DCSupplemental>.

Text S1, DOCX file, 0.01 MB.  
Table S1, DOCX file, 0.02 MB.  
Table S2, DOCX file, 0.01 MB.  
Table S3, DOCX file, 0.02 MB.  
Figure S1, TIF file, 0.6 MB.  
Figure S2, TIF file, 0.6 MB.  
Figure S3, TIF file, 1.7 MB.  
Figure S4, TIF file, 0.7 MB.

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