Biofilm Formation in *Streptococcus agalactiae* Is Inhibited by a Small Regulatory RNA Regulated by the Two-Component System CiaRH

Nancy Jabbour, a Eric Morello, a Emilie Camiade, a Marie-Frédérique Lartigue a,b

aISP, UMR1282, Université de Tours, INRAE, Tours, France  
bCHRU de Tours, Service de Bactériologie-Virologie-Hygiène, Tours, France

**ABSTRACT** Regulatory small RNAs (sRNAs) are involved in the adaptation of bacteria to their environment. CiaR-dependent sRNAs (csRNAs) are controlled by the regulatory two-component system (TCS) CiaRH, which is widely conserved in streptococci. Except for *Streptococcus pneumoniae* and *Streptococcus sanguinis*, the targets of these csRNAs have not yet been investigated. *Streptococcus agalactiae*, the leading cause of neonatal infections, has four conserved csRNA genes, namely, *srn015*, *srn024*, *srn070*, and *srn085*. Here, we demonstrate the importance of the direct repeat TTTAAG-N5-TTTAAG in the regulation of these csRNAs by CiaRH. A 24-nucleotide Srn024-*sap* RNA base-pairing region is predicted in silico. The *sap* gene encodes a LPXTG-cell wall-anchored pullulanase. This protein cleaves α-glucan polysaccharides such as pullulan and glycogen present in the environment to release glucose and is involved in adhesion to human cervical epithelial cells. Inactivation of *S. agalactiae* pullulanase (SAP) leads to no bacterial growth in a medium with only pullulan as a carbon source and reduced biofilm formation, while deletion of *ciaRH* and *srn024* genes significantly increases bacterial growth and biofilm formation. Using a new translational fusion vector, we demonstrated that Srn024 is involved in the posttranscriptional regulation of *sap* expression. Complementary base pair exchanges in *S. agalactiae* suggest that Srn024 interacts directly with *sap* mRNA and that disruption of this RNA pairing is sufficient to yield the biofilm phenotype of Srn024 deletion. These results suggest the involvement of Srn024 in the adaptation of *S. agalactiae* to environmental changes and biofilm formation, likely through the regulation of the *sap* gene.

**IMPORTANCE** Although *Streptococcus agalactiae* is a commensal bacterium of the human digestive and genitourinary tracts, it is also an opportunistic pathogen for humans and other animals. As the main cause of neonatal infections, it is responsible for pneumonia, bacteremia, and meningitis. However, its adaptation to these different ecological niches is not fully understood. Bacterial regulatory networks are involved in this adaptation, and the regulatory TCSs (e.g., CiaRH), as well as the regulatory sRNAs, are part of it. This study is the first step to understand the role of csRNAs in the adaptation of *S. agalactiae*. This bacterium does not currently exhibit extensive antibiotic resistance. However, it is crucial to find alternatives before multidrug resistance emerges. Therefore, we propose that drugs targeting regulatory RNAs with Srn024-like activities would affect pathogens by reducing their abilities to form biofilm and to adapt to host niches.

**KEYWORDS** *Streptococcus agalactiae*, CiaRH, sRNAs, csRNAs, regulation, adaptation, biofilm, SAP pullulanase
invasive infections in neonates, causing pneumonia, bacteremia, and meningitis mainly via maternal transmission, and has emerged as an increasing cause of invasive diseases in immunocompromised and elderly adults (4, 5). The capacity of *S. agalactiae* to colonize and survive in such different environments underlines its ability to adapt to encountered changes. A first line of control is transcriptional, with regulators such as two-component systems (TCSs) that respond to physical and chemical perturbations. It is followed by posttranscriptional regulations in which regulatory small RNAs (sRNAs) may affect RNA elongation, processing/degradation, and translation (6).

The TCS CiaRH in *Streptococcus pneumoniae* regulates the transcription of sRNAs (CiaR-dependent sRNAs [csRNAs]) through the binding of CiaR protein to a direct repeat, TTTAAG-N5-TTTAAG (7). A previous study showed that *S. pneumoniae* csRNAs was involved in lung infection and thus in adaptation to its environment (8). In *S. agalactiae*, several studies described sRNAs (9–13). Among them, four are potential csRNAs. They were first predicted in *silico* and named csRNA10, csRNA11, csRNA12, and csRNA14 (10). Then, they were detected by high-throughput RNA sequencing (RNA-seq) and renamed Sm015, Sm024, Sm070, and Sm085, respectively, and the expression of three of them was confirmed by Northern blotting in *S. agalactiae* strain NEM316 (11).

Here, we examined the role of csRNAs in *S. agalactiae* adaptation to its environment. Our results showed that csRNAs are conserved in *S. agalactiae* and controlled by the TCS CiaRH. We demonstrated that Sm024 is involved in sap posttranscriptional regulation. The sap gene encodes an LPXTG-cell wall-anchored pullulanase that cleaves α-glucan polysaccharides such as pullulan and glycogen found in the environment to release glucose, a nutrient source for bacteria (14). In *S. agalactiae*, *S. agalactiae* pullulanase (SAP) is known to bind to human cervical epithelial cells (15). Adhesion to matrix constitutes a critical step to promote biofilm formation and contributes to bacterium virulence. We demonstrated (i) that inactivation of SAP leads to no bacterial growth in a medium with only pullulan as a carbon source, while deletion of *ciaRH* and *sm024* genes significantly increases bacterial growth versus the wild-type (WT) strain, and (ii) that SAP induces biofilm formation, whereas CiaRH and Sm024 repress biofilm production of *S. agalactiae*. The regulatory network of CiaRH, Sm024, and SAP could thus be involved in the host colonization. We propose that drugs targeting regulatory RNAs with Sm024-like activities would affect pathogens by reducing their ability to produce biofilm and to adapt to host niches.

**RESULTS**

csRNAs are conserved in *S. agalactiae*. We reported the identification of 47 putative trans-acting sRNAs in *S. agalactiae* strain NEM316 (11). Among them, the four csRNAs (Sm015, Sm024, Sm070, and Sm085, which are 140, 90, 112, and 218 nucleotides [nt] long, respectively) were expressed. Their corresponding genes, namely, *srn015*, *srn024*, *srn070*, and *srn085*, are located between *pgi* and *gbs0438*, between *gbs0600* and *gbs0601*, between *gbs1697* and *gbs1698*, and between *gbs2087* and *gbs2088*, respectively. Sequence homology searches using all available *S. agalactiae* shotgun and complete genome sequences reveal that *srn015*, *srn024*, *srn070*, and *srn085* are conserved in *S. agalactiae*, with a low degree of polymorphism (>98% nucleotide identity). Thus, csRNAs belong to *S. agalactiae* regulatory RNA core genes. Interestingly, BLAST analysis did not result in significant matches when performed in other streptococcal genomes, suggesting that *srn015*, *srn024*, *srn070*, and *srn085* are specific to *S. agalactiae*. The csRNA conservation within *S. agalactiae* suggests that they have an important role in bacterial physiology.

csRNAs are regulated by the TCS CiaRH. The presence of a conserved motif, TTTAAG-N5-TTTAAG, was detected within the promoter regions of *srn015*, *srn024*, *srn070*, and *srn085* using WebLogo (Fig. 1A) (16). This sequence was previously identified as a CiaR binding site in *S. pneumoniae* (7). This result suggested CiaRH regulation of these csRNAs. To test this possibility, a strain in which the *ciaRH* operon was inactivated (∆ciaRH) was constructed along with the corresponding complemented strain (∆ciaRH::ciaR*<sub>WT</sub>*). The promoter regions of each of the four csRNA genes were cloned upstream of the *lacZ* gene (encoding β-galactosidase enzyme) into the
promoter probe vector pTCV-lacZ. The resulting plasmids were introduced into NEM316 WT, ΔciaRH, and ΔciaRH::ciaRH<sup>in situ</sup> strains, and the β-galactosidase activity of each strain was determined at the mid-exponential growth phase in Todd-Hewitt (TH) broth. The four tested promoters were active in the NEM316 and ΔciaRH::ciaRH<sup>in situ</sup> strains but had strongly reduced activity in the ΔciaRH strain (Fig. 1B and C). Moreover, an important difference was observed in the promoter activities of the four csRNAs. Indeed, that of Srn024 was 2- to 46-fold higher than that of the others (Fig. 1B). This finding confirmed the involvement of CiaRH in the regulation of these csRNAs.

The direct repeat sequence TTTAAG-N5-TTTAAG is involved in the regulation of Srn024. To evaluate the role of the predicted CiaR binding site in transcriptional regulation of these csRNAs, point mutations were introduced in the CiaR box of the pTCV-P<sub>srm024</sub>-lacZ transcriptional fusion (Fig. 2A); the recombinant plasmids were transformed into the NEM316 strain, and the β-galactosidase activity of the different constructs was measured. Compared to the native promoter (P<sub>srm024</sub>), the β-galactosidase activity was strongly reduced when the second TTTAAG motif was replaced by the CCCGGT sequence (P<sub>srm024</sub>*). However, the β-galactosidase activity was not altered by replacing the second nucleotide of this motif (P<sub>srm024</sub>**) (Fig. 2). These results confirmed the involvement of CiaRH in the transcriptional regulation of Srn024.

Srn024 does not encode a small peptide. A putative open reading frame (ORF) predicted in the srm024 sequence is for a 24-amino acid peptide. Although the sequence is not preceded by a ribosome binding site (RBS), a translational fusion was performed to test the putative synthesis of a peptide. For this, we developed pNJE2, a vector derived from pMSP3545 (see Fig. S1A and B in the supplemental material) (17) containing a nisin-inducible promoter (P<sub>nisy</sub>) followed by (i) a transcriptional start site, (ii) a multiple cloning site, and (iii) a transcriptional terminator. Its derivative, pNJE2::srm024ORF, contains the putative srm024 ORF with its upstream sequence cloned in frame and upstream of the lacZ gene starting from the second codon (see Fig. S2). No
LacZ activity was detected with this construct (see Fig. S2B). Therefore, we conclude that Srn024 likely does not express a peptide; consequently, the phenotypes that we report here are likely due to RNA pairing activities.

Srn024 targets the LPXTG-cell wall-anchored pullulanase SAP by RNA-RNA pairing in silico. To identify putative mRNA targets of Srn024, a genome-wide prediction of Srn024-mRNA interactions was performed using in silico target prediction web servers (CopraRNA, TargetRNA2, and RNApredator) (18–20). A list of the top three ranked candidate target genes for Srn024 is shown in Table 1 and described in Table 2. gbs1288 mRNA, also known as sap mRNA (S. agalactiae pullulanase), was the most probable target of Srn024, with the lowest thermodynamic pairing energy between the two RNA molecules for TargetRNA2 (−214.37 kJ/mol) and RNApredator (−217.41 kJ/mol). It was also predicted in third position by CopraRNA (−217.74 kJ/mol) (Table 1). The putative interaction region of 22 nt between Srn024 and the sap mRNA predicted by IntaRNA is located between the AUG start codon region of sap mRNA and the first 2 to 24 nt of Srn024 (Fig. 3; also see Fig. S3) (18). Thus, in silico predictions suggest that Srn024 affects sap expression by RNA-RNA pairing.

Srn024 does not affect the transcription of sap. To evaluate sap transcription, a strain in which the srn024 gene was inactivated (Δsrn024) was constructed along with a complemented strain containing a recombinant vector carrying srn024 (Δsrn024-pTCV-P_ANT::srn024). The amount of sap transcripts was determined by reverse transcription-quantitative PCR

![Image](image_url)

**FIG 2** Involvement of the CiaR binding site in the regulation of Srn024. (A) Sequences of native (P_{srn024}) and mutated (P_{srn024}*) and P_{srn024}**) promoters of the srn024 gene. The performed substitutions are shown in bold and indicated by stars. In the promoter sequences, the CiaR-related repeat is underlined by arrows and boxed. The transcriptional start site is shown in bold. The putative −35 sequence is shown in red, and the −10 region is boxed. (B) The activity of native and mutated promoters of srn024 was measured in S. agalactiae strain NEM316. Results are presented as means ± standard deviations of three independent experiments. The significance was determined by ANOVAs and Student t tests. ***, P < 0.001.

**TABLE 1** Top three ranked putative target genes for Srn024 in S. agalactiae strain NEM316

| Predicted target rank | Predicted target from CopraRNA (energy [kJ/mol]) | Predicted target from TargetRNA2 (energy [kJ/mol]) | Predicted target from RNApredator (energy [kJ/mol]) |
|-----------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| 1                     | gbs1498 (−18.16)                               | gbs1288 (−14.37)*                              | gbs1288 (−17.41)*                              |
| 2                     | gbs1396 (−18.04)                               | gbs1398 (−13.95)                               | gbs1396 (−17.20)                               |
| 3                     | gbs1288 (−17.74)*                              | gbs0877 (−13.57)                               | gbs0609 (−15.41)                               |

*Gene predicted by the three tools.
(RT-qPCR) in NEM316-pTCV, Δsrn024-pTCV-P, and Δsrn024-pTCV-P strains. Transcription was not significantly different in the three strains (see Fig. S4). We concluded that the role of Sm024 is not likely to affect sap transcription; therefore, Sm024 may act posttranscriptionally.

Posttranscriptional regulation of sap by Sm024. Because sap transcription is not altered by Sm024 but is a predicted Sm024 target, we considered that Sm024 could act posttranscriptionally. In order to decipher any involvement of Sm024 in posttranscriptional regulation of sap mRNA, we constructed a pNJE2: sap vector in which the beginning of the sap gene (RBS and translation start codon) was cloned upstream of the lacZ gene starting from the second codon (see Fig. S1C). NEM316, Δsrn024, and complemented Δsrn024::srn024 in situ strains were transformed with the resulting vector pNJE2::sap. Expression of SAP-reporter was induced by the addition of nisin (25 ng/mL) to the growth medium. At mid-exponential growth phase, β-galactosidase activity was measured. Compared to Δsrn024, the expression of the reporter fusion was about 2- and 3-fold higher in NEM316 and Δsrn024::srn024 strains, respectively (Fig. 4). These results suggest that Sm024 downregulates sap mRNA posttranscriptionally, likely by direct sRNA-mRNA interaction. In order to show the involvement of Sm024 in posttranscriptional regulation of sap mRNA, pNJE2::sap derivative plasmids carrying either a 24-nt deletion (pNJE2::sapD24) or a 7-nt substitution (pNJE2::sapS) in the sequence corresponding to the predicted interaction region of Sm024::sap mRNA were introduced into NEM316, Δsrn024, and Δsrn024::srn024::srn024 in situ strains (Fig. 4A). No difference in β-galactosidase activity was observed between the six strains (Fig. 4B). To confirm the importance of this sequence in the regulation of SAP, we constructed a mutant strain carrying

![FIG 3 Predicted interaction between Sm024 and sap mRNA. (A) Putative secondary structure of the csRNA Sm024, as predicted by RNAfold (43). (B) Putative interaction between csRNA Sm024 and the sap mRNA target as predicted by IntaRNA. The first codon of sap mRNA is boxed.](image-url)
7-nt substitutions in smr024 gene (smr024S) that were complementary to those performed in sap (Fig. 5A). SAP-reporter was less expressed in the srn024 strain than in NEM316 strain. Together, these experiments appear to support the predicted Srn024-sap mRNA interaction.

**Srn024 and CiaRH inhibit S. agalactiae growth in a chemically defined medium and biofilm formation.** Our findings suggesting the effects of Srn024 on SAP expression led us to investigate the roles of Smr024 in *S. agalactiae* growth in a medium with only pullulan as a carbon source and in biofilm formation. To test these hypotheses, we constructed a strain in which the sap gene was inactivated (sap*). In a chemically defined medium (CDM) with only 1% pullulan as a carbon source, the sap* strain did not grow while the Δsrn024 and ΔciaRH strains grew faster than the WT strain (Fig. 6).

Biofilm formation depends on adhesion of microorganisms to each other and to biotic or abiotic surfaces. The NEM316 strain forms biofilms on 96-well plastic plates. The sap* strain showed strongly reduced biofilm formation, which was restored by introducing the sap* allele (Fig. 7A), whereas the strain deficient for Srn024 and CiaRH showed increased biofilm formation, which was also restored by introducing the srn024* and ciaRH* alleles.

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**FIG 4** Posttranscriptional regulation of sap by Srn024. (A) Sequences of native sap mRNA, sapΔ24 (24-nt deletion is boxed), and sapS (7-nt substitutions are shown in bold and underlined). (B) Translation of SAP was measured in *S. agalactiae* NEM316, Δsrn024, and Δsrn024::srn024* strains. Results are presented as means ± standard deviations of three independent experiments. The significance was determined by ANOVAs and Student t tests. **, P < 0.01.

**FIG 5** Posttranscriptional regulation of sap by Srn024. (A) Putative interaction sequence between sap mRNA and Smr024. Performed substitutions are shown in bold and underlined. (B) Translation of SAP was measured in *S. agalactiae* NEM316 and srn024 strains. Results are presented as means ± standard deviations of three independent experiments. The significance was determined by ANOVAs and Student t tests. **, P < 0.01.
alleles (Fig. 7B). Additionally, biofilm formation by the sm024S strain, as the Δsm024 strain, increased compared to the WT strain (Fig. 7B and C).

We conclude that Sm024, likely through its interaction with sap mRNA, contributes to *S. agalactiae* adaptation to environmental changes and biofilm formation.

**DISCUSSION**

Regulatory sRNAs have been recognized to be essential in the regulation of bacterial genes. Their roles in pathogenesis and adaptation of bacteria to the environment are well established. In streptococci, many sRNAs have been predicted by bioinformatics or experimental screenings as next-generation sequencing (RNA-seq) (21). The expression of some sRNAs (csRNAs) is regulated by the CiaRH TCS conserved in streptococci (22). CiaRH controls many cellular processes, including resistance to the immune system, natural competence, and virulence (23–26). However, with the exception of *S. pneumoniae* and *Streptococcus sanguinis*, the function of most of these csRNAs remains unknown. Indeed, in *S. pneumoniae*, csRNAs are involved in competence, autolysis, and lung infection (7, 8, 27). In *S. sanguinis*, csRNA1-1 has been confirmed to inhibit biofilm formation (28).

In *S. agalactiae* NEM316, we previously identified four csRNAs that were bioinformatically predicted (9–13). Here, we showed good conservation (>98% nucleotide identity) of these csRNAs in *S. agalactiae*. Therefore, these genes are part of the core genome of this species, suggesting an important role for bacterial physiology.
To determine their roles in bacterial adaptation to the host, we first confirmed regulation of Smo015, Smo024, Smo070, and Smo085 csRNAs by the CiaRH TCS. In the promoters driving the expression of each of these four csRNAs, we identified the TTTAAG-N5-TTTAAG sequence, which was previously shown to be essential for CiaR binding in S. pneumoniae (7). Here, we confirmed the upregulation of the four csRNAs by the CiaRH TCS, suggesting the strong dependence of these csRNAs on CiaR (Fig. 1). These results were fully consistent with those for S. pneumoniae (7). We observed an important difference in the promoter activity of the four csRNAs (Fig. 1B). This could be due to sequence variability in csRNAs promoters (see Fig. S5 in the supplemental material). The dependence of the activated Smo024 promoter on CiaR is based on the direct repeat of the TTTAAG hexamer. Indeed, the conversion of the second TTTAAG motif to CCCCAG prevented the activity of the Smo024 promoter. However, the modification of TTTAAG to TGTAAG was not sufficient to impact regulation. The regulation of S. agalactiae csRNAs by the CiaRH TCS strongly suggested a role in the adaptation of the bacterium to its environment.

We then searched for genes that might be regulated by Smo024 in S. agalactiae using target prediction web servers (CopraRNA, TargetRNA2, and RNApredator). Among the different candidate target genes, sap, encoding a pullulanase, was the most likely target (Table 1). We demonstrated that Smo024 did not affect the sap mRNA transcription (see Fig. S2). However, Smo024 appeared to base pair with the coding sequence of sap mRNA (Fig. 3B). Using a translational fusion strategy, we confirmed that Smo024 is involved in sap mRNA posttranscriptional regulation. Our results suggest that duplex formation between Smo024 and sap mRNA could occlude the start site, resulting in translation inhibition (Fig. 4 and 5).

Bacteria need carbon sources to colonize the host; the most common are α-glucans such as starches and glycogen. Indeed, during periods of high estrogen availability, glycogen is found in large amounts in the vaginal epithelium, while dietary starches are abundant in the human colon (29, 30). SAP pullulanase is a surface-exposed protein that is able to hydrolyze starches and glycogen (14). In this study, we demonstrated the inability of a strain deficient in SAP to grow with only pullulan as a carbon source. However, ΔciaRH and Δsmo024 strains grow better in the presence of pullulan (Fig. 6). Thus, CiaRH may regulate the expression of SAP pullulanase through Smo024 depending on the availability of carbon sources in the bacterial environment.

Genes homologous to sap were found in S. pneumoniae, Streptococcus pyogenes, and Streptococcus suis, named spuA, pulA, and apuA, respectively. They have been proposed as key factors in the metabolic pathway involved in bacterial adaptation to host niches (31–33). SpuA was shown to be necessary for virulence in a mouse lung model of infection and to bind to alveolar type II cell glycogen in the lung. PulA is responsible for adhesion to human epithelial cells, and ApuA to promote adhesion to porcine epithelium (32–35). SAP was also shown to be necessary for S. agalactiae binding to human cervical epithelial cells (15). We can notice here that pullulanases found in different Streptococcus species play a role in adhesion. Based on these results, we investigated whether CiaRH and Smo024 could play a role in biofilm formation and thus in adhesion by regulating the expression of SAP. We demonstrated that CiaRH and Smo024 downregulate the biofilm formation of S. agalactiae, likely through the interaction between the csRNA and sap mRNA (Fig. 7). CiaRH is known to be involved in biofilm formation in several streptococcal species such as S. pyogenes, Streptococcus mutans, and Streptococcus gordonii (22). This study is the first one that shows the involvement of the TCS CiaRH and the csRNA Smo024 in S. agalactiae biofilm formation.

Here, we suggest an adaptation mechanism in which CiaRH may play a role in the posttranscriptional regulation of SAP by Smo024 in response to extracellular stimuli detected by the TCS. When bacteria would be exposed to large amounts of glycogen and starch or would have to adhere to the cell surface, CiaRH would no longer activate Smo024 expression, thus preventing the inhibition of posttranscriptional regulation of SAP. However, the regulation model that we propose here requires further work.

In this study, we confirmed the regulation of the four S. agalactiae csRNAs by the TCS CiaRH. Next, we identified the sap gene, which is involved in bacterial adaptation to its
different environments, as a target of Sm024 (14). We also demonstrated the implication of CiaRH and Sm024 in biofilm formation of \textit{S. agalactiae}. Although we have confirmed the posttranscriptional regulation of SAP by Sm024, it would also be important to find other targets and characterize the entire role of Sm024 in \textit{S. agalactiae}. In addition, characterization of other csRNAs will provide insight into the sRNA-dependent regulatory network of \textit{S. agalactiae}. This may contribute to the development of drugs targeting regulatory RNAs, reducing the ability of pathogens to adapt to host niches (36).

**MATERIALS AND METHODS**

\textbf{Plasmids, bacterial strains, and growth conditions.} Serotype III \textit{S. agalactiae} strain NEM316 was the parental strain used for this study (37). \textit{Escherichia coli} was used for cloning purposes. All plasmids and bacterial strains used in this study are listed in Table S1 in the supplemental material. \textit{S. agalactiae} and \textit{E. coli} strains were routinely grown at 37°C statically in TH broth (Difco) and under agitation in Luria-Bertani (LB) broth (MP Biomedicals), respectively. For translational fusions, \textit{S. agalactiae} strains were cultured in TH broth with the addition of 25 ng/mL of nisin. For growth kinetics, \textit{S. agalactiae} strains were grown at 37°C without shaking for 18 h in TH broth. Dilution was performed from these cultures to obtain an optical density at 600 nm (OD$_{600}$) of 0.05 in a CDM (38) supplemented with 1% pullulan (Thermo Fisher Scientific). Growth was studied using 96-well microplates (FALCON, USA). The plates were incubated for 24 h at 37°C without shaking in a spectrophotometer (BioTek Instruments, USA). Every 30 min, the OD$_{600}$ was measured after shaking for 5 s. When necessary, \textit{E. coli} and \textit{S. agalactiae} strains were grown with erythromycin (150 \( \mu \)g/mL for \textit{E. coli} or 10 \( \mu \)g/mL for \textit{S. agalactiae}).

\textbf{RNA preparation.} Total RNA was extracted from mid-exponential-phase cells (OD$_{600}$ of 0.6) by using the phenol/Trizol method (39). The quality of total RNA was checked on agarose.

\textbf{RT-qPCR.} RNA samples were treated with TURBO DNase (Ambion) according to the manufacturer’s instructions. The absence of contaminating genomic DNA was checked by PCR using recA primers (all primers used in this study are listed in Table S2 in the supplemental material). Gene selection for normalization, RT-qPCR experiments, and data analyses were performed as described previously (36). The expression levels of the tested genes were normalized using the recA gene. Presented data were obtained from duplicate measures of three independent RNA sample extractions.

\textbf{Construction of ciaRH and sm024 deletion mutants, and sap' inactivation mutant.} Mutant strains were constructed by allelic exchanges via a two-step homologous recombination process using pg+host1 derivatives as described (40).

\textit{S. agalactiae} \textit{ΔciaRH} is a nonpolar mutant of the NEM316 strain with deletion of the coding sequences of 
\textit{ciaRH} genes. Upstream and downstream flanking regions of \textit{ciaRH} were PCR amplified. A fusion between these two regions was obtained using the BsaI restriction site. The resulting sequence was amplified and cloned into the EcorR/BamHI restriction sites of the pg+host1. The recombinant plasmid was electroporated in the NEM316 strain. Allelic exchange was performed as described previously (40). To complement this mutant, the entire coding sequence of \textit{ciaRH} was amplified by PCR and cloned in the pg1+host1, as described above. After allelic exchange, the in situ chromosomal complementation of \textit{ciaRH} was confirmed by sequencing.

The same cloning strategy was applied to obtain the \textit{Δsm024} mutant strain. To complement the \textit{Δsm024} mutant strain, the entire sequence of \textit{sm024} was PCR amplified and the PCR fragment was cloned into the Xbal/PstI restriction sites of the pTCV-P$_{lac}$ vector, a derivative of the shuttle vector pTCV that carries a constitutively expressed Gram-positive promoter sequence (40). To construct the \textit{in situ} complement, \textit{sm024} was amplified by PCR and inserted into pg1+host1, using the same strategy as for \textit{ciaRH}.

To construct sm024 with substitutions of 7 nt in sm024, upstream and downstream flanking regions of \textit{sm024} were PCR amplified. A fusion between the two regions was obtained by using splicing-by-overlap extension PCR. The same strategy was used to construct a SAP inactivation mutant (sap') by adding a stop codon to the beginning of the \textit{sap} sequence.

After ligation, recombinant plasmids were transformed into \textit{E. coli}, verified by DNA sequencing of the cloned regions, and then electroporated into the appropriate NEM316 \textit{S. agalactiae} derivative. For strain constructions, the genomic DNA of mutants was isolated, and the presence of appropriate mutations was checked by sequencing the concerned regions.

\textbf{Construction of vectors for transcriptional fusion assays.} The pTCV-\textit{lacZ} vector was used to construct transcriptional fusions between the \textit{E. coli} lacZ reporter gene and upstream regions of the \textit{srn015}, \textit{srn024}, \textit{srn070}, and \textit{srn085} genes (41). The intergenic regions containing the four csRNAs promoters were PCR amplified (11). The corresponding purified DNA fragments were cloned into the BamHI/EcoRI restriction sites of the pTCV-\textit{lacZ} vector. NEM316, \textit{ΔciaRH}, and \textit{ΔciaRH;ciaRH*} strains were transformed with these constructions. The same cloning strategy was applied to obtain mutations in the \textit{sm024} promoter. A fusion between the two regions of each construction was obtained by using splicing-by-overlap extension PCR.

\textbf{β-Galactosidase transcriptional fusion assays.} For quantification of csRNAs expression, TH broth with 10 \( \mu \)g/mL erythromycin was inoculated to an OD$_{600}$ of 0.05 with an overnight culture. This culture was incubated at 37°C without agitation. Bacteria were harvested at mid-exponential growth phase (OD$_{600}$ of 0.6), and \textbf{β}-galactosidase assays were performed as described previously (38). All experiments were carried out at least three times.

\textbf{Construction of the pNJE2 vector for translational fusion.} The nisin-inducible promoter (P$_{nisin}$) was amplified from the pMSP3545 vector (17). The lacZ gene was amplified from its second codon from the pTCV-\textit{lacZ} vector (41). A fusion between these two fragments was obtained using the Ncol restriction site present in primers. The resulting sequence was amplified and cloned into the BgIII/XbaI restriction sites of the pMSP3545 vector.
To construct the pNJE2::sap vector, the first 90 nt of sap coding sequence were amplified by PCR. To construct pNJE2::sapL24 and pNJE2::sapS vectors, sequences upstream and downstream of the sap coding sequence were amplified by PCR (for primers, see Table S2 in the supplemental material). A fusion between the two regions was obtained by using splicing-by-overlap extension PCR. The resulting sequences were cloned into the EagI/PstI restriction sites of the pNJE2 vector. To construct pNJE2::srn0240RF, the srn024 gene was amplified and cloned into the Ncol/PstI restriction sites of the pNJE2 vector.

β-Galactosidase translational fusion assays. Bacteria were grown in TH broth supplemented with 25 ng/mL of nisin at 37°C without agitation to the mid-exponential growth phase (OD600 of 0.6). The bacteria were harvested, and the β-galactosidase activity was measured as described previously (38). All experiments were carried out at least three times.

Biofilm formation assay. Biofilm formation was studied using 96-well polystyrene microplates (Costar, Washington, DC, USA). Dilution was performed from overnight cultures to obtain an OD600 of 0.05 in the same medium (TH broth supplemented with 1% glucose). Then, 200 μL of this dilution was dispensed into the microplate and incubated at 37°C for 48 h. After incubation, the culture was removed, and the microplate was washed twice with water. Biofilm was stained for 15 min with 0.2% crystal violet (Merck, Darmstadt, Germany). (110 μL per well) and washed four times with water. After air drying, biofilm was dissolved for 1 h at room temperature with ethanol/acetone (80:20 [vol/vol]). The solution was transferred into a new plate, and the OD600 was measured using an Eon thermostatted spectrophotometer plate reader (BioTek Instruments). The biofilm formation index was calculated as the ratio of the OD600 after crystal violet treatment to the OD600 before treatment. Biofilm formation was represented as a percentage of that of the WT strain.

Statistical analyses. Data are presented as the mean ± standard deviation of at least three independent experiments. Analyses of variance (ANOVARs) and Student t tests were used to determine the significance of the differences between means (42).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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