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Rgg-Associated SHP Signaling Peptides Mediate Cross-Talk in Streptococci

Betty Fleuchot¹,²*, Alain Guillot¹,², Christine Mézange¹,², Colette Besset¹,², Emilie Chambellon¹,², Véronique Monnet¹,², Rozenn Gardan¹,²*

¹ INRA, UMR1319 MICALIS, Jouy en Josas, France, ² AgroParistech, UMR MICALIS, Jouy en Josas, France

Abstract

We described a quorum-sensing mechanism in the streptococci genus involving a short hydrophobic peptide (SHP), which acts as a pheromone, and a transcriptional regulator belonging to the Rgg family. The shp/rgg genes, found in nearly all streptococcal genomes and in several copies in some, have been classified into three groups. We used a genetic approach to evaluate the functionality of the SHP/Rgg quorum-sensing mechanism, encoded by three selected shp/rgg loci, in pathogenic and non-pathogenic streptococci. We characterized the mature form of each SHP pheromone by mass-spectrometry. We produced synthetic peptides corresponding to these mature forms, and used them to study functional complementation and cross-talk between these different SHP/Rgg systems. We demonstrate that a SHP pheromone of one system can influence the activity of a different system. Interestingly, this does not seem to be dependent on the SHP/Rgg group and cross-talk between pathogenic and non-pathogenic streptococci is observed.

Introduction

Quorum-sensing (QS) is a cell-cell communication mechanism that allows bacteria to control gene expression in a co-ordinated manner at a population scale. It involves the detection of an autoinducer signal that is synthesized, and actively or passively secreted; it is detected, or triggers a response, when its extracellular concentration reaches a threshold or quorum. This sensing leads cells to modulate expression of the gene targets of the mechanism. QS controls various important functions including for example virulence in Staphylococcus aureus [10,11], ComCDE in S. pneumoniae [12], ComQXPA in B. subtilis [13,14] and PapR/PlcR in B. cereus [15,16]. These studies are of significance for at least two reasons: i) a better knowledge of the interaction between the signaling peptides and their receptors may allow intervention, based on synthetic peptides, and this would be of particular value for the regulation of virulence factors as demonstrated in S. aureus [17]; ii) deciphering this diversity and evolution may help understand ecological adaptation by bacteria [18].

Many of these QS mechanisms have been deciphered in detail by more than 40 years of studies, such that we now understand quite well how a cell communicates with its siblings. A QS issue that has emerged more recently is the communication between different strains of the same species and even between species. Work on this subject has led to the definition of pherotypes or specificity groups, and amino acid sequence polymorphism has been documented for the signaling peptides and their receptors. All bacteria that belong to one pherotype can sense the peptides synthesized by members of the same pherotype but not the peptides synthesized by members of the others. Pherotypes have been defined for different mechanisms: Agr in S. aureus [10,11], ComCDE in S. pneumoniae [12], ComQXPA in B. subtilis [13,14] and PapR/PlcR in B. cereus [15,16]. These studies are of significance for at least two reasons: i) a better knowledge of the interaction between the signaling peptides and their receptors may allow intervention, based on synthetic peptides, and this would be of particular value for the regulation of virulence factors as demonstrated in S. aureus [17]; ii) deciphering this diversity and evolution may help understand ecological adaptation by bacteria [18].

We recently discovered a QS mechanism that relies on a transcriptional regulator of the Rgg family and a small hydrophobic peptide (SHP) detected in the intracellular medium [19,20]. We studied the shp/ster_1358 (rgg1358) locus of Streptococcus thermophilus LMD-9, where the two genes are transcribed divergently, and showed that SHP1358 is secreted, matured and imported back into the cell by the oligopeptide transporter Ami.
Then, the mature form of SHP1358 interacts with Rgg1358 enabling Rgg1358 to control, positively, the expression of two targets, *shp1358* and *ster_1357* [21]. The *ster_1357* gene encodes a secreted cyclic peptide of unknown function. A similar mechanism of action, involving Rgg1358 and SHP1358, has been suggested for the *shp/stu0182* (*rgg0182*) locus of *S. thermophilus* strain LMG18311 [22] and has been confirmed for two SHP/Rgg systems in *Streptococcus pyogenes*: one is an activator and the other a repressor involved in biofilm development [23,24]. The phylogenetic tree of Rgg-like proteins indicates that Rgg are widespread in Gram-positive bacteria but that *shp*-associated *rgg* genes are only found in the streptococci genus. This genus contains various species, including commensal bacteria of the human microbiome, the GRAS (generally recognized as safe) bacterium, *S. thermophilus*, used for the manufacture of dairy products, but also human pathogenic bacteria such as *S. pneumoniae*, *S. agalactiae*, *S. pyogenes* and *S. mutans* [25]. We found 68 *shp*/*rgg* copies, 28 of which encode a unique amino acid sequence, although the sequences of all these SHP pheromones are generally similar. Nearly all streptococci genomes contain one copy, but some streptococci have multiple copies, for example *S. thermophilus* strain LMD-9 has six. This phylogenetic study of Rgg amino acid sequences led to their classification into three groups. In groups I and II, the SHPs have a conserved glutamate and aspartate, respectively, and the *shp* and *rgg* genes are transcribed divergently. In group III, the *shp* genes are located downstream from the *rgg* genes, in a convergent orientation and the SHPs have a glutamate or an aspartate residue [21].

Different streptococci species can meet in raw milk [26], the human oral microbiome [27,28] and gastrointestinal tract [29]. We therefore investigated whether there is interspecies cross-talk via SHP peptides. We first studied the functionality of the SHP/Rgg cell-cell communication mechanism associated with three different *shp/*rgg loci, one from each of the three groups, in three distinct streptococci species. The mature form of each SHP was identified in the extracellular medium. We then used synthetic peptides to study the specificity of the interaction between the SHPs and the Rgg regulators of different groups and species. We demonstrate cross-talk between SHP/Rgg systems belonging to distinct groups and different species.

**Materials and Methods**

**Bacterial Strains and Growth Conditions**

The bacterial strains used in this study are listed in Table 1. *S. thermophilus*, *S. agalactiae* and *S. mutans* strains were grown at 30, 37 or 42°C in M17 medium (Difco) supplemented with 10 g l⁻¹...
lactose (M17lac) or in a chemically defined medium (CDM) without shaking, under atmospheric air and with a ratio of air space to liquid of approximately 90% [30]. *Escherichia coli* strains were grown at 30 or 37°C in Luria-Bertani (LB) broth with shaking. Agar (1.5%) was added to the media as appropriate. When required, antibiotics were added to the media at the following final concentrations: erythromycin, 200 μg ml–1 for *E. coli* and 5 μg ml–1 for *S. thermophilus*; and kanamycin, 1 mg ml–1 for *S. thermophilus*. The optical density at 600 nm of the cultures was measured with an UVikon 931 spectrophotometer (Kontron).

### DNA Manipulation and Sequencing

Restriction enzymes, T4 DNA ligase (New England Biolabs), and Phusion DNA polymerase (Finnzymes) were used according to the manufacturers’ instructions. Standard methods were used for DNA purification, restriction digestion, PCR, ligation and sequencing. The oligonucleotides, purchased from Eurogentec, are listed in Table S1. PCR amplifications were carried out in a GeneAmp PCR System 2720 (Applied Biosystems) and all amplified fragments were purified with a Wizard purification kit (Promega). Plasmids were extracted with QIAprep spin miniprep kits (Qiagen). The *E. coli* strain TG1 *epi*Δ was used as the host for cloning experiments. *S. thermophilus* was transformed using natural competent cells of strain LMD-9 leading to the construction of strains TIL1047 (*shp1299::erm*) and TIL1389 (*amiCDE::erm*). Strain TIL1160 (Δ*ster_1299*) was constructed by deleting an internal fragment of the gene by a double crossover event using pG*host*9. Briefly, oligonucleotides ster_1299-SpeI with ster_1299-EcoRI and ster_1299-EcoRI with ster_1299-HindIII were used to amplify upstream and downstream fragments from the *ster_1299* gene; the resulting two fragments were double digested with the restriction enzymes SpeI+EcoRI and EcoRI+HindIII, respectively, and ligated between the SpeI and HindIII restriction sites of pG*host*9. The resulting plasmid, pG*host*9::updown.ster_1299, was used to transform electrocompetent cells of strain LMD-9. Integration and excision of the plasmid led to the deletion of the *ster_1299* gene.

### Constructions of Strains Containing luxAB Reporters

First, plasmid pGICB004a, a derivative of pGICB004, was constructed to facilitate integration of transcriptional fusions to the luxAB reporter genes into the blp locus in *S. thermophilus* LMD-9 by natural transformation and double crossover events. For this purpose, the *aphA3* cassette was amplified with oligonucleotides AphA3-F and AphA3-R using plasmid pKa as the template [32]. The resulting fragment was inserted at the blp locus in *S. thermophilus* LMD-9.

### Table 2. Plasmids used in this study.

| Plasmid | Descriptiona,b | Source or reference |
|---------|----------------|---------------------|
| pG*host*9 | Erm, Ts plasmid | [41] |
| pG*host*9::updown.ster_1299 | Erm, pG*host*9 derivative, for ster_1299 gene replacement by double cross-over integration | This study |
| pGICB004 | Erm, Ts plasmid allowing the integration of transcriptional fusions to the luxAB reporter genes at the blp locus in *S. thermophilus* | [21] |
| pGICB004a | Erm, pGICB004 derivative containing the aphA3 gene, conferring kanamycin resistance, upstream from the luxAB genes | This study |
| pGICB004a::P<sub>shp1299</sub> | Erm, Km, pGICB004a derivative used to introduce a P<sub>shp1299</sub>luxAB transcriptional fusion into *S. thermophilus* LMD-9 | This study |
| pGICB004a:gbh1555 shp1555 | Erm, Km, pGICB004a derivative used to introduce a gbs1555 shp1555-luxAB transcriptional fusion into *S. thermophilus* LMD-9 | This study |
| pGICB004a::gbh1555 P<sub>shp1555</sub> | Erm, Km, pGICB004a derivative used to introduce a gbs1555 P<sub>shp1555</sub>luxAB transcriptional fusion into *S. thermophilus* LMD-9 | This study |
| pGICB004a::shp1555 | Erm, Km, pGICB004a derivative used to introduce a shp1555-luxAB transcriptional fusion into *S. thermophilus* LMD-9 | This study |
| pGICB004a::shp1509 | Erm, Km, pGICB004a derivative used to introduce a shp1509-luxAB transcriptional fusion into *S. thermophilus* LMD-9 | This study |
| pGICB004a::SMU.1509 shp1509 | Erm, Km, pGICB004a derivative used to introduce a SMU.1509 shp1509-luxAB transcriptional fusion into *S. thermophilus* LMD-9 | This study |
| pGICB004a::SMU.1509 P<sub>shp1509</sub> | Erm, Km, pGICB004a derivative used to introduce a SMU.1509 P<sub>shp1509</sub>luxAB transcriptional fusion into *S. thermophilus* LMD-9 | This study |

*aTis indicates that the plasmid encodes a thermosensitive RepA protein.

*bKm and Erm indicate resistance to kanamycin and erythromycin, respectively.

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fragments of both shp1299 and ami genes were amplified with oligonucleotides shp1299_up-F/shp1299_up-R and shp1299_down-F/shp1299_down-R and amiCDE_up-F/amiCDE_down-R, amiCDE_up-F/amiCDE_down-R. The resulting fused PCR fragments were used to transform natural competent cells of strain LMD-9 leading to the construction of strains TIL1047 (shp1299::erm) and TIL1389 (amiCDE::erm). Strain TIL1160 (Δ*ster_1299*) was constructed by deleting an internal fragment of the gene by a double crossover event using pG*host*9. Briefly, oligonucleotides ster_1299-SpeI with ster_1299-EcoRI and ster_1299-EcoRI with ster_1299-HindIII were used to amplify upstream and downstream fragments from the *ster_1299* gene; the resulting two fragments were double digested with the restriction enzymes SpeI+EcoRI and EcoRI+HindIII, respectively, and ligated between the SpeI and HindIII restriction sites of pG*host*9. The resulting plasmid, pG*host*9::updown.ster_1299, was used to transform electrocompetent cells of strain LMD-9. Integration and excision of the plasmid led to the deletion of the *ster_1299* gene.
the restriction enzymes SspI and EcoRI and ligated between the same restriction sites of pGICB004a. ScaI-linearized pGICB004a::P
shp1299 was used to transform competent cells of strains LMD-9, TIL1047 and TIL1160 leading to strains TIL1038 (blp::P
shp1299-luxAB aphA3), TIL1052 (shp1299::erm blp::P
shp1299-luxAB aphA3) and TIL1048 (Δsterr_1299 blp::P
shp1299-luxAB aphA3), respectively. The plasmids pGICB004a::gsb1555 shp1555, pGICB004a::shp1555 were constructed similarly and in these cases, the PCR fragments ligated into each plasmid were amplified with oligonucleotides GBS-SpeI/GBSshp-EcoRI and GBSrgg-SpeI/GBS-EcoRI, respectively. Natural transformation of strain LMD-9 with each linearized plasmid lead to construction of strains TIL1345 (blp::P
shp1555 shp1555-luxAB aphA3), TIL1382 (blp::P
shp1555 luxAB aphA3) and TIL1380 (blp::P
shp1555 luxAB aphA3), respectively. Similarly, pGICB004a::shp1509 was constructed by ligating a PCR fragment amplified with oligonucleotides SMUrgg-SpeI/SMUshp-EcoRI and double digested with EcoRI and SspI, into pGICB004a. The SMU.1509 gene contains a EcoRI restriction site, so pGICB004a::SMU.1509 shp1509 and pGICB004a::SMU.1509 shp1509 were constructed in two steps. First, the downstream part of SMU.1509 was amplified with oligonucleotides SMU-SpeI/SMU-2, double digested with EcoRI and SspI, and ligated between the same restriction site of pGICB004a. Secondly, the two fragments containing the fusions to the shp promoter were amplified with oligonucleotides SMU-1/SMU-EcoRI and SMU-1/SMUshp-EcoRI, digested with EcoRI and ligated into the same restriction site of pGICB004a already containing the downstream part of SMU.1509. Linearized pGICB004a::shp1509, pGICB004a::SMU.1509 shp1509 and pGICB004a::SMU.1509 P
shp1509 were then used to transform competent cells of strain LMD-9 leading to strains TIL1386 (blp::P
shp1509-luxAB aphA3), TIL1383 (blp::SMU.1509 shp1509-luxAB aphA3) and TIL1384 (blp::SMU.1509 P
shp1509-luxAB aphA3), respectively. Finally, TIL1042 (amiCDE::erm blp::P
shp1299-luxAB aphA3), TIL1381 (amiCDE::erm blp::gsb1555::shp1555-luxAB aphA3) and TIL1385 (amiCDE::erm blp::SMU.1509::shp1509-luxAB aphA3) were constructed by transforming competent cells of strains TIL1038, TIL1345 and TIL1383, respectively, with chromosomal DNA from strain TIL1389 (amiCDE::erm).

**LC-MS/MS**

*S. thermophilus* strain LMD-9 carrying the *shp1299* gene, *S. agalactiae* strain NEM316 carrying the *shp1555* gene, *S. mutans* strain UA159 carrying the *shp1509* gene, *S. thermophilus* strains TIL1345 and TIL1383 carrying the *shp* genes of *S. agalactiae* and *S. mutans*, respectively, were grown in CDM, and the culture supernatants were recovered at the end of the exponential phase. Aliquots of 5 μl of supernatant were loaded on a Pepmap C18 column (length 150 mm, 75 μm ID, 100 Å, Dionex, Voisins-le-Bretonneux) and analyzed on-line by mass spectrometry on a LTQ-Orbitrap Discovery apparatus (Thermo Fischer, San Jose).

The sequences of the synthetic peptides used in this study are underlined.

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The *shp* gene is followed by the Genbank id of the *shp* genes.

The *shp* genes are not annotated in Genbank but were identified using BactgeneSHOW [20], except for the *shp* gene associated with ster_1299, which is annotated ster_1299 in the genome of *S. mutans* strain LMD-9. Consequently, all the *shp* gene products are indicated with the term ‘‘SHP’’ followed by the number of the cognate *rgg* gene in Genbank. To unify the nomenclature, the ster_1298 gene product was renamed SHP1299.

The sequences of the synthetic peptides used in this study are underlined.

**Figure 1. Description of strains containing P
shp-luxAB transcriptional fusions in various genetic backgrounds.** These strains were constructed in *S. thermophilus* strain LMD-9 and used to study the expression of the *shp* genes of *S. agalactiae* strain NEM316 (shp/gsb1555 locus) and *S. mutans* strain UA159 (shp/SMU.1509 locus) in the presence and absence of the corresponding *shp* and *rgg* genes and in the presence and absence of the *ami* genes of *S. thermophilus* strain LMD-9.

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The masses of the separated molecules were first analyzed with the high resolution accuracy (10 ppm) of the Orbitrap mass analyser. Then, selected ions were fragmented in the trap by collision induced dissociation (CID) and the ion daughters were analyzed at low accuracy (250 ppm) in the linear ion trap (LTQ).

We manually extracted the ion current signals (XIC) of the masses of all monocharged peptides corresponding to the C-terminal fragments of the SHP precursors ranging from LIIVGG to FTIIMDILIVGG for SHP1555, from HIIGGG to IVVLE-THIHGG for SHP1509 and from FPPFG to VVIDHFPFFPG for SHP1299. Using the sequences of the three streptococcal genomes, we checked that these peptides could not be encoded by genes other than the shp genes. We also checked that the XIC detected fulfilled two different criteria: (i) the retention time of the XIC detected was compatible with the hydrophobicity (GRAVY index) of the corresponding peptide and (ii), the XIC signal was absent from the supernatant of a strain that did not encode the SHP. Then, selected ions were fragmented. This approach was not successful for S. thermophilus expressing the shp1509 gene of S. mutans (TIL1383), so a more targeted and sensitive approach was used. We searched specifically for the peptide ETIIIIGGG which has a predicted mass of 872.51 Da. First, we fragmented all the ions with a mass of 872.51+/-2 Da during the chromatographic separation and analyzed the fragments in the LTQ. Second, we extracted the MS2 XIC with a mass of 740, corresponding to the fragment b7. This transition was chosen on the basis of previous fragmentation data obtained with SHP1358 of the S. thermophilus strain LMD-9 [21]. The patterns of fragmentation were analyzed to identify one with all ion daughters that fitted well with the sequence of the peptide sought.

To estimate the concentration of mature SHP1358 (EGII-VIVVG) in the supernatant of S. thermophilus strain LMD-9, we used the corresponding heavy form of the mature peptide [NH2-EGII[V13C15N15]IVVG-OH] (Thermo, Scientific) dissolved in 5% CH3CN and 0.1% trifluoroacetic acid, as an internal standard. The heavy peptide was added to S. thermophilus LMD-9 supernatant at a final concentration of 10 ng ml⁻¹. The area obtained with the heavy peptide was measured and used to calculate the amount of the natural peptide.

**Luciferase Assay**

Cells were grown overnight at 42°C in CDM. Cultures of 50 ml of CDM were then inoculated at an OD600 of 0.05 and incubated at the appropriate temperature, i.e. 30, 37 or 42°C. Aliquots of 1 ml were sampled at regular intervals until the culture reached the stationary phase and analyzed as described previously [21]. Synthetic peptides (Table 3), stored in lyophilized form and prepared in 5% formic acid, were added as appropriate to a final concentration of 1 mM at the beginning of the cultures. Purities of crude preparations were above 90%. Results are reported in Relative Luminescence Units divided by the OD600 (RLU/OD600). S. thermophilus strains TIL1345, TIL1383, TIL1038 and TIL1163 were used in cultures at 30, 37 and 42°C to assess which of these was the optimal temperature for the expression of shp1509, shp1509, shp1299 and shp1358, respectively. It appeared to be 30°C for shp1535 and shp1299 and 42°C for shp1299 and shp1358 (data not shown).

**Results**

**Selection of Relevant shp/rgg Loci for the Study of Cross-talk in Streptococci**

To study cross-talk among streptococci via SHP signaling peptides, we chose four shp/rgg loci found in the three SHP-associated Rgg phylogenetic groups [21] (Table 3). For group I, we chose the locus shp/gbs1555 (rgg1555) of Streptococcus agalactiae strain NEM316, present in all sequenced strains of S. agalactiae. The role...
of Gbs1555, also called RovS, in virulence has been studied but without taking into account the existence of its cognate SHP [33]. Moreover, the amino acid sequence of the predicted mature SHP of \textit{S. agalactiae} is identical to those of the SHPs of \textit{Streptococcus dysgalactiae} subsp. \textit{equisimilis} and of SHP2 of \textit{S. pyogenes} (Table S2).

For group II, we chose the locus \textit{shp/ster_1358} (\textit{rgg1358}) of \textit{S. thermophilus} strain LMD-9, already studied in detail [19,21], and the locus \textit{shp/SMU.1509} (\textit{rgg1509}) of \textit{Streptococcus mutans} strain UA159 present in all sequenced strains of \textit{S. mutans}. For group III, we chose the locus \textit{shp/ster_1299} (\textit{rgg1299}) of \textit{S. thermophilus} strain LMD-9 also found in strain JIM8232, in \textit{Streptococcus oralis} strain SK60 and \textit{Streptococcus tigurinus} strain 1368. These loci thus correspond to three SHP/Rgg mechanisms that have not previously been studied, including two in pathogenic streptococci of two different streptococci groups: mutans (\textit{S. mutans}) and pyogenic (\textit{S. agalactiae}). These pathogenic streptococci are found in the same niche in the oral cavity [28] and are therefore likely to encounter each other, and also, at least briefly, \textit{S. thermophilus}, a species of the salivarius group that is one of the two starters used to produce yogurt. First, we studied the QS mechanisms of the loci that had not previously been studied.

**SHP/Rgg Mechanisms in Different Species of Streptococci Function Similarly**

Analysis of the \textit{shp/ster_1358} locus of \textit{S. thermophilus} showed that the SHP1358 peptide, the Rgg1358 transcriptional regulator and the Ami oligopeptide transporter are essential for a QS mechanism that positively controls the transcription of the \textit{shp1358} gene, creating a positive feedback loop [21]. We tested whether the autoinduction of \textit{shp} gene expression was conserved in the SHP/Gbs1555 system of \textit{S. agalactiae} strain NEM316, the SHP/SMU.1509 system of \textit{S. mutans} strain UA159 and the SHP/\textit{Ster_1299} system of \textit{S. thermophilus} strain LMD-9 (Table 3). We evaluated the activity of the \textit{shp} promoter of each locus in \textit{S. thermophilus} strain LMD-9, in the presence and absence of the genes encoding the three partners, SHP, Rgg and Ami. Thus, for locus \textit{shp/ster_1299} of \textit{S. thermophilus}, a \textit{P}_{\text{shp1299}}::\text{luxAB} fusion was introduced into the wild-type strain LMD-9 and the \textit{Δrgg1299}, \textit{Δshp1299} and \textit{ΔamiCDE} isogenic mutants. For \textit{shp/gbs1555} of \textit{S.
agalactiae and shp/SMU.1509 of S. mutans, the promoter of the shp gene and the shp genes were fused, independently, to the luxAB genes with or without the cognate rgg gene and then introduced into S. thermophilus strain LMD-9 or its isogenic DamiCDE mutant (Figure 1). For all three loci, luciferase activity was detected when rgg, shp and amicDE genes were all present (TIL1345, TIL1383, TIL1038, Figure 2A–C). If one of the genes was absent, the expression of the three Pshp promoters was undetectable (Figure 2A–C) except for the shp/gbs1555 locus of S. agalactiae studied in the Dami genetic background (TIL1381, Figure 2A): the relative luciferase activity in this case was one quarter of that in the wild-type genetic background (TIL1345). These experiments clearly demonstrated that the SHP pheromone, the Rgg regulatory protein of each locus and the Ami transporter of strain LMD-9 are required for strong expression of the three shp genes in the condition tested.

The pattern of activity of the different shp promoters differed: expression of luciferase activity started either in the middle (shp/stere_1299, Figure 2A and C) or at the end (shp/SMU.1509 - Figure 2B) of the exponential growth phase. Once the maximal activity was reached, it was maintained until the end of the exponential growth phase for Pshp1555 (Figure 2A) but was transient for the two other promoters studied, Pshp1509 and Pshp1299 (Figure 2B and C).

These analyses indicate that the SHP/Rgg systems of both pathogenic and non-pathogenic streptococci function in a similar way and appear to be temperature (data not shown, see Materials and Methods) and growth phase-dependent in S. thermophilus strain LMD-9.

The Mature Forms of SHP are Released by Cleavage of the C-terminal Part of their Precursor in Front of a Conserved Acid Residue

The mature form of the SHP1299 of S. thermophilus was sought directly in the supernatant of the wild-type strain LMD-9. Without purification or concentration, direct analysis of the supernatant of S. thermophilus strains TIL1345 and TIL1383 by LC-MS/MS identified two masses corresponding to the fragments, DIIFPPFG and FPPFG, of the SHP1299 precursor. These sequences were confirmed by fragmentation (Figure 3A–B). We used mass spectrometry to identify the sequences of the mature forms of the SHP1555 of S. agalactiae and SHP1509 of S. mutans in the supernatants of the S. thermophilus strains TIL1345 and TIL1383. A mass corresponding
to the octapeptide DIIIIVGG was identified as the mature form of the \textit{S. agalactiae} SHP1555 produced by \textit{S. thermophilus}. The sequence of this peptide was also confirmed by fragmentation. No mass corresponding to fragments of the precursor SHP1509 of \textit{S. mutans} was found in supernantant of \textit{S. thermophilus} strain TIL1383. A similar method was used to identify mature SHP peptides in the supernatants of the wild-type strains \textit{S. agalactiae} NEM316 and \textit{S. mutans} UA159. The production of the octapeptide DIIIIVGG was confirmed for \textit{S. agalactiae} (Figure 3C) but once again, we did not find any mass corresponding to fragments of the SHP1509 of \textit{S. mutans}. Therefore, we predicted by analogy, that the mature SHP peptide produced by \textit{S. mutans} was ETIIIIGGG and we used a more sensitive mass spectrometry approach (based on MS2) to detect this peptide in the supernatant of \textit{S. mutans} UA159. This approach successfully detected one mass corresponding to this peptide (Figure 3D).

To check that the longest peptides identified by mass spectrometry for the three loci were active, synthetic peptides with these three sequences were produced. These synthetic peptides were added to cultures of reporter strains containing a \textit{P}\textsubscript{shp}-\textit{lucAB} fusion of the corresponding locus but not encoding the cognate SHP. In all cases, the synthetic peptide functionally complemented the reporter strain (Figure 4 - TIL1052, TIL1382 and TIL1384 hatched bars).

We used mass spectrometry to evaluate the amount of SHP1358 naturally present in cultures of \textit{S. thermophilus} LMD-9 at the end of the exponential phase of growth, which is when its gene is maximally expressed. The heavy form of SHP1358 [NH\textsubscript{2}-EGII[V\textsubscript{C13}N\textsubscript{15}]IVVG-OH] (Thermo, Scientific) was used as an internal standard. The concentration of SHP present in the culture supernatant was estimated to be 7+/-3 ng ml\textsuperscript{-1}.

**The SHP Pheromones Allow Cross-talk between Streptococci**

SHP/Rgg QS mechanisms are widespread among species of streptococci and the amino acid sequences of the various Rgg and SHP proteins are similar (Table S2). We therefore investigated the existence of cross-talk phenomena. We used functional complementation experiments to determine whether \textit{shp}/\textit{rgg} loci can be regulated by mature SHPs with an amino acid sequence different from that of its cognate mature SHP. The four selected loci (Table 3) were studied in four reporter strains that cannot produce the cognate SHP. The four selected loci (Table 3) were studied in four reporter strains that cannot produce the cognate SHP. The four selected loci (Table 3) were studied in four reporter strains that cannot produce the cognate SHP. The four selected loci (Table 3) were studied in four reporter strains that cannot produce the cognate SHP. The four selected loci (Table 3) were studied in four reporter strains that cannot produce the cognate SHP. The four selected loci (Table 3) were studied in four reporter strains that cannot produce the cognate SHP. The four selected loci (Table 3) were studied in four reporter strains that cannot produce the cognate SHP. The four selected loci (Table 3) were studied in four reporter strains that cannot produce the cognate SHP. The four selected loci (Table 3) were studied in four reporter strains that cannot produce the cognate SHP.

The mature forms of SHP1299, SHP1553 and SHP1509 identified directly in \textit{S. thermophilus}, \textit{S. agalactiae} and \textit{S. mutans} supernatants were each the C-terminal part of the precursor and start with an acidic amino acid (Asp or Glu). These amino acids are conserved in nearly all SHP identified from streptococci genome sequences; the exceptions are one in \textit{S. thermophilus} strain LGM18311 (Rgg Stud182-associated SHP) and another in strain CNRZ1066 (Rgg Str0182-associated SHP) that contain a Cys residue at this position [21]. Naturally secreted SHP1358 also starts with a Glu [21]. The activity of SHP2 in \textit{S. pyogenes} is maintained if the Asp amino acid at this position is substituted with a Glu, but not with an amide-bearing residue [23]. Therefore, all mature SHPs are expected to have an Asp or Glu at their N terminus. These conserved residues seem to be required for the recognition of the precursor by the protease involved in their maturation and the activity of the mature SHP. The Eep membrane protease is involved in the production of mature SHP by \textit{S. thermophilus} [21] and \textit{S. pyogenes} [23]. It has not been established whether or not this role is direct. Nevertheless, Eep-encoding genes are present in all streptococci genomes, and map in a conserved environment, so it is highly probable that this role is common to all streptococci. The amino acid sequence of the SHP1555 of \textit{S. agalactiae} produced by \textit{S. thermophilus} and by \textit{S. agalactiae} were identical, consistent with the conservation of the role of Eep, and the maturation more generally. In \textit{Enterococcus faecalis}, the sex pheromones are matured by Eep [34,35,36], but there is no conservation of such acid amino acids. The maturation of the...
XIP, another family of signaling peptides produced by streptococci and that are involved in the triggering of competence, seems less well conserved. Indeed, Eep is involved in the production of the XIP of S. thermophilus but not in that of S. mutans. The sequences of the XIP peptides are less conserved than those of the SHP peptide, and this may explain the involvement of different proteases in their maturation.

We detected a shorter mature form of SHP1299 in the supernatant of S. thermophilus. Shorter forms were not detected in other supernatants but it does not indicate that there are not present in small amounts. This probably means that these linear non-modified peptides are subject to degradation by, at least, aminopeptidase present in the extracellular medium. We have already observed such N-terminal degradation with ComS [37] indicating the existence of a significant aminopeptidase activity at the surface of streptococci.

The cross-talk experiments with four sbp/rgg loci and five synthetic SHPs showed a generally high specificity of the SHP/Rgg interaction. Only one peptide, SHP3 from S. pyogenes, was able to induce the expression of the sbp genes from the other species. This result for S. agalactiae was not surprising because the two peptides differ only at the third residue, and the difference is very minor (DIIVGG/DIIVVGG). S. pyogenes encodes both peptides, and they can stimulate the expression of their targets to similar levels [23]. The cross-talk result with S. mutans was more surprising: the amino acid sequences of the SHPs are more divergent and they do not belong to the same group despite both containing a hydrophobic stretch of isoleucines (DIIVVGG/ETIIIIGGG). The SHP of S. agalactiae was not able to cross talk with the S. mutans system and vice versa indicating that the presence of the four isoleucine residues are critical only for the S. mutans system, and that the specificity of the interaction is complex. Mature SHP3 peptide can be produced by three different species of streptococci i.e. pyogenes, pneumoniae and thermophilus, and the mature SHP peptide of S. agalactiae can be produced by two other species of streptococci, pyogenes (SHP2) and S. dysgalactiae (Table S2). This suggests that if present in the same environment, these streptococci can potentially interact with each other through their SHP/Rgg systems. It would be interesting to investigate this possibility with co-cultures in an ecosystem model. Such interactions may be of great significance to the co-operation or competition between streptococci species.

Supporting Information

Table S1 Primers used in this study.

Table S2 Comparison of the sequences of six similar SHP pheromones.

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Author Contributions

Conceived and designed the experiments: BF RG VM AG. Performed the experiments: BF AG CM CB EC. Analyzed the data: BF RG VM AG EC. Contributed reagents/materials/analysis tools: AG CM CB EM. Wrote the paper: BF RG AG VM.

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