Multiple Length Peptide-Pheromone Variants Produced by *Streptococcus pyogenes* Directly Bind Rgg Proteins to Confer Transcriptional Regulation[^5]^[^5]

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**Background:** Bacterial peptide pheromones control gene expression via Rgg family transcription factors in *Streptococcus*.

**Results:** Pheromone length variants produced by *S. pyogenes* directly bind Rgg receptors with *Kd* values ranging from 0.5 to 5 μM.

**Conclusion:** Peptide variant activities correlate with binding affinities.

**Significance:** Bioactivity and receptor-ligand affinity assays developed herein provide a platform to discover quorum-sensing inhibitors.

*Streptococcus pyogenes*, a human-restricted pathogen, accounts for substantial mortality related to infections worldwide. Recent studies indicate that streptococci produce and respond to several secreted peptide signaling molecules (pheromones), including those known as short hydrophobic peptides (SHPs), to regulate gene expression by a quorum-sensing mechanism. Upon transport into the bacterial cell, pheromones bind to and modulate activity of receptor proteins belonging to the Rgg family of transcription factors. Previously, we reported biofilm regulation by the Rgg2/3 quorum-sensing circuit in *S. pyogenes*. The aim of this study was to identify the composition of mature pheromones from cell-free culture supernatants that facilitate biofilm formation. Bioluminescent reporters were employed to detect active pheromones in culture supernatants fractionated by reverse-phase chromatography, and mass spectrometry was used to characterize their properties. Surprisingly, multiple SHPs that varied by length were detected. Synthetic peptides of each variant were tested individually using bioluminescence reporters and biofilm growth assays, and although activities differed widely among the group, peptides comprising the C-terminal eight amino acids of the full-length native peptide were most active. Direct Rgg/SHP interactions were determined using a fluorescence polarization assay that utilized FITC-labeled peptide ligands. Peptide receptor affinities were seen to be as low as 500 nM and their binding affinities directly correlated with observed bioactivity. Revelation of naturally produced pheromones along with determination of their affinity for cognate receptors are important steps forward in designing compounds whose purpose is positioned for future therapeutics aimed at treating infections through the interference of bacterial communication.

Bacteria coordinate gene expression between members of a group by means of extracellular chemical signaling, commonly referred to as quorum sensing. By producing, secreting, and detecting various signaling molecules, referred to here as pheromones, bacteria manage population scale behaviors using information supplied by means of pheromone identity and abundance. An extraordinary diversity of pheromones continues to be discovered in the pursuit to elucidate regulatory networks controlling a variety of microbial behaviors, including but not limited to production of virulence attributes, horizontal genetic exchange, and biofilm development (1). Because quorum sensing appears to play an important regulatory role for several bacterial pathogens during host colonization, spread, or disease progression (1–3), the therapeutic potential of manipulating bacterial behavior by modulating bacterial communication has been proposed as an attractive alternative to current antimicrobial therapies (4).

*Streptococcus pyogenes* (synonymous with group A *Streptococcus*, GAS[^3]) is a human-restricted bacterium responsible for a wide range of diseases arising from localized (pharyngitis and impetigo) or systemic and invasive infections (necrotizing fasciitis and toxic shock) (12, 13). Adaptive immune responses directed at the bacterium may lead to post-infection sequelae, such as acute rheumatic fever, where serum components initiate self-recognition followed by inflammation (12). Rates of asymptomatic carriage of *S. pyogenes* are based on limited studies and presumably range between a few percent of adults to as many as 25% or more of school-aged children (14). Nonetheless, this bacterium persists in a significant proportion of the

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[^3]: The abbreviations used are: GAS, group A Streptococcus; FP, fluorescence polarization; SHP, short hydrophobic peptide; sSHP, synthetic SHP; CDM, chemically defined medium.
Identification of S. pyogenes SHP Pheromone Variants

TABLE 1
Bacterial strains and plasmids used in this study

| Strain/plasmid       | Description                                                                 | Ref. |
|----------------------|-----------------------------------------------------------------------------|------|
| NZ131                | Wild-type S. pyogenes M49 strain                                             | 27, 28 |
| BNL148               | NZ131 integrated with pBL111 P_{shp2}::luxAB reporter, Erm'                 | 15   |
| BNL193               | NZ131 Δrgg3::cat shp2_{GAG}::shp3_{CAT} Cm'                                 | 19   |
| BNL170               | NZ131 shp2_{GAG}::shp3_{GAG} integrated with pBL111 P_{shp2}::luxAB reporter, Erm' | 11   |
| BNL177               | NZ131 shp2_{GAG}::shp3_{GAG} integrated with pBL111 P_{shp3}::luxAB reporter, Erm' | 11   |
| BNL187               | NZ131 Δrgg3::cat Δshp2::shp3; Cm'                                         | 19   |
| BNL198               | NZ131 Δrgg3::cat Δshp3::shp2, Cm'                                         | 19   |
| JCC177               | NZ131 Δrgg3::cat shp3_{GAG}; Cm' with pBL125                                | 19   |
| p7INT                | Shuttle-suicide vector that integrates at streptococcal bacteriophage T12 attB site; Erm' | 29   |
| pBL111               | DNA fragment containing the shp2 promoter (500 bp) fused to luxAB and cloned into p7INT; Erm' | 15   |
| pBL120               | pEF760-based vector for replacement of shp2 with shp3; Erm'                 | 19   |
| pBL122               | pEF760-based vector for simultaneous rgg::cat and shp3::shp2 mutations; Cm' Erm' | 19   |
| pBL123               | Shuttle vector encoding spectinomycin resistance; pWV01 origin; Spe'        | 30   |
| pLZ12-spec           | Shuttle vector encoding spectinomycin resistance; pWV01 origin; Spe'        | 30   |
| pBL125               | pLZ12 spec based full-length shp2 complementation plasmid, Spe'             |       |
| pED760               | Shuttle vector pGh9-iss1 deleted for iss1 element; temperature-sensitive; Erm' | 7, 31 |
| pJC175               | pED760-based vector for replacement of rgg with cat cassette; Cm' Erm'       | 15   |
| pJC180               | pEF760-based vector for mutation of shp3 start codon to GGG; Erm'           | 11   |
| pJC219               | DNA fragment containing the shp3 promoter (384 bp) fused to luxAB and cloned into p7INT; Erm' | 19   |

Bacterial strains, Plasmids, and Culture Media—Bacterial strains and plasmids used in this study are listed in Table 1. S. pyogenes was routinely grown in Todd-Hewitt medium (BD Biosciences) supplemented with 0.2% (w/v) yeast extract (Amresco) (THY) or in a chemically defined medium (CDM) (15) containing 1% (w/v) glucose. Luciferase reporter assays were performed by growing S. pyogenes reporter strains in CDM. When necessary, antibiotics were included at the following concentrations for S. pyogenes: chloramphenicol, 3 μg ml⁻¹; erythromycin, 0.5 μg ml⁻¹; spectinomycin, 100 μg ml⁻¹.

population without causing disease, and events leading to pathogenesis or carriage remain poorly understood (12).

For low G + C Gram-positive bacteria (Firmicutes), oligopeptides are by far the most common and best understood bacterially produced pheromones. Among the multiple classes of peptide signal receptors is a family of transcription factors known as Rgg (which include proteins annotated as MutR or GadR, whose role in quorum sensing among species of Lactobacillales has recently come into focus (5–9)). Rgg proteins serve as pheromone receptors and directly bind short linear peptides, which thereby modulate Rgg activity. Because Rgg/pheromone interactions occur in the cytoplasm, extracellular peptide signals must be imported across the cell envelope, typically via an oligopeptide permease. Biosynthesis of these pheromones originates from small open reading frames whose molecular switches. For peptide pheromones, the prediction of their composition is greatly facilitated by knowledge of their corresponding gene’s coding sequence; however, predictions of mature products based solely on sequence information may neglect critical signaling properties afforded by naturally produced pheromones of a particular length and composition. Furthermore, understanding the molecular interactions of natural signals could significantly enhance the development of therapeutically active SHP pheromones present in cultures of S. pyogenes. We found that multiple length SHP variants are present in culture supernatants, demonstrating that our prediction that the mature forms of SHP2 and SHP3 pheromones would contain a length of only eight residues offered a rather limited picture of the natural complexity of the system.

**Experimental Procedures**

Bacterial Strains, Plasmids, and Culture Media—Bacterial strains and plasmids used in this study were performed by growing S. pyogenes reporter strains in CDM. When necessary, antibiotics were included at the following concentrations for S. pyogenes: chloramphenicol, 3 μg ml⁻¹; erythromycin, 0.5 μg ml⁻¹; spectinomycin, 100 μg ml⁻¹. Esch-
erichia coli strains DH10β (Invitrogen) and BH10C (17) were used for cloning purposes and were grown in Luria broth (LB) or on LB agar with antibiotics at the following concentrations: chloramphenicol, 10 μg ml⁻¹; erythromycin, 500 μg ml⁻¹; spectinomycin, 100 μg ml⁻¹; ampicillin 100 μg ml⁻¹. The E. coli expression strain C41 (DE3) (18) was maintained on LB agar with ampicillin.

Construction of Mutant Strains and Plasmids—Strains used in this study were derived from S. pyogenes serotype M49 strain NZ131. Construction of derivative strains and luciferase reporters has been discussed in detail previously (11, 15, 19). To generate pBL125 plasmid to complement full-length shp2, primers BL241 (CATGAGATCTGGCTTAAGGTGTTCGAGTTC)T and SHP2-C9-Rev (CATGAGATCTACAAA-CTAAATATAAGGGTTTC) were used to amplify the region encompassing shp2 and its promoter from NZ131 genomic DNA. This amplified fragment was then digested with BglII and ligated into BglIII-digested pLZ12spec. Genotypes were confirmed by PCR and sequencing.

Spent-culture Medium Fractionation—S. pyogenes strains were grown overnight and diluted 1:100 in 50 ml of fresh CDM. Cultures were grown statically at 37 °C until reaching an A₆₀₀ of 0.6, and cells were separated from the liquid phase by centrifugation at 4000 rpm for 15 min, followed by filtration through a 0.2-μm filter (VWR International). Filtered spent-culture supernatants were loaded onto 2000-mg HyperSep C18 cartridge (Thermo Scientific) and washed with 5% acetonitrile and 0.1% trifluoroacetic acid (TFA). Fractions were eluted with a gradient ranging from 5 to 100% acetonitrile was applied over 20 min. The remaining eluates were dried using a SpeedVac Concentrator (Savant SC250EXP, Thermo). The dried, concentrated samples were stored at −20 °C until further analysis.

Synthetic Peptides—Synthetic peptides were synthesized by NeoBioLab (Cambridge, MA) at >95% purity. Synthetic peptides were reconstituted as 2 mM stocks in DMSO and stored at −20 °C. All dilutions for working stocks were made in DMSO and stored at −20 °C.

Luminescence Transcriptional Reporter Assays—To assess the transcription inducing activity of eluted fractions, a luciferase reporter strain BNL177 (Table 1) was used. This strain was grown to an exponential growth phase A₆₀₀ of 0.1 in CDM. 10 μl of each elution fraction was tested for activity in the luciferase reporter assay. The remaining eluates were dried in vacuo using a SpeedVac Concentrator (Savant SC250EXP, Thermo). The dried fractions were sealed and stored at −20 °C until further analysis.

Synthetic Peptides—Synthetic peptides were purchased from NeoBioLab (Cambridge, MA) at >95% purity. Synthetic peptides were reconstituted as 2 mM stocks in DMSO and stored in aliquots at −80 °C. All dilutions for working stocks were made in DMSO and stored at −20 °C.

Identification of S. pyogenes SHP Pheromone Variants—The dried, active eluates were suspended in 50 μl of 5% acetonitrile, 0.1% formic acid. The samples were loaded directly onto a 15 cm × 75 μm reversed phase capillary column (ProteoPep™ II C18, 300 Å, 5 μm size, New Objective, Woburn, MA), and a solvent gradient ranging from 5 to 100% acetonitrile was applied over 100 min by a Proxeon Easy n-LC II HPLC instrument (Thermo Scientific, San Jose, CA). The peptides were directly eluted into an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA) with electrospray ionization at 350 nA/min flow rate. The mass spectrometer was operated in the data-dependent mode, and for each MS1 precursor ion scan, the 10 most intense ions were selected from fragmentation by collision-induced dissociation. Other mass spectrometry analysis parameters were as follows: resolution of MS1 was set at 60,000; normalized collision energy 35%; activation time 10 ms; isolation width 1.5; +4 and higher charge states were rejected.

SH3 and SH2A peptides were fragmented in silico using Prospector’s on-line tool, and the MS spectra were manually searched using a BioTek Synergy 2 plate reader, and the resulting effective concentration for 50% maximum activity (EC₅₀) were determined as described above.

Purification of Recombinant Rgg3 and MBP-Rgg2—Details of purification schemes have been described previously (15, 16). Briefly, His₅-SUMO-Rgg3 was expressed and purified from E. coli using nickel-affinity chromatography. The His₅-SUMO tag was removed using an in-house purified SUMO protease, and untagged Rgg3 was used in all experiments. Recombinant MBP-Rgg2 was also expressed in E. coli and purified with amylose resin (New England Biolabs). Purified MBP-Rgg2 was used for all experiments requiring Rgg2.

Fluorescence Polarization (FP)—For the direct FP assay, the concentration of N-terminal FITC-labeled synthetic peptides was kept constant at 10 nM for all reactions. Purified Rgg proteins were serially diluted, ranging from 10 μM to 5 nM, and mixed with peptide in a final reaction volume of 50 μl in protein storage buffer (20 mM Tris-HCl buffer, pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 20% (v/v) glycerol). For FP, the storage buffer was supplemented with 0.01% Triton X-100 and 0.1 mg/ml BSA. Reactions were transferred to a Corning® 96-well, half-area, black polystyrene plate prior to incubation at 20 °C for 30 min. Polarization values were measured using a BioTek Synergy 2 plate reader, and the resulting millipolarization values were plotted for each protein concentration tested to assess protein/peptide interactions (21).

For competition FP assays, 10 nM FITC-SHP was incubated for 10 min with the concentration of Rgg protein corresponding to the Kᵣ value, as determined from the direct FP assay (500 nM Rgg3 or 1 μM MBP-Rgg2). Reactions were then titrated against serial dilutions of unlabeled peptides. Millipolarization values were determined as described above.

Biofilm Assays—Bacterial strains were grown overnight in THY at 30 °C, diluted 1:20 into fresh CDM, and 0.5 ml was present for 10 min with the concentration of Rgg protein corresponding to the Kᵣ value, as determined from the direct FP assay (500 nM Rgg3 or 1 μM MBP-Rgg2). Reactions were then titrated against serial dilutions of unlabeled peptides. Millipolarization values were determined as described above.

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dispensed, in duplicate, to two wells of a cell culture-treated 24-well polystyrene plate (Greiner Bio-one). Synthetic peptide pheromones were added to concentrations ranging from 1 to 1000 nM. Plates were incubated statically at 37 °C with 5% CO2 for 24 h. Liquid medium was aspirated; wells were washed once with 300 μl of 0.9% NaCl to remove unattached cells, and the remaining biomass was dry-fixed overnight at 37 °C. Biofilms were stained with 0.2% crystal violet solution, washed three times with a solution containing 0.9% NaCl, 10% ethanol, and quantified by measurement of absorbance (595) by area scan of the wells in a Synergy 2 BioTek plate reader. A minimum of three biological replicates was performed for each condition.

RESULTS

Multiple SHP Peptide Length Variants Are Present in GAS Culture Supernatants—As reported previously, S. pyogenes suspended in cell-free culture supernatants of a strain in which the rgg3 regulator was deleted (JCC131, Δrgg3) display highly induced levels of shp2 and shp3 transcripts as compared with cultures grown in medium alone (15). Induction of transcription is due to the presence of mature SHP pheromones that were secreted by producing cells and remained in the supernatant medium. Genetic studies utilizing shp3 gene truncations revealed the minimum length of shp3 capable of activating the Rgg2/3 circuit was a version encoding the C-terminal nine amino acids (notated SHP-C9) (15). Although synthetic peptides comprising the eight C-terminal amino acids encoded by shp genes (sSHP2-C8 and sSHP3-C8) are sufficient to induce transcription (15), the native composition of mature SHP-signaling pheromones remains unsolved. We therefore sought the identity of naturally produced pheromones in spent-culture supernatants using mass spectrometry. Because the C-terminal nine amino acids encoded by both shp genes (MDIIIIVGG and MDIIIVGG) have identical predicted masses, mass spectrometry would be unable to distinguish between SHP types if mature pheromones entailed peptides less than 10 amino acids. Therefore, strains expressing either shp2 or shp3 exclusively were required for analysis. Furthermore, because shp gene dosage influences pheromone yield (19), strains expressing each shp in multiple copies were utilized; strain BNL187 contains two gene copies of shp3 (Δrgg3Δshp2→shp3), and JCC177 (pSar72) is a strain expressing shp2 from a multicopy plasmid (Δrgg3 shp3GGG (pshp2)) (19).

Collected supernatants from cultures of each SHP-producing strain, as well from a strain unable to express either shp2 or shp3 (Δrgg3 shp2GGG shp3GGG, BNL193), were subjected to solid-phase extraction. Eluates were generated from C18 column cartridges with increasing concentration steps of acetonitrile and were assessed for their ability to induce luciferase in a reporter strain responsive to SHP pheromones (Δrgg3 shp3GGGshp2GGG::Pshp2-luxAB; BNL178). 40% acetonitrile eluate from strains producing SHP2 or SHP3 each generated maximum relative luciferase activity (Fig. 1A). These fractions, along with the equivalent 40% acetonitrile fraction from a SHP nonproducing strain (Δrgg3 shp2GGGshp3GGG, BNL193), were further analyzed by liquid chromatography-tandem mass-spectrometry (LC-MS/MS). Taking a nonbiased approach for peptide identification, the total ion chromatograms were surveyed for ion masses corresponding to all theoretically possible pep-
Supplemental Fig. S1

The subsequent determination of effective concentrations (EC50) (Table 3).

SHP3-C7 peptide were capable of inducing P
ecAB reporter strain (BNL148) containing all aspects of SHP signaling. Under laboratory conditions used for the luminescence bioassay, unstimulated wild-type cultures stimulated by several of the SHP variants compared with shp− reporters (Fig. 2B and Table 3). However, elevated and sustained responses were seen in WT cultures stimulated by several of the SHP variants compared with shp− strain (Fig. 2, D versus C), indicating an ability to trigger the feed-forward loop. Although luminescence activity was observable for SHP3-C9 and SHP2-C10 at the concentrations tested, neither peptide was able to reach a critical threshold to maintain positive feedback.

SHP Peptides Bind Rgg Proteins Directly and with Varying Affinities—Central to Rgg-mediated quorum sensing in Firmicutes is the hypothesis that Rgg proteins directly bind to mature peptide pheromones for the modulation of protein activity. Upon identification of several variant SHP lengths with different specific activities, it was imperative to determine how peptide length and composition affected interaction with Rgg2 and Rgg3. To test this, a direct FP binding assay was developed in which fluorescein isothiocyanate (FITC)-labeled sSHP-C8 peptides were combined with purified recombinant Rgg proteins.

### TABLE 2
Integrated areas under curves for SHP variants in extracted ion chromatograms

| Peptide  | Sequence      | Retention time | Area under curve |
|----------|---------------|----------------|------------------|
| SHP2-C10 | IMDILIIVGG    | 46.48          | ND               |
| SHP2-C9  | IMDILIIVGG    | 46.80          | 2,110,058        |
| SHP2-C8  | DILIIVGG      | 50.69          | 178,268,415      |
| SHP2-C7  | IILIIVGG      | 42.28          | 6,311,768        |
| SHP3-C10 | AMDIIVGG      | 51.29          | ND               |
| SHP3-C9  | MDDIIIIVGG    | 46.68          | ND               |
| SHP3-C8  | IIIIVGG       | 51.55          | ND (too broad)   |
| SHP3-C7  | IIIIVGG       | 40.29          | 1,711,157        |

ND means not determined.

### TABLE 3
Peptide variant EC50 values as obtained from luciferase assay dose-response curves

| Peptide | EC50 (nM) |
|---------|-----------|
| SHP2-C8 | 1.1       |
| SHP2-C9 | 2.1       |
| SHP2-C10| 7.7       |
| SHP3-C7 | 499.0     |
| SHP3-C8 | 1.3       |
| SHP3-C9 | 29.9      |
| SHP3-C10| 9.5       |

played similar EC50 values (Fig. 2A and Table 3). sSHP-C7 was found to be slightly active only at the highest concentrations tested. Among the three SHP2 variants longer than seven amino acids, an inverse relationship was observed between peptide length and luminescence activity, with C8 having the greatest activity and C9 being more effective at stimulating transcription than C10 (Fig. 2A). Unexpectedly, SHP3 variants did not display this pattern, and sSHP3-C10 was more active than sSHP3-C9 (Fig. 2A). Likewise, it was surprising to find a 10-fold difference in activity between C9 peptides, considering the basis for their distinction is the same discrepancy between C8 peptides (C-6 position, Leu versus Ile), which contained similar bioactivities.

**SHP Variants Stimulate Positive Feedback to Induce Endogenous SHP Production**—The experiments described above demonstrate that various SHP peptides are produced by GAS and that each variant has a distinct potential to induce Rgg-dependent transcription in reporter strains unable to produce endogenous pheromones. However, to test the effect of each variant on the natural signaling network containing a feed-forward loop of autoinduction of shp genes, individual synthetic peptides were tested in a wild-type reporter strain (BNL148) containing all aspects of SHP signaling. Under laboratory conditions used for the luminescence bioassay, unstimulated wild-type cultures of GAS do not produce sufficient quantities of pheromone to induce Rgg2/3 signaling autonomously, and therefore they require an exogenous supply of pheromone to induce observable luminescence. At initial time points following stimulus with SHP peptide variants, luciferase induction patterns matched those seen in shp− reporters (Fig. 2B).

**SHP Peptides Bind Rgg Proteins Directly and with Varying Affinities**—Central to Rgg-mediated quorum sensing in Firmicutes is the hypothesis that Rgg proteins directly bind to mature peptide pheromones for the modulation of protein activity. Upon identification of several variant SHP lengths with differing specific activities, it was imperative to determine how peptide length and composition affected interaction with Rgg2 and Rgg3. To test this, a direct FP binding assay was developed in which fluorescein isothiocyanate (FITC)-labeled sSHP-C8 peptides were combined with purified recombinant Rgg proteins.

**SHP Peptides**

SHP-C8 Is the Most Active Variant of SHP Peptides—Having identified four lengths of each SHP in the active fraction, we next sought to examine the specific activity of each variant using synthetic peptides (sSHP) titrated to cultures of the shp− bioluminescent reporter strain (shp2ggcshp3ggcPshp−luxAB; BNL177). The relative luminescence activity that followed peptide addition was used to generate dose-response curves and the subsequent determination of effective concentrations resulting in 50% of the log maximum luminescence activity (EC50) (Table 3).

In previous studies investigating the minimum length of SHP peptides that produced bioluminescence activity, neither endogenously expressed shp3(17–23) (C7) nor the synthetic SHP3-C7 peptide were capable of inducing P
luxAB reporters at the concentrations tested (15). However, because we found C7 peptide in the active fraction, sSHP-C7 along with sSHP-C8, -C9, and -C10 variants were fully assessed using luciferase reporters. The peptides generating the lowest EC50 values (greatest bioactivity) were sSHP2-C8 and sSHP3-C8 and dis-
The affinity of this interaction was determined using a constant amount of FITC-labeled peptide probe (10 nM) while titrating increasing amounts of purified Rgg proteins. As expected, both SHP-C8 peptides were able to bind either MBP-Rgg2 (Fig. 3A) or Rgg3 (Fig. 3B) with low micromolar affinities (Table 4). Given the similarity of the two SHP-C8 peptides, it was not surprising that both peptides bound to both Rgg proteins with similar affinity.

To explore the relative affinities of SHP variants, an FP competitive binding assay was employed. In this assay, FITC-SHP-C8 (10 nM) was preincubated with a concentration of purified Rgg3 that generated half-maximal Rgg-SHP complex formation, as was determined from direct-binding FP. Subsequently, unlabeled SHP variants, as well as peptides composed of the reversed sequence of SHP (SHP-C8-rev), were titrated into the reaction and tested for their ability to compete with FITC-SHP-C8 for binding to Rgg3 protein (Fig. 3, C and D). Results indicated that unlabeled peptides were able to displace the already bound FITC-labeled peptides, and the concentrations at which unlabeled SHP peptides disrupted Rgg-FITC-SHP complexes by 50% were considered the IC50 value. For both SHP2-C8 and SHP3-C8, the IC50 values of unlabeled peptides were consistently lower than the apparent Kd values of FITC-SHP-C8 peptides. This modest decrease in affinity for FITC peptides is presumably due to interference by the added fluorescein conjugate. For other SHP variants, affinity for the Rgg proteins was lower than that seen for SHP-C8 peptides (Fig. 3, C and D), and a direct correlation was observed between peptide affinity for Rgg and the activity measured by bioluminescence assay. Interestingly, the SHP3-C7 peptide, like the SHP-C8-rev peptide, was unable to interact with Rgg proteins (Fig. 3D). The inability of SHP3-C7 to bind Rgg is consistent with its inability to induce Pshp expression (8, 15, 22), and this indicates that a negatively charged residue at position C-8 is required for binding, as was suggested previously (8, 15, 22).

Consistent among models of peptide-based intercellular signaling in Gram-positive bacteria is the premise that pheromones are synthesized by the ribosome as inactive precursors (pre-peptides), which undergo processing and/or modification to generate an active signal at a time concomitant or subsequent to peptide secretion. For Rgg signaling pathways, a requirement for maturation outside the cytoplasmic compartment would prevent premature activation of the pathway and avoid direct stimulation of Rgg within the producing cell. Previously, it was determined that the 23-amino acid sSHP3 (full-length SHP) was able to induce the luciferase bio-reporter, albeit to levels much lower than sSHP3-C8 (15). It was therefore pertinent to test the ability of an SHP pre-peptide to engage Rgg proteins. Using the competitive-binding FP assay, we found that full-length SHPs were unable to compete for Rgg binding (Fig. 4). This supports the notion that pre-peptides adopt a
conformation incapable of Rgg interaction and that synthetic, full-length SHPs must undergo some type of processing event outside the cell, prior to their functioning as an active signal.

| SHP variant Kd values as obtained from fluorescence polarization |
|---------------------------------------------------------------|
| **TABLE 4**  |
|                | Direct FP (Kd in μM)                      |                     |                     |
|                | MBP-Rgg2 | Rgg3 |                     |                     |
| FITC-SHP2-C8   | 0.98     | 0.50 |                     |                     |
| FITC-SHP3-C8   | 2.58     | 1.88 |                     |                     |
| **Competition FP with Rgg3 (Kd in μM)**                      |                     |                     |                     |
| SHP2           |          |      |                     |                     |
| C7             | -        | ND   |                     |                     |
| C8             | 0.2      | 0.15 |                     |                     |
| C9             | 0.5      | 4.6  |                     |                     |
| C10            | ND       | 0.3  |                     |                     |
| **Competition FP with MBP-Rgg2 (Kd in μM)**                   |                     |                     |                     |
| C8             | 0.4      | 0.5  |                     |                     |
| C9             | 2.5      | ND   |                     |                     |
| C10            | ND       | ND   |                     |                     |

**FIGURE 3. Synthetic SHP variant interaction with purified Rgg proteins.** A and B, direct FP of 10 nM FITC-labeled synthetic peptides titrated with purified MBP-Rgg2 (A) or Rgg3 (B) proteins. C and D, synthetic SHP variants were assessed for their ability to compete with FITC-labeled SHP2 for binding to Rgg proteins. Complexes of Rgg3:FITC-SHP2-C8 (formed under conditions containing 500 nM Rgg3, 10 nM FITC-SHP2-C8) were titrated with synthetic SHP2 variants (C) or SHP3 variants (D). E and F, synthetic SHP variants were assessed for their ability to compete with FITC-labeled SHP2-C8 for binding to Rggs. Complexes of MBP-Rgg2:FITC-SHP2-C8 (formed under conditions containing 1 μM MBP-Rgg2, 10 nM FITC-SHP2-C8) were titrated with synthetic SHP2 (E) and SHP3 (F) variants. Plots indicate the means of at least three independent experiments. Kd values were determined by applying linear regression on dose-response curves using GraphPad Prism (version 6.01).

**FIGURE 4. Full-length native SHP peptides do not bind Rgg proteins.** Synthetic SHP variants were assessed for their ability to compete with FITC-labeled SHP2 for binding to Rgg proteins. Complexes of Rgg3:FITC-SHP2-C8 (pre-formed under conditions described in Fig. 3) were titrated with synthetic SHP3-C8 or full-length (FL) SHPs.
Identification of S. pyogenes SHP Pheromone Variants

Induction of Biofilm Formation by SHP Variants—As reported previously, SHP-C8 pheromones are capable of stimulating biofilm development in the wild-type strain NZ131 (15). To test functionality of the SHP-pheromone variants in culture conditions conducive to biofilm formation, cells were grown in the presence of increasing concentrations of sSHP-C8, -C9, and -C10. As a first test, a strain incapable of endogenous SHP production (BNL170) was used to monitor biofilm development. Consistent with results obtained for transcriptional reporters, SHP-C8 pheromones were the most efficient at producing biofilms, initiating a measurable response at concentrations between 10 and 25 nM and reaching saturation at ~100 nM (Fig. 5, top). SHP-C9 and -C10 pheromones were able to induce biofilm development only at high nanomolar concentrations (400–1000 nM), and only SHP3-C9 generated biomass comparable with those produced by either SHP-C8 pheromones.

When tested with wild-type cells, which are able to synthesize pheromones de novo in response to exogenously provided signals, SHP-C8 peptides were able to increase biofilm production at concentrations as low as 5 nM (Fig. 5, bottom). The remaining variants, with exception to SHP2-C10, were effective at inducing biofilm formation, albeit at lower concentrations (10 to 25 nM) required for C8 peptides. The SHP2-C10 variant, however, only triggers a weak increase in biofilm production in the concentrations tested.

DISCUSSION

In recent years, the capacity for Rgg protein family members to serve as pheromone receptors has been revealed for several species of Streptococcus (6–9, 11, 15, 22, 23). Whereas many rgg genes are identifiable among streptococci and several other genera of the Firmicutes (8, 24), identification of cognate pheromone genes, if present, has been significantly more difficult due to their small size and a limited amount of information relating to their ability to serve as extracellular signals. To advance an understanding pertaining to the nature of mature signaling pheromones produced by bacteria, we attempted to identify and characterize all potential signaling pheromones of a specific Rgg quorum-sensing pathway. Here, we show that S. pyogenes secretes multiple forms of SHP pheromones displaying varying activities. Luciferase-based bioreporter assays demonstrated that of these multiple forms, SHP-C8 is the most active. With a series of in vitro Rgg-peptide binding experiments, it was established that SHP variants have different affinities for binding to Rgg proteins, and more significantly, their binding affinity correlated with Rgg-dependent transcriptional activity and the ability to induce biofilm formation.

The use of mass spectrometry to identify mature Rgg-related pheromones from culture supernatants has been successful in previous studies investigating SHP peptides produced by Streptococcus thermophilus, Streptococcus agalactiae, and Streptococcus mutans (SHP1358Sth, SHP1299Sag, SHP1555Sag, and SHP1509Smuth) (8, 22) and ComS-derived pheromones of S. thermophilus and S. mutans (25, 26). Evidence that multiple variants of a pheromone signal can be generated by bacteria has also been documented. For example, three forms of ComS (ComS(14–24), ComS(15–24), and ComS(16–24)) were found in culture supernatants of S. thermophilus, one of which (ComS(14–24)) could be verified by MS/MS (26). Similarly, the C9 and C5 (carboxyl nine and five amino acids) variants of SHP1299 of S. thermophilus were identified directly from culture supernatants without the need for purification or concentration (22). It remains unclear, however, how activity of these variants compare with that of the presumed mature peptides in their ability to engage the target receptors and induce their activity.
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Considering the possibility that variant peptides present in culture supernatants may themselves be active or inhibitory, it was our intention to measure specific activities of each identifiable variant in culture supernatants. Each SHP variant contained the same intact C terminus (corresponding with the final codon of the open reading frame), and size variations were due to N-terminal truncations, consistent with previous studies indicating that SHP pre-peptides are processed internally or from their N terminus (8, 15). The protease(s) responsible for generating length variants remains to be identified, but the fact that each variant differs by only one residue supports the likelihood that an amino-peptidase is responsible, as has been hypothesized for ComS variants in S. thermophilus (26). Further studies will be needed to identify the processing factor and its essentiality for extracellular signaling. It also remains unclear whether the production of size variants provides a use in downstream gene regulation. Although the C8 variants were shown to contain the greatest activity for Rgg2/3 responses in S. pyogenes, the potential exists that SHP variants may have differential effects on orthologous Rgg proteins of other species with which S. pyogenes corresponds. It was recently shown that an S. agalactiae Rgg could respond to pheromones generated by S. pyogenes (11), and although size variants were not tested, the SHP3-C8 peptide (a peptide not produced by S. agalactiae) displayed more activity than SHP2-C8 (22). These findings indicate sequence, and possibly size variations, could have unpredicted consequences on receptor activity.

The key step in any cell-cell communication circuit centers on interactions between the signaling molecule and its cognate receptor. Here, we have developed methodology using fluorescence polarization to assess the direct interaction between fluorescently labeled peptide variants with recombinant Rgg protein receptors. By these methodologies, we report Rgg/SHP-C8 interactions occur at submicromolar affinity. We also found that native SHP variant affinities for Rgg proteins directly correlated with their ability to activate the proteins, as determined by culture-based luminescence reporters. Perhaps the greatest benefit in testing each SHP variant was the ability to correlate length and sequence variations to differences in receptor affinity and activity. These correlations highlight important molecular interactions between Rgg receptors and ligands. Because S. pyogenes SHP2 and SHP3 variants differ in sequence at only two positions (C-10 and C-6), the corresponding differences in measured activity can be attributed to only a few variables. Most striking is the difference that position C-10 (the 10th residue from the C terminus, SHP2Ala versus SHP3Ala) has on binding affinity to Rgg proteins. Remarkably, SHP2-C10 induces to only one-tenth of the maximal luciferase expression seen by all other SHP variants (Fig. 2A). This is consistent with a weak SHP2-C10 interaction with Rgg (Fig. 3C). Because SHP3-C10 is relatively more capable of binding Rgg than SHP2-C10, it appears the first residue of the C10 peptides can dramatically affect interactions with the receptor. A related finding is seen in differences between C9 peptides; however, in these variants only the C-6 positions differ, and the SHP2 variant displays greater activity than SHP3, although to a lower differential than seen between C10 variants. Taken together, these results indicate that the C-6 position may potentiate binding differences seen with peptides having extensions from the N terminus. Because binding affinities of SHP-C8 peptides are nearly equivalent (Fig. 3, A and B), differences in binding of C9 and C10 appear due to their added lengths. Interestingly, we also observed that the shortest length peptide, SHP-C7, had the least capacity to engage Rgg proteins (Fig. 3D), and this confirms earlier observations that an N-terminally proximal and negatively charged residue is essential for activity (8, 15, 22). As the only position within any of the SHP variants that contains a charged residue, it seems likely that the amino acid at C-8 would orient the ligand properly to the binding interface. It will be interesting to see the effects of placing the charged residue at other locations along the peptide. Going forward, a systematic approach for substitutions along the polypeptide, together with supplementary studies that identify binding pocket contributions, will greatly enhance an understanding of the intermolecular interactions and may facilitate design of peptides or compounds that have enhanced affinity.

Consistent with models that suggest pre-peptides require processing and/or modification concomitant or subsequent to peptide secretion, we found that full-length SHP pre-peptides were unable to engage Rgg proteins in vitro (Fig. 3E). Whether interaction with Rgg by the pre-peptide is inhibited due to an unfavorable conformation caused by intramolecular interactions of the ligand, or whether an extended N terminus presents a steric clash with the binding pocket of the receptor cannot be distinguished by our current means of assessment. Additional mechanisms may be in place in the cell to forego unintended auto-stimulation by unprocessed peptides, including the possibility for translationally coupled secretion of pre-peptides, as well as selectivity by peptide transporters against importation of longer peptides. Nevertheless, our findings underscore the notion that an unprocessed pre-peptide located in the cytoplasm will not trigger a cellular response.

A deeper understanding of the ligand/receptor interactions presented by Rgg/SHP complexes will facilitate the development of new strategies aimed at disrupting bacterial communication. Development of a straightforward fluorescence-polarization assay allows for quick assessment of compounds that have the potential to interfere with ligand receptors. Fortuitously, we observed relatively quick off-rate kinetics of labeled peptides from Rgg proteins, as based on observations that reaction equilibriums were reached within the short incubation times (15 min) upon adding unlabeled competitor peptides in FP experiments. This finding is a potentially important consideration for the future development of competitor compounds aimed at disrupting Rgg/SHP interactions. Given that Rgg proteins are conserved across streptococci, these proteins present an exciting target for quorum-quenching strategies. The ease by which the FP competition assay can be carried out, even in small volumes, is especially amenable for high throughput screening of compound and peptide libraries for the identification of high affinity ligands.

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