

# Redundant Group A Streptococcus Signaling Peptides Exhibit Unique Activation Potentials

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All bacterial quorum sensing (QS) systems are based on the production, secretion, and detection of small signaling molecules. Gram-positive bacteria typically use small peptides as QS effectors, and each QS circuit generally requires the interaction of a single signaling molecule with a single receptor protein. The recently described Rgg2 and Rgg3 (Rgg2/3) regulatory circuit of *Streptococcus pyogenes* (group A streptococcus [GAS]) is one of only a few QS circuits known to utilize multiple signaling peptides. In this system, two distinct, endogenously produced peptide pheromones (SHP2 and SHP3) both function to activate the QS circuit. The aim of this study was to further define the roles of SHP2 and SHP3 in activation of the Rgg2/3 QS system, specifically with regard to *shp* gene identity and dosage. Results from our studies using transcriptional reporters and isogenic GAS mutants demonstrate that *shp* gene dosage does contribute to Rgg2/3 system induction, as decreased gene dosage results in decreased or absent induction. Beyond this, however, data indicate that the *shp* genes possess distinct potentials for supporting system activation, with *shp3* more readily able to support system activation than *shp2*. Studies using synthetic peptides and *shp* gene mutants indicate that the disparate activities of endogenous SHPs are due to production, rather than signaling, differences and are conferred by the N-terminal regions rather than the C-terminal signaling regions of the peptides. These data provide evidence that the N-terminal, noneffector sequences of SHP pheromones influence their production efficiencies and thereby the relative activation potentials of endogenous SHPs.

Bacteria commonly benefit from a prevalent form of bacterial communication known as quorum sensing (QS), a process which enables the coordination of responses across a population. This process occurs via the production, secretion, and detection of small, secreted signaling molecules which are often referred to as pheromones or autoinducers. The latter term references the common phenomenon wherein the signaling molecule positively regulates its own expression. Gram-positive bacteria often utilize processed oligopeptides as QS molecules, and these are genetically encoded and ribosomally generated within the cell. Either the general secretory (Sec) system or specialized transporters belonging to the ATP-binding cassette (ABC) family are responsible for active transport of these peptides out of the cell due to their inability to permeate the cell membrane (1, 2). At points between translation, export, and detection, peptides are subject to various modification events, including processing and/or cyclization (for a review, see reference 3). Following signal accumulation in the extracellular milieu, detection of the peptide signal can occur either at the surface of the cell via interaction with a sensor kinase or intracellularly via direct interaction with a cytoplasmic regulatory protein.

There are two groups of receptor proteins now known to function in intracellular detection of QS peptides: the RNPP family, named for its four prototypical members (Rap, NprR, and PlcR, each found in several *Bacillus* species, and PrgX of *Enterococcus faecalis*) (4, 5), and the Rgg family of transcriptional regulators widespread among the *Firmicutes* (6–12). Although the majority of these QS systems function with only a single peptide pheromone and single cognate receptor protein, several rely on the coordinated actions of multiple signaling peptides and/or receptors. The best-studied example is the PrgX system of *E. faecalis*. The transcriptional repressor PrgX interacts with both the chromosomally encoded activator peptide cCF10 and the plasmid-en-

coded inhibitor peptide iCF10 (13–15). In simple terms, cCF10 induces expression of plasmid conjugation genes through disruption of intermolecular PrgX interactions, whereas iCF10 promotes repression by stabilizing PrgX tetramer formation. Protein crystallization and modeling studies have revealed that both peptides bind at the same site of PrgX and that the disparity in sequence between these two peptides promotes their antagonistic activities (for a review, see reference 16).

The Rgg2 and Rgg3 (Rgg2/3) regulatory circuit of *Streptococcus pyogenes* that was recently described by our laboratory is another QS system that utilizes two distinct endogenously produced peptide pheromones in a single QS circuit (6). This system utilizes two Rgg-family regulators (Rgg2 and Rgg3) that respond to adjacently encoded signaling peptides (SHP2 and SHP3) to control gene expression and biofilm formation. Rgg2 is a transcriptional activator of target genes, including the *shp* genes themselves, whereas Rgg3 represses expression of these genes. The C-terminal eight amino acids of the SHP peptides are identical at seven of eight positions and comprise the active signaling molecules (Fig. 1A), and both mature peptides function intracellularly to induce system activation through derepression of Rgg3 and activation of Rgg2 (6, 10). Interestingly, this is the only Rgg-mediated QS system thus far identified to involve the coordinated action of multiple Rgg proteins and natively produced signaling peptides rather than a single receptor-peptide pair.

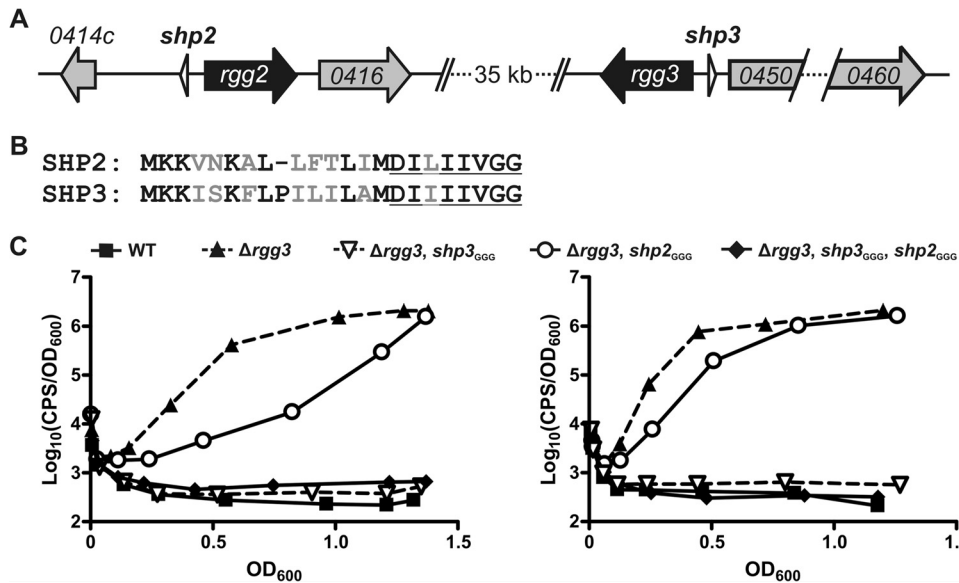
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**FIG 1** Inactivation of *shp* genes differentially affects Rgg2/3 system autoinduction in the  $\Delta$ rgg3 background. (A) Organization of the *rgg2* and *rgg3* loci in the NZ131 genome. (B) Alignment of full-length prepeptides SHP2 and SHP3. Nonidentical amino acids are in gray font, and the C-terminal eight residues comprising the mature, active peptides are underlined. (C) Luciferase expression from  $P_{shp2}$  reporter integrated into WT (BNL148),  $\Delta$ rgg3 (BNL149),  $\Delta$ rgg3 *shp3*<sub>GGG</sub> (BNL203),  $\Delta$ rgg3 *shp2*<sub>GGG</sub> (BNL176), and  $\Delta$ rgg3 *shp3*<sub>GGG</sub> *shp2*<sub>GGG</sub> (BNL199) isogenic mutant strains. Panels show results obtained with biological replicates using the same strains on separate occasions, and expression trends are representative of at least three independent experiments.

Despite both utilizing two endogenously produced peptide pheromones, the PrgX and Rgg2/3 systems are distinct in several ways. Unlike the case in *E. faecalis*, where only a single peptide-responsive regulator, PrgX, governs system activation, the Rgg2 and Rgg3 regulators of *S. pyogenes* both appear to respond to peptide signals. Unlike the PrgX system, where the two peptides have opposite activities, both SHP2 and SHP3 function to activate the Rgg2/3 system. Finally, unlike cCF10 and iCF10, which share less than 50% identity, the C-terminal eight amino acids that comprise the active portions of SHP2 and SHP3 differ by only a single residue (possessing leucine and isoleucine, respectively).

The aim of this study was to further define the precise roles of SHP2 and SHP3 in activation of the Rgg2/3 QS system. More specifically, we sought to determine if the SHP peptides possess distinct activities and/or if *shp* gene dosage is a factor for system activation. As expected, *shp* gene dosage does contribute to system activation. Unexpectedly, however, the data reveal that the *shp2* and *shp3* genes possess distinct potentials for supporting system activation in a manner independent of their C-terminal active regions, with *shp3* possessing enhanced activity compared to *shp2*. Comparable activities of exogenously added synthetic peptides indicate that the disparate activities of endogenous SHPs result from differential production and/or secretion, providing evidence that the N-terminal, noneffector sequences of SHP pheromones influence their production efficiencies and thereby the relative activation potentials of endogenous SHPs.

## MATERIALS AND METHODS

**Strains, plasmids, and culture media.** All strains used in this study are listed in Table 1, and construction of mutant strains is described in detail below. All plasmids and primers used in this study are listed in Tables 2 and 3, respectively. Group A streptococcus (GAS) was routinely grown in Todd-Hewitt medium (TH; BD Biosciences) supplemented with 2% (wt/vol) yeast extract (Y; Amresco). For all reporter expression studies, GAS

was grown in chemically defined medium (CDM) (6) made no more than 24 h prior to the experiment and containing 1% (wt/vol) glucose at 37°C. When necessary, antibiotics were included at the following concentrations: chloramphenicol (Cm), 3  $\mu$ g ml<sup>-1</sup>; erythromycin (Erm), 0.5  $\mu$ g ml<sup>-1</sup>; and spectinomycin (Spec), 100  $\mu$ g ml<sup>-1</sup>. *Escherichia coli* strains DH10 $\beta$  (Invitrogen) and BH10C (17) were used for cloning and were grown in Luria-Bertani (LB) broth or on LB agar with antibiotics at the

**TABLE 1** Bacterial strains used in this study

Strain	Description or genotype; phenotype <sup>a</sup>	Reference or source
NZ131	Wild-type M49 <i>S. pyogenes</i> isolate	18, 31
BNL148	NZ131 with integrated pBL111 $P_{shp2}$ reporter; Erm <sup>r</sup>	6
BNL149	JCC131 with integrated pBL111 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	6
BNL169	NZ131 $\Delta$ rgg3::cat <i>shp2</i> <sub>GGG</sub> ; Cm <sup>r</sup>	This study
BNL170	NZ131 <i>shp2</i> <sub>GGG</sub> <i>shp3</i> <sub>GGG</sub> ; unmarked	19
BNL176	BNL169 with integrated pBL111 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	This study
BNL177	BNL170 with integrated pBL111 $P_{shp2}$ reporter; Erm <sup>r</sup>	19
BNL186	NZ131 $\Delta$ rgg3::cat <i>shp2</i> <sub>leu171q</sub> ; Cm <sup>r</sup>	This study
BNL187	NZ131 $\Delta$ rgg3::cat $\Delta$ shp2→shp3; Cm <sup>r</sup>	This study
BNL192	BNL187 with integrated pBL111 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	This study
BNL193	NZ131 $\Delta$ rgg3::cat <i>shp2</i> <sub>GGG</sub> <i>shp3</i> <sub>GGG</sub> ; Cm <sup>r</sup>	This study
BNL196	BNL186 with integrated pBL111 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	This study
BNL198	NZ131 $\Delta$ rgg3::cat $\Delta$ shp3→shp2; Cm <sup>r</sup>	This study
BNL199	BNL193 with integrated pBL111 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	This study
BNL202	BNL198 with integrated pBL111 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	This study
BNL203	JCC177 with integrated pBL111 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	This study
BNL206	BNL170 with integrated pJC219 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	This study
BNL208	NZ131 $\Delta$ rgg3::cat <i>shp3</i> <sub>GGG</sub> $\Delta$ shp2→shp3; Cm <sup>r</sup>	This study
BNL210	NZ131 $\Delta$ rgg3::cat <i>shp2</i> <sub>GGG</sub> $\Delta$ shp3→shp2; Cm <sup>r</sup>	This study
BNL212	NZ131 $\Delta$ rgg3::cat $\Delta$ shp3→shp2 $\Delta$ shp2→shp3; Cm <sup>r</sup>	This study
BNL216	BNL208 with integrated pBL111 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	This study
BNL220	BNL210 with integrated pBL111 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	This study
BNL222	BNL212 with integrated pBL111 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	This study
BNL225	NZ131 $\Delta$ rgg3::cat <i>shp3</i> <sub>le181eu</sub> ; Cm <sup>r</sup>	This study
BNL227	BNL225 with integrated pBL111 $P_{shp2}$ reporter; Cm <sup>r</sup> Erm <sup>r</sup>	This study
JCC131	NZ131 $\Delta$ rgg3::cat; Cm <sup>r</sup>	6
JCC177	NZ131 $\Delta$ rgg3::cat <i>shp3</i> <sub>GGG</sub> ; Cm <sup>r</sup>	This study

<sup>a</sup> Cm, chloramphenicol; Erm, erythromycin.

TABLE 2 Plasmids used in this study

Plasmid	Description; phenotype <sup>a</sup>	Reference or source
p7INT	Shuttle-suicide vector that integrates at streptococcal bacteriophage T12 <i>attB</i> site; Erm <sup>r</sup>	23
pBL111	DNA fragment containing the <i>shp2</i> promoter (500 bp) fused to <i>luxAB</i> and cloned into p7INT; Erm <sup>r</sup>	6
pBL119	pFED760 containing DNA fragment encompassing <i>shp2</i> and flanking DNA; Erm <sup>r</sup>	19
pBL120	pFED760-based vector for mutation of <i>shp2</i> start codon to GGG; Erm <sup>r</sup>	19
pBL121	pBL119 with <i>shp2</i> leu17 codon mutated to code for ile; Erm <sup>r</sup>	This study
pBL122	pFED760-based vector for replacement of <i>shp2</i> with <i>shp3</i> ; Erm <sup>r</sup>	This study
pBL123	pFED760-based vector for simultaneous <i>rgg3::cat</i> and <i>shp3</i> → <i>shp2</i> mutations; Cm <sup>r</sup> Erm <sup>r</sup>	This study
pBL124	pJC175 with <i>shp3</i> ile18 codon mutated to code for leu; Cm <sup>r</sup> Erm <sup>r</sup>	This study
pFED760	Shuttle vector pGh9-ISS1 deleted for ISS1 element; temp-sensitive; Erm <sup>r</sup>	11, 20
pJC175	pFED760-based vector for replacement of <i>rgg3</i> with <i>cat</i> cassette; Cm <sup>r</sup> Erm <sup>r</sup>	6
pJC180	pFED760-based vector for mutation of <i>shp3</i> start codon to GGG; Erm <sup>r</sup>	19
pJC219	DNA fragment containing the <i>shp3</i> promoter (384 bp) fused to <i>luxAB</i> and cloned into p7INT; Erm <sup>r</sup>	This study

<sup>a</sup> Cm, chloramphenicol; Erm, erythromycin.

following concentrations: chloramphenicol, 10 µg ml<sup>-1</sup>; erythromycin, 500 µg ml<sup>-1</sup>; and spectinomycin, 100 µg ml<sup>-1</sup>.

**Construction of mutant GAS strains.** All *S. pyogenes* strains used in this study were derived from the serotype M49 strain NZ131 (18). The plasmids used to delete *rgg3* (pJC175) and to mutate the translational start codons of *shp2* (pBL120) and *shp3* (pJC180) were described previously (6, 19).

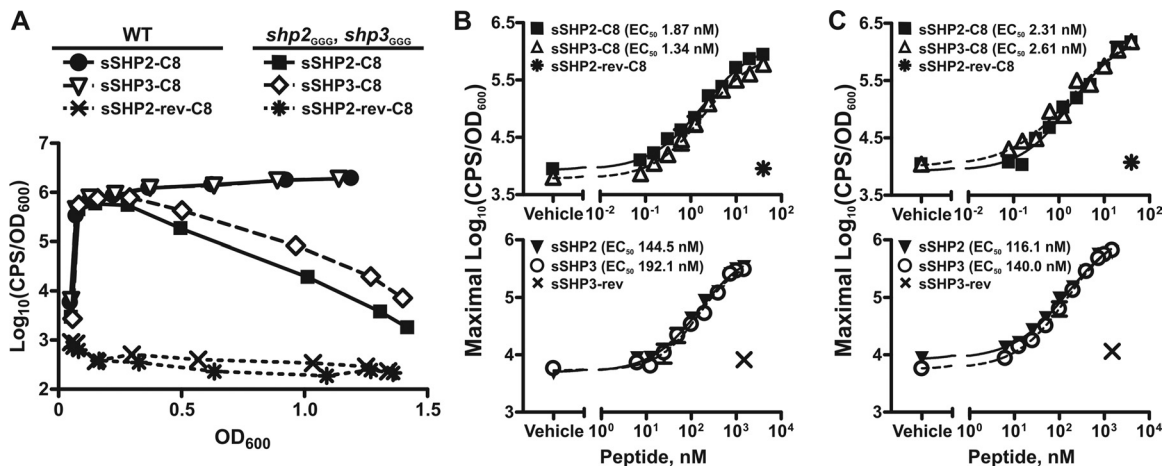
Vectors used for the allelic replacement of *shp2* were generated as

follows. To construct a plasmid in which the *shp2* codon encoding leucine at amino acid position 17 was mutated to encode isoleucine, pBL119 was used as the template in an inverse PCR with primer pair BL84 and BL85, resulting in plasmid pBL121. For the vector used in allelic replacement of *shp2* with *shp3*, upstream and downstream regions flanking the *shp2* gene were amplified by PCR with primer pair BL80 and BL86 and primer pair BL87 and BL81 using NZ131 DNA as the template, with the antisense and sense primers for the upstream and downstream regions, respectively,

TABLE 3 Primers used in this study

Constructed plasmid	Primer Sequence <sup>a</sup>	Description
pBL121	BL84 CTTTTTACATTGATCATGGATATCAT <u>C</u> ATTATCGTTGGTGGATAACC	Inverse PCR S primer to mutate <i>shp2</i> leu17 codon with nucleotide changes underlined
	BL85 GGTATCCACCAACGATAATG <u>A</u> TGATATCCATGATCAATGTA AAAAG	Inverse PCR AS primer to mutate <i>shp2</i> leu17 codon with nucleotide changes underlined
pBL122	BL80 GCGTG <b>C</b> CCGGGCCACTTTAAATAAATAATCTGTGAG	S primer for <i>shp2</i> and flanking regions; XmaI
	BL81 GCGTG <b>C</b> CCGGGATCGGTTTATCTGTAATAATTTTTTGACAAAGGTT	AS primer for <i>shp2</i> and flanking regions; XmaI
	BL86 <u>ATTTTCTTCAT</u> GACTGTCTCCTTTCTGATTTTCTATTTTGTACT	AS primer for <i>shp2</i> upstream flanking region with complementary sequence to <i>shp3</i> underlined
	BL87 <u>TTGTTGGTGGG</u> TAACCTTTCGCTTAAAAACAATCCACTTTG	S primer for <i>shp2</i> downstream flanking region with complementary sequence to <i>shp3</i> underlined
	BL88 <u>AGGAGACAGT</u> CATGAAGAAAATTTCAAAATTTTTCGCGATTTTAA	S primer for <i>shp3</i> with complementary sequence to <i>shp2</i> upstream region underlined
	BL89 <u>GCGAAAGGTT</u> ACCACCAACAATAATGATAATATCCATTGCTAAA	AS primer for <i>shp3</i> with complementary sequence to <i>shp2</i> downstream region underlined
pBL123	BL90 CATGG <b>C</b> GGCCGCAAAGCGAAAAAATGACACATTTATTC	S primer for <i>shp3</i> and flanking regions; NotI
	BL91 CATGG <b>C</b> GGCCGCGAAAAAATATTACCATCGTTAA	AS primer for <i>shp3</i> and flanking regions; NotI
	BL92 <u>AACTTTTTTCATA</u> AGTGGACTTCCTTTCAGTTTTTATTATTGTA	AS primer for <i>shp3</i> upstream flanking region with complementary sequence to <i>shp2</i> underlined
	BL93 <u>ATCGTTGGTGG</u> AATAAGTTACTTAGGTGTATACCTAAGTCTTTAGATACTCTA	S primer for <i>shp3</i> downstream flanking region with complementary sequence to <i>shp2</i> underlined
	BL94 <u>GAAGTCCACT</u> TATGAAAAAGTTAATAAAGCTTTGCTTTTACA	S primer for <i>shp2</i> with complementary sequence to <i>shp3</i> upstream region underlined
	BL95 <u>TAAGTAACTT</u> ATCCACCAACGATAATCAGGATATCCATGAT	AS primer for <i>shp2</i> with complementary sequence to <i>shp3</i> downstream region underlined
pBL124	BL96 ATTTTAGCAATGGATATTCTGATTATTGTTGGTGGG	Inverse PCR S primer to mutate <i>shp3</i> ile18 codon with nucleotide changes underlined
	BL97 CCCACCAACAATAATCAGAAATATCCATTGCTAAAAT	Inverse PCR AS primer to mutate <i>shp3</i> ile18 codon with nucleotide changes underlined
pJC219	BL27 GCGTG <b>G</b> AATTCGCCTTTAATTTTATTATGGT	AS primer for <i>luxAB</i> ; EcoRI
	JC156 ATGAAGTTTGGAAATATTTGTTTTTCG	S primer for <i>luxAB</i>
	JC238 CATGG <b>G</b> ATCCGTGTCGGAAAGTAAACATGC	S primer for <i>shp3</i> promoter; BamHI
	JC239 <u>CAAAATATTTCCAA</u> ACTTCATAAGTGGACTTCCTTTCAG	AS primer for <i>shp3</i> promoter with complementary sequence to <i>luxAB</i> underlined

<sup>a</sup> Restriction sites are in bold.



**FIG 2** Synthetic SHP peptides are equivalently able to independently support Rgg2/3 activation. (A) Luciferase expression from  $P_{shp2}$  reporter integrated into WT (BNL148) and  $shp3_{GGG} shp2_{GGG}$  (BNL177) strains grown in the presence of 5 nM sSHP2-C8, sSHP3-C8, or control peptide sSHP2-rev-C8. Data shown are representative of at least three independent experiments. (B and C) Dose-response curves for maximal relative luminescence from  $P_{shp2}$  (B) and  $P_{shp3}$  (C) reporters integrated into the  $shp3_{GGG} shp2_{GGG}$  strain (BNL177 and BNL206, respectively) in response to synthetic C8 (top panels) or full-length (bottom panels) SHP peptides. Single concentrations of reverse peptides were included as controls. Data shown represent means and standard errors of the means (error bars) of the results of three independent experiments, with  $EC_{50}$ s calculated from dose-response curves (GraphPad Prism, version 5.0).

containing sequence complementary to the *shp3* gene. A separate PCR was used to amplify *shp3* with primers BL88 and BL89, each of which contained complementary sequence to the upstream or downstream flanking region of *shp2*. The upstream region and *shp3* PCR products were fused together by overlap extension PCR using the primers BL80 and BL89. The product of this fusion was then used together with the downstream region PCR fragment in a second overlap extension PCR using the primers BL80 and BL81. This final fusion product was inserted at the XmaI site of the temperature-sensitive vector pFED760 (11, 20) to generate pBL122.

Vectors used for the allelic replacement of *shp3* simultaneously with *rgg3::cat* were generated as follows. For the vector used in allelic replacement of *shp3* with *shp2*, upstream and downstream regions flanking the *shp3* gene were amplified by PCR with primer pair BL90 and BL92 and primer pair BL91 and BL93 using JCC131 DNA as the template, with the antisense and sense primers for the upstream and downstream regions, respectively, containing sequence complementary to the *shp2* gene. A separate PCR was used to amplify *shp2* with primers BL94 and BL95, each of which contained complementary sequence to the upstream or downstream flanking region of *shp3*. The upstream region and *shp2* PCR products were fused together by overlap extension PCR using the primers BL90 and BL95, the product of which was then used together with the downstream region PCR fragment in a second overlap extension PCR using primers BL90 and BL91. This final fusion product was inserted at the NotI site of pFED760 to generate pBL123. To construct a plasmid in which the *shp3* codon encoding isoleucine at amino acid position 18 was mutated to encode leucine, pJC175 was used as the template in an inverse PCR with primer pair BL96 and BL97, resulting in plasmid pBL124.

All deletion vectors were electroporated into NZ131, and a two-step temperature-dependent selection process was used to isolate mutants of interest (21). Briefly, cells containing each deletion construct were grown at the permissive temperature (30°C) and then shifted to 37°C and plated on the appropriate antibiotic to select for bacteria in which the plasmid had integrated at one of the flanking regions. Cells were then grown at the permissive temperature to allow the plasmid to recombine out of the chromosome, and loss of Erm resistance was used to identify a successful second crossover event and loss of the deletion vector. Genotypes were confirmed by PCR and sequencing. This process was repeated to construct double and triple mutants.

**Construction of luciferase transcriptional reporters.** Construction of  $P_{shp2}$ -*lux* reporter plasmid pBL111 was reported previously (6). For the

$P_{shp3}$ -*lux* reporter plasmid, a 384-bp region directly upstream of *shp3*, but not including the *shp3* gene itself, was amplified from NZ131 genomic DNA using primers JC238 and JC239, and *Vibrio fischeri luxAB* genes were amplified from pCN58 (22) using primers JC156 and BL27. The promoter product was fused to *luxAB* in an overlap extension PCR using JC238 and BL27, and this product was inserted into EcoRI and BamHI sites of p7INT (23) to generate pJC219. All reporter plasmids were electroporated into GAS, and site-specific integration of the plasmids at a *tRNA(Ser)* gene was confirmed by PCR.

**Luciferase transcriptional reporter assays.** For luciferase assays monitoring autoinduction, cells from overnight cultures grown in THY at 30°C were diluted 160-fold into CDM and incubated at 37°C. At each time point, 50  $\mu$ l of each culture was removed to an opaque 96-well plate, samples were exposed to decyl aldehyde (Sigma) fumes for 30 s, and luminescence was quantified (in counts per second [cps]) using a Turner Biosystems Veritas Microplate Luminometer. The optical density of the culture at 600 nm ( $OD_{600}$ ) was also measured at each time point using a Spectronic 20D spectrophotometer (Milton Roy). Relative luminescence was calculated by normalizing cps to  $OD_{600}$ .

Luminescence experiments with synthetic peptides were performed in two different manners. For Fig. 2A, reporter strains were grown in CDM to an  $OD_{600}$  of 0.3 to 0.5 and then diluted 1:11 into fresh CDM containing peptide of interest, and  $OD_{600}$  and luminescence were monitored as described above. For Fig. 2B and C, reporter strains were grown in CDM to an  $OD_{600}$  of 0.3 to 0.5 and then diluted 1:11 into fresh CDM. Aliquots of the dilute culture were then distributed into the wells of a clear-bottom 96-well plate containing peptides of interest. Volatile decyl aldehyde was provided as a 1% solution in mineral oil between the wells of the microplate as previously described (24), and the plate was lidged and parafilmmed to ensure entrapment of the volatile content. Cells were grown at 37°C in a Synergy 2 plate reader (BioTek) with continuous fast shaking to deter cell sedimentation and facilitate accurate evaluation of the culture density, and  $OD_{600}$  and cps were measured every 10 min throughout growth. The maximum cps/ $OD_{600}$  value was determined for each well and plotted against peptide concentration to determine peptide-specific activities.

**Synthetic peptides.** Crude preparations of synthetic peptides used in luciferase assays were purchased from Neo-Peptide (Cambridge, MA), with purities ranging from 31% to 51%. Synthetic peptides were recon-

stituted as 2 mM stocks in dimethyl sulfoxide (DMSO) and stored at  $-80^{\circ}\text{C}$ .

## RESULTS

**Disruption of *shp2* and disruption of *shp3* have differential impacts on system autoinduction in the absence of *rgg3*.** It was previously shown that in the absence of *rgg3*, the Rgg2/3 system is activated in an Rgg2-dependent manner and results in high-level expression from the *shp* promoters (6). It was also shown that simultaneous deletion of *shp3* and *rgg3* abolished system activation (6), indicating that removal of *rgg3* was not sufficient and that SHPs, or at least SHP3, were required for Rgg2-mediated system activation. We questioned if the abolition of autoinduction in the  $\Delta\text{rgg3-}shp3$  mutant occurred because both *shp* genes are required due to peptide dosage or if only *shp3* is required because SHP3 possesses distinct activity that cannot be compensated for by SHP2. To assess these possibilities, we systematically generated  $\Delta\text{rgg3}$  mutants that also harbored mutations, changing the translation initiation codons of *shp2* or *shp3* or both *shp* genes to GGG (*shp2*<sub>GGG</sub>, *shp3*<sub>GGG</sub>) and then observed luminescence production from the  $P_{shp2}$  reporter in the various mutant strains. Mutation of the start codon was anticipated to abolish translation of the peptide, while use of the  $\Delta\text{rgg3}$  background allowed us to observe endogenous system activation without adding synthetic peptide, since the lack of a repressor in this strain allows for natural autoinduction of the system (6). In agreement with previous results, deletion of *rgg3* ( $\Delta\text{rgg3}$ ; BNL149) resulted in high-level luminescence production whereas there was no detectable reporter induction in the wild-type (WT) (BNL148) reporter strain. Also in agreement with previous results, mutation of *shp3* abolished reporter induction in the  $\Delta\text{rgg3}$  strain ( $\Delta\text{rgg3}$ , *shp3*<sub>GGG</sub>; BNL203) (Fig. 1B). It should be noted that the ability of the start codon mutation to abolish reporter induction in a manner equivalent to that seen when the entire *shp3* gene was deleted (6) indirectly confirms that the start codon mutation is sufficient to prevent SHP production or at least reduces SHP production to levels below that required for detection by the system. Unexpectedly, mutation of *shp2* in the  $\Delta\text{rgg3}$  strain ( $\Delta\text{rgg3}$ , *shp2*<sub>GGG</sub>; BNL176) did not abolish reporter induction; however, there was a difference in the timings of reporter induction, with luminescence induction in the  $\Delta\text{rgg3}$  *shp2*<sub>GGG</sub> mutant reproducibly slower than that in the  $\Delta\text{rgg3}$  strain. A reporter strain lacking *rgg3* and carrying initiation codon mutations in both *shp* genes ( $\Delta\text{rgg3}$ , *shp2*<sub>GGG</sub>, *shp3*<sub>GGG</sub>; BNL199) produced no detectable luminescence, akin to the profiles of WT and  $\Delta\text{rgg3}$   $\Delta\text{shp3}$ <sub>GGG</sub> reporter strains (Fig. 1B). Expression profiles determined using a  $P_{shp3}$  reporter were comparable to those seen with the  $P_{shp2}$  reporter in the various strains (data not shown). Overall, these data suggest that SHP3, but not SHP2, is required for system activation in the absence of *rgg3* under these growth conditions. They also indicate that although *shp2* alone is not sufficient for system activation, SHP2 does contribute to system induction, as indicated by the timing difference between activation in the  $\Delta\text{rgg3}$  and activation in the  $\Delta\text{rgg3}$  *shp2*<sub>GGG</sub> reporter strains (Fig. 1B).

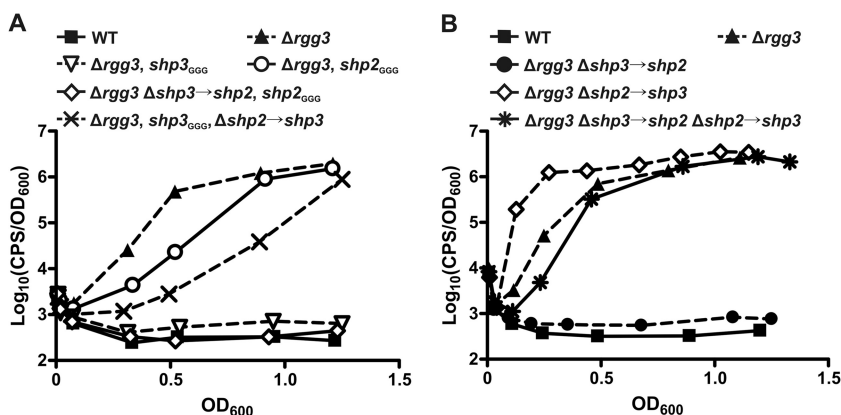
As can be noted in the replicate panels in Fig. 1B, we found that although the luminescence trend of each strain was consistent, the exact timing of the reporter induction varied slightly throughout our experiments, especially in *shp* mutant strains. We have found that the timing of full induction correlates, at least in part, with the age of the CDM, such that the older the CDM, the later the lumi-

nescence plateau is reached in strains that autoinduce successfully (data not shown). Unfortunately, despite efforts to optimize medium preparation and culture conditions to maximize reproducibility in our experiments, there remained variability in the timing of induction, although we were able to achieve values correlating to relative light production consistently plateauing between OD<sub>600</sub> of 0.5 and 1.0 in the  $\Delta\text{rgg3}$  mutant. Therefore, in order to make conclusions regarding system induction differences in the various strains, we included both WT and  $\Delta\text{rgg3}$  reporter strains as controls in all experiments to provide reference curves against which to compare the mutant expression patterns. As such, all conclusions regarding the timing of system induction should be regarded as qualitative rather than quantitative.

**sSHP2-C8 and sSHP3-C8 can independently induce the Rgg2/3 circuit with comparable specific activities.** The lack of autoinduction in the  $\Delta\text{rgg3}$  *shp3*<sub>GGG</sub> strain but presence of autoinduction in the  $\Delta\text{rgg3}$  *shp2*<sub>GGG</sub> strain (Fig. 1B) indicated that the two pheromones possess distinct abilities to activate the Rgg2/3 system, with SHP3 being required for system induction but not SHP2. This appeared to conflict with previous studies in which both synthetic SHP2 and SHP3 C8 peptides (sSHP2-C8 and sSHP3-C8) were able to activate the Rgg2/Rgg3 QS system in a wild-type (WT) background (6). However, in the WT background, endogenous production of both SHPs resulting from positive autoregulation of the *shp* genes in response to synthetic SHPs precluded the determination of independent SHP activities. Thus, we sought to examine if synthetic SHPs could independently support system activation using transcriptional reporter strains in which the translation initiation codons of *shp2* and *shp3* were mutated from ATG to GGG (*shp2*<sub>GGG</sub>, *shp3*<sub>GGG</sub>; BNL177 and BNL206). We reasoned that if SHP3, but not SHP2, were indeed required for activation of the system, addition of sSHP3 to a *shp*-null strain should result in system activation whereas addition of sSHP2 should not.

It was previously demonstrated that the C-terminal 8 amino acids of the SHP peptides comprise the active signaling molecules. Addition of either sSHP2-C8 or sSHP3-C8 to the *shp*-null strain carrying the  $P_{shp2}$  reporter (BNL177) at a concentration of 5 nM resulted in robust luminescence expression, whereas a control peptide with the reverse sequence of SHP2-C8 (sSHP2-rev-C8) had no inducing activity (Fig. 2A). These data demonstrate that the active portions of both SHP2 and SHP3 can in fact activate the Rgg2/3 system independently and that SHP3 is not required for system induction when SHP2-C8 is provided exogenously. Of note, when the sSHP-induced luminescence profiles of the *shp2*<sub>GGG</sub> *shp3*<sub>GGG</sub> mutant were compared to those of a WT strain carrying the same reporter (BNL148), it was evident that an intact positive-feedback loop contributed to overall system activation in the WT strain. Following addition of synthetic peptide, luminescence was highly induced in both strains but declined over time in the double *shp* mutant whereas it remained high throughout growth in the WT reporter assay (Fig. 2A). These results confirm the importance of endogenous peptide production for sustained induction following initial system activation. Comparable luminescence expression patterns were observed in WT and *shp2*<sub>GGG</sub> *shp3*<sub>GGG</sub> strains carrying a comparably constructed  $P_{shp3}$  transcriptional reporter (JCC181 and BNL206, respectively), indicating that peptide-mediated system activation is not promoter specific in nature under these conditions (data not shown).

A possible reason that the *shp* genes have disparate abilities to



**FIG 3** The enhanced system induction potential of *shp3* compared to *shp2* is not a consequence of differential promoter strengths. (A) Luciferase expression from a  $P_{shp2}$  reporter integrated into WT (BNL148),  $\Delta rgg3$  (BNL149),  $\Delta rgg3$  *shp3*<sub>GGG</sub> (BNL203),  $\Delta rgg3$  *shp2*<sub>GGG</sub> (BNL176),  $\Delta rgg3$   $\Delta shp3 \rightarrow shp2$  *shp2*<sub>GGG</sub> (BNL220), and  $\Delta rgg3$   $\Delta shp2 \rightarrow shp3$  *shp3*<sub>GGG</sub> (BNL216) strains. (B) Luciferase expression from  $P_{shp2}$  reporter integrated into WT (BNL148),  $\Delta rgg3$  (BNL149),  $\Delta rgg3$   $\Delta shp3 \rightarrow shp2$  (BNL202),  $\Delta rgg3$   $\Delta shp2 \rightarrow shp3$  (BNL192), and  $\Delta rgg3$   $\Delta shp3 \rightarrow shp2$   $\Delta shp2 \rightarrow shp3$  (BNL222) strains. Expression trends are representative of at least three independent experiments.

independently support autoinduction but are equivalently able to induce the system when added exogenously is that the synthetic peptide concentrations being used were greater than those natively produced by the bacteria. This could potentially mask differential peptide activities detectable only at lower peptide concentrations. Thus, we performed synthetic peptide titrations using both full-length and C8 peptides to determine if the specific induction activities of SHP2 and SHP3 were indeed different. We performed these studies using both the  $P_{shp2}$  and  $P_{shp3}$  reporters in the *shp2*<sub>GGG</sub> *shp3*<sub>GGG</sub> background (BNL177 and BNL206, respectively) to simultaneously determine if there were peptide-specific and/or promoter-specific differences in induction potential at the lower peptide concentrations. In agreement with our earlier synthetic peptide studies, there were no statistical differences between the effective peptide concentrations of sSHP2-C8 and sSHP3-C8 that elicited half-maximal relative luminescence induction (50% effective concentrations [EC<sub>50</sub>s]) using either reporter (Fig. 2B and C, top panels). Likewise, full-length sSHP2 and sSHP3 also had comparable EC<sub>50</sub>s with both reporters (Fig. 2B and C, bottom panels). These results further confirm that SHP2 and SHP3 are functionally redundant for system activation but fail to explain why the *shp2* gene alone is incapable of supporting autoinduction in the  $\Delta rgg3$  *shp3*<sub>GGG</sub> mutant.

**Differential induction potentials of the *shp* genes are not due to differences in promoter strength.** During the completion of the experiments described above, we noticed that basal and maximal luminescence levels from the  $P_{shp3}$  reporter were slightly but reproducibly higher than those seen with the  $P_{shp2}$  reporter. This led us to investigate if differences in promoter strength could account for the differences in induction potential of the *shp2* and *shp3* genes. If the *shp3* promoter was indeed stronger than the *shp2* promoter, it could be that more SHP3 than SHP2 is produced by cells, resulting in nonequivalent abilities of the two genes to support autoinduction. To test this, we generated additional  $\Delta rgg3$  strains in which one *shp* gene harbors the GGG start codon mutation and the second *shp* gene is replaced in the chromosome with the open reading frame of the first *shp* gene ( $\Delta rgg3$ , *shp3*<sub>GGG</sub>,  $\Delta shp2 \rightarrow shp3$ , BNL208;  $\Delta rgg3$ , *shp2*<sub>GGG</sub>,  $\Delta shp3 \rightarrow shp2$ , BNL210). These strains still only have one functional *shp* gene, but it is ex-

pressed from the promoter of the other *shp* gene. We then compared  $P_{shp2}$  reporter expression in these new strains to that in the parent  $\Delta rgg3$  strain and in strains with a single *shp* gene under the control of its native promoter. When we did this, we found that a single copy of *shp2* was unable to support any detectable autoinduction regardless of whether the gene was expressed from its own promoter ( $\Delta rgg3$ , *shp3*<sub>GGG</sub>, BNL203) or the *shp3* promoter ( $\Delta rgg3$ , *shp2*<sub>GGG</sub>,  $\Delta shp3 \rightarrow shp2$ , BNL220) (Fig. 3A). In contrast, a single *shp3* gene successfully induced luminescence when expressed from either the native *shp3* promoter ( $\Delta rgg3$ , *shp2*<sub>GGG</sub>, BNL176) or from the *shp2* promoter ( $\Delta rgg3$ , *shp3*<sub>GGG</sub>,  $\Delta shp2 \rightarrow shp3$ , BNL216), both being delayed for induction compared to the  $\Delta rgg3$  strain (Fig. 3A). It should be noted that *shp3* was better able to induce autoinduction when expressed from its native promoter, which again indicates some low-level differences in promoter strength, in agreement with the basal luminescence levels detected from our reporters. However, whatever this difference may be does not account for the differential induction capacities of *shp2* and *shp3*, given that *shp3* is sufficient to support detectable autoinduction whereas *shp2* is not, regardless of the promoter driving its expression.

To further confirm that promoter strength disparities could not fully account for the disparate induction potentials of the *shp* genes, we generated additional strains in the  $\Delta rgg3$  background in which we replaced one *shp* gene with a second copy of the other *shp* gene ( $\Delta rgg3$ ,  $\Delta shp2 \rightarrow shp3$ , BNL187;  $\Delta rgg3$ ,  $\Delta shp3 \rightarrow shp2$ , BNL198). We also generated a strain in which the *shp* genes are swapped ( $\Delta rgg3$ ,  $\Delta shp3 \rightarrow shp2$ ,  $\Delta shp2 \rightarrow shp3$ , BNL212) such that there was still one copy of each *shp* gene but they were each under the control of the opposite gene's promoter. We then compared reporter expression in these *shp*-swap strains to that in the  $\Delta rgg3$  parent strain. When *shp2* was replaced by *shp3*, resulting in a strain carrying two copies of *shp3* ( $\Delta rgg3$ ,  $\Delta shp2 \rightarrow shp3$ , BNL192), the  $P_{shp2}$  reporter was induced earlier than in the parent  $\Delta rgg3$  strain (BNL149) (Fig. 3B). Surprisingly, a strain carrying two copies of *shp2* was unable to support autoinduction ( $\Delta rgg3$ ,  $\Delta shp3 \rightarrow shp2$ , BNL202), further pointing to an inherent difference in the induction potentials of endogenous SHP2 and SHP3. Notably, the strain in which both *shp* genes were swapped ( $\Delta rgg3$ ,

$\Delta shp3 \rightarrow shp2$ ,  $\Delta shp2 \rightarrow shp3$ , BNL222) had a luminescence profile similar to that of the parent  $\Delta rgg3$  strain (Fig. 3B). These data confirm that promoter strength does not account for the differential abilities of *shp2* and *shp3* to support autoinduction: *shp2* cannot support autoinduction even when present in two copies and expressed from the *shp3* promoter whereas *shp3* is better able to induce the system than *shp2* even when expressed from the *shp2* promoter. These data also confirm that *shp* gene dosage contributes to system induction, at least for *shp3*, as two copies of *shp3* resulted in premature activation of the reporter (Fig. 3B) whereas one copy of the gene resulted in delayed activation compared to the  $\Delta rgg3$  strain results (Fig. 3A).

**Differential induction potentials of the *shp* genes reside in the N termini of the peptides.** The disparate ability of *shp3* but not *shp2* to individually support system activation in the  $\Delta rgg3$  background (Fig. 1B and 3A), and the premature reporter induction seen when *shp2* was replaced by *shp3* in the  $\Delta rgg3$  strain versus the lack of reporter induction in a strain carrying only two copies of *shp2* (Fig. 3B), conflicted with the ability of both sSHP2 and sSHP3 to induce system activation when added exogenously (Fig. 2) and led us to question if the SHP pro peptides possess distinct activities apart from the activities of the active portion of the peptides. As mentioned previously, the C-terminal active portions of SHP2 and SHP3 are highly similar whereas the N-terminal regions are much more disparate (Fig. 1A), and thus we postulated that the N-terminal regions of these peptides may have been contributing to the differences we were observing.

To examine this possibility, we generated strains in which only the C8 region of the *shp* gene was swapped for that of the other *shp* gene. Since the C8 peptides differ by only a single residue (leucine and isoleucine), this was achieved by site-directed mutagenesis of a single codon within each *shp* gene. The codon encoding leucine at position 17 of SHP2 was changed to encode isoleucine (*shp2<sub>leu17ile</sub>*), and the codon encoding isoleucine at position 18 of the SHP3 peptide was altered to encode leucine (*shp3<sub>ile18leu</sub>*). A strain carrying the *shp2<sub>leu17ile</sub>* allele will in effect produce WT SHP3 peptide from the *shp3* locus and a SHP2<sub>Nterm</sub>-SHP3<sub>C8</sub> hybrid peptide from the *shp2* locus. Similarly, a strain with the *shp3<sub>ile18leu</sub>* allele will produce WT SHP2 from the *shp2* locus and a SHP3<sub>Nterm</sub>-SHP2<sub>C8</sub> hybrid peptide from the *shp3* locus. We again engineered these mutations in the  $\Delta rgg3$  background (BNL186 and BNL225) and then introduced the  $P_{shp2}$  reporter and assessed the timing of system autoinduction compared to that seen with other reporter strains.

Unlike the strain carrying two copies of *shp3* ( $\Delta rgg3 \Delta shp2 \rightarrow shp3$ , BNL192), swapping of only the C-terminal isoleucine of SHP3 ( $\Delta rgg3, shp2_{leu17ile}$ , BNL196) did not result in early reporter induction (Fig. 4). It is evident that the hybrid SHP2<sub>Nterm</sub>-SHP3<sub>C8</sub> peptide is produced, however, given that the timing of system induction in the  $\Delta rgg3 shp2_{leu17ile}$  strain mirrors that of the  $\Delta rgg3$  strain (Fig. 4) and is not delayed as in the  $\Delta rgg3 shp2_{GGG}$  strain (Fig. 1B). Likewise, replacement of the C-terminal isoleucine of SHP3 with leucine ( $\Delta rgg3, shp3_{ile18leu}$ , BNL227) failed to abolish reporter induction, unlike the case when the entire *shp3* gene was replaced with *shp2* ( $\Delta rgg3 \Delta shp3 \rightarrow shp2$ , BNL202) (Fig. 4). The cause of the slight delay in the  $\Delta rgg3 shp3_{ile18leu}$  strain compared to the  $\Delta rgg3$  parent strain remains unclear. Overall, these data indicate that the N-terminal portions of the SHPs are responsible for the differential induction activities of *shp2* and *shp3*, as swapping of only the active C-terminal region

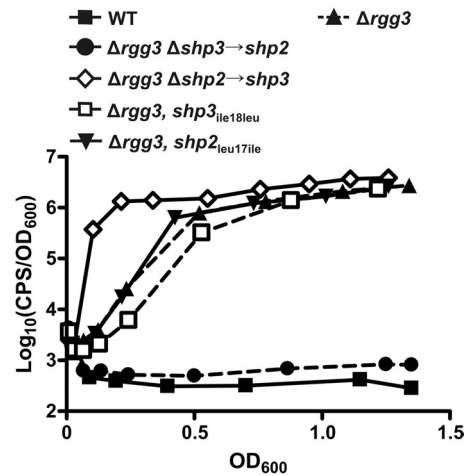


FIG 4 The N-terminal regions of the peptides confer the differential system induction potentials of *shp2* and *shp3*. Data represent luciferase expression from a  $P_{shp2}$  reporter integrated into WT (BNL148),  $\Delta rgg3$  (BNL149),  $\Delta rgg3 \Delta shp3 \rightarrow shp2$  (BNL202),  $\Delta rgg3 \Delta shp2 \rightarrow shp3$  (BNL192),  $\Delta rgg3 shp3_{ile18leu}$  (BNL227), and  $\Delta rgg3 shp2_{leu17ile}$  (BNL196) strains. Expression trends are representative of at least three independent experiments.

does not reproduce the luminescence profiles seen when the entire *shp* gene is swapped. These results are the first evidence that the N-terminal, nonactive portions of the endogenously produced SHP peptides factor into their native signaling potentials.

## DISCUSSION

It has become evident that peptide-mediated QS systems have diverse circuit architectures, with most systems utilizing only a single peptide pheromone but others requiring multiple signals that interact with a single or multiple receptor proteins. Clear examples of the latter are PrgX, which interacts with peptides cCF10 and iCF10 (13–15), and the more recently discovered Rgg2 and Rgg3 regulators that each interact with both SHP2 and SHP3 (6, 10). The previous evidence of redundancy in SHP stimulatory activities (6, 10) and the conservation of both *shp* genes in all sequenced GAS genomes led us to investigate the roles of SHP peptide specificity and signal dosage in the Rgg2/3 QS system. Our genetic studies have led to three general conclusions. The mature SHP2 and SHP3 peptides are redundant in their ability to activate the Rgg2/3 system (Fig. 2). Separately, *shp* gene dosage contributes to the timing of Rgg2/3 system induction, with a reduced gene copy number resulting in delayed system induction (Fig. 1B and 3). Lastly, and perhaps most intriguingly, full-length endogenous SHP2 and SHP3 possess distinct potentials for supporting system activation independently of their highly similar C-terminal active regions (Fig. 4). Overall, these data support our hypothesis that both SHP identity and signal dosage contribute to activation of the Rgg2/3 system.

The data presented herein represent the first evidence that the noneffector, N-terminal regions of SHP2 and SHP3 influence their overall system activation potential in GAS. As mentioned above, the N-terminal regions of SHP2 and SHP3 are much more divergent than the nearly identical C-terminal region which contains the active signaling portion of the molecule (Fig. 1A). Although not directly involved in activation of their cognate receptors, the N-terminal regions of signaling peptides could be

imagined to indirectly affect signaling potential at several steps of the circuit, namely, SHP maturation and/or export, and we hypothesize that the differential activation potentials of *shp2* and *shp3* indeed result from one or both of these steps. The possibility cannot be ruled out, however, that the production differences between the SHPs occur at the mRNA level rather than the peptide level, perhaps due to disparate translation efficiencies of the N termini or differential interactions of small RNAs with the *shp* mRNAs. Additional studies will be needed to address these possibilities.

Very little is known regarding processing of SHP peptides. Evidence from our laboratory supports the idea of a prominent role for the membrane-associated metalloprotease Eep in the Rgg2/3 system; however, Eep was not essential for activation of the system when SHP3 was overexpressed, indicating that additional enzymes can participate in processing of at least SHP3 under certain conditions (6). Redundancy in peptide processing may be a conserved trend in intracellular peptide signaling systems, as is the case in *B. subtilis*, where three proteases (subtilisin, Epr, and Vpr) have been implicated in the maturation of Phr peptides (25). In *E. faecalis*, Eep has been demonstrated to function in the maturation of both cCF10 and iCF10 from their precursor molecules (26, 27), but cCF10 production is additionally believed to involve signal II peptidase and a yet-to-be-identified carboxy peptidase which are required to fully separate the mature signaling peptide from its lipoprotein precursor (27). Given that Eep recognizes determinants in *E. faecalis* in the N-terminal, nonsignal portion of signal precursor molecules (27), it is plausible that the disparate N termini of SHP2 and SHP3 are differentially recognized by Eep, resulting in superior processing of SHP3 compared to SHP2. However, the possibility cannot be excluded that native processing of SHP2 and that of SHP3 are performed by different and/or multiple proteolytic enzymes. Genetic and biochemical experiments are under way to examine native SHP processing, assess potential SHP substrate specificity corresponding to Eep, and identify the potential secondary pathway that facilitates processing of SHP3 when overexpressed in the absence of Eep.

Identification of the mechanism(s) by which cognate peptides of Rgg regulators are exported from the cell also remains elusive. The amino acid sequences of SHP2 and SHP3 (Fig. 1A) resemble only vaguely the general signal peptides used by Gram-positive bacteria to target proteins for export via Sec (28), and online prediction algorithms designed to identify Sec-targeting sequences disagree as to whether SHP2 and/or SHP3 are Sec substrates, depending on the program being used (data not shown). Regardless of the identity of the export system, be it Sec or a designated ABC transporter, it is possible that the N-terminal regions of the SHP peptides result in unequal degrees of recognition by the systems, thereby resulting in preferential export of one peptide, presumably SHP3, over the other. It will be interesting to determine how SHP peptides are exported from the cell, the sequence determinants that facilitate recognition by the export system, and if export is coupled to peptide maturation or if these are temporally and/or spatially distinct steps in the circuit.

With regard to the functional redundancy of SHP2 and SHP3 in system activation, it should be noted that the EC<sub>50</sub>s for the full-length synthetic peptides were 50 to 140 times less than those for their C8 counterparts when tested for induction activity in a *shp*-null background. Additionally, the maximum induction by C8 peptides was always higher than that for the full-length pep-

tides (Fig. 2B and C). Given that GAS secretes numerous proteases (29), it is possible that the activation by exogenously added, full-length synthetic SHPs results from some degree of nonspecific but productive peptide cleavage by proteases not normally involved in SHP peptide maturation. Such nonspecific cleavage would likely also result in the nonproductive degradation of a percentage of the added sSHPs, thereby reducing the effective peptide concentration available for system induction and resulting in higher effective EC<sub>50</sub>s. The C8 peptides, which presumably do not require any additional processing steps to be active, may be less-optimal substrates for these proteases or may be imported into the cell more efficiently, resulting in overall lower EC<sub>50</sub>s. Hopefully, further elucidation of SHP maturation will help address such uncertainties.

On a final note, we find it interesting that GAS has evolved to utilize two Rgg-SHP pairs in a single circuit when other systems appear to be fully functional with just one. It is appealing to speculate that having two peptide-encoding genes with disparate activation potentials confers some type of advantage for the bacteria, especially given that both pairs are conserved among all sequenced GAS strains. One possibility is that it is energetically costly to have the system in an “on” state, and requiring two peptides to be sufficiently produced prior to robust induction may help prevent premature system activation. Another possibility is that having multiple peptides driven by separate promoters could allow more regulatory inputs into the system than if just one *shp* gene were involved. Although the conserved Rgg binding site is highly conserved in the two *shp* promoters, the remainder of the intergenic regions between each *shp* and *rgg* gene is highly divergent (10) and could support differential regulation by other regulatory proteins. There is evidence supporting this idea, at least in part, as the metal-dependent regulator MtsR is known to regulate the *shp3* promoter but is not known to have any role in *shp2* regulation (30). Taking this idea farther, if regulatory inputs are indeed distinct at the two *shp* promoters, it may be that *shp2* is in fact sufficient to support autoinduction under specific, yet-to-be determined conditions despite not having sufficient activity under the growth conditions used for these studies. Differential regulation of processing enzymes and/or export machinery in response to cellular and/or environmental stimuli may also be a factor in determining if and when the Rgg2/3 system is activated, further expanding the levels at which other signals could influence this system.

## ACKNOWLEDGMENTS

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