SUPPLEMENTAL INFORMATION

The proteomic and transcriptomic landscapes altered by Rgg2/3 activity in *Streptococcus pyogenes.*

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Running Head: Rgg2/3 disruption alters *S. pyogenes* expression landscape.

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Contents

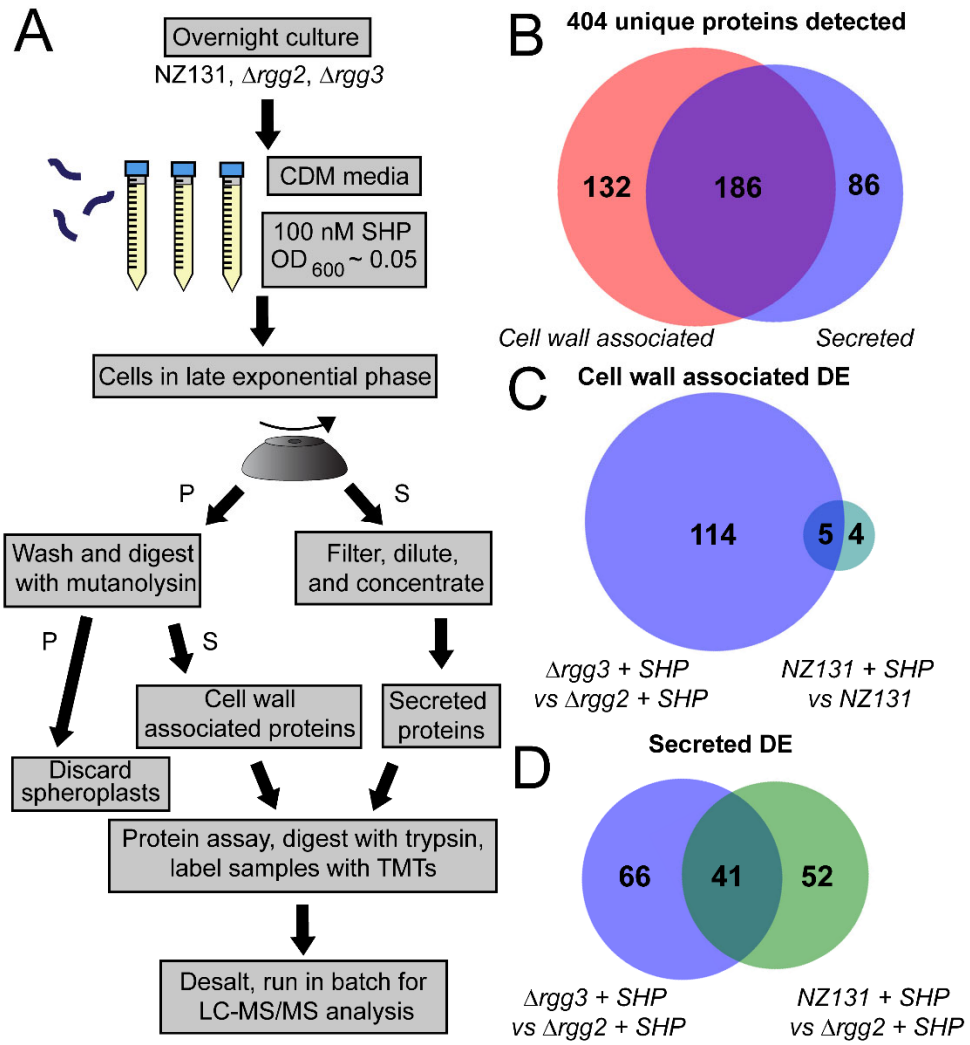

FIGURE S1: Proteomics workflow and summary of results. For a complete list of differentially expressed proteins, TMT-LC-MS/MS results, and comparison to Wilk *et. al,* 2018 proteomics data set see Data Sets S2-S3. A) Overnight cultures were diluted 1:100 into CDM, grown statically at 37°C until OD_{600} ~0.05 at which time 100 nM SHP was added to appropriate cultures. Cells were harvested by centrifugation in late exponential phase (OD_{600} ~0.8-1.0) and split into pellet (P) and supernatant (S) fractions. To obtain the "cell wall associated proteins" cell pellets were washed with cold buffers and digested with mutanolysin 18 hrs at 37°C. The "secreted proteins" were obtained from culture supernatants that were filtered, diluted with PBS, and concentrated several times. All

samples were digested with trypsin, labeled with tandem mass tags, and analyzed in batch via LC-MS/MS. For further details see *Materials and Methods*. B) Number of unique proteins detected via TMT-LC-MS/MS. The pink circle indicates number of proteins detected in the cell wall associated fraction, whereas the blue circle indicates the number of proteins detected in the supernatant fraction. The area of overlap indicates the number of unique proteins detected in both samples. C) Number of differentially expressed cell wall associated proteins between Δ*rgg3* and Δ*rgg2* cultures stimulated with 100 nM SHP (blue) and wild-type NZ131 + SHP vs NZ131 (green). The overlap indicates the number of proteins that were differentially expressed in both data sets. D) Number of differentially expressed secreted proteins between Δ*rgg3* and Δ*rgg2* cultures stimulated with 100 nM SHP (blue) and wild-type NZ131 + SHP vs Δ*rgg2* + SHP (green).

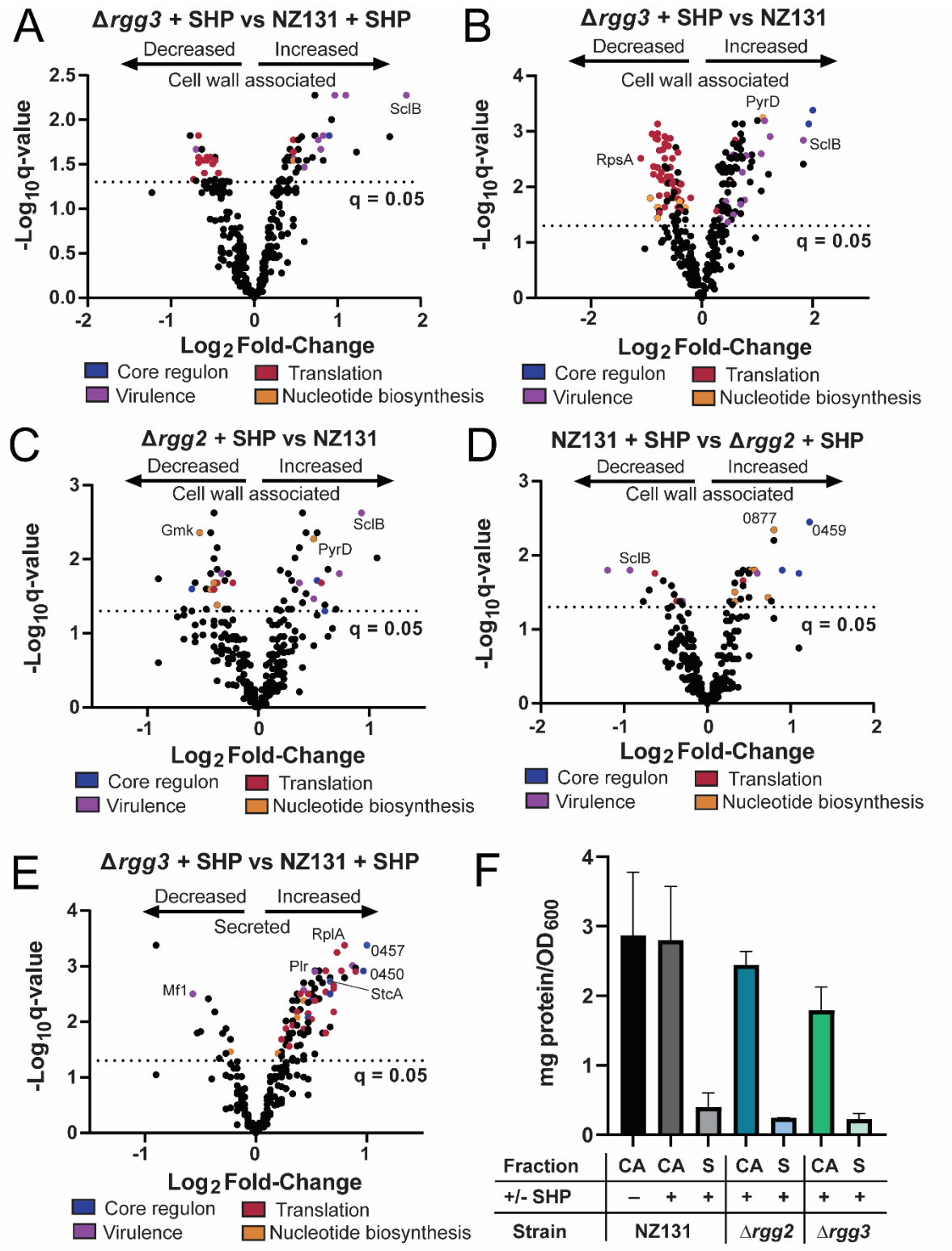

FIGURE S2: Data from proteomics analysis. A-E: Additional volcano plots of proteomics results. The legends below the volcano plots indicate processes or operons in which

proteins are involved in. Blue, core Rgg2/3 regulon; purple, virulence; red, translation; orange, nucleotide biosynthesis. Proteins of particular interest are also indicated on each volcano plot. For a complete list of differentially expressed proteins, TMT-LC-MS/MS results, and comparison to Wilk *et. al,* 2018 data set, see Data Sets S2-S3. A) Differentially expressed cell wall associated proteins in Δr*gg3* + SHP vs wild-type NZ131 + SHP. B) Differentially expressed cell wall associated proteins in Δ*rgg3* + SHP vs wild-type NZ131. C) Differentially expressed cell wall associated proteins in Δ*rgg2* + SHP vs wild-type NZ131. D) Differentially expressed cell wall associated proteins in wild-type NZ131 + SHP vs Δ*rgg2* + SHP. E) Differentially expressed secreted proteins in Δ*rgg3* + SHP vs wild-type NZ131 + SHP. F) Graph of total mg of protein per OD_{600} harvested for each proteomics sample. Samples were isolated in biological triplicate. CA indicates cell wall associated, S indicates secreted. +/- indicates if 100 nM SHP was added to samples. No significant differences in protein harvest were observed between strains for either the cell associated or secreted fractions by One-way ANOVA with Tukey's Multiple Comparisons Post-Test.

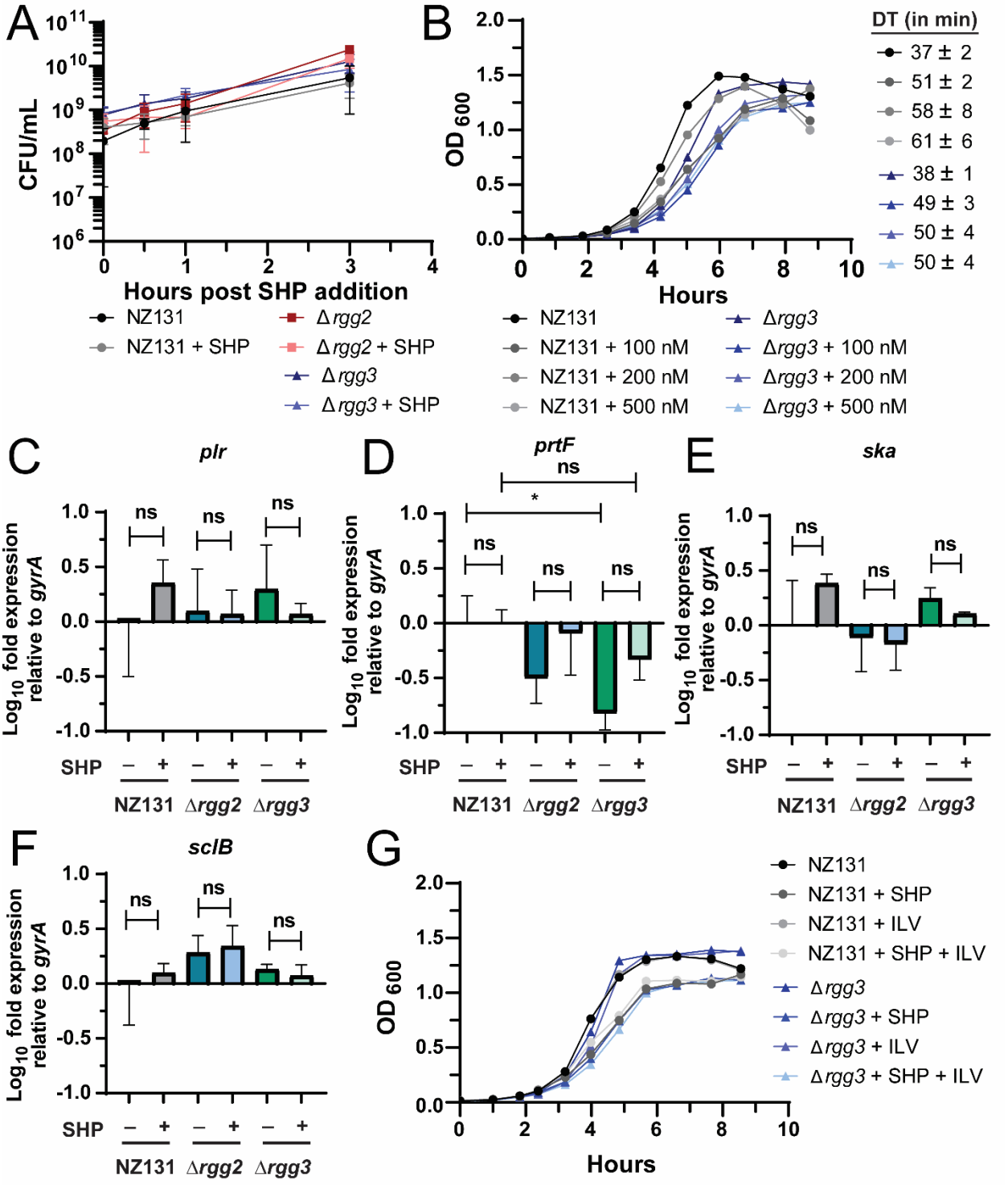

FIGURE S3: Results examining effects of Rgg2/3 QS induction on cells and qRT-PCR results. A) CFU/mL over time of wild-type NZ131, Δ*rgg2*, and Δ*rgg3* grown with or without 100 nM SHP peptide. Strains and conditions are indicated in the legend below the graph.

Graph represents the sum of three independent experiments. B) Growth curve of wild-type NZ131 and Δ*rgg3* grown with increasing amounts of SHP peptide (100 nM, 200 nM, 500 nM). Strains and conditions are indicated in the legend below the graph. Mean doubling times plus/minus S.E.M. in minutes for each strain and condition are listed beside the graph. Graph is representative of four independent experiments. C-F) qRT-PCR results verifying RNA-seq and examining if proteomics targets have altered transcript levels. Wild-type NZ131, Δ*rgg2*, and Δ*rgg3* strains were grown in biological triplicate with or without 100 nM SHP peptide (indicated by + or – sign below each graph) and RNA was harvested at late exponential phase (OD_{600} ~0.8-1.0). RNA was processed for qRT-PCR and transcript levels were determine relative to the *gyrA* reference gene. Significance of transcript level changes were determined using a One-way ANOVA with Tukey's Multiple Comparisons Post-test. *, $p < 0.05$; ns, non-significant. For further experimental details, see *Materials and Methods*. C) Relative transcript levels of *plr* (*spy49_0234*). D) Relative transcript levels of *prtF* (*spy49_0119*). E) Relative transcript levels of *ska* (*spy49_1630*). F) Relative transcript levels of *sclB* (*spy49_0830*). G) Examination of the supplementation of additional branched-chain amino acids. Growth curve of wild-type NZ131 and Δ*rgg3* grown with or without 100 nM SHP and/or ~380 μM L-Ile, L-Leu, and L-Val. Strains and conditions are indicated in the legend below the graph. Mean doubling times plus/minus S.E.M. in minutes for each strain and condition are listed beside the graph. Graph is representative of two independent experiments. For further experimental details, see *Materials and Methods*.

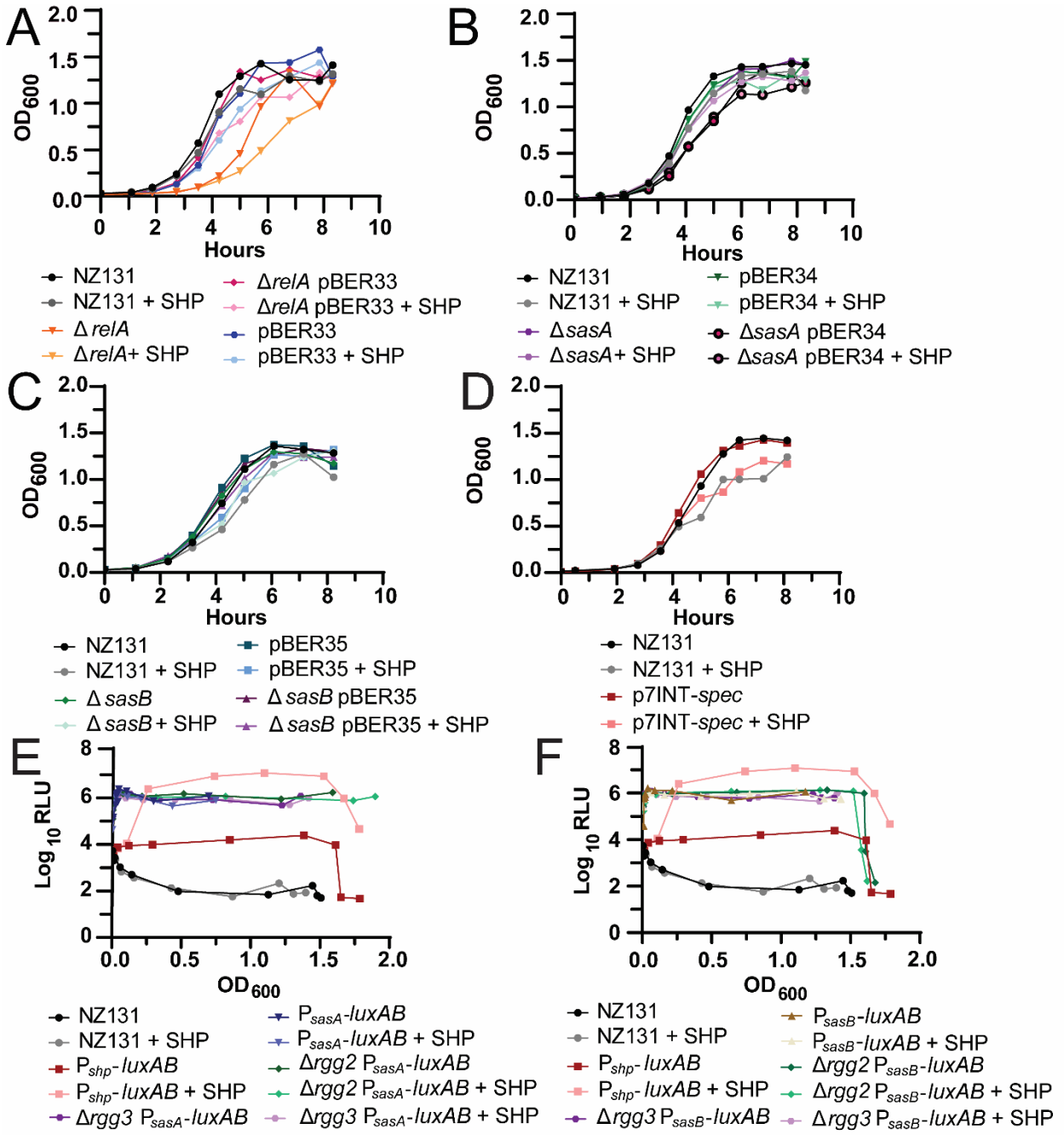

FIGURE S4: Growth curves of complementation and multi-copy strains for putative stringent response enzymes, and examination of transcriptional response to the Rgg2/3 system. All experiments were performed a minimum of three times. A) Growth curve of wild-type NZ131, Δ*relA*, complementation, and multi-copy strains in the presence or absence of 100 nM SHP. Strains and conditions are indicated in the legend below the

graph. pBER33 indicates the *relA* complementation plasmid (p7INT-*spec*-*relA*). B) Growth curve of wild-type NZ131, Δ*sasA*, complementation, and multi-copy strains in the presence or absence of 100 nM SHP. Strains and conditions are indicated in the legend below the graph. pBER34 indicates the *sasA* complementation plasmid (p7INT-*spec*-*sasA*). C) Growth curve of wild-type NZ131, Δ*sasB*, complementation, and multi-copy strains in the presence or absence of 100 nM SHP. Strains and conditions are indicated in the legend below the graph. pBER35 indicates the *sasB* complementation plasmid (p7INT-*spec*-*sasB*). D) Growth curve of wild-type NZ131 and p7INT-*spec* empty vector. Strains were grown with or without 100 nM SHP peptide. Strains and conditions are indicated in the legend below the graph. E) Luciferase assay examining the response of *sasA* promoter to SHP induction or disruption of the Rgg2/3 system. Wild-type NZ131, Δ*rgg2*, or Δ*rgg3* strains containing *luxAB* reporters to P_{shp} or P_{sasA} were grown with or without 100 nM SHP peptide. Strains and conditions are indicated in the legend below the graph. F) Luciferase assay examining the response of the *sasB* promoter to SHP induction or disruption of the Rgg2/3 system. Wild-type NZ131, Δ*rgg2*, or Δ*rgg3* strains containing *luxAB* reporters to P_{shp} or P_{sasB} were grown with or without 100 nM SHP peptide. Strains and conditions are indicated in the legend below the graph.

TABLE S1. Bacterial strains, plasmids, and primers used in this study.[a]

***S. pyogenes* strains**			
Strain/Plasmid	**Description**	**Antibiotic Resistance[b]**	**Reference**
NZ131	*S. pyogenes* wild-type reference strain; M49 isolate isolated from a case of acute post-streptococcal glomerulonephritis	NR	(1)
BNL145	NZ131 Δ*rgg2* Δ*rgg3*::*cat*	Cm^R	(1)
BNL206	NZ131 $shp2_{GGG}$ $shp3_{GGG}$ pJC219	Cm^R, Erm^R	(2)
BRSP13	NZ131 Δ*relA*	NR	This study
BRSP15	NZ131 Δ*sasB*	NR	This study
BRSP20	NZ131 Δ*sasA*	NR	This study
BRSP25	NZ131 Δ*relA* pJC219	Erm^R	This study
BRSP27	NZ131 Δ*sasA* pJC219	Erm^R	This study
BRSP29	NZ131 Δ*sasB* pJC219	Erm^R	This study
BRSP39	NZ131 Δ*relA* Δ*sasA*	NR	This study
BRSP42	NZ131 Δ*relA* Δ*sasB*	NR	This study
BRSP44	NZ131 pBER33	$Spec^R$	This study
BRSP48	NZ131 Δ*relA* pBER33	$Spec^R$	This study
BRSP50	NZ131 pBER34	$Spec^R$	This study
BRSP52	NZ131 pBER35	$Spec^R$	This study
BRSP55	NZ131 Δ*sasB* pBER35	$Spec^R$	This study
BRSP57	NZ131 Δ*sasA* Δ*sasB*	NR	This study
BRSP58	NZ131 Δ*sasA* pBER34	$Spec^R$	This study
BRSP60	NZ131 pBER37	Erm^R	This study
BRSP63	NZ131 pBER38	Erm^R	This study
BRSP66	NZ131 Δ*relA* Δ*sasA* Δ*sasB*	NR	This study
BRSP67	NZ131 p7INT-*spec*	$Spec^R$	This study
BRSP69	NZ131 Δ*rgg2* pBER37	Erm^R	This study
BRSP72	NZ131 Δ*rgg2* pBER38	Erm^R	This study
BRSP79	NZ131 Δ*rgg3*::*cat* pBER37	Cm^R; Erm^R	This study
BRSP82	NZ131 Δ*rgg3*::*cat* pBER38	Cm^R; Erm^R	This study
BRSP92	NZ131 pBER40	$Spec^R$	This study
BRSP94	NZ131 pBER41	$Spec^R$	This study
BRSP100	NZ131 pLZ12-Sp	$Spec^R$	This study
BRSP102	NZ131 Δ*rgg2* pBER41	$Spec^R$	This study
BRSP105	NZ131 Δ*rgg3*::*cat* pBER41	$Spec^R$	This study
BRSP107	NZ131 Δ*rgg2* pBER40	$Spec^R$	This study
BRSP110	NZ131 Δ*rgg3*::*cat* pBER40	$Spec^R$	This study
BRSP116	HSC5 pFED630	Cm^R	This study
HSC5	*S. pyogenes* wild-type reference strain; M14 isolate	NR	(3, 4)
JCC131	NZ131 Δ*rgg3*::*cat*	Cm^R	(1)
JCC131 pJC219	NZ131 Δ*rgg3*::*cat* pJC219	Cm^R; Erm^R	(4)
JCC137	NZ131 Δ*rgg2*	NR	(1)
JCC137 pJC219	NZ131 Δ*rgg2* pJC219	Erm^R	(4)
JCC181	NZ131 pJC219	Erm^R	(2)
***E. coli* strains**			
DH5α	*Escherchia coli* strain for cloning	NR	NEB

TX2737	*E. coli* K12 containing pALS13 (P_{tac}-relA'$_{1-455}$ lacI^{q}); IPTG inducible constitutive (p)ppGpp synthetase allele *relA*'			Amp^{R}		Gift from M.E. Winkler; (5, 6)
Plasmids						
Plasmid Name	**Description**	**Template[c]**	**Method of Const.[d]**	**RE Used[e]**	**Antibiotic Resistance[b]**	**Reference**
p7INT	Shuttle-suicide vector that integrates at streptococcal bacteriophage T12 *attB* site	N/A	N/A	N/A	Erm^{R}	(7)
p7INT-*spec*	Shuttle-suicide vector that integrates at streptococcal bacteriophage T12 *attB* site; Erm cassette replaced with Spec cassette from pLZ12-Sp	pLZ12-Sp; p7INT; primers	RE	NcoI	Spec^{R}	This study
pALS13	P_{tac}-relA'$_{1-455}$ lacI^{q}; IPTG inducible constitutive (p)ppGpp synthetase allele *relA*'	N/A	N/A	N/A	Amp^{R}	Gift from M.E. Winkler; (5)
pBER16	To construct unmarked deletion of *relA;* in pFED760	pFED760; NZ131 gDNA; primers	Gibson + RE	NotI; EcoRI	Erm^{R}	This study
pBER17	To construct unmarked deletion of *sasB*; in pFED760	pFED760; NZ131 gDNA; primers	Gibson + RE	NotI; EcoRI	Erm^{R}	This study
pBER18	To construct unmarked deletion of *sasA*; in pFED760	pFED760; NZ131 gDNA; primers	Gibson + RE	NotI; EcoRI	Erm^{R}	This study
pBER23	p7INT-*spec-L-sfGFP*	p7INT-*spec*; pY71-sfGFP; primers	Gibson	N/A	Spec^{R}	This study
pBER25	p7INT-*spec-aroE2-L-sfGFP*	pBER23; NZ131 gDNA; primers	Gibson	N/A	Spec^{R}	This study
pBER30	pLZ12-Sp-*aroE.2-L-sfGFP*	pLZ12-Sp; pBER25; primers	Gibson	N/A	Spec^{R}	This study
pBER33	p7INT-*spec-relA*	p7INT-*spec*; NZ131 gDNA; primers	Gibson	N/A	Spec^{R}	This study
pBER34	p7INT-*spec-sasA*	p7INT-*spec*; NZ131 gDNA; primers	Gibson	N/A	Spec^{R}	This study

pBER35	p7INT-*spec-sasB*	p7INT-*spec*; NZ131 gDNA; primers	Gibson	N/A	$Spec^R$	This study
pBER37	p7INT-P_{sasB}-*luxAB;* 219 bp DNA fragment from region before *sasB* (*spy49_0687*) start ATG	pJC219; NZ131 gDNA; primers	Gibson	N/A	Erm^R	This study
pBER38	p7INT-P_{sasA}-*luxAB;* 150 bp DNA fragment from region before *sasA* (*spy49_0877*) start ATG	pJC219; NZ131 gDNA; primers	Gibson	N/A	Erm^R	This study
pBER40	pLZ12-Sp-*aroE.2-L-sfGFP shp3*(ATG→GGG)	pBER30; primers	QC[f]	N/A	$Spec^R$	This study
pBER41	pLZ12-Sp-*sasA-L-sfGFP*	pBER30; NZ131 gDNA; primers	Gibson	N/A	$Spec^R$	This study
pCN52	*E. coli*-staphylococcal shuttle vector containing *cop-wt-repC*, *gfpmut2*, and *blaZ* transcriptional terminator	N/A	N/A	N/A	Amp^R, Erm^R	(8)
pEVP3	Plasmid encoding synthetic promoter and *cat* chloramphenicol resistance cassette	N/A	N/A	N/A	Cm^R	(9)
pFED322	Shuttle vector encoding chloramphenicol resistance, derived from pLZ12-Sp; pWV01 origin	pLZ12-Sp, pEVP3; primers	RE	PacI	Cm^R	This study
pFED576	pCN52 with P_{rrnB} from *S. pyogenes* driving *gfp*	pCN52; NZ131 gDNA; primers	RE	EcoRI; ScaI	Amp^R, Erm^R	This study
pFED601	pFED576 with *luxS* from *S.pyogenes*, P_{rrnB} *-luxS-gfp*	pFED576; NZ131 gDNA; primers	RE	EcoRI	Amp^R, Erm^R	This study
pFED630	pFED322 with P_{rrnB}-*luxS* and *gfp*;	pFED322; pFED601	RE	PacI; XbaI	Cm^R	This study
pFED760	pGh9-ISS1 derivative deleted for ISS1	N/A	N/A	N/A	Erm^R	(10)
pJC219	p7INT-P_{shp3}-*luxAB* (384 bp DNA fragment containing *shp3* promoter fused to *luxAB*)	N/A	N/A	N/A	Erm^R	(2)

pLZ12-Sp	Shuttle vector encoding spectinomycin resistance; pWV01 origin	N/A	N/A	N/A	$Spec^R$	(11)
pY71-sfGFP	P_{T7}::*sfGFP*, C-terminal Strep-tag	N/A	N/A	N/A	Kan^R	Gift from S. Mankin and N. Vazquez-Laslop; (12)

Primers used for strain construction				
Primer	**Sequence (5' to 3')**	**Template[c]**	**RE Used[e]**	**Amplicon or Plasmid Product**
For construction of p7INT-*spec*				
JC601	CATG**CCATGG**CACGTTACTAAAGGGAATGTAG	p7INT	NcoI	p7INT backbone without Erm cassette
JC602	CATG**CCATGG**TTTGCTTCTAAGTCTTATTTCC			
JJ89	CATG**CCATGG**TAACGTGACTGGCAAGA	pLZ12-Sp	NcoI	Spec cassette
JJ90	CATG**CCATGG**GAATGAATATTTCCCAAATATT			
For construction of pBER16 to construct Δ*relA*, BRSP13				
BR114	GGATCCCACCGCGGTG**GCGGCCGC**GTGAACAAGGAATCCAAGG	NZ131 gDNA	NotI	5' flanking region of *relA*
BR198	TTATCCTTTCTTCGTTCCTCATCTAGCATTTCTTTTC		N/A	
BR199	GAGGAACGAAGAAAGGATAAAAACATGAAACTTGTC	NZ131 gDNA	N/A	3' flanking region of *relA*
BR119	AAGCTTGATAATTCG**GAATTC**TATTTCTGAGAAATATTACGTCCTTAA		EcoRI	
For construction of pBER17 to construct Δ*sasB*, BRSP15				
BR108	CCCACCGCGGTG**GCGGCCGC**ACACCATCTTTACTTGTTGC	NZ131 gDNA	NotI	5' region flanking region of *sasB*
BR196	ATTTTCATGGACGCTTATTATAACAAATTTTCTCAAC		N/A	
BR197	TAATAAGCGTCCATGAAAATTTTGTTAGCTGAAGACG	NZ131 gDNA	N/A	3' flanking region of *sasB*
BR113	AGCTTGATAATTCG**GAATTC**GTAACTGCCTTTTTCTTCCC		EcoRI	
For construction of pBER18 to construct Δ*sasA*, BRSP20				
BR102	GGATCCCACCGCGGTG**GCGGCCGC**TCATTGACAGGAAAACTAGTG	NZ131 gDNA	NotI	5' flanking region of *sasA*
BR194	TCTGTGTCATCTATTGTCTCCTTTCTTGTCAAAGC		N/A	
BR195	GAGACAATAGATGACACAGATGAATTATACAGGTAAGGTAAAG	NZ131 gDNA	N/A	3' flanking region of *sasA*
BR107	AAGCTTGATAATTCG**GAATTC**ATGGCTTCAAGCTTTTCAAA		EcoRI	

BR208	TCCTGTCAATGA**GCGGCCGC**CACCGCG GTG	Plasmid backbone	NotI	Intermediate plasmid backbone
For construction of pBER23 (p7INT-*spec-L-sfGFP*)				
BR271	CAGCTGAACCCCCGGGGTACCGAATTC C	p7INT-Sp	N/A	p7INT-Sp backbone
BR272	CGAAAAATAATTCGAAATCGATAAGCTT G			
BR273	GTACCCCGGGGGTTCAGCTGGTTCAGC TGCTGGTTCAGGTGAATTCATGAGCAAA GGTGAAGA	pY71-sfGFP		L-sfGFP
BR274	CGATTTCGAATTATTTTTCGAACTGCGG ATGG			
To construct pBER25 (p7INT-*spec-aroE.2-L-sfGFP*)				
BR279	ATTAGTCTGTCCCGGGGTACCGAATTCC	pBER23	N/A	pBER23 backbone
BR280	AAAAGGAAAAGGTTCAGCTGGTTCAGCT G			
BR281	GTACCCCGGGACAGACTAATTTGCTTTC C	NZ131 gDNA		AroE.2
BR282	CAGCTGAACCTTTTCCTTTTATCTTCCCT TC			
To construct pBER30 (pLZ12-Sp-*aroE.2-L-sfGFP*)				
BR293	ATTAGTCTGTGGCACGACAGGTTTCCCG	pLZ12-Sp	N/A	pLZ12-Sp backbone
BR294	CGAAAAATAAAGGACCAGACATTACGAA C			
BR295	CTGTCGTGCCACAGACTAATTTGCTTTC C	pBER25		AroE.2-L-sfGFP
BR296	GTCTGGTCCTTTATTTTTCGAACTGCGG			
To construct pBER33 (p7INT-*spec-relA*)				
BR333	CTCTCAGGCGGAATTCCTCGAGTCTAGA G	p7INT-*spec*	N/A	p7INT-*spec* backbone
BR334	CAATGGCTAAGGTACCCCGGGTTCGAA ATCGATAAG			
BR335	CGAGGAATTCCGCCTGAGAGATCCCAA AAG	NZ131 gDNA		*relA* CDS
BR336	CCGGGGTACCTTAGCCATTGGTCCGCTT C			
To construct pBER34 (p7INT-*spec-sasA*)				
BR337	CCGTCTTTTAGAATTCCTCGAGTCTAGA G	p7INT-*spec*	N/A	p7INT-*spec* backbone
BR338	ATACAGGTAAGGTACCCCGGGTTCGAAA TC			
BR339	CGAGGAATTCTAAAAGACGGTTATACTC ATTTTTTG	NZ131 gDNA		*sasA* CDS
BR340	CCGGGGTACCTTACCTGTATAATTCATC TGTG			
To construct pBER35 (p7INT-*spec-sasB*)				
BR341	TTAAAAATACGAATTCCTCGAGTCTAGA G	p7INT-*spec*	N/A	p7INT-*spec* backbone
BR342	GGAAGAGTAAGGTACCCCGGGTTCGAA ATC			
BR343	CGAGGAATTCGTATTTTTAAAAAATAACG TACAAAAAATATATGAAAG	NZ131 gDNA		*sasB* CDS

BR344	CCGGGGTACCTTACTCTTCCTCCGTCGTTTC			
To construct pBER37 (p7INT-P*sasB*-*luxAB*)				
BR359	AGGAGATTTTGGATCCGGAGAGCTCCCAAC	pJC219	N/A	pJC219 backbone
BR360	TAATAAGCGTATGAAGTTTGGAAATATTTGTTTTTC			
BR361	CTCCGGATCCAAAATCTCCTAATAAGTTAACGTAATC	NZ131 gDNA		*sasB* predicted promoter
BR362	CAAACTTCATACGCTTATTATAACAAATTTTCTC			
To construct pBER38 (p7INT-P*sasA*-*luxAB*)				
BR363	CCGTCTTTTAGGATCCGGAGAGCTCCCAAC	pJC219	N/A	pJC219 backbone
BR364	GAGACAATAGATGAAGTTTGGAAATATTTGTTTTTC			
BR365	CTCCGGATCCTAAAAGACGGTTATACTCATTTTTTG	NZ131 gDNA		*sasA* predicted promoter
BR366	CAAACTTCATCTATTGTCTCCTTTCTTGTC			
To construct pBER40 (pLZ12-Sp-*aroE.2-L-sfGFP shp3*(ATG→GGG))				
JC139	CAATAAATAAAAACTGAAAGGAAGTCCACTTGGGAAGAAAATTTCAAAATTTTTGCCGATTTTA	pBER30	N/A	QC[f] of pBER30
JC140	TAAAATCGGCAAAAATTTTGAAATTTTCTTCCCAAGTGGACTTCCTTTCAGTTTTTATTTATTG			
To construct pBER41 (pLZ12-Sp-*sasA-L-sfGFP*)				
BR301	GGAATTTCCCGGCACGACAGGTTTCCCG	pBER30	N/A	pBER30 backbone
BR302	ATTATACAGGGGTTCAGCTGGTTCAGCTG			
BR303	CTGTCGTGCCGGGAAATTCCTTTTAATTTGTG	NZ131 gDNA	N/A	*sasA* CDS without stop codon
BR304	CAGCTGAACCCCTGTATAATTCATCTGTGTC			
To construct pFED322				
pLZ12-S3	CCTCCTCACTATTTTGATTAGTACC	pLZ12-Sp	PacI	pLZ12-Sp backbone
pLZ12-A2	GCGTG**TTAATTAA**GGAGAGAATATTGAATGGACT			
To construct pFED576 (P*rrnB*-*gfp*)				
PrrnB-S1-EcoRI	GCGTG**GAATTC**CCTAGCGGGAACACTCATCAT	NZ131 gDNA	EcoRI	*rrnB* promoter
PrrnB-A1-ScaI	GCGTG**AGTACT**CCCTCACGTTTGGTTCGT		ScaI	
To construct pFED601 (P*rrnB*-*luxS-gfp*)				
luxS-EXT-S-Eco	GCGTG**GAATTC**GTGTAAAACAAAGGAGATTGAAATG	NZ131 gDNA	EcoRI	*luxS* CDS
luxS-EXT-A-Eco	GCGTG**GAATTC**ACACTAGTATCAGATGACAT			
Primers for qRT-PCR				
Primer	Sequence (5' to 3')	Description		
KMT043	GTTATCGAGATTCGTCGAG	Forward primer for *gyrA* (*spy49_0905*)		
KMT044	CACACCATTTTCAATAGCC	Reverse primer for *gyrA* (*spy49_0905*)		

BR188	TCACAGCTCCTGGTGGAAAC	Forward primer for *plr* (*spy49_0234*)
BR189	GTCACCAGTGTAAGCGTGGA	Reverse primer for *plr* (*spy49_0234*)
BR120	AAACCTGCCGAACAGATGGT	Forward primer for *prtF* (*spy49_0119*)
BR121	CTTTGCTGGGCGTTGATCTG	Reverse primer for *prtF* (*spy49_0119*)
BR134	GCGTCAACGACAAGACATGA	Forward primer for *sasA* (*spy49_0877*)
BR135	CAGGGTACTCAACCACCACA	Reverse primer for *sasA* (*spy49_0877*)
BR130	ACGTGGTGACAAGGGTGAAA	Forward primer for *sclB* (*spy49_0830*)
BR131	GGACCTACTGGACCACGTTC	Reverse primer for *sclB* (*spy49_0830*)
BR124	CGAGTTTCAAGCCCCTGAGT	Forward primer for *sfbX49* (*spy49_1683c*)
BR125	CGTTAGCTGGGTCACTTGGT	Reverse primer for *sfbX49* (*spy49_1683c*)
BR122	CGACCCAACCTGTCCAAGAA	Forward primer for *ska* (*spy49_1630*)
BR123	GCAGAGTTGTGAACGGCTTT	Reverse primer for *ska* (*spy49_1630*)
BR128	GGTGGTTCGGCTATCTTAGCA	Forward primer for *upp* (*spy49_0322*)
BR129	CCCTTCTGGTGCTGCAACTA	Reverse primer for *upp* (*spy49_0322*)
LC074	CCTAATAATCCTGCGGATGTGTTTG	Forward primer for *slo* (*spy49_0146*)
LC075	GTTCGACCATAGGCTACGTTAC	Reverse primer for *slo* (*spy49_0146*)
KL1	CAGCCCTAAACCACCATTCC	Forward primer for *stcA* (*spy49_0414c*)
KL2	GATTTGATAGCGCTGTCCCC	Reverse primer for *stcA* (*spy49_0414c*)
spy49_0450_F1_RW	CAGGAACTAATACTGATTGGAAAGG	Forward primer for *aroE.2* (*spy49_0450*)
spy49_0450_R1_RW	CAACTGTTGGTGAGATTTGTAGTT	Reverse primer for *aroE.2* (*spy49_0450*)

[a]Strains were constructed as described in *Materials and Methods*.

[b]Antibiotic resistance markers: NR, no resistance markers; Amp^R, ampicillin resistance; $Spec^R$, spectinomycin resistance; Erm^R, erythromycin resistance; Kan^R, kanamycin resistance; Cm^R, chloramphenicol resistance.

[c]Templates indicates plasmid or gDNA (genomic DNA) templates used to construct plasmids or constructs. Primers indicates that primers were used to amplify templates during construction of plasmid. N/A indicates plasmid has already been published, see reference for construction.

[d]Method of Const. indicates method of construction used to obtain plasmids. Gibson indicates plasmids were assembled by PCR amplification of fragments and resulting Gibson assembly, whereas RE indicates plasmids were obtained by restriction enzyme digest and resulting ligation of fragments. Gibson + RE indicates both methods were used. N/A indicates plasmid has already been published, see reference. For further details, see *Materials and Methods.*

[e]RE used indicated restriction enzymes or site used. N/A not applicable or plasmid already published, see reference. Site is highlighted in primers as underlined and bolded nucleotides.

[f]QC indicates quick change mutagenesis.

TABLE S2. Doubling times of strains with or without 100 nM SHP or revSHP peptide.[a]

Genotype	**Doubling Time ± S.E.M.[b] (in min)**	**Sig.[c] vs NZ131[d]**	**Sig. vs. NZ131 + SHP[d]**	**Sig. vs No SHP condition[d,e]**
NZ131	36 ± 1	N/A	****	N/A
NZ131 + SHP	47 ± 1	****	N/A	****
NZ131 + revSHP	36 ± 2	ns	ns	ns
Δ*rgg2*	35 ± 1	ns	**	N/A
Δ*rgg2* + SHP	34 ± 2	ns	***	ns
Δ*rgg3*::*cat*	38 ± 2	ns	**	N/A
Δ*rgg3*::*cat* + SHP	52 ± 2	****	ns	****
Δ*rgg2* Δ*rgg3*::*cat*	40 ± 3	ns	ns	N/A
Δ*rgg2* Δ*rgg3*::*cat* + SHP	36 ± 3	ns	ns	ns
Δ*relA*	41 ± 1	ns	ns	N/A
Δ*relA* + SHP	62 ± 2	****	****	****
Δ*sasA*	43 ± 2	*	ns	N/A
Δ*sasA* + SHP	47 ± 2	****	ns	ns
Δ*sasB*	37 ± 2	ns	**	N/A
Δ*sasB* + SHP	47 ± 3	***	ns	*
Δ*relA* Δ*sasA*	37 ± 2	ns	ns	N/A
Δ*relA* Δ*sasA* + SHP	47 ± 3	ns	ns	ns
Δ*relA* Δ*sasB*	38 ± 3	ns	ns	N/A
Δ*relA* Δ*sasB* + SHP	50 ± 3	**	ns	ns
Δ*sasA* Δ*sasB*	36 ± 1	ns	ns	N/A
Δ*sasA* Δ*sasB* + SHP	39 ± 2	ns	ns	ns
Δ*relA* Δ*sasA* Δ*sasB*	62 ± 8	****	**	N/A
Δ*relA* Δ*sasA* Δ*sasB* + SHP	72 ± 5	****	****	ns
Δ*relA* pBER33 (p7INT-*spec-relA*)	34 ± 2	ns	ns	N/A
Δ*relA* pBER33 (p7INT-*spec-relA*) + SHP	42 ± 2	ns	ns	ns
Δ*sasA* pBER34 (p7INT-*spec-sasA*)	38 ± 1	ns	ns	N/A
Δ*sasA* pBER34 (p7INT-*spec-sasA*) + SHP	42 ± 0.1	ns	ns	ns
Δ*sasB* pBER35 (p7INT-*spec-sasB*)	39 ± 6	ns	ns	N/A
Δ*sasB* pBER35 (p7INT-*spec-sasB*) + SHP	47 ± 5	ns	ns	ns
NZ131 p7INT-*spec*	32 ± 3	ns	ns	N/A
NZ131 p7INT-*spec* + SHP	35 ± 4	ns	ns	ns
pBER33 (p7INT-*spec-relA*)	36 ± 3	ns	ns	N/A

pBER33 (p7INT-*spec-relA*) + SHP	43 ± 2	ns	ns	ns
pBER34 (p7INT-*spec-sasA*)	38 ± 2	ns	ns	N/A
pBER34 (p7INT-*spec-sasA*) + SHP	45 ± 4	ns	ns	ns
pBER35 (p7INT-*spec-sasB*)	40 ± 4	ns	ns	N/A
pBER35 (p7INT-*spec-sasB*) + SHP	53 ± 9	**	ns	ns

[a]SHP refers to SHP3-C8 peptide and revSHP refers to reverse sequence peptide of SHP3-C8 peptide, see *Materials and Methods* for further details.

[b]S.E.M. is Standard Error of Mean.

[c]Sig. stands for significantly.

[d]Stars indicate statistical significance via a One-way Anova with a Šidák's or Dunnett's Multiple Comparisons Post-Test. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.0001$.

[e]Versus the doubling time for the same strain, without addition of SHP peptide.

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