Supplementary Table 1. Plasmids, strains, and selected primers used in this study

Plasmid	Description	Backbone/Source
pPP2	htrA'-'lacZ intergrative reporter	Bruckner et al
pHDY06	pPP2, bga::P_shp1518-LacZ, AmpR (P_shp1518 sequence: ATTGTTCCTCCTAGAAAAATGTGAATATTTTCTACATTTATAAAAAA CGTAGAAAAAGGTGGTATTATTATATCAAAAAGGAGGGAAAAAGTC)	pPP2
pMalC9	MBP-MarC9 transposae, AmpR	Opijnen, 2014
pMagellan6	transposon plasmid, KanR	Opijnen, 2014
pET28a	N/C-terminal His•Tag®/thrombin/T7	Novagen™
pHDY13	pET28a::N'-His-[HindIII]pepO[BamHI], KanR	pET28a
pHDY16	pHDY13, pepO::E479Q pepO, KanR	pHDY13

Strain	Description	Source
	S. pneumoniae	
CP2000	Rx, but Dcps, hex', mal', str-1, bgl-1; CPS-, Hex-, Mal-, Bga-, StrR	[1]
CP2532 (P_shp1518-LacZ)	CP2000, but bga::pHDY06; TetR, StrR, Bga(+)	this study
CP2554	CP2532, but Δrgg0112::cmR; TetR, CmR, StrR, Bga(+)	this study
CP2555	CP2532, but Δrgg0999::cmR; TetR, CmR, StrR, Bga(+)	this study
CP2556	CP2532, but Δrgg1518::cmR; TetR, CmR, StrR, Bga(+)	this study
CP2557	CP2532, but Δrgg1786::cmR; TetR, CmR, StrR, Bga(+)	this study
CP2558	CP2532, but Δrgg1916::cmR; TetR, CmR, StrR, Bga(+)	this study
CP2559	CP2532, but Δrgg1952::cmR; TetR, CmR, StrR, Bga(+)	this study
CP2562	CP2532, but Δrgg0939::cmR; TetR, CmR, StrR, Bga(+)	this study
CP2565	CP2532, but Δrgg0144::cmR; TetR, CmR, StrR, Bga(+)	this study
CP2569	CP2532, but ΔpepO; TetR, StrR, Bga(+)	this study
CP2574	CP2532, but pepO::E479Q pepO; TetR, StrR, Bga(+)	this study
CP2576	CP2532, but Δ*spxB*; TetR, StrR, Bga(+)	this study
	E.coli	
DH5α	F– Φ80lacZ, ΔM15, Δ(lacZYA-argF), endA1, recA1, hsdR17(rK–, mK+), phoA, supE44 λ– thi-1, relA1, gyrA96	Thermo Scientific™
BL21 (DE3)	F– ompT hsdSB (rB–, mB–) gal dcm (DE3)	Thermo Scientific™

Primer	Sequence (5'-3')	Location
DH026	AGGCGATTAAGTTGGGTAAC	pHDY13
DH030	ACTCAAATTGCGAGATTTGG	pHDY13
DH058	GTTCAGAGTTCTACAGTCCGACGATCACACNN	Adapter A
DH059	/5Phos/GTGTGATCGTCGGACTGTAGAACTCTGAACCTGTC/3Phos/	Adapter B
DH060	GTTCAGAGTTCTACAGTCCGA	transposon
DH061	ATCGAGCTGTATGCGGAGTG	transposon

Supplementary Table 2. Summary of blue/white screening of transposon insertion mutants grown in CDM sandwich X-gal agar or on TSB-SB X-gal agar plates.

Screening in CDM sandwich agar,under 37°C, 16 hours of incubation

Library #	Cell# input	Blue CFU# output	*P_LacZ*	*pepO*	*Others**
L1	4 x 3,000	5	2	2	1
L2	4 x 3,000	0	0	0	0
L3	4 x 3,000	4	3	1	0
L4	4 x 3,000	3	0	0	3
L5	4 x 3,000	8	0	5	3
Total#	**60,000**	**20**	**5**	**8**	**7**

Screening on TSB-SB agar, under 37°C, 5% CO_2, 16 hours of incubation

Library #	Cell# input	Blue CFU# output	P_LacZ	pepO	spxB	Others*
L6	10 x 3,000	46	5	9	18	11
L7	10 x 3,000	21	0	5	16	0
L8	10 x 3,000	40	1	10	1	10
L9	10 x 3,000	21	0	11	52 (unmapped)	
L10	10 x 3,000	23	0	2		
Total#	**150,000**	**151**	**6**	**37**	**108**	

* randomly appeared blue colonies that do not have a kanamycin-resistant (transposon insertion) phenotype. These mutants were no longer recovered after back-cross transformation.

Supplementary Table 3. Summary of putative RRNPP quorum-sensing systems in *S. pneumoniae* (top) and their cognate pheromone sequences (bottom).

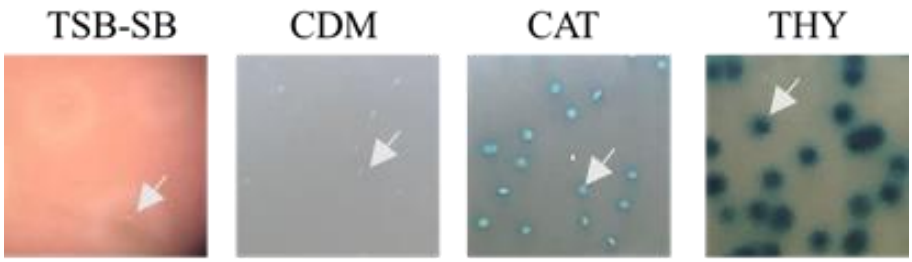
D39 RRNPP homolog	Ortholog (in other strains)	Regulated operon	Preferred carbon source	Possible functions	upstream regulators	Reference
spd_0112 (rgg112)	*rtgR (Sp9-BS68)*	*spd_0113-spd_0116*		colonization		[2]
spd_0144 (rgg144)	*sp_0141 (TIGR4)*	*vp1, vpoBCD*	mannose	adaptation, virulence	CodY, GlnR	[3-8]
spd_0939 (rgg939)		*spd_0940-spd_0950*	mannose	capsule synthesis		[9, 10]
spd_0999 (rgg999)	*sp_1115 (TIGR4)*					[3, 4, 9]
spd_1518 (rgg1518)		*spd_1513-spd_1517*	galactose	ABC transporter	PepO, SpxB	[7, 9]; this study
spd_1745 (tprA)	*sp_1745 (TIGR4)*	*spd_1744-spd_1756*	galactose	virulence	CcpA	[3, 4, 7, 11]
spd_1786 (rgg1786)[*]	*sp_1989 (TIGR4)*					[3, 4]
spd_1916 (rgg1916)	*sp_2090 (TIGR4)*					this study
spd_1952 (rgg1952)				stress response		[9, 12]

D39 RRNPP homolog	(putative) Precursor-**SHP**	Reference
spd_0112 (rgg112)	MNKKIFCILVCIILLI**SLAIIFPWGWPI**	[2]
spd_0144 (rgg144)	MKKQILTLLKIVA**EIIIILPFLTNL**	[3-8]
spd_0939 (rgg939)	MKKISKFLPILFLVM**DIIIIVGG**	[9, 10]
spd_0999 (rgg999)	N.A.	
spd_1518 (rgg1518)	MGFKKYLKNLPKNSGFLIWSW**IQLIWFETWFWG**	[7, 9]; this study
spd_1745 (tprA)	MFSNTIPYQQFIQKNKQLEIRVQSQK**KSNGLDVGKAD (PhrA)**	[3, 4, 7, 11]
*spd_1786 (rgg1786)**	(MSKNIYKHSESVTKMSLSSYQNSSKVRL)	this study,
spd_1916 (rgg1916)	N.A.	
spd_1952 (rgg1952)	(MKKIRNQGEVYGADWKSL)	this study [9, 12]

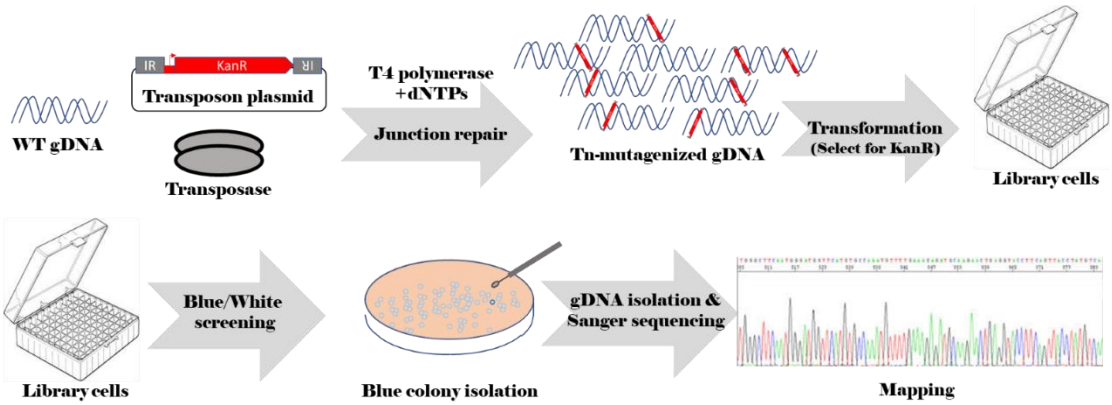
[*] Some studies refer Spd_1786 as a PlcR-like regulator

Supplementary Table 4. Differentially expressed genes of *rgg* knockouts

	# of genes differentially expressed more than 2-fold		**Log2 [fold change] of *spd_1514-1517* expression**			
mutants	**up**	**down**	***spd_1514***	***spd_1515***	***spd_1516***	***spd_1517***
Δrgg112	70	56	-2.88	-2.7	-2.51	-2.32
Δrgg999	62	16	-0.43	-0.48	-0.41	-0.01
Δrgg1518	38	34	-4.75	-4.93	-4.92	-5.03
Δrgg1786	85	87	-1.57	-1.33	-1.18	-0.81
Δrgg1916	28	26	-1.23	-1.15	-1.18	-1.29
Δrgg1952	31	44	-1.26	-1.16	-0.91	-0.69

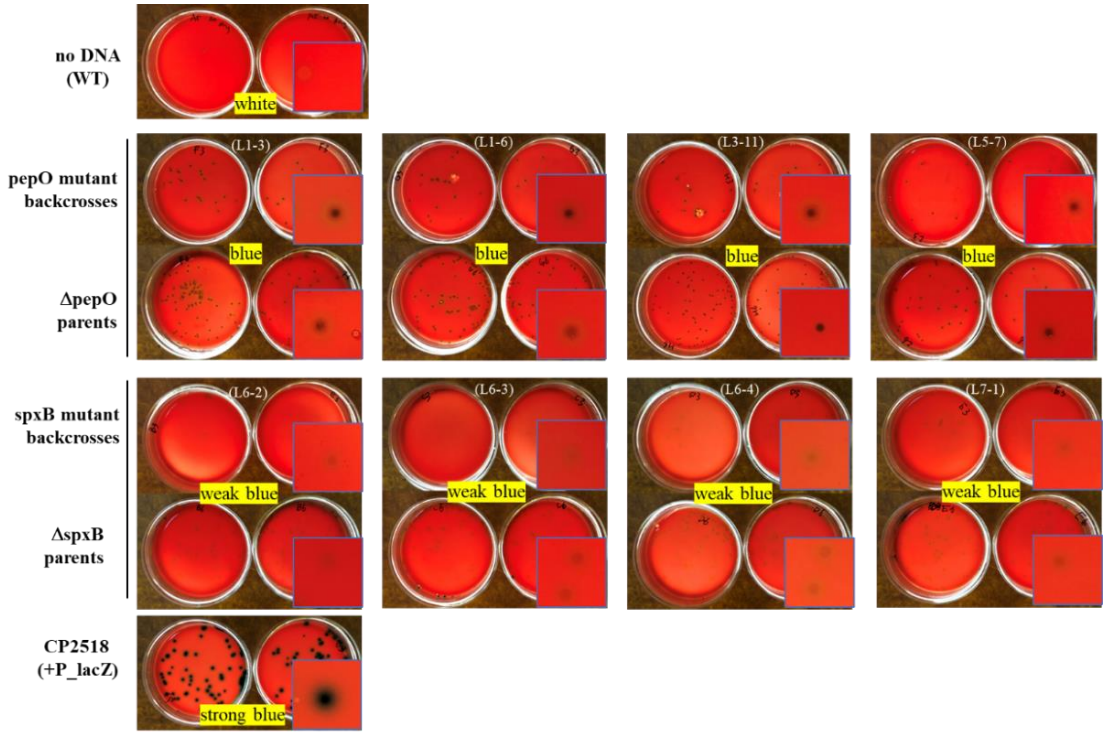


Supplementary Figure 1. Colony color phenotypes of P_shp1518-LacZ reporter strain in four agar plates. Examples of one colony in each agar are indicated by white arrows



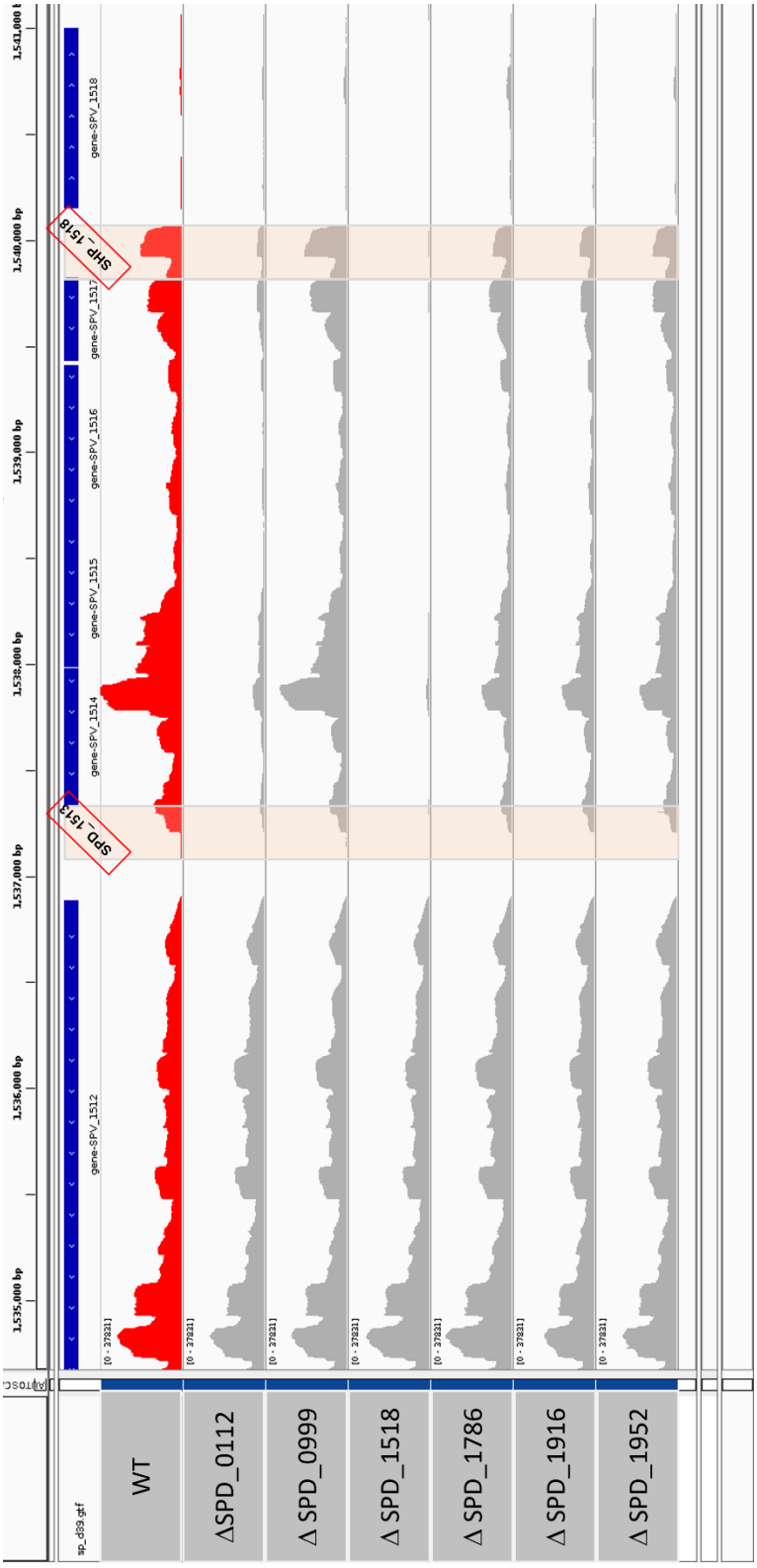
Supplementary Figure 2. Diagram of using transposon mutagenesis to screen Rgg/SHP QS upstream regulators.

WT gDNA: genomic DNA of P_shp1518-LacZ strain; Transposon plasmid: pMagellan6 (KanR); Transposase: purified from E. coli BL21 expressing pMalC9 plasmid; Tn-mutagenized gDNA contained the transposon element (KanR); Library cells were KanR transformants of P_shp1518-LacZ; Blue/White screenings were performed on either TSB-SB agar or in CDM sandwich agar supplemented with 400 μg/ml X-gal; isolated genomic DNA were MmeI-digested and Adapter-ligated before proceeding to Sanger sequencing.



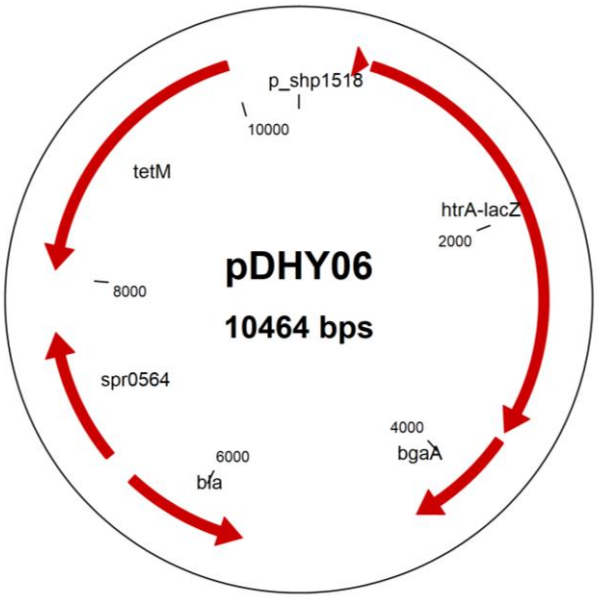
Supplementary Figure 3. Backcross verification of transposon hits in pepO and spxB.

The genomic DNA from Tn-pepO mutants (L1-3, L1-6, L3-11, and L5-7) were exacted and used for transforming the parental CP2532 reporter cells. Likewise, the genomic DNA from Tn-spxB mutants (L6-2, L6-3, L6-4, and L7-1) were exacted and used. Transformants' blue colony phenotype was compared to their cognate parents. CP2518 was used as the positive control, where the KanR promoter of the transposon drives the LacZ expression.



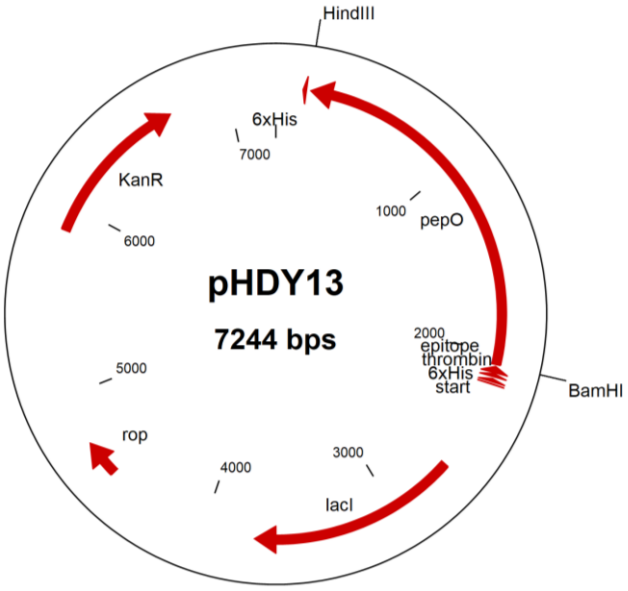
Supplementary Figure 4. RNA expression of the spd_1513-1517 operon, shp1518, and rgg1518(spd_1518) in wild-type and rgg knockout strains.

The *spd_1513* and *shp1518* gene were not indexed (unexpected) in mapping the RNA sequences, and they are indicated by a red box at their loci. Y-axis represents the RNA expression amount.



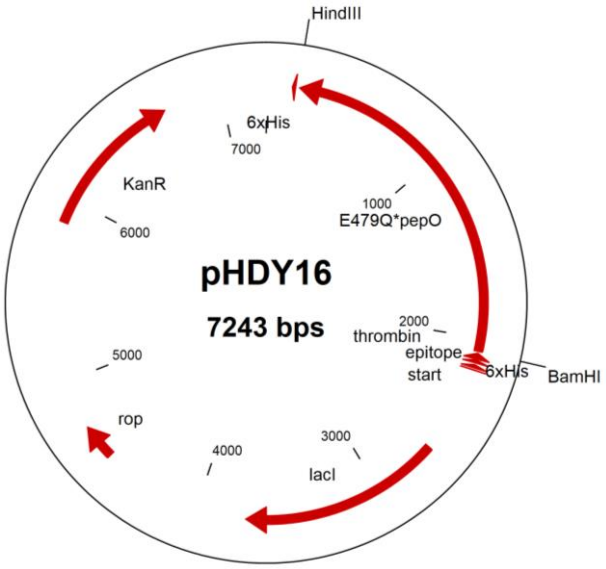
Appendix A. Figure. 1 Construction of pHDY06.

To clone pHDY06, *P_shp1518* was PCR amplified from pneumococcal strain CP2000 with primers DH118 and DH127. The PCR fragment and the vector pPP2 were digested with *BamHI* and *KpnI*. Double-digested vector and insert were ligated by T4 ligase. The ligated plasmid was transformed into E. coli DH5α and selected for AmpR transformants. The correct insert was verified by Sanger sequencing.



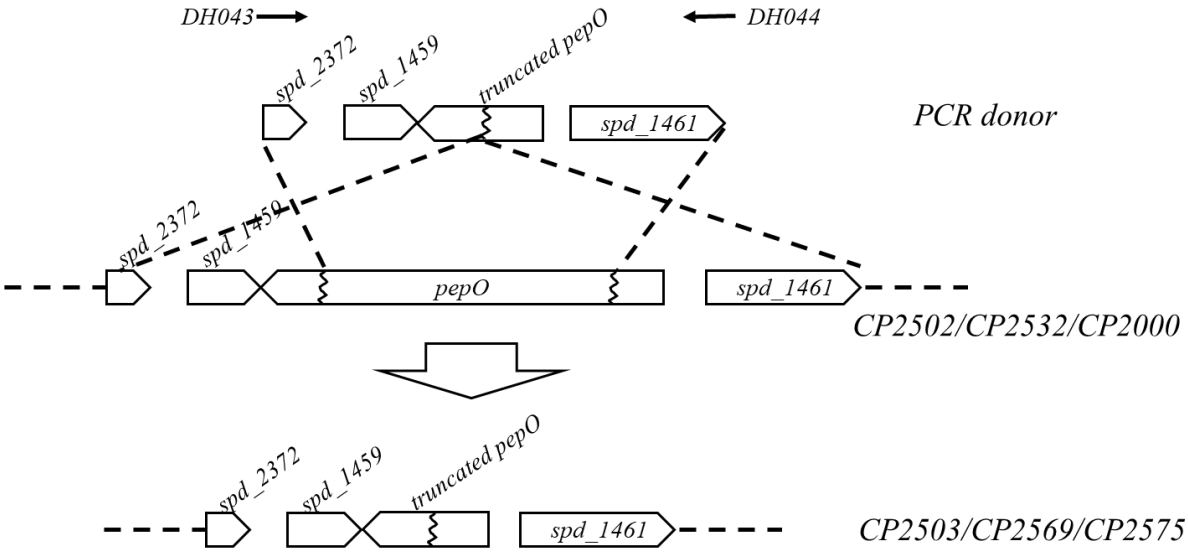
Appendix A. Figure. 2 Construction of pHDY13.

To clone pHDY13, the coding sequence of *pepO* was PCR amplified from pneumococcal strain CP2000 with primers DH200X and DH201. The PCR fragment and the vector pET28a were digested with *BamHI* and *HindIII*. Double-digested vector and insert were ligated by T4 ligase. The ligated plasmid was transformed into E. coli DH5α and selected for KanR transformants. The correct insert was verified by Sanger sequencing.

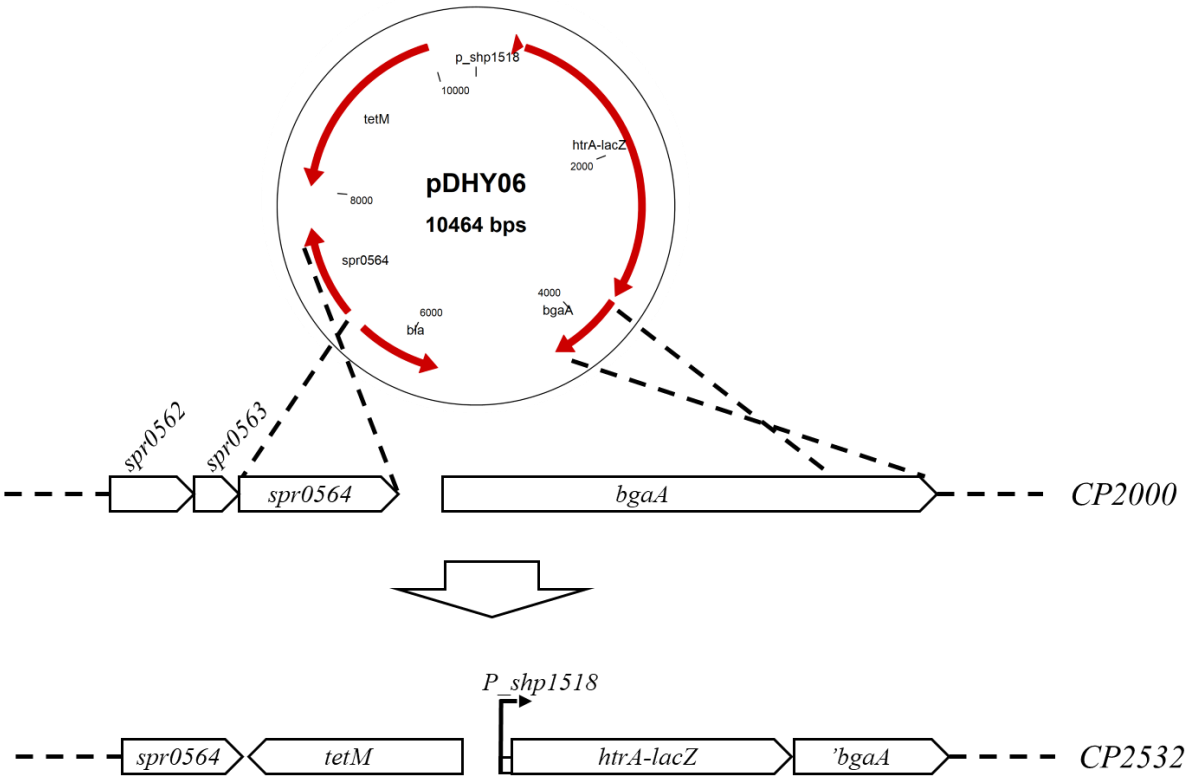


Appendix A. Figure. 3 Construction of pHDY16.

To clone pHDY16, the coding sequence of E479QPepO was PCR amplified from pneumococcal strain CP2574 with primers DH200X and DH201. The PCR fragment and the vector pET28a were digested with *BamHI* and *HindIII*. Double-digested vector and insert were ligated by T4 ligase. The ligated plasmid was transformed into *E. coli* DH5α and selected for KanR transformants. The correct insert was verified by Sanger sequencing.

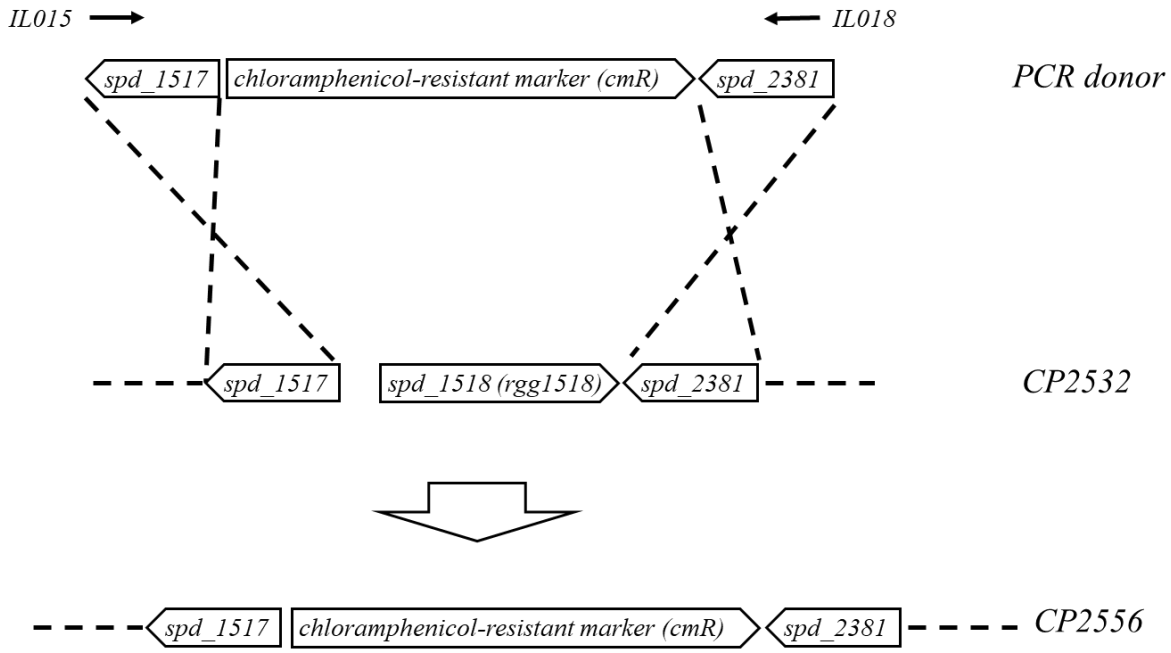


Appendix B. Figure. 1. Construction of CP2503, CP2569, and CP2575.
To clone CP2503, CP2569, and CP2575, whose *pepO* gene was replaced by a truncated, non-function *pepO*. A PCR donor consisting of the truncated *pepO* and flanking neighboring genes with approximately 2000 bp length was generated by overlapping extension PCR. First, the overlapping PCR fragments were assembled using primers DH043 and DH044. Then, the PCR donor was transformed into CP2502 to yield CP2503, into CP2532 to yield CP2569, and into CP2000 to yield CP2575. These transformants were verified by colony PCR using primers DH043 and DH044. Transformants with the truncated *pepO* yielded PCR fragments with a reduced size. Transformants validated by colony PCR were isolated and restreaked. The extracted genomic DNAs of these transformants were used as templates to generate a PCR fragment containing the truncated *pepO*, and their truncated *pepO* sequences were verified by Sanger sequencing.



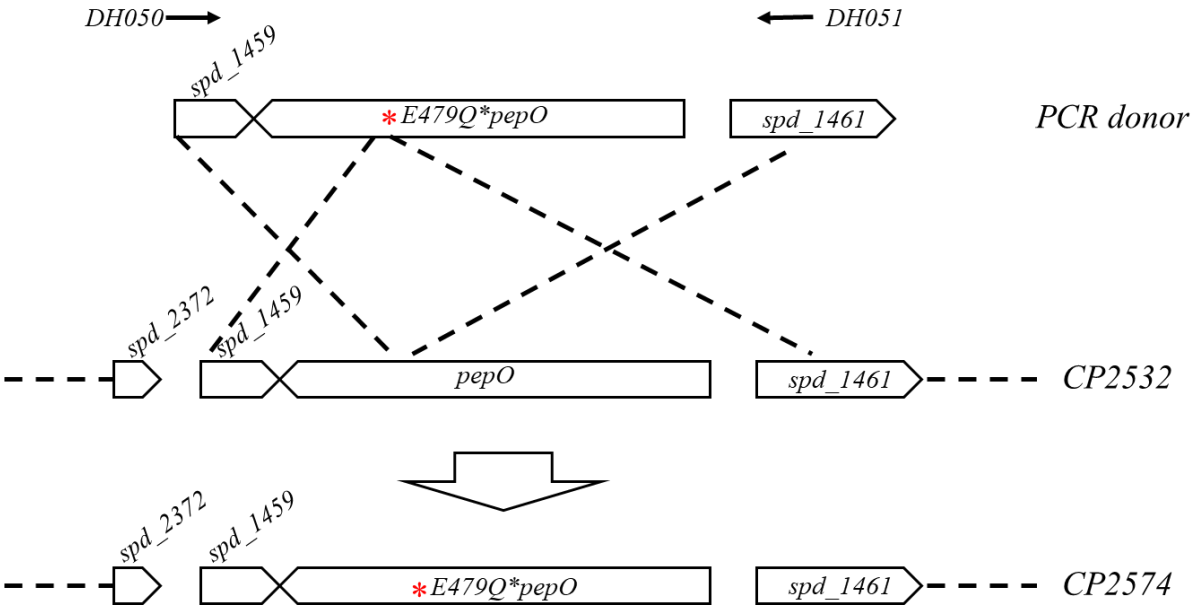
Appendix B. Figure. 2. Construction of CP2532.

To clone a LacZ reporter strain where the LacZ reporter was driving by *P_shp1518*. The plasmid pHDY06 was transformed into CP2000 and selected for TetR transformants. Three TetR colonies were isolated and restreaked. The extracted genomic DNAs of these transformants were used as templates to generate a PCR fragment containing the LacZ insert. The insert-naïve junction sequences at the two ends were verified by Sanger sequencing.



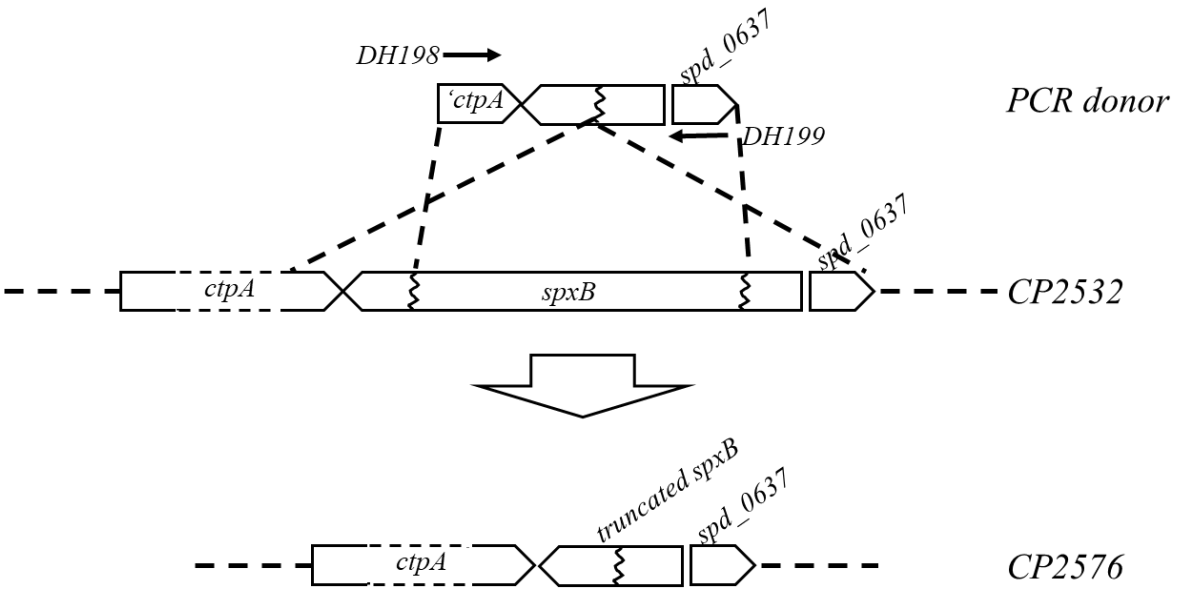
Appendix B. Figure. 3. Construction of CP2556.

To clone CP2556, whose Rgg1518 gene was replaced by a chloramphenicol-resistant marker, CmR. A PCR donor consisting of the CmR and flanking neighboring genes of Rgg1518 with approximately 2000 bp length was generated by overlapping extension PCR. First, the overlapping PCR fragments were assembled using primers IL015 and IL018. Then, the PCR donor was transformed into CP2532 and selected for CmR to yield CP2556. Next, three CmR colonies were isolated and restreaked. The extracted genomic DNAs of these transformants were used as templates to generate a PCR fragment containing the CmR and junction sequences. Finally, these junction sequences were verified by Sanger sequencing.



Appendix B. Figure. 4 Construction of CP2574.

To clone CP2574, whose *pepO* gene was replaced by a mutated *pepO* (E479Q*). A PCR donor consisting of the E479QPepO and flanking neighboring genes with approximately 2000 bp length was generated by overlapping extension PCR. First, the overlapping PCR fragments were assembled using primers DH050 and DH051. Then, the PCR donor was transformed into CP2532 to yield CP2574. Transformants were plated into CDM sandwich agar supplemented with 400 μg/ml X-gal. CP2532 yielded white colonies, and the transformants with E479QPepO mutation yielded blue colonies. Three blue colonies were isolated and restreaked. The extracted genomic DNAs of these transformants were used as templates to generate a PCR fragment containing the E479Q**pepO*. Mutated *pepO* sequences were verified by Sanger sequencing.



Appendix B. Figure. 5Construction of CP2576.

To clone CP2576, whose spxB gene was replaced by a truncated, non-function spxB. A PCR donor consisting of the truncated spxB and flanking neighboring genes with approximately 2000 bp length was generated by overlapping extension PCR. First, the overlapping PCR fragments were assembled using primers DH198 and DH199. Then, the PCR donor was transformed into CP2532 to yield CP2576. Transformants were plated into TSB-SB agar supplemented with 400 μg/ml X-gal. CP2532 yielded white colonies, and the transformants with ΔspxB yielded blue colonies. Three blue colonies were isolated and restreaked. The extracted genomic DNAs of these transformants were used as templates to generate a PCR fragment containing the truncated spxB. Junction sequences of spxB were verified by Sanger sequencing.

References

1. Weng, L., A. Piotrowski, and D.A. Morrison, *Exit from competence for genetic transformation in Streptococcus pneumoniae is regulated at multiple levels.* PLoS One, 2013. **8**(5): p. e64197.
2. Wang, C.Y., et al., *Molecular Determinants of Substrate Selectivity of a Pneumococcal Rgg-Regulated Peptidase-Containing ABC Transporter.* mBio, 2020. **11**(1).
3. Hava, D. and A. Camilli, *Large-scale identificationof serotype 4 Streptococcus pneumoniae virulence factors.* Molecular Microbiology, 2002. **45**(5): p. 1389-1405.
4. van Opijnen, T. and A. Camilli, *A fine scale phenotype-genotype virulence map of a bacterial pathogen.* Genome Res, 2012. **22**(12): p. 2541-51.
5. Hendriksen, W.T., et al., *CodY of Streptococcus pneumoniae: Link between nutritional gene regulation and colonization.* Journal of Bacteriology, 2008. **190**(2): p. 590-601.
6. Hendriksen, W.T., et al., *Site-specific contributions of glutamine-dependent regulator GlnR and GlnR-regulated genes to virulence of Streptococcus pneumoniae.* Infection and Immunity, 2008. **76**(3): p. 1230-1238.
7. Hoover, S.E., et al., *A new quorum-sensing system (TprA/PhrA) for Streptococcus pneumoniae D39 that regulates a lantibiotic biosynthesis gene cluster.* Mol Microbiol, 2015. **97**(2): p. 229-43.
8. Cuevas, R.A., et al., *A novel streptococcal cell-cell communication peptide promotes pneumococcal virulence and biofilm formation.* Mol Microbiol, 2017. **105**(4): p. 554-571.
9. Zhi, X., et al., *Rgg-Shp regulators are important for pneumococcal colonization and invasion through their effect on mannose utilization and capsule synthesis.* Sci Rep, 2018. **8**(1): p. 6369.
10. Junges, R., et al., *A Quorum-Sensing System That Regulates Streptococcus pneumoniae Biofilm Formation and Surface Polysaccharide Production.* mSphere, 2017. **2**(5).
11. Carvalho, S.M., et al., *CcpA Ensures Optimal Metabolic Fitness of Streptococcus pneumoniae.* Plos One, 2011. **6**(10).
12. Bortoni, M.E., et al., *The pneumococcal response to oxidative stress includes a role for Rgg.* Microbiology-Sgm, 2009. **155**: p. 4123-4134.