DprA-dependent exit from the competent state benefits *Streptococcus pneumoniae* virulence

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Supplementary Materials

All *in vitro* experiments were independently performed at least three times. Results from a typical experiment are presented. Statistical analyses were performed using the GraphPad Prism statistical package, and expressed as mean ± standard deviation. A p value ≤ 0.05 was considered significant.

Supplementary Experimental Procedures

PCR, gene splicing and cloning: PCR amplification were performed using the Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) following manufacturer's instructions. DNA fragments was spliced together using the NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA). Genes were cloned following restrictive enzyme digests and ligated with T4 DNA ligase (New England Biolabs, Ipswich, MA). Purification of PCR products and plasmids were carried out following manufacturer's instructions (ZYMO Research, Irvine, CA).

Generation of the Δ*dprA*Δ*cbpD*Δ*lytA* triple deletion mutant. A Δ*dprA*Δ*cbpD*Δ*lytA* deletion mutant was obtained by transforming GW1941 (Δ*cbpD*Δ*lytA*) with chromosomal DNA of AD1122 (Δ*dprA*) with the selection for kanamycin resistance (Kan^R) (Table S1). All the Δ*dprA*-related mutants were generated using similar methods.

Construction of the *rplL lacZ* reporter strain. An amplicon with *rplL* gene was generated through PCR using a pair of primers rplLF and rplLR (Table S3). PCR products

were digested with KpnI/BamHI and ligated into the pEVP3 vector, and used to transform *E. coli* DH5α. The resultant plasmid pEVP3-*rplL* was verified by restriction digest and used to transform the *S. pneumoniae* strain D39 (WT) with the selection for chloramphenicol resistant (Cm^R) colonies to generate the *rplL-lacZ* expressing strain.

Generation of the *cbpD* Sweet Janus Cassette insertional mutant. An amplicon with the promoter and *sacB-kan-rpsL*$^+$ (Sweet Janus Cassette) was generated through PCR with primers SJCF and SJCR, using chromosomal DNA from the strain SpnYL001 (Table S1) as template. An amplicon for the upstream sequence of the *cbpD* combox was generated through PCR with primers cbpDSJCU1 and cbpDSJCU2 (Table S3). A second amplicon for the downstream sequence of *cbpD* combox was generated through PCR with primers cbpDSJCD1 and cbpDSJCD2. Primers cbpDSJCU2 and cbpDSJCD1 each overlaps (Tm > 48˚C) with the Sweet Janus Cassette amplicon. Three amplicons were purified and then assembled using NEBuilder® HiFi DNA Assembly Master Mix. Then, a pair of nesting primers, cbpDSJCFx and cbpDSJCRx (Table S3), were used to amplify the assembled product. The assembled product was used to transform the WT with the selection for kanamycin resistance (Kan^R), to produce *cbpD*::SJC (Table S1).

Construction of the *cbpD* combox deletion mutant. An amplicon with the upstream sequence of the *cbpD* combox was PCR amplified with primers cbpDSJCU1 and cbpDSJCx1 (Table S3). A second amplicon with the downstream sequence of the *cbpD* combox was PCR amplified with primers cbpDSJCx2 and cbpDSJCD2 (Table S3). Primers cbpDSJCx1 and cbpDSJCx2 overlap (Tm > 48˚C) with each other. Two

amplicons were purified and then assembled using NEBuilder® HiFi DNA Assembly Master Mix. Then a pair of nesting primers, cbpDSJCFx and cbpDSJCRx (Table S3), were used to amplify the assembled product. The assembled product was used to transform the *cbpD*::SJC strain by sucrose selection (Su^R) to produce ΔP_{comX}*cbpD* strain.

Construction of *cibAB* SJC and combox deletion mutant. An amplicon with both the promoter of *cibAB* and *sacB-kan-rpsL*$^+$ (Sweet Janus Cassette) was generated by PCR with primers SJCF and SJCR (Table S3), using SpnYL001 (Table S1) chromosomal DNA as template. An amplicon upstream of the *cibAB* combox was generated by PCR with primers cibABSJCU1 and cibABSJCU2 (Table S3). An amplicon downstream of *cibAB* combox was generated by PCR with primers cibABSJCD1 and cibABSJCD2 (Table S3). Primers cibABSJCU2 and cibABSJCD1 each has overlapping sequence (Tm > 48˚C) with the Sweet Janus Cassette amplicon. All three amplicons were purified and assembled using the NEBuilder® HiFi DNA Assembly Master Mix. Then a pair of nesting primers, cibABSJCFx and cibABSJCRx (Table S3), were used to amplify the assembled products. The final construct was transformed into recipient pneumococcal cells by selecting for kanamycin resistance (Kan^R) transformants, to produce *cibAB*::SJC.

Construction of the *cibAB* combox deletion mutant. An amplicon upstream of the *cibAB* combox was generated by PCR with primers cibABSJCU1 and cibABSJCx1 (Table S3). A second amplicon downstream of the *cibAB* combox was generated by PCR with primers cibABSJCx2 and cibABSJCD2 (Table S3). Primer cibABSJCx1 and cibABSJCx2

have overlapping sequence (Tm > 48˚C). Two amplicons were gel-purified and then assembled using the NEBuilder® HiFi DNA Assembly Master Mix. A pair of nesting primers, cibABSJCFx and cibABSJCRx (Table S3), were used to amplify and assemble the final product. The assembled product was used to transform the *cibAB*::SJC strain (Table S1) with sucrose selection (Su^R) to produce ΔP_{comX}*cibAB* (Table S1).

Construction of *comM* deletion mutant. An amplicon upstream of the *comM* gene was generated by PCR with primers comMUPF and comMUPR (Table S3). Another amplicon downstream of the *comM* gene was generated by PCR using primers comMDNF and comMDNR (Table S3). An amplicon containing erythromycin resistance gene was generated by PCR with primers ermF and ermR (Table S3) using the genomic DNA from strain AD0636 (*spxB*::*erm*) as template. All three amplicons were assembled using the NEBuilder® HiFi DNA Assembly Master Mix. Then, a pair of nesting primers, comMFx and comMRx (Table S3), were used to amplify and assemble the final construct. The assembled construct was used to transform the pneumococcus strains by selecting erythromycin resistance (Erm^R) transformants.

Construction of pneumococcal firefly luciferase reporter strains. An amplicon containing part of pEVP3 plasmid (Table S2) was generated using primers ropF-luc and BamHR-luc (Table S3). An amplicon containing firefly luciferase was generated by using primers lucF and lucR (Table S3). Both amplicons were assembled using the NEBuilder® HiFi DNA Assembly Master Mix, and transformed into *E. coli* DH5α. The correct plasmid

pEVP3-*luc* (Table S2) was verified by restriction digest and sequencing. An amplicon of the *ssbB* gene was generated by PCR using primers ssbF and ssbR (Table S3) and cloned into the pEVP3-*luc* plasmid via BamHI/KpnI digestion and ligation. The resultant plasmid pEVP3-*luc-ssbB* was used to transform the pneumococcal strains by selecting for Cm^R transformants. Similarly, an amplicon of *comM* gene was generated by PCR using primers comMF and comMR1 (Table S3) and cloned into the pEVP3-*luc* plasmid via BamHI/KpnI digestion and ligation. The resultant plasmid pEVP3-*luc-comM* was used to transform the pneumococcal strains by selecting for Cm^R transformants.

Construction of the *luxABCDE* reporter strain. Amplicons containing each of *luxABCDE* genes with Gram-positive ribosomal binding site were generated by PCR with the following primer sets luxAF/luxAR, luxBF/luxBR, luxCF/luxCR, luxDF/luxDR, and luxEF/luxER (Table S3) (1), using pMS402(2) as template. Amplicons were cloned sequentially into pUCP18 plasmid as described (1). After assembly, a DNA fragment containing *luxABCDE* was generated by BamHI/BsiWI digestion and cloned into pEVP3 plasmid (Table S2), and the result plasmid was named pEVP3-*luxABCDE* (Table S2). Then, a *ssbB* gene was transcriptionally fused into the pEVP3-*luxABCDE* plasmid using a KpnI/BamHI digestion, followed by ligation with T4 ligase. The resultant pEVP3-*luxABCDE-ssbB* plasmid (Table S2) was used to transform the pneumococcal strains by selecting for Cm^R resistant reporter strains.

Construction of 3xFLAG tag and *luxABCDE* dual reporter strains in pneumococcus. The dual reporter strains were generated by placing the ribosomal binding sequence of *S. pneumoniae rplL* gene for *luxABCDE* genes. Each of the five *luxABCDE* genes was cloned in sequence into pUCP18 plasmid using the method described above, using the following primer sets: luxnAF/luxnAR, luxnBF/luxnBR, luxnCF/luxnCR, luxnDF/luxnDR, and luxnEF/luxnER (Table S3). After assembly, an amplicon containing the *luxABCDE* genes was generated by PCR using the rLXAEF and rLXAER primers (Table S3). A single strand oligonucleotide named 3xFLAG-FLX3 containing 3xFLAG tag and overlapping regions of the pEVP3 amplicon and *luxA* was synthesized. An amplicon containing part of pEVP3-*luxABCDE* plasmid was generated by PCR using primers RopF-FLX3 and BamR-FLX3 (Table S3). The two fragments were assembled using the NEBuilder® HiFi DNA Assembly Master Mix, and the resultant plasmid was named pEVP3-FLX3 (Table S2), which has a 3xFLAG tag translationally-fused to BamHI site and a *luxABCDE* reporter transcriptionally-fused after the 3xFLAG tag. Then, the *lytA* gene was translationally cloned into this pEVP3-FLX3 by KpnI/BamHI digestion and T4 ligation. The resultant pEVP3-FLX3-*lytA* (Table S2) was used to transform the pneumococcus strains by selecting for Cm^R resistant transformants, for construction of *lytA*-FLX3-expressing pneumococcal reporter strains. For construction of the *cbpD*-FLX3-expressing pneumococcal reporter strains, a DNA fragment of *cbpD* was generated by PCR using primers cbpDp3F/cbpDp3R (Table S3), and assembled into a KpnI/BamHI-digested pEVP3-FLX3 fragment using the NEBuilder® HiFi DNA Assembly Master Mix. The

resulting plasmid pEVP3-FLX3-*cbpD* (Table S2) was transformed into the pneumococcal strains by selecting for Cm^R resistant transformants.

Construction of the ComM-3xFLAG reporter strains in pneumococcus. An amplicon containing part of pEVP3 plasmid (Table S2) was generated by PCR using primers ropF-FLAG and BamR-FLX3 (table S3), and assembled with the single strand oligonucleotide named 3xFLAG-FLX3 (Table S3) by using the NEBuilder® HiFi DNA Assembly Master Mix. The resultant plasmid was named pEVP3-3xFLAG (Table S2). A *comM* gene was amplified by PCR with chromosomal DNA from D39 strain as template by using primers comMF and comMR (Table S3), and translationally-fused into this pEVP3-3xFLAG plasmid using KpnI/BamHI digestion and T4 ligation. The resultant pEVP3-3xFLAG-*comM* plasmid was transformed into the pneumococcal strains by selecting for Cm^R resistant transformants.

Construction of ComM-3xFLAG-based knockout strains. A Δ*comCDE-comM*-3xFLAG strain was obtained by transforming WT-*comM*-3xFLAG strain with chromosomal DNA of AD2064 (Δ*comCDE*) with the selection for kanamycin resistance (Kan^R) and chloramphenicol (Cm^R). A Δ*comX1*Δ*comX2-comM*-3xFLAG strain was obtained by transforming WT-*comM*-3xFLAG strain with chromosomal DNA of FS1975 (Δ*comX1*Δ*comX2*) twice with the first-time selection for Kan^R and the second-time selection for Kan^R, Erm^R, and Cm^R. Similarly, Δ*dprA-comM*-3xFLAG strain, Δ*lytA-comM*-3xFLAG strain, Δ*cbpD-comM*-3xFLAG strain, Δ*cibAB-comM*-3xFLAG strain were

obtained by transforming WT-*comM*-3xFLAG strain with chromosomal DNA from AD1122 (Δ*dprA*), AD1737 (Δ*lytA*), AD2028 (Δ*cbpD*), and AD0133 (Δ*cibAB*), respectively. To construct Δ*lytA*, Δ*cbpD*, and Δ*cibAB* knockout in Δ*dprA comM*-3xFLAG background, similar transformation strategy was used, except that we used Δ*dprA* (Erm^R) instead of Δ*dprA* (Kan^R). To construct the Δ*dprA* (Erm^R) comM-3xFLAG strain, a strategy similar to *comM* deletion mentioned above was used. An amplicon upstream of the *dprA* gene was generated by PCR with primers dprAUPF and dprAUPR (Table S3). Another amplicon downstream of the *comM* gene was generated by PCR using primers dprADNF and dprADNR (Table S3). An amplicon containing erythromycin resistance gene was generated by PCR with primers ermF and ermR (Table S3) using the genomic DNA from strain AD0636 (*spxB*::*erm*) as template. All three amplicons were assembled using the NEBuilder® HiFi DNA Assembly Master Mix. Then, a pair of nesting primers, dprAFx and dprARx (Table S3), were used to amplify and assemble the final construct. The assembled construct was used to transform the pneumococcus strains, WT-*comM*-3xFLAG, Δ*lytA*-*comM*-3xFLAG, Δ*cbpD*-*comM*-3xFLAG, and Δ*cibAB*-*comM*-3xFLAG, by selecting Erm^R and Cm^R transformants, to generate Δ*dprA*(Erm^R)-*comM*-3xFLAG, Δ*dprA*Δ*lytA*-*comM*-3xFLAG, Δ*dprA*Δ*cbpD*-*comM*-3xFLAG, and Δ*dprA*Δ*cibAB*-*comM*-3xFLAG, respectively.

Construction of the *rplL-luxABCDE* reporter strain. An amplicon with *rplL* gene was generated through PCR using a pair of primers named rplLF and rplLR (Table S3). PCR products were digested with KpnI/BamHI and ligated into the pEVP3-*luxABCDE* vector

and used to transform *E. coli* DH5α. The resultant plasmid pEVP3-*luxABCDE-rplL* was verified by restriction digest and used to transform the *S. pneumoniae* strain D39 strain with the selection for chloramphenicol resistant (Cm^R) colonies to generate the WT-*rplL-luxABCDE* expressing strain.

Table S1. *Streptococcus pneumoniae* strains used in this study.

Strain	Relevant Properties	Origin/Ref.
D39 (WT)	Wild-type *S. pneumoniae*, serotype 2	(3)
CP1250	A non-encapsulated derivative of *S. pneumoniae* strain Rx, which itself was a derivative of D39	(4)
AD1122 (Δ*dprA*)	D39, but Δ*dprA*::Kan, Kan^R	(5)
GW1941 (Δ*cbpD*Δ*lytA*)	D39, but Δ*cbpD*::pEVP3, Δ*lytA*::Erm; Cm^R, Erm^R	(5)
Δ*dprA*Δ*cbpD*Δ*lytA*	D39, but Δ*dprA*::Kan, Δ*cbpD*::pEVP3, Δ*lytA*::Erm; Kan^R, Cm^R, Erm^R	This work
WT-*rplL-lacZ*	D39, but a promoterless *lacZ* reporter gene from pEVP3 transcriptionally-fused behind the *rplL* gene; Kan^R	This work
D39-Δ*dprA-rplL-lacZ*	D39, but Δ*dprA*::Kan, a promoterless *lacZ* reporter gene from pEVP3 transcriptionally-fused behind the *rplL* gene; Kan^R, Cm^R	This work
CP1250-*rplL-lacZ*	CP1250, but with a promoterless *lacZ* reporter gene from pEVP3 transcriptionally-fused behind the *rplL* gene; Kan^R	This work
CP1250-Δ*dprA-rplL-lacZ*	CP1250, but with a Δ*dprA*::Kan, a promoterless *lacZ* reporter gene from pEVP3 transcriptionally-fused behind the *rplL* gene; Kan^R, Cm^R	This work
SpnYL001 (Sweet Janus Cassette, SJC)	Sm^R TIGR4 (serotype 4 isolate) by selection of mutations, but *cps*::*sacB-kan-rpsL*$^+$; Km^R, Sm^S, Su^S	(6)
cbpD::SJC	D39, *cbpD*::*sacB-kan-rpsL*$^+$; Km^R, Sm^S, Su^S	This work

cibAB::SJC	D39, *cibAB*::*sacB-kan-rpsL*$^{+}$; Km^{R}, Sm^{S}, Su^{S}	This work
WTΔP$_{comX}$*cbpD*	D39, but ΔP$_{comX}$*cbpD*	This work
Δ*dprA*ΔP$_{comX}$*cbpD*	D39, but Δ*dprA*::Kan, ΔP$_{comX}$*cbpD*; Kan^{R}	This work
WTΔP$_{comX}$*cibAB*	D39, but ΔP$_{comX}$*cibAB*	This work
Δ*dprA*ΔP$_{comX}$*cibAB*	D39, but Δ*dprA*::Kan, ΔP$_{comX}$*cibAB*; Kan^{R}	This work
AD1737 (Δ*lytA*)	D39, but Δ*lytA*::Kan, Kan^{R}	(5)
AD2028 (Δ*cbpD*)	D39, but Δ*cbpD*::Kan, Kan^{R}	(5)
AD0133 (Δ*cibAB*)	D39, but Δ*cibAB*::Kan, Kan^{R}	(5)
AD2064 (Δ*comCDE*)	D39, but Δ*comCDE*::Kan, Kan^{R}	(5)
FS1975 (Δ*comX1*Δ*comX2*)	D39, but Δ*comX1*::Kan, Δ*comX2*::Erm; Kan^{R}, Erm^{R}	(5)
AD0636 (Δ*spxB*)	D39, but Δ*spxB*::Erm, Erm^{R}	(5)
Δ*comM*	D39, but Δ*comM*::Erm, Erm^{R}	This work
Δ*dprA*Δ*comM*	D39, but Δ*dprA*::Kan, Δ*comM*::Erm; Kan^{R}, Erm^{R}	This work
WT-*ssbB-luxABCDE*	D39, but promoterless bioluminescence reporter genes *luxABCDE* transcriptionally-fused behind the *ssbB* gene; Cm^{R}	This work
Δ*dprA-ssbB-luxABCDE*	D39, but Δ*dprA*::Kan, promoterless bioluminescence reporter genes *luxABCDE* transcriptionally-fused behind the *ssbB* gene; Kan^{R}, Cm^{R}	This work
D39-*ssbB-luc*	D39, but with a promoterless firefly luciferase *luc* reporter gene transcriptionally-fused behind the *ssbB* gene; Cm^{R}	This work

D39-Δ*dprA-ssbB-luc*	D39, but Δ*dprA*::Kan, with a promoterless firefly luciferase *luc* reporter gene transcriptionally-fused behind the *ssbB* gene; Kan^R, Cm^R	This work
CP1250-*ssbB-luc*	CP1250, but with a promoterless firefly luciferase *luc* reporter gene transcriptionally-fused behind the *ssbB* gene; Cm^R	This work
CP1250-Δ*dprA-ssbB-luc*	CP1250, but Δ*dprA*::Kan, with a promoterless firefly luciferase *luc* reporter gene transcriptionally-fused behind the *ssbB* gene; Kan^R, Cm^R	This work
WT-*lytA*-FLX3	D39, but with 3xFLAG tag translationally-fused to C-terminus of *lytA*, followed by transcriptionally-fused promoterless bioluminescence reporter genes *luxABCDE*; Cm^R	This work
Δ*dprA-lytA*-FLX3	D39, but Δ*dprA*::Kan, with 3xFLAG tag translationally-fused to C-terminus of *lytA*, followed by transcriptionally fused promoterless bioluminescent reporter genes *luxABCDE*; Kan^R, Cm^R	This work
WT-*cbpD*-FLX3	D39, but 3xFLAG tag was translationally fused to C-terminus of *cbpD*, followed by transcriptionally-fused promoterless bioluminescence reporter genes *luxABCDE*; Cm^R	This work
Δ*dprA-cbpD*-FLX3	D39, but Δ*dprA*::Kan, 3xFLAG tag was translationally fused to C-terminus of *cbpD*, followed by transcriptionally fused promoterless bioluminescence reporter genes *luxABCDE*; Kan^R, Cm^R	This work
WT-ΔP_{comX}*cbpD-cbpD*-FLX3	D39, but ΔP_{comX}*cbpD*, 3xFLAG tag was translationally fused to C-terminus of *cbpD*, followed by transcriptionally fused promoterless bioluminescence reporter genes *luxABCDE*; Cm^R	This work

Δ*dprA*-ΔP_{comX}*cbpD-cbpD*-FLX3	D39, but Δ*dprA*::Kan, ΔP_{comX}*cbpD*, 3xFLAG tag was translationally fused to C-terminus of *cbpD*, followed by transcriptionally fused promoterless bioluminescence reporter genes *luxABCDE*; Cm^R	This work
WT-*comM-luc*	D39, but a promoterless firefly luciferase *luc* reporter gene was transcriptionally-fused behind the *comM* gene; Cm^R	This work
Δ*dprA-comM-luc*	D39, but Δ*dprA*::Kan, with a promoterless firefly luciferase *luc* reporter gene transcriptionally-fused behind the *comM* gene; Kan^R, Cm^R	This work
WT-*comM*-3xFLAG	D39, but 3xFLAG tag was translationally-fused to the C-terminus of *comM*; Cm^R	This work
Δ*dprA-comM*-3xFLAG	D39, but Δ*dprA*::Kan, and 3xFLAG tag was translationally-fused to the C-terminus of *comM*; Kan^R, Cm^R	This work
Δ*comCDE-comM*-3xFLAG	D39, but Δ*comCDE*::Kan, and 3xFLAG tag was translationally-fused to the C-terminus of *comM*; Kan^R, Cm^R	This work
Δ*comX1*Δ*comX2-comM*-3xFLAG	D39, but Δ*comX1*::Kan, Δ*comX2*::Erm, and 3xFLAG tag was translationally-fused to the C-terminus of *comM*; Kan^R, Erm^R, Cm^R	This work
Δ*lytA-comM*-3xFLAG	D39, but Δ*lytA*::Kan, and 3xFLAG tag was translationally-fused to the C-terminus of *comM*; Kan^R, Cm^R	This work
Δ*cbpD-comM*-3xFLAG	D39, but Δ*cbpD*::Kan, and 3xFLAG tag was translationally-fused to the C-terminus of *comM*; Kan^R, Cm^R	This work

Δ*cibAB-comM*-3xFLAG	D39, but Δ*cibAB*::Kan, and 3xFLAG tag was translationally-fused to the C-terminus of *comM*; Kan^R, Cm^R	This work
Δ*dprA*(Erm^R)-comM-3xFLAG	D39, but Δ*dprA*::Erm, and 3xFLAG tag was translationally-fused to the C-terminus of *comM*; Erm^R, Cm^R	This work
Δ*dprA*Δ*lytA-comM*-3xFLAG	D39, but Δ*dprA*::Erm, Δ*lytA*::Kan, and 3xFLAG tag was translationally-fused to the C-terminus of *comM*; Erm^R, Kan^R, Cm^R	This work
Δ*dprA*Δ*cbpD-comM*-3xFLAG	D39, but Δ*dprA*::Erm, Δ*cbpD*::Kan, and 3xFLAG tag was translationally-fused to the C-terminus of *comM*; Erm^R, Kan^R, Cm^R	This work
Δ*dprA*Δ*cibAB-comM*-3xFLAG	D39, but Δ*dprA*::Erm, Δ*cibAB*::Kan, and 3xFLAG tag was translationally-fused to the C-terminus of *comM*; Erm^R, Kan^R, Cm^R	This work
WT-*rplL-luxABCDE*	D39, but promoterless bioluminescence reporter genes *luxABCDE* transcriptionally-fused behind the *rplL* gene; Cm^R	This work
Δ*dprA-rplL-luxABCDE*	D39, but Δ*dprA*::Kan, promoterless bioluminescence reporter genes *luxABCDE* transcriptionally-fused behind the *rplL* gene; Kan^R, Cm^R	This work

Table S2. Plasmids used in this study.

Plasmid	Relevant Properties	Origin/Reference
pEVP3	Stable in *E. coli*, contains *lacZ* transcriptional reporter; Cm^R	(7)
pEVP3-*rplL*	pEVP3, but has *lacZ* transcriptionally-fused behind the *rplL* gene; Cm^R	This work
pEVP3-*luc*	pEVP3, but has *lacZ* gene replaced by a transcriptional firefly luciferase reporter *luc*; Cm^R	This work
pEVP3-*luc-ssbB*	pEVP3-*luc*, but with the *luc* gene transcriptionally-fused behind the *ssbB* gene; Cm^R	This work
pEVP3-*luxABCDE*	pEVP3, but has *lacZ* gene replaced by the transcriptionally-fused bioluminescence reporter *luxABCDE*; Cm^R	This work
pEVP3-*luxABCDE-ssbB*	pEVP3-*luxABCDE*, but with *luxABCDE* transcriptionally-fused behind the *ssbB* gene; Cm^R	This work
pEVP3-FLX3	pEVP3, but has *lacZ* gene replaced by a translationally-fused 3xFLAG tag, followed by a transcriptionally-fused bioluminescence reporter *luxABCDE*; Cm^R	This work
pEVP3-FLX3-*lytA*	pEVP3-FLX3, but has 3xFLAG tag translationally-fused to the C-terminus of the *lytA* gene, followed by the bioluminescence reporter *luxABCDE* genes transcriptionally-fused to the 3xFLAG-tagged *lytA* gene; Cm^R	This work
pEVP3-FLX3-*cbpD*	pEVP3-FLX3, but has a 3xFLAG tag translationally-fused to the C-terminus of *cbpD*, and with the bioluminescence reporter *luxABCDE* transcriptionally-fused to the 3xFLAG-tagged *cbpD* gene; Cm^R	This work

pEVP3-*luc-comM*	pEVP3-*luc*, but with the *luc* gene transcriptionally-fused to the *comM* gene; Cm^R	This work
pEVP3-3xFLAG	pEVP3, but has the *lacZ* gene replaced by a translational-fused 3xFLAG tag; Cm^R	This work
pEVP3-3xFLAG-*comM*	pEVP3-3xFLAG, but has a 3xFLAG tag translationally-fused to the C-terminus of the *comM* gene; Cm^R	This work
pEVP3-*rplL-luxABCDE*	pEVP3-*luxABCDE*, but with *luxABCDE* transcriptionally-fused behind the *rplL* gene; Cm^R	This work

Plasmids were maintained in DH5α with chloramphenicol antibiotics at 4 ug ml^{-1}.

Table S3 Oligonucleotides used in this study

Oligonucleotides	Sequence (5' to 3')	Used in*
rplLF	CGGGGTACCCTTTCTATGCTCCTTTCTGTACTTC	pEVP3-rplL/ pEVP3-*luxABCDE-rplL*
rplLR	CGCGGATCCACTGGTAAATAGGGATTCCGCA	pEVP3-rplL/ pEVP3-*luxABCDE-rplL*
SJCF	TCTGTCTTGATTGAAAACACCCTA	*cbpD*::SJC / *cibAB*::SJC
SJCR	TTATGCTTTTGGACGTTTAGTACC	*cbpD*::SJC / *cibAB*::SJC
cbpDSJCU1	TATTGGTGGCTTGTCTTACTTGGTTG	*cbpD*::SJC / ΔP_{comX}*cbpD*
cbpDSJCU2	GTGTTTTCAATCAAGACAGAGTCATTTCTACGACTTGTC CCTCATG	*cbpD*::SJC
cbpDSJCD1	CTAAACGTCCAAAAGCATAAGCTTATGGAAATGCGAAT GAATGGG	*cbpD*::SJC
cbpDSJCD2	CCCAGATAATACCACGAGCCATTC	*cbpD*::SJC / ΔP_{comX}*cbpD*
cbpDFx	TGGAAGCTCGCTCGCAACAAACGAC	*cbpD*::SJC / ΔP_{comX}*cbpD*
cbpDRx	CAGCTAGAACCGTCTTTCAGCCATCCTGT	*cbpD*::SJC / ΔP_{comX}*cbpD*
cbpDx1	CCTTCACCTAAATGAAAAAGTGAGAAAATTTCTCACTTT AGTCAAG	ΔP_{comX}*cbpD*
cbpDx2	TCACTTTTTCATTTAGGTGAAGGCAATCATCGCT	ΔP_{comX}*cbpD*
cibABSJCU1	CGATAACTGCCATCTTTTCTTTCTTGTA	*cibAB*::SJC / ΔP_{comX}*cibAB*
cibABSJCU2	GTGTTTTCAATCAAGACAGACGGAAAAGAAAAGGAAAA GTGAGCAT	*cibAB*::SJC

cibABSJCD1	CTAAACGTCCAAAAGCATAAGTTGGTATCGCTTTATTTATGGCAGGT	*cibAB*::SJC
cibABSJCD2	CGACTTCTTCAGGTTTAAGAGTGTAGG	*cibAB*::SJC / ΔP_{comX}*cibAB*
cibABFx	CTTCTGCTTCAAAGAGAGTCTCCTG	*cibAB*::SJC / ΔP_{comX}*cibAB*
cibABRx	GACAAGTTGGCGAGCAGTGATA	*cibAB*::SJC / ΔP_{comX}*cibAB*
cibABx1	CTTGTTCACTTTTAAGGAAAAGTGAGCATTTTTTTTAAATTATTTTTAGTTAAG	ΔP_{comX}*cibAB*
cibABx2	TCACTTTTCCTTAAAAGTGAACAAGAAAAAAGGAGGAAAGTTCAATG	ΔP_{comX}*cibAB*
comMUPF	TTGAGACTCTCCCAACAAACTCTTG	Δ*comM*
comMUPR	AACAAATTTTGGGCCCGGATCTTCTCTCTCCCTTCCTACCAAT	Δ*comM*
comMDNF	ATGAGTCGCTGCCGACTCTTGTATTGGTCATTGTTACGCTCA	Δ*comM*
comMDNR	TTGTGCCCACTCTATCGCTTCTT	Δ*comM*
ermF	CCGGGCCCAAAATTTGTTTGATTT	Δ*comM*
ermR	AGTCGGCAGCGACTCATAGAAT	Δ*comM*
comMFx	GAAGACCGAACTTACCTTGAATGGA	Δ*comM*
comMRx	GCAAGTAAGGTTATCTGATTGTCCG	Δ*comM*
ropF-luc	TCGCCGTGTAACAAGTGACCAAACAGGAAAAAACC	pEVP3-*luc*
BamHR-luc	AGAGAGTCCTCCTCGGATCCATATGACGTCGACGC	pEVP3-*luc*
lucF	TCATATGGATCCGAGGAGGACTCTCTATGGAAGACGCCAAAAACATAAAGAAAGGC	pEVP3-*luc*
lucR	GTTTGGTCACTTGTTACACGGCGATCTTTCCGCCCTTC	pEVP3-*luc*
luxAF	CCCGGATCCGAATTCAGGAGGACTCTCTATGAAATTTG	pEVP3-*luxABCDE*

luxAR	GGCGGATCCGTCGACTTAATATAATAGCGAACGTTG	pEVP3-*luxABCDE*
luxBF	GGGAATTCTCGAGGAGGAGAAAGAAATGAAATTTGGA	pEVP3-*luxABCDE*
luxBR	GGCGGATCCGTCGACTTAGGTATATTCCATGTGGTAC	pEVP3-*luxABCDE*
luxCF	GGGAATTCTCGAGGAGGATGGCAAATATGACTAA	pEVP3-*luxABCDE*
luxCR	GGCGGATCCGTCGACTTATGGGACAAATACAAGGAAC	pEVP3-*luxABCDE*
luxDF	GGGAATTCTCGAGGAGGAGTAAAAGTATGGAAAATGA	pEVP3-*luxABCDE*
luxDR	GGCGGATCCGTCGACTTAAGACAGAGAAATTGCTTGA	pEVP3-*luxABCDE*
luxEF	GGGAATTCTCGAGGAGGAAAACAGGTATGACTTCATATG	pEVP3-*luxABCDE*
luxER	GGCGTCGACGTACGTTAACTATCAAACGCTTCGGTTA	pEVP3-*luxABCDE*
ssbF	GAGAGAGGTACCATTGGGCGTTTAACGTCTACACC	pEVP3-*luc-ssbB* / pEVP3-*luxABCDE-ssbB*
ssbR	CTCTCTGGATCCTCTTAAAATGGCAATTCTTCCTCTT	pEVP3-*luc-ssbB* / pEVP3-*luxABCDE-ssbB*
luxnAF	CCCGGATCCGAATTCGGAGGAAGAAAATATGAAATTTG	pEVP3-FLX3
luxnAR	CGCGGATCCGTCGACTTATCTTATATAATAGCGAACGTTG	pEVP3-FLX3
luxnBF	CGGGGTACCTCGAGGAGGAAGAAAATATGAAATTTGGATTGTTCTTC	pEVP3-FLX3
luxnBR	CGCGGATCCGTCGACTTATCTTAGGTATATTCCATGTGGTAC	pEVP3-FLX3
luxnCF	CGGGGTACCTCGAGGAGGAAGAAAATATGACTAAAAAAATTTCATTC	pEVP3-FLX3

luxnCR	CGCGGATCCGTCGACTTATCTTATGGGACAAATACAAG GAAC	pEVP3-FLX3
luxnDF	CGGGGTACCTCGAGGAGGAAGAAAATATGGAAAATGA	pEVP3-FLX3
luxnDR	CGCGGATCCGTCGACTTATCTTAGACAGAGAAATTGCT TGA	pEVP3-FLX3
luxnEF	CGGGGTACCTCGAGGAGGAAGAAAATATGACTTCATAT G	pEVP3-FLX3
luxnER	GGCGTCGACGTACGTTAACTATCAAACGCTTCGGTTA	pEVP3-FLX3
RopF-FLX3	TTAACGTACGCAAGTGACCAAACAGGAAAAAACC	pEVP3-FLX3
BamR-FLX3	GTCCTTATAGTCGGATCCATATGACGTCGACGC	pEVP3-FLX3 / pEVP3-3xFLAG
3xFLAG-FLX3	ATGGATCCGACTATAAGGACCATGATGGCGACTATAAG GATCATGACATTGACTATAAGGATGACGACGATAAGTAA GCTAGCAGGA	pEVP3-FLX3 / pEVP3-3xFLAG
rLXAEF	CGATAAGTAAGCTAGCAGGAGGAAGAAAATATGAAATT TGGAAACT	pEVP3-FLX3
rLXAER	TGGTCACTTGCGTACGTTAACTATCAAACGCTTCG	pEVP3-FLX3
lytAp3F	CGGGGTACCTGTAAGGTAGAAATAAAGGAGTTTC	pEVP3-FLX3-*lytA*
lytAp3R	CGCGGATCCTTTTACTGTAATCAAGCCATCTG	pEVP3-FLX3-*lytA*
cbpDp3F	TCGATGCATGCCATGGTACTGTCCATGCCTATTCCAGA GG	pEVP3-FLX3-*cbpD*
cbpDp3R	CATCATGGTCCTTATAGTCGACTCGTTCTCCATCACTGT TTACT	pEVP3-FLX3-*cbpD*
ropF-FLAG	GATAAGTAAGCTAGCAGGACAAGTGACCAAACAGGAAA AAACC	pEVP3-3xFLAG
comMF	GAGAGAGGTACCGAAGATGAAATCAATGAGAATCTTAT TTTTGT	pEVP3-*luc-comM* / pEVP3-3xFLAG-*comM*
comMR1	CTCTCTGGATCCGCTTTCTCTAAAAGAAAATGAGCGT	pEVP3-*luc-comM*
comMR	CTCTCTGGATCCAAAGAAAATGAGCGTAACAATGACCA	pEVP3-3xFLAG-*comM*

dprAUPF	TCTACATTGGGATTCTCTGTGCTTT	Δ*dprA* (Erm) *comM*-3xFLAG
dprAUPR	AACAAATTTTGGGCCCGGAAAGCTCCTGATCAACATTTTCACC	Δ*dprA* (Erm) *comM*-3xFLAG
dprADNF	ATGAGTCGCTGCCGACTGAGCAAAATTGGTCACCAGTGGGCA	Δ*dprA* (Erm) *comM*-3xFLAG
dprADNR	CCCACTCCCAGAATAGGTGTCATCA	Δ*dprA* (Erm) *comM*-3xFLAG
dprAFx	AGGCTACAGGCTTGAACGTGAGCTA	Δ*dprA* (Erm) *comM*-3xFLAG
dprARx	TTCCTTTCCCAGCTAACTAGTCGCT	Δ*dprA* (Erm) *comM*-3xFLAG

* Used in construction of the strains or plasmids.

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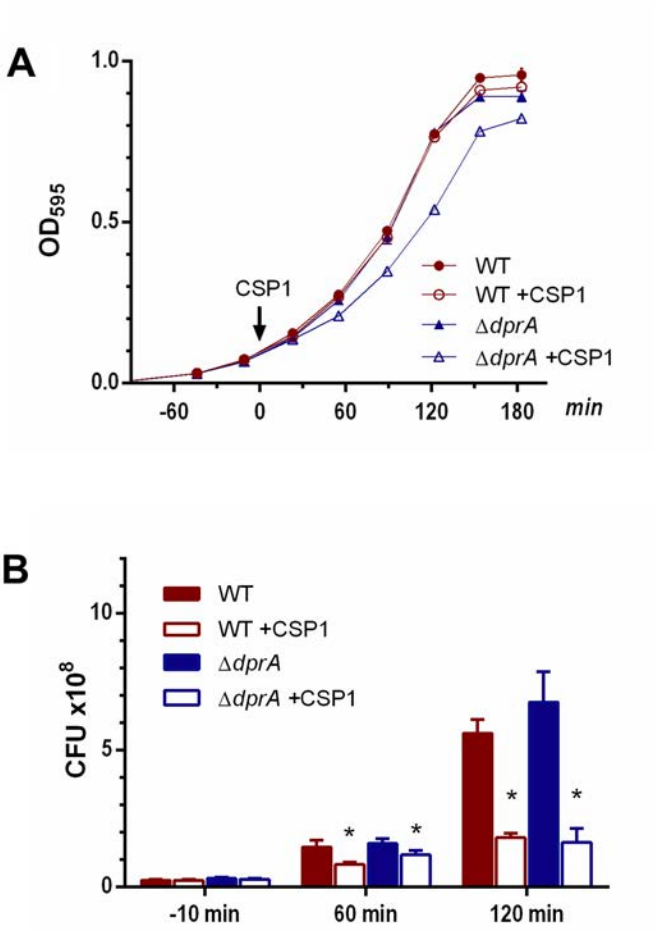

FIG S1. Δ*dprA* exhibits reduced growth *in vitro* after competence induction. (A) Δ*dprA* showed growth delay after competence induction. Both WT and Δ*dprA* were cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract at 37°C. CSP1 was added at OD_{595nm} of ~ 0.1 to a final concentration of 10 ng ml^{-1}. (B) Both WT and Δ*dprA* showed reduced colony forming units (CFU) upon CSP1 induction. CFUs were measured by serial dilution plating in parallel to the growth assays (A) at designated time points. Two-tailed unpaired Student's *t*-test were used to determine the significance of differences in CFU ($p < 0.05$).

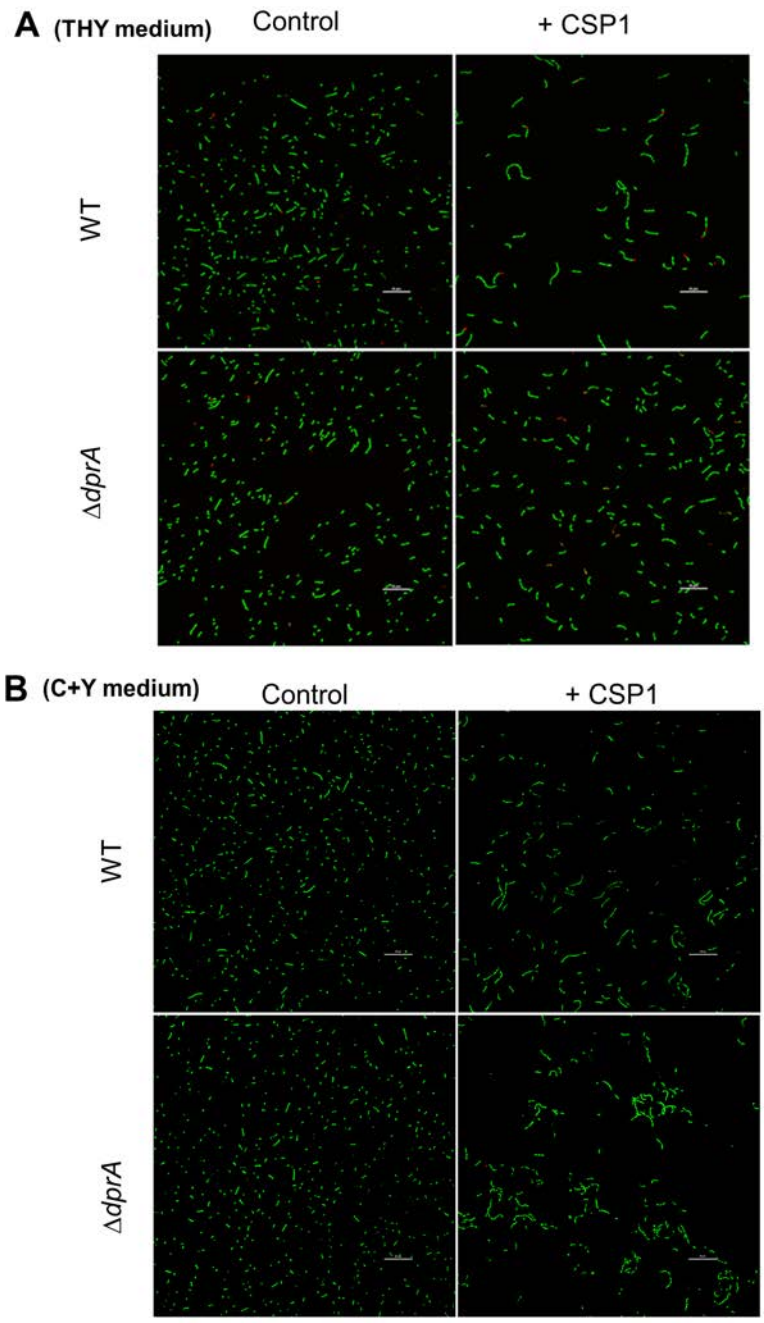

FIG S2. Δ*dprA* does not exhibit more cell death after competence induction *in vitro*. CSP1 (100 ng ml^{-1}) was added at OD_{595nm} of ~ 0.1 to WT and dprA cells cultured in THY medium (A) and C+Y medium (B). After 1 hour (A) or 3 hours (B) of incubation at 37°C, cells were assessed for viability with a Live/Dead staining kit and imaged under a fluorescence microscope.

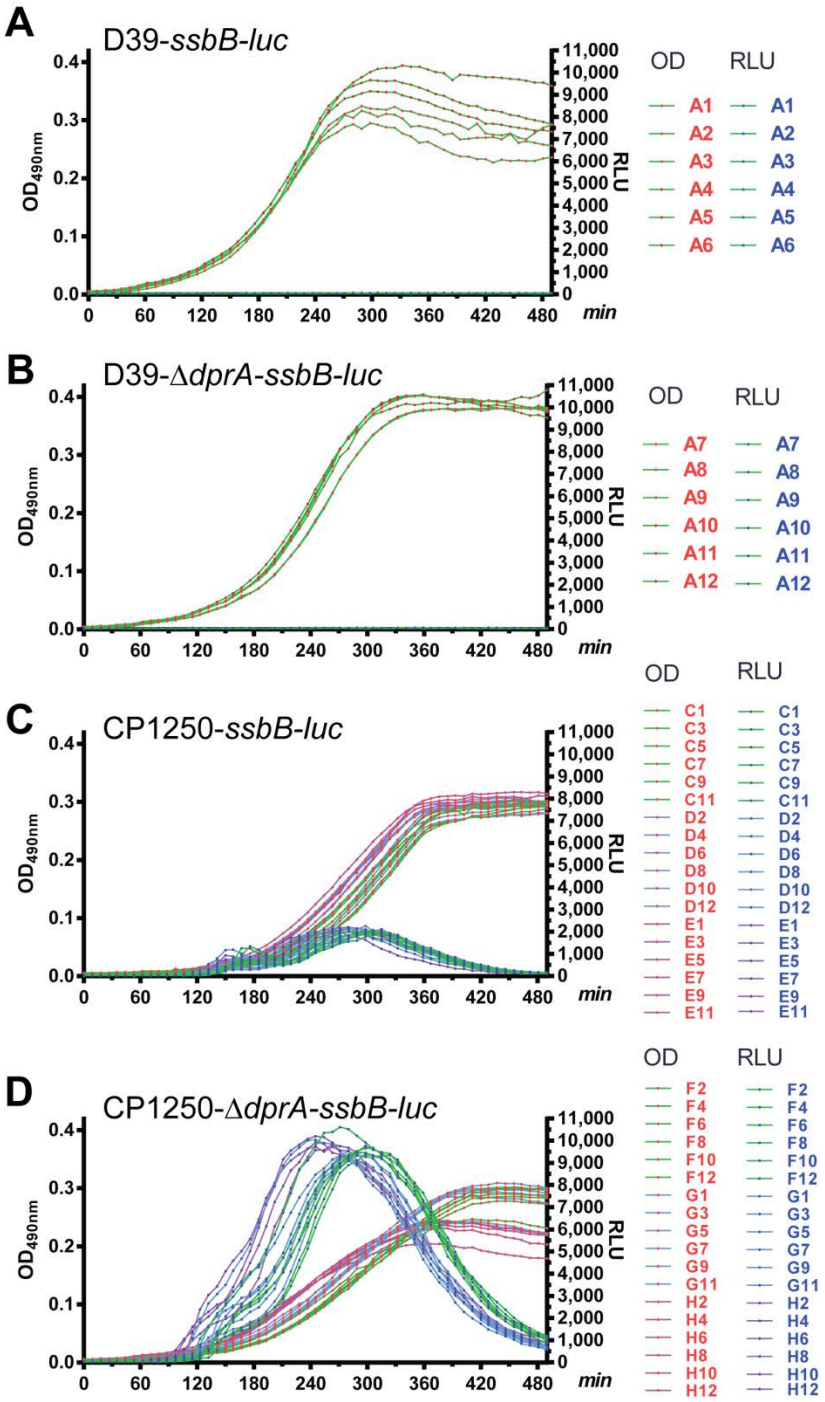

FIG S3. CP1250 strains enter the competent state spontaneously in THY medium. Single colonies from overnight cultures were diluted in the THY medium and distributed into 6 wells each containing 200μl in a 96-well plate. For A1-A6, A7-A12, C1-11, D2-12, E1-11, F2-12, G1-11, and H2-12, each set was from one single colony. D39-*ssbB-luc* (A) and D39-Δ*dprA-ssbB-luc* (B) did not enter the competent state during duration of culturing. CP1250 *ssbB-luc* (C) and CP1250-Δ*dprA-ssbB-luc* (D) in each well entered the competent state.

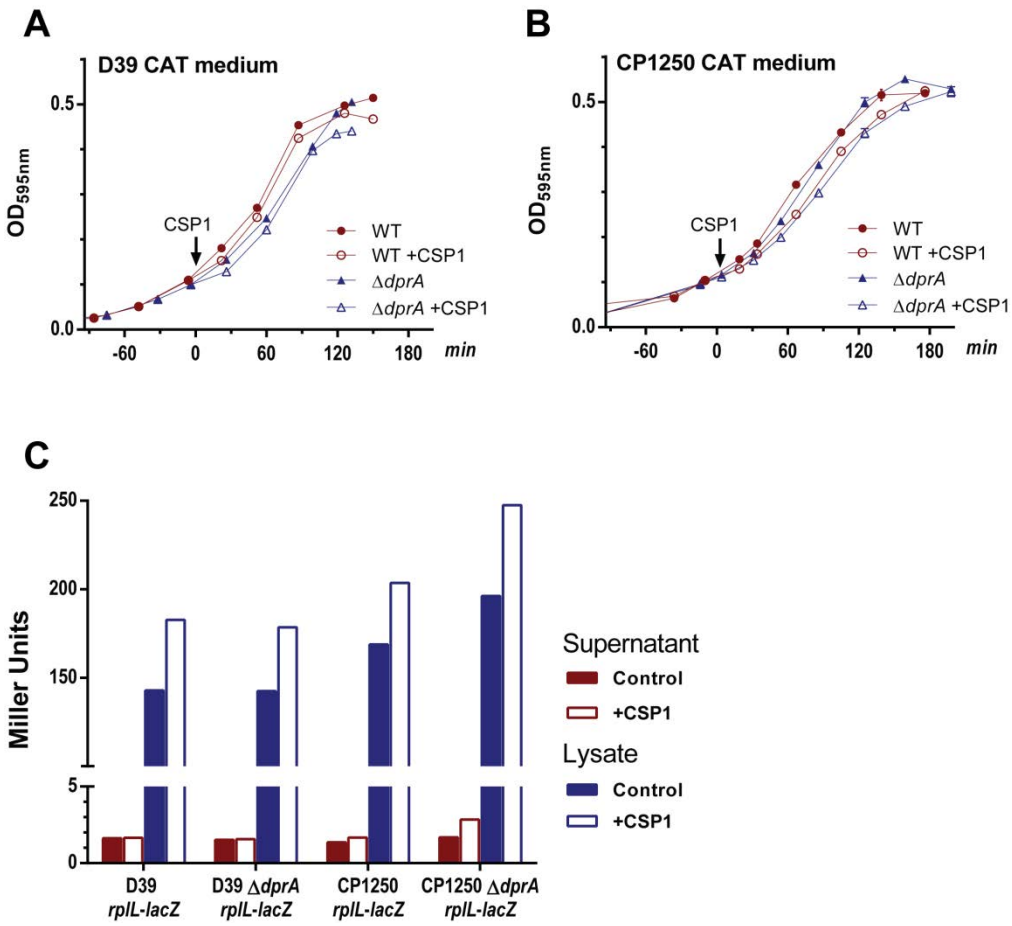

FIG S4. DprA deficient D39 and CP1250 strains do not exhibit significant growth reduction and cell lysis after competence induction in the CAT medium. (A, B) WT and Δ*dprA* cells for both D39 (A) and CP1250 (B) cultured in CAT medium containing 10 mM HCl were induced to competent state by adding $CaCl_2$ (to 0.5mM), BSA (to 0.002%) and CSP1 (to 250 ng ml^{-1}) at the OD_{595nm} of ~ 0.1. (C) Pneumococcal strains harboring constitutively expressing *lacZ* reporter gene were cultured in CAT medium and induced to competence state using conditions described above. Beta-galactosidase activities in supernatants and lysates were measured.

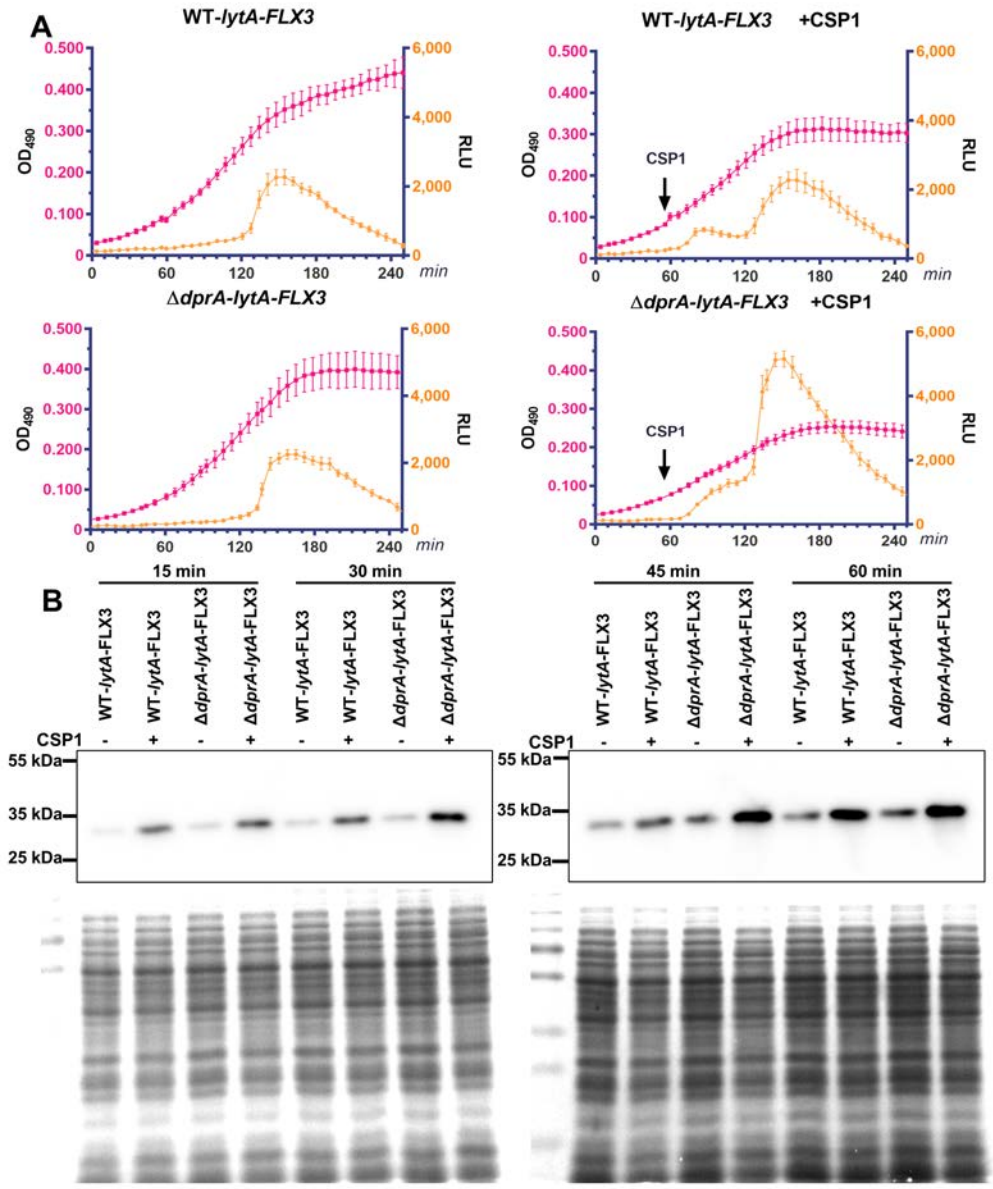

FIG S5. Δ*dprA* expresses higher amount of the allolytic gene *lytA* during competence induction. (A) Pneumococcal growth and *lytA* gene expression were tracked in WT and Δ*dprA* using the *FLX3* dual reporters. The FLX3 dual reporter harbors a 3xFLAG tag translationally-fused to the C-terminus of the *lytA* gene, immediately followed by a transcriptionally-fused promoterless bioluminescent reporter *luxABCDE* (see Table S1). CSP1 (100 ng ml^{-1}) was added at OD_{490nm} ~0.1 (Time 56 min). (B) Expression of LytA protein in WT and Δ*dprA* reporter strains (A) were tracked in parallel after addition of CSP1 for 15, 30, 45, and 60 minutes. Equal amounts of bacterial lysates were loaded onto two 10% polyacrylamide gels. One gel was used for Western blotting and the other one stained with Coomassie blue to demonstrate equal loading.

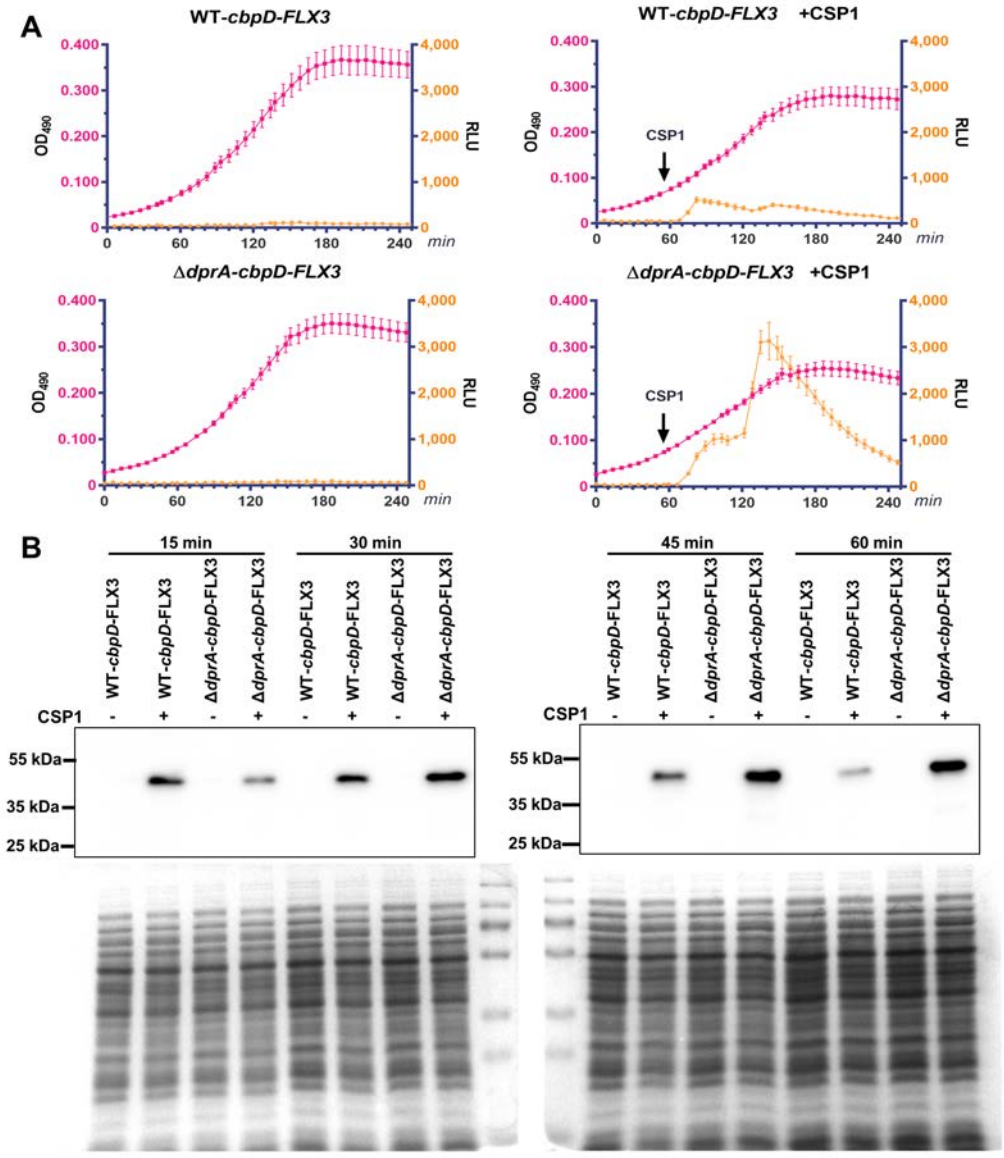

FIG S6. Δ*dprA* expresses higher amount of the allolytic gene *cbpD* during competence induction. (A) Pneumococcal growth and *cbpD* gene expression were tracked in WT and Δ*dprA* using the *FLX3* dual reporters, which harbors a 3xFLAG tag translationally fused to the C-terminus of the *cbpD* gene, immediately followed by a promoterless transcriptionally-fused luminescent reporter *luxABCDE* (see Table S1). CSP1 (100 ng ml^{-1}) was added at OD_{490nm} ~0.1 (Time 56 min). (B) Expression of CbpD protein in WT and Δ*dprA* reporter strains (A) were tracked in parallel after addition of CSP1 for 15, 30, 45, and 60 minutes. Bacterial lysates were loaded onto two 10% polyacrylamide gels. One gel was used for Western blotting and the other one stained with Coomassie blue to demonstrate equal loading.

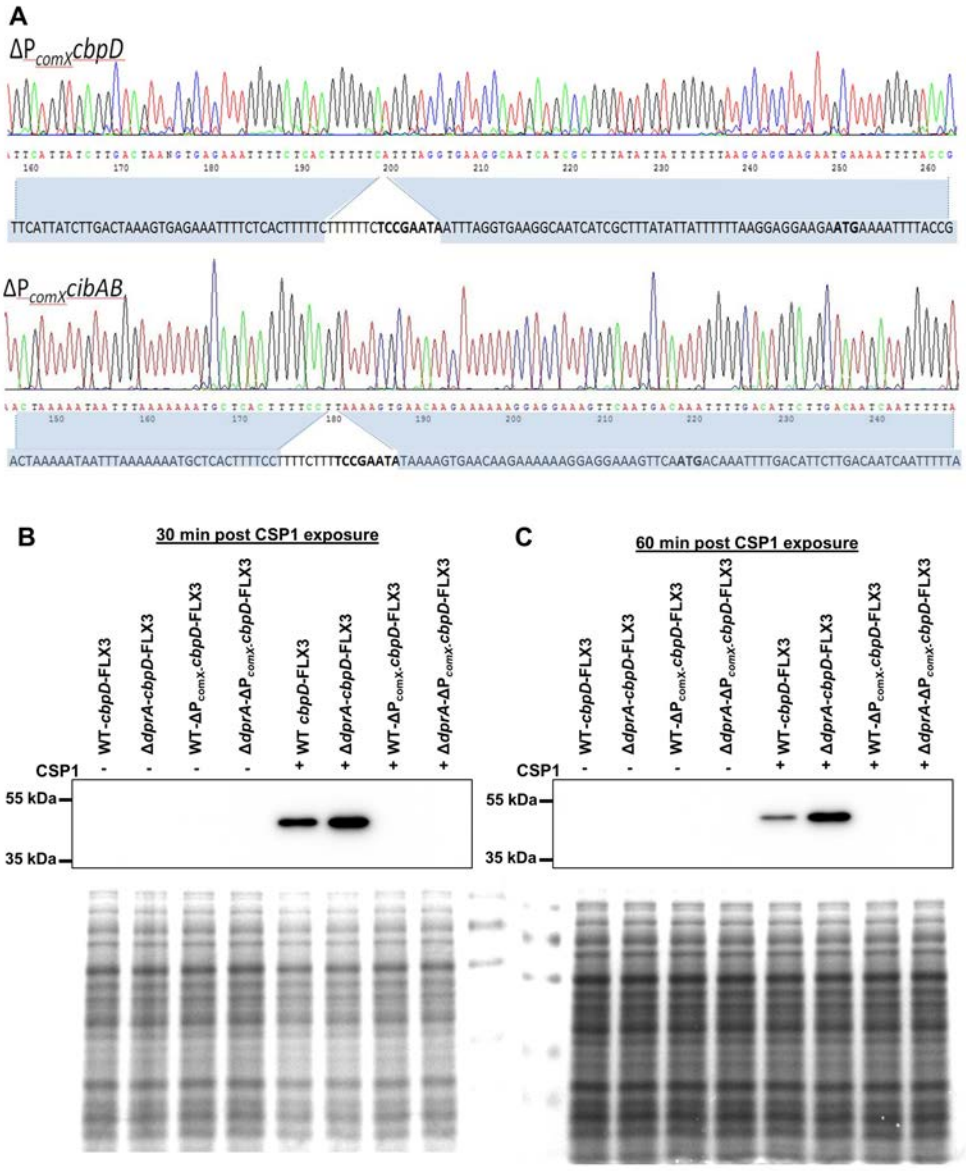

FIG S7. Deletion of combox from the promoter of both *cbpD* and *cibAB* genes abolishes CSP1-mediated induction of both allolytic genes. (A) Deletion of ComX-binding site (combox) from the promoter of *cbpD* and *cibAB* genes. Sequencing results of ΔP_{comX}*cbpD* or ΔP_{comX}*cibAB* at the promoter *cbpD* and *cibAB* genes. The combox (TCCGAATA), and the preceding consensus sequence (TTTTTT or TTCTTT) before *combox* were deleted. (B) Deletion of combox in promoter abolishes CSP1-induced CbpD expression. Pneumococcal strains were transformed with FLX3 dual reporter (see Fig. S6). CSP1 was added at OD_{595nm} ~ 0.1. CbpD expression was tracked after induction with CSP1 (100 ng ml^{-1}) for 30 and 60 minutes. Bacterial lysates were loaded onto two 10% polyacrylamide gels. One gel was used for Western blotting with anti-FLAG antibody and the other one stained with Coomassie blue to demonstrate equal loading.

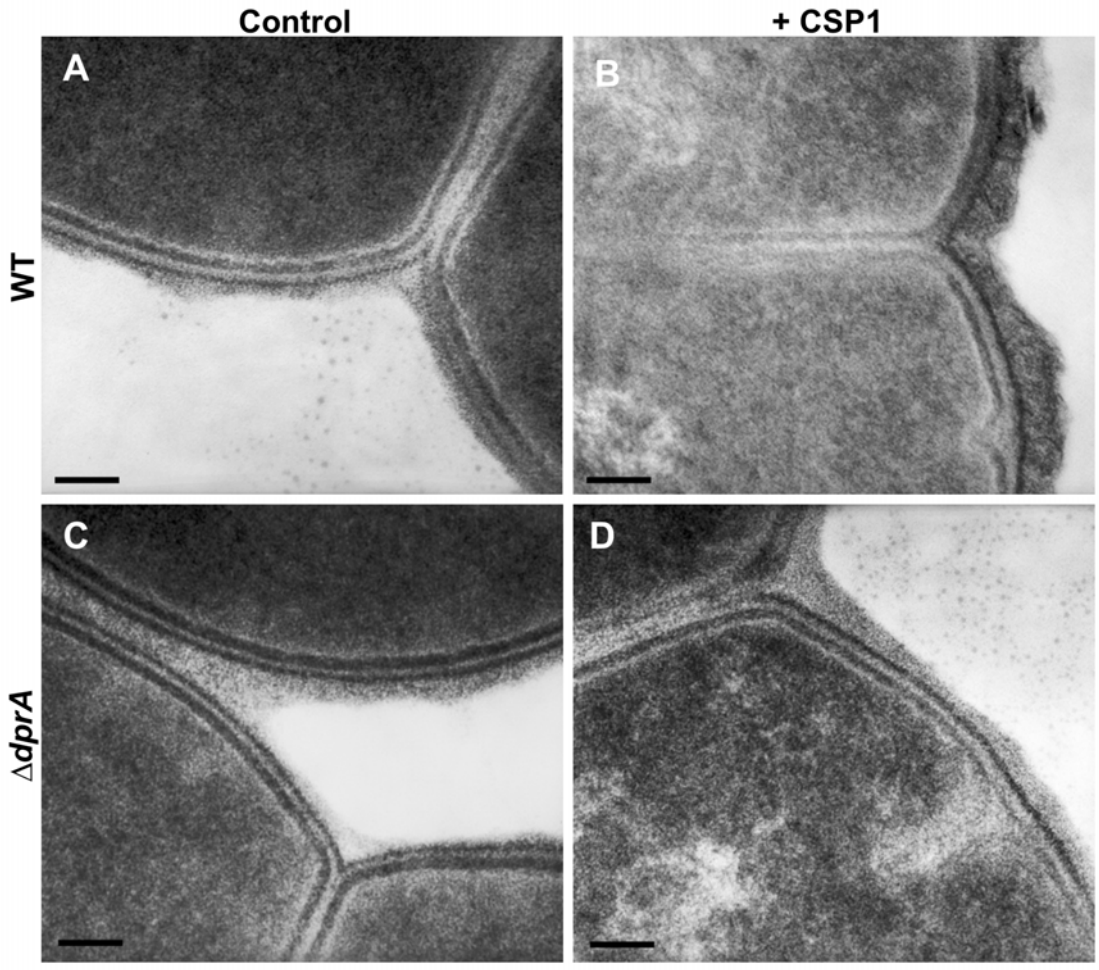

FIG S8. Δ*dprA* does not exhibit aberrant cell wall structures after competence induction. Transmission electron micrographs showing the morphology of WT (A), WT + 100 ng ml^{-1} CSP1 (1 hr) (B), Δ*dprA* (C), and Δ*dprA* + 100 ng ml^{-1} CSP1 (1 hr) (D). Scale bar: 50 nm.

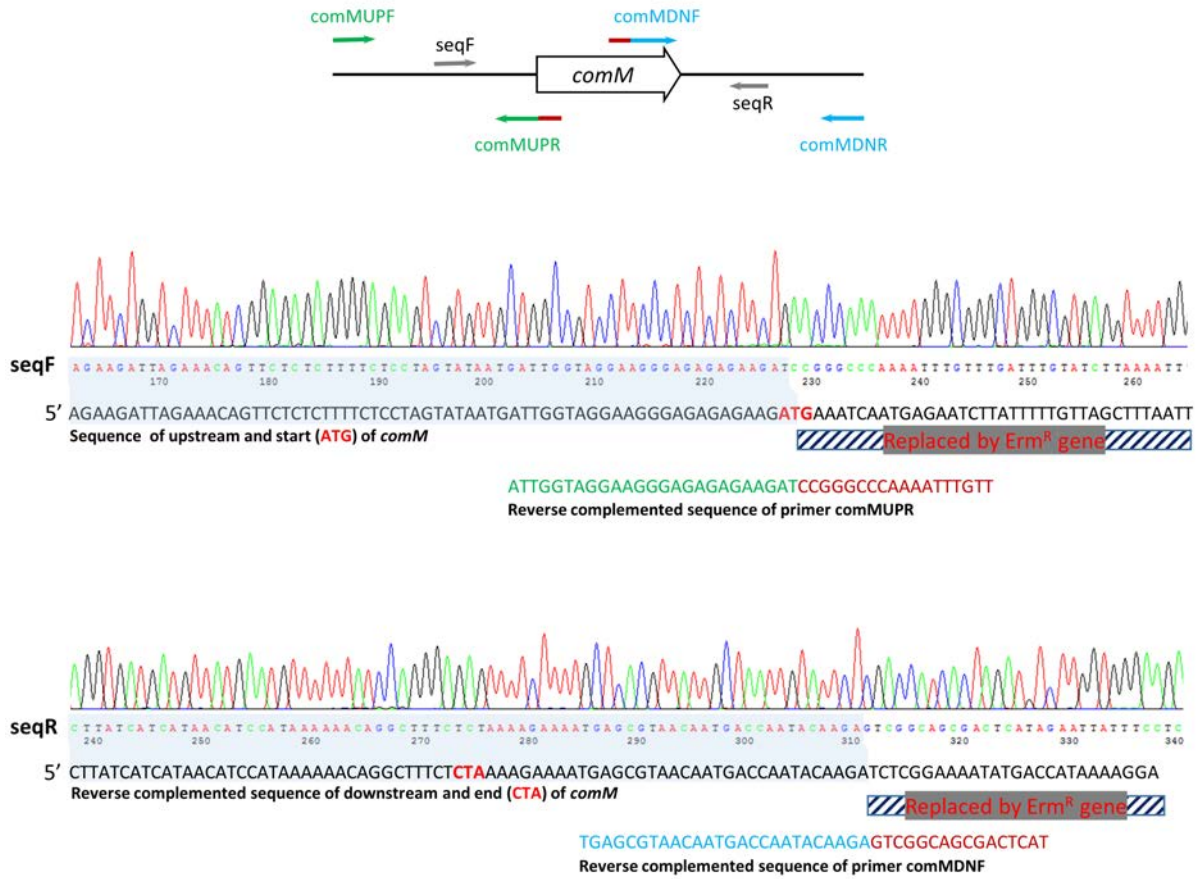

FIG S9. Gene replace of *comM* with the erythromycin resistance gene. Schematic depiction of *comM* deletion and confirmation by DNA sequencing. Sequencing results of Δ*comM* using the upstream primer seqF and the downstream primer seqR. Coding region between 3 - 584 bp nucleotides of the *comM* gene (full length 621 bp) were replaced by erythromycin resistance gene.

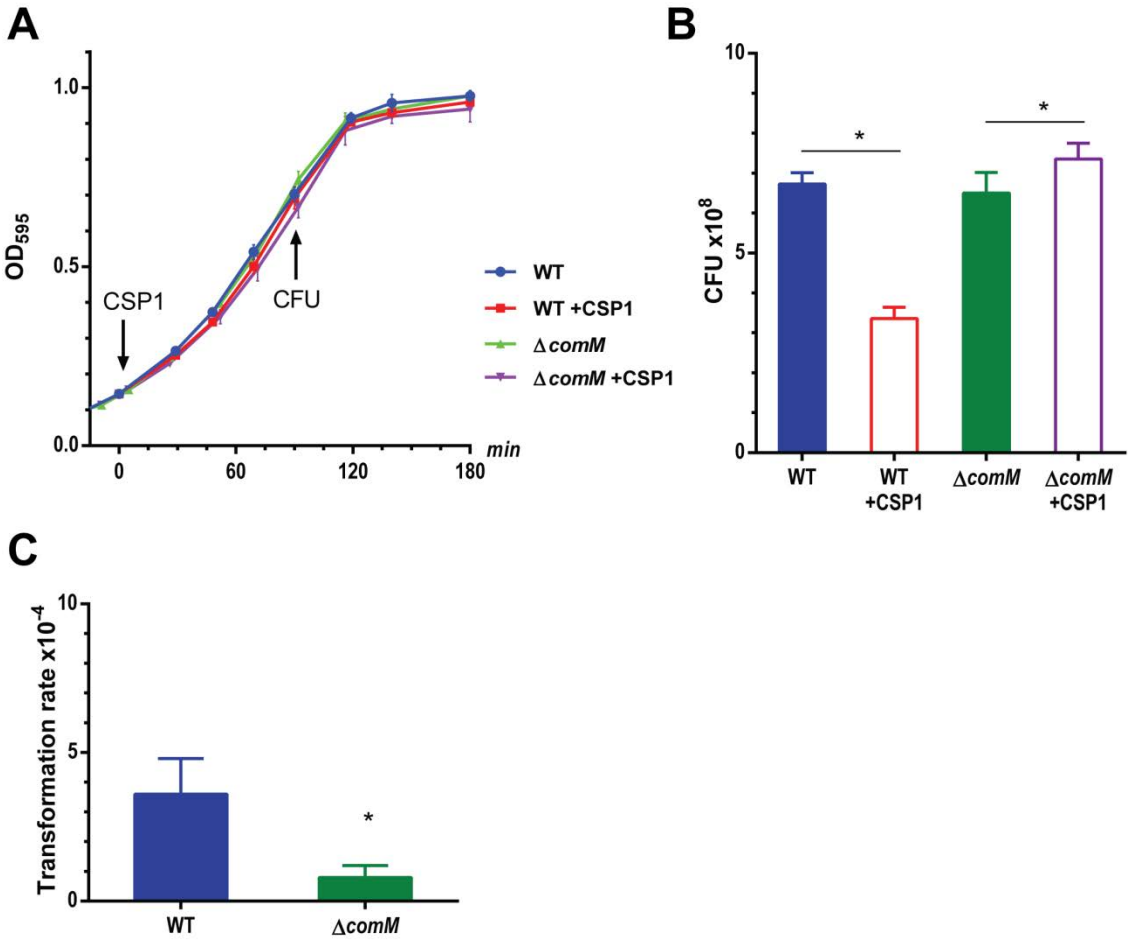

FIG S10. Growth phenotype, CSP1 susceptibility and transformation rate of Δ*comM*. (A) Δ*comM* showed similar growth kinetics as WT with or without competence induction by CSP1 (Time 0 min). Both WT and Δ*dprA* were cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract at 37°C. CSP1 was added at OD_{595nm} of ~ 0.14 to a final concentration of 100 ng ml^{-1}. (B) Δ*comM* is resistant to CSP1-mediated colony forming units (CFU) reduction. CFUs were measured by serial dilution plating in parallel to the growth assays (A) at the 90 min post CSP1 exposure. Two-tailed unpaired Student's *t*-test were used to determine the significance of CFU differences ($p < 0.05$). (C) Δ*comM* has lower transformation rate than WT. WT and Δ*comM* were cultured in C+Y medium to ~OD 0.15 and treated with 100 ng mL^{-1} CSP1 and 50 ng mL^{-1} of a 1.6kbp rpsL+ PCR fragment (Streptomycin resistance) (Zhu, 2014). After 1 hour of CSP1 treatment, the number of transformants and total CFU were determined by serial dilutions and plating on THY plates with or without Streptomycin. The transformation frequency was calculated by dividing #transformants by total CFU. Two-tailed unpaired Student's *t*-test were used to determine the significance differences between transformation rate ($p = 0.0186$).

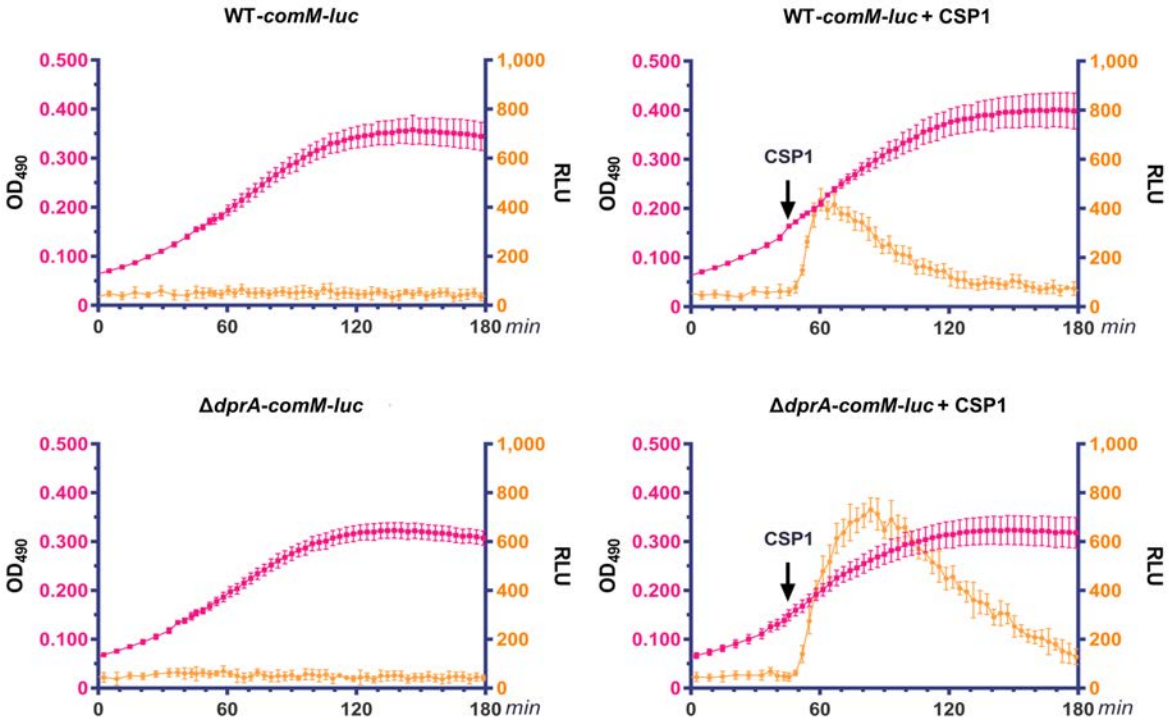

FIG S11. The "early" competence gene *comM* is expressed at higher levels in competent state Δ*dprA*. Expression of *comM* was tracked in both WT and Δ*dprA* using the *comM-luc*, which is a firefly luciferase reporter transcriptionally fused to the promoter of *comM* gene. CSP1 (100 ng ml^{-1}) was added at OD_{490nm} ~0.1 (Time 45 min). Notice that competence induction resulted in lower growth rate and higher expression of *comM* in Δ*dprA* than in WT.

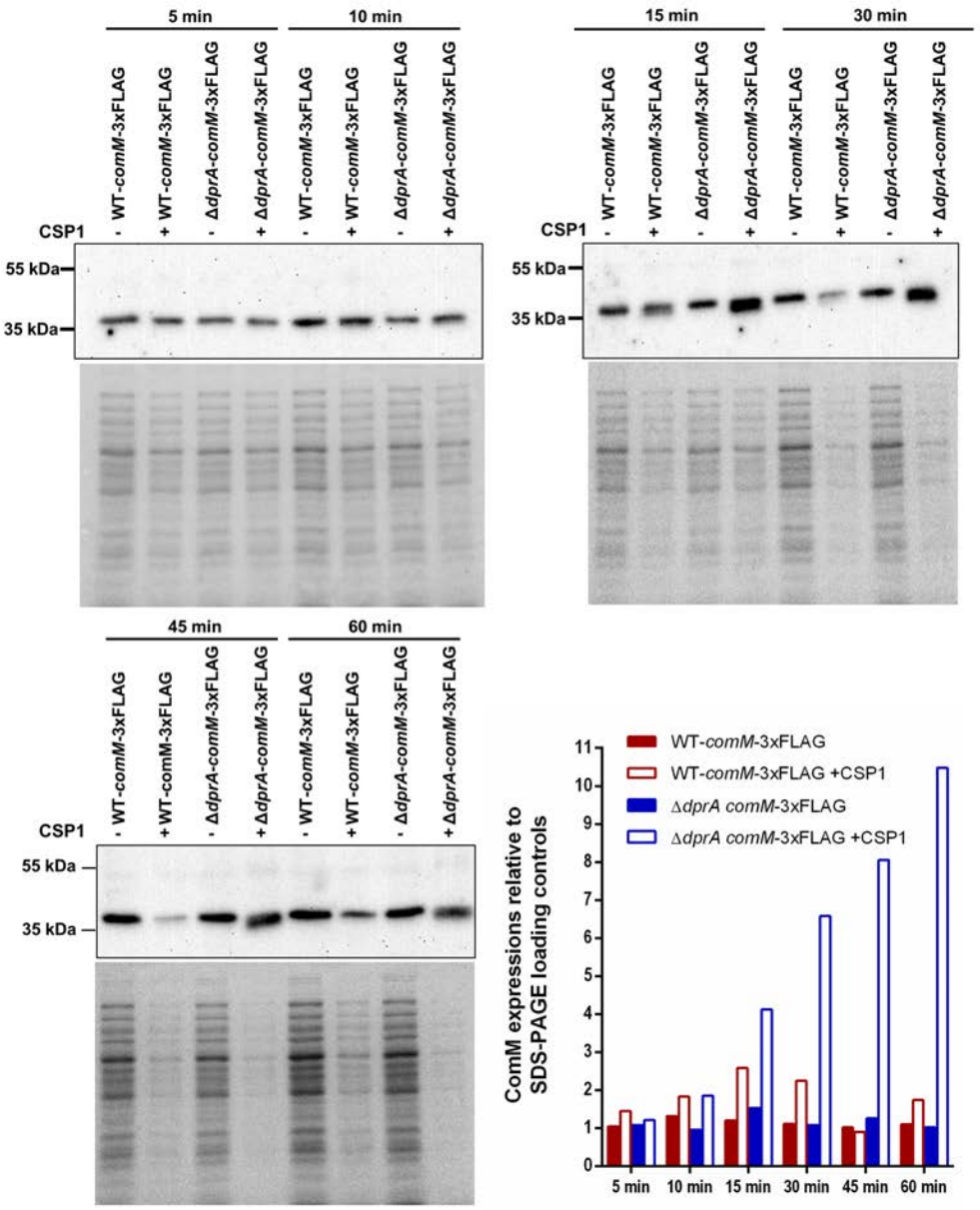

FIG S12. Δ*dprA* overexpresses ComM protein during competence induction. Expression of ComM protein in WT and Δ*dprA* were tracked using the a *comM*-3xFLAG reporter, where a 3xFLAG tag was translationally-fused to the C-terminus of the *comM* gene. Culture samples (1 mL) were collected at 5, 10, 15, 30, 45 and 60 minutes post-CSP1 (100 ng ml^{-1}) treatment at OD_{595nm} ~0.1. Bacterial lysates were loaded onto two 10% polyacrylamide gels. One gel was used for Western blotting with anti-FLAG antibody and the other one stained with Coomassie blue as loading control. Densitometry analyses of ComM expression were performed using the ImageJ software (NIH) and normalized against the loading controls.

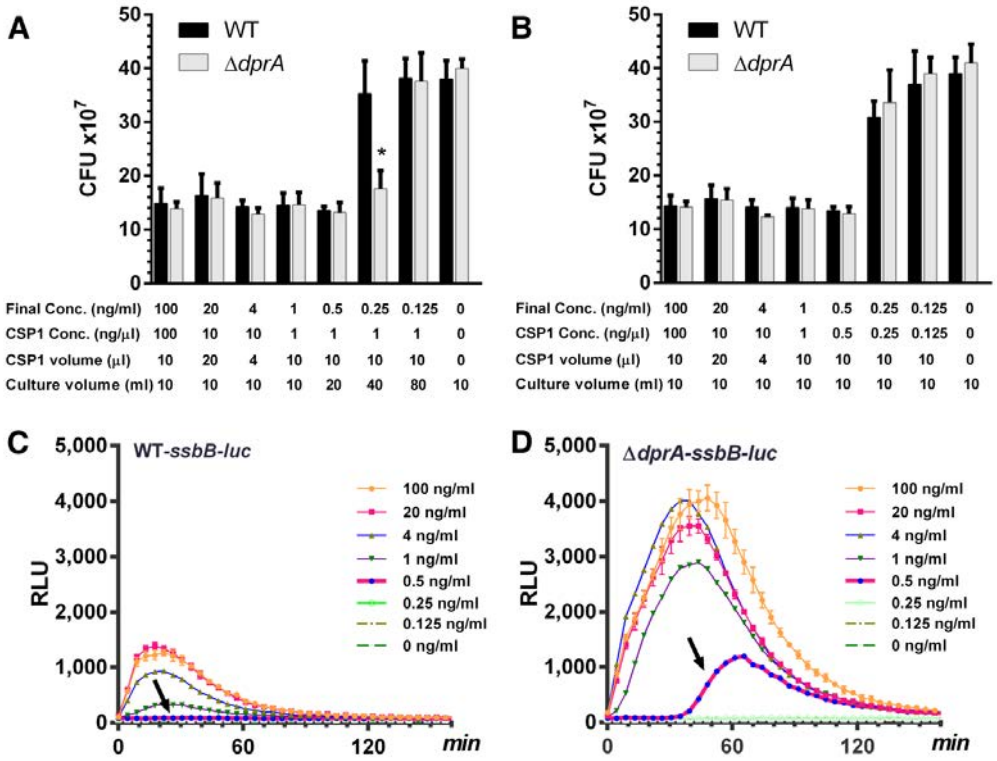

FIG S13. Δ*dprA* is more responsive to competence induction by lower concentrations of CSP1. (A-B) CFU reduction induced by addition of CSP1 stock solution to pneumococcal cultures at OD_{595nm} ~ 0.1 using schemes described. After 1.5 hours, CFU was determined by serial dilution plating. Two-tailed unpaired Student's *t*-test were used to determine the significance of CFU differences ($p < 0.05$). (C-D). The expression of the ComX-regulated "late" gene *ssbB* in WT (C) versus Δ*dprA* (D) in response to different concentrations of CSP1. WT-*ssbB-luc* and Δ*dprA-ssbB-luc* were grown to OD_{595nm} ~ 0.1 in Todd Hewitt Broth supplemented with 0.5% yeast extract, and exposed to different amounts of CSP1 to achieve designated final concentrations. The expression of *ssbB* was measured by using a luminometer.

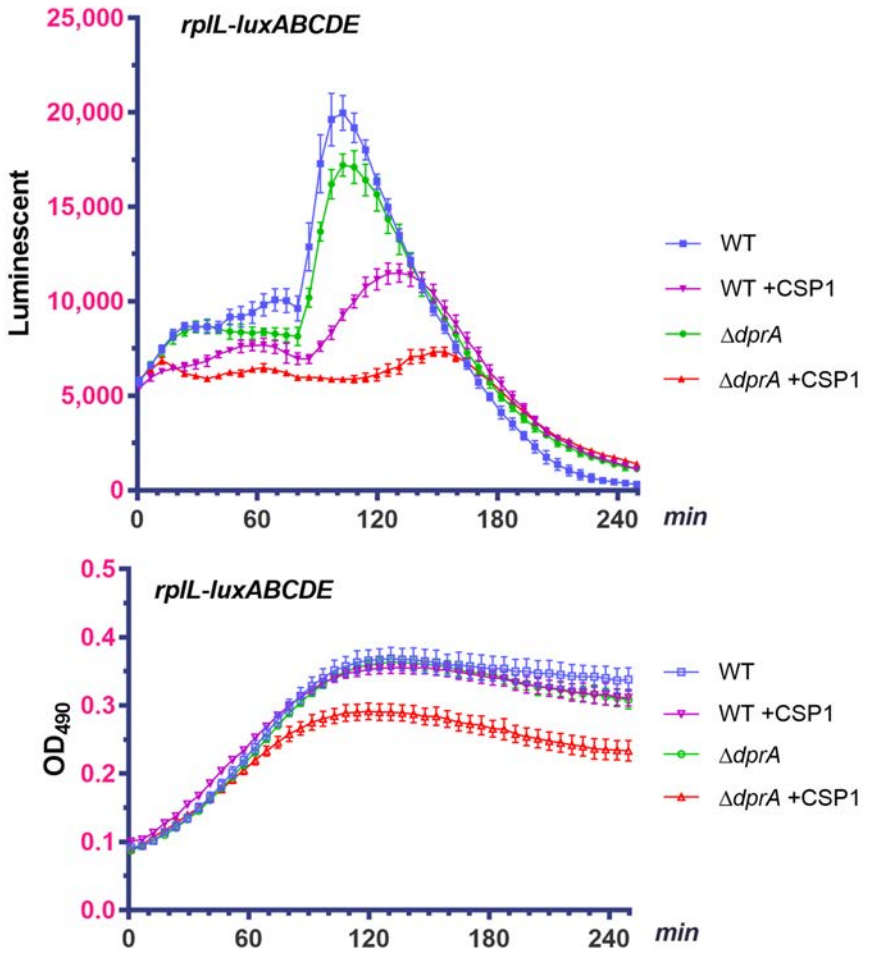

FIG S14. Competence induction in Δ*dprA* drains resources dedicated for cellular growth. WT and Δ*dprA* cells harboring *luxABCDE* transcriptionally fused to constitutively expressing *rplL* gene were treated with CSP1 (100 ng ml^{-1}) at Time 0. Both bioluminescence and growth were monitored for 4 hours. Competence induction by CSP1 caused a more severe reduction of light output in Δ*dprA* than in WT, indicating less *luxABCDE* protein production and/or less energy was available for bioluminescence reaction.