Supplementary data.

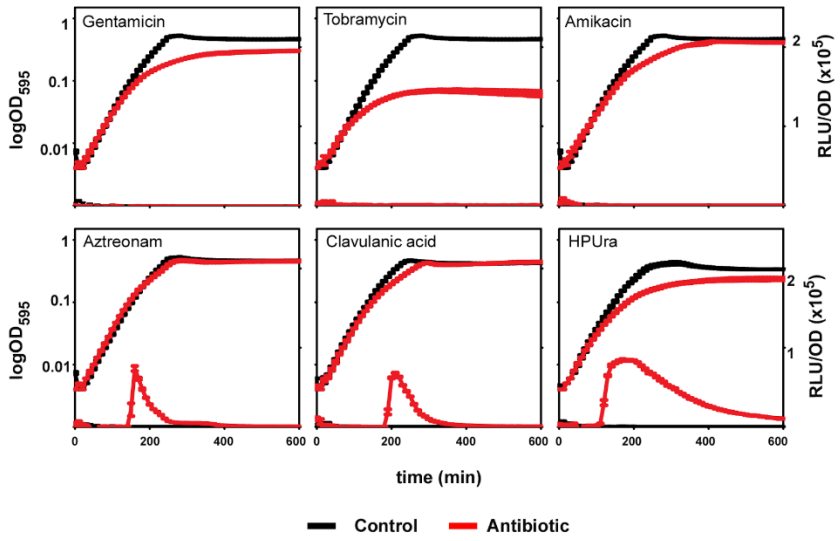

Figure S1. Growth curves (OD_{595}) and bioluminescence activity (RLU/OD_{595}) of *htrA* mutant in presence of several antibiotics. Strain ADP1 (*P_{ssbB}-luc*, *htrA::ery*) was grown in C+Y medium at pH 7.35 with (red lines) or without (black lines) addition of the antibiotics. Average of 3 replicates and Standard Error of the Mean (SEM) are plotted. Antibiotics used: 10 µg/ml gentamicin, 28 µg/ml tobramycin, 28 µg/ml amikacin, 28 µg/ml aztreonam, 2 µg/ml clavulanic acid, and 0.15 µg/ml HPUra. Aminoglycosides are not able to induce competence in the *htrA* mutant, as suggested before (Stevens et al. 2011). However, both aztreonam and clavulanic acid induce competence by another mechanism, as well as HPUra (Slager et al. 2014).

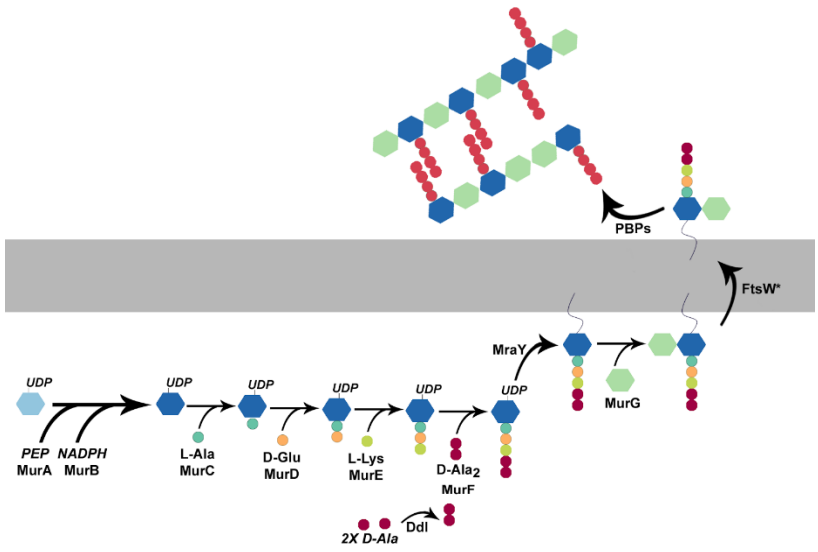

Figure S2. Representation of the cell wall synthesis in *Streptococcus pneumoniae*. The first stage of the peptidoglycan synthesis is the assembling of the pentapeptide, constituted by six enzymes (MurA-F). The second stage, which occurs on the intracellular part of the membrane, drives the synthesis of the lipid II (*mraY* and MurG). Then, the

lipid II is flipped and showed at the external part of the membrane, potentially by FtsW. The final stage occurs on the extracellular face of the membrane, and involves the continuous transglycosylation and transpeptidation activities of the Penicillin-Binding Proteins (PBPs).

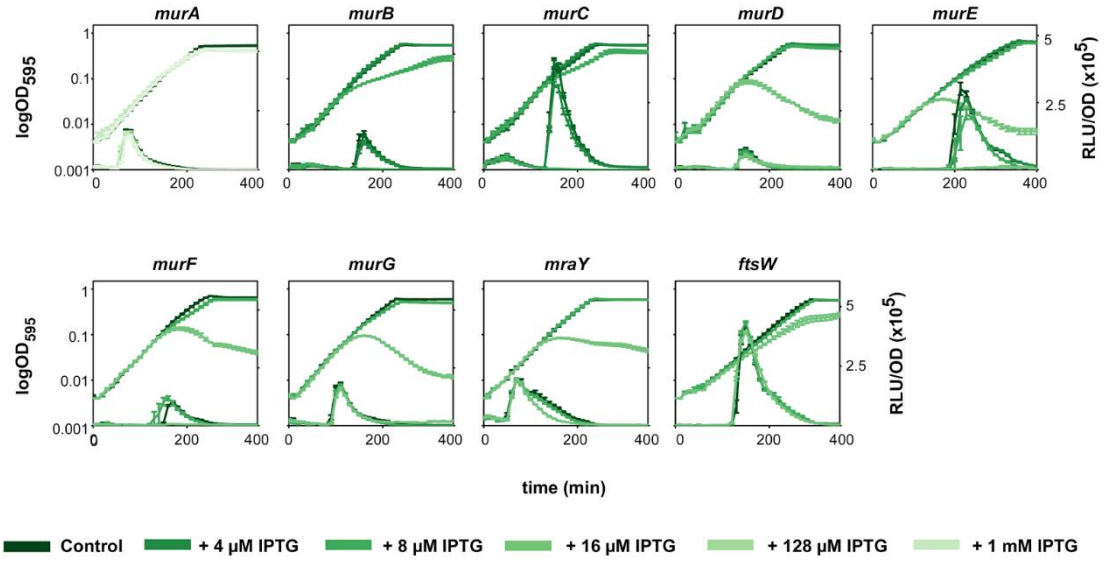

Figure S3. Repression of genes involved in pentapeptide (*murA-F*) and lipid II (*murG* and *mraY*) formation, and its putative flippase (*ftsW*) by CRISPRi. No effect on competence was observed after depleting the individual genes. Detection of competence development was performed in C+Y medium at a permissive pH 7.5 for natural competence. IPTG was added to the medium at the beginning, at different final concentrations (128 µM and 1 mM for *murA*; 4 µM and 8 µM for *murB* and *murC*; 8 µM and 16 µM for the other genes). The values represent averages of three replicates with SEM. Experiments were also reproduced at pH 7.35 (non-permissive) to confirm that the depletion of none of these genes cause an upregulation of competence (data not shown).

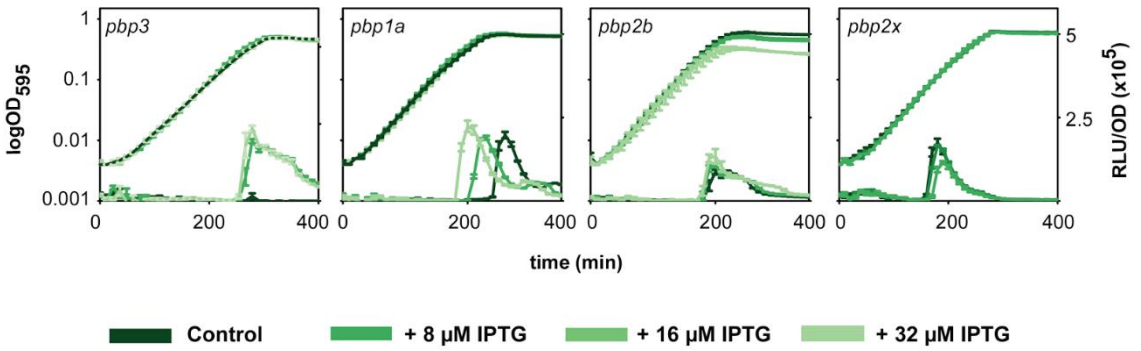

Figure S4. CRISPRi-dependent downregulation of *pbp1a* and *pbp3* leads to competence induction. Depletion of *pbp1a* and *pbp3* by induction of dCas9 with IPTG upregulates competence development. In contrast, depletion of *pbp2b* and *pbp2x* does not have any effect on the regulation of this process. Detection of competence development was performed in C+Y medium at a permissive pH (pH 7.5). IPTG was added to the medium at the beginning, at different final concentrations (8 µM and 16 µM for *pbp1a*; 16 µM and 32 µM for the other *pbp* genes). The values represent averages of three replicates with SEM.

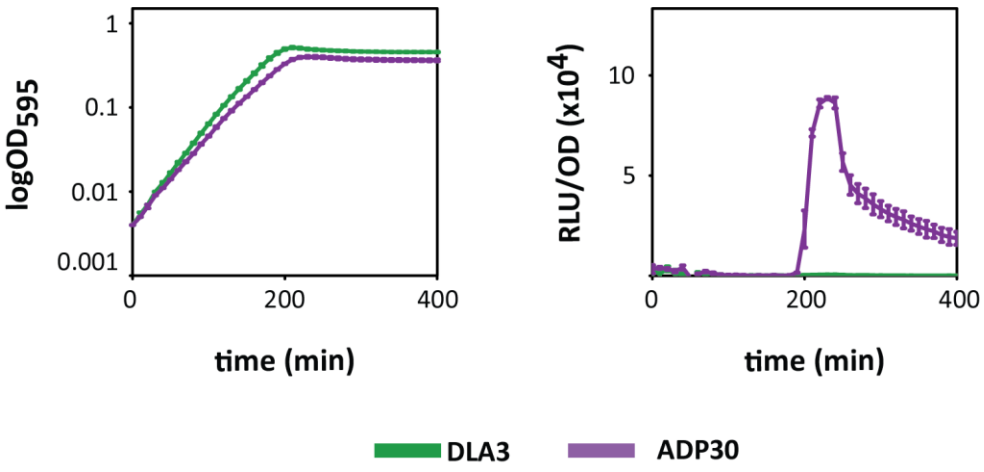

Figure S5. Natural competence in DLA3 (*P*$_{ssbB}$-*luc*) and ADP30 (*P*$_{ssbB}$-*luc*, *pbp3::chl*). To confirm that *pbp3* is involved in competence development, we compared the natural competence activation of the wild type (DLA3) with the *pbp3* mutant (ADP30). At pH 7.35, only the mutant was able to become competent, confirming the upregulation of this pathway in absence of PBP3.

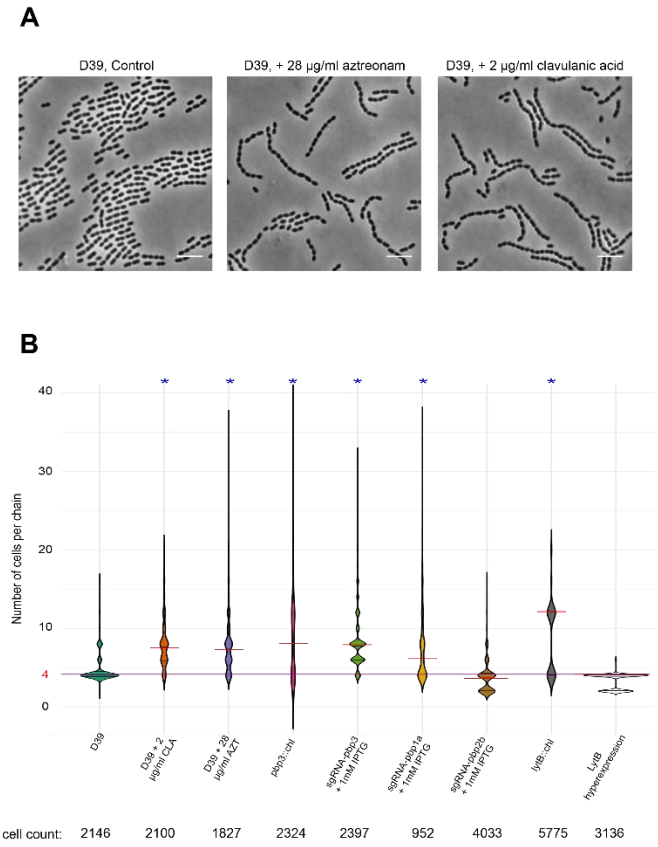

Figure S6. AZT and CLA induce chain formation. Cells were grown in C+Y acid medium until OD 0.4 (stationary fase). **A) Phase-contrast images**. Scale: 6 µm. **B) Length of the chains.** Horizontal red line means the average of the number of cells per chain while the purple line highlights the control condition. The addition of AZT or CLA results in the presence of longer chains, as does the deletion and depletion of *pbp3*. Depletion of *pbp1a* induces chain

formation, contrary to the *pbp2b* depletion phenotype. The absence of *lytB* also resulted in an increase of chain length, while its overexpression restores normal chain length.

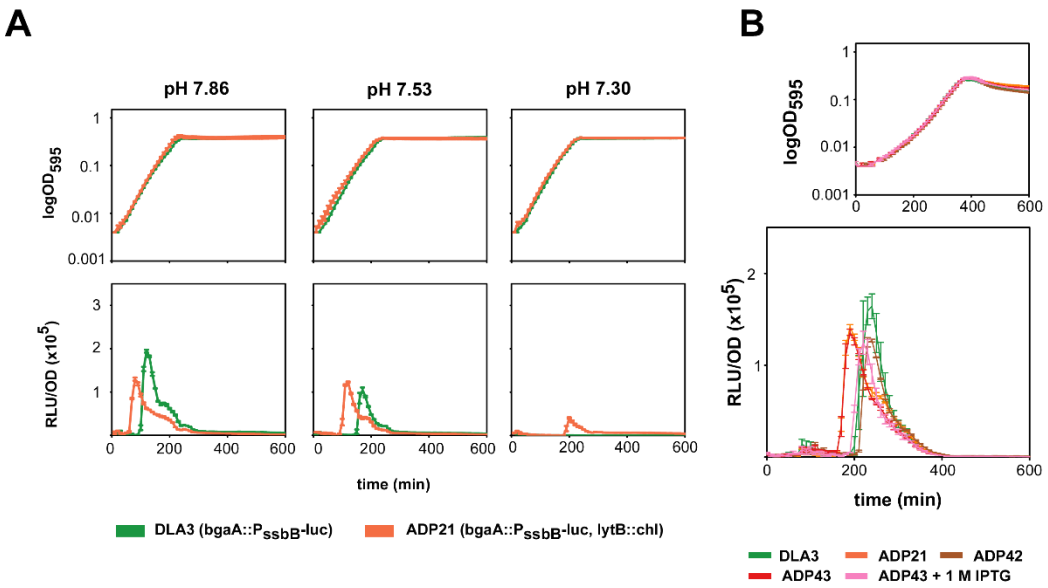

Figure S7. A) Natural competence in DLA3 (*P*$_{ssbB}$-*luc*) and ADP21 (*P*$_{ssbB}$-*luc, lytB::chl*), in a range of three different pHs. In all the conditions, the *lytB* mutant strain showed an earlier development of competence. Even at pH 7.30, where the wild type strain did not become competent, the *lytB* mutant showed bioluminescence activity, implying competence activation. **B) LytB complementation restores normal natural competence.** Strains showing a chainy phenotype [ADP21 (*lytB::chl*) and ADP43 (inducible *lytB* in *lytB::chl* background without presence of the inducer IPTG)] were overcompetent compared with strains showing a wild type phenotype [DLA3 (control), ADP42 (constitutive expression of LytB) or the induction of LytB in ADP43 with 1M IPTG].

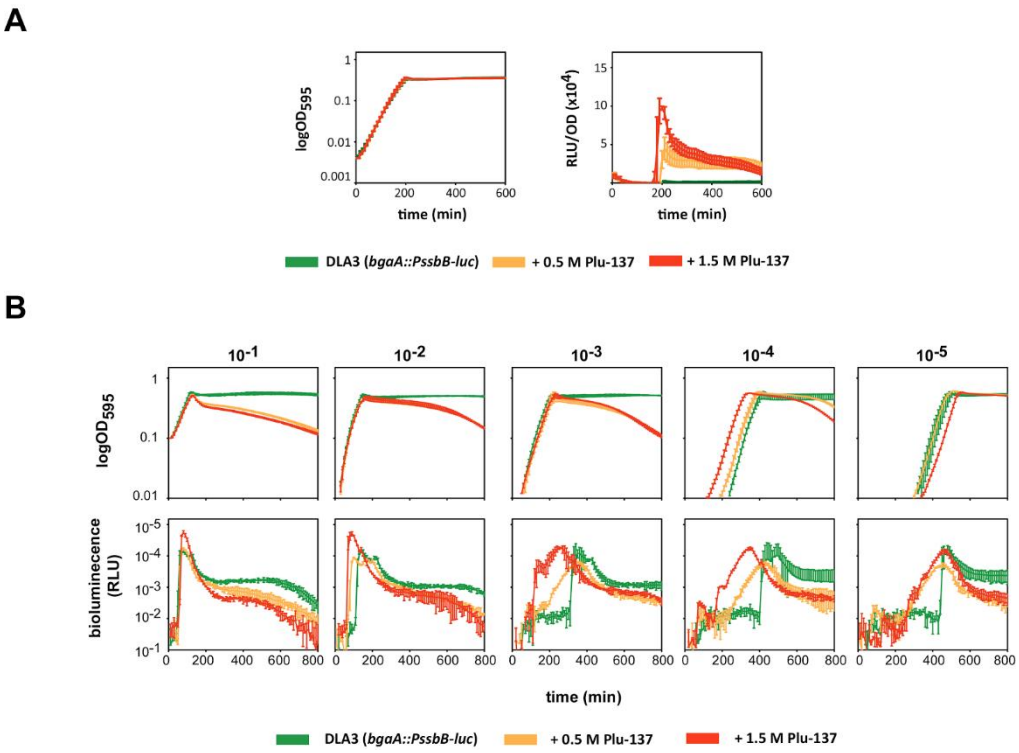

Figure S8. A) Effect of medium density on natural competence development. The addition of increasing concentration of Pluronic-137 results in a lower diffusion of the CSP and thereby a strong and earlier natural competence induction. Cells were grown in C+Y pH 7.3 with or without the indicated concentration of Pluronic 137. **B) Synchronization of competence is affected by a reduction of CSP diffusion**. Growth curves (OD_{595}; up) and competence expression (relative luminescence units; down) in a range of initial densities. Cell were grown in C+Y no acid (green), or in presence of 0.5 M (orange) or 1.5 M (red) of the polymer Pluronic-137 (P-137). Average of 3 replicates and Standard Error of the Mean (SEM) are plotted for each of five inoculation densities: OD_{595} 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}.

77 **Table S1. List of antibiotics tested for competence induction.**

Bacterial target	Antibiotic class	Antibiotic*	Concentration range (µg/mL)
Cell-wall inhibitors	Beta-lactams (carbapenems)	Imipenem	0.015 - 0.12
		Meropenem	0.015 - 0.12
	Beta-lactams (monobactams)	**Aztreonam**	12.5 – 100
	Beta-lactams (amino-penicillins)	Methicillin	0.016 - 2
		Ampicillin	0.0015 – 0.25
		Amoxicillin	0.0015 – 0.25
		Amoxicillin/Clavulanic acid	0.0015/2 – 0.25/2
	Beta-lactams (Cephalosporins)	Cephalexin (1st generation)	0.006 - 1
		Cefaclor (2nd generation)	0.006 – 1
		Cefuroxime (2nd generation)	0.006 – 1
		Cefotaxime (3rd generation)	0.006 – 1
		Cefepime (4th generation)	0.006 – 1
	Beta-lactams (antipseudomonal)	Piperacillin	0.003 – 0.12
	Beta-lactamase inhibitors	**Clavulanic acid**	0.5 – 8
DNA replication inhibition	Fluoroquinolones	**Ciprofloxacin**	0.4
	-	**HPUra**	0.15
Protein synthesis inhibition	Aminoglycosides	**Gentamicin**	5 – 100
		Amikacin	5 – 100
	Macrolides	Clarithromycin (14 carbons)	0.003 – 0.12
		Azithromycin (15 carbons)	0.003 – 0.12
		Josamycin (16 carbons)	0.003 – 0.12
	Oxazolidinones	Linezolid	0.06 - 0.5

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79 *In bold, antibiotics that induce competence in C+Y pH 7.35.

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Table S2. Intraspecific *in vitro* horizontal gene transfer (HGT) of antimicrobial resistance determinants*.

pH	Experiment	No. of transformants (cfu/ml)*	Total viable count (cfu/ml)	Transformation efficiency (%)
7.3	DLA3 + MK134 (control)	0 ± 0 cfu/ml	$1.4 \cdot 10^{11} \pm 4.0 \cdot 10^{10}$ cfu/ml	0 ± 0 %
	DLA3 + MK134 + AZT	$6.2 \cdot 10^{4} \pm 4.1 \cdot 10^{3}$ cfu/ml	$9.0 \cdot 10^{10} \pm 2.7 \cdot 10^{10}$ cfu/ml	$7.0 \cdot 10^{-5} \pm 2.3 \cdot 10^{-5}$ %
	DLA3 + MK134 + CLA	$2.1 \cdot 10^{4} \pm 1.0 \cdot 10^{4}$ cfu/ml	$9.3 \cdot 10^{10} \pm 1.5 \cdot 10^{10}$ cfu/ml	$2.3 \cdot 10^{-5} \pm 1.4 \cdot 10^{-5}$ %
7.5	DLA3 + MK134 (control)	$2.0 \cdot 10^{3} \pm 1.0 \cdot 10^{3}$ cfu/ml	$1.4 \cdot 10^{11} \pm 3.1 \cdot 10^{10}$ cfu/ml	$1.3 \cdot 10^{-5} \pm 4.3 \cdot 10^{-7}$ %
	DLA3 + MK134 + AZT	$8.4 \cdot 10^{4} \pm 4.9 \cdot 10^{3}$ cfu/ml	$9.0 \cdot 10^{10} \pm 2.6 \cdot 10^{10}$ cfu/ml	$9.9 \cdot 10^{-5} \pm 3.0 \cdot 10^{-5}$ %
	DLA3 + MK134 + CLA	$5.3 \cdot 10^{4} \pm 7.5 \cdot 10^{3}$ cfu/ml	$9.3 \cdot 10^{10} \pm 1.5 \cdot 10^{10}$ cfu/ml	$5.9 \cdot 10^{-5} \pm 1.7 \cdot 10^{-5}$ %

* Three independent replicates per condition were performed. Strains: DLA3 (D39, *bgaA::*P_{ssbB}*-luc*, tetracycline resistance marker), MK134 (D39, *ssbB-luc*, kanamycin resistance marker). Abbreviations: AZT = 28 µg/ml of aztreonam, CLA = 2 µg/ml of clavulanic acid.

Table S3. Interspecific transfer of DNA from *E. coli* to *S. pneumonia*e promoted by aztreonam.

Experiment	No. of transformants (cfu/ml)*	Total viable count (cfu/ml)	Transformation efficiency (%)
pLA18 *E. coli* + D39 SPNE	0 ± 0 cfu/ml	$1.9 \cdot 10^{8} \pm 7.8 \cdot 10^{6}$ cfu/ml	0 ± 0
pLA18 *E. coli* + D39 SPNE + AZT	$3.4 \cdot 10^{4} \pm 8.5 \cdot 10^{3}$ cfu/ml	$1.0 \cdot 10^{8} \pm 3.5 \cdot 10^{6}$ cfu/ml	$3.3 \cdot 10^{-2} \pm 9.3 \cdot 10^{-3}$ %

* Three independent replicates per condition were performed. Abbreviations: AZT: 28 µg/ml of aztreonam; SPNE: *Streptococcus pneumoniae*; *E. coli*: *Escherichia coli* DH5α carrying the high-copy plasmid pLA18, with the tetracycline resistance marker *tetM*. Both SPNE and *E. coli* were co-incubated at the same initial concentration (OD_{595} 0.04).

Table S4. Functional analysis of the microarray experiments of *S. pneumoniae* ADP62 (*comC::chl*) grown in presence or absence of antibiotics

Condition	Gene regulation	Class	Single list	Class Size	Description
AZT (RE)	upregulation	-			
	downregulation	COG	0.00 (3)	209	Cell wall/membrane/envelope biogenesis
		COG	0.00 (3)	159	Inorganic ion transport and metabolism
		GO	0.00022 (2)	7	GO:0005315 - phosphate transmembrane transporter activity
		GO	0.00051 (3)	92	GO:0006810 - transport
		GO	0.00194 (3)	164	GO:0016020 - membrane
		KEYWORDS	0.00258 (3)	121	IPR003439 - ABC transporter-like
		KEYWORDS	0.00082 (3)	66	IPR003445 - Cation transporter
AZT (AE)	upregulation	-			
	downregulation	COG	0.000 (3)	209	Cell wall/membrane/envelope biogenesis
		GO	0.00022 (2)	7	GO:0005315 - phosphate transmembrane transporter activity
		Others	0.0e+00 (4)	23	t_RNA-Ser
		Others	2.1e-06 (2)	2	Serine protease
CLA (RE)	upregulation	-			
	downregulation	-			
CLA (AE)	upregulation	-			
	downregulation	COG	0.00 (5)	209	Cell wall/membrane/envelope biogenesis
		COG	0.00 (4)	159	Inorganic ion transport and metabolism
		GO	7.4e-05 (2)	7	GO:0005315 - inorganic phosphate transmembrane transporter
		GO	0.0e+00 (4)	92	GO:0006810 - transport
		GO	0.0e+00 (5)	164	GO:0016020 - membrane

	IPR	0.00 (3)	37	IPR000515 - MetI-like domain
	KEYWORDS	0.0e+00 (4)	121	IPR003439 - ABC transporter-like
	KEYWORDS	6.9e-05 (3)	42	IPR000515 - MetI-like domain
	KEYWORDS	0.0e+00 (4)	66	IPR003445 - Cation transporter
	Pfam	0.00 (3)	37	PF00528 - Binding-transport system inner membrane
	Other	0.00 (3)	37	SSF161098 - MetI-like

Abbreviations: AZT: aztreonam, CLA: clavulanic acid, RE: rapid exposure, AE: adaptive exposure; COG: clusters of orthologous groups, GO: gene ontology, IPR: interpro

Table S5. Summary of gene expression changes in transcriptome comparison of *S. pneumoniae* D39 grown in presence or absence of antibiotics

Condition	gene modulation	locus	gene description	log. fold change
AZT (RE)	downregulation	**SPD_0741**	Putative deoxyribose-specific ABC transporter permease protein	- 1.10
		SPD_1231	Phosphate transport system permease protein PstC2	- 1.12
		SPD_1393	pyridine nucleotide-disulfide oxidoreductase family protein	- 1.32
		SPD_1230	Phosphate transport system permease protein PstA2	- 1.35
		SPD_0555	Antibiotic ABC transporter permease protein	- 1.48
AZT (AE)	downregulation	SPD_1682	tRNA-Ser2	-1,01
		SPD_1231	Phosphate transport system permease protein PstC2	-1,01
		SPD_1695	tRNA-Leu3	-1,13
		SPD_1685	tRNA-Phe1	-1,13
		SPD_1230	Phosphate transport system permease protein PstA2	-1,19

		SPD_1760	tRNA-Cys1	-1,22
		SPD_2069	SpoJ protein	-1,34
		SPD_0555	Antibiotic ABC transporter permease protein	-1,36
		SPD_2068	Serine protease	-1,40
		SPD_0913	Hypothetical protein	-1,46
		SPD_1697	tRNA-Asp-GTC	-1,98
		SPD_1874	LysM domain-containing protein	-2,37
CLA (RE)	upregulation	SPD_0620	Lysyl-tRNA synthetase	2.31
		SPD_2028	Choline binding protein D	1.90
		SPD_1654	Ribosomal large subunit pseudouridine synthase B	1.08
		SPD_0919	Hypothetical protein	1.04
	downregulation	SPD_1697	tRNA-Asp-GTC	- 1.01
		SPD_0361	Transcriptional regulon	- 1.10
CLA (AE)	upregulation	SPD_1181	Hypothetical protein	1.19
		SPD_2007	Transporter major facilitator family protein	1.16
		SPD_0141	Hypothetical protein	1.04
		SPD_0293	PTS system transporter subunit IIA	1.02
		SPD_0934	Tn5252, ORF 10 protein	1.01
	downregulation	SPD_1738	MATE efflux family protein DinF	- 1.01
		SPD_1220	Spermidine/putrescine ABC transporter permease	- 1.01
		SPD_0741	Putative deoxyribose-specific ABC transporter permease protein	- 1.15
		SPD_1231	Phosphate transport system permease protein PstC2	- 1.23
		SPD_1230	Phosphate transport system permease protein PstA2	- 1.43
		SPD_0555	Antibiotic ABC transporter permease protein	- 1.53
		SPD_1697	tRNA-Asp-GTC	- 1.57

Abbreviations: AZT: aztreonam, CLA: clavulanic acid, RE: rapid exposure, AE: adaptive exposure. In bold, genes with transcriptome changes in more than one condition.

Table S6. List of strains used.

***S. pneumoniae* strains**	**Relevant genotype**	**Reference**
D39	Serotype 2 strain	Avery et al. 1944
DLA3	Δ*bgaA::*P_{ssbB}*-luc-gfp*	Slager et al. 2014
MK134	*ssbB-luc*	(Slager et al. 2014
ADP21	Δ*bgaA::*P_{ssbB}*-luc-gfp, lytB::chl*	This study
ADP30	Δ*bgaA::*P_{ssbB}*-luc-gfp, dacC::chl*	This study
ADP42	Δ*bgaA::*P_{ssbB}*-luc-gfp, cep::*P_{Lac}*-lytB , lytB::chl*	This study
ADP43	Δ*bgaA::*P_{ssbB}*-luc-gfp, cep::*P_{Lac}*-lytB , lytB::chl, prsA::*P_{F6}*-lacI, ssbB-luc*	This study
ADP62	Δ*bgaA::*P_{ssbB}*-luc-gfp, comC::chl*	Moreno-Gámez et al. 2017
ADP157	Δ*cep::*P_3*-sgRNA-pbp1A,* Δ*bgaA::*P_{Lac}*-dCas9, prsA::*P_{F6}*-lacI, ssbB-luc*	This study
ADP161	Δ*cep::*P_3*-sgRNA-pbp2b,* Δ*bgaA::*P_{Lac}*-dCas9, prsA::*P_{F6}*-lacI, ssbB-luc*	This study
ADP165	Δ*cep::*P_3*-sgRNA-murB,* Δ*bgaA::*P_{Lac}*-dCas9, prsA::*P_{F6}*-lacI, ssbB-luc*	This study
ADP173	Δ*cep::*P_3*-sgRNA-murA-2,* Δ*bgaA::*P_{Lac}*-dCas9, prsA::*P_{F6}*-lacI, ssbB-luc*	This study
ADP177	Δ*cep::*P_3*-sgRNA-pbp2x,* Δ*bgaA::*P_{Lac}*-dCas9, prsA::*P_{F6}*-lacI, ssbB-luc*	This study
ADP178	Δ*cep::*P_3*-sgRNA-mraY,* Δ*bgaA::*P_{Lac}*-dCas9, prsA::*P_{F6}*-lacI, ssbB-luc*	This study

ADP179	Δ*cep::P*$_{3}$*-sgRNA-murD,* Δ*bgaA::P*$_{Lac}$*-dCas9, prsA::P*$_{F6}$*-lacI, ssbB-luc*	This study
ADP180	Δ*cep::P*$_{3}$*-sgRNA-murG,* Δ*bgaA::P*$_{Lac}$*-dCas9, prsA::P*$_{F6}$*-lacI, ssbB-luc*	This study
ADP185	Δ*cep::P*$_{3}$*-sgRNA-ftsW,,* Δ*bgaA::P*$_{Lac}$*-dCas9, prsA::P*$_{F6}$*-lacI, ssbB-luc*	This study
ADP187	Δ*cep::P*$_{3}$*-sgRNA-murE,* Δ*bgaA::P*$_{Lac}$*-dCas9, prsA::P*$_{F6}$*-lacI, ssbB-luc*	This study
ADP190	Δ*cep::P*$_{3}$*-sgRNA-murF,* Δ*bgaA::P*$_{Lac}$*-dCas9, prsA::P*$_{F6}$*-lacI, ssbB-luc*	This study
ADP203	Δ*cep::P*$_{3}$*-sgRNA-murA-1,* Δ*bgaA::P*$_{Lac}$*-dCas9, prsA::P*$_{F6}$*-lacI, ssbB-luc*	This study
ADP207	Δ*cep::P*$_{3}$*-sgRNA-murC,* Δ*bgaA::P*$_{Lac}$*-dCas9, prsA::P*$_{F6}$*-lacI, ssbB-luc*	This study
ADP245	*Δcep::P*$_{3}$*-mkate2, P*$_{ssbB}$*-ssbB-gfp*	Moreno-Gámez et al. 2017
ADP264	Δ*cep::P*$_{3}$*-sgRNA-pbp3,* Δ*bgaA::P*$_{Lac}$*-dCas9, prsA::P*$_{F6}$*-lacI, ssbB-luc*	This study
E. coli pLA18	*DH5α,* plasmid *amp*^{R}*, bgaA', tet*^{R}*, P*$_{ssbB}$*-luc_gfp, 'bgaA*	Slager et al. 2014

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Supplementary methods

CRISPRi library: to monitor the competence effect of the downregulation of genes involved in the cell wall synthesis, the PCR product of the fragment *ssbB–luc–kan* from strain MK134 (Slager et al. 2014) into the CRISPRi library of the indicated genes (Slager et al. 2014; Liu et al. 2017). Transformants were selected on Columbia blood agar containing 250 µg/ml kanamycin resulting in strains listed on table S6.

ADP21 strain: to monitor the effect of the chain formation on competence induction, the gene *lytB* encoding for the Autolysin B was replaced by the chloramphenicol resistance marker. The upstream region was amplified using primers ADP1/34 (GATGTGGTGAAAGCAGCTGTGGAAG) and ADP1/35+AscI (CGATGGCGCGCCTCCTCTGTTCTTATTTATTTTATTG), the downstream region with primers ADP1/36+NotI (CGATGCGGCCGCTACTATAAGTGAATATGATTTGAGTG) and ADP1/37 (GTGTAGAAACCGTCCTCAACCAAG), and the chloramphenicol resistance marker with sPG11+AscI (ACGTGGCGCGCCAGGAGGCATATCAAATGAAC) and sPG12+NotI (ACGTGCGGCCGCTTATAAAAGCCAGTCATTAG). All three fragments were digested with the proper restriction enzymes (*Asc*I and/or *Not*I) and ligated. The *ΔlytB::chl* fragment containing the chloramphenicol resistance marker flanked by the sequence up- and downstream of *lytB* was transformed into DLA3 resulting in ADP21 strain (*ΔbgaA::*P_{ssbB}*–luc, ΔlytB::chl*). Transformants were selected on Columbia blood agar containing 4.5 µg/ml chloramphenicol. Correct deletion was verified by PCR and sequencing.

ADP30 strain: to monitor the effect of the PBP3 on competence, the related gene was replaced by the chloramphenicol resistance marker. The upstream region was amplified using primers ADP1/59 (GCCCTCAACTCAGCAGTATGG) and ADP1/60+AscI (CGATGGCGCGCCTTATCCAACTGATCCCTCCATTTC), the downstream region with primers ADP1/61+NotI (CGATGCGGCCGCGAGGTAACTCATGTTTCGTAG) and ADP1/62 (AAGCCTGCAATATGCAAGCGATCC), and the chloramphenicol resistance marker with sPG11+AscI (ACGTGGCGCGCCAGGAGGCATATCAAATGAAC) and sPG12+NotI (ACGTGCGGCCGCTTATAAAAGCCAGTCATTAG). All three fragments were digested with the proper restriction enzymes (*Asc*I and/or *Not*I) and ligated. The *Δpbp3::chl* fragment containing the chloramphenicol resistance marker flanked by the sequence up- and downstream of *pbp3* was transformed into DLA3 resulting in ADP30 strain (*ΔbgaA::*P_{ssbB}*–luc, Δpbp3::chl*). Transformants were selected on Columbia blood agar containing 4.5 µg/ml chloramphenicol. Correct deletion was verified by PCR and sequencing.

ADP42 and ADP43 strains: to test whether the ectopic hyperexpression of LytB in the Δ*lytB* mutant restored the normal diplococcus phenotype and restored competence development to wild type, we created an inducible expression of LytB. The inducible system was created using BglFusion cloning (Sorg et al. 2015). To amplify the *lytB* fragment, primers ADP1/71+BglII (ACGTAGATCTAGAGGAAGAAGGTTGATGAAGAAAG) and ADP1/72+XhoI (CATGCTCGAGTTACTGGAGGGATCCAGTACTAATCTTTG) were used using D39 chromosomal DNA as a template. The construction was transformed to strain ADP21 and transformants (ADP42) were selected on Columbia blood agar containing 100 µg/ml spectinomycin. Correct deletion was verified by PCR and sequencing. ADP42 shows a constitutive expression of LytB since it lacks the lacI repressor of the IPTG-inducible system. To control the expression of the LytB, we then transformed the codon optimized *lacI* gene into strain ADP42. For that, we PCR-ed the fragment including *lacI* integrated into the *prsA*-locus together with a gentamycin resistance cassette from chromosomal DNA of strain ADP95 (Moreno-Gámez et al. 2017) using primers OLI40 (CCATGGCATCAGCGAGAAGGTGATAC) and OLI41 (GCGGCCGCAGGATAGAAAGGCGAGAG).