Antibiotic-induced cell chaining triggers pneumococcal competence by reshaping quorum sensing to autocrine signaling

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Abstract

Streptococcus pneumoniae can acquire antibiotic resistance by activation of competence and subsequent DNA uptake. Several antibiotics induce competence by disrupting protein-quality control or perturbing DNA replication. Here, we demonstrate that aztreonam (AZT) and clavulanic acid (CLA) also promote competence. We show that both compounds induce cell chain formation by targeting the D,D-carboxypeptidase PBP3. In support of the hypothesis that chain formation promotes competence, we demonstrate that an autolysin mutant (*lytB*) is hypercompetent. As competence is initiated by the binding of a small extracellular peptide (CSP) to a membrane-anchored receptor (ComD), we wondered if chain formation alters CSP diffusion and thereby sensing by ComD. Indeed, the presence of AZT or CLA affects competence synchronization by switching CSP-based quorum sensing to autocrine-like signaling, as CSP is retained to chained cells and no longer shared in a common pool. Together, these insights demonstrate the versatility of quorum sensing in integrating different stresses and highlight that certain antibiotics should be prescribed with care not to drive the spread of antibiotic resistance.

Keywords: Clavulanic acid, aztreonam, *Streptococcus pneumoniae*, antibiotics, competence, transformation, bacterial stress response, CSP, Quorum Sensing, autocrine signaling, contact-dependent signaling.

Introduction

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive diplococcus, member of the commensal microbiota of the human nasopharynx. However, the pneumococcus is also considered one of the leading bacterial causes of morbidity and mortality worldwide, being responsible for a wide variety of invasive and non-invasive diseases (O'Brien et al. 2009).

Non-invasive infections (e.g. otitis media, non-bacteremic pneumonia or acute exacerbations of chronic respiratory diseases) are typically treated with antibiotics, which are considered a risk factor for the acquisition of resistant pneumococci in the nasopharynx. Actually, non-invasive infections are frequently caused by pneumococci with higher levels of antibiotic resistance than invasive strains (Kyaw et al. 2002). The colonization of antibiotic resistant pneumococci in the human body can occur by several mechanisms such as the replacement of susceptible pneumococci by a resistant community-acquired strain, by spontaneous mutation prior to or during antibiotic therapy, or by the acquisition of a new antibiotic resistance allele from other pneumococci or closely-related Streptococci by the process of transformation (Schrag et al. 2000).

Transformation, defined as the uptake and assimilation of exogenous DNA, is an important mechanism of genome plasticity throughout evolutionary history, and is largely responsible for the rapid spread of antimicrobial resistance in the pneumococcus (Croucher et al. 2011). This process is regulated by competence (Figure 1A), a physiological state that involves about 2-10% of the pneumococcal genome (Claverys et al. 2009); Slager et al., in preparation). Competence is induced by a classical two-component quorum sensing system in which the *comC*-encoded competence-stimulating peptide (CSP), is cleaved and exported by the membrane transporter ComAB to the extracellular space. CSP can then diffuse and can stimulate autophosphorylation of the membrane-bound histidine-kinase ComD, which subsequently activates the cognate response regulator ComE (Figure 1A) (Martin et al. 2013; Pestova et al. 1996). Upon a certain threshold CSP concentration, a positive feedback loop overcomes counteracting processes and the competent state is fully activated. One of the genes regulated by ComE, *comX*, encodes a sigma factor, which activates the genes required for DNA repair, DNA uptake, and transformation. CSP can be retained by producing cells (Prudhomme et al. 2016), but CSP also diffuses and can induce competence in neighboring cells (Christie 2016; Havarstein et al. 1995; Moreno-Gámez et al. 2017). The diffusibility of the growth medium also influences competence development (Yang et al. 2010). Thus, the initiation of competence can be considered as a combination of diffusion sensing and autocrine signaling (or self-sensing) (Doğaner et al. 2016; Moreno-Gámez et al. 2017).

The competent state is activated in response to several antibiotics, which thereby allow the bacterium to take up foreign DNA and potentially acquire antimicrobial resistance determinants (Prudhomme 2006; Slager et al. 2014; Stevens et al. 2011). Hence, when antibiotic therapy is not appropriate or inadequate, competence activation of the commensal pneumococci resident in the nasopharynx can lead to the acquisition of resistant genes or virulence factors from its environment. Spread of antibiotic resistance is exacerbated by the fact that, coregulated with competence, *S. pneumoniae* expresses several bacterial killing factors, thereby using interbacterial predation to acquire foreign DNA (Veening & Blokesch 2017; Kjos et al. 2016; Wholey et al. 2016).

We have shown previously that antimicrobials targeting DNA replication, such as fluoroquinolones, cause an increase in the copy number of genes proximal to the origin of replication (*oriC*) due to replication stalling (Slager et al. 2014). As the competence operons *comAB* and *comCDE* are located near *oriC*, these antibiotics induce competence. Aminoglycoside antibiotics such as kanamycin are thought to activate competence by causing the accumulation of misfolded proteins via mistranslation. As these misfolded proteins are targeted by the HtrA chaperone/protease, the natural HtrA substrate CSP can accumulate and competence is activated (Stevens et al. 2011). While several classes of antibiotics have been tested for their ability to induce competence (Slager et al. 2014; Prudhomme 2006), a systematic analysis of clinically relevant antibiotics and their effects on competence is lacking.

Here, we tested a panel of commonly prescribed antibiotics for their potential to induce competence. We find that the antibiotic aztreonam (AZT) and the beta-lactamase inhibitor clavulanic acid (CLA) induce competence. We show that both compounds bind to the non-essential D,D-carboxypeptidase PBP3. Consequently, cells are perturbed in their ability to separate, leading to the formation of long chains of cells. Cell chaining decreases diffusion of CSP into the extracellular milieu, thereby facilitating CSP's interaction with membrane-bound ComD receptors on the producing cell itself and on daughter cells. This effectively changes the dynamics and shifts the major regulatory mode of competence from quorum sensing to autocrine-like signaling, subsequently enhancing local competence induction and promoting horizontal gene transfer.

Results

Identification of clinically relevant antibiotics that induce competence

To monitor competence development, we utilized the ComX-dependent promoter P_{ssbB}, driving expression of firefly luciferase (*luc*). We selected antibiotics on basis of their use for the treatment of several pneumococcal respiratory infections (otitis media, pneumonia or exacerbations of chronic respiratory diseases), as well as for the treatment of respiratory infections with other bacterial etiologies (Table S1). Cells were grown in C+Y medium at pH 7.35, a pH non-permissive for natural competence development under our experimental conditions (Moreno-Gámez et al. 2017), and antibiotics were added at concentrations below the minimum inhibitory concentration (MIC) to prevent large growth defects and cell killing. Only when antibiotics induce competence, the *ssbB* promoter is activated and firefly luciferase is produced. In line with previous reports, four antibiotics belonging to the fluoroquinolone and aminoglycoside classes of antibiotics robustly induced competence (Figure 1B) (Slager et al. 2014; Prudhomme 2006; Stevens et al. 2011; Moreno-Gámez et al. 2017). Macrolides and linezolids classes of antibiotics were not able to induce competence (Table S1). The beta-lactam subclass antibiotics, carbapenems and cephalosporins, also did not induce competence at any of the concentrations tested (Table S1). In contrast, the addition of aztreonam (AZT) and the combination of amoxicillin and clavulanic acid resulted in activation of P_{ssbB}-*luc*. To test whether amoxicillin, clavulanic acid (CLA) or the combination of amoxicillin-CLA was responsible for competence induction, the compounds were also tested individually. Surprisingly, competence was not induced by the beta-lactam amoxicillin, but by clavulanic acid, an inhibitor of beta-lactamases. Together, this now extends the list of antibiotics capable of inducing competence to the following compounds: HPUra, aminoglycosides, fluoroquinolones, trimethoprim, the beta-lactam aztreonam, and the inhibitor of beta-lactamases clavulanic acid.

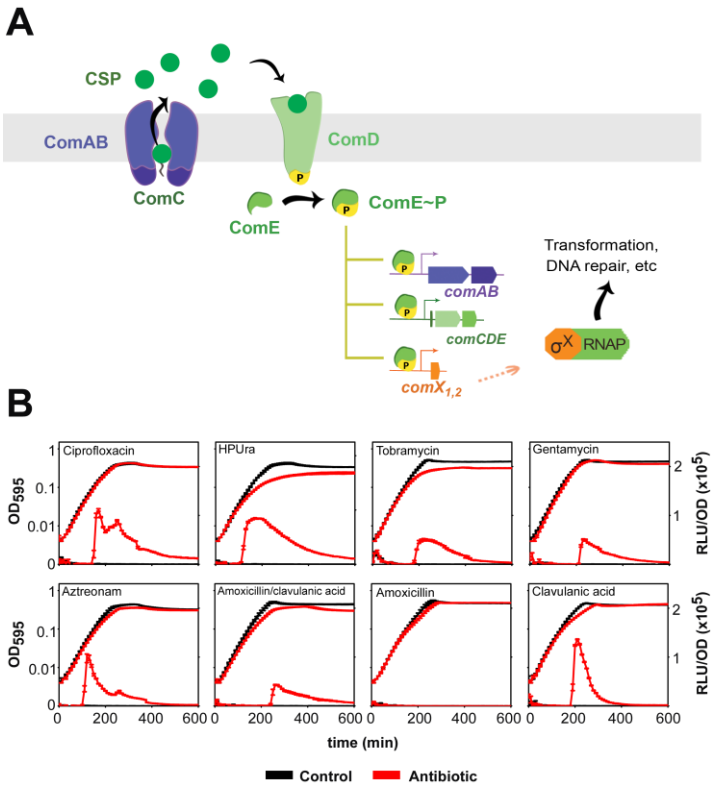

Fig. 1 A) Competence in *S. pneumoniae* is activated by several classes of antibiotics. ComC binds the membrane protein complex ComAB, and is processed and exported as CSP to the extracellular space. CSP then binds to the histidine kinase ComD, which is in the membrane as a dimer. Upon CSP binding, ComD autophosphorylates and transfers the phosphate group to the response regulator ComE (Pestova *et al.,* 1996; Martin *et al.,* 2013). The phosphorylated form of ComE (ComE~P) dimerizes and activates transcription of *comAB*, *comCDE* and *comX* by binding to their promoters (Havarstein *et al.,* 1995; Pestova *et al.,* 1996). Synthesis of the alternative sigma factor ComX directs transcription of genes required for genetic transformation as well as other functions. **B) Growth curves (OD_{595}nm) and bioluminescence activity (RLU/OD_{595}nm) of *S. pneumoniae* in the presence of several antibiotics.** Strain DLA3 (P_{ssbB}-luc) was grown in C+Y medium at pH 7.35, which is non-permissive for natural competence initiation, with (red lines) or without (black lines) addition of antibiotics. Average of 3 replicates and Standard Error of the Mean (SEM) are plotted. Concentrations of the antibiotics used: 0.4 µg/ml ciprofloxacin, 0.15 µg/ml HPUra, 28 µg/ml amikacin, 10 µg/ml gentamicin, 28 µg/ml aztreonam, 0.12 µg/ml amoxicillin, and 2 µg/ml clavulanic acid. Induction of competence by ciprofloxacin and HPUra was shown before (Slager *et al.,* 2014; Prudhomme *et al.,* 2006).

Aztreonam (AZT) and clavulanic acid (CLA) promote horizontal gene transfer

To examine whether competence induction by AZT and CLA leads to increased horizontal gene transfer (HGT), we co-incubated two pneumococcal strains that are genetically identical except for a unique antibiotic resistance marker (tetracycline and kanamycin) integrated at different genomic locations. Since the extracellular pH is an important factor for natural competence development (Prudhomme 2006; Moreno-Gámez et al. 2017; Chen & Morrison 1987), we performed this experiment in two different growth conditions (pH 7.3 and pH 7.5, non-permissive and permissive conditions for natural competence, respectively) in presence or absence of AZT or CLA. As expected, at pH 7.3 no transformants were detected in the control condition. However, cells treated with either AZT or CLA showed a high proportion of HGT events $(7.3 \pm 2.3) \cdot 10^{5}$ % and $(2.3 \pm 1.4) \cdot 10^{-5}$ %,

respectively. In addition, both AZT and CLA potentiated HGT at pH 7.5 (Figure 2, Table S2).

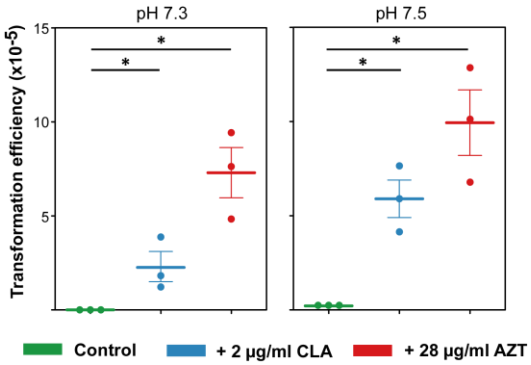

Fig. 2. Induction of horizontal gene transfer by aztreonam (AZT) and clavulanic acid (CLA). DLA3 (tetracycline resistant) and MK134 (kanamycin resistant), were individually grown to OD595nm 0.4 in C+Y pH 6.8 at 37°C. Then, a mixed 100-fold dilution of both strains were grown in C+Y pH 7.3 (non-permissive conditions) or pH 7.5 (permissive conditions) to OD595nm 0.3 to promote the transfer of genes. Antibiotics were added when indicated (2 µg/ml clavulanic acid or 28 µg/ml aztreonam). Afterwards, serial dilutions of cultures were plated with or without antibiotics (for the recovery of the total viable counts) and with the combination of 125 µg/ml of kanamycin plus 1 µg/ml tetracycline and the ratio between total viable cells and transformants was calculated. *Statistically significant longer chains then wild type, mean comparison test p<0.05.

AZT is mainly used to treat infections caused by Gram-negative bacteria as most Gram-positive bacteria, such as *S. pneumoniae*, are less susceptible to AZT. To test whether AZT could promote HGT between a Gram-negative and *S. pneumoniae*, we co-incubated pneumococcal strain D39 with *Escherichia coli* strain DH5α. The *E. coli* strain used in this experiment carries the high-copy number plasmid, pLA18 (Slager et al. 2014), containing a tetracycline-resistance allele flanked by homology regions with the non-essential pneumococcal *bgaA* locus. At 28 µg/ml of AZT, *E. coli* is readily lysed while competence is induced in *S. pneumoniae* (Figure 1B). Importantly, a high percentage of *S. pneumoniae* transformants with the integration plasmid was observed, demonstrating that AZT not only promotes competence, but can also enhance HGT by killing AZT-susceptible donors (Table S3).

AZT and CLA do not induce competence via HtrA or altering gene dosage

So far, two different molecular mechanisms of competence induction by antibiotics have been described. The first mechanism is via substrate competition of the HtrA protease, which degrades both CSP and misfolded proteins (Stevens et al. 2011; Cassone et al. 2012). An increase of misfolded proteins induced by aminoglycosides would derepress competence, because HtrA is occupied and cannot degrade CSP anymore. The second mechanism described applies to antibiotics that stall DNA replication elongation such as fluoroquinolones or HPUra. These drugs cause replication forks to stall, while DNA replication initiation continues, resulting in an increase in copy numbers of genes close to the origin of replication (including both early competence operons *comAB* and *comCDE*) (Slager et al. 2014).
We confirmed that the aminoglycosides gentamicin and tobramycin are not able to induce competence in an *htrA* mutant strain (Figure S1). However, competence was still induced in the *htrA* mutant by AZT, CLA and HPUra. Notably, the *htrA* mutant on its own does not show upregulated competence (Figure S1) (Moreno-Gámez et al. 2017).
To test whether AZT and CLA induce competence via altering the gene dosage of the early competence operons, we performed marker frequency analysis. As shown in Figure 3A, a shift in origin to terminus ratio was observed after the addition of HPUra; however, the presence of AZT or CLA did not lead to an increase of the ori-ter ratio. To uncover potential transcriptional changes upon AZT or CLA treatment, we performed transcriptome profiling using DNA microarrays.
We analyzed the rapid (15 minutes after addition) and adaptive (cells growing with the compound) transcriptional exposures to AZT and CLA. These analyses validated the marker frequency experiments and no differential gene expression of origin-proximal genes was observed (Figure 3B). Furthermore, both compounds, at competence-inducing concentrations, had minor effects on the global transcriptome, suggesting that their effects are post-transcriptional (Tables S4 and S5).

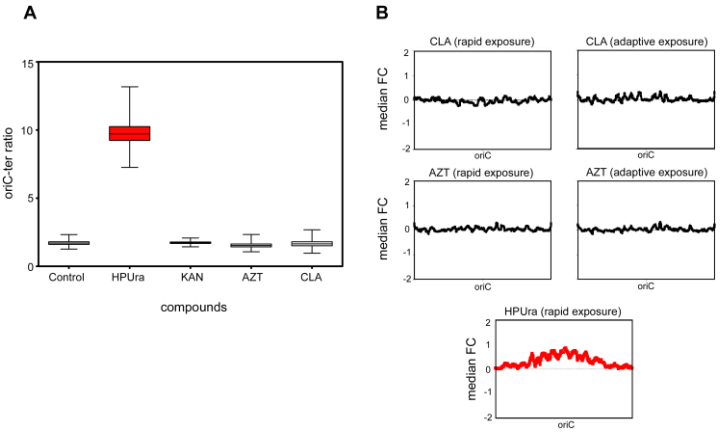

Figure 3. A) Antibiotic-induced shifts in origin-terminus ratio. Boxplots represent *oriC-ter* ratios as determined by real-time qPCR. Whiskers represent the 10th and 90th percentile of data from Monte Carlo simulations. Strain DLA3 (P_{ssbB}-*luc*) was grown in medium without (control) or with the following compounds: 0.15 µg/ml HPUra, 28 µg/ml kanamycin, 28 µg/ml of aztreonam and 2 µg/ml of clavulanic acid. Red box (HPura) matches with previous data showing an increase of the *oriC-ter* ratio (Slager *et al.,* 2014). **B) Transcript copy number changes.** The median fold change in a 2027 genes window is plotted for RNA as a function of the central gene's position. Both AZT and CLA do not affect the *oriC-ter* ratio. HPUra analysis from Slager *et al.,* is shown in red, as a positive control of oriC-ter ratio shift (Slager *et al.,* 2014).

AZT and CLA target PBP3 and induce cell chaining

It is well known that both AZT and CLA have an impact on cell wall synthesis. Specifically, it has been shown that they can directly interact with PBP3 (Kocaoglu et al. 2015; Severin et al. 1997). To assess whether perturbing cell wall synthesis could lead to activation of competence, we employed CRISPR interference (CRISPRi) allowing us to downregulate essential genes involved in cell wall biosynthesis (Liu et al. 2017). Downregulation of the expression of genes involved in peptidoglycan precursor synthesis (*murA-F*, *ftsW*) did not influence competence development (Figures S2-S3), as well as the class B PBPs (transpeptidase only) *pbp2b* and *pbp2x*. However, when the class A (dual transglycosylase and transpeptidase) PBP1A, or the D,D-carboxypeptidase PBP3 were repressed using CRISPRi, competence was strongly induced under otherwise non-permissive conditions (Figure 4A).

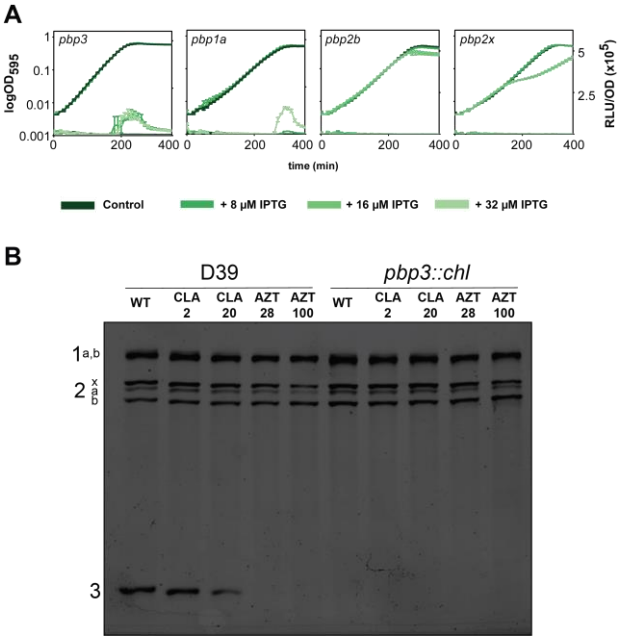

Figure 4. A) CRISPRi-dependent downregulation of *pbp1a* and *pbp3* leads to competence induction. Depletion of *pbp1a* and *pbp3* by induction of dCas9 with IPTG upregulates competence. In contrast, *pbp2b* and *pbp2x* do not have any effect on the regulation of this process. Detection of competence development was performed in C+Y medium at a non-permissive pH (pH 7.35). 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. **B) Representation of the PBP profiles of whole cells.** D39 and a *pbp3* mutant were treated with 2 µg/ml and 20 µg/ml of clavulanic acid (CLA), and 28 µg/ml and 100 µg/ml of aztreonam (AZT) and subsequently labeled with Bocillin-FL. Both AZT and CLA bind PBP3 in the D39 strain. Numbers indicate the different PBPs (i.e. 2b = PBP2B, 3 = PBP3).

To confirm that *pbp2b* and *pbp2x* do not upregulate competence, we repeated the same experiment in a permissive pH for natural competence. As expected, *pbp1a* and *pbp3* repression resulted in a stronger induction of competence, while *pbp2b* and *pbp2x* did not influence competence development (Figure S4).

To confirm that AZT and CLA bind PBP1A and/or PBP3, we used fluorescently labelled Bocillin (Bocillin-FL). As shown before (Kocaoglu et al. 2015; Severin et al. 1997), AZT and CLA bind PBP3, with AZT having a higher affinity to PBP3 than CLA (Figure 4B). As we were not able to clearly separate PBP1A and PBP1B, we cannot conclude whether AZT and/or CLA also bind to one of these PBPs. As *pbp3* is not essential, we constructed a replacement mutant. In line with the CRISPRi results, the *pbp3* mutant (strain ADP30) displayed a 'hypercompetent' behavior (Figure S5).

To examine the effects of AZT and CLA and of downregulation of *pbp1a* and *pbp3* on cell morphology, we performed microscopy analysis on exponentially growing cells (OD 0.1), when the wild type D39 strain becomes naturally competent (Moreno-Gámez et al. 2017). In contrast to downregulated *pbp2b* or *pbp2x* (Liu et al. 2017; Berg et al. 2013; Peters et al. 2014; Land et al. 2013), individual cell size and morphology was nearly unaltered by AZT, CLA or *pbp1a* and *pbp3* perturbation. However, in all cases, pneumococci formed longer chains of unseparated cells (Figure 5). When cells were grown until stationary phase (OD 0.4), chain formation was even more evident (Figure S6).

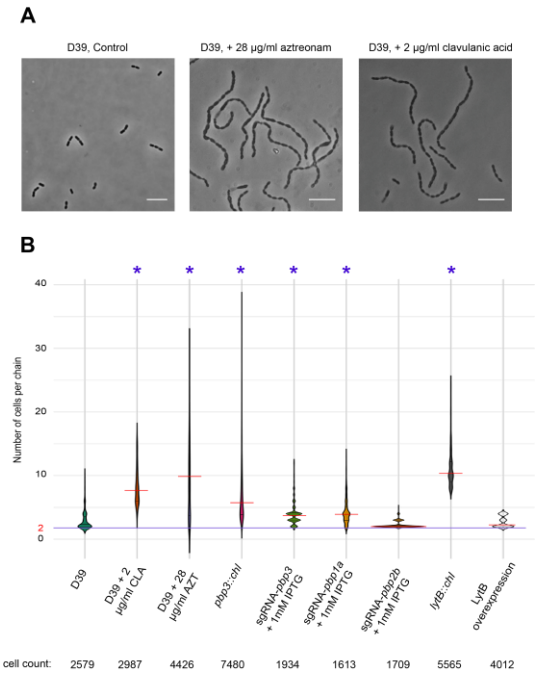

Figure 5. AZT and CLA induce chain formation. Cells were grown in C+Y acid medium until OD 0.1 (density at which cells become naturally competent). **A) Phase-contrast images**. Scale: 6 µm. **B) Length of the chains.** Horizontal red line means the average of the number of cells per chain while the purple line highlights the typical diplococcus state. 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. *Statistically significant longer chains then wild type, mean comparison test p<0.05.

Cell chaining is responsible for AZT- and CLA-induced competence

To test whether AZT and CLA induce competence by specific binding to PBP3 or because of cell chaining, we generated a knockout of the gene encoding the major autolysin LytB (strain ADP21). LytB mutants are well known to form long chains of cells due to their lack in muralytic activity at cell poles (De Las Rivas et al. 2002; Rico-Lastres et al. 2015; Berg et al. 2013; García et al. 1999). In line with the hypothesis that cell chaining induces competence, the Δ*lytB* mutant showed a hypercompetent phenotype, and readily developed competence even at a pH (pH 7.3) in which wild type cells do not become naturally competent (Figure S7A). Importantly, ectopic overexpression of LytB in the Δ*lytB* mutant (ADP43), restored the normal diplococcus phenotype and restored competence development to wild-type-like (Figure 5 and S7B).

Cell chaining modifies quorum sensing into autocrine-like signaling

We hypothesized that antibiotic-induced chaining reduces diffusion of CSP, thus altering synchronization of competence within the population. Under our standard plate-reader conditions (at the population level), encapsulated D39 *S. pneumoniae* cells release CSP in the medium, and when the CSP concentration reaches a critical threshold, all cells activate competence in a synchronized manner

(Moreno-Gámez et al. 2017). This results in a very steep RLU (Relative Luminescence Units) slope from the P_{ssbB}-*luc* reporter at different inoculation densities (Figure 6A, green line). In contrast, in the presence of AZT or CLA, the RLU increase at lower densities starts earlier, since both compounds induce competence; however, the slope of light production is less steep, indicating reduced synchronization of competence among the population (Figure 6A).

To confirm whether this observation occurs at a single-cell level as well, we performed flow cytometry using a D39 variant with a full translational SsbB-GFP fusion that is under ComX control. The presence of AZT resulted in an earlier GFP expression due to competence induction; however, the percentage of positive cells increased slower compared with the control condition, which strongly correlates with our observations at the population level (Figure 6B). Finally, similar results were obtained by fluorescence microscopy and AZT resulted in earlier but less synchronous competence activation (Figure 6C).

Reduced diffusion stimulates competence development

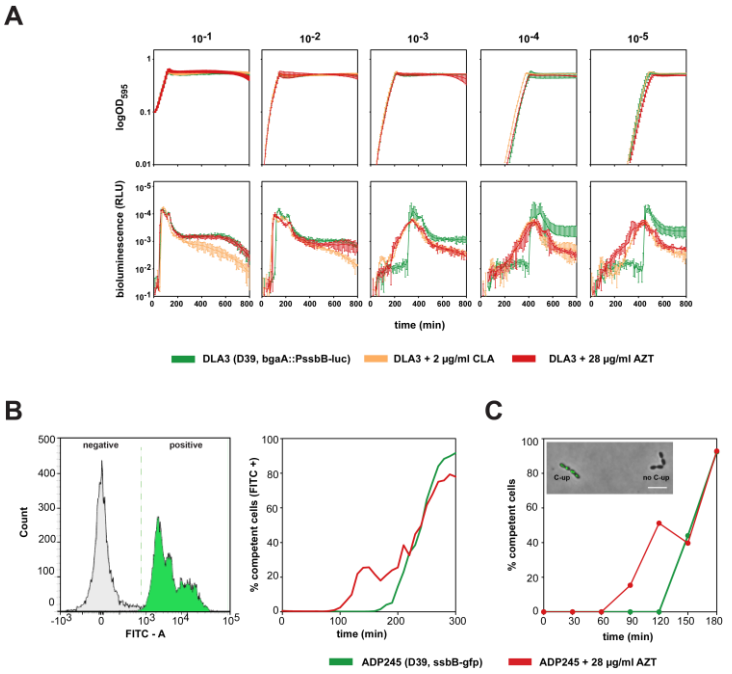

Figure 6. Synchronization of competence is affected by chain formation. A) Growth curves (OD_{595nm}; up) and competence expression (relative luminescence units; down) in a range of initial cell densities. Cells were grown in C+Y at a competence-permissive pH without antibiotics (green lines), in the presence of 2 µg/ml clavulanic acid (orange lines) or with 28 µg/ml of aztreonam (red lines). Average of 3 replicates and Standard Error of the Mean (SEM) are plotted for each of five inoculation densities: OD_{595nm} 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. **B)** At left, cutoff value for FITC positive detection (competence activation). Cells untreated (grey) and pretreated with CSP to fully activate competence (green). **B)** Left, cells untreated (grey) and treated with CSP (green) were used to stablish the cutoff value for FITC positive (competence activation). Right, competence (FITC +) was induced earlier when aztreonam was present (red) than the control condition (green). The slope of competence induction was drastically less steep with AZT, confirming a loose of synchronization within the population. **C)** Single cells were observed on fluorescent microscope every 30 minutes, observing the same trend than the FACS experiment (panel B, right). An average of 250 colonies were counted in every time point. White scale: 4 µm. C-up: competence upregulation.

As competence is initiated by recognition of extracellular CSP by its cognate membrane-anchored receptor (ComD), we hypothesize that chain formation alters CSP diffusion and thereby sensing by ComD. To test whether a reduction of CSP diffusion in the medium can also trigger and desynchronize competence, we decreased the diffusion coefficient of the growth medium by making it more viscous. To do so, we grew cells in a range of concentrations of Pluronic-137, an innocuous polymer that increases the medium density without affecting cell metabolism (Yang et al. 2010). As expected, the addition of this polymer resulted in an earlier competence development (Figure S8A). Interestingly, pneumococcal cell morphology and cell chain size are unaltered by the addition of Pluronic 137, with the exception of the highest inoculum (Figure S8B). However, we observed a reduced RLU slope of the P_{ssbB}-luc reporter at several inoculation densities at both Pluronic concentrations tested. Together, these data suggest that a reduced diffusion of the CSP pheromone in the medium results in earlier activation of the CSP-ComD-ComE positive feedback, but at the same time desynchronizes population-wide competence activation.

Discussion

Many clinically used antibiotics are able to induce competence, which can subsequently lead to the acquisition of antibiotic resistance. Two molecular mechanisms underlying antibiotic-induced competence have been described: altered gene-dosage by DNA targeting antibiotics (Slager et al. 2014), and reduced degradation of CSP by HtrA under mistranslation conditions (Stevens et al. 2011). The principal contribution of this work is the identification of a third mechanism by which certain cell wall targeting antibiotics can induce competence. Specifically, the commonly prescribed antibiotic aztreonam (AZT), which is used to treat respiratory infections caused by Gram-negative bacteria, and clavulanic acid (CLA), which is frequently co-administered with the broad spectrum antibiotic amoxicillin, induce competence (Figure 1B). Both AZT and CLA target the non-essential PBP3 of *S. pneumoniae* (Figure 4B) (Kocaoglu et al. 2015; Severin et al. 1997), and we show that this causes cell chaining (Figure 5).

Pneumococcal competence is a noise-based quorum sensing process, where single cells produce and sense CSP at different rates. Neighbor communication between cells is crucial in the modulation of the relationship between the number of cells and the CSP concentration, rather than the heterogeneity of the CSP production by single cells (Figure 7, left). However, several factors, such as pH or antibiotics, can modify the rates at which single cells produce and sense CSP (Moreno-Gámez et al. 2017; Prudhomme et al. 2016). Our results suggest that chain formation by the presence of AZT or CLA, modifies the balance between CSP production and sensing, increasing the self-sensing of CSP between cells in the same chain. Thus, single cells that produce more CSP than average, are more likely to share this CSP with cells of the same chain (autocrine-like signaling), reducing the shared pool of CSP (Figure 7, right)(Bareia et al. 2017). At the population and single-cell level, this creates local positive-feedbacks that result in an earlier timed competence response, which is also less synchronized (Figure 5). In addition, the presence of chains could decrease the diffusivity of the CSP in the medium, enhancing autocrine signaling (Figure S8).

It is interesting to note that our observations reconcile observations made across different laboratories concerning the dynamics of pneumococcal competence. Recently we showed that unencapsulated strains are less synchronized in competence development, which would explain the difference between the findings reported by (Prudhomme et al. 2016) and our previous study (Moreno-Gámez et al. 2017). Strains R6 and D39 do not form chains under normal growth conditions, but this difference could be explained by the presence of a polysaccharide

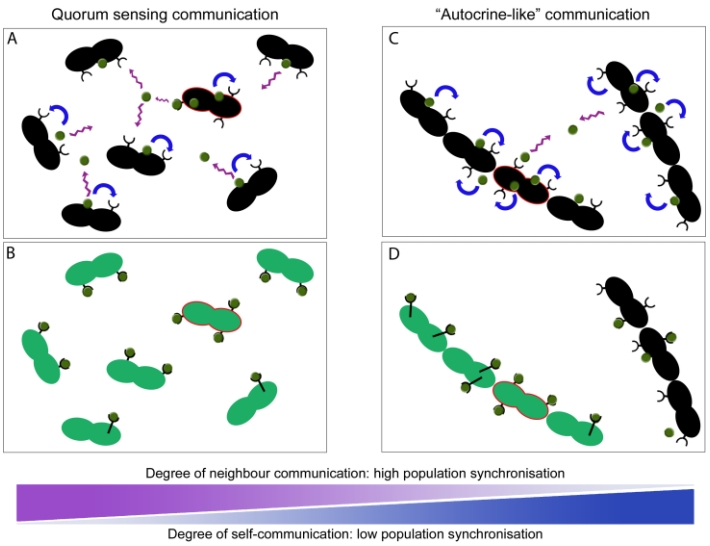

Figure 7. Models of Quorum sensing signaling (left) and contact-dependent autocrine-like signaling (right). A) *Streptococcus pneumoniae* secretes CSP to communicate with other cells (purple arrows) and to synchronize competence once a critical CSP (green dots) threshold is reached. In addition, self-sensing CSP (blue arrows) plays a role, as part of the CSP pool is retained to the producer (Prudhomme et al. 2016). Diplococci producing more CSP than average (highlighted in red) contribute to the increase of the extracellular CSP pool. **B)** Because of neighbor communication, when the CSP threshold is reached, all diplococci synchronize and become competent at the same time (green cells). **C)** Chain formation modifies the quorum sensing signaling mechanism to an autocrine-like signaling system. CSP released by cells present in the same chain is retained and sensed by the same chain. **D)** As a result of the autocrine-like signaling, the extracellular pool is lower thereby reducing the communication with other cells and decreasing competence synchronization (not all the cells become competent at the same time). However, as stochastic fluctuations are not buffered because of the shared pool of CSP, individual chains of cells will initiate competence earlier than well mixed populations consisting of diplococci.

capsule decreasing sensing efficiency. The work presented here suggests that differences in medium diffusion or the extent of cell chaining present under the tested experimental conditions could also explain these discrepancies.

Noteworthy, other clinical beta-lactam antibiotics such as aminopenicillins or cephalosporins are not able to induce competence as they do not bind PBP3, but PBP2B and/or PBP2X (Kocaoglu et al. 2015). Contrary to PBP3, the depletion of PBP2B and PBP2X is not related with chain formation, and thereby to upregulation of competence (Figure 4A).

Amoxicillin/clavulanic acid (Augmentin) has been available for over 20 years, and continues to be one of the most widely used antibiotics, especially in the treatment of respiratory tract infections. However, CLA is a beta-lactamase inhibitor that is useless for the specific treatment of pneumococcal infections, as there have been no reports of *S. pneumoniae* producing beta-lactamases. Our study suggests that in such cases clavulanic acid can best be omitted for antibiotic therapy as it would drive pneumococcal evolution and potentiate antibiotic resistance development by upregulating competence. Additionally, it has been described that the presence of pneumococcal chains enhances adhesion and colonization (Rodriguez et al. 2012), facilitating the persistence in the nasopharynx, increasing the probability of spreading and dissemination.

It will be interesting to see how competence is synchronized in more realistic environments, closely resembling the polymicrobial environment that is present in the human nasopharynx. Furthermore, continued molecular epidemiology studies will be crucial to determine the role and long-term effects of antibiotic therapy and vaccination on pneumococcal prevalence and antibiotic resistance.

Materials and Methods

Bacterial strains and growth conditions.

All pneumococcal strains used in this study are derivatives of the clinical isolate *S. pneumoniae* D39 (Avery et al. 1944) unless specified otherwise. To monitor competence development, strains either contain a transcriptional fusion of the firefly *luc* and the *gfp* gene with the late competence gene *ssbB* or a full translational *ssbB-gfp* fusion. See Table S6 for a list of the strains used and the Supplemental information for details on the construction of the strains. *S. pneumoniae* was always grown in C+Y medium at 37ºC. C+Y was adapted from Adams and Roe (Adams & Roe 1945) and contained the following compounds: adenosine (68.2 µM), uridine (74.6 µM), L-asparagine (302 µM), L-cysteine (84.6 µM), L-glutamine (137 µM), L-tryptophan (26.8 µM), casein hydrolysate (4.56 g L^{-1}), BSA (729 mg L^{-1}), biotin (2.24 µM), nicotinic acid (4.44 µM), pyridoxine (3.10 µM), calcium pantothenate (4.59 µM), thiamin (1.73 µM), riboflavin (0.678 µM), choline (43.7 µM), $CaCl_2$ (103 µM), K_2HPO_4 (44.5 mM), $MgCl_2$ (2.24 mM), $FeSO_4$ (1.64 µM), $CuSO_4$ (1.82 µM), $ZnSO_4$ (1.58 µM), $MnCl_2$ (1.29 µM), glucose (10.1 mM), sodium pyruvate (2.48 mM), saccharose (861 µM), sodium acetate (22.2 mM) and yeast extract (2.28 g L^{-1}).

Luminescence assays of competence induction.

Cells were pre-cultured in C+Y (pH 6.8) at 37ºC to an OD_{595nm} of 0.4. Right before inoculation, cells were collected by centrifugation (8000 rpm for 3 minutes) and resuspended in fresh C+Y at pH ~7.35, which is non-permissive for natural spontaneous competence under these experimental conditions. All experiments were started with an inoculation density of OD_{595nm} 0.004, unless indicated. Luciferase assays were performed in 96-wells plates with a Tecan Infinite 200 PRO illuminometer at 37ºC as described before (Slager et al. 2014). Luciferin was added at a concentration of 0.45 mg/mL to monitor competence by means of luciferase activity. Optical density (OD_{595nm}) and luminescence (relative luminescence units [RLU]) were measured every 10 minutes. For the CRISPRi experiments, cells were grown as above, and diluted 100x at pH 7.5 (allowing for natural competence) in the presence of a range of IPTG indicated for each condition.

Detection of the PBPs using Bocillin-FL.

Samples were prepared as described before (Kocaoglu et al. 2015) with slight modifications. Briefly, 4 ml of cells were grown in C+Y pH 6.8 until OD 0.15 and harvested by centrifugation (16,000 × *g* for 2 min at 4 ºC). Cell pellets were washed in 1 ml PBS, pH 7.4. Cells were pelleted and resuspended in 50 µl PBS with or without the indicated concentration of CLA or AZT. After 30 min of incubation at room temperature, cells were pelleted, washed in 1 ml PBS, and resuspended in 50 µl PBS containing 5 µg/ml Bocillin-FL. After 10 min of incubation at room temperature, cells were washed again in 1 ml PBS. Next, cells were sonicated on ice (power 30%, three cycles of 10 seconds interval with a 10 seconds cooling time on ice (Sonoplus, Bandelin). Then samples were centrifuged at max speed for 15 min at 4°C and pellets were resuspended in 100 µl cold PBS. The protein concentration was adjusted to 2 mg/ml as determined by Bradford by diluting with PBS. 5× SDS-PAGE loading buffer was added to each

sample and heated 10 minutes at 95 ºC. Proteins were separated by gel electrophoresis (10% acrylamide) for 2.5 h at 180 V, 400 mA, and 60 W. The gel was scanned using a Typhoon gel scanner (Amersham Biosciences, Pittsburgh, PA) with a 526-nm short-pass filter at a 25-µm resolution.

Intraspecies horizontal gene transfer (HGT).

We calculated the *in vitro* HGT efficiency using two genetically identical pneumococcal strains, differing only with the integration of two antibiotic resistance markers at two different locations of the genome. Strains DLA3 and MK134 (tetracycline and kanamycin resistant, respectively), (Slager et al. 2014) were grown to OD_{595nm} 0.4 in C+Y pH 6.8 at 37°C (non-permissive conditions for natural competence activation). Then, a mixed 100-fold dilution of both strains were grown in C+Y pH 7.3 (non-permissive conditions) and pH 7.5 (permissive conditions) to OD_{595nm} to promote the transfer of genes. Afterwards, serial dilution of cultures were plated with or without antibiotics (for the recovery of the total viable counts) and with the combination of 125 µg/ml of kanamycin plus 1 µg/ml tetracycline.

Interspecies horizontal gene transfer.

S. pneumoniae strain D39 was grown to OD_{595nm} 0.4 in C+Y pH 6.8 at 37°C, and *E. coli* carrying the plasmid pLA18 (integrates the tetracycline resistant marker *tetM*, via double crossover at the non-essential *bgaA* gene in *S. pneumoniae*, and contains a high copy Gram-negative origin of replication; Slager et al. 2014) was grown overnight with shaking, in LB supplemented with 100 µg/ml of ampicillin (resistant marker also contained in the plasmid, outside the double integration region). Both strains were diluted to OD_{595} 0.004 and co-incubated with or without 28 µg/ml of AZT in C+Y pH 7.35. After 3h, serial dilutions were plated either with 1 µg/ml of tetracycline (to recover transformants) or 50 µg/ml of aztreonam (to recover only the total viable pneumococci). Transformation efficiency was calculated by dividing the number of transformants by the total number of viable count. Three independent replicates of each condition were performed.

Microarray experiments.

Pneumococcal transcriptome profiles in the presence or absence of antibiotics were tested under conditions that do not support natural competence development to avoid differences in gene expression due to the activation of the competence pathway. We used strain *S. pneumoniae* ADP62 (D39 non-competent variant, *comC*::*chl*), grown in two biological replicates in C+Y (pH 7.6). Two kind of experiments were performed to detect rapid and adaptive exposures to the antibiotics. For the fast response, cells were collected during mid-exponential growth phase (OD 0.15) and incubated 15 minutes with or without 2 µg/ml of CLA or 28 µg/ml of AZT. For the adaptive response, cells at OD 0.15 were diluted 100X with or without the same concentration of antibiotics and grown again until OD 0.15. Results were compared using DNA microarray analysis, as previously described. (Shafeeq et al. 2015). For the identification of differentially expressed genes a Bayesian $p< 0.001$ and a fold change cut-off ≥ 2 was applied. Microarray data is available at Gene Expression Omnibus (GEO) with accession number GSE111562.

oriC-ter ratio determination by Real-Time qPCR.

Cells were grown as described above in the presence of antibiotics. In the real-time qPCR experiments, samples were prepared as previously detailed (Slager et al. 2014). Amplification was performed on a iQ5 Real-Time PCR Detection System (Bio-Rad). To find the amplification efficiencies, Monte Carlo simulations were performed with Mathematica (Wolfram Research); average CT-values and their corresponding standard deviations were used to simulate 10,000 new sets of CT-values, and with those the amplification efficiencies were computed for each set.

Chain formation detection.

To detect morphological changes, we incubated the different strains in C+Y acid medium (~pH 6.8) until OD_{595nm} 0.1 and OD_{595nm} 0.4. Antibiotics or IPTG were added when indicated. 1 µl of cells at the indicated optical density was spotted onto a PBS agarose pad on microscope slides, and phase contrast images were acquired with a Leica DMi8 microscope. Microscopy images conversions were done using Fiji and analysis of the length of the chains was done using MicrobeJ (Ducret et al. 2016). Plotting was performed using the BactMAP package (Van Raaphorst R, in preparation)

Fluorescence microscopy.

Cells were pre-cultured in acid C+Y (pH 6.8) until OD 0.1 and diluted 1000 times in fresh C+Y (pH 7.9) with or without 28 µg/ml of aztreonam. Samples were collected every 30 minutes, and phase contrast and GFP images were taken using a Leica DMi8 microscope as explained before.

Flow cytometry.

ADP245 cells were pre-cultured in C+Y (pH 6.8) at 37ºC to an OD_{595nm} of 0.1 and diluted as explained before in C+Y (pH 7.9). Cells were thoroughly vortexed to avoid possible chains. Experiments were started with an inoculation density of OD_{595nm} 0.0001, with or without 28 µg/ml of AZT. Optical density (OD_{595nm}) was measured every 10 minutes in 96-wells plates with a Tecan Infinite 200 PRO luminometer at 37ºC. Right after every measurement, a sample was taken and measured on a Novocyte Flow Cytometer (ACEA Biosciences). The pneumococci were gated to exclude debris. Twelve thousand bacteria were analyzed for FITC fluorescence (GFP expression) with a flow rate of 9 µl/min. Cells pretreated with CSP and cells untreated were used to establish the cutoff value for FITC positive (competence activation). Results were analyzed by Novoexpress software (ACEA Biosciences).

Author Contributions and Notes

A.D. and J.W.V. designed research, A.D. and J.S performed research, A.D. and J.S. analyzed data; and A.D. and J.W.V. wrote the paper.
The authors declare no conflict of interest.
This article contains supporting information online.

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References

Adams MH, Roe AS. (1945). A Partially Defined Medium for Cultivation of Pneumococcus. J Bacteriol. 49, 401-9. https://www.ncbi.nlm.nih.gov/pubmed/16560933.

Avery OT, Macleod CM, McCarty M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types: Induction of transformation by a

desoxyribonucleic acid fraction isolated from pneumococcus type III. J Exp Med. 79, 137-58. https://www.ncbi.nlm.nih.gov/pubmed/19871359.

Bareia T, Pollak S, Eldar A. (2018). Self-sensing in *Bacillus subtilis* quorum-sensing systems. Nat Microbiol. 3, 83-89. http://dx.doi.org/10.1038/s41564-017-0044-z.

Berg KH, Stamsås GA, Straume D, Håvarstein LS. (2013). Effects of low PBP2b levels on cell morphology and peptidoglycan composition in *Streptococcus pneumoniae* R6. J Bacteriol. 195, 4342-54. http://dx.doi.org/10.1128/JB.00184-13.

Cassone M, Gagne AL, Spruce LA, Seeholzer SH, Sebert ME. (2012). The HtrA protease from *Streptococcus pneumoniae.* digests both denatured proteins and the competence-stimulating peptide. J Biol Chem. 287, 38449-59. http://dx.doi.org/10.1074/jbc.M112.391482.

Chen JD, Morrison DA. (1987). Modulation of competence for genetic transformation in *Streptococcus pneumoniae*. J Gen Microbiol. 133, 1959-67. http://dx.doi.org/10.1099/00221287-133-7-1959.

Christie PJ. (2016). Classic Spotlight: the Physiological State of Competence and So Much More. J Bacteriol. 198, 1005–6. http://dx.doi.org/10.1128/JB.00078-16.

Claverys J-P, Martin B, Polard P. (2009). The genetic transformation machinery: composition, localization, and mechanism. FEMS Microbiol Rev. 33, 643-56. http://dx.doi.org/10.1111/j.1574-6976.2009.00164.x.

Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, van der Linden M, McGee L, von Gottberg A, Song JH, Ko KS, Pichon B, Baker S, Parry CM, Lambertsen LM, Shahinas D, Pillai DR, Mitchell TJ, Dougan G, Tomasz A, Klugman KP, Parkhill J, Hanage WP, Bentley SD. (2011). Rapid pneumococcal evolution in response to clinical interventions. Science. 331, 430-4. http://dx.doi.org/10.1126/science.1198545.

De Las Rivas B, Garcia JL, Lopez R, Garcia P. (2002). Purification and Polar Localization of Pneumococcal LytB, a Putative Endo- -N-Acetylglucosaminidase: the Chain-Dispersing Murein Hydrolase. J Bacteriol. 184, 4988-5000. http://dx.doi.org/10.1128/jb.184.18.4988-5000.2002.

Doğaner BA, Yan LKQ, Youk H. (2016). Autocrine Signaling and Quorum Sensing: Extreme Ends of a Common Spectrum. Trends Cell Biol. 26, 262-71. http://dx.doi.org/10.1016/j.tcb.2015.11.002.

Ducret A, Quardokus EM, Brun YV. (2016). MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. Nat Microbiol. 1(7):16077. http://dx.doi.org/10.1038/nmicrobiol.2016.77.

García P, González MP, García E, López R, García JL. (1999). LytB, a novel pneumococcal murein hydrolase essential for cell separation. Mol Microbiol. 31, 1275-81. https://www.ncbi.nlm.nih.gov/pubmed/10096093.

Havarstein LS, Coomaraswamy G, Morrison DA. (1995). An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. PNAS. 92, 11140–4. PMID: 7479953.

Kjos M, Miller E, Slager J, Lake FB, Gericke O, Roberts IS, Rozen DE, Veening JW. (2016). Expression of *Streptococcus pneumoniae* Bacteriocins Is Induced by Antibiotics via Regulatory Interplay with the Competence System. PLoS Pathog. 12(2): e1005422. http://dx.doi.org/10.1371/journal.ppat.1005422.

Kocaoglu O, Tsui H-CT, Winkler ME, Carlson EE. (2015). Profiling of β-lactam selectivity for penicillin-binding proteins in *Streptococcus pneumoniae* D39. Antimicrob Agents Chemother. 59, 3548-55. http://dx.doi.org/10.1128/AAC.05142-14.

Kyaw MH, Clarke S, Jones IG, Campbell H. (2002). Non-invasive pneumococcal disease and antimicrobial resistance: vaccine implications. Epidemiol Infect. 128, 21-7. https://www.ncbi.nlm.nih.gov/pubmed/11895087.

Land AD, Tsui H-CT, Kocaoglu O, Vella SA, Shaw SL, Keen SK, Sham LT, Carlson EE, Winkler ME. (2013). Requirement of essential Pbp2x and GpsB for septal ring closure in *Streptococcus pneumoniae* D39. Mol Microbiol. 90, 939-55. http://dx.doi.org/10.1111/mmi.12408.

Liu X, Gallay C, Kjos M, Domenech A, Slager J, van Kessel SP, Knoops K, Sorg RA, Zhang JR, Veening JW. (2017). High-throughput CRISPRi phenotyping identifies new essential genes in *Streptococcus pneumoniae*. Mol Syst Biol. 13, 931. http://dx.doi.org/10.15252/msb.20167449.

Martin B, Soulet A-L, Mirouze N, Prudhomme M, Mortier-Barrière I, Granadel C, Noirot-Gros MF, Noirot P, Polard P, Claverys JP. (2013). ComE/ComE~P interplay dictates activation or extinction status of pneumococcal X-state (competence). Mol Microbiol. 87, 394-411. http://dx.doi.org/10.1111/mmi.12104.

Moreno-Gámez S, Sorg RA, Domenech A, Kjos M, Weissing FJ, van Doorn GS, Veening JW. (2017). Quorum sensing integrates environmental cues, cell density and cell history to control bacterial competence. Nat Commun. 8, 854. http://dx.doi.org/10.1038/s41467-017-00903-y.

O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, Lee E, Mulholland K, Levine OS, Cherian T; Hib and Pneumococcal Global Burden of Disease Study Team. (2009). Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet. 374, 893-902. http://dx.doi.org/10.1016/S0140-6736(09)61204-6.

Pestova EV, Håvarstein LS, Morrison DA. (1996). Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. Mol Microbiol. 21, 853–62. https://www.ncbi.nlm.nih.gov/pubmed/8878046.

Peters K, Schweizer I, Beilharz K, Stahlmann C, Veening JW, Hakenbeck R, Denapaite D. (2014). *Streptococcus pneumoniae* PBP2x mid-cell localization requires the C-terminal PASTA domains and is essential for cell shape maintenance. Mol Microbiol. 92, 733-55. http://dx.doi.org/10.1111/mmi.12588.

Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys J-P. (2006). Antibiotic Stress Induces Genetic Transformability in the Human Pathogen *Streptococcus pneumoniae*. Science. 313, 89–92. http://dx.doi.org/10.1126/science.1127912.

Prudhomme M, Berge M, Martin B, Polard P. (2016). Pneumococcal Competence Coordination Relies on a Cell-Contact Sensing Mechanism. PLoS Genet. 12(6):e1006113. http://dx.doi.org/10.1371/journal.pgen.1006113.

Rico-Lastres P, Díez-Martínez R, Iglesias-Bexiga M, Bustamante N, Aldridge C, Hesek D, Lee M, Mobashery S, Gray J, Vollmer W, García P, Menéndez M. (2015). Substrate recognition and catalysis by LytB, a pneumococcal peptidoglycan hydrolase involved in virulence. Sci Rep. 5, 16198. http://dx.doi.org/10.1038/srep16198.

Rodriguez JL, Dalia AB, Weiser JN. (2012). Increased chain length promotes pneumococcal adherence and colonization. Infect Immun. 80, 3454-9. http://dx.doi.org/10.1128/IAI.00587-12.

Schrag SJ, Beall B, Dowell SF. (2000). Limiting the spread of resistant pneumococci: biological and epidemiologic evidence for the effectiveness of alternative interventions. Clin Microbiol Rev. 13, 588-601. https://www.ncbi.nlm.nih.gov/pubmed/11023959.

Severin A, Severina E, Tomasz A. (1997). Abnormal physiological properties and altered cell wall composition in *Streptococcus pneumoniae* grown in the presence of clavulanic acid. Antimicrob Agents Chemother. 41, 504-10. https://www.ncbi.nlm.nih.gov/pubmed/9055983.

Shafeeq S, Afzal M, Henriques-Normark B, Kuipers OP. (2015). Transcriptional profiling of UlaR-regulated genes in *Streptococcus pneumoniae*. Genom Data. 4, 57-9. http://dx.doi.org/10.1016/j.gdata.2015.02.004.

Slager J, Kjos M, Attaiech L, Veening J-W. (2014). Antibiotic-induced replication stress triggers bacterial competence by increasing gene dosage near the origin. Cell. 157, 395-406. http://dx.doi.org/10.1016/j.cell.2014.01.068.

Sorg RA, Kuipers OP, Veening JW. (2015). Gene expression platform for synthetic biology in the human pathogen *Streptococcus pneumoniae*. ACS Synth Biol. 4, 228-39. http://dx.doi.org/10.1021/sb500229s.

Stevens KE, Chang D, Zwack EE, Sebert ME. (2011). Competence in *Streptococcus pneumoniae* is regulated by the rate of ribosomal decoding errors. MBio. 20;2(5). http://dx.doi.org/10.1128/mBio.00071-11.

Yang J, Evans BA, Rozen DE. (2010). Signal diffusion and the mitigation of social exploitation in pneumococcal competence signalling. Proc Biol Sci. 277, 2991-9. http://dx.doi.org/10.1098/rspb.2010.0659.

Veening J-W, Blokesch M. (2017). Interbacterial predation as a strategy for DNA acquisition in naturally competent bacteria. Nat Rev Microbiol. 15, 621-629. http://dx.doi.org/10.1038/nrmicro.2017.89.

Wholey W-Y, Kochan TJ, Storck DN, Dawid S. (2016). Coordinated Bacteriocin Expression and Competence in *Streptococcus pneumoniae* Contributes to Genetic Adaptation through Neighbor Predation. PLoS Pathog. 12(2): e1005413. http://dx.doi.org/10.1371/journal.ppat.1005413.