RESEARCH ARTICLE

Expression of *Streptococcus pneumoniae* Bacteriocins Is Induced by Antibiotics via Regulatory Interplay with the Competence System

Morten Kjos, Eric Miller, Jelle Slager, Frank B. Lake, Oliver Gericke, Ian S. Roberts, Daniel E. Rozen*, Jan-Willem Veening

1 Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, Centre for Synthetic Biology, University of Groningen, Groningen, The Netherlands, 2 Institute of Biology, Leiden University, Leiden, the Netherlands, 3 Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom

☯ These authors contributed equally to this work.

* d.e.rozen@biology.leidenuniv.nl (DER); j.w.veening@rug.nl (JWV)

Abstract

Pneumococcal bacteriocins (pneumocins) are antibacterial toxins that mediate intra-species competition within the human host. However, the triggers of pneumocin expression are poorly understood. Using RNA-sequencing, we mapped the regulon of the pneumocin cluster (*blp*) of *Streptococcus pneumoniae* D39. Furthermore, by analogy with pneumococcal competence, we show that several antibiotics activate the *blp*-genes. Using real-time gene expression measurements we show that while the promoter driving expression of the two-component regulatory system *blpR/H* is constitutive, the remaining *blp*-promoters that control pneumocin expression, immunity and the inducer peptide BlpC, are pH-dependent and induced in the late exponential phase. Intriguingly, competence for genetic transformation, mediated by the paralogous ComD/E two-component quorum system, is induced by the same environmental cues. To test for interplay between these regulatory systems, we quantified the regulatory response to the addition of synthetic BlpC and competence-stimulating peptide (CSP). Supporting the idea of such interplay, we found that immediately upon addition of CSP, the *blp*-promoters were activated in a *comD/E*-dependent manner. After a delay, *blp*-expression was highly induced and was strictly dependent on *blpRH* and *blpC*. This raised the question of the mechanism of BlpC export, since bioinformatic analysis showed that the genes encoding the putative exporter for BlpC, *blpAB*, are not intact in strain D39 and most other strains. By contrast, all sequenced pneumococcal strains contain intact *comAB* genes, encoding the transport system for CSP. Consistent with the idea that *comAB* mediate BlpC export, we finally show that high-level expression of the *blp*-genes requires *comAB*. Together, our results demonstrate that regulation of pneumocin expression is intertwined with competence, explaining why certain antibiotics induce *blp*-expression. Antibiotic-induced pneumocin expression might therefore have unpredictable consequences on pneumococcal colonization dynamics by activating genes that mediate intra-specific interference competition.
Author Summary

*Streptococcus pneumoniae* is an opportunist pathogen with high carriage rates in children. Pneumococci express pneumocins that kill competing bacteria. Pneumocin expression is controlled by a pheromone-induced two-component system (BlpR/H) but the triggers for the system are poorly understood. We show that the pheromone-induced two-component system driving competence for genetic transformation, ComD/E, also controls expression of BlpC, the peptide pheromone activating BlpR/H-dependent gene expression. Importantly, we show that the competence pheromone exporter, ComAB, also exports BlpC. Since antibiotics that disrupt protein quality control or DNA replication trigger competence, it follows that the same antibiotics activate pneumocin expression. Our experiments show that this dual-quorum sensing system ensures that pneumocins are expressed at the end of exponential growth when nutrients become limiting. Pneumocin expression might thus be used to liberate nutrients by lysing competing bacteria. Antibiopic-induced pneumocin production might also aid in clearing the niche after antibiotic stress. Any free DNA can then be used for transformation to acquire antibiotic-resistance.

Introduction

*Streptococcus pneumoniae* is a Gram-positive opportunistic pathogen that resides in the human nasopharynx. Pneumococci can cause invasive and non-invasive infections to which children, the elderly and the immunocompromised are particularly susceptible. The carriage rate of *S. pneumoniae* in the human population can be very high. Up to 80% of children under the age of 5 are colonized [1], and colonization with multiple strains simultaneously is widespread. Competition between strains in the human nasopharynx during co-colonization has important implications for the epidemiology of the pneumococcus, potentially influencing strain prevalence, serotype distributions and disease progression.

Among the most important potential drivers of intraspecific competition are several diverse classes of pneumococcal bacteriocins (small antimicrobial peptides), including the CibAB two-peptide bacteriocin [2], a lantibiotic [3] and the Blp bacteriocins (pneumocins) [4–6]. The *blp* locus is ubiquitous in pneumococcal genomes, and the operon is exceptionally diverse, suggesting that these toxins have evolved via diversifying selection. Puzzlingly, however, in most pneumococcal strains the genes mediating export of the *blp* peptides, encoding the ABC transporter BlpAB, carry frameshift mutation(s) that render these genes non-functional. This paradox raises two questions: 1) are Blp bacteriocins exported in strains lacking a functional transporter? and 2) if so, by which mechanism does this occur? One possibility is that bacteriocins are not exported, but that strains with interrupted *blpAB* alleles constitute so-called cheater sub-populations, which cannot export pneumocins alone but are able to express immunity in response to co-colonizing strains [7]. Such cheaters would therefore only activate *blp* expression in response to secreted BlpC in their surroundings. A second possibility is that Blp bacteriocins have an alternative mode of export and that strains with non-functional BlpAB transporters can activate their own *blp* expression. Here we provide direct evidence for this second hypothesis and show that *blp* bacteriocins are co-regulated with competence for transformation. Moreover, we show that environmental cues regulating competence overlap with those that induce Blp secretion. These functional data clarify the mechanisms of *blp* regulation and cast doubt on the validity of the *blpA* cheater hypothesis.

Genes for the production and regulation of Blp bacteriocins are organized in gene clusters with several operons, typically flanked by the genes *ecsB* and a putative choline kinase located...
at approximately 400–600 kb from the origin of replication. Pneumocin expression is regulated by a classical quorum sensing two-component regulatory system [5,8] which is conserved among diverse S. pneumoniae genomes [9]. The inducer peptide BlpC, which contains an N-terminal double-glycine leader sequence, is putatively processed and exported by an ABC transporter system, BlpAB. Indeed, Kochan and Dawid showed that an engineered laboratory strain with intact BlpAB efficiently processes BlpC but not in the absence of BlpA [10]. When the external concentration of BlpC exceeds a certain threshold, it binds specifically to a membrane-located histidine protein kinase BlpH [9], which activates the DNA binding response regulator BlpR by phosphorylation (BlpR-P). BlpR-P binds to specific sequence sites in the promoter region of blp to activate their expression. In addition to genes for regulation and transport, the blp gene cluster of S. pneumoniae also contains two genes of unknown function (blpS and blpT) along with genes encoding (putative) pneumocins (known as blpD, blpE, blpI, blpJ, blpK, blpM, blpN, blpO, blpQ, pncT and pncW) and cognate immunity genes (known as blpL, blpX, blpY, blpZ and pncP) [11]. Similar to BlpC, the pneumocins contain an N-terminal double-glycine leader, which is processed upon export via the ABC-transporter system. The region encoding pneumocins and immunity proteins (referred to as the BIR, bacteriocin immunity region) is highly variable and the number of pneumocin genes differs greatly between strains [7,12]. However, despite significant variation in the BIR region, a superficial analysis indicates that the cluster is intact even in strains with a degenerated blpA.

A likely candidate mechanism for Blp peptide export is the paralogous quorum-based two-component signaling system regulating competence for natural transformation (comCDE). com regulation is mediated by the export of a quorum sensing peptide (CSP) via an ABC transporter ComAB, followed by CSP concentration-dependent activation of downstream late competence genes. Importantly, the blp genes have been shown to be weakly upregulated during pneumococcus competence [13]. In addition to the Blp bacteriocins, competent S. pneumoniae also express CbpD and CibAB, a murein hydrolase and a two-peptide bacteriocin, respectively, which both cause lysis of non-competent cells [2].

The competence regulatory system is highly sensitive to environmental cues such as pH and exposure to certain antibiotics [14–16]. For example, sub-MIC concentrations of antibiotics that perturb DNA replication elongation (e.g. ciprofloxacin, trimethoprim and mitomycin C), induce competence by increasing the gene dosage of the comCDE genes, which are located close to the replication origin [16]. Moreover, aminoglycosides (e.g. kanamycin and streptomycin), which target protein synthesis and lead to high numbers of misfolded proteins, induce competence by decreasing the degradation rate of CSP in the external environment. This occurs because in cells exposed to aminoglycosides, the protease HtrA, which normally degrades CSP, is occupied with targeting misfolded proteins [17].

Our aims here are twofold. First, we critically evaluate hypotheses derived from the proposal that strains carrying degenerate blpA genes are cheaters that lack Blp secretion. Secondly, failing to find support for this idea, we seek mechanisms that could offset the consequences of blpA lesions, focusing in particular on the roles of the paralogous com operon and the influence of environmental cues. Briefly, we demonstrate that blp expression is co-regulated with the competence regulatory system of S. pneumoniae and show that the ‘cheaters’-hypothesis [7] is inconsistent with both bioinformatics and experimental data.

**Results**

**Strains with degenerate blpAB do not lose blp bacteriocins**

We identified blp bacteriocin open reading frames (ORFs) in 4,096 S. pneumoniae genomes, as well as all ORFs that had reciprocal best BLAST hits to blpA, blpB, comA and comB. We
identified striking diversity in the length of blpA/B across these genomes, with only 23.5% of genomes containing full-length blpA and blpB sequences (Fig 1A). We found a large diversity of indel and stop codon mutations that lead to interrupted ORFs in both of these genes. The entire blpAB region is completely deleted along with the adjoining blpC in 21 strains, while an additional 27 strains have no blpAB but still contain blpC. Thus, the majority of pneumococcal strains retain blpAB pseudogenes in the genome. If strains lacking a functional blpAB are incapable of exporting bacteriocins, then we would predict that these strains would lose bacteriocin genes by genetic drift. However, we only found a difference of 0.14 bacteriocins between the genomes with a full-length blpA (average = 4.46 bacteriocins) compared to the number of bacteriocins found in genomes with interrupted blpA ORFs (average = 4.32 bacteriocins; Wilcoxon rank test, $1.55 \times 10^{-8}$; Fig 1B). Although non-functional bacteriocin genes may be retained in the genome, this small difference in average number of bacteriocins between genomes with and without blpA suggests that bacteriocin secretion is not exclusively dependent on a functional BlpAB.

In order to examine the co-occurrence of interrupted ORFs in blpA and blpB, we mapped these gene variants onto a phylogenetic tree assembled using full genome SNPs (Fig 2). Using this phylogeny and a maximum likelihood approach, we found that genomes with an interrupted blpA are more likely to have an interrupted blpB, even after accounting for phylogeny (log ratio test, $p = 7.86 \times 10^{-4}$).

The ABC transporter system involved in competence, comAB, is the closest homolog to blpAB in S. pneumoniae. To examine if comA or comB variation is linked to co-occurrence of either full-length or interrupted ORFs of blpA and blpB, we divided comA and comB amino acid variants into similarity-based clusters termed phylotypes. We found no association between the phylotypes of either comA or comB and the structure of either blpA (log ratio test, $p > 0.133$ for comA, $p > 0.139$ for comB) or blpB (log ratio test, $p > 0.0889$ for comA, $p > 0.537$ for comB).

**Expression of blp genes in S. pneumoniae D39 is induced by antibiotics and is pH-dependent**

The bioinformatics results strongly suggest that pneumococcal strains with degenerate blpAB alleles are still able to express pneumocins but it remains unknown what factors stimulate natural blp expression. Exposure to antibiotics can lead to global changes in gene expression and can induce the competent state in S. pneumoniae [14–16]. To test whether antibiotics also trigger pneumocin gene expression, we re-examined our earlier RNA-seq data [16] of S. pneumoniae D39 cells treated with sub-MIC levels of HPura (6-(p-hydroxyphenylazo)-uracil) which blocks DNA replication [18] and kanamycin, which induces mistranslation [17]. In the absence of these agents, the blp genes were not expressed in C+Y medium at pH 7.4 (S1 Table). However, under the same pH conditions, both HPura and kanamycin weakly induced expression of some blp genes (S1 Table). To test if other antibiotics also induce blp gene expression, we performed transcriptome sequencing on RNA isolated from cells treated with ciprofloxacin (topoisomerase IV/DNA-gyrase inhibitor), hydroxyurea (decreases the cellular pool of dNTP via inhibition of ribonucleotide reductase) and rifampicin (RNA polymerase inhibitor). Similar to the earlier antibiotic exposure experiments [16], cells were harvested for RNA sequencing in early exponential phase. As previously observed, competence was activated by ciprofloxacin and hydroxyurea but not by rifampicin (S1 Table) [16]. Strikingly, ciprofloxacin and hydroxyurea also induced expression of some blp genes, although to a lesser degree (S1 Table). Together, the RNA-seq data shows that the same antibiotics that trigger competence also activate expression of blp genes.
Fig 1. The blpAB genes are frameshifted in the majority of pneumococcal genomes. (A) Distribution of blpAB ORF lengths in a set of 4,096 genomes. Amino acid residue numbers are shown at the top. Variants found in less than 0.5% of the genomes are not shown. Frameshifted regions encoding non-BlpA ORFs are...
The blp gene cluster of strain D39 contains four transcriptional units [5]; the regulatory operon blpSRH (promoter P_{blpS}), the transport operon blpABC (promoter P_{blpA}), the bacteriocin/immunity operon pncW-blpYZ-pncP (promoter P_{pncW}) and finally a transcriptional unit expressing a single gene of unknown function, blpT (promoter P_{blpT}) (Fig 3A). In addition, another blp promoter (P_{blpK}) is located outside the blp gene cluster in *S. pneumoniae* D39 (Fig 3A) [19] and controls the expression of BlpK, which encodes a putative bacteriocin. Each of the transcriptional units is likely under control by BlpR since the promoters contain an extended -10 element and upstream 9-bp tandem direct repeats (Fig 3B) [5]. Of the five promoters, P_{blpS} is the least conserved compared to P_{pncW}, P_{blpT}, P_{blpA} and P_{blpK}.

Fig 2. Association between blpAB length and comAB phylotypes. The phylogenetic tree of *S. pneumoniae* genomes is surrounded by three rings; moving outwards, these show the ORF lengths for blpAB, and the phylotypes for comA and comB, respectively. Partial length ORFs and missing data are also shown for comA and comB. comB phylotypes found in less than 0.5% of genomes are colored grey to indicate their status as full-length variants.

doi:10.1371/journal.ppat.1005422.g002
In order to investigate the conditions that induce blp expression, we developed a novel tripartite reporter cassette containing firefly luciferase (luc), superfolder gfp (gfp) and β-galactosidase (lacZ) in the BglBrick-compatible vector pPEP1 [20] (Fig 3C). Using these different reporters, expression can be monitored in cultures through time (luc), at the single cell level (gfp) or in colonies on agar plates (lacZ). Each of the five blp promoters identified above were fused upstream of this cassette, and the reporter constructs were chromosomally integrated at an ectopic locus (Fig 3C and Materials and Methods).
Using luc we could follow expression of the blp promoters in a time-resolved manner. S. pneumoniae was grown in C+Y medium and expression was monitored by luc activity. Expression was observed from all five blp promoters at pH 8.0 but not at pH 7.0 (see below). For four of the promoters, P<sub>blpT</sub>, P<sub>blpA</sub>, P<sub>pncW</sub> and P<sub>blpK</sub>, expression was activated simultaneously in late exponential phase and switched off when the population reached stationary phase (Fig 3D, S1 Fig). A second shallow peak of expression was observed in late stationary phase (Fig 3D, S1 Fig). Interestingly, P<sub>blpS</sub>, encoding the regulatory system, was active from early exponential phase, and displayed expression dynamics similar to known constitutive promoters in S. pneumoniae, such as the synthetic promoter P<sub>3</sub> (S2 Fig) [20]. The observed expression pattern allows for continuous expression of the regulatory genes, blpSRH, during all growth stages (Fig 3E). It should be noted that the P<sub>blpS</sub> sequence (Fig 3B) is the least conserved of the promoters and that this sequence is located 100 bp upstream of the blpS start codon; thus there might be an unidentified, non-blp promoter that drives the seemingly constitutive expression of blpSRH. BlpSRH together with BlpC constitute a quorum sensing regulatory system, and we can therefore assume that the extracellular BlpC concentration required to activate BlpR is reached by the end of exponential phase. Accordingly, no expression could be observed from the regulated promoters (P<sub>blpT</sub>, P<sub>blpA</sub>, P<sub>pncW</sub> and P<sub>blpK</sub>) when the regulatory genes blpSRH were deleted (Fig 3D and 3E, S1 Fig). Furthermore, deletion of only blpC also abolished promoter activity, demonstrating that induction is mediated via BlpC (S1 Fig), and blp expression in this strain could be activated by addition of synthetic BlpC (S1 Fig). Although the expression dynamics from the four inducible promoters were the same, their expression strengths were variable; P<sub>blpK</sub> was by far the strongest, showing approximately 3-fold higher maximum luc expression compared to P<sub>blpT</sub>, which again was stronger than P<sub>pncW</sub> and P<sub>blpA</sub> (Fig 3D, S1 Fig).

By adjusting the pH of the growth medium, we observed that natural induction of all the regulated blp promoters in D39 only occurs above a threshold medium pH of 7.4 (Fig 4A and S3 Fig). pH-dependent expression is also seen in cells grown as colonies on agar plates (Fig 4A, bottom panel). Importantly, expression from P<sub>blpS</sub> was independent of the initial pH of the medium (Fig 4B), confirming that this promoter is active across a broader range of environmental conditions.

To validate the RNA-seq results, which showed antibiotic-induced blp gene expression (S1 Table), we grew the reporter strains in C+Y pH 7.4 (which does not support blp induction) with and without sub-lethal concentrations of HPUra, ciprofloxacin and streptomycin. As shown in Fig 4C and S4 Fig, exposure to these competence-inducing antibiotics induced expression from all the regulated blp promoters (P<sub>blpT</sub>, P<sub>blpA</sub>, P<sub>pncW</sub>, P<sub>blpK</sub>). Importantly, exposure to rifampicin, which does not induce competence, also did not induce blp expression (S4 Fig). Moreover, the blpS promoter still displays activity throughout all growth stages, also when exposed to antibiotics (Fig 4D).

**Competence stimulating peptide (CSP) induces blp expression**

The pH dependency we observed for the blp promoters (Fig 4A) was previously shown for the promoters of the competence regulatory system [15,16,21]. Furthermore, as described above, blp expression was induced by exposure to the same antibiotics that induce com expression (Fig 4C, S4 Fig, S1 Table). This prompted us to further investigate the putative interplay between the blp system and the com system. The com system can be induced by external addition of the competence stimulating peptide, CSP. By addition of CSP to cultures growing in C + Y pH 7, which normally does not allow for blp expression, we observed as expected that expression of the com gene ssbB was immediately induced [13,15] (Fig 5A). Crucially, addition of CSP to the blp reporter strains also led to high expression from all regulated blp promoters.
in the late exponential phase, approximately 100 min after addition of CSP (Fig 5B and S5 Fig). Notably, this delayed high induction of blp via CSP still depended on the blp regulatory system, as CSP-activated induction could not be observed when blpSRHC was deleted (S5 Fig).

The onset of high blp expression occurred 100 min after addition of CSP, prompting us to investigate in detail the more immediate consequences of exogenous CSP on blp promoters.

We observed that addition of CSP caused a weak but consistent induction of the regulated blp promoters (Fig 5B–5D, S5 Fig). Importantly, the promoter controlling the inducer peptide blpC, P_{blpC}, was also induced by CSP (Fig 5D). The same effects were observed in strains with or without blpSRHC deletion strains, which rule out any effect of binding by BlpR. The weak
Fig 5. blp expression is induced by CSP. (A-B) Competence and blp expression are both stimulated by CSP addition, although with different kinetics. Competence reporter strain P_{ssbB-luc} (A) and blp reporter strain P_{blpT-luc} (B) grown in C+Y pH 7 with or without addition of 100 ng/ml CSP after 100 min (indicated by an arrow). Similar CSP induction of promoters P_{blpA}, P_{pncW} and P_{blpK} are shown in S5 Fig. (C-D) CSP causes a weak but immediate activation of the blp promoters independent of blpSRHC. Reporter strains for P_{blpT} (C) and P_{blpA} (D), in which blpSRHC are deleted, grown in C+Y pH 7. (E) The immediate inducing effect is dependent on the ComCDE regulatory system. Expression from P_{blpT} in a strain with ΔblpSRHC and ΔcomCDE grown in C+Y pH 7 is not induced by CSP addition. For extended plots of C-E, see S5 Fig. For all plots, gene expression as measured by luciferase activity (RLU/OD) is shown on the left axis and growth as measured by absorbance at 595 nm (OD595) is shown on the right axis. Averages of three replicates with the standard deviation are plotted. (F) blp expression is activated after competence in all cells. Time-lapse fluorescence microscopy of dual reporter strains for competence (P_{ssbB-rfp}) and blp expression (P_{blpK-gfp}) grown on C+Y agarose pH 8. PC; phase contrast.

doi:10.1371/journal.ppat.1005422.g005
but immediate effects of the addition of CSP on the blp promoter suggest that the competence regulatory system (comCDE) can activate the blp promoters. To test this more specifically, we therefore deleted comCDE, and in this case, no direct effect or any delayed induction of blp expression was observed (Fig 5E, S5 Fig).

To further study the dynamics of com- and blp activation, we constructed a double reporter strain where (i) the ssbB promoter was transcriptionally fused to mKate2, a red fluorescent protein, and (ii) P_{blpK} was fused to gfp. Using fluorescence time-lapse microscopy under conditions that allowed natural blp and competence expression (pH 8.0), we observed that blp expression was activated after cells became competent (Fig 5F). This single cell analysis showed that all cells in the population turn on expression of com or blp synchronously once the systems are activated (Fig 5F).

ComAB is responsible for processing/export of BlpC and thereby blp activation

First, to investigate whether the frameshifted blpAB locus could still be responsible for export and processing of BlpC, we replaced the blpAB pseudogenes with an antibiotic resistance marker, while keeping the promoter and blpC intact. As shown in Fig 6A, the ΔblpAB strain did not change expression of blp from any of the promoters (Fig 6 and S6 Fig) thereby demonstrating that, under these conditions, the remnants of blpAB in strain D39 are not involved in BlpC processing and export.

Next, we tested if BlpC utilizes the ComAB complex for processing and export. ComAB is highly similar to BlpAB (protein sequences show 61.9% identity between ComA/BlpA, and 29.0% identity between ComB/BlpB) and is located downstream (within 2.2 kb) of the BlpR-regulated blpK gene. Our bioinformatics analysis showed that ComAB are highly conserved and intact in most pneumococcal strains (Fig 2). Indeed, when deleting comAB, or even comA or comB separately, blp expression could no longer be induced by CSP (Fig 6B) and, natural blp expression at pH 8.0 was also abolished (Fig 6C). Addition of extracellular BlpC, however, still induced blp expression in the comAB deletion strain (S7 Fig).

When expressing comA, comB or comAB from an inducible promoter at an ectopic locus, we observed that comAB (but not comA or comB alone) caused onset of blp expression independent of blpAB, but again this occurred in late exponential phase (Fig 6D and S7 Fig). Ectopic expression of comAB also induced blp expression when the native comAB genes were deleted, but to lower levels (Fig 6E). Finally, the comAB-mediated induction was dependent on comCDE (Fig 6F), showing that ComCDE-stimulated blpC expression is also essential for natural blp activation.

Discussion

We and others recently discovered that exposing the human pathogen S. pneumoniae to antibiotics that either stall replication forks [16] or increase the misfolding of proteins [17] can induce competence, leading to increased transformation rates [15,16]. Here we show that exposing S. pneumoniae to the same types of antibiotics can also activate expression of the blp genes, encoding antimicrobial peptides as well as genes necessary for self-immunity, processing, transport and regulation. Because blp bacteriocins are involved in pneumococcal interstrain competition [4], antibiotic exposure therefore has the potential to indirectly modify competitive interactions among coexisting pneumococcal strains within the human nasopharynx.

We show that regulation of blp expression is tightly linked with the competence regulatory system. blp and competence expression is regulated by paralogous quorum sensing based two-
component systems. Over 75% of all pneumococcal strains sequenced to date (Fig 1) contain frameshift mutations in the ABC-transporter genes of the \textit{blp} locus (i.e. \textit{blpA} and \textit{blpB}) thus rendering them inoperative and incapable of bacteriocin transport. Nevertheless, natural \textit{blp} expression can only occur if \textit{comAB} is intact. Strains with the \textit{P_{blpT}} reporter were grown in C+Y pH 7.4 and CSP (100 ng/ml) was added as indicated by the arrow. (C) Natural induction of \textit{blp} expression can only occur if \textit{comAB} is intact. Strains with the \textit{P_{blpT}} reporter were grown in C+Y pH 8. (D) Ecotopic, inducible expression of \textit{comAB}, but not \textit{comA} or \textit{comB} alone, activate \textit{blp} expression. Strains with the \textit{P_{blpT}} reporter were grown in C+Y pH 7.4 with 0.1 mM ZnCl$_2$ and 0.01 mM MnCl$_2$ for induction. (E) Complementation of \textit{blp} expression in a \textit{ΔcomAB} mutant by ecotopic expression of \textit{comAB} from a Zn-inducible promoter. (F) \textit{comCDE} is required for \textit{blp} activation in late exponential phase. Ecotopic expression of \textit{comAB} cannot activate \textit{blp} expression when \textit{comCDE} is absent suggesting that the weak activation of \textit{blpC} transcription by \textit{comE} is necessary for \textit{blp} expression. For E and F, reporter strains were grown with or without 0.1 mM ZnCl$_2$ and 0.01 mM MnCl$_2$ in C+Y pH 7.1. For all plots, gene expression as measured by luciferase activity (RLU/OD) is shown on the left axis and growth as measured by absorbance at 595 nm (OD$_{595}$) is shown on the right axis. Averages of three replicates with the standard deviation are plotted.

doi:10.1371/journal.ppat.1005422.g006
genes which have a low basal expression level; therefore processing and export of CSP by the dedicated ComAB transporter can initiate in the early growth stages. By contrast, the gene encoding the blp induction peptide, blpC, is co-transcribed with the transporter genes blpAB and requires initial activation, probably via ComE-P, for basal expression. BlpC also needs to use the non-cognate transporter ComAB for processing and export (Fig 7), and this process may be inefficient compared to ComC export. Thus the accumulation of extracellular BlpC might be slow (or BlpH might require high levels of BlpC for autophosphorylation to occur), and this may explain the long delay between activation of competence and activation of blp expression (approximately 100 min). Another reason for slow BlpC accumulation may be that the number of active ComAB proteins present in cells is low after escape from the competent state. Furthermore, we cannot exclude that also other factors, for example unidentified σX-dependent genes, are also involved in the late exponential phase activation of blp expression. Nevertheless, activation of blp expression is dependent on accumulation of two distinct quorum sensing peptides (CSP and BlpC), and this is, to the best of our knowledge, the first example of such a dual quorum sensing system. By hard-wiring bacteriocin expression with the competence system, expression is only activated at high cell densities when nutrients are scarce. Whether this timing is important for actual predation on competing bacteria, and how this
occurs in strains with intact BlpAB remains to be determined. Furthermore, the mechanism responsible for shut-down of blp-expression as cultures enter stationary phase are also unknown, and future research should show whether competence genes are also involved in this process.

In a previous study, Son et al. [7] suggested that strains with interrupted blpAB are so-called cheater strains that are unable to secrete pneumocins and BlpC but still respond to peptides produced by coexisting strains. While cross-induction between strains may be possible via response to different allelic variants of secreted BlpC, our results clarify this is not required in interrupted blpAB strains; induction in these strains can be entirely due to endogenous BlpC production. We show that BlpC and possibly also pneumocins, can be exported via ComAB, suggesting that strains carrying BlpAB lesions are not cheaters. Importantly, however, autoinduction and cross-induction of blp expression within cells are not mutually exclusive and cross-induction can possibly occur under environmental conditions not allowing autoinduction. Many questions remain about the evolution of this apparently redundant BlpAB transport system. Our results suggest that once blpA frameshift mutations arise, deletions in blpB likely follow (Fig 1A). However, it is puzzling that the genes themselves are retained in most pneumococcal genomes despite the prediction that they would become further degraded by genetic drift since they are apparently not required for BlpC or bacteriocin transport. Our preliminary analysis suggests that blpA mutations have occurred independently several times during pneumococcal evolution (S8 Fig). Thus, an intriguing possibility is that our snapshot in time of this operon has captured the slow progression of the loss of this gene as its functions are overtaken by comAB. Alternatively, the kinetics of blp activation may be different in the minority (23.5%) of strains with intact blpAB alleles (Fig 1A), or (parts of) blpAB may retain functions in the export of bacteriocins under certain conditions, and this may further vary as a function of the position of the blpA frameshift. It is worth noting from Fig 1A that some parts of the blpAB genes (for example amino acid 1–159 encoding a peptidase domain) is retained in almost all strains. At the same time, since blpC is located on the same transcriptional unit as blpAB, it is possible that the operon is not further degraded to avoid interference with blpC transcription and translation.

During the competent state, pneumococci are able to lyse non-competent siblings in order to gain access to their DNA for natural transformation [19]. In contrast to the murein hydrolase CbpD and the CibAB bacteriocins, the Blp bacteriocins have not been implicated in fratricide, partially because their induction level upon competence activation by CSP appear low [19,23]. In the present work we show that although the initial blp activation in early exponential phase is indeed low, a strong induction of blp expression occurs approximately 100 minutes after competence, potentially causing high production of bacteriocins. The competence regulon is considered to consist of two sets of genes; those directly regulated by binding of ComE-P (early competence genes) and those that are activated by the alternative sigma factor, σX, which is encoded by the ComE-P induced gene comX (late competence genes). Expression of both early and late genes occurs in the early exponential growth phase. The blp genes are also part of the competence regulon [13,16,23], but their dependence on the competence system is not via σX and also not solely via ComE-P, based on the mechanism described here (Fig 7). Therefore, our results clarify that the blp genes represents a separate subgroup within the competence regulon. Furthermore, since high level blp activation is competence-dependent and Blp bacteriocins have been shown to function in inter-strain competition [4], it remains possible that Blp-mediated cell lysis, together with CibAB and CbpD, can also contribute to the fratricide mechanism by releasing DNA from competitor strains [19]. One requirement for this proposed mechanism would be that competence is also activated. We observe in our in vitro experiments that the peak of bacteriocin export, and therefore potentially DNA release from dead cells, does not coincide with the peak of competence. This is the case both for natural expression and for
antibiotic-induced expression of com and blp. Notably, however, it was recently shown that competence is constitutive in pneumococcal biofilms and in vivo [24], suggesting that pneumocin activity during competence is possible. However, further experimentation is required to test this hypothesis.

**Materials and Methods**

**Bioinformatics**

We compiled 4,418 *S. pneumoniae* genomes from several data sets: 295 genomes from GenBank, which include 121 genomes from Georgia [25]; 3017 genomes from Myanmar refugees [26]; 616 genomes from Massachusetts [27]; 82 genomes from Complex 3 strains [28]; 240 genomes from PMEN-1 strains [29], 142 genomes from strains isolated from The Netherlands (European Nucleotide Archive study PRJEB10892) and 26 additional PMEN strains (European Nucleotide Archive study PRJEB10893). All sources collected strains without regard to strain identity, except for the Complex 3 and PMEN-1 research, which purposely focused on sampling subclades of *S. pneumoniae*. Therefore, excluding these two sources, we considered 4,096 of these genomes as randomly sampled from global populations.

We aligned the genomes to GenBank R6_uid57859 using Stampy 1.0.23 [30] with a substitution rate of 0.01. Based on the SNP data, we used FastTree 2.1.7 [31] with the ‘no maximum likelihood’ option, Jukes-Cantor nucleotide distances, and minimum 75% Shimodaira-Hasegawa local support to construct the full-genome phylogeny. We excluded any sites with more than 5% gaps. We included 69 *Streptococcus* sp. *viridans* as an outgroup clade for this phylogeny.

We used a reciprocal best-hit BLAST criterion to find sequences more similar to *blpA*, *blpB*, *comA*, and *comB* than other annotated *S. pneumoniae* genes. In this search, we only examined ORFs longer than 150 bp. We considered full-length alleles to be at least 717, 435, 717, and 449 residues long for each gene, respectively. *comA* and *comB* had 180 (34 over 0.5% proportion) and 121 (31 over 0.5% proportion) amino acid variants, respectively; we grouped the amino acid sequences by similarity as phylotypes. Amino acid sequences for each gene were aligned, and a neighbor-joining tree was created using Geneious 7.1.5 [32]. These gene trees were impartially divided into subtrees based on three restrictions: branches over 3.5 standard deviations in length from the mean branch length for the entire tree were cut; branches with length over 0.025 were cut; and clades were divided so the maximum intra-clade distance was 0.05. This lead to 5 phylotypes of *comA* over 0.5% proportion (A: 73.1%; B: 15.0%; C: 6.3%; D: 3.7%; E: 1.5%) and 2 phylotypes of *comB* over 0.5% proportion (A: 97.5%; B: 2.3%).

To find bacteriocins, we used a reciprocal best-hit BLAST criterion with annotated *blp* bacteriocins; we also examined all ORFs containing M[DN][TK]K leader sequence upstream of a GG site and all ORFs containing a 'TMLS' leader sequence upstream of a GG site. We classified any sequences with this feature that did not have a BLAST hit in the GenBank database as a bacteriocin. Afterwards, we found any resulting sequences mapped to either the *blp* locus or to the *comAB* locus, as expected of *blp* bacteriocins.

In order to test associations between gene types along the phylogeny, we calculated the maximum likelihood of two different models for each pair of gene lengths or phylotypes using BayesTraits 2.0 [33] and our phylogenetic tree. One model allowed the genes to mutate independently of each other; the other model had the mutation rate of each gene depend on the state of the other gene. We only considered gene length or phylotype pairs that co-occur in at least 0.5% of the 4,418 genomes. In all cases, strains with no allele of a gene were treated as missing data. We used a log ratio test between the two likelihood models to evaluate significance.

To reconstruct the phylogeny of the *blpA* region, a GTR+I+G model of evolution was employed, using Geneious 7.1.5 [32] and MrBayes 3.2.2 [34].
Growth conditions and transformation

*S. pneumoniae* was grown in C+Y medium [35] at 37°C. For transformation, *S. pneumoniae* was grown until OD_{600} = 0.1 before cells were washed and incubated 12 min at 37°C with 100 ng/ml synthetic CSP-1. DNA to be transformed was added to the cells, followed by 20 min incubation at 30°C. Cells were then diluted 10 times in fresh medium and incubated for 1.5 hours at 37°C. The transformations were plated in Columbia agar supplemented with 2% (v/v) defibrinated sheep blood (Johnny Rottier, Kloosterzade, The Netherlands). For selection, the following concentrations of antibiotics were used: 1 μg/ml tetracycline, 100 μg/ml spectinomycin, 0.25 μg/ml erythromycin, 2 μg/ml chloramphenicol.

*Escherichia coli* was grown in LB at 37°C with shaking. *E. coli* was transformed with heat-shock of chemically competent cells according to standard protocols [36]. When appropriate, 100 μg/ml ampicillin or 100 μg/ml spectinomycin was used for selection.

Strain construction

All strains and plasmids used in this study are listed in S2 Table. Oligonucleotides are listed in S3 Table.

Construction of triple reporter cassette

A reporter cassette containing three reporter genes, firefly luciferase (*luc*), superfolder GFP, ((sf)gfp), and β-galactosidase (*lacZ*) was amplified from plasmid pAD4 (A. Domenech and J.-W. Veening) using primers OG48 and OG50. The fragments were digested with restriction enzymes Asel and BamHI and ligated into the corresponding site of plasmid pPEP1 [20], a vector which replicates in *E. coli* and integrates into the cep-locus of *S. pneumoniae* D39 by double crossover. The resulting reporter plasmid (Fig 3C), suitable for insertion of promoter fragments upstream of the triple reporter system, was called pPEP1-LGZ.

Construction of promoter fusions to reporter cassette

For construction of pPEP1-P_{blpT}-LGZ, the promoter fragment P_{blpT} was amplified from genomic DNA of *S. pneumoniae* D39 using primers OG56 and OG26. The fragment was digested with enzymes Nhel and BglII and ligated into the corresponding restriction sites of plasmid pPEP1-LGZ. Using the same template DNA, promoter fragments P_{blpK} was amplified with primers Pspsd_0046-F+NheI+NotI and Pspsd_0046-R+BglII, P_{blpS} with primers PblpS_F_Nhel_NotI and PblaS_R_BglII, P_{blpA} with primers PblpA_F_Nhel_NotI and PblaA_R_BglII and P_{pncW} with primers PblpU_F_NotI_NheI and PblaU-R+BamHI. For all promoter fragments, we amplified a region of 250 bp, containing 200 bp upstream and 50 bp downstream of the startcodon of the first gene in each transcriptional unit. Fragments were ligated into pPEP1-P_{blpT}-LGZ using restriction sites Nhel and BglII to construct vectors pPEP1-P_{blpK}-LGZ, pPEP1-P_{blpS}-LGZ, pPEP1-P_{blpA}-LGZ and pPEP1-P_{pncW}-LGZ, respectively. The ligations were transformed into *E. coli* DH5a and transformants were selected with 100 μg/ml spectinomycin. Correct plasmids were verified by sequencing and transformation into *S. pneumoniae* D39. Correct integration of the plasmids via double crossover in the pneumococcal genome was verified by PCR.

Deletion of *blpSRHC*

The regulatory genes of the *blp* operon, *blpSRHC*, were deleted using allelic replacement with an erythromycin resistance cassette. The erythromycin resistance cassette was amplified from strain MK110 [16], using primers eryR-up_F_BamHI and eryR-down+Not. The region upstream of *blpS* was amplified using primers Blp_SRHC_up_F and
Blp_SRHC_up_R_BamHI, while the region downstream of blpC was amplified with primers Blp_SRHC_dn_F_NotI and Blp_SRHC_dn_R. The upstream fragment was digested with BamHI, the downstream fragments with NotI and the fragments containing the erythromycin cassette with both BamHI and NotI. The three fragments were ligated and transformed into S. pneumoniae D39 using 0.25 μg/ml erythromycin for selection. Correct deletion of blpSRHC was confirmed by PCR and sequencing.

Deletion of bgaA

The S. pneumoniae β-galactosidase gene bgaA was deleted by transforming the plasmid pMK11 (see below), containing a tetracycline resistance gene and the PZn promoter, into S. pneumoniae. Transformants were selected using 1 μg/ml tetracycline.

Deletion of comA, comB and comAB

comA, comB and comAB were deleted by allelic replacement with an erythromycin resistance cassette. Briefly, the region upstream of comA was amplified with primers comA1 and comA2 +AscI and the region downstream of comA was amplified with comA3+NotI and comA4. Genomic DNA from S. pneumoniae D39 was used as template. The erythromycin resistance cassette was amplified from genomic DNA of strain ΔhexA::ery [37] using primers trmp-F +AscI and sPG20_eryR+NotI. The comA-up fragment was digested with restriction enzyme AscI, the erythromycin resistance cassette fragment with AscI and NotI and the comA-down fragment with NotI. The three fragments were ligated and transformed into S. pneumoniae. Transformants were selected with 0.25 μg/ml erythromycin and correct transformants were verified by PCR and sequencing.

comB was deleted in a similar fashion. The comB-up fragment was amplified using primers comB_up_R+AscI and ComA1, the comB-down fragment with primers comB_down_F+NotI and comB_down_R, and the ery fragment was amplified using primers trmp_F+Ascl and SPG20_eryR+NotI with ΔcomA::ery strain as a template. For deletion of comAB, a fragment containing the comAB-up region and the erythromycin resistance gene was amplified with primers comA1 and SPG20_eryR+NotI using the ΔcomA::ery strain as a template. The comAB-down fragment was amplified using primers pr225comB_down_F+NotI and pr226comB_down_R. Genomic DNA was used as template in all cases. Digestion, ligation and transformation was performed in the same manner as for the ΔcomA::ery deletion, to generate strains ΔcomB::ery and ΔcomAB::ery.

Deletion of comCDE

The competence regulatory operon comCDE was deleted with allelic replacement with a chloramphenicol resistance cassette, as described previously [16].

Construction of pMK11, a plasmid for Zn-inducible expression

A Zn^{2+}-inducible promoter was amplified from plasmid pJWV25 [38] using primers pr27 and pr28. The fragment was digested with SphI and Spel and ligated into the corresponding sites in plasmid pJWV100 [39] to create pMK11.

Constructs for inducible expression of comA, comB and comAB

For controlled expression of comA, comB or comAB, the gene(s) were inserted downstream of a Zn^{2+} inducible promoter and integrated in the genome at the bgaA-locus. comA was amplified using primers start-comA+EcoRI and end-comA+SpeI, comB with primers start-comB+EcoRI
and end-comB+SpeI and comAB with primers start-comA+EcoRI and end-comB+SpeI. The fragments were digested with restriction enzymes EcoRI and SpeI/BcuI, and ligated into the corresponding sites of plasmid pMK1, to generate plasmids pMK11-P_Zn-comA, pMK11-P_Zn-comB and pMK11-P_Zn-comAB. The ligation was transformed into E. coli using ampicillin selection (100 μg/ml). Correct plasmids were verified by sequencing and then transformed in S. pneumoniae using tetracycline selection (1 μg/ml).

Deletion of blpAB pseudogenes

The blpAB pseudogenes in S. pneumoniae D39 were deleted by replacement with an erythromycin resistance cassette. The promoter P_{blpA} and blpC were kept intact. The region downstream of blpAB was amplified using primers blpB-down-F and blpB-down-R-NotI, while the region upstream of blpAB was amplified with primers blpA-up-F-BamHI and blpA-up-R. In both cases, genomic DNA from S. pneumoniae D39 was used as template. The erythromycin resistance gene was amplified from strain MK304 using primers Ery-For-BamHI and sPG20_eryR+NotI. The up- and downstream fragments were digested with BamHI and NotI, respectively, while the fragment containing the erythromycin resistance gene was digested with both BamHI and NotI. The fragments were ligated and the construct was transformed into S. pneumoniae D39. In all steps of the transformation reaction, 500 ng/ml CSP-1 was added to induce expression from the P_{blpA} promoter driving expression of the erythromycin resistance gene. Cells were plated on C+Y agar plates containing erythromycin for selection and 500 ng/ml CSP-1. The construct in the resulting strain was confirmed by PCR and sequencing.

Construction of double-labeled reporter strain P_{ssbB-rfp} and P_{blpK-gfp} for time-lapse microscopy

D39 was transformed with plasmid pPEP1-P_{blpK}-LGZ (including the gfp reporter), which integrates in the cep-locus. Transformants were selected on plates with 100 μg/ml spectinomycin. This new strain was subsequently transformed with plasmid pLA21 [37] containing the P_{ssbB-rfp} fusion which integrates in the bgaA-locus. In the second transformation round, colonies were selected on 1 μg/ml tetracycline.

RNA-sequencing

For RNA-sequencing, samples of S. pneumoniae, strain DLA3 (bgaA::P_{ssbB-luc}) were grown to OD$_{600}$ = 0.4 in 5 ml tubes and diluted 1:100 in fresh C+Y medium (pH 7.4). To study the effects of antibiotics, cells grown without antibiotics were compared to cells grown with 0.4 μg/ml ciprofloxacin, 608 μg/ml hydroxyurea, or 0.04 μg/ml rifampicin. Cells were grown in microtiter plates and growth and competence development (lac-expression) were followed. For all the samples, when one-third of the maximum OD$_{600}$ was reached, cells were harvested by centrifugation (7,500 rcf for 5 min) and frozen. For RNA isolation, cells were lysed by bead beating and RNA was purified using phenol–chloroform extractions and ethanol precipitations. DNA was removed from the sample with RNase-free DNase I (Fermentas) treatment for 45 min. Ribolock (Fermentas) was added to avoid RNA degradation.

Library preparation and whole-genome sequencing were performed by vertis Biotechnologie AG (Freising, Germany). Ribosomal RNA was removed using the Ribo-Zero rRNA Removal Kit (Epicenter) prior to preparation of cDNA libraries. Sequencing of the cDNA libraries was performed with an Illumina HiSeq 2000 machine with 100 nt read length paired end.

Sequence reads were mapped to the S. pneumoniae D39 genome (NC_008533) using Rockhopper version 2.03 [40,41], using default parameters. Reads Per Kilobase of exon per
Megabase of library size (RPKM) were calculated using a protocol from Chepelev et al. [42]. In short, exons from all isoforms of a gene were merged to create one meta-transcript. The number of reads falling in the exons of this meta-transcript were counted and normalized by the size of the meta-transcript and by the size of the library. This was done internally by Rockhopper version 2.03 after aligning reads. Upper quartile normalization (to be able to compare expression between samples) was used to transform RPKM (reads per kilo base per million) values into expression values, performed by Rockhopper. Finally, Rockhopper was also used for differential gene expression analysis, using default parameters.

Sequencing data used in this paper have been deposited in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE54199 and GSE69729.

**Luminescence assays**

*S. pneumoniae* were pre-grown to OD$_{600}$ 0.4 and diluted 100-fold in C+Y medium containing 340 μg/ml luciferin prior to the assay. pH of the medium was adjusted with HCl or NaOH. Production of firefly luciferase (encoded by *luc*) cause emission of light when the medium contains luciferin [43]. Luminescence assays were performed in 96-wells plates at 37°C. Absorbance (OD$_{595}$) and luminescence (as relative luminescence units, RLU) were measured every 10 min for at least 13 hours using a Tecan Infinite 200 PRO instrument. When appropriate, 100 ng/ml synthetic peptide CSP-1 or 500 ng/ml BlpC purchased from Genscript (Piscataway, NJ) were added to the plates after 100 min. Concentrations were selected based on previous studies [9,16].

**LacZ reporter assay**

β-galactosidase activity was assayed on agar plates. X-gal (40 μl of 40 mg/ml stock solution) was added on top of C+Y agar with adjusted pH. Twenty μl of *S. pneumoniae* at OD$_{600} = 0.4$ (approx. 10$^7$ cells/μl) was then spotted. The drops with bacterial culture were allowed to dry prior to incubation overnight in a 5% CO$_2$ incubator at 37°C.

**Time-lapse fluorescence microscopy**

Time-lapse fluorescence microscopy was performed using a Deltavision Elite (GE Healthcare, USA) as described before [44,45]. In short, cells were grown until OD$_{600} = 0.08$ before they were spotted onto C+Y (pH 8) agarose slides. The slides were kept in a temperature controlled chamber at 37°C, and images (GFP, RFP and phase contrast) were acquired with a sCMOS camera every 10 min.

**Supporting Information**

S1 Fig. Natural induction of *blp* promoters. Reporter strains for *blp* promoters (A) P$_{blpA}$, (B) P$_{pncW}$ and (C) P$_{blpK}$ grown in C+Y pH 8. (D) Strains containing the reporter P$_{blpT}$ grown in C+Y pH 8. When *blpC* is deleted, no natural induction is observed, however by external addition of BlpC (timing indicated by arrow), *blp* expression is immediately switched on. For all plots, gene expression as measured by luciferase activity (RLU/OD) is shown on the left axis and growth as measured by absorbance at 595 nm (OD$_{595}$) is shown on the right axis. Averages of three replicates with the standard deviation are plotted. (TIF)

S2 Fig. Comparison of expression dynamics of the synthetic, constitutive promoter P3 and promoter P$_{blpS}$. Strains harboring the reporter construct P3-luc (A) or P$_{blpS}$-luc (B) were
pre-grown to OD<sub>600</sub> = 0.4 and inoculated at four different initial cell densities (10<sup>-2</sup> in black, 10<sup>-3</sup> in red, 10<sup>-4</sup> in green and 10<sup>-5</sup> in blue) in C+Y pH 8. Growth curves (OD<sub>595</sub>, upper panels) and gene expression (RLU/OD, lower panels) over time are shown. The expression dynamics from the two promoters appear similar; expression starts when cells enter the exponential growth phase and the promoters remain active until stationary phase. Averages of three replicates with the standard deviation are plotted.

**S3 Fig. pH-dependent blp expression.** Strains containing the promoter reporter fusions for P<sub>blpA</sub> (A), P<sub>pncW</sub> (B) and P<sub>blpK</sub> (C) were grown in C+Y with different initial pH. Natural induction for all promoters is only observed for pH > 7.4. Gene expression as measured by luciferase activity (RLU/OD) is shown on the left axis and growth as measured by absorbance at 595 nm (OD<sub>595</sub>) is shown on the right axis. Averages of three replicates with the standard deviation are plotted.

**(TIF)**

**S4 Fig. Antibiotic-induced blp expression.** blp reporter strain for P<sub>blpA</sub> (A), P<sub>pncW</sub> (B) and P<sub>blpK</sub> (C) grown with or without sub-lethal concentrations of HPUra (0.15 μg/ml), ciprofloxacin (0.4 μg/ml) or streptomycin (6 μg/ml) shows that competence-inducing antibiotics also induce blp expression. (D) When P<sub>blpK</sub> was grown with sub-lethal concentrations of rifampicin (0.04 μg/ml), which does not induce competence, blp expression was also not induced. For all plots, cells were grown in C+Y pH 7.4. Gene expression as measured by luciferase activity (RLU/OD) is shown on the left axis and growth as measured by absorbance at 595 nm (OD<sub>595</sub>) is shown on the right axis. Averages of three replicates with the standard deviation are plotted.

**(TIF)**

**S5 Fig. Effect of CSP on blp expression.** CSP induces expression from regulated blp promoters P<sub>blpA</sub> (A), P<sub>pncW</sub> (B) and P<sub>blpK</sub> (C), but does not affect expression from P<sub>blpS</sub> (D). Upon addition of CSP an immediate (weak) induction of the promoter fusions in A-C were observed, while a delayed full activation of these promoters were observed in late exponential phase. The immediate activation is independent of blpSRHC, but the delayed full activation is dependent of blpSRHC, since reporter strains for P<sub>blpT</sub> (E) or P<sub>blpA</sub> (F) with deleted blp regulatory genes (ΔblpSRHC) still show similar levels of immediate induction, but no delayed induction (compare Fig 5B with panel E and panels A with F). (G) No CSP response is observed for P<sub>blpT</sub> when blpSRHC and comCDE are deleted. For all plots, strains were grown in C+Y pH 7 with or without addition of CSP after 100 min (indicated by an arrow). Gene expression as measured by luciferase activity (RLU/OD) is shown on the left axis and growth as measured by absorbance at 595 nm (OD<sub>595</sub>) is shown on the right axis. Averages of three replicates with the standard deviation are plotted.

**(TIF)**

**S6 Fig. Deletion of blpAB pseudogenes is not detrimental for natural blp expression.** Reporter strains for the promoters P<sub>blpA</sub> (A), P<sub>pncW</sub> (B), P<sub>blpK</sub> (C) and P<sub>blpS</sub> (D) with and without deleted blpAB pseudogenes show no differences in activity when grown in C+Y pH 8. Gene expression as measured by luciferase activity (RLU/OD) is shown on the left axis and growth as measured by absorbance at 595 nm (OD<sub>595</sub>) is shown on the right axis. Averages of three replicates with the standard deviation are plotted.

**(TIF)**

**S7 Fig. Induction of blp expression by external BlpC or over-expression of comAB.** (A) blp expression can still be induced by external addition of BlpC in a comAB deletion strain. The
(B) Overexpression of comAB induces blp expression also in a blpAB deletion strain. The P<sub>blpT</sub> reporter strain with Zn<sup>2+</sup>-inducible comAB-expression was grown in C+Y pH 7.4 with or without the presence of 0.1 mM ZnCl<sub>2</sub> and 0.01 mM MnCl<sub>2</sub> for induction. Gene expression as measured by luciferase activity (RLU/OD) is shown on the left axis and growth as measured by absorbance at 595 nm (OD<sub>595</sub>) is shown on the right axis. Averages of three replicates with the standard deviation are plotted.

(TIF)

S8 Fig. Phylogeny of the blpA region. We aligned the nucleotides of the blpA locus regardless of the presence of ORFs with 4 alleles of comA as an outgroup. Sites with more than 5% gaps were removed. Using a GTR+I+G model of evolution, we used Geneious 7.1.5 and MrBayes 3.2.2 to reconstruct the phylogeny of blpA. We collapsed clades with a posterior probability of less than 0.95. In red are two clades with full-length blpA alleles as the parsimonious ancestor that contain derived interrupted blpA alleles. Alleles coding for full-length blpA are in black circles; alleles producing interrupted blpA are colored by the length classes found in Fig 1A. Alleles producing interrupted blpA with the 1–195 fragment as in Fig 1A are shown as half-colored circles.

(TIF)

S1 Table. Transcriptional response of blp genes of S. pneumoniae D39 after exposure to sub-lethal level of antibiotics as determined by RNA sequencing.

(DOCX)

S2 Table. Strains and plasmids used in this study.

(DOCX)

S3 Table. Oligonucleotides used in this study.

(DOCX)

Acknowledgments
We thank Laetitia Attaiech for constructing the ΔcomA::ery deletion strain.

Author Contributions
Conceived and designed the experiments: MK EM DER JWV. Performed the experiments: MK EM JS FBL OG. Analyzed the data: MK EM DER JWV. Contributed reagents/materials/analysis tools: ISR. Wrote the paper: MK EM DER JWV.

References
1. Regev-Yochay G, Raz M, Dagan R, Porat N, Shainberg B, Pinco E, et al. Nasopharyngeal carriage of Streptococcus pneumoniae by adults and children in community and family settings. Clin Infect Dis. 2004; 38: 632–639. doi:10.1086/381547 PMID: 14986245
2. Guiral S, Mitchell TJ, Martin B, Claverys J-P. Competence-programmed predation of noncompetent cells in the human pathogen Streptococcus pneumoniae: genetic requirements. Proc Natl Acad Sci USA. 2005; 102: 8710–8715. doi: 10.1073/pnas.0500879102 PMID: 15928084
3. Hoover SE, Perez AJ, Tsui H-CT, Sinha D, Smiley DL, DiMarchi RD, et al. A new quorum sensing system (TprA/PhrA) for Streptococcus pneumoniae D39 that regulates a lantibiotic biosynthesis gene cluster. Mol Microbiol. 2015; 97: 229–43. doi: 10.1111/mmi.13029 PMID: 25869931
4. Dawid S, Roche AM, Weiser JN. The blp bacteriocins of Streptococcus pneumoniae mediate intraspecies competition both in vitro and in vivo. Infect Immun. 2007; 75: 443–451. doi: 10.1128/IAI.01775-05 PMID: 17074857
5. de Saizieu A, Gardés C, Flint N, Wagner C, Kamber M, Mitchell TJ, et al. Microarray-based identification of a novel *Streptococcus pneumoniae* regulon controlled by an autoinduced peptide. J Bacteriol. 2000; 182: 4696–4703. doi: 10.1128/JB.182.17.4696–4703.2000 PMID: 10940007

6. Reichmann P, Hakenbeck R. Allelic variation in a peptide-inducible two-component system of *Streptococcus pneumoniae*. FEMS Microbiol Lett. 2000; 190: 231–236. doi: 10.1111/j.1574-6968.2000.tb09291.x PMID: 11034284

7. Son MR, Shchepetov M, Adrian PV, Madhi SA, de Gouveia L, von Gottberg A, et al. Conserved mutations in the pneumococcal bacteriocin transporter gene, *bipA*, result in a complex population consisting of producers and cheaters. MBio. 2011; 2. doi: 10.1128/mBio.00179-11

8. Lange R, Wagner C, de Saizieu A, Flint N, Molnos J, Stieger M, et al. Domain organization and molecular characterization of 13 two-component systems identified by genome sequencing of *Streptococcus pneumoniae*. Gene. 1999; 237: 223–234. doi: 10.1016/S0378-1119(99)00266-8 PMID: 10524254

9. Pinchas MD, LaCross NC, Dawid S. An electrostatic interaction between BlpC and BlpH dictates pheromone specificity in the control of bacteriocin production and immunity in *Streptococcus pneumoniae*. J Bacteriol. 2015; 197: 1236–1248. doi: 10.1128/JB.02432-14 PMID: 25622617

10. Steens KE, Chang D, Zwack EE, Sebert ME. Competence in *Streptococcus pneumoniae* is regulated by the rate of ribosomal decoding errors. MBio. 2014; 5: 406. doi: 10.1016/j.cell.2014.01.068 PMID: 24725406

11. Charpentier X, Polard P, Claverys J-P. Induction of competence for genetic transformation by antibiotic stress and CiaR regulons in *Streptococcus pneumoniae*: competence triggers stationary phase autolysis of CiaR mutant cells. Mol Microbiol. 2004; 51: 1071–1086. doi: 10.1111/j.1365-2958.2003.03892.x PMID: 14763981

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

13. Prudhomme M, Attaiieh L, Sanchez G, Martin B, Claverys J-P. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science. 2006; 313: 89–92. doi: 10.1126/science.1127912 PMID: 16825569

14. Slager J, Kjos M, Attaiieh L, Veening J-W. Antibiotic-induced replication stress triggers bacterial competence by increasing gene dosage near the origin. Cell. 2014; 157: 395–406. doi: 10.1016/j.cell.2014.01.068 PMID: 24725406

15. Charpentier X, Polard P, Claverys J-P. Induction of competence for genetic transformation by antibiotics: convergent evolution of stress response systems in distant bacterial species lacking SOS? Curr Opin Microbiol. 2012; 15: 570–576. doi: 10.1016/j.mib.2012.08.001 PMID: 22910199

16. Prudhomme M, Attaiieh L, Sanchez G, Martin B, Claverys J-P. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science. 2006; 313: 89–92. doi: 10.1126/science.1127912 PMID: 16825569

17. Slager J, Kjos M, Attaiieh L, Veening J-W. Antibiotic-induced replication stress triggers bacterial competence by increasing gene dosage near the origin. Cell. 2014; 157: 395–406. doi: 10.1016/j.cell.2014.01.068 PMID: 24725406

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

19. Brown NC. 6-(p-hydroxyphenylazo)-uracil: a selective inhibitor of host DNA replication in phage-infected *Bacillus subtilis*. Proc Natl Acad Sci USA. 1970; 67: 1454–1461. PMID: 4992015

20. Sorg RA, Kuipers OP, Veening J-W. Gene expression platform for synthetic biology in the human pathogen *Streptococcus pneumoniae*. ACS Synth Biol. 2015; 4: 228–239. doi: 10.1021/sb500229s PMID: 24845455

21. Tomasz A, Mosser JL. On the nature of the pneumococcal activator substance. Proc Natl Acad Sci U S A. 1966; 55: 58–66. PMID: 4380138

22. Knutsen E, Ween O, Hävarstein LS. Two separate quorum-sensing systems upregulate transcription of the same ABC transporter in *Streptococcus pneumoniae*. J Bacteriol. 2004; 186: 3078–3085. doi: 10.1128/JB.186.10.3078–3085.2004 PMID: 15126469

23. Peterson SN, Sung CK, Cline R, Desai BV, Snesrud EC, Luo P, et al. Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. Mol Microbiol. 2004; 51: 1051–1070. doi: 10.1046/j.1365-2958.2003.03907.x PMID: 14763980

24. Marks LR, Reddinger RM, Hakansson AP. High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in *Streptococcus pneumoniae*. MBio. 2012; 3: e00200–12. doi: 10.1128/mBio.00200-12 PMID: 23015736

25. Chancey ST, Agrawal S, Schroeder MR, Farley MM, Tettelin H, Stephens DS. Composite mobile genetic elements disseminating macrolide resistance in *Streptococcus pneumoniae*. Front Microbiol. 2015; 6: 26. doi: 10.3389/fmicb.2015.00026 PMID: 25709602
26. Chewapreecha C, Harris SR, Croucher NJ, Turner C, Marttinen P, Cheng L, et al. Dense genomic sampling identifies highways of pneumococcal recombination. Nat Genet. 2014; 46: 305–309. doi: 10.1038/ng.2895 PMID: 24509479

27. Croucher NJ, Finkelstein JA, Pelton SI, Mitchell PK, Lee GM, Parkhill J, et al. Population genomics of post-vaccine changes in pneumococcal epidemiology. Nat Genet. 2013; 45: 656–663. doi: 10.1038/ng.2625 PMID: 23644493

28. Croucher NJ, Mitchell AM, Gould KA, Inverarity D, Barquist L, Feltwell T, et al. Dominant role of nucleotide substitution in the diversification of serotype 3 pneumococci over decades and during a single infection. PLoS Genet. 2013; 9: e1003868. doi: 10.1371/journal.pgen.1003868 PMID: 24130509

29. Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, van der Linden M, et al. Rapid pneumococcal evolution in response to clinical interventions. Science. 2011; 331: 430–434. doi: 10.1126/science.1203008 PMID: 21273480

30. Lunter G, Goodson M. Stampy: a statistical algorithm for sensitive and fast mapping of Illumina sequence reads. Genome Res. 2011; 21: 936–939. doi: 10.1101/gr.111120.110 PMID: 20980556

31. Price MN, Dehal PS, Arkin AP. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol. 2009; 26: 1641–1650. doi: 10.1093/molbev/msp077 PMID: 19377059

32. Page M, Meade A. BayesTraits v.2 [Internet]. 2013. Available: http://www.evolution.rdg.ac.uk/BayesTraits.html

33. Huelsenbeck JP, Ronquist F. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics. 2001; 17: 754–755. PMID: 11524383

34. Kjos M, Veening J-W. Tracking of chromosome dynamics in live Streptococcus pneumoniae reveals that transcription promotes chromosome segregation. Mol Microbiol. 2014; 91: 1088–1105. doi: 10.1111/mmi.12517 PMID: 24417389

40. McClure R, Balasubramanian D, Sun Y, Bobrovskyy M, Sumby P, Genco CA, et al. Computational analysis of bacterial RNA-Seq data. Nucl Acids Res. 2013; 41: e140–e140. doi: 10.1093/nar/gkt444 PMID: 23716638

41. Tjaden B. De novo assembly of bacterial transcriptomes from RNA-seq data. Genome Biol. 2015; 16: 1. doi: 10.1186/s13059-014-0572-2 PMID: 25583448

44. de Jong IG, Beilharz K, Kuipers OP, Veening J-W. Live cell imaging of Bacillus subtilis and Streptococcus pneumoniae using automated time-lapse microscopy. J Vis Exp. 2011; 53: 3145. doi: 10.3791/3145 PMID: 21841760