



ABC transporter content diversity in *Streptococcus pneumoniae* impacts competence regulation and bacteriocin production

Charles Y. Wang^a, Nisha Patel^a, Wei-Yun Wholey^b, and Suzanne Dawid^{a,b,1}

^aDepartment of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109; and ^bDepartment of Pediatrics, University of Michigan Medical School, Ann Arbor, MI 48109

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The opportunistic pathogen *Streptococcus pneumoniae* (pneumococcus) uses natural genetic competence to increase its adaptability through horizontal gene transfer. One method of acquiring DNA is through predation of neighboring strains with antimicrobial peptides called “bacteriocins.” Competence and production of the major family of pneumococcal bacteriocins, pneumocins, are regulated by the quorum-sensing systems *com* and *blp*, respectively. In the classical paradigm, the ABC transporters ComAB and BlpAB each secretes its own system’s signaling pheromone and in the case of BlpAB also secretes the pneumocins. While ComAB is found in all pneumococci, only 25% of strains encode an intact version of BlpAB [BlpAB(+)] while the rest do not [BlpAB(-)]. Contrary to the classical paradigm, it was previously shown that BlpAB(-) strains can activate *blp* through ComAB-mediated secretion of the *blp* pheromone during brief periods of competence. To better understand the full extent of *com*-*blp* crosstalk, we examined the contribution of each transporter to competence development and pneumocin secretion. We found that BlpAB(+) strains have a greater capacity for competence activation through BlpAB-mediated secretion of the *com* pheromone. Similarly, we show that ComAB and BlpAB are promiscuous and both can secrete pneumocins. Consequently, differences in pneumocin secretion between BlpAB(+) and BlpAB(-) strains derive from the regulation and kinetics of transporter expression rather than substrate specificity. We speculate that BlpAB(-) strains (opportunists) use pneumocins mainly in a narrowly tailored role for DNA acquisition and defense during competence while BlpAB(+) strains (aggressors) expand their use for the general inhibition of rival strains.

pneumococcus | competence | bacteriocin | regulation | ABC transporter

The opportunistic pathogen, *Streptococcus pneumoniae* (pneumococcus) can cause serious illnesses such as pneumonia, meningitis, and bacteremia, with the greatest disease burden in the very young and the elderly. The natural niche of pneumococcus is the human nasopharynx, and colonization of this niche is a prerequisite for invasive pneumococcal disease. Pneumococcus colonizes up to 60% of young children (1, 2). As many as half of those who are colonized carry multiple pneumococcal strains (3).

Pneumococcus, a naturally competent bacterium (4), can exploit the large pool of genetic material available to it (1, 2, 5) in the nasopharynx. Natural competence allows pneumococcus to take up new genetic material through horizontal gene transfer and recombination. Multiple studies have documented that recombination occurs with great frequency in pneumococcal lineages that are globally distributed (6), geographically isolated (7), and even confined to a single patient (8). Additionally, to compete with other bacteria found in the nasopharynx, pneumococcus produces small antimicrobial peptides called “bacteriocins.” Pneumocins are the major family of bacteriocins encoded by pneumococcus. The pneumocin locus, *blp*, is found in all sequenced strains of pneumococcus (9). Pneumocin-producing organisms inhibit sensitive strains and have a fitness advantage in both in vitro biofilms and competitive colonization of the mouse nasopharynx (10, 11).

Both competence and pneumocin production in pneumococcus are under strict regulation by two separate but similar systems (Fig. 1). The *com* system regulates competence. In this system, a peptide prepheromone, ComC, is processed and secreted by a transporter complex ComAB (12, 13). After processing and secretion, the mature pheromone, now called “competence-stimulating peptide” (CSP), accumulates extracellularly. Once a threshold concentration is reached, CSP signals through the ComDE two-component system to up-regulate the set of so-called “early (competence) genes” (14). The early genes include *comAB* and *comCDE*, creating a positive feedback loop. Up-regulation of early gene expression starts a regulatory cascade mediated by the alternative sigma factor ComX that ultimately leads to competence development (15). The *com* system integrates many environmental and physiological signals, such as cell density (16), pH (17), antibiotic stress (18), and protein mistranslation (19). As a result, the propensity for competence activation can differ greatly from one set of conditions to another.

Meanwhile, the *blp* locus regulates pneumocin production in a manner similar to *com* and competence (20). In the prototypical case, a small peptide pheromone, BlpC, is processed and secreted by the BlpAB transporter complex. Mature BlpC then

Significance

The opportunistic pathogen *Streptococcus pneumoniae* (pneumococcus) participates in horizontal gene transfer through genetic competence and produces antimicrobial peptides called “bacteriocins.” Here, we show that the competence and bacteriocin-related ABC transporters ComAB and BlpAB share the same substrate pool, resulting in bidirectional crosstalk between competence and bacteriocin regulation. We also clarify the role of each transporter in bacteriocin secretion and show that, based on their transporter content, pneumococcal strains can be separated into a majority opportunist group that uses bacteriocins only to support competence and a minority aggressor group that uses bacteriocins in broader contexts. Our findings will impact how bacteriocin regulation and production is modeled in the many other bacterial species that use ComAB/BlpAB-type transporters.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. [MH304212](https://www.ncbi.nlm.nih.gov/nuclseq/MH304212/) (PcomAB Nluc), [MH304213](https://www.ncbi.nlm.nih.gov/nuclseq/MH304213/) (PBIR RFluc), and [MH304214](https://www.ncbi.nlm.nih.gov/nuclseq/MH304214/) (Sweet Janus)].

¹To whom correspondence should be addressed. Email: sdawid@med.umich.edu.

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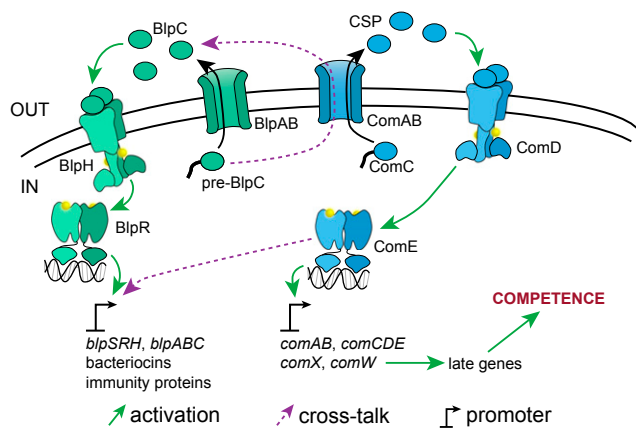


Fig. 1. *com* and *blp* regulation in pneumococcus.

signals through the BlpHR two-component system to up-regulate the entire *blp* locus. Unlike *com*, the entire *blp* regulon is directly controlled by BlpR. The up-regulation of the regulatory system forms another positive feedback loop, while the up-regulation of the so-called “bacteriocin immunity region” (BIR) within the *blp* locus results in the production of a diverse array of pneumocins and their immunity proteins. As with competence, the propensity for *blp* activation is environment- and context-dependent.

Due to the autoinducing nature of *com* and *blp*, activation tends to proceed synchronously among all cells within a population once the pheromone concentration threshold is reached (13). However, different strains can encode different phenotypes of CSP and BlpC along with matched cognate ComD and BlpH receptors (21, 22). In general, each phenotype efficiently activates only its cognate receptor type. Therefore, CSP/BlpC signaling and synchronous *com/blp* activation is restricted by phenotype. Phenotype diversity among pneumococci may have evolved as a method for cells to privilege clonal or closely related cells which are more likely to have a matched phenotype. Such cells would then share in the benefits of competence activation or be protected from pneumocin-mediated killing while strains with mismatched phenotypes would not.

While *com* and *blp* were originally thought to operate independently, two recent studies have shown that *com* positively influences the regulation of *blp* (11, 23). This occurs through two mechanisms (Fig. 1, purple dotted arrows): (i) ComE directly up-regulates the transcription of *blp* genes, and (ii) ComAB processes and secretes BlpC in addition to ComC/CSP. As a result, *com* activation also induces *blp* activation. This crosstalk from *com* to *blp* allows the coordinated regulation of the two systems. In a biofilm model, pneumocin production resulted in an increase in transformation efficiency (11). The competence regulon itself encodes so-called “fratricide effectors” which target and kill noncompetent pneumococci (24). The role of fratricide seems to be to increase access to extracellular DNA during competence (25, 26). Therefore, pneumococcus may have evolved *com-to-blp* crosstalk to increase access to genetic material during competence by using pneumocins to augment fratricide.

While the *com* regulatory system is found intact in nearly all strains of pneumococcus, 75% of sequenced pneumococcal strains contain mutations that disrupt the *blpA* and/or *blpB* genes (27). Strains harboring these mutations are predicted to be incapable of making functional BlpAB transporter. These BlpAB(–) strains are dependent on *com* activation to activate their own *blp* system (*com*-dependent *blp* activation) via the *com-to-blp* crosstalk described above (11, 23). They can also respond to BlpC pheromone secreted by other strains, since their BlpHR sensory ap-

paratus remains intact. However, it is not clear if BlpAB(–) strains can effect pneumocin-mediated killing. Pneumocins, similar to the pheromones CSP and BlpC, need to be processed and secreted before they can exert their effects. BlpAB is the putative transporter for pneumocins, so the loss of BlpAB would be expected to render cells incapable of secreting active pneumocins. Two previous studies have reported conflicting results on whether BlpAB(–) strains exhibit pneumocin-mediated inhibitory activity (27, 28).

At the heart of both the *com* and *blp* systems lie the peptide transporters ComAB and BlpAB, which belong to the superfamily of ATP-binding cassette (ABC) transporters. ComAB and BlpAB are formed from A and B subunits. The B subunits, encoded by *comB* and *blpB*, serve unknown functions. The A subunits, encoded by *comA* and *blpA*, contain all the known functional domains. Each A subunit contains an N-terminal peptidase domain followed by the channel-forming transmembrane domain and a C-terminal nucleotide-binding domain (NBD) (29). The peptidase domain catalyzes the first step of transport called “processing”: the cleavage of the peptide substrate immediately C-terminal to a conserved double-glycine (GG) motif (sometimes also GA or GS), thereby removing the substrate’s N-terminal signal sequence (29, 30). After processing, the now-mature peptide is secreted out of the cell using energy provided by the ATP-hydrolyzing NBD. While ComAB’s ability to secrete both ComC and BlpC has revealed some degree of promiscuity in its substrate selection process, it is unknown if BlpAB shares this property or just how far it extends.

In this study, we demonstrate that BlpAB can secrete CSP and show the functional significance of this in the 25% of strains that are BlpAB(+). In addition, we demonstrate that ComAB can process and secrete pneumocins, suggesting that BlpAB(–) strains can support pneumocin-mediated inhibition but only under competence-permissive conditions. We also show that temporal regulation of ComAB and BlpAB expression leads to differences in pneumocin transport efficiency in BlpAB(+) and BlpAB(–) strains. Finally, we demonstrate that BlpAB(+) strains are more effective competitors than BlpAB(–) strains in a mouse nasopharyngeal colonization model. These findings suggest that the diversification of the pneumococcal population into BlpAB(+) and BlpAB(–) types occurred to allow for a small pool of costly antagonistic strains that can eliminate competitors when environmental conditions mandate this approach.

Results

Development of a *com/blp* Dual Luciferase Reporter. To facilitate the study of *com-blp* crosstalk, we developed a dual luciferase reporter system to monitor *com* and *blp* activation in the same population of cells in real time. For our two luciferases, we chose the NanoLuc luciferase (Nluc; Promega) and the red-emitting *Luciola italica* luciferase (RFluc; Targeting Systems). We selected these two candidates because their emission spectra have little overlap and can be conveniently separated using optical filters (Fig. 2B), and they use different substrates with no cross-reactivity (31, 32). We also validated that the Nluc substrate furimazine (also known as “Nano-Glo substrate”) was suitable for use in live pneumococcal cultures (*SI Appendix*, Fig. S1).

We created a transcriptional fusion of Nluc to the ComE-regulated *comAB* promoter (P_{comAB}) and a transcriptional fusion of RFluc to the BlpR-regulated proximal BIR promoter (P_{BIR}) (Fig. 2A). These two constructs were then transformed into wild-type unencapsulated (R6) and encapsulated (D39) strains engineered to encode intact versions of *blpAB*. In these dual reporters Nluc activity correlates with *com* activation, and RFluc activity correlates with *blp* activation (*SI Appendix*).

We confirmed that the dual reporter successfully recapitulates what has been previously published regarding the response of the *com* and *blp* systems to CSP treatment (11, 23, 33, 34) (Fig. 2C

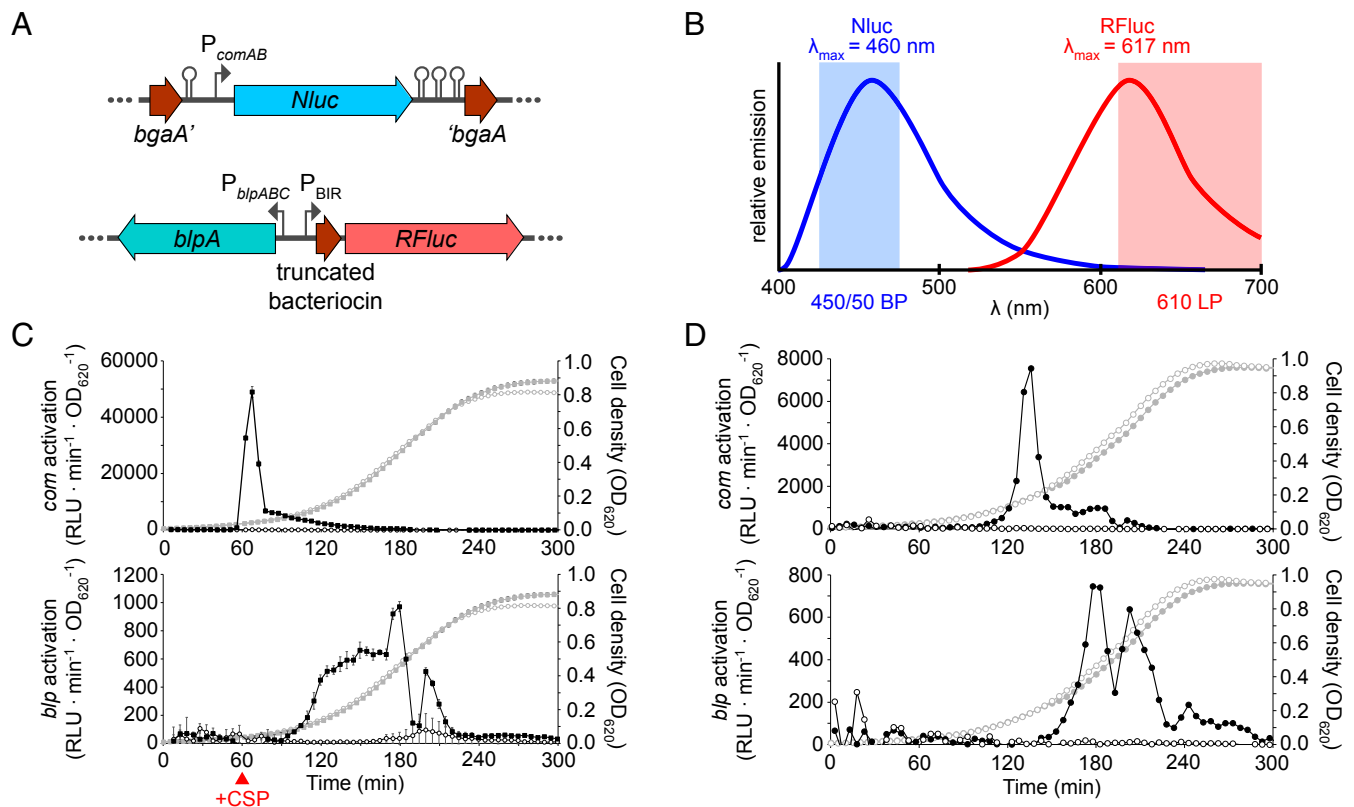


Fig. 2. The dual *com/blp* luciferase reporter allows simultaneous monitoring of *com* and *blp* activation kinetics. (A) Schematic representation of P_{comAB} -*Nluc* and P_{BIR} -*RFluc* constructs. Bent arrows denote promoters. Hairpins denote transcriptional terminators. (B) Diagram of the emission spectra of *Nluc* (blue) and *RFluc* (red) and the optical filter sets used to separate their signals. (C) An R6-derived wild-type dual reporter strain was grown in a 96-well plate in THY broth at pH 7.1. Cells were monitored for growth (right y axes, light shading), *com* activation (Upper, left y axis, dark shading), and *blp* activation (Lower, left y axis, dark shading). At $t = 60$ min, cells were treated with either mock treatment (open circles) or 100 ng/mL CSP (closed squares). Data are plotted as the average \pm SD of four wells. (D) An R6-derived wild-type dual reporter strain was grown in a 96-well plate in THY broth at pH 7.4 without treatment. Cells were monitored as in C. Data from two representative wells are shown, one with spontaneous *com* and *blp* activation (filled circles), and one without (open circles). RLU, relative luminescence units.

and *SI Appendix, Fig. S2A*). Using it, we are also able to detect spontaneous *com* and *blp* activation events when cells are grown under permissive conditions (Fig. 2D and *SI Appendix, Fig. S2B*). Therefore, the dual reporter provides accurate and convenient simultaneous readouts of *com* and *blp* activation in live cells.

BlpAB Processes and Secretes ComC/CSP. We used the dual luciferase reporter to assay the response of cells to treatment with synthetic BlpC pheromone during growth in THY broth (Todd Hewitt broth + 0.5% yeast extract) at pH 7.1, which is non-permissive for spontaneous *com* activation. We noticed that BlpC treatment led to activation of the *com* system (Fig. 3A and B). This BlpC-induced *com* activation was delayed by roughly 30 min compared with activation of the *blp* system, which responds almost immediately to BlpC. Given this delay, we reasoned that it was unlikely this *com* activation resulted from direct up-regulation of P_{comAB} by BlpR. Moreover, BlpC-induced *com* activation is completely abolished in the $\Delta blpA$ and $\Delta comC$ mutants but persists in the $\Delta comAB$ and $\Delta blpC$ mutants (Fig. 3A and B). These data led us to hypothesize that the mechanism underlying the cross-activation was BlpAB-mediated CSP secretion.

To test our hypothesis, we employed a previously developed peptide-processing assay (35). Using FLAG-tagged peptides, the unprocessed and processed forms can be separated by size using SDS/PAGE and detected via Western blot. FLAG-tagged peptides cannot be secreted by ComAB/BlpAB and are retained within the cytoplasm (35), most likely because the high charge

density of the FLAG tag interferes with loading into the transporter channel. Therefore, this assay assesses only the processing step of transport.

We engineered R6 strains expressing C-terminally FLAG-tagged ComC (ComC-FLAG) in place of wild-type ComC from the native *comC* locus. Using these strains, we assayed for ComC processing in the wild-type, $\Delta comAB$, $\Delta blpA$, and $\Delta comAB\Delta blpA$ backgrounds 30 min after induction with CSP and BlpC in THY broth (pH 7.1) (Fig. 3C). The double mutant was included as a control to evaluate for the presence of a transporter or transporters other than ComAB or BlpAB that might contribute to ComC processing. The double mutant showed only a negligible amount of ComC processing, suggesting there are no other such transporters. Meanwhile, we confirmed that ComAB can process ComC, consistent with established models of *com* regulation. We also observed ComC processing in the $\Delta comAB$ strain expressing only BlpAB. These results indicate that BlpAB can complete at least the first step of ComC transport (processing) and when taken together with the *com* activation kinetics data (Fig. 3A and B) strongly suggest that BlpAB can carry out the second step (secretion) as well. Therefore, we conclude that BlpAB can transport ComC/CSP and that this mechanism is responsible for the observed BlpC-induced *com* activation.

Both ComAB and BlpAB Process and Secrete Pneumocins. Having established that ComAB and BlpAB both secrete CSP and BlpC (11, 23), we sought to determine if the promiscuity of these

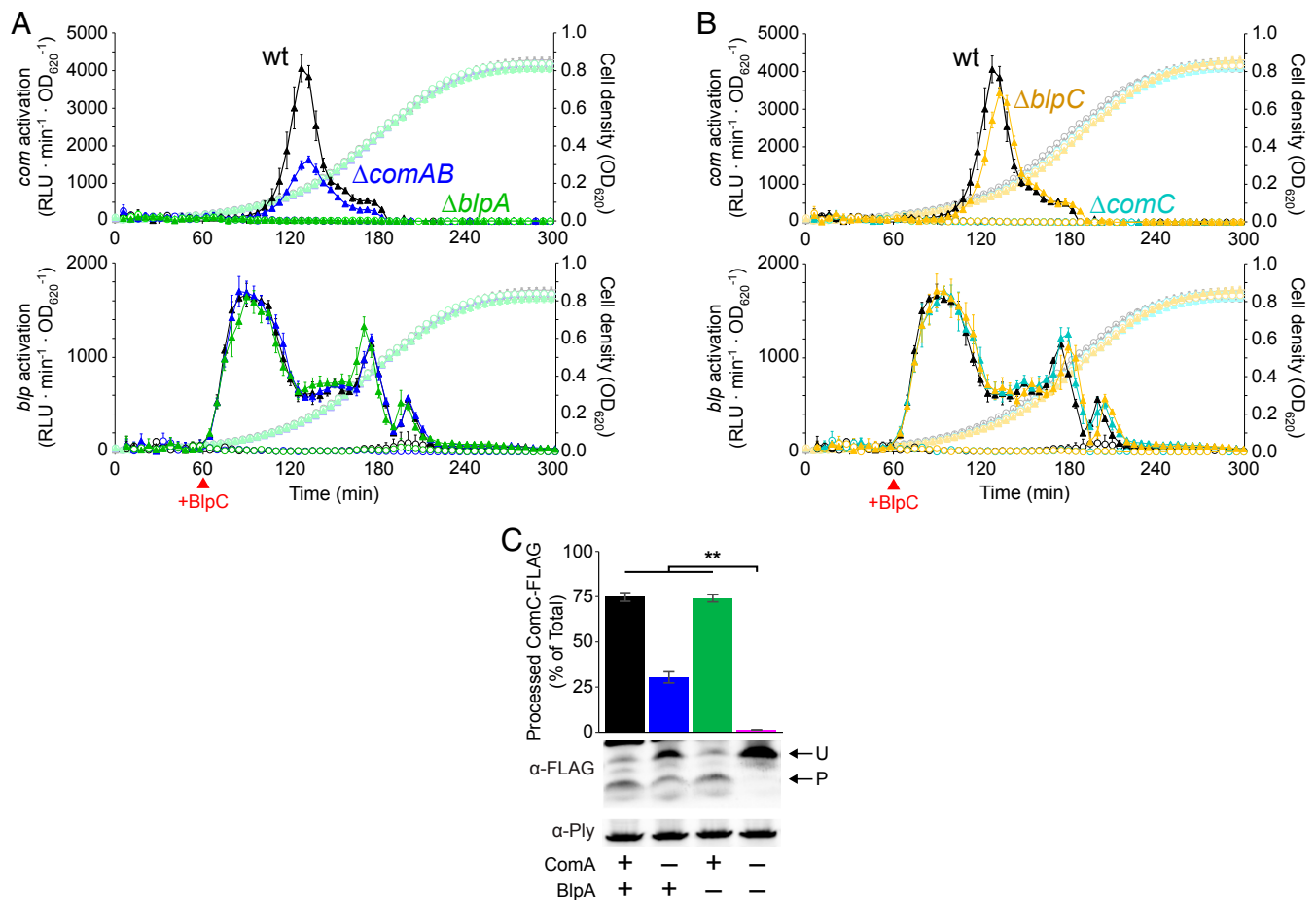


Fig. 3. BlpAB processes and secretes ComC/CSP. (A and B) R6-derived dual reporter strains were grown in 96-well plates in THY broth at pH 7.1. Cells were monitored for growth (right y axes, light shading), *com* activation (Upper, left y axis, dark shading), and *blp* activation (Lower, left y axis, dark shading). At $t = 60$ min, cells were treated with either mock treatment (open circles) or 100 ng/mL BlpC (closed triangles). Data from one representative experiment are shown and are plotted as the average \pm SD of four wells. (C) ComC-FLAG processing in wild-type (black), $\Delta comAB$ (blue), $\Delta blpA$ (green), and $\Delta comAB \Delta blpA$ (magenta) strains. Cells were grown in THY broth at pH 7.1, induced with 100 ng/mL CSP and 100 ng/mL BlpC, and whole-cell lysates were collected 30 min later for Western blot. Representative blots are presented, showing the unprocessed (U) and processed (P) forms of ComC-FLAG and a pneumolysin (Ply) loading control. The amount of processed ComC-FLAG as a percentage of total (processed and unprocessed) ComC-FLAG is quantified and plotted as the average \pm SE of four independent experiments. ****** $P < 0.01$, ANOVA with Tukey's HSD test.

transporters extended to other substrates as well. The current model of substrate recognition by ComAB/BlpAB posits that the peptidase domain of the transporters interacts with the substrates' N-terminal signal sequences. In addition to the double-glycine motif, four specific hydrophobic residues in the signal sequence (SI Appendix, Fig. S3A, yellow highlights) are important for this interaction (30). These residues are conserved across all *com*- and *blp*-regulated double-glycine peptides found in pneumococcus. Moreover, the residues in the transporter peptidase domain that are thought to participate in substrate recognition (36) are also highly conserved in both ComA and BlpA (SI Appendix, Fig. S3C, yellow highlights). Given this, we hypothesized that ComAB and BlpAB recognize and transport the same pool of substrates, including the pheromones ComC/CSP and BlpC, the pneumococci, and the competence-induced bacteriocins CibAB.

We were particularly interested in testing this hypothesis on the pneumococci for several reasons. First, while long suspected to be BlpAB, the transporter or transporters responsible for pneumococin secretion have never been definitively identified. Second, the question of whether ComAB can secrete the pneumococins is an important unanswered question given the ability of the *com* system to up-regulate pneumococin expression and the absence of BlpAB in 75% of strains. Since all pneumococins share

very similar signal sequences (SI Appendix, Fig. S3A), we reasoned that the details of transport would be similar, if not identical, across the different pneumococins. As such, for use in subsequent experiments we chose a representative pneumococin, BlpI_{P133} (henceforth referred to as "BlpI"), which has been previously shown to inhibit sensitive strains when expressed with its partner, BlpJ (27).

To assess BlpI secretion, we chose to employ the HiBiT tag detection system (Promega). The HiBiT tag is a short, 11-residue peptide tag (VSGWRLFKKIS) that associates with the inactive LgBiT luciferase fragment with subnanomolar affinity to complement the latter's luciferase activity (37, 38). This system allows highly sensitive detection of HiBiT-tagged peptides and proteins using a bioluminescence assay (38).

After validating the assay system (SI Appendix, Fig. S4), we used R6 strains expressing BlpI-HiBiT from the native, BlpR-regulated proximal BIR promoter to evaluate BlpI secretion 60 min after BlpC and CSP induction in the wild-type and transporter-deletion backgrounds (Fig. 4A). The amount of BlpI-HiBiT detected in the supernatant of the double mutant was 180-fold less than that of the wild type, confirming that ComAB and BlpAB are the primary contributors to BlpI secretion. We also found that both single mutants secreted significantly more BlpI-HiBiT than the double

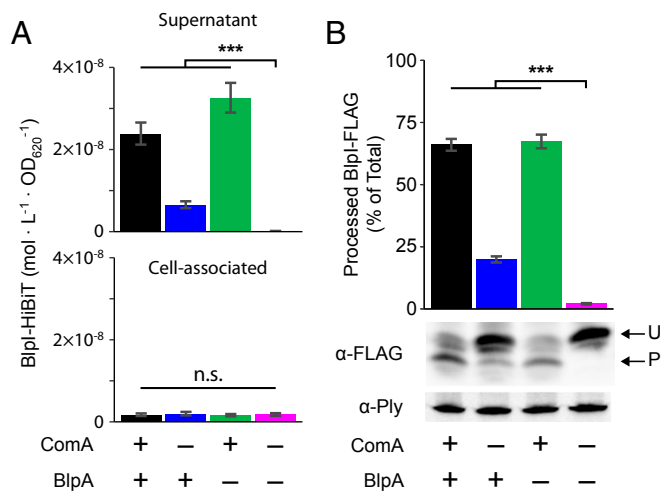


Fig. 4. ComAB and BlpAB process and secrete the pneumocin BlpI. (A) BlpI-HiBIT secretion in wild-type (black), $\Delta comAB$ (blue), $\Delta blpA$ (green), and $\Delta comAB\Delta blpA$ (magenta) strains. Concentration of BlpI-HiBIT normalized to cell density detected in the supernatant (Upper) and cell-associated (Lower) fractions 60 min after induction with 200 ng/mL CSP and 200 ng/mL BlpC in THY broth (pH 7.1). Data are plotted as the average \pm SE of three independent experiments. (B) BlpI-FLAG processing. Cells were grown in THY broth at pH 7.1 and were induced with 100 ng/mL CSP and 100 ng/mL BlpC; whole-cell lysates were collected 30 min later. Representative blots are presented, showing the unprocessed (U) and processed (P) forms of BlpI-FLAG and a pneumolysin (Ply) loading control. The amount of processed BlpI-FLAG as a percentage of total (processed and unprocessed) BlpI-FLAG is quantified and plotted as the average \pm SE of four independent experiments. n.s., not significant; *** $P < 0.001$; ANOVA with Tukey's HSD test.

mutant. In contrast, we detected similar levels of BlpI in the cell-associated fractions of all strains (Fig. 4A, Lower). We corroborated these findings using a BlpI-FLAG processing assay (Fig. 4B), which showed high levels of processing in both single transporter mutants. Therefore, we conclude that both ComAB and BlpAB can process and secrete BlpI and likely the rest of the pneumocins as well.

Pheromone Secretion by BlpAB Enhances Competence Activation and Allows Competence-Independent *blp* Activation. Having determined that ComAB and BlpAB are functionally redundant for secretion of pheromones and pneumocins, we sought to determine what advantages, if any, intact BlpAB confers to pneumococcus. First, we hypothesized that ComC/CSP secretion by BlpAB could drive spontaneous *com* activation, similar to the way BlpC secretion by ComAB can drive spontaneous *blp* activation; if true, then we should be able to observe spontaneous *com* activation following *blp* activation in a $\Delta comAB$ mutant. To test this, we used our dual reporter to monitor spontaneous *com* activation in a panel of transporter- and pheromone-deletion mutants grown in THY broth at pH 7.4 in a 96-well plate. Each well was independently assessed for *com* activation events, defined as when the *com* activation level of the well rose above an empirically determined threshold value. If a well reached its maximum observed cell density as measured by OD_{620} before a *com* activation event occurred, then it was censored. Finally, the event data were fit to Kaplan-Meier estimators and plotted as the cumulative probability of *com* activation versus cell density.

Under these conditions, we observed within-strain stochasticity in the pattern of *com* activation; the unencapsulated R6 wild-type strain activated *com* in only a subset of identically inoculated wells, and there was large variation in the timing of *com* activation among those wells in which it did occur (Fig. 5A).

Contrary to our hypothesis, we did not observe *com* activation in the $\Delta comAB$ mutant. However, we did observe a defect in *com* activation in the $\Delta blpA$ mutant compared with the wild-type strain. This defect was not caused by an upstream deficiency in *blp* activation, since *blp* activation was not observed before *com* activation in any strain. Due to conflicting reports about the effect of capsule on CSP signaling (39, 40), we tested whether we could reproduce this phenotype in an encapsulated strain. Indeed, we observed the same competence defect in the $\Delta blpA$ mutant compared with the wild-type strain in the encapsulated D39 background (Fig. 5B). Therefore, under these conditions the presence of a capsule does not appreciably affect BlpAB-mediated *blp*-to-*com* crosstalk.

To assess the contribution of *com* and *blp* activation states of the starter cultures used to inoculate media at the beginning of the assay, we repeated the previous experiment using inocula from starter cultures of R6 strains grown in THY broth at pH 6.8, a condition under which neither *com* nor *blp* activation occurs. This produced results that closely resemble those obtained using pH 7.4 starter cultures (SI Appendix, Fig. S5A). When analyzing these data, we noticed that two wells of the $\Delta comAB$ mutant activated *com* (SI Appendix, Fig. S5A, blue line). We reasoned that this must represent *blp*-dependent *com* activation. Indeed, the *com* and *blp* activation kinetic curves of the two wells show peaks of *blp* activation that are concurrent with the peaks of *com* activation (SI Appendix, Fig. S5C). We also observed the same phenomenon in one well containing the D39 $\Delta comAB$ mutant (Fig. 5B, blue line). These data show that BlpAB is sufficient to drive *com* activation during spontaneous *blp* activation under certain conditions.

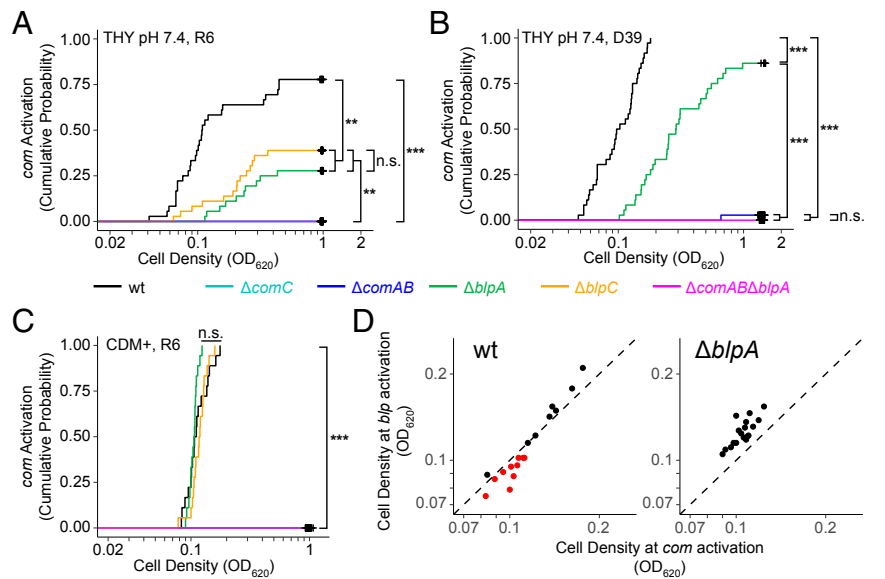
We next tested whether enhanced *com* activation by BlpAB occurred under other growth conditions. We repeated the spontaneous *com* activation experiment using CDM+ medium, a more minimal medium than THY broth that is used for maintaining pneumococcal biofilms (41). Unlike in THY broth, we did not observe a *com* activation defect in the R6 $\Delta blpA$ mutant in CDM+ medium compared with the wild type (Fig. 5C).

From the same experiment in CDM+ medium, we were also able to gather data on *blp* activation using the dual reporter. When analyzing these data, we noticed that a significant number of wells of the wild-type strain activated *blp* before *com* (Fig. 5D, Left, red dots). This *com*-independent *blp* activation occurred in just over half of the wells (10/18) in the wild-type strain compared with zero wells in the $\Delta blpA$ mutant (Fig. 5D, Right). Consistent with previous work (11), these results show that BlpAB can promote and is required for *com*-independent *blp* activation.

BlpAB Ensures Efficient Transport of Pneumocins. Next, we wanted to investigate whether BlpAB(+) strains, which possess both ComAB and BlpAB, enjoy advantages in pneumocin secretion over BlpAB(-) strains, which possess only ComAB. The previous BlpI secretion assay (Fig. 4A) was performed on cells simultaneously induced with saturating concentrations of both CSP and BlpC. These conditions are unlikely to resemble what occurs during natural, spontaneous activation of *com* and *blp*.

First, we found that the pheromone concentrations required to induce strains to levels and with kinetics similar to spontaneous activation were 10 ng/mL for CSP and 25 ng/mL for BlpC (SI Appendix, Fig. S6 A-C), much lower than the 100–200 ng/mL used in previous processing and secretion assays. Second, in the absence of an exogenous source of BlpC, BlpAB(-) strains can activate *blp* only in a *com*-dependent fashion. This *com*-dependent *blp* activation also happens in BlpAB(+) strains. In both strain backgrounds, *blp* activation in this manner occurs after *com* activation following a cell density-dependent delay (Fig. 6A), the length of which is not affected by the presence of BlpAB ($P > 0.05$, ANCOVA). Third, we observed that ComA protein levels following CSP induction decrease rapidly after 45 min

Fig. 5. BlpAB enhances spontaneous *com* activation and drives *com*-independent *blp* activation. (A–C) Spontaneous *com* activation in THY broth and CDM+ medium. R6 (A and C) and D39 (B) dual reporter strains were grown in 96-well plates in THY broth at pH 7.4 (A and B) or in CDM+ medium at pH 7.0 (C). All six (wild-type, pheromone, and transporter deletion) strains are presented in A and C; only the wild-type and transporter deletion strains are presented in B. Each well was continuously monitored for growth and *com* activation. Data were fit to the Kaplan–Meier estimator. Wells that did not experience a *com* activation event before cells reached their maximum density were censored (crosses). $n = 36$ (A and B) or 18 (C) wells per strain, pooled from three independent experiments. $***P < 0.001$; $**P < 0.01$; n.s., not significant; log-rank test with Holm’s correction for multiple comparisons. (D) Cell density at *com* activation plotted against cell density at *blp* activation in individual wells from C of the wild-type and $\Delta blpA$ strains. Points that fall on the dotted line represent wells in which *blp* activation occurred at the same time as *com* activation. Points that fall to the left of the dotted line represent *blp* activation occurring after *com* activation. Points that fall to the right of the dotted line (highlighted in red) represent *blp* activation occurring before *com* activation.



(Fig. 6B). This is consistent with the rapid shut-off in transcription activity from P_{comAB} seen with the luciferase reporter (*SI Appendix, Fig. S6C*). Given these data, we hypothesized that BlpAB(–) strains, which must rely on ComAB for secretion, secrete less pneumocins during *com*-dependent *blp* activation than BlpAB(+) strains, which can use both ComAB and BlpAB.

To test this hypothesis, we assayed BlpI secretion under conditions similar to spontaneous *com* and *blp* activation. The strains used in this experiment were engineered to express pheromone-receptor mismatched CSP2–ComD1 and BlpC_{6A}–BlpH_{R6} pairs. Therefore, these strains are deficient in autoactivation of both *com* and *blp* to ensure that differences in transporter content (and hence pheromone secretion) did not affect activation kinetics. Cells were induced with either BlpC alone (to mimic *com*-independent *blp* activation), with CSP and BlpC together (“simultaneous treatment”), or with CSP followed by BlpC 45 min later (“staggered treatment,” to mimic *com*-dependent *blp* activation) (*SI Appendix, Fig. S6D*). Consistent with our hypothesis, we observed a small but statistically significant increase in BlpI secretion from the wild-type strain compared with the $\Delta blpA$ mutant in the staggered-treatment group (*SI Appendix, Fig. S6E*). This difference was not seen in the simultaneous-treatment group. Additionally, when comparing the staggered- with the simultaneous-treatment group, a large decrease in BlpI secretion was seen only in the strains possessing ComAB, and of these two strains the wild-type strain, which possesses both ComAB and BlpAB, suffered the smaller decrease.

The differences seen between the wild-type and $\Delta blpA$ strains in the previous experiment were small when assessed at 105 min after CSP treatment. However, given our observation that ComAB levels decrease continuously past 45 min post-CSP treatment, the differences should increase over time. To test this hypothesis, we monitored BlpI–HiBiT secretion over time following the BlpC-only or staggered treatments (Fig. 6C). Consistent with falling ComAB levels, beginning at 60 min post-BlpC treatment (105 min post-CSP treatment) the $\Delta blpA$ mutant in the staggered-treatment group showed increasingly large defects in BlpI–HiBiT secretion over time compared with the wild-type strain in the same treatment group. The same was seen when comparing the $\Delta blpA$ mutant in the staggered-treatment group with the wild-type strain in the BlpC-only treatment group.

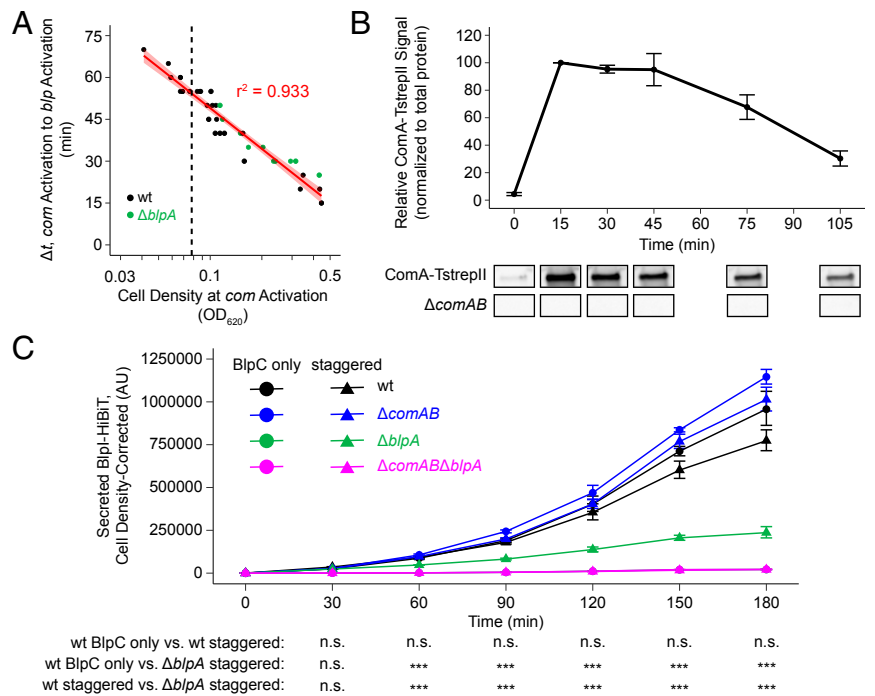
Last, in the BlpC-only treatment group, only BlpAB-containing strains secreted BlpI at levels higher than the double transporter mutant, and the wild-type strain did not secrete more than the $\Delta comAB$ mutant ($P > 0.05$ at all time points). These data show that BlpI secretion through ComAB (but not BlpAB) decreases rapidly with time during *com*-dependent *blp* activation and is negligible during *com*-independent *blp* activation.

BlpAB(+) Strains Enjoy a Competitive Advantage over BlpAB(–) Strains During Nasopharyngeal Colonization. We hypothesized that BlpAB(+) strains’ ability to activate *blp* independently of *com* and to secrete greater amounts of pneumocins would give them a competitive advantage over BlpAB(–) strains during nasopharyngeal colonization. To test this, we coinoculated mice with either a BlpAB(+) or BlpAB(–) pneumocin-expressing “killer” strain and a Δblp pneumocin-sensitive strain and assessed competitive indices at 4 d postinoculation. The BlpAB(+) killer strain outcompeted the sensitive strain to a greater extent than the BlpAB(–) killer strain did (Fig. 7A). We also assessed how BlpAB(+) and BlpAB(–) strains fare in direct competition. We coinoculated mice with pairs of either BlpC pherotype-matched or mismatched pneumocin-expressing BlpAB(+) and BlpAB(–) strains and assessed competitive indices at 4 d postinoculation. The coinoculated strains had identical BIRs; therefore, either strain could develop immunity to the other’s pneumocins provided it activates *blp* at the appropriate time. We observed that the BlpAB(+) strain had a competitive advantage over the BlpAB(–) strain but only when the two strains had mismatched BlpC phenotypes (Fig. 7B). All strains used in these competition assays colonize to similar levels when inoculated alone (*SI Appendix, Fig. S8*), indicating none have intrinsic colonization defects.

Discussion

We have presented evidence showing that competence and bacteriocin regulation in pneumococcus are more entwined than previously thought. While it was known that *com* could send positive inputs to the *blp* system, we show here that signals can also travel in the opposite direction due to secretion of CSP by BlpAB. While we found this *blp*-to-*com* crosstalk could drive *com* activation following or concurrently with *blp* activation (*SI Appendix, Fig. S5C*), the more common effect of the crosstalk

Fig. 6. BlpAB promotes efficient pneumocin secretion. (A) Correlation of the time delay between spontaneous *com* and *blp* activation with cell density at the point of *com* activation in wild-type (black) and $\Delta blpA$ (green) strains in THY broth at pH 7.4. Data were taken from the same experiments as in Fig. 5A and represent all wells in which *blp* activation was observed. The red line and shading represent the linear regression estimate \pm 95% CI. The dashed vertical line is placed at OD₆₂₀ 0.078. (B) Twin-Strep-tagged ComA (ComA-Tstrepll) protein levels following treatment with 10 ng/mL CSP at OD₆₂₀ 0.078 in THY broth at pH 7.1. Samples were collected at the indicated time points, and membrane fractions were assayed by quantitative Western blot with anti-Strep-tag II antibody. Representative blots of samples from the strain expressing ComA-Tstrepll (*Upper*) and from a $\Delta comAB$ negative control (*Lower*) are shown. For quantification, ComA signal was normalized to total protein and expressed relative to the 15-min time point, which was set to a value of 100. Data are plotted as the average \pm SE of three independent experiments. (C) BlpI-HiBiT secretion over time in wild-type (black), $\Delta comAB$ (blue), $\Delta blpA$ (green), and $\Delta comAB\Delta blpA$ (magenta) strains grown in THY broth at pH 7.1. Beginning at OD₆₂₀ 0.02 ($t = -45$ min), cells were given two treatments at -45 and 0 min: BlpC-only (mock treatment followed by 25 ng/mL BlpC) and staggered treatment (10 ng/mL CSP followed by 25 ng/mL BlpC). Samples were collected every 30 min and assayed for BlpI-HiBiT in the supernatant fractions. Data were corrected for differences in cell density between samples independently per time point and are plotted as the average \pm SE of three independent experiments. Green circles ($\Delta blpA$, BlpC only) are obscured behind the magenta plots. n.s., not significant; *** $P < 0.001$; ANOVA with Tukey's HSD test.



was activation of *com* at higher frequencies and lower cell densities, even in the absence of overt *blp* activation (Fig. 5A and B). This highlights the importance of the basal level of transporter expression as the rate-limiting factor for CSP secretion and *com* activation; the addition of a second transporter (BlpAB) to augment basal CSP secretion by ComAB is sufficient to increase *com* activation. This phenotype was medium dependent; it was found in THY broth but not CDM+ medium. We postulate that the threshold for *com* activation in CDM+ medium is lower than in THY broth because of increased rates of CSP secretion, increased CSP stability in the medium, increased responsiveness to CSP, or some combination of these. This allows ComAB alone to secrete enough CSP to activate *com* efficiently without the help of BlpAB. Therefore, BlpAB's role in enhancing *com* activation depends on environmental conditions and is most pronounced when the threshold for *com* activation is relatively high. We also found that in THY broth *com* activation suffered the same defect in the absence of BlpC signaling as in the absence of the BlpAB transporter (Fig. 5A; compare $\Delta blpC$ with $\Delta blpA$). We speculate that basal levels of BlpC secretion and signaling lead to noise in *blp* activation during the preactivation period. This noise results in transient elevated expression of BlpAB in a subpopulation of cells that leads to bursts of CSP secretion which over time help push the total extracellular CSP concentration above the threshold required for *com* activation. Further studies will be needed to confirm this hypothesis and to further elucidate the determinants of transporter-expression dynamics in the preactivation period and their effects on *com* and *blp* activation.

We have also shown that the ability of both ComAB and BlpAB to secrete the other system's pheromone is not a special case but rather is a natural consequence of the transporters' promiscuity toward substrates. It was previously shown that purified peptidase domain from BlpA could cleave a synthetic CSP-like analog (42). We confirm here that full-length BlpAB can secrete native ComC/CSP in a live-cell context. We have also observed that both transporters can process and secrete the pneumocin BlpI (Fig. 4).

These data support the conclusion that ComAB and BlpAB share the same substrate pool. Finally, our data indicate that the signal sequence-peptidase domain interaction is the primary factor that determines whether a peptide is secreted by ComAB/BlpAB. Both transporters tolerate a wide variety of mature peptides for secretion: small and amphipathic (CSP), charged (BlpC), and large and highly hydrophobic (pneumocins).

The promiscuity of ComAB and BlpAB has multiple functional consequences, which are given added importance by the fact that 75% of pneumococcal strains lack a functional BlpAB but nearly all strains produce a functional ComAB. It was previously unclear whether BlpAB(-) strains could secrete pneumocins. We provide evidence here that these strains can in fact secrete pneumocins during *com*-dependent *blp* activation. Over short time frames, the amount of pneumocin secreted by BlpAB(-) strains in this manner is comparable to the amount secreted by BlpAB(+) strains during either *com*-independent or *com*-dependent *blp* activation (Fig. 6C and *SI Appendix*, Fig. S6E). This suggests that BlpAB(-) strains theoretically can effect pneumocin-mediated inhibition during *com*-dependent *blp* activation. This would be true for all but a small minority of strains that cannot activate *com* or produce ComAB due to acquired mutations in *com* regulatory genes (43). Obtaining direct evidence for this will be a priority for future studies.

Despite the above, BlpAB(+) strains still enjoy multiple advantages in pneumocin secretion over their BlpAB(-) counterparts due to differences in the regulation and kinetics of ComAB and BlpAB expression. First, BlpAB(+) strains can secrete more pneumocins than BlpAB(-) strains during *com*-dependent *blp* activation in a time-dependent fashion (Fig. 6C). The transient nature of ComAB expression during *com* activation limits pneumocin secretion in BlpAB(-) strains to short bursts following *com*-dependent *blp* activation. In contrast, *blp* activation—and therefore BlpAB expression—is not subject to a rapid shut-off mechanism; in broth culture BlpR-regulated promoters remain highly active after initial activation throughout the

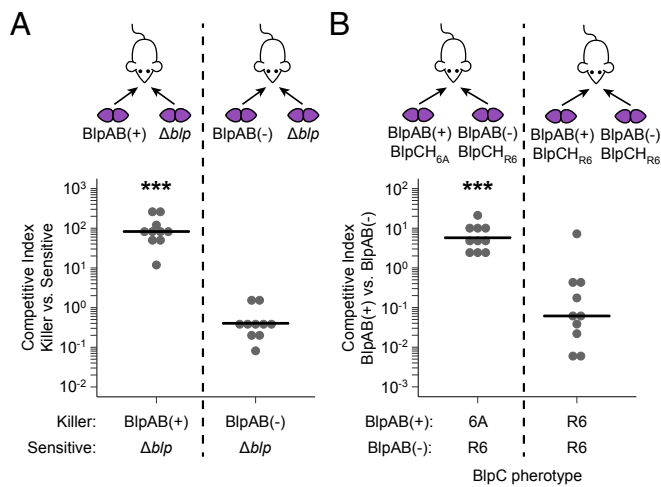


Fig. 7. BlpAB(+) strains have a competitive advantage over BlpAB(-) strains during mouse nasopharyngeal colonization. All pneumococci are mouse-adapted serotype 19A strains. (A) Competition of BlpAB(+) and BlpAB(-) strains against a pneumocin-sensitive strain. BALB/c mice were coinoculated in a 1:1 ratio with either a BlpAB(+) or BlpAB(-) pneumocin-expressing killer strain and a Δblp (whole locus) sensitive strain. Both killer strains encode a P164-type BIR. The cfu counts from the nasopharynx were obtained at 4 d postinoculation and used to calculate competitive indices. $n = 10$ mice per competition. (B) Direct competition of BlpAB(+) strains against BlpAB(-) strains. Mice were coinoculated as in A with either a BlpCH_{6A}- or BlpCH_{R6}-expressing BlpAB(+) strain and a BlpCH_{R6}-expressing BlpAB(-) strain. All strains encode a P133-type BIR. Competitive indices were calculated as in A. $n = 10$ mice per competition. Black bars represent medians. *** $P < 0.001$ (left-hand vs. right-hand group); Mann-Whitney U test.

exponential phase (Fig. 3A and B). Thus, BlpAB(+) strains can sustain pneumocin secretion for longer periods of time and generate higher extracellular pneumocin concentrations due to secretion through BlpAB. Second, unlike BlpAB(-) strains, BlpAB(+) strains can activate *blp* independently of *com* (Fig. 5D and ref. 11) with no penalty to pneumocin secretion capacity despite ComAB being unavailable to contribute to secretion (Fig. 6C).

Consistent with our *in vitro* findings, BlpAB(+) strains enjoy a competitive advantage over BlpAB(-) strains during nasopharyngeal colonization in mice. During cocolonization, BlpAB(+) strains were better able than BlpAB(-) strains to outcompete a pneumocin-sensitive strain (Fig. 7A). Moreover, a BlpAB(+) strain directly outcompeted its BlpAB(-) counterpart when both expressed the same pneumocins, but only when the two strains could not cross-activate each other's *blp* systems (Fig. 7B). These data indicate that, compared with BlpAB(-) strains, BlpAB(+) strains can use pneumocins more effectively in competition during colonization due to more frequent activation of *blp* and/or secretion of greater amounts of pneumocins. Accordingly, we propose that BlpAB(+) strains leverage these abilities to act as aggressors, wielding pneumocins as weapons for generalized antibacterial competition. In contrast, BlpAB(-) strains act primarily as opportunists, using pneumocins in a limited capacity to augment fratricide and/or inhibit competitors while they are in a potentially vulnerable state during competence.

Linkage analysis indicates the predominant BlpAB-inactivating mutation moves from strain to strain via recombination and that BlpAB(-) strains occasionally revert to a BlpAB(+) genotype in the same manner (27). This suggests that there are costs and benefits to maintaining an intact BlpAB, and BlpAB(+) and BlpAB(-) strains represent dynamic populations that switch their *blpAB* genotype in response to different selective pressures favoring an aggressor phenotype over opportunist or vice versa.

Currently, genomics data do not indicate that invasion is such a selective pressure; in a collection of human isolates from South Africa (27), the distribution of BlpAB(+) strains did not differ between the invasive and colonizing groups (8/21 invasive vs. 11/30 colonizing, $P = 1$, Fisher's exact test). Another set of potential selective pressures is that associated with competence development. The increased propensity for competence activation in BlpAB(+) strains (Fig. 5A and B) would provide them with greater access to DNA for horizontal gene transfer and an improved ability to cope with DNA-damaging stress. On the other hand, competence is a costly, energy-intensive process that interferes with normal cell metabolism and proliferation (44, 45). The stringent regulation of competence and its rapid shut-off once activated likely are strategies to mitigate its negative impacts. Therefore, the increased frequency of competence activation conferred by an intact BlpAB may be detrimental to fitness in certain cases. Finally, BlpAB(+) strains may incur a fitness cost from frequent *blp* activation. Consistent with this, we observed a dose-dependent, small but reproducible and statistically significant growth defect in THY broth-grown strains following BlpC treatment (SI Appendix, Fig. S9). Validating these and other pressures in physiologic settings to further define how environmental and genetic contexts influence the adaptive value of maintaining an intact BlpAB presents an attractive target for future studies.

Many streptococci are naturally competent, but only members of the *Streptococcus mitis* (Mitis) group (to which pneumococcus belongs) and the closely related *Streptococcus anginosus* group (SAG) employ a ComCDE-type system to regulate competence (46). The ability of non-ComAB ABC transporters to secrete ComC/CSP and influence competence development is of direct relevance in other species of Mitis/Anginosus groups. For instance, *Sil* (*Streptococcus invasion locus*) is a bacteriocin-encoding locus (47) found in SAG that is structurally and functionally similar to pneumococcal *blp*. The regulation of *Sil* is effectively identical to that of *blp*, with SilE/D/CR/B/A taking the roles of BlpA/B/C/H/R, respectively. Importantly, SilED is the only ComAB/BlpAB-type transporter found in many SAG strains and therefore is the only potential transporter for ComC/CSP. SAG SilE and SilCR/ComC share the same sequence motifs important for substrate recognition as those found in pneumococcal BlpA/ComA and BlpC/ComC, respectively (SI Appendix, Fig. S3B and D). Therefore, it is likely that SilED can secrete CSP. Given this, competence in SAG may be chiefly regulated by a bacteriocin locus (*Sil*) through secretion of CSP through a non-ComAB transporter.

Looking beyond streptococci, ComAB/BlpAB-type transporters are widely distributed among Gram-positive and Gram-negative bacteria, where they primarily function to export bacteriocins and in some cases also the signaling peptide that induces expression of said bacteriocins (29, 48–51). With few exceptions, these peptides and transporters share the same conserved sequence motifs found to be important for substrate recognition in their pneumococcal counterparts (Fig. 3B and D). This implies that most if not all ABC transporters of this family recognize their substrates not only in the same manner but also using the same sequence motifs. Therefore, the promiscuity of pneumococcal ComAB and BlpAB is likely a general feature of this transporter family. Consistent with this, others have shown that in *Lactococcus lactis*, the lactococin Q transporter LaqD can secrete the related but distinct bacteriocin lactococin G (48), and in *Enterococcus faecium*, the enterocin transporter EnkT recognizes the signal sequences of a number of bacteriocins from other species (52). This transporter promiscuity has wide-ranging implications for the regulation and biosynthesis of bacteriocins in many different bacterial species. Understanding the details of pheromone and bacteriocin secretion by these ABC transporters will provide key insights into the dynamics of interbacterial communication and competition.

Methods

Bacterial Strains and Growth Conditions. All strains are derived from the R6 strain P654 (35) (referred to as “PSD100” in ref. 35) or the D39 strain P2055 (*SI Appendix*). For experiments, pneumococcus was grown in either filter-sterilized THY broth or CDM+ medium (41) at 37 °C. Except where noted otherwise, pneumococcal cultures used for all experiments were inoculated from starter cultures grown in THY broth at pH 7.4 to an OD₆₂₀ of 0.275 and were frozen at –80 °C in 13% glycerol. Transformations were carried out as previously described (53). Unmarked chromosomal mutations were created via Janus or Sweet Janus exchange (54, 55). See *SI Appendix* for details. All transformants were verified by PCR fragment length analysis and Sanger sequencing. Antibiotics were used at the following concentrations: chloramphenicol, 2 µg/mL; kanamycin, 500 µg/mL; spectinomycin, 200 µg/mL; and streptomycin, 100 µg/mL.

DNA Manipulation. PCR for downstream Gibson assembly, transformation, or sequencing applications was performed using Phusion polymerase (E0553; New England Biolabs). All other PCR reactions were performed using Taq polymerase (M0273; New England Biolabs). Primers were designed using primer3 (56, 57) and synthesized by IDT. PCR products were purified using silica columns (28106; Qiagen). Gibson assembly was performed using NEBuilder HiFi DNA Assembly master mix (E2621; New England Biolabs). All codon optimization was performed using OPTIMIZER (58).

com/blp Activation Kinetics Assays. Dual reporter strains were inoculated 1:150 into filter-sterilized THY broth + 25 mM Hepes, 5 µg/mL catalase, 165 µM D-luciferin (88294; Thermo Fisher Scientific), 1:10,000 Nano-Glo substrate (N113B; Promega), or CDM+ medium + 5 µg/mL catalase, 330 µM D-luciferin, and 1:5,000 Nano-Glo substrate. Cultures were aliquoted into a white, clear-bottomed 96-well plate (301012; Corvair), 200 µL (if no treatment was to be added) or 198 µL (if treatment was to be added) per well. The plate was incubated at 37 °C in a BioTek Synergy HTX plate reader. Every 5 min, the plate was shaken (linear shake setting, 587 cycles/min, 5 s), and absorbance (620 nm) and luminescence (bottom-read mode, 0.90-s integration time, gain 150) were read. The Nluc signal was isolated using a 450/50-nm band-pass (BP) filter (7082208; BioTek); The RFluc signal was isolated using a 610-nm long-pass (LP) filter (7092209; BioTek). For experiments in which pheromone treatments were added to the cells, 2 µL sterile medium containing the appropriate pheromone (treatment) or 2 µL sterile medium alone (mock treatment) was added to each well. Luminescence data were used to calculate *com/blp* activation levels (*SI Appendix*).

For analysis of spontaneous *com* and *blp* activation events, individual wells were assessed for activation events. An activation event was defined as the *com/blp* activation level exceeding a threshold value for at least two consecutive time points (*SI Appendix*). Wells without an activation event before cell density reached its maximum observed value were censored at the time point at which maximum cell density was reached. Once generated, activation event data were fit to the Kaplan–Meier estimator using the survfit() function in the R survival package (v2.41-3). The resulting survival curves were compared with the log-rank test using the survdiff() function in the R survival package (v2.41-3) and were adjusted for multiple comparisons with the Holm correction using the p.adjust() function in R 3.4.2.

Peptide Processing Assays. R6 strains expressing FLAG-tagged peptides were inoculated 1:150 into THY broth + 25 mM Hepes (pH 7.1) and 5 µg/mL catalase and were grown statically at 37 °C. At OD₆₂₀ 0.078, cells were induced with 100 ng/mL CSP1 and 100 ng/mL BlpC_{R6}. Thirty minutes later, a sample of cells, equivalent to OD₆₂₀ 1.175 × 1 mL, was taken from each culture and pelleted by centrifugation at 5,000 × g for 5 min at 4 °C. The pellets were washed with 1 mL PBS and pelleted again using the same method. The washed pellets were then resuspended in 25 µL 1% SDS, 0.1% Triton X-100 and mixed with an equal volume of 4× Laemmli sample buffer (1610747; Bio-Rad) supplemented with 10% (vol/vol) 2-mercaptoethanol. The resulting mixture was boiled at 95 °C for 10 min and then was stored at –20 °C. The samples were later analyzed by peptide Western blot (*SI Appendix*). Blots were quantified using Image Studio v5.2. Percent peptide processing was calculated by dividing the signal from the processed peptide band by the sum of the signals from the processed and unprocessed peptide bands. Experiments were repeated four times, and differences between groups were evaluated by applying ANOVA and Tukey’s honest significant difference (HSD) test to the percentage of peptide processing values using the aov() and TukeyHSD() functions in R 3.4.2.

Blp-HiBIT Secretion Assays. R6 strains expressing BlpI-HiBIT along with a background control strain expressing Strep-tag II-tagged BlpI were inoculated 1:150 into THY broth + 25 mM Hepes (pH 7.1) and 5 µg/mL catalase and were grown statically at 37 °C. These strains expressed a mismatched BlpC_{6A}–BlpH_{R6} pair to prevent spontaneous *blp* activation. At OD₆₂₀ 0.078, cells were induced with 200 ng/mL CSP1 and 200 ng/mL BlpC_{R6}. Sixty minutes later, a sample of culture supernatant of each strain was filter sterilized through a 0.22-µm centrifugal filter (8160; Costar) and diluted 10-fold in sterile THY broth + 25 mM Hepes (pH 7.1) and 5 µg/mL catalase. Samples of mock-treated cultures were not diluted. At the same time, a sample of cells of each strain was taken and pelleted by centrifugation at 5,000 × g, for 5 min at 4 °C. The pellet was washed once with 1× culture volume PBS and pelleted again by centrifugation at 5,000 × g for 5 min at 4 °C. The washed pellet was then resuspended in cell wall digest buffer [20 mM MES (pH 6.5), 0.5 M sucrose, 20 mM MgCl₂, 50 U/mL mutanolysin (M9901; Sigma), 0.5 mg/mL lysozyme; adapted from ref. 59] and incubated at 37 °C for 30 min. After incubation, the protoplast suspension was diluted fivefold in 0.25% Triton X-100 and incubated at room temperature for 15 min.

For experiments simulating spontaneous activation, cells expressing mismatched CSP2–ComD1 and BlpC_{6A}–BlpH_{R6} pairs were used. The background control strain was Δ*comC* in lieu of a mismatched CSP–ComD pair. For the single-time point assay, cells were induced starting at OD₆₂₀ 0.078 as follows: BlpC-only: mock treatment followed by 25 ng/mL BlpC_{R6} 45 min later; simultaneous treatment: mock treatment followed by 10 ng/mL CSP1 and 25 ng/mL BlpC_{R6} 45 min later; staggered treatment: 10 ng/mL CSP1 followed by 25 ng/mL BlpC_{R6} 45 min later. Supernatant and cell samples were collected 60 min after BlpC induction in all cases and were processed as above. For the multiple-time point assay, cells were induced starting at OD₆₂₀ 0.02 with either the BlpC-only or staggered treatment as above. Supernatant samples were collected just before BlpC treatment (0 min) and every 30 min thereafter for 3 h and were clarified by centrifugation at 2,750 × g for 5 min at 4 °C. All samples after the 0-min time point were diluted 1:10 in sterile THY broth + 25 mM Hepes (pH 7.1) and 5 µg/mL catalase.

Supernatant and cell lysate samples of BlpI-HiBIT-expressing strains along with standards (*SI Appendix*) were aliquoted into a white 96-well plate (3917; Costar), 50 µL per well, *n* = 3 wells (single-time point assays) or *n* = 1 well (multiple-time point assays) per sample/standard. The samples and standards were then assayed for HiBIT tag by adding 50 µL HiBIT Extracellular Detection Reagent (N2421; Promega) to each well. Luminescence was then read using a BioTek Synergy HTX plate reader with the following settings: top read mode (1.00-mm height), 2-s integration time, gain 135. The BlpI-HiBIT concentrations in the samples were calculated using their luminescence values and the appropriate standard curve. Experiments were repeated three times, and differences between groups were evaluated by applying ANOVA and Tukey’s HSD to the log-transformed BlpI-HiBIT concentrations using the aov() and TukeyHSD() functions in R 3.4.2.

ComA Protein Level Kinetics Assay. R6 strains expressing Twin-Strep-tagged (60) ComA (ComA-TstrepII) (*SI Appendix*, Fig. S7) or no ComAB (Δ*comAB*) and mismatched CSP2–ComD1 and BlpC_{R6}–BlpH_{6A} pairs were inoculated 1:150 into THY broth + 25 mM Hepes (pH 7.1) and 5 µg/mL catalase, grown statically at 37 °C, and induced with 10 ng/mL CSP1 at OD₆₂₀ 0.078. Samples were taken just before induction (0 min) and at 15, 30, 45, 75, and 105 min postinduction. Membrane fractions were isolated using a protocol adapted from ref. 59 and were analyzed by Western blot (*SI Appendix*). Blots were quantified using Image Studio v5.2. The Strep-tag II signal was normalized to total protein before being expressed as relative to the signal at the 15-min time point.

Mouse Nasopharyngeal Colonization Assays. Mouse colonization was performed as previously described (27). Briefly, each strain (see *SI Appendix* for strain details) was grown in THY broth to an OD₆₂₀ of 0.5. Cells were gently pelleted and resuspended in sterile PBS at one-fifth the original volume. For coinoculations, PBS mixtures were combined in a 1:1 ratio; for single inoculations, an additional equal volume of sterile PBS was added. Ten microliters of dual- or single-strain mixtures were inoculated into the nasopharynx of unanesthetized 5- to 7-wk-old female BALB/c mice (Taconic). Inocula were plated on selective medium after inoculation to ensure that 1:1 ratios were maintained. No mouse developed evidence of sepsis during the experimental period. Mice were killed with CO₂ overdose on day 4, and nasopharyngeal colonization was sampled by cannulation of the trachea toward the nasopharynx and administration of 200 µL of sterile PBS. Samples were collected from the nares and used to obtain colony counts (*SI Appendix*).

Mice were housed in a specific pathogen-free facility, and experiments were performed under an approved protocol in compliance with the University of Michigan Institutional Animal Care and Use Committee recommendations. Differences in colonization densities and competitive indices between groups were evaluated by the Mann–Whitney (two groups) and Kruskal–Wallis (more than groups) tests using the `wilcox.test()` and `kruskal.test()` functions in R 3.4.2.

Nucleotide Accession Numbers. The nucleotide sequences of the P_{comAB}-Nluc and P_{BIR}-Rfluc reporters are deposited in GenBank with accession numbers MH304212 and MH304213, respectively.

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