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

Charles Y. Wang*, Nisha Patel², Wei-Yun Wholey³, and Suzanne Dawid*²,³

*Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109; and *Department of Pediatrics, University of Michigan Medical School, Ann Arbor, MI 48109

Edited by Richard P. Novick, New York University School of Medicine, New York, NY, and approved May 16, 2018 (received for review March 16, 2018)

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 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.

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 pheromone, 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 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 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.

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.

Significance

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.” Pneumococci 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).

Author contributions: C.Y.W. and S.D. designed research; C.Y.W., N.P., and W.-Y.W. performed research; C.Y.W. and S.D. analyzed data; and C.Y.W. and S.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-NDD).

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. MH304212 (PomAB Niuc), MH304213 (PbRII Pfus), and MH304214 (Sweet Janus)]

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

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1804668115/-/DCSupplemental.

Published online June 4, 2018.
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 pherotypes 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 pherotype. Pherotype 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 pherotypes 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 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 pherotype. Pherotype 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 pherotypes 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 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 pherotype. Pherotype 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 pherotypes 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 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 pherotype. Pherotype 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 pherotypes 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 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 pherotype. Pherotype 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 pherotypes 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 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 pherotype. Pherotype 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 pherotypes 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 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 pherotype. Pherotype 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 pherotypes would not.
BlaB 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. 3 A 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 ΔblpA and ΔcomC mutants but persists in the ΔcomAB and ΔblpAΔcomC mutants (Fig. 3 A 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/BlaB and are retained within the cytoplasm (35), most likely because the high charge density of the FLAG tag interferes with loading into the transport 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, ΔcomAB, ΔblpA, and ΔcomABΔblpA 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 ΔcomAB 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. 3 A 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
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 pneumocins, and the competence-induced bacteriocins CibAB.

We were particularly interested in testing this hypothesis on the pneumocins for several reasons. First, while long suspected to be BlpAB, the transporter or transporters responsible for pneumocin secretion have never been definitively identified. Second, the question of whether ComAB can secrete the pneumocins is an important unanswered question given the ability of the com system to up-regulate pneumocin expression and the absence of BlpAB in 75% of strains. Since all pneumocins 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 pneumocins. As such, for use in subsequent experiments we chose a representative pneumocin, BlpI (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

transports 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 ± SD of four wells. (C) ComC-FLAG processing in wild-type (black), ΔcomAB (blue), ΔblpA (green), and ΔcomABΔblpA (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 ± SE of four independent experiments. **P < 0.01, ANOVA with Tukey’s HSD test.

**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 ± SD of four wells. (C) ComC-FLAG processing in wild-type (black), ΔcomAB (blue), ΔblpA (green), and ΔcomABΔblpA (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 ± SE of four independent experiments. **P < 0.01, ANOVA with Tukey’s HSD test.
Contrary to our hypothesis, we did not observe com activation in the ΔcomAB mutant. However, we did observe a defect in com activation in the ΔblpA 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 ΔblpA 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 ΔcomAB 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 ΔcomAB 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 ΔblpA 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 none of the wells in the ΔblpA 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 pneumococcal 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 pneumococcal 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. 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_{6B} pairs. Therefore, these strains are deficient in autoactivation of both com and blp to ensure that differences in transporter content (and hence pheromone secretion) do 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 Δblp{sub A} mutant in the staggered-treatment group (SI Appendix, Fig. S6F). This difference was not seen in the simultaneous-treatment group. Additionally, when comparing the staggered—without com-dependence—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 difference seen between the wild-type and Δblp{sub A} 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 Δblp{sub A} mutant in the staggered-treatment group showed increasing 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 Δblp{sub A} 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 ΔcomAB 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(+) strain outcompeted the sensitive strain to a greater extent than the BlpAB(−) 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 pherotypes (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
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 for the efficient secretion of CSP, which is dependent per time point and are plotted as the average ± SE of three independent experiments. (C) Blp-C-HiBiT secretion over time in wild-type (black), ΔblpA (blue), ΔΔblpA (green), and ΔΔcomABΔΔblpA (magenta) strains grown in THY broth at pH 7.1. Beginning at OD₆0₀ 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 BlpC-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 ± SE of three independent experiments. Green circles (ΔΔblpA, BlpC only) are obscured behind the magenta plots. n.s., not significant; ***p < 0.001; ANOVA with Tukey’s HSD test.

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 this, 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
exponential phase (Fig. 3.A 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 coccolization, 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 competition.

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 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. 7 A 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 Streplococcus 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 Com/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 SilIE/D/CR/B/A taking the roles of BlpA/B/C/H/R, respectively. Importantly, SilIED is the only ComAB/BlpAB-type transporter found in many SAG strains and therefore is the only potential transporter for Com/CSP. SAG SilIE 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. S3 B and D). Therefore, it is likely that SilIED 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. 3 B 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 lactococcin O transporter LaqD can secrete the related but distinct bacteriocin lactococcin G (48), and in Enterococcus faecium, the enterocin transporter EmK 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 DCM+ medium (41) at 37 °C. Except where noted otherwise, pneumococcal cultures were grown for all experiments and inoculated from starter cultures grown in THY broth at pH 7.4 to an OD$_{620}$ of 0.275 and were frozen at −80 °C in 1.5% glycerol. Transformants were carried out as previously described (33). Unmarked chromosomal mutations were created via Janus or Sweet Janus exchange (54, 55). See SI Appendix for details.

**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 (281021; 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 μg/mL α-luciferin (88294; Thermo Fisher Scientific), and 25 ng/mL BlpC. Thirty minutes later, a sample of culture supernatant was collected 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. Samples were resuspended in 1× culture volume PBS at 37 °C for 30 min. After incubation, the protein content was determined using a BCA protein assay kit (Pierce). Peptides were extracted from bacteria using a 610-nm long-pass (LP) filter (7092209; BioTek). For experiments in which peptide treatments were added to the cells, 25 ng/mL BlpC was added to the culture and was grown statically at 37 °C. These strains expressed a mismatched BlpC<sub>R</sub>–BlpH<sub>Δ</sub> pair to prevent spontaneous blp activation. At OD$_{620}$ of 0.078, cells were induced with 200 ng/mL CSP1 and 200 ng/mL BlpC<sub>Δ</sub>. 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.

**Blp-HiBiT Secretion Assays.** R6 strains expressing Blp-HiBiT along with a background control strain expressing Strept-tag II-tagged Blp were inoculated 1:150 into THY broth + 25 mM Hepes and were grown statically at 37 °C. These strains expressed a mismatched BlpC<sub>R</sub>–BlpH<sub>Δ</sub> pair to prevent spontaneous blp activation. At OD$_{620}$ of 0.078, cells were induced with 200 ng/mL CSP1 and 200 ng/mL BlpC<sub>Δ</sub>. 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.

**Activation Kinetics Assays.** For experiments simulating spontaneous activation, cells expressing mismatched CSP2–ComD1 and BlpC<sub>R</sub>–BlpH<sub>Δ</sub> pairs were used as the background control strains. The background control strain was ΔcomC<sub>R</sub> in lieu of a mismatched CSP–ComD pair. For the single-time point assay, cells were induced starting at OD$_{620}$ of 0.078 as follows: BlpC-only treatment: mock treatment followed by 25 ng/mL BlpC<sub>R</sub> 45 min later; simultaneous treatment: mock treatment followed by 10 ng/mL CSP1 and 25 μg/mL BlpC<sub>R</sub> 45 min later; staggered treatment: 10 ng/mL CSP1 followed by 25 ng/mL BlpC<sub>R</sub> 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 OD$_{620}$ 0.078 with the BlpC-only or staggered treatment as above. Supernatant and cell samples were collected just before BlpC treatment (0 min) and every 30 min thereafter for 3 h. Both supernatant and cell samples 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.

**Mouse Nasopharyngeal Colonization Assays.** For experiments simulating spontaneous activation, cells expressing mismatched CSP2–ComD1 and BlpC<sub>R</sub>–BlpH<sub>Δ</sub> pairs were used as the background control strains. The background control strain was ΔcomC<sub>R</sub> in lieu of a mismatched CSP–ComD pair. For the single-time point assay, cells were induced starting at OD$_{620}$ of 0.078 as follows: BlpC-only treatment: mock treatment followed by 25 ng/mL BlpC<sub>R</sub> 45 min later; simultaneous treatment: mock treatment followed by 10 ng/mL CSP1 and 25 μg/mL BlpC<sub>R</sub> 45 min later; staggered treatment: 10 ng/mL CSP1 followed by 25 ng/mL BlpC<sub>R</sub> 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 OD$_{620}$ 0.078 with the BlpC-only or staggered treatment as above. Supernatant and cell samples were collected just before BlpC treatment (0 min) and every 30 min thereafter for 3 h. Both supernatant and cell samples 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.

**ComA Protein Level Kinetics Assay.** For experiments simulating spontaneous activation, cells expressing mismatched CSP2–ComD1 and BlpC<sub>R</sub>–BlpH<sub>Δ</sub> pairs were used as the background control strains. The background control strain was ΔcomC<sub>R</sub> in lieu of a mismatched CSP–ComD pair. For the single-time point assay, cells were induced starting at OD$_{620}$ of 0.078 as follows: BlpC-only treatment: mock treatment followed by 25 ng/mL BlpC<sub>R</sub> 45 min later; simultaneous treatment: mock treatment followed by 10 ng/mL CSP1 and 25 μg/mL BlpC<sub>R</sub> 45 min later; staggered treatment: 10 ng/mL CSP1 followed by 25 ng/mL BlpC<sub>R</sub> 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 OD$_{620}$ 0.078 with the BlpC-only or staggered treatment as above. Supernatant and cell samples were collected just before BlpC treatment (0 min) and every 30 min thereafter for 3 h. Both supernatant and cell samples 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.

**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 OD$_{620}$ 0.078, cells were induced with 100 ng/mL CSP1 and 100 ng/mL BlpC<sub>R</sub>. Thirty minutes later, a sample of cells, equivalent to OD$_{620}$ 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 5 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.

**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 OD$_{620}$ of 0.5. Cells were gently pelleted and resuspended in sterile PBS at one-fifth the original volume. For coinfections, 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-week-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 experiment. Mice were killed by CO$_2$ inhalation after the mental period. Mice were killed with CO$_2$ inhalation after the mental period. Mice were killed with CO$_2$ inhalation after the mental period. Mice were killed with CO$_2$ inhalation after the mental period.
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 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 two groups) tests using the wilcox.test() and kruskal.test() functions in R 3.4.2.

Nucleotide Accession Numbers. The nucleotide sequences of the P_comA-Nluc and P_Rfluc reporters are deposited in GenBank with accession numbers MH304212 and MH304213, respectively.

ACKNOWLEDGMENTS. We thank Schyler Bennett for assistance in obtaining the ComA-Tsreptil protein kinetics data. This work was supported by NIH Grants R01AI101285, T32GM007863, and T32AI007528.