Characterization of a Multipeptide Lantibiotic Locus in *Streptococcus pneumoniae*

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**ABSTRACT**  
Bacterial communities are established through a combination of cooperative and antagonistic interactions between the inhabitants. Competitive interactions often involve the production of antimicrobial substances, including bacteriocins, which are small antimicrobial peptides that target other community members. Despite the nearly ubiquitous presence of bacteriocin-encoding loci, inhibitory activity has been attributed to only a small fraction of gene clusters. In this study, we characterized a novel locus (the *pld* locus) in the pathogen *Streptococcus pneumoniae* that drives the production of a bacteriocin called pneumolancidin, which has broad antimicrobial activity. The locus encodes an unusual tandem array of four inhibitory peptides, three of which are absolutely required for antibacterial activity. The three peptide sequences are similar but appear to play distinct roles in regulation and inhibition. A modification enzyme typically found in loci encoding a class of highly modified bacteriocins called lantibiotics was required for inhibitory activity. The production of pneumolancidin is controlled by a two-component regulatory system that is activated by the accumulation of modified peptides. The locus is located on a mobile element that has been found in many pneumococcal lineages, although not all elements carry the *pld* genes. Intriguingly, a minimal region containing only the genes required for pneumolancidin immunity was found in several *Streptococcus mitis* strains. The pneumolancidin-producing strain can inhibit nearly all pneumococci tested to date and provided a competitive advantage in vivo. These peptides not only represent a unique strategy for bacterial competition but also are an important resource to guide the development of new antimicrobials.

**IMPORTANCE**  
Successful colonization of a polymicrobial host surface is a prerequisite for the subsequent development of disease for many bacterial pathogens. Bacterial factors that directly inhibit the growth of neighbors may provide an advantage during colonization if the inhibition of competitors outweighs the energy for production. In this work, we found that production of a potent antimicrobial called pneumolancidin conferred a competitive advantage to the pathogen *Streptococcus pneumoniae*. *S. pneumoniae* secreting pneumolancidin inhibits a wide array of Gram-positive organisms, including all but one tested pneumococcal strain. The pneumolancidin genetic locus is of particular interest because it encodes three similar modified peptides (lantibiotics), each of which has a distinct role in the function of the locus. Lantibiotics represent a relatively untapped resource for the development of clinically useful antibiotics which are desperately needed. The broad inhibitory activity of pneumolancidin makes it an ideal candidate for further characterization and development.

*Streptococcus pneumoniae* is a common colonizer of the human nasopharynx, a highly diverse polymicrobial environment (1–3). Bacterial competition between members of the microbiome is often mediated by antimicrobial peptides called bacteriocins. In pneumococci, competition is enhanced in strains with a functional bacteriocin locus during nasal colonization of the mouse (4). Bacteriocin production in pneumococci is controlled by the *blp* locus, which has been identified in all sequenced strains (4–6). Most pneumococcal genomes also contain the *cil* locus, which encodes a highly conserved two-peptide bacteriocin, C1hAB, that has been shown to play a partial role in competence-mediated fratricide, resulting in alolysis of non-competent cells (7). Both the *blp* and *cil* loci are predicted to encode class II, or unmodified, peptide bacteriocins.

Lantibiotics are a specific class of bacteriocins that are characterized by their extensive posttranslational modifications. Secretion of functional lantibiotics has not been described for pneumococci. Precursor lantibiotic peptides are modified intracellularly by one or more enzymes encoded by genes contained within the specific *lan* locus. The most common modification involves the dehydration of serines and threonines and, through a thioster linkage, cyclization of the dehydrated amino acids to cysteines, creating either lanthionine or methyllanthionine, respectively (8–12). Following modification, the prelantibiotic is transported out of the cell by a dedicated ABC transporter that recognizes a conserved signal sequence (9). The signal peptide is cleaved either concomitantly with secretion by the transporter or by an alterna-
tive protease (13). Cleavage of the signal sequence renders the lantibiotic active and able to exert an antimicrobial effect on susceptible cells by either binding to lipid II and blocking wall synthesis and/or forming pores (14). This dual mechanism of action can be combined in a single lantibiotic peptide or split among two peptides. Modification of a two-peptide lantibiotic typically requires two LanM enzymes, each one specific for one peptide (15–19). Self-immunity to lantibiotics can occur through expression of a lipoprotein that is thought to competitively bind to the lantibiotic or through production of an efflux ABC transporter. Some loci encode both immunity strategies; in these cases, partial immunity defects are noted when one gene is deleted. In most cases, the lantibiotics also function as signaling peptides and interact with a cognate histidine kinase, LanK. Binding of the lantibiotic to the histidine kinase results in upregulation of the locus via a two-component regulatory cascade (20–22). Some potential lantibiotic-encoding clusters have been observed in pneumococci; however, no activity has been attributed to these loci. Some members of the pandemic pneumococcal strain Spain23F of the sequence type 81 lineage, which at one point was found to be responsible for 40% of pneumococcal disease in America, carry a locus that appears to have all elements required for lantibiotic expression, but no inhibitory activity has been detected in strains carrying this locus (23, 24). In this study, we report the identification of the first functional lantibiotic locus in pneumococcus. The activity derived from this locus inhibits a significant number of pneumococci and other Gram-positive pathogens. It requires the concerted action of three similar but distinct structural peptides, all of which appear to be modified by the same LanM enzyme. To our knowledge, the requirement for three homologous peptides for full inhibitory activity of a lantibiotic has not been described previously and represents a unique approach to bacterial antago-

**RESULTS**

**Identification of a functional lantibiotic locus in P174.** During previous work on the blp locus, we noted that an invasive serotype 23F isolate derived from a patient in South Africa had significant antibacterial activity against all but one pneumococcal isolate despite containing an inactivating mutation in the blp bacteriocin locus (6). Deletion of the upstream regulator of the cib or blp bacteriocins (comDE or blpC, respectively) did not abrogate inhibitory activity, suggesting that the inhibitory activity was derived from a previously undescribed locus (Fig. 1A). Transposon mutagenesis localized the activity to a locus predicted to encode a series of lantibiotic peptides. A schematic of the locus is shown in Fig. 1B. We chose to designate this lantibiotic locus the pneumocandin locus, and the corresponding genes are designated pld per the standards of nomenclature for lantibiotics. A nucleotide BLASTn search of the pld locus found in P174 demonstrated that a homologous gene cluster is found in a pneumococcal strain designated PN1, isolated from Papua New Guinea in the 1970s (25). The locus encodes two ABC transporters: the 5′-most ABC transporter consists of two open reading frames (ORFs), pldFE, which encode an ATP binding protein and a permease, respectively. These genes share 99% amino acid identity with an ABC transporter identified in PN1 and 97% similarity with a transporter encoded in the Streptococcus mitis B6 genome. Based on conserved domains, PldF serves as the nucleotide-binding domain of an immunity ABC transporter. Although it does not contain a typical E loop, which is conserved in all LanF proteins of immunity ABC transporters, we chose to name the genes PldFE based on the location of these genes in the locus, homology, and likely immunity function (26). The proteins also share homology to bacitracin ABC transporters found in a number of streptococcal species that provide resistance to bacitracin and can provide cross-resistance to heterologous lantibiotics such as nisin and gallidermin (27–29). A second ABC transporter, pldIT, was identified in the 3′ region of the locus. The second ABC transporter shared 31% identity at the amino acid level to the mersacidin ABC transporter, MrST, in Bacillus sp. and contains conserved domains involved in lantibiotic export and cleavage. The only modification enzyme found in the locus is encoded by the pldM gene. PldM is a member of the LanM family of modification enzymes. The pld locus contains coding sequences for a two-component regulatory system, pldKR, which is homologous to genes found in the S. mitis B6 strain. Homologues of S. mitis B6 pldKR are adjacent to the immunity pldFE homologues, but the surrounding region lacks the remainder of the pneumococcal pld locus (Fig. 1B). Flanking either end of the pld locus in P174 are regions of significant homology to the integrative and conjugative element (ICE) found in the S. pneumoniae strain ATCC 700669. This strain has a lantibiotic locus in the precise location of the pld locus, although the genes in the two loci share no homology (Fig. 1B).

A region encoding the putative structural proteins was identified upstream of pldFE. A tandem array of four possible ORFs were identified (pldA1 to pldA4), each encoding a peptide with a signal sequence followed by a sequence with a large number of serines, threonines, and cysteines typically found in lantibiotic peptides. The predicted Pld peptides do not share homology to known lantibiotic peptides. The four ORFs are homologous to each other, and the N-terminal leader sequence of each peptide is followed by a double-glycine, GA, GS, or double-alanine motif, suggesting the point of peptidase cleavage. The active peptides of PldA1 and PldA2 would be predicted to differ by only two amino acids (Fig. 1C).

**Identification of the genes required for lantibiotic activity and immunity.** To determine the role of each gene in the pld locus of P174, individual deletions were constructed and assayed for inhibition and immunity to the P174 secreted lantibiotic. Deletions in pldM, pldIT, and pldK, encoding the modification enzyme, lantibiotic transporter, and histidine kinase, respectively, all resulted in loss of inhibition (Fig. 1D). When tested for immunity, the pldM and pldIT deletion strains retained immunity to P174, while the pldK mutant lost immunity, confirming the requirement for the regulatory proteins for activation of lantibiotic immunity. To determine the role of pldFE, an in-frame, unmarked mutation of both genes was introduced into P174. This mutant, P174ΔpldFE, lacked inhibition in overlay assays and was sensitive to wild-type (WT) secreted lantibiotic peptide, suggesting that this ABC transporter is required to initiate immunity or is directly involved in protection from its own lantibiotic (Fig. 1D). As expected, Pld peptides are required for inhibition but not for immunity, since a strain carrying a deletion of all 4 peptides had loss of inhibition but retained immunity (Fig. 1D). P174 displays an interesting self-inhibitory phenotype in which the formation of a halo of decreased density of growth can be seen when P174 is plated against itself, suggesting that there is a lapse in the development of immunity in this assay which allows some degree of self-inhibition in the presence of functional lantibiotic peptides. This
FIG 1  Inhibitory activity and genetic structure of the pld lantibiotic locus. (A) Overlay assays were performed using either P174 wild-type or deletion mutants of either upstream regulators of the blp or cib locus or a deletion of the pldM gene that was identified in the transposon mutagenesis screen. A TIGR4 strain was used as the overlay strain. (B) The pld locus of P174 and the corresponding loci of S. mitis B6 and S. pneumoniae ATCC 700669 are shown. The percent amino acid identity between the predicted proteins found in S. mitis B6 and P174 homologues is noted above the B6 ORFs. Presumed functional designations are indicated by the color of the ORF. Regions of DNA homology between sequences are in grey. (C) Amino acid alignment demonstrating the homology between predicted structural proteins PldA1 to 4. The proposed signal peptide sequence cleavage point is shown with an arrow. Shared amino acid residues in the functional peptide are highlighted in yellow. Amino acid residues in red are sites of possible modification catalyzed by PldM. (D) Deletions of various genes in the pld locus of P174 were constructed and assayed for inhibitory and signaling activity as well as immunity to WT lantibiotic. Inhibition and signal secretion were tested by stabbing the strain of interest and overlaying with the sensitive indicator strain TIGR4 or the reporter strain P174pldM-lacZ, respectively. The chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was included in the overlay mixture for signaling assay. Immunity was determined by stabbing P174 and overlaying with each of the deletion mutants.
phenomenon is also seen when P174 is grown as a single-strain lawn, where it is noted to form occasional plaque-like structures that are characterized by areas of partial clearing. Although the mechanism for the development of plaques is unknown, we hypothesize that these are areas in which the pld locus is spontaneously activated in a portion of the population, resulting in the inhibition of any neighboring cells that are delayed in their production of immunity.

**Modified lantibiotic peptides are required for activation of the locus.** In addition to their inhibitory activity, lantibiotic peptides also often serve as inducers by interaction with the cognate histidine kinase, leading to upregulation of the entire *lan* locus. Upregulation of the locus is dependent upon the concentration of the lantibiotic peptides. We found that, similar to other lantibiotic loci, the Pld peptides are required for activation of transcription of the *pld* locus. We compared the activation of a *pld* reporter construct to the signal secreted by P174 in the P174 and P174Δ*pldA1-4* backgrounds (see Fig. S1 in the supplemental material). The reporter strain in the wild-type background produced a large zone of signaling when plated over a peptide-secreting strain, while only a small zone was seen when the reporter in the P174Δ*pldA1-4* background was tested. Because the stabbed strain secretes the same amount of peptide in each overlay, this result suggests that propagation of the signal within the overlay results in a larger activation zone and further suggests that this propagation requires the lantibiotic peptides. Similarly, P174Δ*pldA1-4* does not secrete a signal that can stimulate the reporter strain P174Δ*pldA1-4-lacZ*, further supporting the role of Pld peptides as inducers of the *pld* locus (Fig. 1D). To determine if the Pld peptides need to be modified and secreted to act as inducers, P174Δ*pldM* and P174Δ*pldT* were stabbed and overlaid with the WT reporter (Fig. 1D). Stimulation of the *pld* locus was not seen in either overlay confirming that the induction signal of the locus is one or more extracellular, modified peptides. As expected, the strain P174Δ*pldK*, which lacks the histidine kinase regulator, was not able to secrete a signal, consistent with a loss of both inhibition and immunity in this strain (Fig. 1D). Surprisingly, the strain carrying the unmarked *pldFE* mutation, missing the genes that are proposed to play a role in immunity, also did not secrete a signal despite retaining the genes encoding the peptides and the regulatory, modification, and secretion proteins (Fig. 1D). This may be due to development of a compensatory mutation that prevents the activation of the locus in the setting of reduced immunity or because the immunity transporter plays an undefined role in regulation. We attempted to evaluate the kinetics of activation of the locus in broth culture using the *pldM-lacZ* reporter strain, but this strain failed to show any induction of the locus during growth in broth, presumably because the concentration of peptides never reaches the level required to support activation of the locus under these conditions (data not shown).

Given the unusual presence of four homologous but not identical putative functional peptides, we wanted to determine the function of each peptide individually in inhibition and signaling. Individual, in-frame, unmarked peptide deletions were tested for inhibition, immunity, and evidence of signal secretion (Fig. 2). P174 lacking *pldA1, pldA2,* or *pldA3* had identical phenotypes in that all three deletion strains had a loss of inhibitory activity and signal secretion (Fig. 2A), suggesting that all three peptides are required for activation of the locus. These strains had nearly wild-type levels of immunity to P174, with only a small zone of clearing that most likely represents some degree of delayed production of immunity. P174Δ*pldA4* was fully inhibitory and secreted a signal that was indistinguishable from that of P174, suggesting that this gene is dispensable for inhibition (Fig. 2A).

**A hyperinducible variant of P174 has a decreased threshold for locus activation.** Surprisingly, we found that certain isolates derived from allelic exchange of the counterselectable Janus cassette with a cloned copy of the peptide region that was being used for constructing peptide deletions had an altered lantibiotic production phenotype which we have designated P174act. When the *pldM* reporter plasmid was integrated into this strain, it exhibited evidence of greater *pldM* transcription in overlay assays when stimulated with P174 and produced a large number of plaque-like formations in the overlay lawn compared with the reporter in the wild-type background (Fig. 3A). When P174act was stabbed into plates, there was no significant difference in inhibition or signaling compared with the wild-type strain (Fig. 2A and B). To determine the mutation responsible for the hyperinducible phenotype of P174act, we sequenced the entire fragment that was used for allelic exchange. Sequencing revealed a single base pair mutation presumably attributable to a PCR error that was located in the intergenic region between *pldA4* and *pldFE* (Fig. 3B). The mutation was found in the cloned fragment used for transformation and, by means of linkage analysis on transformants that resulted from allelic exchange with the plasmid carrying the mutation, was found to be absolutely linked to the hyperinducible phenotype (data not shown). The mutation resulted in a change of a thymidine to a cytosine at the first T of the proposed TATA box preceding *pldFE* (Fig. 3B). Because this mutation was in an intergenic region downstream of the peptide ORFs, we reasoned that this single base pair change was affecting transcription of key genes in the locus by either disrupting a transcriptional attenuator (allowing increased read-through) or affecting a promoter element (in particular by altering RNA polymerase binding at the −10 region), either of which might result in changes in downstream gene expression. DNA analysis failed to demonstrate any sequences likely to form a stem-loop typical of a transcriptional attenuator. To further examine this, a 4-bp deletion that included the site of the mutation was constructed in P174, and both the inhibition and immunity phenotypes of the resulting strain were assessed (Fig. 3C). The strain carrying this deletion lost both inhibition and immunity, making the presence of a transcriptional terminator at this site unlikely and suggesting that the mutation that results in the P174act phenotype affected the activity of a promoter element preceding *pldFE*.

**Genes involved in immunity, regulation, and peptide modification are not part of an operon.** We attempted to determine the transcriptional units of the locus and to compare RNA levels in wild-type and hyperinducible backgrounds using both reverse transcription PCR (RT-PCR) and Northern blotting; however, transcripts could not be detected in broth grown organisms, and RNA isolated from plate grown organisms was too degraded for use in RT experiments (data not shown). As an alternative approach to determining minimal transcriptional units and relative activity of various genes in the *pld* locus, we constructed three additional reporters in either the P174 or P174act background through plasmid integration in which the reporter gene lacZ was fused to the region upstream of either *pldK, pldFE*, or *pldA1*. All constructed reporter strains were tested for inhibition and signal secretion. All of the fusion constructs retained the wild-type inhibitory phenotype (see Fig. S2A in the supplemental material), demonstrating that insertion of the reporter plasmid at either of these locations did not disrupt the function of the locus. Like
The pldM reporter, the pldFE and pldA1 reporters demonstrated signal detection when overlaid over P174, suggesting that both promoters are autoregulated (see Fig. S2A in the supplemental material). Although the pldK reporter can secrete wild-type levels of peptide by signal secretion assay and inhibits the TIGR4 strain in overlay assays, this reporter in either the P174 or P174act background was not activated by P174 when used in an overlay assays (see Fig. S2B in the supplemental material). This indicates that the genes involved in regulation are not inducible with exogenous peptides and that the pldFE, pldKR, and pldM genes are all controlled by separate promoters, since the peptide-inducible promoters of pldFE and pldM flank the noninduced pldFE genes (Fig. 1B). The lack of appreciable lacZ expression in the pldKR reporter suggests that the regulatory genes are produced at very low levels and are not part of the positive feedback regulation in response to secreted peptides. In addition, the lack of appreciable activity of this reporter in the P174act background suggests that the alteration of the promoter in front of pldF is responsible for the hyperinducible phenotype does not appreciably alter expression of the downstream regulatory genes.

The hyperinducible mutation functions only when directly upstream of pldFE. To better determine the role of the mutation in the hyperinducible phenotype, we created two additional reporter plasmid integrations into the promoter of pldF in which the P174act mutation was placed upstream of lacZ only or upstream of pldF only (Fig. 4A). The activity of these strains in reporter overlay assays was compared with that of the wild-type reporter strain, P174pldF-lacZ, by using cell-free supernatant preparations of cultures of P174act to activate the locus (Fig. 4A). Only the reporter that had the mutation directly upstream of the pldF gene had the hyperinducible (multiple-plaque-forming) phenotype, as seen with the multiple spots of lacZ expression (Fig. 4A). When the relative activity of the wild-type reporter strain was compared with that of the strain containing the mutation only in front of lacZ, it appeared that the reporter with the mutation had less induction, suggesting that the mutation may decrease the activity of the pldF promoter (Fig. 4A).

To better understand the hyperinducible phenotype, we used cell-free supernatant preparations of cultures of P174act as an inducer to measure β-galactosidase activity (Fig. 4B). Twofold dilutions of the cell free supernatant were used to induce the wild-type P174pldF-lacZ reporter strain and the same reporter with the P174act mutation in front of lacZ (P174PactpldF-lacZ) or in front of pldF (P174act P174pldF-lacZ). The P174act P174pldF-lacZ strain showed a clear dose response to increasing concentrations of supernatant (Fig. 4B). P174pldF-lacZ and P174P174actpldF-lacZ

**FIG 2** Deletion of the lantibiotic peptides in either P174 or the hyperinducible P174act background. In-frame, unmarked peptide deletion mutants were constructed and assayed for inhibitory and signaling activity as well as immunity to WT lantibiotic. Evidence of inhibition and signal secretion was tested for by stabbing the strain of interest and overlaying with the sensitive indicator strain TIGR4 or the reporter strain P174pldM-lacZ, respectively. Immunity was determined by stabbing P174 and overlaying with each of the deletion mutants. Peptide deletion mutants were made in either the P174 background (A) or the P174act background (B). (C) Phenotypic complementation was assayed using P174actΔpldA3 and P174actΔpldA1. Both strains were stabbed progressively more closely to each other and the plate subsequently overlaid with TIGR4. Pictures were taken at a higher magnification (×2) than other overlays to better appreciate the inhibitory effect.
strains showed no appreciable activation of the locus (Fig. 4B). These findings suggest that the P174act mutation results in a decreased threshold for signaling, resulting in enhanced transcription of the locus even at low peptide concentrations, and confirm our observations on plates that the mutation must lie directly 5’ to the start codon of pldF to demonstrate the hyperinducible phenotype.

Three distinct phenotypes for peptides found in the P174act background. Given the decreased threshold for signaling in the hyperinducible background, the three unmarked peptide mutations that lacked inhibition in overlay assays in the wild-type background were moved into this background and assessed for secretion of signaling and inhibitory peptides (Fig. 2B). In this background, the three deletion strains had three distinct phenotypes in overlay assays. The strain with the pldA1 deletion in the P174act background retained signal secretion but lost inhibition. The strain carrying the pldA2 deletion was indistinguishable from the P174 wild-type strain. The P174act ΔpldA3 deletion strain had the most dramatic phenotype and lost all signal secretion and inhibition. The inhibitory defect in the P174actΔpldA1 strain could be phenotypically complemented by placing an adjacent stab of the P174act ΔpldA3 strain, confirming the overall integrity of the locus in each deletion mutant (Fig. 2C). It is important to note here that the active peptide sequences of PldA1 and PldA2 differ by only two conserved amino acids. The difference in the phenotypes resulting from the pldA1 and pldA2 knockouts in the P174 and P174act backgrounds suggests that both are required for signaling when thresholds are at wild-type levels but that PldA2 plays less of a role in inhibition than PldA1 when the signaling threshold is low.

Broad spectrum of inhibitory activity derived from the pld locus. P174 antibiotic-derived inhibitory activity was examined using a previously described collection of 50 pneumococcal isolates from South Africa and the alternative antibiotic-expressing strain ATCC 700669 (6). Additionally, a selection of nonpneumococcal strains was tested for sensitivity to P174. Inhibitory activity was found against all but one of the pneumococcal strains as well as isolates of Listeria monocytogenes, Streptococcus pyogenes, Streptococcus agalactiae, and Lactococcus lactis (Table 1). Further information on the pneumococcal and streptococcal strains tested can be found in Table S1 in the supplemental material. Strains that were sensitive to P174 were also tested against P174ΔpldK to confirm that activity was attributed to the pld locus. A single strain, P130, was the only pneumococcal isolate that had immunity when tested against P174 in the overlay assay.

Pneumolancidin-producing strains have an advantage in invasion of colonization in vivo. To determine if pneumolancidin production provides a competitive advantage during colonization, we compared the ability of a producing strain to invade a colonized mucosal surface to an otherwise isogenic nonproducer. Using a staggered inoculation model, colonization was first established with the sensitive P174ΔpldK strain for 3 days followed by...
introduction of either the producer strain P174st\textsuperscript{th} or the nonproducer strain P174Δ\textit{pld}A1–4. P174st\textsuperscript{th} was able to invade the established community of P174Δ\textit{pldK} in the nasopharynx better than P174Δ\textit{pldA1}–4 (Fig. 5). Levels of P174Δ\textit{pldK} were lower in the mice inoculated with the wild-type strain, suggesting that colonization results in the eviction of some resident organisms; however, this difference was not statistically significant. To account for difference in overall colonization density, we assessed the ratio of invading to resident (I/R) CFU per mouse under each condition. While only 1 of 20 of the P174Δ\textit{pldA1}–4 inoculated mice had an I/R greater than 1, 7 of 19 mice inoculated with the wild-type strain had a predominance of the invading strain (see Fig. S3 in the supplemental material).

**Identification and characterization of additional strains with a \textit{pld}-like locus.** P130, which has immunity to pneumolancidin, was shown to encode a \textit{pld} locus by PCR and sequencing. The locus in P130 has the same gene content as P174, but overlay assays failed to demonstrate any evidence of lantibiotic-mediated inhibition. The \textit{pld} locus of P130 is nearly identical to the locus in the previously identified PN1 strain and the fully sequenced BH418 strain and does not contain any large deletions or frame-shift mutations that might account for the lack of activity. Multi-locus sequence typing (MLST) analysis of the \textit{pld}-positive strains P174, P130, BH418, and PN1 was determined to assess their genetic similarity (see Table S2 in the supplemental material). P130 and BH418 differed by only a single MLST allele, while P174 did not share any alleles with these strains. It appears that acquisition of the \textit{pld} locus is a unique property of P174, because the locus is not found in three publicly available, fully sequenced strains (GA05245, GA17227, and GA41301) that share the same founder ST as P174 (ST242). In fact, these strains lack the associated ICE altogether, suggesting that P174 may have recently acquired the element.

To determine if more pneumococcal strains could be identified that contain the locus, we screened a collection of over 400 clinical isolates for the presence of the \textit{pldM} gene using PCR. Four strains were identified and were categorized by serotyping and MLST analysis (see Table S2 in the supplemental material). One of the newly identified clinical isolates was found to be from the same clonal complex as BH418 and P130, but this isolate was neither active nor immune to P174. Three nontypeable (NT) isolates with the locus were identified as belonging to the 448 sequence type. A genome-sequenced isolate from the same ST, MNZ14, was found to have a \textit{pld} locus that encodes all of the elements found in P174 with the exception of an apparent disruption of the gene encoding the peptide transporter, \textit{pldT} (30). The three \textit{pldM}-positive NT isolates from our collection lacked both inhibition and immunity to P174-mediated inhibition (data not shown).

**P130 contains an inactive locus due to a mutation in \textit{pldM}**. Using allelic replacement, lysates from P130 were used to transform P174 containing an exchangeable cassette replacing the entire \textit{pld} locus. All of the resulting transformants lacked inhibitory activity, suggesting that a mutation in the \textit{pld} locus was responsible for the lack of P130-derived inhibitory activity. Amino acid sequence alignment of the \textit{PldM} gene products from both strains demonstrated that the P130 strain contained a C867Y mutation in one of the critical residues required for zinc coordination (see Fig. S4 in the supplemental material). It was shown previously that mutating any one of the conserved cysteine residues resulted in a loss of cyclization activity in the NisC enzyme (31). To confirm that the mutation alone would result in a strain with the P130 phenotype, the region of the \textit{pldM} gene in P174 containing the site of the mutation was exchanged with the P130 allele. The resulting strain, P174 \textit{pldM} C869Y, was unable to inhibit (see Fig. S4 in the supplemental material). Replacement of the P174 locus with the P130 \textit{pld} versions of \textit{pldA1}–4, \textit{pldF}, and \textit{pldK} resulted in a fully inhibitory strain (data not shown), suggesting that the \textit{pldM} mutation alone was responsible for the P130 phenotype. Reciprocal gain-of-function experiments in P130 could not be performed because P130 was not transformable.

**DISCUSSION**

This work describes the first functional lantibiotic locus found in pneumococci. The locus contains the genes required for inhibitory activity against other pneumococci and closely related Gram-positive organisms. It is found as cargo of an ICE and has been identified in unrelated isolates, suggesting that the locus is moving through the pneumococcal population via either conjugation or horizontal gene transfer. Not only has this locus disseminated in distinct pneumococcal lineages, but the two-component system and \textit{pld}FE genes of the locus also show significant homology to a region in the \textit{S. mitis} B6

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### TABLE 1 Spectrum of inhibitory activity for P174

| Strain                              | Inhibition by P174<sup>a</sup> |
|-------------------------------------|---------------------------------|
| pld-negative \textit{Streptococcus pneumoniae} | + (50/50)                       |
| pld-positive \textit{Streptococcus pneumoniae} | + (4/5)                         |
| \textit{Streptococcus agalactiae} clinical isolates and lab strains | + (6/15)                        |
| \textit{Streptococcus pyogenes} clinical isolates and lab strains | + (12/12)                       |
| \textit{Listeria monocytogenes} 10403S | +                              |
| \textit{Lactococcus lactis} ATCC 14365 | +                              |
| \textit{Staphylococcus mitis} ATCC 49456 | –                              |
| \textit{Staphylococcus aureus} clinical isolate | –                              |
| \textit{Enterococcus faecalis} ATCC 29212 | –                              |
| Vancomycin-resistant \textit{Enterococcus faecalis} ATCC 51299 | –                              |

<sup>a</sup> +, zone of clearance was detected; –, no inhibitory activity was detected. Numbers in parentheses are numbers of strains killed/total number of strains tested. See Table S1 in the supplemental material for details.

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**FIG 5 Competitive advantage of the \textit{pld} locus in vivo**. Mice were either colonized with P174\textit{pldK} or treated with sterile PBS at day 0. At day 3, mice were challenged intranasally with either P174 or P174\textit{pldA1}–4. Nasal washes were obtained 3 days postinoculation, and the number of CFU was calculated by differential plating. Medians and interquartile ranges are shown. The dotted line indicates the limit of detection (LOD). Statistical analysis was performed using an unpaired Mann-Whitney test. *P < 0.05; **P < 0.01.
Genetic exchange between *S. pneumoniae* and *S. mitis* is common and contributes to the genomic diversity of the species (32). The region of homology in the *S. mitis* B6 genome is sufficient for immunity to the Pld (non italicized and capital P) peptides via the PldKR-mediated upregulation of the PldFE ABC transporter. *S. mitis* ATCC 49456 was found to contain the *pldK* gene by PCR (data not shown). This isolate was immune to inhibition by P174, which may be due to the presence of the four *pldA* genes, although without deletion analysis, the requirement for the *pld* homologues for immunity cannot be verified.

All pneumococcal strains that we have identified apart from P174 that contain the *pld* locus lack inhibitory activity. P130 has *pld*-mediated immunity that seems to derive from the *pld* locus. The mutation responsible for lack of inhibition was localized to an SNP within the *pldM* gene that results in the mutation of a critical residue involved in zinc coordination which is required for enzymatic activity of the modification enzyme. This mutation is found in the sequenced constructs in plate assays in an attempt to determine the likely transcriptional units. Unlike many other lantibiotic loci, in which the entire cluster of *lan* genes is located on a single transcript, we have shown that the genes clusters downstream of *pldA1-4* are controlled by separate promoters. The separate control of each of the gene clusters is further supported by the fact that plasmid integrations into the presumed promoters of *pldA1, pldFE, pldKR*, and *pldM* did not disrupt inhibitory activity. In fact, unlike the *pldFE* and *pldM* genes, the intervening *pldKR* genes are not up-regulated in response to P174 peptides at all. The lack of peptide responsiveness of these genes may serve to damp the positive feedback loop that occurs during peptide stimulation, by limiting the amount of regulatory proteins on the cell surface.

We describe the identification of a serendipitously isolated hyper-inducible strain. Based on the location of the mutation in the presumed TATA box of the promoter preceding *pldFE* and our results with the *pldFE* reporter strains, the most likely explanation of the phenotype is that the mutation affects expression of only *pldFE*, the proposed immunity transporter. Based on preliminary data, the variant phenotype of P174act seems to be attributable to decreased levels of *PldFE*, leading to increased sensitivity of the cell to exogenous peptides. The *pldFEKR* cluster falls into the family of Bce regulatory/transporter proteins typified by the bacitracin resistance gene cluster BceRS-BceAB. In these cases, the transporter appears have a dual role, functioning as a resistance protein by pumping out antibiotics and as a regulator interacting with the two-component system to upregulate gene expression (36–38). One possible interpretation of our findings is that altering the ratio of immunity transporter to regulatory gene products results in increased sensitivity to signaling peptide. Alternatively, it is possible that PldFE binds the Pld peptides with different affinity. If this is true, then decreased production of the immunity transporter complex may change effective concentrations of the peptides.

We have not yet tested whether the *pld* locus in P174 provides resistance against other cell wall-targeting antimicrobials. Since bacitracin transporters often mediate resistance to other antibiotics, this could explain the maintenance of the *pld* locus in strains that do not produce functional lantibiotics (27–29).

Wild-type P174 produces appreciable *pld*-mediated inhibition only in plate-grown organisms. This property has hampered our attempts at large-scale purification. We have noted, however, that by a halo of decreased growth around the stab. This phenotype is seen only when strains that can produce inhibition themselves are grown in the overlay. This may be indicative of a lapse in developing immunity in response to exogenous peptides when broth-grown organisms are applied over stabs in which the locus has already been upregulated. When the overlay strain is placed over an actively secreting stab, lantibiotic-mediated signaling derived from the stabbed strain activates some cells in the overlay and kills others. The activated overlay strains in turn kill or activate surrounding strains, resulting in a wave of combined signaling and inhibition. The fact that the wave of signaling and inhibition requires signal propagation within the overlay strain is supported by the activity of the three reporter strains (*pldA1-4* knockout, P174, and P174act) with an increasing ability to amplify the response to secreted signals and correlated increased zones of signaling when placed in overlay over P174.

Although our inability to examine *pld*-specific transcripts directly has hampered characterization of transcriptional control of the locus, we have described the activity of a series of reporter constructs in plate assays in an attempt to determine the likely transcriptional units. Unlike many other lantibiotic loci, in which the entire cluster of *lan* genes is located on a single transcript, we have shown that the genes clusters downstream of *pldA1-4* are controlled by separate promoters. The separate control of each of the gene clusters is further supported by the fact that plasmid integrations into the presumed promoters of *pldA1, pldFE, pldKR*, and *pldM* did not disrupt inhibitory activity. In fact, unlike the *pldFE* and *pldM* genes, the intervening *pldKR* genes are not up-regulated in response to P174 peptides at all. The lack of peptide responsiveness of these genes may serve to damp the positive feedback loop that occurs during peptide stimulation, by limiting the amount of regulatory proteins on the cell surface.
the P174act strain can produce appreciable quantities of peptides during growth in broth, presumably due to the decreased threshold for signaling. This mutant may serve as a useful tool for large-scale purification of this potent lantibiotic that inhibits nearly all pneumococcal strains tested. In addition the manipulation of immunity transporter quantities for increased yield of inhibitory peptides may be translatable for use in other lan systems with a bacitracin-like immunity transporter.

We have shown that the P174 pld locus has the interesting property of requiring the presence of three very similar peptides for self-signaling to occur in a wild-type background. This may be a result of a gene dosage effect, such that three copies of nearly identical genes are required to reach the threshold for activation; this assumes that all three peptides have the same function. Alternatively, the three peptides may form a complex and each peptide of the complex is uniquely required for signaling. The potential for separate roles of the individual peptides could not be assessed in the P174 background because the block in signaling does not allow us to assess differences in inhibition. In contrast to the individual peptide knockouts in the wild-type background, the individual knockouts in the P174act background each had a distinct phenotype, most likely due to the lower threshold for activation noted in this strain. The phenotypes of individual peptide knockouts noted in this background confirm the absolute requirement for PldA3 for signaling. Of note, PldA3 is the most divergent of the three peptides, with two more cysteine amino acids in the active domain than in PldA1 and PldA2, and would be predicted to have a very different structure. In addition, the single peptide deletions in the P174act background demonstrated that either PldA1 or PldA2 is sufficient to promote signal secretion in combination with PldA3 but that PldA1 is more important for inhibition than PldA2. This may be either because more PldA1 is made than PldA2 or because the two peptides differ in their inhibitory activity on the target cell surface. We are currently working to isolate each of the three peptides to better understand their contribution to the remarkable antipneumococcal activity of pneumolancidin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All pneumococcal strains used are described in Table S3 in the supplemental material, and all primers used are described in Table S4 in the supplemental material. Growth conditions and construction of strains are described in detail in Text S1 in the supplemental material. All pneumococcal strains were plated on either 5% sheep’s blood agar (SBA) or tryptic soy agar (TSA) plates with 0.5% catalase (Worthington, Lakewood, NJ) (4,741 U) and incubated at 37°C with 5% CO₂. For growth in liquid culture, all pneumococcal strains were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY). Escherichia coli strains were grown in Luria-Bertani (LB) broth or LB agar.

Miller assays. Reporter strains containing pldF-lacZ in either P174 or P174act background were grown to an optical density (OD) of 0.2, and 40 μl was added to a microtiter plate containing catalase and 40 μl of supernatants either from P174ΔpldK or from P174act and then diluted in 2-fold serial dilutions in THY. Plates were incubated for 1.5 h, and activity (in Miller units) was determined as previously described (39, 40).

Staggered mouse colonization assays. All mice were purchased from Jackson Laboratories and were housed in accordance with Institutional Animal Care and Use Committee protocols. This protocol was described previously with some modifications (4). Ten 6- to 7-week-old BALB/c mice were inoculated intranasally with 10 μl containing 4 × 10⁷ CFU of animal-passaged strain P174ΔpldK resuspended in phosphate-buffered saline (PBS). At 3 days postinoculation, approximately 10⁷ CFU of either animal-passaged P174αt or P174ΔpldA1-4 stα was intranasally inoculated into mice that had previously been either colonized with P174ΔpldK or treated with PBS. Five control mice received PBS followed by the two invading strains. After 3 days, mice were sacrificed, and nasal washes were collected by tracheal lavage using 200 μl of PBS. The lavage fluid was diluted in PBS and plated on TSA with selection. All plates contained neomycin to prevent growth of the natural flora. Strains were differentiated on streptomycin- or spectinomycin-containing medium. The experiment was repeated once, and the cumulative data from both experiments are shown. One mouse in the producer arm died during the course of the experiment; colonization for this mouse was not determined.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01656-15/-/DCSupplemental.

ACKNOWLEDGMENTS

We acknowledge Keith Klugman for graciously providing the South African clinical isolate collection and Michael Bachman, Michael Watson, Betsy Foxman, and Mary O’Riordan for providing nonpneumococcal species for sensitivity testing. We also thank Michael Watson and Adam Ratner for their critical reading of the manuscript and helpful suggestions.

This work was supported by grants from the NIH (R01AI078538 to S.D.), the Amendt-Heller Award (to S.D.), and the Cellular Biotechnology Training Program (T32GM008353 to N.M.). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

FUNDING INFORMATION

Amendt-Heller Award provided funding to Suzanne Dawid. HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Suzanne Dawid under grant number R01AI078538. HHS | NIH | National Institute of General Medical Sciences (NIGMS) provided funding to Natalie Maricic under grant number T32GM008353. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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