Title

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Journal

Molecular Microbiology, 97(2)

ISSN

0950-382X

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Publication Date

2015-07-01

DOI

10.1111/mmi.13029

A new quorum sensing system (TprA/PhrA) for *Streptococcus pneumoniae* D39 that regulates a lantibiotic biosynthesis gene cluster

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Running Title: Phr-peptide signaling by *S. pneumoniae*
Keywords: Quorum sensing, Phr peptides, Lantibiotics, *Pneumococcus*

ABSTRACT

The Phr peptides of *Bacillus* species mediate quorum sensing, but their identification and function in other species of bacteria has not been determined. We have identified a Phr peptide quorum sensing system (TprA/PhrA) that controls the expression of a lantibiotic gene cluster in the Gram-positive human pathogen, *Streptococcus pneumoniae*. Lantibiotics are highly modified peptides that are part of the bacteriocin family of antimicrobial peptides. We have characterized the basic mechanism for a Phr peptide signaling system in *S. pneumoniae* and found that it induces expression of the lantibiotic genes when pneumococcal cells are at high density in the presence of galactose, a main sugar of the human nasopharynx, a highly competitive microbial environment. Activity of the Phr peptide system is not seen when pneumococcal cells are grown with glucose, the preferred carbon source and the most prevalent sugar encountered by *S. pneumoniae* during invasive disease. Thus, the lantibiotic genes are expressed under the control of both cell density signals via the Phr peptide system and nutritional signals from the carbon source present, suggesting that quorum sensing and the lantibiotic machinery may help pneumococcal cells compete for space and resources during colonization of the nasopharynx.

INTRODUCTION

Quorum sensing is carried out by small secreted molecules that diffuse through the environment, eliciting responses in proximal cells when the signaling molecules reach a critical threshold concentration (Keller & Surette, 2006, Waters & Bassler, 2005). In Gram-positive bacteria, quorum sensing often relies on recognition of small secreted peptides that signal through two-component regulatory systems (Rutherford & Bassler, 2012, Pottathil & Lazazzera, 2003, Lyon & Novick, 2004). An alternative group of quorum sensing peptides are those that signal to cells by interacting with an oligopeptide transporter and then a cytoplasmic receptor protein, which is either a member of the RNPP or the Rgg family of proteins (Rocha-Estrada *et al.*, 2010, Dunny, 2007, Jimenez & Federle, 2014, Fleuchot *et al.*, 2011, Pottathil & Lazazzera, 2003). The Phr family of signaling peptides of Bacilli belongs to the latter group, and these peptides regulate a number of important processes such as sporulation, development of genetic competence, virulence gene expression, biofilm formation, and excision and transfer of mobile genetic elements (Rocha-Estrada *et al.*, 2010, Slamti & Lereclus, 2002, Lazazzera *et al.*, 1997, Auchtung *et al.*, 2005, Bongiorni *et al.*, 2005, Perego & Hoch, 1996). However, the role that the Phr signaling peptides play outside of the Gram-positive endospore forming bacterial species has not yet been established.

The Phr family of quorum sensing peptide of Bacilli are secreted through the Sec-dependent export pathway and processed from a small precursor protein to a mature, 5-7 residue, polar peptide (Mirouze *et al.*, 2011, Aceves-Diez *et al.*, 2007, Ogura *et al.*, 2003, Perego, 1997, Solomon *et al.*, 1996, Bongiorni *et al.*, 2006). These peptides are encoded by genes typically found immediately adjacent to genes encoding their cytoplasmic target proteins, forming a quorum-sensing cassette (Pottathil & Lazazzera, 2003, Rocha-Estrada *et al.*, 2010). A classic example of the Phr peptides is **c**ompetence and **s**porulation **f**actor (CSF) of *Bacillus subtilis* (Pottathil & Lazazzera, 2003). The precursor protein for CSF, PhrC, has an N-terminal signal sequence for Sec-dependent export, and the CSF pentapeptide is derived from the C-terminus of the protein through processing by extracellular proteases (Solomon *et al.*, 1996, Lanigan-Gerdes *et al.*, 2007, Lanigan-Gerdes *et al.*, 2008). Once CSF has reached a critical concentration, it is transported into the cell by an oligopeptide permease (Opp) and interacts with at least two intracellular receptors, RapC and RapB, to inhibit their activity (Core & Perego, 2003, Lazazzera *et al.*, 1997, Perego, 1997). Both RapB and RapC control the activity of response regulators involved in the regulation of sporulation and genetic competence, respectively (Core & Perego, 2003, Ishikawa *et al.*, 2002, Parashar *et al.*, 2013). A number of Phr peptide signaling cassettes have been found in the genomes of other Gram-positive endospore forming *Bacilli* and *Clostridia* species (Perego & Brannigan, 2001, Pottathil & Lazazzera, 2003, Slamti & Lereclus, 2005, Bongiorni *et al.*, 2006, Rocha-Estrada *et al.*, 2010). Of the non-*B. subtilis* cassettes, the best studied is the PlcR/PapR cassette of *B. cereus* and *B. thuringiensis*, in which the secreted Phr signaling peptide produced by *papR* activates the transcription factor PlcR to simulate virulence gene expression (Slamti & Lereclus, 2002, Grenha *et al.*, 2013, Gohar *et al.*, 2008, Sastalla *et al.*, 2010).

Several lines of evidence suggested that Phr peptides may play a role in the physiology of *Streptococcus pneumoniae* (pneumococcus), an important Gram-positive commensal bacterium that colonizes the human nasopharynx and is a serious opportunistic pathogen, causing significant health and financial burdens worldwide (2007, O'Brien *et al.*, 2009, Huang *et al.*, 2011). Pneumococcal Opp (encoded by *amiACDEF*), the homologue to the transporter necessary for import of *phr* peptides in Bacilli, is important for colonization and virulence in this organism (Chen *et al.*, 2008, Hava & Camilli, 2002, Molzen *et al.*, 2011, Orihuela *et al.*, 2004, Song *et al.*, 2008). How Opp influences these processes is unknown, but knockout of Opp in *S. pneumoniae* has highly pleiotropic effects, influencing amino acid uptake, adherence to human epithelial cells, and development of genetic competence (Claverys *et al.*, 2000, Trombe *et al.*, 1984, Trombe *et al.*, 1979, Alloing *et al.*, 1996, Cundell *et al.*, 1995). Additionally, Opp is upregulated in pneumococcal cells exposed to human lung epithelial cells (Song *et al.*, 2008). As Opp plays such a central role in the physiology of *S. pneumoniae* and is essential for Phr peptide uptake in Bacilli, we hypothesized that Phr-type peptides could play a role in *S. pneumoniae* colonization or virulence.

Here we report the identification of genes involved in Phr-peptide signaling from *S. pneumoniae*. One of the Phr-peptide signaling systems, termed TprA/PhrA, is highly conserved amongst different pneumococcal serotype strains. Analysis of TprA/PhrA in *S. pneumoniae* serotype 2 strain D39 revealed that this system mediates quorum sensing and is active in media containing galactose, one of the main carbon sources encountered by pneumococcal cells during colonization of the nasopharynx. Additionally, we determined that the system controls expression of a lantibiotic biosynthesis gene cluster with the potential to produce antimicrobial peptides that may be important for competitive fitness during nasopharynx colonization.

RESULTS

In silico identification of a conserved *phr* peptide quorum sensing cassette in *S. pneumoniae*

To identify Phr peptide sensing systems in different species of *Streptococcus*, we blasted sequenced Streptococcal genomes for gene encoding proteins with similarities to the PlcR/Rap regulatory proteins of Bacilli. The region neighboring these genes was then searched for adjacent small open reading frames with the features of Phr peptides, encoding 30 to 100 residue peptide with an N-terminal signal sequence for export and a polar C-terminal region (see *Supplemental Information* for details). Using this method, we identified 53 potential Phr peptide-signaling cassettes in sequenced Streptococcal genomes (see Tables S1-3 for the complete list). In all of the cassettes identified, only homologues to PlcR from the *B. cereus* group were identified, and no homologues to the Rap proteins of *B. subtilis* were found. These PlcR-like proteins were also suggested previously to be putative Rgg transcription factors (Fleuchot *et al.*, 2011). However, these proteins lacked the adjacent gene for a small hydrophobic peptide (SHP) that is typically associated with Rgg proteins. Instead, we determined that these proteins were adjacent to small genes that have the features of Phr-type peptides (an N-terminal secretion signal and C-terminal polar region). To further determine if these cassettes could be best described as orthologs of the PlcR/PapR system of Bacilli, the secondary and tertiary structure predictions for one cassette from *S. pneumoniae* were determined using Phyre^2 (Kelley & Sternberg, 2009). The protein structure that most closely resembled the predict structure of the protein from *S. pneumoniae* was PlcR of *B. thuringiensis* (Table S4 and Figure S1). Additionally, pairwise Blast analysis revealed that these *S. pneumoniae* proteins were more similar to PlcR of *B. cereus* (E-value of $2e^{-12}$) than Rgg2 of *Streptococcus pyogenes* (E-value of $3e^{-05}$). Thus, these gene cassettes of *S. pneumoniae* appear to be orthologs of PlcR/PapR system.

PlcR orthologs were found in several Streptococcal species; however, in *S. pneumoniae* strains, the orthologs were mainly encoded adjacent to a *phr* gene (Tables S1-3). Three distinct Phr peptide-signaling cassettes were found in *S. pneumoniae* strains. Each of these cassettes has a gene for a secreted Phr peptide and a gene for a transcription factor, the PlcR ortholog, which we refer to as *tpr*, for **t**ranscription factor regulated by a **P**h**r** peptide. The first cassette (TprA/PhrA; Table S1) is conserved across 60% of sequenced pneumococcal genomes. The two other peptide systems (TprB/PhrB; Table S2 and TprC/PhrC; Table S3) are present in islands of horizontally transferred genes, including pathogenicity island 1 (PI1) and region of diversity 2 (RD2) both in TIGR4 (Blomberg *et al.*, 2009, Brown *et al.*, 2004, Obert *et al.*, 2006).

The *tprA*/*phrA* cassette of *S. pneumoniae* differs from the typical Phr-signaling cassettes. The *tprA* and *phrA* genes are oriented in opposite directions, as compared to all known Phr-signaling cassettes that have the genes oriented in the same direction (Pottathil & Lazazzera, 2003, Pomerantsev *et al.*, 2009). Interestingly, this is similar to the arrangement of the some of the Rgg/SHP peptide signaling cassettes (Chang *et al.*, 2011, Fleuchot *et al.*, 2011). Additionally, *phrA* encodes a 56-residue peptide, which is only surpassed in length by the 57-residue PhrH of *B. subtilis* and is longer than the other characterized Phr peptides of 38 to 48 residues (Slamti & Lereclus, 2002, Pottathil & Lazazzera, 2003, Mirouze *et al.*, 2011). These differences are indicative of the distinct evolutionary trajectory of this *S. pneumoniae* Phr-signaling cassette.

TprA is an inhibitor of *phrA* expression

To begin to understand the role of the TprA/PhrA cassette in *S. pneumoniae*, we constructed mutant strains that contained deletions of *tprA* or *phrA*. These deletion strains showed no significant deviation from wild-type during growth *in vitro* or virulence in a murine model for invasive disease, indicating that the deletion mutations do not alter the overall physiology of the bacteria (Figure S2). Many quorum sensing regulatory circuits, including those regulated by Phr peptides, contain positive feedback loops to rapidly increase expression of the genes that produce the signaling molecules (Lazazzera *et al.*, 1999, Lereclus *et al.*, 1996, Kleerebezem *et al.*, 1997). To assess the activity of the TprA/PhrA system and whether *phrA* was regulated by the system, we constructed a *lacZ* transcriptional fusion to the promoter of *phrA* by fusing the intergenic region between *tprA* and *phrA* to *lacZ* and integrating this construct in single copy on the *S. pneumoniae* chromosome at *bgaA.* The gene construct results in the disruption of *bgaA,* which is the endogenous gene for ß-galactosidase (Halfmann *et al.*, 2007). During exponential growth in rich media, the levels of *phrA-lacZ* were low in the wild-type strain background are not statistically different from the levels in the Δ*phrA* mutant (Figure 1A), indicating that the TprA/PhrA system was not active under these growth conditions. In the Δ*tprA* strain background, *phrA* expression was elevated 34-fold relative to the wild-type strain (Figure 1A). The increased expression of *phrA* was due to the absence of TprA as *phrA-lacZ* levels dropped back to wild-type levels in the complemented strain (i.e. Δ*tprA* strain background with *tprA* at an ectopic locus) (Figure 1A). The same pattern of expression was seen when *phrA* expression levels were monitored directly using qRT-PCR in exponentially growing cells (Figure 1B). These data indicate that TprA serves as an inhibitor of PhrA expression and that the TprA/PhrA system was not active during growth in rich media.

PhrA can induce expression of the TprA regulon by antagonizing TprA activity

Next, we tested whether PhrA encodes a signaling peptide that can influence expression of the TprA-regulated genes. If PhrA were a co-inhibitor of the TprA regulon, an increase in *phrA*-*lacZ* expression would be expected in the Δ*phrA* strain background, which was not observed (Figure 1A). Alternatively, PhrA could serve as an antagonist to TprA activity, and if so, we would expect to see an increase in *phrA*-*lacZ* in response to PhrA. To that end, full-length *phrA* was placed under the control of a fucose-inducible promoter (P_{fcsK}), and this construct was integrated as a single copy in a strain that contained *phrA*-*lacZ*. We monitored the levels of *phrA*-*lacZ* in the presence and absence of fucose and found that *phrA*-*lacZ* levels were indeed increased in a statistically significant manner when PhrA was overexpressed (Figure 2A), indicating that PhrA can activate expression of the TprA regulon and participate in autoregulation.

Based on the work with Phr peptides in Bacilli (Slamti & Lereclus, 2002, Bouillaut *et al.*, 2008, Pottathil & Lazazzera, 2003), we predicted that the active PhrA signaling peptide would be a small peptide derived from the C-terminus of the PhrA precursor protein. To see if such a peptide was capable of activating the TprA/PhrA system, we treated cells containing the *phrA*-*lacZ* reporter with a synthetic peptide corresponding to last 10 residues of PhrA. Expression of *phrA* was approximately 15-fold higher in the presence of this synthetic peptide compared to the levels obtained by addition of the peptide-resuspension buffer (Figure 2B-C). A similar 45-fold increase in *phrA* expression was measured by qRT-PCR of wild-type cells treated with the 10-residue peptide (Figure S3). To test whether the PhrA peptide acts through TprA, we examined levels of the *phrA*-*lacZ* reporter in a Δ*tprA* background. In the presence and absence of the 10-residue synthetic peptide, the levels of *phrA* expression were high in the absence of TprA (Figure 2C). Taken together, these data show that PhrA can induce expression of TprA-regulated genes, ostensibly by antagonizing the inhibitory activity of TprA.

Identification of the minimal peptide form of the PhrA signaling peptide

To begin to elucidate the nature of the mature PhrA signaling peptide, we took two complementary approaches. First, we overexpressed PhrA proteins that lacked various portions of the C-terminus. If all or part of the mature peptide were located in the deleted portions of *phrA*, we would not observe induction of *phrA-lacZ*. Constructs of *phrA*, under the control of P_{fcsK}, were created that lacked the last 15, 10, 5 or 1 residues of PhrA (PhrAΔ42-56, PhrAΔ47-56, PhrAΔ52-56, and PhrAΔ56, respectively). Each of these truncated proteins failed to induce *phrA-lacZ* (Figure 2A), consistent with idea that the mature signaling peptide is derived from the very C-terminus of the precursor protein. Additionally, cells treated with 5 µM of synthetic peptides corresponding to the last 6, 7, or 10 residues of PhrA resulted in induction of *phrA-lacZ*. In contrast, treatment with synthetic peptides corresponding to the last 5 residues or a 6-residue internal fragment (i.e. the active 7-residue peptide but lacking the very last aspartic acid) did not induce *phrA-lacZ* (Figure 2C). These data demonstrate that the minimal peptide corresponds to the very C-terminal 6 amino acids of PhrA. Interestingly, the longer synthetic 7- and 10-residue PhrA peptides showed a greater ability to induce system activity (Figure 2C). Thus, the mature PhrA peptide produced by cells could be longer than the minimal 6-residue peptide identified in this study.

Oligopeptide permease is required for cells to respond to the synthetic PhrA peptide

Internalization of the extracellular PhrA-signaling peptide is central to the mechanism whereby the extracellular PhrA peptide signals to cells by interacting directly with TprA. Thus, we asked whether the oligopeptide permease encoded by *amiABCDE* of *S. pneumoniae* is required for responding to the PhrA-signaling peptide. To this end, expression of *phrA-lacZ* was measured in a strain that lacked *amiC* in the presence and absence of the 10-residue synthetic

PhrA peptide (Figure 3). Expression of *phrA* in the strain deleted for Δ*amiC* was low and indistinguishable from isogenic wild-type cells in the absence of peptide, but failed to show induction in response to PhrA peptide treatment. A simple model for the role of Opp is to import the PhrA peptide into the cell to antagonize TprA activity. Consistent with this model, *phrA-lacZ* expression increased 21-fold in the strain lacking both *amiC* and *tprA*, as compared to the strain just lacking *amiC*, and reached a level of expression comparable to the *tprA* mutant strain (Figure 3). As expected, the strain lacking *amiC* and *tprA* was unable to respond to the 10-residue PhrA peptide. These data are in agreement with Opp functioning upstream of TprA to transport mature PhrA peptide into the cell to antagonize TprA control of gene expression.

The TprA/PhrA system is active in media containing galactose.

During growth under laboratory conditions in rich media, the TprA/PhrA signaling system is not active (Figure 1). The promoter for *phrA* is predicted to contain a CRE element for binding the carbon-catabolite repressor CcpA in *S. pneumoniae*, and expression of both *tprA* and *phrA* were found to be elevated in a Δ*ccpA* background in a microarray study (Carvalho *et al.*, 2011). These data suggest that the lack of activity for the TprA/PhrA system in the rich BHI media may be due to the inhibitory effects of glucose in the formulated Becton-Dickinson BHI media as it contains 0.2 % glucose in this media. Thus, we examined *phrA-lacZ* activity when cells were grown in chemically defined media made with either glucose or galactose as the sole carbon source (CDM-glucose or CDM-galactose, respectively) (Figure 4A). When cells containing the *phrA-lacZ* reporter were grown in CDM-glucose, the activity of the reporter remained very low throughout the growth curve (Figure 4B). However, when the same cells were grown in CDM-galactose, expression of the reporter was induced during mid-exponential growth and continued to increase throughout the growth of the culture as expected for a cell density monitoring system (Figure 4A & B). Similar results for *phrA* expression were observed by qRT-PCR (Figure S4). These data indicate that the TprA/PhrA system is active in cells grown in the presence of galactose, but not glucose.

As *phrA* expression was induced when cells were grown in CDM-galactose, we asked if a PhrA signaling peptide was produced and exported in this same media. Wild-type cells containing *phrA-lacZ* were grown in CDM-galactose to exponential phase (OD_{620} ~ 0.1) when they were collected by centrifugation and resuspended in CDM-galactose media conditioned by the growth of wild-type cells (WT CM) or Δ*phrA* mutant cells (Δ*phrA* CM). As a control, these cells were also resuspended in the original CDM-galactose growth media (untreated). Incubation of cells with WT CM resulted in a strong induction of *phrA-lacZ* after 4 hours, which was significantly higher than the induction observed with the untreated media. The inducing activity of the conditioned media was dependent on PhrA, as ∆*phrA* CM did not induce *phrA-lacZ* (Figure 4C). These data indicate that a mature PhrA signaling peptide was present in the conditioned media from wild-type cells and that this signaling peptide is able to activate the TprA regulon when added to cells in trans.

TprA and PhrA regulate lantibiotic biosynthesis machinery

TprA is predicted to be a transcription factor, and thus, to begin to identify genes controlled by the TprA/PhrA quorum sensing system in *S. pneumoniae*, we performed whole-genome expression profiling using RNA-sequencing (RNA-seq) on cells lacking *tprA*, *phrA* or both, and on cells treated with 10-residue synthetic PhrA peptide (PhrA(10)). RNA was isolated from Δ*tprA* cells, Δ*phrA* cells, and Δ(*tprA*-*phrA*) cells and compared to RNA isolated from wild-type cells, and RNA isolated from wild-type cells treated with 10 µM peptide was compared to RNA isolated from cells lacking peptide treatment. Genes that were significantly differentially expressed were identified as those that had an up- or down-fold change of at least 2.0 with a false detection rate of 0.001 or lower (Table 1). Transcript levels for *tprA* (spd1745) and *phrA* (spd1746) were increased on average 10- and 28-fold, respectively, in cells lacking *tprA* or induced with PhrA(10). While *tprA* was only identified to be induced in cells treated with PhrA(10), *tprA* was not identified as differentially expressed in the strains Δ*tprA* and Δ(*tprA*-*phrA*) in Table 1. In strains with a deletion of *tprA* mutant, we observed an increase of transcription just 5' to the location of the *tprA* deletion (Figure S6), indicating that TprA negatively regulates its own expression. However, due to the lack of *tprA* transcription in the deleted region, the total sum of reads was below the 2-fold difference threshold through *tprA* in the Δ*tprA* or Δ(*tprA*-*phrA*) mutant strains compared to the wild-type strain. Thus, these data revealed *tprA* and *phrA* are transcribed divergently and autoregulate their own production.

Besides *phrA* and *tprA*, 22 genes were found to be differentially expressed in at least one of the mutant strains or cells treated with PhrA(10) (Table 1). Eleven of these genes flank the *tprA, phrA* region and were differentially expressed to similar extents in both the PhrA(10)-treated cells, as well as the Δ*tprA* single and Δ(*tprA*-*phrA*) double mutants (Table 1; Figure 5). None of these genes was differentially expressed in the Δ*phrA* mutant*,* consistent with the levels of *phrA* expression in wild-type cells being insufficient to activate expression of these genes. Four genes, extending from spd0769 to spd0773 were all up-regulated in PhrA(10)-treated cells and in the Δ(*tprA*-*phrA*) mutant, with the exception of spd0769 that was only induced in the PhrA(10)-treated cells. Why these genes were not also induced in either the Δ*tprA* or Δ*phrA* single mutant is unknown. The remaining seven genes were only regulated under one of the conditions tested. While these genes controlled by one or two of the conditions tested are potentially interesting, we choose to focus on the genes, spd1744 to spd1756, consistently identified as differentially expressed under conditions of PhrA(10) treatment or loss of *tprA* (i.e. the single Δ*tprA* or double Δ(*tprA*-*phrA*) mutant).

Of the thirteen genes extending from spd1744 to spd1756, at least nine of these genes (spd1747 to spd1755) are predicted to be involved in synthesis of or immunity to a lantibiotic peptide (Figure 5, Table 1). All of the genes in this region were upregulated in strains lacking *tprA* at least 3-fold and as much as 50-fold over the levels found in the wild-type strain, and were induced to a slightly lesser extent in the presence of the PhrA peptide. Four promoters are predicted in this region from RNA-seq data of the wild-type strain by comparing expression levels of neighboring genes (data not shown) (Figure 5). One of these predicted promoters is upstream of spd1744, a gene of unknown function. A second promoter is located upstream of *tprA*, which is predicted to be in a single gene operon, and this promoter most likely contributes to its autoregulation. A third promoter is in located upstream of *phrA*, which is predicted to be in an operon with spd1747 and spd1748, which encode lantipeptide precursor proteins. The last promoter is located upstream of spd1754, which appears to be in an operon with spd1755 and possibly spd1756, and encodes a putative lantipeptide transporter, an immunity protein, and a protein of unknown function, respectively. The genes extending from spd1749 to spd1753, which encode putative lantipeptide modifying enzymes, were expressed at levels that were below the threshold for promoter prediction in the wild-type strain. However, the DOOR database for predicted operons (Mao *et al.*, 2009) indicates that potential promoters are upstream of spd1749 and spd1750. We were unable to identify any conserved inverted repeats in the putative promoter regions (see Supplemental Material and Methods). Thus, the binding-site for TprA is unknown, and future research is necessary to identify its binding site.

To validate the RNA-seq data, qRT-PCR was performed to confirm the regulation by the TprA on several of the key lantibiotic biosynthesis genes: the lantibiotic precursor peptides, spd1747 and spd1748 (these genes were probed together in the qRT-PCR analysis due to their small size), the bifunctional modification enzyme encoding by spd1749, a second predicted lantibiotic biosynthesis enzyme encoded by spd1750, and the gene that encodes the predicted immunity protein, spd1754 (Figure 5). RNA from wild-type, Δ*tprA* and Δ*tprA*-complemented strains were isolated from mid-exponentially growing cells and subjected to qRT-PCR analysis. TprA-dependent inhibition of expression was observed for all 4 sets of genes as probe levels increased significantly in the Δ*tprA* strain compared to wild-type and dropped again to near-wild-type levels in the Δ*tprA*-complemented strain (Figure S5).

To confirm that the extracellular PhrA peptide could also activate expression of the lantibiotic machinery, we performed qRT-PCR analysis for these same 4 sets of genes on RNA isolated from mid-exponential wild-type cells that had been treated with the synthetic 10-residue PhrA peptide. Again, we observed significant upregulation of all 4 sets of genes in the cells that were treated with the synthetic peptide compared to those that had been treated with the peptide-resuspension buffer alone (Figure S5). The data from the RNA-seq analysis and subsequent validation of the regulation of several key lantibiotic genes by qRT-PCR indicate that the TprA/PhrA signaling system controls expression of the adjacent putative lantibiotic biosynthesis gene cluster.

DISCUSSION

We report here the identification and characterization of a new quorum-sensing cassette in *S. pneumoniae* D39 that belongs to the Phr family of quorum-sensing systems, which had previously only been identified in Gram-positive, endospore-forming bacteria. Through RNA-seq analysis, the PhrA peptide was found to induce genes that appear to process and provide immunity to a lantibiotic peptide. The expression of *phrA* and the corresponding lantibiotic genes were shown to be inhibited by the presence of glucose and to be induced in the presence of galactose. Galactose is the major sugar in the human nasopharynx (King, 2010, Yesilkaya *et al.*, 2008), consistent with a role for TprA/PhrA and the lantibiotic peptide in colonization of the host.

Lantibiotics are highly modified peptides that fall in the bacteriocin family of small antimicrobial peptides produced by many bacterial species and have been implicated in inter- and intra-species competition. These peptides are ribosomally synthesized and can have broad- or narrow-spectrum antimicrobial activity against other bacteria (Cotter *et al.*, 2005, Cotter *et al.*, 2013). *S. pneumoniae* is already known to produce the Blp bacteriocin that is responsible for mediating intra-species competition amongst a few *S. pneumoniae* clinical isolates during host nasopharynx colonization (Dawid *et al.*, 2007). Antibacterial activity of the lantibiotic system controlled by pneumococcal TprA/PhrA was suggested by experiments in a heterologous system. When the two lantibiotic-precursor peptides from *S. pneumoniae* were produced and modified using the nisin machinery from *Lactococcus lactus*, the peptides exhibited antimicrobial activity against *Micrococcus flavus*, an organism that colonizes humans (Majchrzykiewicz *et al.*, 2010). Given the necessity for inter- as well as intra-species competition during nasopharynx colonization, lantibiotic biosynthesis machinery may represent a mechanism for interspecies competition in the nasopharynx. At this time, we cannot rule out other possible functions for the lantibiotic gene cluster, spd1744-spd1756. Lantipeptides produced by *Streptomyces* spp. have surfactant activity and are used to reduce surface tension for aerial hyphae formation (Willey & van der Donk, 2007, Kodani *et al.*, 2004, Kodani *et al.*, 2005). Surfactants have also been implicated in biofilm formation in a number of bacterial species (Otto, 2013, Raaijmakers *et al.*, 2010), and biofilm formation has recently been implicated in colonization of the nasopharynx by *S. pneumoniae* (Munoz-Elias *et al.*, 2008, Marks *et al.*, 2012a, Marks *et al.*, 2012b). Experiments are currently underway to identify the biological role for this lantibiotic biosynthesis gene cluster.

A model for the mechanism by which TprA/PhrA induce the lantibiotic biosynthesis gene cluster is presented in Figure 6. The PhrA signaling peptide is derived from a precursor protein that is predicted to be exported through the Sec pathway and processed outside the cell by proteases to release the mature peptide, whose minimal form is the C-terminal 6 residues of the PhrA-precursor protein. Once the PhrA peptide has reached a sufficient extracellular concentration, it interacts with Opp whereby it is predicted to be brought into the cytoplasm and antagonize the inhibitory activity of TprA to induce expression of the peptide-encoding gene itself, *tprA,* and the lantibiotic biosynthesis gene cluster. At this time, we cannot rule out that TprA and/or PhrA may control more genes than those identified through the growth condition used in this study. Consistent with this possibility, the *tprA*/*phrA* genes are highly conserved in other *S. pneumoniae* serotype strains, but the lantibiotic biosynthetic cluster genes are not.

We found that the TprA/PhrA system is active and capable of signaling between cells when grown in media that contains galactose, but not glucose. The differential expression of these genes in response to glucose versus galactose appears to be mediated via the canonical, carbon catabolite control mechanism of Gram-positive bacteria, in which the CcpA protein binds to CRE elements in promoters when cells are grown in the presence of glucose (Sonenshein, 2007). The promoter for *phrA* is predicted to contain a CRE-binding site for the pneumococcal CcpA (Carvalho *et al.*, 2011). In this context, it is interesting to note that genes involved in sugar metabolism, including PTS system components (e.g. spd0771-0773), were differentially expressed in PhrA peptide treated cells, but the significance of this regulation is unknown as a Δ*tprA* mutation did not significantly induce the expression of these same genes. The expression levels for *tprA*, *phrA*, and many members of the putative lantibiotic biosynthesis gene cluster were upregulated in a strain that lacked CcpA compared to a wild-type strain when the cells were grown in the presence of glucose (Carvalho *et al.*, 2011). Expression of the lantibiotic gene cluster has also been shown to be inhibited by the CiaRH two-component regulatory system (Mascher *et al.*, 2003); although at this time there is no data to suggest that CiaRH mediates catabolite control of this gene cluster. These data indicate that the production of the lantibiotic biosynthesis machinery is part of the carbon-catabolite response of *S. pneumoniae*, and is the first example, to our knowledge, of a lantibiotic incorporated into the carbon-catabolite regulon.

The differential regulation of the TprA-regulon based on available carbon source is interesting when placed in the context of where the pneumococcal cells may encounter glucose or galactose in the human host. Glucose is the preferred carbon source for pneumococcal cells and found in large amounts in the bloodstream and respiratory tract of the host, areas which are normally low in bacterial counts (Philips *et al.*, 2003). Galactose, in contrast, is one of the main carbon sources that pneumococcal cells encounter in the nasopharynx during colonization where they need to compete with other strains of *S. pneumoniae* and other bacterial species for space and resources (King, 2010, Yesilkaya *et al.*, 2008). These data are consistent with recent Tn-Seq analysis that showed that transposon insertions in *tprA* or in some of the genes of the lantibiotic biosynthesis operon significantly reduced the fitness of *S. pneumoniae* serotype 4 strain TIGR4 for nasopharynx colonization in a murine model (van Opijnen & Camilli, 2012). Thus, the TprA/PhrA system and its controlled lantibiotic genes are important for *S. pneumoniae* to colonize the nasopharynx.

Consistent with a role for the TprA/PhrA system in colonization is the finding that these genes and the lantibiotic gene cluster are not required for invasive disease. The Tn-Seq study previously mentioned did not detect a role for the TprA/PhrA system in murine model of invasive disease (van Opijnen & Camilli, 2012). Likewise, we found that deletion of either *tprA* or *phrA* has no overt effect on a murine pneumonia model (Fig S2). Consistent with the lack of a role for antimicrobial activity during invasive disease by *S. pneumoniae*, others have observed that Blp bacteriocin production is down regulated in mice during invasive pneumococcal disease compared to growth *in vitro* (Orihuela *et al.*, 2004).

In order to effectively combat pneumococcal disease, a detailed understanding of the mechanisms that mediate *S. pneumoniae* inter- and intra-species interactions is essential, yet little is known about the genetic and molecular basis for these exchanges. The TprA/PhrA quorum sensing system control of lantibiotic production may be one method employed by pneumococcal cells to ensure competitive fitness during colonization of the human host. Further characterization of this system will increase our understanding of pneumococcal colonization and possibly lead to new targets for antimicrobial therapies.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Strains used in this study are derivatives of encapsulated, virulent serotype 2 strain D39 (Table S5). Details of mutant strains and constructs generated for this work are found in the *Supplemental Information.* Pneumococcal strains were grown at 37°C with 5% CO_2, either on Trypticase Soy Agar II plates (modified; Becton-Dickinson) with 5% (vol/vol) defibrinated sheep blood (TSAII-BA), in Becton-Dickinson brain heart infusion (BHI) broth, or in chemically defined media (Kazmierczak *et al.*, 2009) with 1% glucose (wt/vol) (CDM-glucose) or 1% (wt/vol) galactose (CDM-galactose) as the sole carbon source. Liquid cultures were grown statically and monitored by optical density at 620 nm (OD_{620}) using a Spectronic 20 or a Teysche 100 spectrophotometer. Starter cultures of pneumococcal strains were inoculated from frozen glycerol stocks into BHI broth, serial diluted in the same media, and propagated for 8-16 hours. For growth in BHI broth, starter cultures still in exponential phase (OD_{620} of 0.1 to 0.4) were diluted to an OD_{620} of 0.002 to 0.005 to start final cultures. For the overexpression of PhrA, the final cultures were grown in BHI broth that contained 1% (wt/vol) L-fucose. For growth in CDM-glucose and CDM-galactose, the cells of starter cultures were collected by microcentrifugation at 3000 x *g* for 10 minutes at room temperature. The cells were washed, resuspended, and diluted in CDM-glucose or CDM-galactose to an OD_{620} of 0.001 to 0.005 to start final cultures. For antibiotic selections, TSAII-BA plates or BHI broth cultures were supplemented with 0.3 µg/ml erythromycin, 250 µg/ml kanamycin, 250 µg/ml streptomycin, or 0.25 µg/ml tetracycline.

β-galactosidase assays

β-galactosidase specific activity ([A_{420} per min per ml of culture per OD_{620}] x 1,000) was determined essentially as described previously (Hoover *et al.*, 2010), except that cell lysis was achieved by incubating cells in lysis buffer containing TritonX-100 to induce autolysis as in (Zahner & Hakenbeck, 2000). Conditioned media for use in these assays was produced by culturing *S. pneumoniae* strains in CDM-galactose to a high cell density (OD_{620} of > 1.0), removing the cells by centrifugation at 7000 x *g* for 5 minutes at room temperature, and sterilization of the supernatant through 0.22 μm filters. For the assays in conditioned media, 30 mL cultures of exponentially growing cells (OD_{620} of ~0.3) were divided into 6 mL aliquots, the cells collected by centrifugation at 7000 x *g* at room temperature for 5 minutes, and the cell pellets resuspended in the appropriate conditioned media. Samples were removed at the indicated time for β-galactosidase assays. Reporter construct expression was compared as indicated by performing unpaired two-tailed t tests.

RNA extraction

To isolate RNA for qRT-PCR analysis, starter cultures were used to inoculate 6 ml cultures of BHI broth or CDM-galactose. RNA was extracted from 4 mL of exponentially growing cultures (OD_{620} of ~0.2) using a hot-lysis, acid-phenol extraction followed by purification using an RNeasy minikit (Qiagen) and on-column DNase I treatment as described in (Barendt *et al.*, 2009, Kazmierczak *et al.*, 2009, Ramos-Montanez *et al.*, 2008). 5 µg of total RNA was further digested with DNase using a DNA-free kit (Ambion) prior to qRT-PCR analysis. To isolate RNA for RNA-sequencing analysis, starter cultures were used to inoculate 30 ml cultures of BHI broth in 50 mL conical tubes. RNA was extracted from 23 mL of exponentially growing culture (OD_{620} of ~0.15) using the FastRNA Pro Blue Kit (MP Bio) according to the manufacturer's guidelines. Briefly, cells were isolated by centrifugation at 14,500 x *g* for 5 minutes at 4℃. The cells were resuspended in 1 ml RNA*pro* (MP Bio) and processed twice in the Fast Prep Instrument (MP Bio) for 40 seconds at a setting of 6.0. Chloroform and 100% ethanol were used to extract and precipitate the RNA from the resulting lysate and the miRNeasy minikit (Qiagen) and on-column DNase I treatments were used to purify the RNA as above. The amount and purity of all RNA samples isolated were assessed by NanoDrop spectroscopy (Thermo Fisher). RNA integrity was assessed using the Agilent 2100 BioAnalyzer (Aligent Technologies).

qRT-PCR

qRT-PCR was performed using a two-step protocol as in (Ramos-Montanez *et al.*, 2008, Kazmierczak *et al.*, 2009). Specifically, cDNA was synthesized from 100 ng of total RNA and random primers using the qScript Flex cDNA Kit (Quanta BioSciences). RT-PCR was performed using the Brilliant SYBR Green qPCR Master Mix (Stratagene), the Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent), or the FastStart Universal SYBR Green Master Mix (Roche) and appropriate primers (see Table S6) as in (Kazmierczak *et al.*, 2009, Ramos-Montanez *et al.*, 2008). Reactions were performed in duplicate and normalized to 16S rRNA amounts. The 16S rRNA was quantified using the same cDNA samples except that the samples were diluted 100-

fold further. Data were collected on an MX3000P thermocycler (Stratagene) or on a CFX96 thermocycler (Bio Rad) and analyzed with the SYBR Green (with dissociation curve) program associated with each machine. Four dilutions of cDNA from *S. pneumoniae* strains wild-type for *tprA* and *phrA* (either IU1781 or Spn049) were used to generate standard curves for each primer set. Normalized transcript amounts were compared as indicated by performing pairwise unpaired two-tailed t tests.

Synthetic peptides

Peptides were synthesized on a modified Applied Biosystems 430A peptide synthesizer using 0.2 mmol of 4-hydroxymethyl-phenylacetamidomethyl (PAM) resin (Midwest Biotech) and subsequently purified using reverse phase HPLC (RP-HPLC). For complete details, see *Supplementary Information*. Synthetic peptides were resuspended in 10 mM Tris pH 7.4 with 50 µg/ml BSA to a concentration of 5 mM. For β-galactosidase or qRT-PCR assays, the indicated synthetic peptide (or the peptide-resuspension buffer) was added at final concentration of 5 µM to each 6 ml cell aliquot.

Library construction and RNA-sequencing

cDNA libraries were prepared from total RNA by the University of Wisconsin-Madison Biotechnology Center. The mRNA was enriched from two micrograms total RNA using RiboZeroTM rRNA Removal (Gram-positive bacteria) Kit (EpiCentre Inc.). rRNA-depleted mRNA samples were purified by ethanol precipitation and quantified by fluorometry with the Qubit® RNA assay kit (Invitrogen). Double stranded cDNA synthesis was performed following ScriptSeqTM v2 RNA-Seq Library Preparation guide (EpiCentre Inc.) in accordance with the manufacturer’s standard protocol. Thirty nanograms of enriched mRNA were fragmented using divalent cations via incubation for 5 min at 85°C. The first strand of cDNA was synthesized by reverse transcription using random-sequence primers containing a tagging sequence at their 5' ends. Di-tagged cDNA was synthesized by random annealing of a terminal-Tagging Oligo (TTO) to the 3' end of the cDNA for extension of the cDNA by DNA polymerase. Di-tagged cDNA was purified using Agencourt AMPure® XP beads (Beckman Coulter) followed by PCR amplification for 15 cycles using FailsafeTM PCR enzyme and ScriptSeq Index DNA primer set (EpiCentre Inc.). This step generated the second strand of cDNA and completed the addition of Illumina adapter sequences incorporating a user-defined barcode. The amplified libraries were purified using Agencourt AMPure® XP beads. Quality and quantity were assessed using an Agilent DNA 1000 chip (Agilent) and Qubit® dsDNA HS assay kit (Invitrogen), respectively. Libraries were standardized to 2 μM. Cluster generation was performed using standard Cluster kits (v3) and Illumina Cluster Station. Single-end 100 bp sequencing was performed using standard SBS chemistry (v3) on an Illumina HiSeq2000 sequencer. Images were analyzed using the standard Illumina pipeline, version 1.8.2.

RNA-seq analysis

The raw sequencing reads were quality and adapter trimmed using Trimmomatic (Lohse *et al.*, 2012) with a minimum length of 90. The trimmed reads were mapped on the *Streptococcus pneumoniae* D39 (RefSeq NC_008533) genome and D39 plasmid pDP1 sequence (RefSeq NC_005022) using bowtie2 (Langmead & Salzberg, 2012). Custom PERL scripts were used to generate read counts for the genes and 100 bp non-overlapping intergenic regions of the genome. Differential gene expression was identified using EdgeR (version 3.6.2) using default parameters (Robinson *et al.*, 2010). The false discovery rate (FDR) was calculated using Benjamini and Hochberg's algorithm (Benjamini & Hochberg, 1995) and a gene or region was defined as differentially expressed if it had an up- or down-fold change of 2.0 and their FDR was less than 0.001.

ACKNOWLEDGMENTS

We thank Reinhold Brückner for pPP2, Kyle J. Wayne for bacterial strains, and John Lisher for instruction in CDM preparation. We thank Kurt Zimmer and Doug Rusch for assistance in analysis of RNA-seq data. We thank the members of the Lazazzera laboratory, members of the Winkler laboratory, and many generous colleagues for helpful discussions.

This work was supported by NIH grant AI48616 to B.A.L. and AI095814 and AI107075 to M.E.W. and by funds from the Indiana University Bloomington METACyt Initiative, funded in part by a major grant from the Lilly Endowment (M.E.W). S.E.H. was supported by an NIH postdoctoral training grant (5T32AI007323). A.J.P. was supported by an NIH IMSD grant (R25GM055052). The authors declare that they have no conflict of interest with the research presented.

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Table 1. Changes in relative transcript amounts caused by Δ*tprA* and Δ*phrA* mutations or the addition of the 10-residue synthetic PhrA peptide[a]

Effect on expression & gene tag	Known or Predicted Function & Gene Name	+PhrA(10) vs. untreated[b]		Δ*tprA* vs. WT[c]		Δ(*tprA*-*phrA*) vs. WT[d]		Δ*phrA* vs. WT[e]	
		Log$_2$ fold change	FDR[f]	Log$_2$ fold change	FDR[f]	Log$_2$ fold change	FDR[f]	Log$_2$ fold change	FDR[f]
tprA, phrA gene region									
spd1744	lipoprotein, putative	2.7	3.0E-20	3.3	1.5E-27	3.2	5.0E-26		
spd1745	PlcR-family transcription factor, *tprA*	3.3	4.2E-88						
spd1746	Phr-family peptide signaling precursor protein, *phrA*	4.7	4.5E-81	5.0	3.7E-103	-4.0	2.4E-19	-4.5	9.1E-22
spd1747	lantibiotic precursor peptide	4.7	1.7E-133	4.7	5.3E-115	5.7	6.7E-217		
spd1748	lantibiotic precursor peptide	4.8	2.6E-80	4.8	2.5E-72	5.7	3.4E-123		
spd1749	bifunctional lantibiotic modification enzyme	3.5	4.3E-37	4.3	4.0E-52	3.9	3.8E-19		
spd1750	FAD-dependent flavoprotein	3.3	4.6E-35	4.2	2.2E-51	3.8	3.4E-24		
spd1751	membrane protein, putative	2.9	8.8E-17	3.8	1.1E-24	3.4	8.7E-14		
spd1752	toxin secretion ABC transporter, ATP-binding/permease protein	3.1	6.3E-18	4.0	1.4E-26	3.6	2.7E-13		
spd1753	serine protease, putative	3.0	4.3E-26	3.8	1.3E-37	3.4	1.3E-13		
spd1754	lantibiotic immunity protein	1.7	2.6E-15	2.4	3.2E-27	2.1	7.7E-11		
spd1755	ABC transporter, ATP-binding protein	1.7	4.4E-16	2.3	6.0E-24	2.0	4.9E-10		
spd1756	unknown	1.6	4.8E-05	2.5	2.7E-12	2.3	1.1E-09		
spd0769-0773 region									
spd0769	tmRNA, *ssrA*	2.0	1.5E-06						
spd0771	lactose phosphotransferase system repressor, *lacR1*	1.5	3.4E-14			1.1	1.4E-05		
spd0772	1-phosphofructokinase	1.6	7.7E-23			1.2	3.7E-12		
spd0773	PTS system fructose specific transporter subunit IIABC	1.5	1.5E-20			1.0	9.5E-06		
Other Genes									

spd0096	transcriptional regulator, PadR family protein	-1.1	3.4E-04						
spd0104	LysM domain-containing protein							-1.1	3.3E-05
spd0391	conserved hypothetical protein							0.99	2.1E-04
spd0450	type I restriction-modification system subunit S							-1.5	3.1E-06
spd0452	integrase/recombinase, phage integrase family protein							1.3	2.4E-04
spd0460	molecular chaperone, *dnaK*							-1.2	3.3E-05
spd2013	glycerol kinase, *glpK*					-1.1	1.8E-04		

[a] Strain construction, growth and RNA-Seq analysis are described in Experimental procedures. RNA was prepared from exponential cultures grown in BHI media at 37°C to $OD_{620} \approx 0.15$ to 0.2. The Log_2 of the fold changes and FDR values are based on three independent biological replicates. Cut-offs for this table were 2.0-fold change and FDR value < 0.001. Empty boxes indicate that there was not a significant change in expression of a gene under one of the four conditions.

[b] The RNA transcript levels from strain IU1781 treated with 10 µM 10-residue synthetic PhrA compared to strain IU1781 receiving no treatment. The peptide was added to cells at an OD_{620} of 0.03 and then allowed to incubate to the required OD before harvesting.

[c] The RNA transcript levels from Δ*tprA* mutant strain IU4955 were compared to the RNA transcripts from an isogenic strain IU1781.

[d] The RNA transcript levels from Δ*tprA* Δ*phrA* mutant strain IU6118 were compared to the RNA transcripts from an isogenic strain IU1781.

[e] The RNA transcript levels from Δ*phrA* mutant strain IU4957 were compared to the RNA transcripts from an isogenic strain IU1781.

[f] FDR is the false-discovery rate (Benjamini & Hochberg, 1995).

FIGURE LEGENDS

Fig 1. TprA serves as an inhibitor of *phrA* expression.

(A) The effect of Δ*tprA* and Δ*phrA* mutations on *phrA-lacZ* expression. Strains: Spn007, "wild-type" parental strain used in this experiment; Spn013, Δ*tprA*; Spn019, Δ*phrA*; Spn195, Δ*tprA* CEP::TprA (complemented strain). Results shown are averages of 2-5 independent experiments and error bars depict the standard error of the mean. ***, significant at $P < 0.001$ compared to "wild type." +++, significant at $P < 0.001$ compared to Δ*tprA*. (B) *phrA* mRNA levels in a Δ*tprA* mutant strain. Strains: IU1781 & Spn049, "wild-type" parental strains used in this experiment; Spn052, Δ*tprA*; Spn197, Δ*tprA* CEP::TprA (complemented strain). mRNA levels were normalized to 16S RNA levels, from 2 independent experiments, and are shown as a ratio relative to the wild-type levels. Error bars depict the standard error of the mean. *, significant at $P < 0.05$ compared to "wild type." +, significant at $P < 0.05$ compared to Δ*tprA*. Different parent strains were used in these approaches containing wild-type alleles for the genes of interest.

Fig 2. Identification of the minimal PhrA-signaling peptide.

(A) *phrA-lacZ* reporter expression is elevated when the full length *phrA* gene is overexpressed. Strains: Spn065, full length PhrA; Spn191, PhrAΔ42-56 ; Spn189, PhrAΔ47-56 ; Spn187, PhrAΔ52-56 ; Spn243, PhrAΔ56 . Cells were grown in BHI or BHI+1% fucose (inducer) to mid-exponential phase (OD_{620} of between 0.15 to 0.35) when samples were removed for β-galactosidase activity assays. Results shown are the averages of at least 3 independent replicates and error bars indicate the standard error of the mean for each set. ***, significant at $P < 0.001$ compared to uninduced strain containing the full-length PhrA construct. (B) Synthetic peptides corresponding to the C-terminus of PhrA used in (C) below. (C) Induction of the *phrA-lacZ* reporter was observed when cells were treated with the last 6, 7, or 10 amino acids of PhrA. Early exponential phase (OD_{620} of ~0.1) wild-type cells (Spn007) were incubated with synthetic peptides at a final concentration of 5 µM or peptide-resuspension buffer for two hours prior to analysis by β-galactosidase assays. Results shown are the averages of at least 3 independent replicates and error bars indicate the standard error of the mean for each set. *, significant at *P* < 0.05 and **, significant at *P* < 0.01 compared to the “wild type” strain incubated with buffer.

Fig 3. Oligopeptide permease is required for induction of *phrA-lacZ* in response to synthetic peptide.

Strains lacking *amiC* in a wild-type or a Δ*tprA* mutant background were tested for their ability to induce *phrA-lacZ* expression in response to the 10-residue PhrA peptide. Strains: Spn007, “wild-type” parental strain used in this experiment; Spn013, Δ*tprA*; Spn141, Δ*amiC*; Spn165, Δ*tprA* Δ*amiC*. Early exponential phase cells (OD_{620} of ~0.1) grown in BHI were incubated with 5 µM synthetic peptide or peptide-resuspension buffer for two hours prior to analysis by β-galactosidase activity assays. Results shown are the average of at least two independent trials, and error bars represent the standard error of the mean. ***, significant at *P* < 0.001 compared to the strain treated with buffer.

Fig 4. PhrA can signal between cells when grown to high cell density in media containing galactose.

Cells (Spn007, wild-type) grown in CDM-glucose (closed squares) or CDM-galactose (open circles). Panel A shows a representative growth curve of these cells on these media. Note that after inoculation of the cultures several hours pass before there is a measurable level of cells, and this lag phase is longer in CDM-galactose. Panel B shows expression of *phrA-lacZ* in the Spn007 cells. At least two independent experiments were performed; the results from one representative experiment are shown. Panel C shows induction of *phrA-lacZ* when these were resuspended in conditioned media from wild-type cells (IU1781) compared to untreated media, and no induction was observed in conditioned media from cells lacking *phrA* (IU4957). The results shown are the average of at least two independent trials and the error bars depict the standard error of the mean. *, significant at $P < 0.05$ compared to untreated media. +, significant at $P < 0.05$ compared to wild-type conditioned media.

Fig 5. The TprA/PhrA system regulates a putative lantibiotic biosynthesis operon.

ORFs are represented by dark grey arrows (in the case of the TprA/PhrA system) or light gray arrows (for the putative lantibiotic biosynthesis genes) and the D39 gene identification numbers are indicated in the arrows, with genes spd1747 and spd1748 shorten to 47 and 48, respectively. Known or predicted functions of each gene are indicated above the arrows, and -- indicates that the function of the genes is unknown. The putative promoters, predicted by the results with RNA-seq, are represented by bent black arrows, and putative promoters predicted by the DOOR database are shown as bent gray arrows. Small black boxes are predicted CRE-binding sites of CcpA (Carvalho *et al.*, 2011). Genes whose expression has been found to be increased in either a Δ*tprA* mutant or by the addition of the PhrA peptide are denoted by + under the gene.

Fig 6. Model for the mechanism by which PhrA and TprA control gene expression in *S. pneumoniae*.

The mature PhrA peptide is encoded by *phrA* producing a precursor protein in the absence of glucose. Glucose repression occurs through a CRE element that is in *phrA* promoter region. The PhrA precursor is exported and processed to release the mature PhrA peptide (dark gray, small ovals). When at a sufficient concentration, the PhrA peptide interacts with oligopeptide permease and is transported into the cell where it inhibits the activity of TprA leading to de-repression of *phrA, tprA,* and a change in transcription of lantibiotic genes (wide arrows). Only three of the eight lantibiotic biosynthesis cluster genes are shown here for simplicity (black outlined, wide arrows with one not shown to scale (angled lines)). Bent arrows indicate the location of data-supported (black) or predicted (gray) promoters, all of which are negatively regulated by TprA (denoted by lines that end with a horizontal line).