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

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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.

56 **INTRODUCTION**

57 Quorum sensing is carried out by small secreted molecules that diffuse through
58 the environment, eliciting responses in proximal cells when the signaling molecules
59 reach a critical threshold concentration (Keller & Surette, 2006, Waters & Bassler,
60 2005). In Gram-positive bacteria, quorum sensing often relies on recognition of small
61 secreted peptides that signal through two-component regulatory systems (Rutherford &
62 Bassler, 2012, Pottathil & Lazazzera, 2003, Lyon & Novick, 2004). An alternative group of
63 quorum sensing peptides are those that signal to cells by interacting with an oligopeptide
64 transporter and then a cytoplasmic receptor protein, which is either a member of the RNPP or
65 the Rgg family of proteins (Rocha-Estrada *et al.*, 2010, Dunny, 2007, Jimenez & Federle, 2014,
66 Fleuchot *et al.*, 2011, Pottathil & Lazazzera, 2003). The Phr family of signaling peptides of Bacilli
67 belongs to the latter group, and these peptides regulate a number of important processes such
68 as sporulation, development of genetic competence, virulence gene expression, biofilm
69 formation, and excision and transfer of mobile genetic elements (Rocha-Estrada *et al.*, 2010,
70 Slamti & Lereclus, 2002, Lazazzera *et al.*, 1997, Auchtung *et al.*, 2005, Bongiorni *et al.*, 2005,
71 Perego & Hoch, 1996). However, the role that the Phr signaling peptides play outside of the
72 Gram-positive endospore forming bacterial species has not yet been established.

73 The Phr family of quorum sensing peptide of Bacilli are secreted through the
74 Sec-dependent export pathway and processed from a small precursor protein to a
75 mature, 5-7 residue, polar peptide (Mirouze *et al.*, 2011, Aceves-Diez *et al.*, 2007,
76 Ogura *et al.*, 2003, Perego, 1997, Solomon *et al.*, 1996, Bongiorni *et al.*, 2006). These
77 peptides are encoded by genes typically found immediately adjacent to genes encoding
78 their cytoplasmic target proteins, forming a quorum-sensing cassette (Pottathil &
79 Lazazzera, 2003, Rocha-Estrada *et al.*, 2010). A classic example of the Phr peptides is
80 **c**ompetence and **s**porulation **f**actor (CSF) of *Bacillus subtilis* (Pottathil & Lazazzera, 2003). The

81 precursor protein for CSF, PhrC, has an N-terminal signal sequence for Sec-dependent export,
82 and the CSF pentapeptide is derived from the C-terminus of the protein through processing by
83 extracellular proteases (Solomon *et al.*, 1996, Lanigan-Gerdes *et al.*, 2007, Lanigan-Gerdes *et*
84 *al.*, 2008). Once CSF has reached a critical concentration, it is transported into the cell by an
85 oligopeptide permease (Opp) and interacts with at least two intracellular receptors, RapC and
86 RapB, to inhibit their activity (Core & Perego, 2003, Lazazzera *et al.*, 1997, Perego, 1997). Both
87 RapB and RapC control the activity of response regulators involved in the regulation of
88 sporulation and genetic competence, respectively (Core & Perego, 2003, Ishikawa *et al.*, 2002,
89 Parashar *et al.*, 2013). A number of Phr peptide signaling cassettes have been found in the
90 genomes of other Gram-positive endospore forming *Bacilli* and *Clostridia* species (Perego &
91 Brannigan, 2001, Pottathil & Lazazzera, 2003, Slamti & Lereclus, 2005, Bongiorni *et al.*, 2006,
92 Rocha-Estrada *et al.*, 2010). Of the non-*B. subtilis* cassettes, the best studied is the PlcR/PapR
93 cassette of *B. cereus* and *B. thuringiensis*, in which the secreted Phr signaling peptide produced
94 by *papR* activates the transcription factor PlcR to simulate virulence gene expression (Slamti &
95 Lereclus, 2002, Grenha *et al.*, 2013, Gohar *et al.*, 2008, Sastalla *et al.*, 2010).
96 Several lines of evidence suggested that Phr peptides may play a role in the physiology
97 of *Streptococcus pneumoniae* (pneumococcus), an important Gram-positive commensal
98 bacterium that colonizes the human nasopharynx and is a serious opportunistic pathogen,
99 causing significant health and financial burdens worldwide (2007, O'Brien *et al.*, 2009, Huang *et*
100 *al.*, 2011). Pneumococcal Opp (encoded by *amiACDEF*), the homologue to the transporter
101 necessary for import of *phr* peptides in Bacilli, is important for colonization and virulence in this
102 organism (Chen *et al.*, 2008, Hava & Camilli, 2002, Molzen *et al.*, 2011, Orihuela *et al.*, 2004,
103 Song *et al.*, 2008). How Opp influences these processes is unknown, but knockout of Opp in *S.*
104 *pneumoniae* has highly pleiotropic effects, influencing amino acid uptake, adherence to human
105 epithelial cells, and development of genetic competence (Claverys *et al.*, 2000, Trombe *et al.*,
106 1984, Trombe *et al.*, 1979, Alloing *et al.*, 1996, Cundell *et al.*, 1995). Additionally, Opp is

107 upregulated in pneumococcal cells exposed to human lung epithelial cells (Song *et al.*, 2008).
108 As Opp plays such a central role in the physiology of *S. pneumoniae* and is essential for Phr
109 peptide uptake in Bacilli, we hypothesized that Phr-type peptides could play a role in *S.*
110 *pneumoniae* colonization or virulence.

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

119 **RESULTS**

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

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

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

153 The *tprA*/*phrA* cassette of *S. pneumoniae* differs from the typical Phr-signaling cassettes.
154 The *tprA* and *phrA* genes are oriented in opposite directions, as compared to all known Phr-
155 signaling cassettes that have the genes oriented in the same direction (Pottathil & Lazazzera,
156 2003, Pomerantsev *et al.*, 2009). Interestingly, this is similar to the arrangement of the some of
157 the Rgg/SHP peptide signaling cassettes (Chang *et al.*, 2011, Fleuchot *et al.*, 2011).
158 Additionally, *phrA* encodes a 56-residue peptide, which is only surpassed in length by the 57-
159 residue PhrH of *B. subtilis* and is longer than the other characterized Phr peptides of 38 to 48
160 residues (Slamti & Lereclus, 2002, Pottathil & Lazazzera, 2003, Mirouze *et al.*, 2011). These
161 differences are indicative of the distinct evolutionary trajectory of this *S. pneumoniae* Phr-
162 signaling cassette.
163
164 **TprA is an inhibitor of *phrA* expression**

165 To begin to understand the role of the TprA/PhrA cassette in *S. pneumoniae*, we
166 constructed mutant strains that contained deletions of *tprA* or *phrA*. These deletion strains
167 showed no significant deviation from wild-type during growth *in vitro* or virulence in a murine
168 model for invasive disease, indicating that the deletion mutations do not alter the overall
169 physiology of the bacteria (Figure S2). Many quorum sensing regulatory circuits, including those
170 regulated by Phr peptides, contain positive feedback loops to rapidly increase expression of the
171 genes that produce the signaling molecules (Lazazzera *et al.*, 1999, Lereclus *et al.*, 1996,
172 Kleerebezem *et al.*, 1997). To assess the activity of the TprA/PhrA system and whether *phrA*
173 was regulated by the system, we constructed a *lacZ* transcriptional fusion to the promoter of
174 *phrA* by fusing the intergenic region between *tprA* and *phrA* to *lacZ* and integrating this construct
175 in single copy on the *S. pneumoniae* chromosome at *bgaA.* The gene construct results in the
176 disruption of *bgaA,* which is the endogenous gene for ß-galactosidase (Halfmann *et al.*, 2007).
177 During exponential growth in rich media, the levels of *phrA-lacZ* were low in the wild-type strain
178 background are not statistically different from the levels in the Δ*phrA* mutant (Figure 1A),
179 indicating that the TprA/PhrA system was not active under these growth conditions. In the Δ*tprA*
180 strain background, *phrA* expression was elevated 34-fold relative to the wild-type strain (Figure
181 1A). The increased expression of *phrA* was due to the absence of TprA as *phrA-lacZ* levels
182 dropped back to wild-type levels in the complemented strain (i.e. Δ*tprA* strain background with
183 *tprA* at an ectopic locus) (Figure 1A). The same pattern of expression was seen when *phrA*
184 expression levels were monitored directly using qRT-PCR in exponentially growing cells (Figure
185 1B). These data indicate that TprA serves as an inhibitor of PhrA expression and that the
186 TprA/PhrA system was not active during growth in rich media.
187

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

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

200 Based on the work with Phr peptides in Bacilli (Slamti & Lereclus, 2002, Bouillaut *et al.*,
201 2008, Pottathil & Lazazzera, 2003), we predicted that the active PhrA signaling peptide would
202 be a small peptide derived from the C-terminus of the PhrA precursor protein. To see if such a
203 peptide was capable of activating the TprA/PhrA system, we treated cells containing the *phrA-*
204 *lacZ* reporter with a synthetic peptide corresponding to last 10 residues of PhrA. Expression of
205 *phrA* was approximately 15-fold higher in the presence of this synthetic peptide compared to the
206 levels obtained by addition of the peptide-resuspension buffer (Figure 2B-C). A similar 45-fold
207 increase in *phrA* expression was measured by qRT-PCR of wild-type cells treated with the 10-
208 residue peptide (Figure S3). To test whether the PhrA peptide acts through TprA, we examined
209 levels of the *phrA-lacZ* reporter in a Δ*tprA* background. In the presence and absence of the 10-
210 residue synthetic peptide, the levels of *phrA* expression were high in the absence of TprA
211 (Figure 2C). Taken together, these data show that PhrA can induce expression of TprA-
212 regulated genes, ostensibly by antagonizing the inhibitory activity of TprA.

213

214 **Identification of the minimal peptide form of the PhrA signaling peptide**

215 To begin to elucidate the nature of the mature PhrA signaling peptide, we took two
216 complementary approaches. First, we overexpressed PhrA proteins that lacked various portions
217 of the C-terminus. If all or part of the mature peptide were located in the deleted portions of
218 *phrA*, we would not observe induction of *phrA-lacZ*. Constructs of *phrA*, under the control of
219 P_{fcsK}, were created that lacked the last 15, 10, 5 or 1 residues of PhrA (PhrAΔ42-56, PhrAΔ47-
220 56, PhrAΔ52-56, and PhrAΔ56, respectively). Each of these truncated proteins failed to induce
221 *phrA-lacZ* (Figure 2A), consistent with idea that the mature signaling peptide is derived from the
222 very C-terminus of the precursor protein. Additionally, cells treated with 5 µM of synthetic
223 peptides corresponding to the last 6, 7, or 10 residues of PhrA resulted in induction of *phrA-*
224 *lacZ*. In contrast, treatment with synthetic peptides corresponding to the last 5 residues or a 6-
225 residue internal fragment (i.e. the active 7-residue peptide but lacking the very last aspartic acid)
226 did not induce *phrA-lacZ* (Figure 2C). These data demonstrate that the minimal peptide
227 corresponds to the very C-terminal 6 amino acids of PhrA. Interestingly, the longer synthetic 7-
228 and 10-residue PhrA peptides showed a greater ability to induce system activity (Figure 2C).
229 Thus, the mature PhrA peptide produced by cells could be longer than the minimal 6-residue
230 peptide identified in this study.

231

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

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

238 PhrA peptide (Figure 3). Expression of *phrA* in the strain deleted for Δ*amiC* was low and
239 indistinguishable from isogenic wild-type cells in the absence of peptide, but failed to show
240 induction in response to PhrA peptide treatment. A simple model for the role of Opp is to import
241 the PhrA peptide into the cell to antagonize TprA activity. Consistent with this model, *phrA-lacZ*
242 expression increased 21-fold in the strain lacking both *amiC* and *tprA*, as compared to the strain
243 just lacking *amiC*, and reached a level of expression comparable to the *tprA* mutant strain
244 (Figure 3). As expected, the strain lacking *amiC* and *tprA* was unable to respond to the 10-
245 residue PhrA peptide. These data are in agreement with Opp functioning upstream of TprA to
246 transport mature PhrA peptide into the cell to antagonize TprA control of gene expression.
247

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

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

265 As *phrA* expression was induced when cells were grown in CDM-galactose, we asked if
266 a PhrA signaling peptide was produced and exported in this same media. Wild-type cells
267 containing *phrA-lacZ* were grown in CDM-galactose to exponential phase (OD_{620} ~ 0.1) when
268 they were collected by centrifugation and resuspended in CDM-galactose media conditioned by
269 the growth of wild-type cells (WT CM) or Δ*phrA* mutant cells (Δ*phrA* CM). As a control, these
270 cells were also resuspended in the original CDM-galactose growth media (untreated).
271 Incubation of cells with WT CM resulted in a strong induction of *phrA-lacZ* after 4 hours, which
272 was significantly higher than the induction observed with the untreated media. The inducing

273 activity of the conditioned media was dependent on PhrA, as Δ*phrA* CM did not induce *phrA-*

274 *lacZ* (Figure 4C). These data indicate that a mature PhrA signaling peptide was present in the

275 conditioned media from wild-type cells and that this signaling peptide is able to activate the TprA

276 regulon when added to cells in trans.

277

278 **TprA and PhrA regulate lantibiotic biosynthesis machinery**

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

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

311 Of the thirteen genes extending from spd1744 to spd1756, at least nine of these genes
312 (spd1747 to spd1755) are predicted to be involved in synthesis of or immunity to a lantibiotic
313 peptide (Figure 5, Table 1). All of the genes in this region were upregulated in strains lacking
314 *tprA* at least 3-fold and as much as 50-fold over the levels found in the wild-type strain, and
315 were induced to a slightly lesser extent in the presence of the PhrA peptide. Four promoters are
316 predicted in this region from RNA-seq data of the wild-type strain by comparing expression
317 levels of neighboring genes (data not shown) (Figure 5). One of these predicted promoters is
318 upstream of spd1744, a gene of unknown function. A second promoter is located upstream of
319 *tprA*, which is predicted to be in a single gene operon, and this promoter most likely contributes
320 to its autoregulation. A third promoter is in located upstream of *phrA*, which is predicted to be in
321 an operon with spd1747 and spd1748, which encode lantipeptide precursor proteins. The last
322 promoter is located upstream of spd1754, which appears to be in an operon with spd1755 and
323 possibly spd1756, and encodes a putative lantipeptide transporter, an immunity protein, and a
324 protein of unknown function, respectively. The genes extending from spd1749 to spd1753,
325 which encode putative lantipeptide modifying enzymes, were expressed at levels that were
326 below the threshold for promoter prediction in the wild-type strain. However, the DOOR
327 database for predicted operons (Mao *et al.*, 2009) indicates that potential promoters are
328 upstream of spd1749 and spd1750. We were unable to identify any conserved inverted repeats
329 in the putative promoter regions (see Supplemental Material and Methods). Thus, the binding-
330 site for TprA is unknown, and future research is necessary to identify its binding site.
331 To validate the RNA-seq data, qRT-PCR was performed to confirm the regulation by the
332 TprA on several of the key lantibiotic biosynthesis genes: the lantibiotic precursor peptides,
333 spd1747 and spd1748 (these genes were probed together in the qRT-PCR analysis due to their
334 small size), the bifunctional modification enzyme encoding by spd1749, a second predicted
335 lantibiotic biosynthesis enzyme encoded by spd1750, and the gene that encodes the predicted
336 immunity protein, spd1754 (Figure 5). RNA from wild-type, Δ*tprA* and Δ*tprA*-complemented

337 strains were isolated from mid-exponentially growing cells and subjected to qRT-PCR analysis.

338 TprA-dependent inhibition of expression was observed for all 4 sets of genes as probe levels

339 increased significantly in the Δ*tprA* strain compared to wild-type and dropped again to near-wild-

340 type levels in the Δ*tprA*-complemented strain (Figure S5).

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

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

359 Lantibiotics are highly modified peptides that fall in the bacteriocin family of small
360 antimicrobial peptides produced by many bacterial species and have been implicated in inter-
361 and intra-species competition. These peptides are ribosomally synthesized and can have broad-
362 or narrow-spectrum antimicrobial activity against other bacteria (Cotter *et al.*, 2005, Cotter *et al.*,
363 2013). *S. pneumoniae* is already known to produce the Blp bacteriocin that is responsible for
364 mediating intra-species competition amongst a few *S. pneumoniae* clinical isolates during host
365 nasopharynx colonization (Dawid *et al.*, 2007). Antibacterial activity of the lantibiotic system
366 controlled by pneumococcal TprA/PhrA was suggested by experiments in a heterologous

367 system. When the two lantibiotic-precursor peptides from *S. pneumoniae* were produced and
368 modified using the nisin machinery from *Lactococcus lactus*, the peptides exhibited antimicrobial
369 activity against *Micrococcus flavus*, an organism that colonizes humans (Majchrzykiewicz *et al.*,
370 2010). Given the necessity for inter- as well as intra-species competition during nasopharynx
371 colonization, lantibiotic biosynthesis machinery may represent a mechanism for interspecies
372 competition in the nasopharynx. At this time, we cannot rule out other possible functions for the
373 lantibiotic gene cluster, spd1744-spd1756. Lantipeptides produced by *Streptomyces* spp. have
374 surfactant activity and are used to reduce surface tension for aerial hyphae formation (Willey &
375 van der Donk, 2007, Kodani *et al.*, 2004, Kodani *et al.*, 2005). Surfactants have also been
376 implicated in biofilm formation in a number of bacterial species (Otto, 2013, Raaijmakers *et al.*,
377 2010), and biofilm formation has recently been implicated in colonization of the nasopharynx by
378 *S. pneumoniae* (Munoz-Elias *et al.*, 2008, Marks *et al.*, 2012a, Marks *et al.*, 2012b).
379 Experiments are currently underway to identify the biological role for this lantibiotic biosynthesis
380 gene cluster.

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

392 We found that the TprA/PhrA system is active and capable of signaling between cells
393 when grown in media that contains galactose, but not glucose. The differential expression of
394 these genes in response to glucose versus galactose appears to be mediated via the canonical,
395 carbon catabolite control mechanism of Gram-positive bacteria, in which the CcpA protein binds
396 to CRE elements in promoters when cells are grown in the presence of glucose (Sonenshein,
397 2007). The promoter for *phrA* is predicted to contain a CRE-binding site for the pneumococcal
398 CcpA (Carvalho *et al.*, 2011). In this context, it is interesting to note that genes involved in sugar
399 metabolism, including PTS system components (e.g. spd0771-0773), were differentially
400 expressed in PhrA peptide treated cells, but the significance of this regulation is unknown as a
401 Δ*tprA* mutation did not significantly induce the expression of these same genes. The expression
402 levels for *tprA*, *phrA*, and many members of the putative lantibiotic biosynthesis gene cluster
403 were upregulated in a strain that lacked CcpA compared to a wild-type strain when the cells
404 were grown in the presence of glucose (Carvalho *et al.*, 2011). Expression of the lantibiotic
405 gene cluster has also been shown to be inhibited by the CiaRH two-component regulatory
406 system (Mascher *et al.*, 2003); although at this time there is no data to suggest that CiaRH
407 mediates catabolite control of this gene cluster. These data indicate that the production of the
408 lantibiotic biosynthesis machinery is part of the carbon-catabolite response of *S. pneumoniae*,
409 and is the first example, to our knowledge, of a lantibiotic incorporated into the carbon-catabolite
410 regulon.
411 The differential regulation of the TprA-regulon based on available carbon source is
412 interesting when placed in the context of where the pneumococcal cells may encounter glucose
413 or galactose in the human host. Glucose is the preferred carbon source for pneumococcal cells
414 and found in large amounts in the bloodstream and respiratory tract of the host, areas which are
415 normally low in bacterial counts (Philips *et al.*, 2003). Galactose, in contrast, is one of the main
416 carbon sources that pneumococcal cells encounter in the nasopharynx during colonization
417 where they need to compete with other strains of *S. pneumoniae* and other bacterial species for

418 space and resources (King, 2010, Yesilkaya *et al.*, 2008). These data are consistent with recent
419 Tn-Seq analysis that showed that transposon insertions in *tprA* or in some of the genes of the
420 lantibiotic biosynthesis operon significantly reduced the fitness of *S. pneumoniae* serotype 4
421 strain TIGR4 for nasopharynx colonization in a murine model (van Opijnen & Camilli, 2012).
422 Thus, the TprA/PhrA system and its controlled lantibiotic genes are important for *S. pneumoniae*
423 to colonize the nasopharynx.

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

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

439 **EXPERIMENTAL PROCEDURES**

440 **Bacterial strains and growth conditions**

441 Strains used in this study are derivatives of encapsulated, virulent serotype 2 strain D39
442 (Table S5). Details of mutant strains and constructs generated for this work are found in the
443 *Supplemental Information.* Pneumococcal strains were grown at 37°C with 5% CO_2, either on
444 Trypticase Soy Agar II plates (modified; Becton-Dickinson) with 5% (vol/vol) defibrinated sheep
445 blood (TSAII-BA), in Becton-Dickinson brain heart infusion (BHI) broth, or in chemically defined
446 media (Kazmierczak *et al.*, 2009) with 1% glucose (wt/vol) (CDM-glucose) or 1% (wt/vol)
447 galactose (CDM-galactose) as the sole carbon source. Liquid cultures were grown statically
448 and monitored by optical density at 620 nm (OD_{620}) using a Spectronic 20 or a Teysche 100
449 spectrophotometer. Starter cultures of pneumococcal strains were inoculated from frozen
450 glycerol stocks into BHI broth, serial diluted in the same media, and propagated for 8-16 hours.
451 For growth in BHI broth, starter cultures still in exponential phase (OD_{620} of 0.1 to 0.4) were
452 diluted to an OD_{620} of 0.002 to 0.005 to start final cultures. For the overexpression of PhrA, the
453 final cultures were grown in BHI broth that contained 1% (wt/vol) L-fucose. For growth in CDM-
454 glucose and CDM-galactose, the cells of starter cultures were collected by microcentrifugation
455 at 3000 x *g* for 10 minutes at room temperature. The cells were washed, resuspended, and
456 diluted in CDM-glucose or CDM-galactose to an OD_{620} of 0.001 to 0.005 to start final cultures.
457 For antibiotic selections, TSAII-BA plates or BHI broth cultures were supplemented with 0.3
458 μg/ml erythromycin, 250 μg/ml kanamycin, 250 μg/ml streptomycin, or 0.25 μg/ml tetracycline.
459

460 **β-galactosidase assays**

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

473

474 **RNA extraction**

475 To isolate RNA for qRT-PCR analysis, starter cultures were used to inoculate 6 ml
476 cultures of BHI broth or CDM-galactose. RNA was extracted from 4 mL of exponentially growing
477 cultures (OD_{620} of ~0.2) using a hot-lysis, acid-phenol extraction followed by purification using an
478 RNeasy minikit (Qiagen) and on-column DNase I treatment as described in (Barendt *et al.*,
479 2009, Kazmierczak *et al.*, 2009, Ramos-Montanez *et al.*, 2008). 5 µg of total RNA was further
480 digested with DNase using a DNA-free kit (Ambion) prior to qRT-PCR analysis. To isolate RNA
481 for RNA-sequencing analysis, starter cultures were used to inoculate 30 ml cultures of BHI broth
482 in 50 mL conical tubes. RNA was extracted from 23 mL of exponentially growing culture (OD_{620}
483 of ~0.15) using the FastRNA Pro Blue Kit (MP Bio) according to the manufacturer's guidelines.
484 Briefly, cells were isolated by centrifugation at 14,500 x *g* for 5 minutes at 4℃. The cells were
485 resuspended in 1 ml RNA*pro* (MP Bio) and processed twice in the Fast Prep Instrument (MP
486 Bio) for 40 seconds at a setting of 6.0. Chloroform and 100% ethanol were used to extract and
487 precipitate the RNA from the resulting lysate and the miRNeasy minikit (Qiagen) and on-column
488 DNase I treatments were used to purify the RNA as above. The amount and purity of all RNA
489 samples isolated were assessed by NanoDrop spectroscopy (Thermo Fisher). RNA integrity
490 was assessed using the Agilent 2100 BioAnalyzer (Aligent Technologies).
491
492 **qRT-PCR**
493 qRT-PCR was performed using a two-step protocol as in (Ramos-Montanez *et al.*, 2008,
494 Kazmierczak *et al.*, 2009). Specifically, cDNA was synthesized from 100 ng of total RNA and
495 random primers using the qScript Flex cDNA Kit (Quanta BioSciences). RT-PCR was performed
496 using the Brilliant SYBR Green qPCR Master Mix (Stratagene), the Brilliant III Ultra-Fast SYBR
497 Green qPCR Master Mix (Agilent), or the FastStart Universal SYBR Green Master Mix (Roche)
498 and appropriate primers (see Table S6) as in (Kazmierczak *et al.*, 2009, Ramos-Montanez *et al.*,
499 2008). Reactions were performed in duplicate and normalized to 16S rRNA amounts. The 16S
500 rRNA was quantified using the same cDNA samples except that the samples were diluted 100-

501 fold further. Data were collected on an MX3000P thermocycler (Stratagene) or on a CFX96
502 thermocycler (Bio Rad) and analyzed with the SYBR Green (with dissociation curve) program
503 associated with each machine. Four dilutions of cDNA from *S. pneumoniae* strains wild-type for
504 *tprA* and *phrA* (either IU1781 or Spn049) were used to generate standard curves for each
505 primer set. Normalized transcript amounts were compared as indicated by performing pairwise
506 unpaired two-tailed t tests.
507
508 **Synthetic peptides**
509 Peptides were synthesized on a modified Applied Biosystems 430A peptide synthesizer
510 using 0.2 mmol of 4-hydroxymethyl-phenylacetamidomethyl (PAM) resin (Midwest Biotech) and
511 subsequently purified using reverse phase HPLC (RP-HPLC). For complete details, see
512 *Supplementary Information*. Synthetic peptides were resuspended in 10 mM Tris pH 7.4 with 50
513 µg/ml BSA to a concentration of 5 mM. For β-galactosidase or qRT-PCR assays, the indicated
514 synthetic peptide (or the peptide-resuspension buffer) was added at final concentration of 5 µM
515 to each 6 ml cell aliquot.
516
517 **Library construction and RNA-sequencing**
518 cDNA libraries were prepared from total RNA by the University of Wisconsin-Madison
519 Biotechnology Center. The mRNA was enriched from two micrograms total RNA using
520 RiboZeroTM rRNA Removal (Gram-positive bacteria) Kit (EpiCentre Inc.). rRNA-depleted mRNA
521 samples were purified by ethanol precipitation and quantified by fluorometry with the Qubit®
522 RNA assay kit (Invitrogen). Double stranded cDNA synthesis was performed following
523 ScriptSeqTM v2 RNA-Seq Library Preparation guide (EpiCentre Inc.) in accordance with the
524 manufacturer’s standard protocol. Thirty nanograms of enriched mRNA were fragmented using
525 divalent cations via incubation for 5 min at 85℃. The first strand of cDNA was synthesized by

526 reverse transcription using random-sequence primers containing a tagging sequence at their 5'
527 ends. Di-tagged cDNA was synthesized by random annealing of a terminal-Tagging Oligo (TTO)
528 to the 3' end of the cDNA for extension of the cDNA by DNA polymerase. Di-tagged cDNA was
529 purified using Agencourt AMPure® XP beads (Beckman Coulter) followed by PCR amplification
530 for 15 cycles using FailsafeTM PCR enzyme and ScriptSeq Index DNA primer set (EpiCentre
531 Inc.). This step generated the second strand of cDNA and completed the addition of Illumina
532 adapter sequences incorporating a user-defined barcode. The amplified libraries were purified
533 using Agencourt AMPure® XP beads. Quality and quantity were assessed using an Agilent DNA
534 1000 chip (Agilent) and Qubit® dsDNA HS assay kit (Invitrogen), respectively. Libraries were
535 standardized to 2 µM. Cluster generation was performed using standard Cluster kits (v3) and
536 Illumina Cluster Station. Single-end 100 bp sequencing was performed using standard SBS
537 chemistry (v3) on an Illumina HiSeq2000 sequencer. Images were analyzed using the standard
538 Illumina pipeline, version 1.8.2.
539
540 **RNA-seq analysis**
541 The raw sequencing reads were quality and adapter trimmed using Trimmomatic (Lohse
542 *et al.*, 2012) with a minimum length of 90. The trimmed reads were mapped on the
543 *Streptococcus pneumoniae* D39 (RefSeq NC_008533) genome and D39 plasmid pDP1
544 sequence (RefSeq NC_005022) using bowtie2 (Langmead & Salzberg, 2012). Custom PERL
545 scripts were used to generate read counts for the genes and 100 bp non-overlapping intergenic
546 regions of the genome. Differential gene expression was identified using EdgeR (version 3.6.2)
547 using default parameters (Robinson *et al.*, 2010). The false discovery rate (FDR) was
548 calculated using Benjamini and Hochberg's algorithm (Benjamini & Hochberg, 1995) and a gene
549 or region was defined as differentially expressed if it had an up- or down-fold change of 2.0 and
550 their FDR was less than 0.001.
551

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791

792

793 **Table 1**. Changes in relative transcript amounts caused by Δ*tprA* and Δ*phrA* mutations or the addition of the 10-residue synthetic
794 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		

795 [a] Strain construction, growth and RNA-Seq analysis are described in Experimental procedures. RNA was prepared from
796 exponential cultures grown in BHI media at 37°C to OD_{620} ≈0.15 to 0.2. The Log_2 of the fold changes and FDR values are based on
797 three independent biological replicates. Cut-offs for this table were 2.0-fold change and FDR value < 0.001. Empty boxes indicate
798 that there was not a significant change in expression of a gene under one of the four conditions.
799 [b] The RNA transcript levels from strain IU1781 treated with 10 µM 10-residue synthetic PhrA compared to strain IU1781
800 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
801 harvesting.
802 [c] The RNA transcript levels from Δ*tprA* mutant strain IU4955 were compared to the RNA transcripts from an isogenic strain IU1781.
803 [d] The RNA transcript levels from Δ*tprA* Δ*phrA* mutant strain IU6118 were compared to the RNA transcripts from an isogenic strain
804 IU1781.
805 [e] The RNA transcript levels from Δ*phrA* mutant strain IU4957 were compared to the RNA transcripts from an isogenic strain IU1781.
806 [f] FDR is the false-discovery rate (Benjamini & Hochberg, 1995).

807 **FIGURE LEGENDS**

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

809 (A) The effect of Δ*tprA* and Δ*phrA* mutations on *phrA-lacZ* expression. Strains: Spn007, “wild-
810 type” parental strain used in this experiment; Spn013, Δ*tprA*; Spn019, Δ*phrA*; Spn195, Δ*tprA*
811 CEP::TprA (complemented strain). Results shown are averages of 2-5 independent experiments
812 and error bars depict the standard error of the mean. ***, significant at $P < 0.001$ compared to
813 “wild type.” +++, significant at $P < 0.001$ compared to Δ*tprA*. (B) *phrA* mRNA levels in a Δ*tprA*
814 mutant strain. Strains: IU1781 & Spn049, “wild-type” parental strains used in this experiment;
815 Spn052, Δ*tprA*; Spn197, Δ*tprA* CEP::TprA (complemented strain). mRNA levels were
816 normalized to 16S RNA levels, from 2 independent experiments, and are shown as a ratio
817 relative to the wild-type levels. Error bars depict the standard error of the mean. *, significant at
818 $P < 0.05$ compared to “wild type.” +, significant at $P < 0.05$ compared to Δ*tprA*. Different parent
819 strains were used in these approaches containing wild-type alleles for the genes of interest.

820

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

822 (A) *phrA-lacZ* reporter expression is elevated when the full length *phrA* gene is overexpressed.
823 Strains: Spn065, full length PhrA; Spn191, PhrAΔ42-56 ; Spn189, PhrAΔ47-56 ; Spn187,
824 PhrAΔ52-56 ; Spn243, PhrAΔ56 . Cells were grown in BHI or BHI+1% fucose (inducer) to mid-
825 exponential phase (OD_{620} of between 0.15 to 0.35) when samples were removed for β-
826 galactosidase activity assays. Results shown are the averages of at least 3 independent
827 replicates and error bars indicate the standard error of the mean for each set. ***, significant at
828 $P < 0.001$ compared to uninduced strain containing the full-length PhrA construct. (B) Synthetic
829 peptides corresponding to the C-terminus of PhrA used in (C) below. (C) Induction of the *phrA-*
830 *lacZ* reporter was observed when cells were treated with the last 6, 7, or 10 amino acids of
831 PhrA. Early exponential phase (OD_{620} of ~0.1) wild-type cells (Spn007) were incubated with

832 synthetic peptides at a final concentration of 5 µM or peptide-resuspension buffer for two hours
833 prior to analysis by β-galactosidase assays. Results shown are the averages of at least 3
834 independent replicates and error bars indicate the standard error of the mean for each set. *,
835 significant at $P < 0.05$ and **, significant at $P < 0.01$ compared to the "wild type" strain incubated
836 with buffer.
837
838 **Fig 3. Oligopeptide permease is required for induction of *phrA-lacZ* in response to**
839 **synthetic peptide.**
840 Strains lacking *amiC* in a wild-type or a Δ*tprA* mutant background were tested for their ability to
841 induce *phrA-lacZ* expression in response to the 10-residue PhrA peptide. Strains: Spn007, "wild-
842 type" parental strain used in this experiment; Spn013, Δ*tprA*; Spn141, Δ*amiC*; Spn165, Δ*tprA*
843 Δ*amiC*. Early exponential phase cells (OD_{620} of ~0.1) grown in BHI were incubated with 5 µM
844 synthetic peptide or peptide-resuspension buffer for two hours prior to analysis by β-
845 galactosidase activity assays. Results shown are the average of at least two independent trials,
846 and error bars represent the standard error of the mean. ***, significant at $P < 0.001$ compared
847 to the strain treated with buffer.
848
849 **Fig 4. PhrA can signal between cells when grown to high cell density in media containing**
850 **galactose.**
851 Cells (Spn007, wild-type) grown in CDM-glucose (closed squares) or CDM-galactose (open
852 circles). Panel A shows a representative growth curve of these cells on these media. Note that
853 after inoculation of the cultures several hours pass before there is a measurable level of cells,
854 and this lag phase is longer in CDM-galactose. Panel B shows expression of *phrA-lacZ* in the
855 Spn007 cells. At least two independent experiments were performed; the results from one
856 representative experiment are shown. Panel C shows induction of *phrA-lacZ* when these were

857 resuspended in conditioned media from wild-type cells (IU1781) compared to untreated media,
858 and no induction was observed in conditioned media from cells lacking *phrA* (IU4957). The
859 results shown are the average of at least two independent trials and the error bars depict the
860 standard error of the mean. *, significant at $P < 0.05$ compared to untreated media. +, significant
861 at $P < 0.05$ compared to wild-type conditioned media.

862

863 **Fig 5. The TprA/PhrA system regulates a putative lantibiotic biosynthesis operon.**
864 ORFs are represented by dark grey arrows (in the case of the TprA/PhrA system) or light gray
865 arrows (for the putative lantibiotic biosynthesis genes) and the D39 gene identification numbers
866 are indicated in the arrows, with genes spd1747 and spd1748 shorten to 47 and 48,
867 respectively. Known or predicted functions of each gene are indicated above the arrows, and --
868 indicates that the function of the genes is unknown. The putative promoters, predicted by the
869 results with RNA-seq, are represented by bent black arrows, and putative promoters predicted
870 by the DOOR database are shown as bent gray arrows. Small black boxes are predicted CRE-
871 binding sites of CcpA (Carvalho *et al.*, 2011). Genes whose expression has been found to be
872 increased in either a Δ*tprA* mutant or by the addition of the PhrA peptide are denoted by + under
873 the gene.

874

875 **Fig 6. Model for the mechanism by which PhrA and TprA control gene expression in *S.***
876 ***pneumoniae*.**
877 The mature PhrA peptide is encoded by *phrA* producing a precursor protein in the absence of
878 glucose. Glucose repression occurs through a CRE element that is in *phrA* promoter region.
879 The PhrA precursor is exported and processed to release the mature PhrA peptide (dark gray,
880 small ovals). When at a sufficient concentration, the PhrA peptide interacts with oligopeptide
881 permease and is transported into the cell where it inhibits the activity of TprA leading to de-
882 repression of *phrA, tprA,* and a change in transcription of lantibiotic genes (wide arrows). Only

883 three of the eight lantibiotic biosynthesis cluster genes are shown here for simplicity (black
884 outlined, wide arrows with one not shown to scale (angled lines)). Bent arrows indicate the
885 location of data-supported (black) or predicted (gray) promoters, all of which are negatively
886 regulated by TprA (denoted by lines that end with a horizontal line).