Pneumococcal attachment to epithelial cells is enhanced by the secreted peptide VP1 via its control of hyaluronic acid processing

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9 ABSTRACT

10 The Gram-positive bacterium *Streptococcus pneumoniae* (pneumococcus) is an
 11 important human pathogen. It can either asymptotically colonize the nasopharynx or spread
 12 to other tissues to cause mild to severe diseases. Nasopharyngeal colonization is a
 13 prerequisite for all pneumococcal diseases. We describe a molecular pathway utilized by
 14 pneumococcus to adhere to host cells and promote colonization. We demonstrate that the
 15 secreted peptide VP1 enhances pneumococcal attachment to epithelial cells. Transcriptional
 16 studies reveal that VP1 triggers the expression of operons involved in the transport and
 17 metabolism of hyaluronic acid (HA), a glycosaminoglycan present in the host extracellular
 18 matrix. Genetic experiments in the pneumococcus reveal that HA processing locus (HAL)
 19 promotes attachment. Further, overexpression of HAL genes in the $\Delta vp1$ background, reveal
 20 that the influence of VP1 on attachment is mediated via its effect on HA. In addition, VP1 also
 21 enhances degradation of the HA polymer, in a process that depends on the HAL genes. siRNA
 22 experiments to knockdown host HA synthesis support this conclusion. In these knockdown
 23 cells, attachment of wild-type pneumococci is decreased, and VP1 and HAL genes no longer
 24 contribute to the attachment. Finally, experiments in a murine model of colonization reveal that
 25 VP1 and HAL genes are significant contributors to colonization. Our working model, which
 26 combines our previous and current work, is that changes in nutrient availability that influence
 27 CodY and Rgg144 lead to changes in the levels of VP1. In turn, VP1 controls the expression of
 28 a genomic region involved in the transport and metabolism of HA, and these HAL genes
 29 promote adherence in an HA-dependent manner. VP1 is encoded by a core gene, which is
 30 highly induced *in vivo* and is a major contributor to host adhesion, biofilm development,
 31 colonization, and virulence. In conclusion, the VP1 peptide plays a central role in a pathway

32 that connects nutrient availability, population-level signaling, adhesion, biofilm formation,
33 colonization, and virulence.

34 AUTHOR SUMMARY

35 *Streptococcus pneumoniae* (the pneumococcus) is a major human pathogen. This
 36 bacterium asymptotically colonizes the human upper respiratory tract from where it can
 37 disseminate to other tissues causing mild to severe disease. Colonization is a prerequisite for
 38 dissemination and disease, such that the molecules that control colonization are high-value
 39 candidates for therapeutic interventions. Pneumococcal colonization is a population-level
 40 response, which requires attachment to host cells and biofilm development. VP1 is a signaling
 41 peptide, highly induced in the presence of host cells and *in vivo*, promotes biofilm
 42 development, and serves as a potent virulence determinant. In this study, we build on the
 43 molecular mechanism of VP1 function to reveal novel bacterial and host molecules that
 44 enhance adherence and colonization. Our findings suggest that host hyaluronic acid serves as
 45 an anchor for pneumococcal cells, and that genes involved in the transport and metabolism of
 46 HA promote adherence. These genes are triggered by VP1, which in turn, is controlled by
 47 regulators that respond to nutrient status of the host. Finally, our results are strongly supported
 48 by studies in a murine model of colonization. We propose that VP1 serves as a marker for
 49 colonization and a target for drug design.

INTRODUCTION

The Gram-positive bacterium *Streptococcus pneumoniae* (also known as the pneumococcus) is an important human pathogen. A recent global study on lower respiratory infections determined that the pneumococcus contributed to morbidity more than all other etiologies combined: it was responsible for an estimated 1.18 million deaths (1,2). This pathogen can either asymptomatically colonize the nasopharynx or spread to other tissues to cause mild to severe diseases. It can spread to the middle ear and sinus, leading to otitis media or sinus infections, and the lungs causing pneumonia (3). It can also disseminate into the bloodstream, brain or heart causing sepsis, meningitis, or heart disease, respectively (4,5). It is well established that nasopharyngeal colonization is a prerequisite for all pneumococcal diseases. In this study, we explore the mechanisms of colonization and virulence.

Pneumococcus secretes many small peptides, which influence colonization, virulence, and adaptation via effects on competence, intra-species competition, and biofilm development (6–18). The **Virulence Peptide 1** (VP1) is a member of a family of peptides with a conserved N-terminal sequence characterized by a double glycine motif, which directs its export into the extracellular milieu via ABC transporters (7,19). The *vp1* gene is widely distributed across pneumococcal strains, as well as encoded in related streptococcal species. The gene encoding VP1 is a virulence determinant and is highly upregulated in the presence of host cells where it promotes biofilm development (7,20). Pneumococcal biofilms have been documented in the nasopharynx, the middle ear, and the sinus (21–24). This mode of growth enhances pneumococcal dissemination and pathogenesis (25,26). Further, biofilms promote pneumococcal colonization in multiple ways: they facilitate immune evasion, increase resistance to antimicrobials, and provide a platform for DNA acquisition (27–29,29–32). In

summary, VP1 is a secreted peptide, induced in the presence of human cells, that promotes biofilm development and virulence.

Glycosaminoglycans (GAGs) are major components of the extracellular matrix. GAGs are acidic linear polysaccharides with a repeated disaccharide unit. The disaccharides consist of an amino sugar (*N*-acetylglucosamine or *N*-acetylgalactosamine) and a uronic acid (glucuronic acid or iduronic acid) or galactose. These polymers are classified into groups based on the nature of the disaccharides, the mode of glycoside bond, and the sulfation levels (33). The main groups are heparin and heparan sulfates (HS), chondroitin and dermatan sulfate (CS), keratan sulfate, and finally hyaluronic acid (HA) (also known as hyaluronate or hyaluronan) (34). Their molecular weights range from 15 to over 100 kDa, and their synthesis takes place in the Golgi apparatus, with the exception of HA, which is synthesized at the membrane (35). GAGs participate in numerous biological processes of the host and the bacteria. In the host, they influence cell adhesion and growth, cell proliferation and differentiation, and tissue formation (for comprehensive reviews, please see Iozzo and Schaefer, 2015; Pomin and Mulloy, 2018; Taylor and Gallo, 2006). In the context of microbial infections, they serve as sources of nutrients and modulators of the immune response (38–41). In addition, many bacteria utilize GAGs to adhere to host cells (42). This includes the pneumococcus, where treatment of lung cells to decrease HS and CS levels reduces pneumococcal adherence (43). In this manner, GAGs are critical components of the bacterial environment during biofilm formation and host interactions.

HA consists of disaccharides of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcUA). This GAG is present on the apical surface of human bronchial epithelial cells and in the airway mucosa (44,45). It is also abundant in the synovial fluid, skin, umbilical cord, and

99 vitreous body and is the only non-sulfated glycosaminoglycan in the lung (46–48). This
100 unbranched polysaccharide provides mechanical support, activates immunity, and modulates
101 cell proliferation, migration, and intracellular signaling within the host (49–51). Many
102 streptococcal genomes encode multiple operons for HA transport and metabolism. In the
103 pneumococcus, it has been shown that HA processing and internalization is carried out by a
104 unique set of enzymes including a hyaluronidase, a transporter system, and a hydrolase
105 (52,53). The current model suggests that HA is digested into disaccharides by a hyaluronidase
106 (*hysA* or *hylA*), predicted to be covalently linked to the cell wall via an LPxTG motif. The
107 disaccharides are imported into the bacteria and phosphorylated via a phosphoenolpyruvate-
108 dependent phosphotransferase system (PTS) system. The PTS system is composed of
109 general and substrate-specific components. Enzyme I (EI) and the histidine-containing
110 phosphocarrier protein (HPr) are cytoplasmic subunits, shared by multiple PTS systems. In
111 contrast, Enzyme II (EII) provides specificity to the PTS via substrate recognition. EII has four
112 enzymes: EIIA and EIIB are cytosolic, and EIIC and EIID are the transmembrane components
113 and form a pore. During internalization via the PTS, the HA disaccharides are phosphorylated.
114 These disaccharides are processed by an unsaturated glucuronyl hydrolase (*ugl*) into the
115 monosaccharide components. Finally, the GlcUA can be further processed into glyceraldehyde
116 3-phosphate and pyruvate by a set of four genes (the isomerase *kduL*, the reductase *kduD*,
117 the kinase *kdgK*, and the aldolase *kdgA*). Operons for the hydrolase, the PTS-EII system, and
118 the GlcUA metabolism enzymes are all adjacent in the genome, and under the control of
119 RegR, a negative regulator located immediately downstream (54). Note that the unsaturated
120 glucuronyl hydrolase not only cleaves HA into GlcNAc and GlcUA, it can also cleave
121 chondroitin sulfate into their component monosaccharides (53,55). The HA pathway has been
122 shown to influence pneumococcal biology. Specifically, *hysA* and the genes in the PTS-EII-

encoding operon (EIIB, EIIC, EIID, and *ugl*), are required for pneumococcal growth on human HA, and contribute to pneumococcal biofilm formation and virulence (54,56,57).

In this study, we show that VP1 promotes pneumococcal adherence via activation of HA transport and metabolism. Our experiments in a murine model of pneumococcal carriage demonstrate that both VP1 and HA processing promote colonization. We propose a model where the nutritional status of the host induces expression of the pneumococcal secreted peptide VP1, via CodY and Rgg144 (7,18,58). Signaling by VP1 triggers HA metabolism in the bacteria, which in turn, regulates attachment to host cells and colonization in the upper airways.

RESULTS

***vp1* enhances pneumococcal attachment to epithelial cells**

We have previously shown that *vp1* plays a role in biofilm development (7). Thus, we investigated whether *vp1* influences attachment to epithelial cells, an early step in biofilm formation. For these studies, we utilized strain PN4595-T23, a representative of the drug-resistant and pandemic PMEN1 pneumococcal lineage (59–62). To show that the results extend beyond one lineage, we also employed TIGR4, a clinical isolate frequently used as a model strain (63). For host cells, we utilized the human lung epithelial cells line A549. These cells have been extensively used to study pneumococcal behavior (20,64,65). Early works demonstrated that A549 cells display features of type II alveolar pneumocytes: they are capable of surfactant production (66–68), they produce cell surface-associated MUC1 and secrete MUC5AC mucin in air-liquid cultures (69,70), and they produce HA. Further, our experiments on adhesion are extended to NIH-3T3 fibroblasts as they express HA and chinchilla middle ear epithelial cells (CMEE) as these primary cells were used in the original characterization of VP1 function (7,71).

We established that the wild-type strain displays strong attachment to A549 cells after one hour of exposure (Fig S1A). Thus, we used this time point to compare adhesion between wild-type, *vp1* deletion mutant ($\Delta vp1$) and *vp1* complement strains ($\Delta vp1:vp1$) by using spot plating assays and enumerating the total bound bacteria on TSA plates. The $\Delta vp1$ displays a reduction in attachment compared to the wild-type cells. Furthermore, the wild-type phenotype is rescued in a *vp1* complement strain ($\Delta vp1:vp1$) (Fig 1A). These data suggest that VP1 promotes attachment to the lung epithelia. This role for the *vp1* gene product is not specific to A549 cells, as *vp1* also promotes adherence to chinchilla middle ear epithelial cells (CMEE)

and NIH-3T3 fibroblasts (Figs S1B and S1C). Similarly, these results extend beyond the PMEN1 lineage, deletion of the operon encoding *vp1* (*vpo*) in a TIGR4 background also displays decreased attachment relative to the wild-type strain and this phenotype is restored to wild-type levels in a *vpo* complement strain (Fig S1D). Adherent bacteria were visualized by fluorescence microscopy of A549 cells inoculated with PMEN1 cells (Fig 1B). The confocal orthogonal view shows localization at the surface of A549 cells (Fig S1E). Finally, the difference in attachment is not the result of variation in growth rate between strains, as growth in DMEM media is the same for wild-type, $\Delta vp1$, and $\Delta vp1:vp1$ strains, as previously reported (7). We conclude that *vp1* promotes pneumococcal attachment to mammalian epithelial cells.

The *vp1* product positively regulates expression of HA transport and metabolism genes.

The VP1 pro-peptide possesses a double glycine motif common to secreted peptides that bind surface-bound histidine kinase receptors from pneumococcal two-component signal transduction system (72). Further, synthetic VP1 binds to the pneumococcal surface (7). Thus, we hypothesized that VP1 signals to producing and neighboring cells and induces expression of genes that participate in the attachment.

To test this hypothesis, we compared the genome-wide expression of the wild-type PN4595-T23 strain and the isogenic $\Delta vp1$ mutant using the pangenome pneumococcal array (13) (Fig 2A). Strains were grown in chemically defined media (CDM) supplemented with glucose (CDM-Glu), as *vp1* expression is induced in this media relative to rich media (7). In support of the hypothesis, twenty-eight genes displayed a 3-fold difference in expression between the wild-type strain and the $\Delta vp1$ strain (Fig 2A and S1 Table). Many of these genes

are organized into three genomic regions (Fig 2B). Microarray results were validated by qRT-PCR for a subset of genes (S2 Table). Taken together, we observed a significant number of genes with transcription levels that respond to *vp1*.

Our analysis shows that two neighboring operons display higher expression in the wild-type strain relative to the $\Delta vp1$; these operons have been previously implicated in the processing of HA (53). Together, these two operons encode eleven genes that displayed an average 4.28-fold decrease in expression in the $\Delta vp1$ relative to the wild-type strain (Figs 2B and 3A). One operon is on the positive strand and encodes seven genes. In this study, for simplicity, we refer to this as the PTS-HAL operon as it encodes the four EIIABCD enzymes that form the PTS importer (Fig 3A). This importer is required for phosphorylation and internalization of HA (EII genes are: SPN23F_02950, and SPN23F_02970 to SPN23F_02990). The operon also contains the *ugt* gene that encodes an unsaturated glucuronyl hydrolase (SPN23F_02960), the preprotein translocase YajC that is a subunit of the bacterial holo-translocon (SPN23F_03000) (73), and finally a predicted heparinase (SPN23F_3010). Our analysis with Phyre2 (74), indicates that the heparinase is a glycosidase that belongs to the superfamily of heparinases type II/III; member of this family bind and degrade heparin and heparin sulfate (75). It is noteworthy, that 60 base-pairs downstream of the end of *hep*, is the negative regulator RegR (SPN23F_3020) (76). Analyses of the region upstream of RegR with the promoter identification programs BDPG and BPRM suggests the presence of an active promoter with the -35 within the *hep* sequence (77,78). The second operon regulated by VP1 is immediately upstream of the PTS-HAL operon on the negative strand, it encodes four enzymes predicted to metabolize glucuronic acid into glyceraldehyde-3 phosphate and

pyruvate (SPN23F_02910 to SPN23F_02940). These transcriptional findings suggest that *vp1* triggers the expression of genes implicated in the transport and metabolism of HA.

The *vp1* product promotes degradation of the HA polymer via its control of the HA processing and transport operon.

Once we established that VP1 stimulates expression of multiple genes involved in HA metabolism, we tested whether VP1 could influence the processing of the HA polymer. To this end, we measured the hyaluronidase activity in pellets and supernatants of the PN4595-T23 strain carrying deletions of *vp1*, PTS-HAL, and various genes involved in HA processing (Fig 3B). In this assay, bacterial pellets or supernatants were mixed with the HA polymer, degradation of HA was measured as a change in optical density at OD₄₀₀ and reported as hyaluronidase concentration (76). As controls, we employed the $\Delta hysA$, as this gene encodes for a hyaluronidase and its deletion should result in reduced hyaluronidase relative to the wild-type strain (53,79). As predicted, this strain displayed 2.6-fold lower hyaluronidase levels ($p < 0.0001$ relative to the wild-type strain). We also employed a $\Delta regR$ mutant, as this gene encodes for a repressor of *hysA* and its deletion should lead to increased hyaluronidase activity (54). The $\Delta regR$ strain exhibited elevated hyaluronidase activity ($p = 0.0006$). The $\Delta vp1$ strain displayed 1.7-fold lower hyaluronidase compared to the wild-type strain ($p < 0.0001$). This defect was rescued in the $\Delta vp1:vp1$ ($p < 0.0001$ relative to $\Delta vp1$ strain). The $\Delta PTS-HAL$ mutant and the $\Delta vp1-\Delta PTS-HAL$ double mutant showed a 1.4- and 1.5-fold reduction in hyaluronidase relative to the wild-type strain ($p < 0.0005$). This reduction was similar to the $\Delta vp1$ strain compared to the wild-type strain. The majority of the hyaluronidase activity was captured within the pellets, suggesting that the majority of the degradation enzymes are

associated with the cell and not secreted into the supernatant. These data suggest that *vp1* and the PTS-HAL processing and transport operon promote degradation of HA.

To determine whether the ability to break down HA is conserved beyond the PMEN1 strain PN4595-T23, we performed the same set of experiments in a TIGR4 background. The locus in TIGR4 resembles that of the PMEN1 strain, with the addition of a predicted transposon at the end of the operon (see graphic Fig S2A). The results were similar in that the genes encoded by the *vp1* operon (*vpo*) and HA processing and transport operon (PTS-HAL) contributed to the degradation of the HA polymer. In contrast to PN4595-T23, the majority of the TIGR4 activity was localized to the supernatant (Fig S3C), suggesting differences in the subcellular localization of the hyaluronidase activity between strains.

Finally, to investigate whether VP1 exerts its influence via genes encoded in the HA processing and transport operon, we tested whether overexpression of the PTS-HAL operon in a $\Delta vp1$ background could rescue the wild-type phenotype in both TIGR4 and PN4595-T23 strains. To this end, using the $\Delta vp1$ backgrounds, we replaced the native promoter of PTS-HAL with a constitutive promoter (from *amiA* gene) (strain $\Delta vp1$ -OE PTS-HAL)(80). These strains displayed HA degradation comparable to the isogenic wild-type strains ($p = 0.21$) (Fig 3B and S2C). These data suggest that *vp1* exerts its effect on HA metabolism via control of the gene products encoded in the PTS-HAL operon.

Pneumococcus attaches to host HA in a process controlled by VP1 and mediated by molecules involved in HA processing.

We have demonstrated that the *vp1* product enhances attachment of pneumococci to lung epithelial cells (Fig 1), and promotes transcription and activity of genes in the HA processing and transport operon associated with HA metabolism (Figs 2 and 3). Thus, we hypothesized that *vp1* influences attachment via its effect on the PTS-HAL operon. To test this hypothesis, we performed attachment assays in a strain with a deletion in the PTS-HAL operon (Δ PTS-HAL); it displayed decreased attachment relative to the wild-type strain (Fig 4A). Moreover, the attachment defect observed in the Δ *vp1* mutant was rescued to wild-type levels in the Δ *vp1*-OE PTS-HAL strain where the genes in the PTS-HAL operon are expressed from a constitutive promoter. These data strongly suggest that the products of *vp1* and PTS-HAL promote adhesion and that the effect of *vp1* is exerted via its influence on PTS-HAL. Further, attachment levels for the double mutant Δ *vp1* Δ PTS-HAL resembled that of the Δ PTS-HAL strain consistent with these gene products acting within the same pathway (Figs S3A and S3B). The Δ *regR* strain, with increased levels of expression for genes encoded in the PTS-HAL operon, resembled wild-type levels. The Δ *hysA* strain displayed similar attachment to the wild-type strain, suggesting that genes in PTS-HAL are not promoting attachment via indirect effects on HysA (Fig S3A). The attachment of the wild-type and mutant strains were also visualized by confocal microscopy, and representative images are displayed in Fig 4B. Together, these data demonstrate that the *vp1* promotes attachment via its control of the genes in the PTS-HAL operon, responsible for processing and transport of HA.

HA production in mammalian cells is controlled by at least three different, membrane-anchored hyaluronan synthases: *HAS1*, *HAS2*, and *HAS3* (35). Previous transcriptomic studies have reported that A549 cells exclusively express *HAS2* and *HAS3*, while *HAS1* remains undetectable (81). Our qPCR analysis corroborates these findings (Fig S3C). To

evaluate whether the HA produced by the A549 cells is implicated in pneumococcal attachment, we employed siRNA to knockdown the expression of *HAS2* and *HAS3* (independently and together) on A549 cells, and 48 h later, assessed the attachment strength of the wild-type strain. As a control for treatment, we used a scrambled construct for siRNA experiments, as well as *HAS1* because it has the low expression levels in A549 (Fig S3C and as described by Chow et al., 2010). At the 48h time point, the siRNA treatment reduced levels of *HAS2* and *HAS3* to below 20% when compared to the scrambled control (Fig S3D). Our attachment assays revealed a significant reduction in pneumococcal attachment to host cells where *HAS2* or *HAS3* were knockdown, with maximum reduction in the double knockdown cells (Fig 4C). These data suggest that pneumococcus binding is enhanced in the presence of host HA.

If products of the *vp1* gene and the PTS-HAL operon exert their influence on attachment via HA, a dramatic decrease in host HA levels should reduce the effect of these pneumococcal molecules on host attachment. Consistently, the $\Delta vp1$ strain displayed similar levels of attachment on host cells independent of the levels of *HAS2* *HAS3*. Further, the Δ PTS-HAL strain also displayed the same levels of attachment in host cells regardless of the levels of *HAS2* and *HAS3* (Fig 4C). Moreover, attachment of the Δ PTS-HAL strain resembled levels of a wild-type strain on cells with reduced *HAS2* and *HAS3*, that is, substantially lower than attachment to wild type cells. Finally, a strain overexpressing the PTS-HAL operon in the $\Delta vp1$ background attached better in host cells that express HA. Thus, pneumococcus binding is enhanced by host HA, in a process that depends on the products of *vp1* and PTS-HAL.

The influence of HA in promoting pneumococcal binding is further corroborated by an experiment with exogenous HA. In one experiment, the addition of exogenous HA diminishes binding to A549, consistent with bacteria binding to detached HA (Fig S3E). In a second experiment, we coated 6-well plates with HA from *S. equis* (Fig S4A). A spot assay revealed that pneumococcal attachment was substantially higher on a surface coated with HA, relative to those without HA (Fig S4B). As observed on cells, the levels of attachment for $\Delta vp1$ or $\Delta PTS\text{-}HAL$ strains were lower than that of the wild-type strain in HA-coated surfaces, and overexpression of the PTS-HAL operon in the $\Delta vp1$ restored attachment to wild-type levels (Fig 4D). Moreover, these genetic changes had no significant effect on binding on surfaces that were not treated with HA. All together, these data demonstrate that the products of genes involved in the HA processing and transport promote the binding of pneumococcus to host HA in a process regulated by VP1.

VP1 and the HA processing and transport operon promote pneumococcal colonization

To establish the role of VP1 and of the PTS-HAL operon in pneumococcal survival in the nasopharynx, we employed the murine model of colonization. Cohorts of five mice were inoculated with single strains, using an inoculation dose of 20 μ l, where the wild-type strain colonizes the nasopharynx, but not the lungs (83,84). The bacterial load was measured two hours and seven days post-inoculation. Enumeration of pneumococci in the nasopharyngeal lavages two hours post-infection did not show any difference in bacterial load among the strains, indicating the accuracy of infection and the consistency in obtaining nasopharyngeal content (Fig 5A). Throughout the course of infection, the number of recovered wild-type pneumococci remained constant (Figs 5A and 5b).

Samples recovered seven days post-inoculation revealed significant differences in colony counts across the pneumococcal strains (Fig 5B). Our previous work, in the chinchilla model of pneumococcal disease, established that *vp1* is a virulence determinant based on the reduced mortality and decreased dissemination associated with the $\Delta vp1$ strain relative to the wild-type strain (7). Akin to the chinchilla model, the $\Delta vp1$ strain displayed a dramatic decrease in the murine model, where four out of five animals cleared the infection ($p < 0.0001$). Complementation of *vp1* ($\Delta vp1:vp1$) partially rescued the wild-type phenotype. The difference between wild-type and complement ($\Delta vp1:vp1$) is likely due to changes in the dose driven by variation of the promoter. These data suggest that VP1 contributes to infection in multiple tissue types, as well as in carriage and disease.

By the seventh day post-inoculation, we also detected a significant reduction in colony counts in the ΔPTS -HAL and the double $\Delta vp1$ - ΔPTS -HAL mutants compared to the wild-type strain ($p < 0.0001$). Moreover, overexpression of the PTS-HAL operon in the $\Delta vp1$ background significantly increased the recovered pneumococci compare to $\Delta vp1$ ($p < 0.001$). Yet, overexpression of PTS-HAL in the wild-type background had no effect ($p = 0.542$). The observation that the PTS-HAL operon can compensate for the absence of *vp1* is consistent with the model where VP1 acts upstream of HA processing (Figs 5 and 6). These results strongly suggest that VP1 and HA transport and metabolism play a critical role in pneumococcal colonization of the upper airways.

DISCUSSION

The binding of pneumococci to epithelial cells of the host mucosal surfaces is a crucial step during colonization and precedes dissemination to other tissues (3,85,86). In this study, we reveal a pneumococcal pathway that promotes adherence via host HA. We previously documented that the signaling peptide *vp1* is dramatically induced during host infection, enhances biofilm formation, and is a potent virulence determinant in a chinchilla model of middle ear infection (7). Here, we build on this work, providing mechanistic details that reveal links across signaling, adherence, and colonization. Specifically, we demonstrate that VP1 controls genes involved in the transport and metabolism of the host glycosaminoglycan, HA. Our *in vitro* studies demonstrate that genes in the PTS-HAL operon enhance attachment of the pneumococcus to epithelial cells. Our *in vivo* studies on a murine model of colonization suggest that VP1 and the operon for HA transport and metabolism play critical roles in nasopharyngeal colonization.

VP1 is part of a regulatory network involved in nutritional sensing. Previous work demonstrated that the master nutritional regulator CodY negatively regulates the level of *vp1* transcripts, and positively regulated by the transcriptional regulator Rgg144 (18,87). The activity of Rgg144 (nomenclature based on gene name in D39, SPD_0144) is regulated by a cognate peptide SHP144 (short hydrophobic peptide 144), which is secreted from cells and internalized into producing and neighboring cells where it binds to Rgg144 (7,18). Rgg144 is a negative regulator of the capsule: it directly binds a predicted promoter upstream of the capsular genes, inhibits the expression of capsular genes, and leads to a reduction in the size of the type 2 capsule (18). The bacterium capsule hinders cell adhesion by concealing bacterial receptors (88). In addition, our work on murine models shows that the Rgg144-

SHP144 system serves as a colonization factor in the nasopharynx and contributes to pathogenesis in pneumonia (18). These data are consistent with a model where the Rgg144-SHP144 system plays an essential role in colonization by decreasing the capsule and inducing *vp1*, and that together these events promote adhesion to epithelial cells in the nasopharynx and the lungs. Of note, adhesion experiments in this study were performed in CDM supplemented with glucose. In this condition, levels of *rgg144* are low, and levels of *vp1* are high (7,18). Thus, while Rgg144-SHP144 likely plays a key role in multiple aspects of colonization in our animal model, it is unlikely to be a major contributor *in vitro* experiments presented in this study. We propose that VP1 is a critical component of a complex cellular response to changes in nutritional status.

Our studies in the murine model of colonization reveal a dramatic defect for the *vp1* deletion mutant, where four out of five mice cleared the infection (Fig 5). The strain with a deletion of the HA transport and metabolism operon also displayed a decrease in bacterial load, but not of the same magnitude as the $\Delta vp1$. These data suggest that: (1) VP1 mediates attachment via control of the HA transport and metabolism operon, and (2) VP1 plays additional roles in colonization, which are mediated by yet unknown factors.

Many pneumococcal surface molecules serve as adhesins, mediating attachment of the bacteria to host cells (40,89,90). A subset of adhesins binds to extracellular matrix components. Pneumococcal virulence protein A and B (PavA and PavB) (91–93) and enolase (94,95) bind to fibronectin and plasminogen. Similarly, the plasmin- and fibronectin-binding proteins A and B (PfbA and PfbB) bind to the extracellular matrix and are required for adherence to lung and laryngeal epithelial cells (96,97). The choline-binding protein A (CbpA

or PspC) binds vitronectin (98), and PsrP binds to keratin (99). *Pneumococcus* also encodes surface-exposed glycosidases, which have been shown to contribute to adhesion not only via modification of the host surface that reveal receptors but also via direct binding (100). The glycosidase NanA (64) and the β -galactosidase BgaA (101), both process GAGs. NanA releases terminal sialic acid residues and BgaA releases terminal galactose residues from glycoconjugates. However, these enzymes promote attachment to host cells in a manner independent of their enzymatic activity. This observation is particularly evident for BgaA, where binding is mediated via a carbohydrate-binding module (102). Thus, the pneumococcal can employ glycosidases in host attachment. In this study, deletion of the surface exposed HysA strongly influenced HA processing (Fig 3), but not attachment (Fig 4). Thus, we deduce that the glycosidase HysA does not mediate the VP1-regulated defect in adhesion.

The data presented in this study strongly suggest that the transport and processing of host HA plays an essential role in adhesion. However, we have not yet established the molecular mechanism responsible for this outcome. Levels of regulators, carbohydrates, and transporters are interconnected in the bacterial cells via highly complex networks. Regulators control gene expression, including levels of transport systems and biosynthesis enzymes. Carbohydrates can function as substrates, and can positively or negatively influence the activity of regulators. Transporters not only control the import of carbohydrates, but the extent and position of phosphorylated residues on the cytoplasmic Hpr in the PTS system also controls CcpA-dependent carbon catabolite repression (103). CcpA influences the levels of almost one-fifth of pneumococcal genes, including ABC transporters and regulators (103,104). Thus, many molecular mechanisms may explain the connections between transporters and adhesions.

412

413 The HA transport and metabolism locus manipulated in this study encodes all the PTS-
414 EII components (EIIB, EIIC, and EIID) that specifically mediate HA import. The locus also
415 encodes two additional genes for a putative heparinase and YajC, a component of the
416 machinery associated with the secretion of proteins with N-terminal signal sequences (73),
417 Data presented in Fig 4, where host HA levels are decreased using SiRNA knockdowns,
418 strongly suggest that the PTS and VP1 mediate attachment, via a yet uncharacterized effector,
419 in an HA-dependent manner.

420

421 Multiple lines of evidence suggest that GAGs facilitate attachment of multiple bacterial
422 species to host cells. Several groups have explored and advanced the utilization of HA to
423 facilitate attachment of bacteria to abiotic surfaces (49,105,106). A recent report showed that
424 multidrug-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* form more robust
425 biofilms on surfaces coated with collagen and HA than on uncoated control surface (42). In
426 addition, levels of GAGs on A549 cells were positively associated with the attachment of
427 several pathogens, including *Staphylococcus aureus*, *Streptococcus pyogenes*, and the
428 pneumococcus (43). Similarly, attachment of multiple bacterial species (both Gram-positive
429 and Gram-negative) was positively associated with GAG levels on corneal epithelial cells HCE-
430 2, well known for expressing a variety of GAGs on their surface (40). In yet another example,
431 HA is the primary attachment site of *M. tuberculosis* in A549 cells (107). While HA has not
432 been directly implicated in the attachment of pneumococcus, previous studies demonstrate
433 that the pneumococcus can utilize HA as a carbon source. In particular, *hysA* and genes in the
434 PTS-EII-encoding operon (EIIB, EIIC, EIID, and *ugt*), are required for pneumococcal growth in
435 media where HA is the only carbohydrate (56). Furthermore, HA supports *in vitro*

pneumococcal biofilm formation (57). These studies suggest that multiple bacteria may utilize host HA for attachment or nutrients and that control of each or both of these processes influences colonization and virulence.

In this study, we reveal a novel pathway involved in the attachment of the pneumococcus to host epithelial cells. Our working model is that Rgg144 and CodY monitor nutrient availability and, both of these transcription factors influence levels of the secreted peptide *vp1*. VP1, in turn, controls a genomic region involved in the transport and metabolism of HA, and these genes promote adherence in an HA-dependent manner. VP1 is encoded by a core gene, is highly expressed during infection, and is a significant contributor to both colonization and virulence. Thus, this molecule is a high-value candidate for the development of anti-pneumococcal therapies.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Wild-type *S. pneumoniae* strain PN4595-T23 (GenBank ABX001), was selected as a representative of the PMEN1 lineage (59–62). The clinical isolate TIGR4 (The Institute for Genomic Research, Aaberge et al., 1995), was graciously provided by Dr. Jason Rosch. The construction of the *vp1* deletion mutant ($\Delta vp1$) and the *vp1* complemented strain ($\Delta vp1:\Delta vp1$) in the PN4595-T23 background was reported previously (7). Strain PN4595-T23 was modified to also generate the PTS-HAL operon deletion (Δ PTS-HAL), the *hysA* deletion ($\Delta hysA$), and the *regR* deletion ($\Delta regR$). We generated a double mutant *vp1*-PTS ($\Delta vp1-\Delta$ PTS-HAL) and a PTS-HAL overexpressor in the $\Delta vp1$ and wild-type backgrounds ($\Delta vp1$ -OE PTS-HAL and OE PTS-HAL, respectively) (S5 Table). A similar set of strains was constructed in the TIGR4 background (S5 Table). In this set, we generated a deletion mutant strain of the operon encoding *vp1* (*vpo*) and the *vpo* complemented strain ($\Delta vpo::\Delta vpo$). For growth on solid media, strains were streaked onto Trypticase Soy Agar II plates (TSA) containing 5% sheep blood (BD BBL, New Jersey, USA). For growth in liquid culture, colonies from a frozen stock were grown overnight on TSA plates and inoculated into Columbia broth (Remel Microbiology Products, Thermo Fisher Scientific, USA). The media was supplemented with antibiotics as needed. Cultures were incubated at 37°C and 5% CO₂ without shaking. Experiments in chemically defined medium (CDM) were performed utilizing a published recipe (104), and glucose was used at a final concentration of 55mM. Growth in CDM was initiated by growing a pre-culture in Columbia broth for 2-3 hours and then diluted to an absorbance of 0.1 at 600 nm, this culture was then grown in CDM-Glu.

Strain construction and transformation. To generate the Δ PTS-HAL mutant strain we used site-directed homologous recombination to replace genes SPN23F_02950 to SPN23F_0310

with a kanamycin-resistance gene (*kan*). Likewise, the PTS-HAL operon in the TIGR4 strain (SP_0321 to SP_0327) was replaced with *kan*. The *kan* region was originally amplified from an *rpsL* cassette (80). Gene IDs for PN4595-T23 and TIGR4 are listed in S3 Table. The same strategy was used for the single gene replacements, of *regR* (SPN23F_03020) and *hysA* (SPN23F_02890). To decrease the likelihood of polar effects in all our constructs, the strong artificial transcriptional terminator B1002 was inserted downstream of *kan* (109). Briefly, the transforming DNA was prepared by ligation of the kanamycin gene and promoter, with DNA (one to two kilobases) from the flanking regions upstream and downstream of the PTS-HAL operon. Flanking regions were amplified from the parental strains using either Q5 polymerase or OneTag polymerase (New England Biolabs). The OE PTS-HAL was generated in the $\Delta vp1$ and wild-type backgrounds by replacing the native PTS operon promoter with an *amiA* promoter and *kan* gene. Assembly of these transforming fragments was achieved by either sticky-end ligation of restriction enzyme-cut PCR products or by Gibson Assembly using NEBuilder HiFi DNA Assembly Cloning Kit. The resulting constructs were transformed into PN4595-T23 or TIGR4. Primers used to generate the constructs are listed in S4 Table. PN4595-T23 and TIGR4 strains were transformed with approximately 1 μ g of DNA. Liquid cultures were supplemented with 125 μ g ml⁻¹ of CSP1 (EMRLSKFFRDFILQRKK, for TIGR4) or CSP2 (EMRISRIILDFLFLRKK, for PN4595-T23), at an absorbance of 0.05 at 600 nm (GenScript, NJ, USA), and incubated at 37°C. After 2 hours, the treated cultures were plated on Columbia agar containing the appropriate concentration of antibiotic for selection, spectinomycin, 100 μ g ml⁻¹, kanamycin 150 μ g ml⁻¹). Resistant colonies were cultured in Columbia broth. Sequences were confirmed using DNA sequencing of the PCR amplimers (Genewiz, Inc., USA). All strains generated in this study are listed in S5 Table.

RNA purification, reverse transcription (RT) and qPCR. Bacterial samples were collected on RNALater (Thermofisher), and pellets were lysed with 1x lysis mix containing 2 mg ml⁻¹ of proteinase K, 10 mg ml⁻¹ of lysozyme, and 20 µg ml⁻¹ mutanolysin in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) for 20 min. Total RNA was isolated using the RNeasy (Zymo Research) following manufacturer instructions. Contaminant DNA was removed by incubating total RNA samples with DNase (2U/µl) at 37°C for at least 15 min and then checked by amplification of *gapdh* (no visible band should be observable in RNA only samples). RT reaction was performed using 1 µg of total RNA using SuperscriptVILO kit for 1 h. Five ng of total cDNA was subjected to real-time PCR using PowerUp SYBR Green Master Mix in the ABI 7300 Real-Time PCR system (Applied Biosystems) according to the manufacturer's instructions. All qRT-PCR amplification was normalized to pneumococcal *gapdh* and expressed as fold change with respect to the wild-type strain. qPCR primers are listed in S4 Table. Primers were obtained from IDT (Integrated DNA Technologies).

Spot assays and CFU counts. Bacteria were inoculated from overnight plates into 20 ml of Columbia broth. Liquid cultures were grown statically and monitored at an optical density of 600 nm using Nanodrop 2000c spectrophotometer (Thermo Scientific). Bacterial counts were assessed by streaking 10-fold dilutions on TSA or TSA-antibiotic containing plates. Spot assays were performed with 5 µl of ten-fold dilutions.

Microarray Analysis: We utilized the Pneumococcal Supragenome Hybridization Array (SpSGH) to compare gene expression between the wild-type PN4595-T23 strain and the $\Delta vp1$ as described previously (13). Pneumococcal cultures were collected and normalized to an absorbance of 0.3 at 600 nm. RNA extraction, cDNA preparation, and cDNA labeling were

performed as described previously (110). Cyber T was used for data analysis (111). Microarray experiments were performed in triplicate. Genes with at least a 3-fold difference between strains and a Bayesian p -value < 0.05 were selected for further analysis. Microarray data for selected genes was confirmed by qPCR.

Preparation of HA-coated surfaces. Hyaluronic acid from *Streptococcus equi* (Sigma-Aldrich) was bound to tissue culture 24-wells plates following a previously described method with modifications (49). Briefly, twenty-four well plates (VWR) were treated with 0.5 ml of concentrated sulfuric acid for 10 min at 60°C, washed extensively with distilled water, then incubated for 2 h at 37°C with 0.5 ml 5 mg ml⁻¹ of hyaluronic acid. Finally, the plates were rinsed with distilled water and allowed to stand for 1h at room temperature. Plates were not dried during the entire procedure. Control plates were treated solely with either sulfuric acid or HA alone. Hyaluronic acid binding was assessed with 0.5 ml of 1% of Alcian Blue 8GX (Sigma-Aldrich) for 10 min at room temperature, then washed extensively with distilled water.

Hyaluronidase activity measurement. Hyaluronidase activity was measured as described previously (76,112). Briefly, pneumococcal cultures were grown in Columbia broth to an absorbance of 0.7 at 600 nm. Then the bacterial pellet and supernatant were obtained by centrifugation. Prior to the reaction, bacterial pellets were washed thoroughly with PBS at room temperature then diluted in 250 µl of assay buffer while supernatant samples were diluted 1:1 in assay buffer. Hyaluronic acid isolated from *Streptococcus equi* (Sigma Aldrich) was utilized at a final concentration of 10 ug ml⁻¹ in assay buffer (150 mM NaCl, 200 mM sodium acetate, pH 6) for 15 min at 37°C. Stop buffer (2% NaOH, 2.5% cetrimide) was added to stop the reaction, and the absorbance at 400 nm was determined. Commercial hyaluronidase from

bovine testes (Sigma-Aldrich) was used as positive control. Assay results were calculated using the standard curve from the positive controls and by comparison to that of a blank comprised of assay buffer solely or without bacterial material.

Mammalian cells and media growth conditions. Epithelial lung human carcinoma A549 and fibroblast NIH-3T3 cells were propagated and maintained at 37°C and supplemented of 5% carbon dioxide at pH 7 in DMEM media contained 1% L-glutamine, 5% fetal bovine serum (FBS) without antibiotics. Chinchilla middle ear CMEE cells were maintained as previously reported (7,71).

siRNA knockdown. All transfection reagents were provided by Sigma-Aldrich. Subconfluent A549 cultures (20% to 30%) were seed in 24-wells plates and transfected with 3 pmol of a mixture containing pre-designed siRNA. Each mixture consisted of multiple siRNA targeting specifically either *HAS1*, *HAS2*, and *HAS3*. Universal control siRNA was used as a negative control. siRNA was transfected in a final volume of 100 µl of OptiMEM containing 3 µl of MISSION siRNA transfection reagent and no antibiotics. Twenty-four hours later the medium was replaced by DMEM supplemented with fetal bovine serum (FBS). Forty-eight hours after transfection, cells were used in adherence assays. To verify the reduction in *HAS* expression, A549 cells were lysed using the RNeasy kit and relative *HAS1*, *HAS2*, *HAS3*, and *ACTINB* expression was verified by qPCR, as described above.

Pneumococcal host cell adherence assay. A549, NIH-3T3 or CMEE were seed in 24-wells plates or on Lab-Tek II Chamber Slides (Thermo Fisher) at a density of 1×10^5 , and

maintained without antibiotic until confluence. Before infection, the cell monolayer medium is replaced with fresh medium and then infected with pneumococci resuspended in DMEM supplemented with 1% FBS using a multiplicity of infection (MOI) of 50 bacteria per cell. Infected cells were incubated at 37°C in 5% CO₂ for 1 h. Then the media of post-infected cells was aspirated and washed thoroughly three times with PBS to remove unbound bacteria. The total number of adherent bacteria was released with saponin (1% w/v) for 15 minutes at 37 °C and plated on TSA plates followed by colony formation and enumeration. The effect of exogenous HA on attachment interference was performed through the addition of HA from *Streptococcus equi* at concentrations ranging between 0.01 to 1 mg/ml to the A549 monolayers before the addition of the bacteria. Each experiment was repeated at least four times, and results were expressed as mean ± S.E.M.

Confocal microscopy. A549 cells were seeded on Nunc Lab-Tek 4-chamber (VWR) and inoculated with pneumococcal strains with a MOI of 50 as described above. After 1 hour of infection, cells were washed 3-times with PBS and fixed with 4% PFA, washed with PBS and stained with BODIPY FL Vancomycin (Thermo Fisher Scientific), Alexa Fluor 568-Phalloidin (Thermo Fisher Scientific) following manufacturer's instructions. Finally, cells were covered with Vectashield mounting media with DAPI (Vector Laboratories). Confocal microscopy was performed on the stage of Carl Zeiss LSM-880 META FCS, using 488 nm laser line for the fluorescence tag and 561 nm laser line for the fluorescence Alexa tag. Images were analyzed using ZEN Black software (Carl Zeiss Microscopy, Thornwood, NY) and assembled in Fiji (ImageJ. 2.0.0rc48). Laser intensity and gain were kept the same for all stacks and images.

590 ***In vivo* studies:** Colonization ability of pneumococcal strains were tested essentially as
 591 previously described (83,84). Nine-week-old CD1 mice were raised in the pre-clinical facility of
 592 the Leicester University. Mice were anesthetized lightly with 2.5% isoflurane over oxygen (1.5
 593 to 2 liter/min), and they were inoculated intranasally with 20 μ l PBS, pH 7.0, containing
 594 approximately 5×10^5 CFU/mouse. When inoculating, mice were kept horizontally to prevent the
 595 dissemination of inoculum into the lower respiratory tract. A group of mice ($n = 5$) for each
 596 cohort were killed by cervical dislocation at 2 hours and 7 days after infection. After removing
 597 the mandible, nasopharyngeal cavity was accessed using a 18G needle through the soft
 598 palate, and the content of nasopharynx was collected by injecting 500 μ l PBS, pH 7.0. This
 599 process was repeated once more using the same lavage fluid in order to optimize the recovery
 600 of bacteria. Undiluted and serially diluted lavage fluid was plated on blood agar base plates
 601 and CFU/nasopharynx was calculated. The results were analyzed by two-way ANOVA
 602 followed by Tukey's multiple comparisons test.

603

604 **Ethics statement:** Mouse experiments were done under the UK Home Office approved
 605 project (permit no: P7B01C07A) and personal (permit no. I66A9D84D) licenses. The protocols
 606 used were approved by both the UK Home Office and the University of Leicester ethics
 607 committee. We used anesthetic during the procedure to minimize the suffering. Moreover, the
 608 animals were kept in individually ventilated cages, had access to water and feed *ad libido*, and
 609 their health were checked regularly in accordance with the legal and institutional requirements.

610

611 **Statistical analysis.** Statistical significance was assessed using Student's two-tailed *t*-test for
 612 independent samples, one-way ANOVA and Bonferroni's multiple comparisons test for

613 independent samples, and two-way ANOVA and Tukey's multiple comparisons test for animal
614 data. p -values less than 0.05 were considered statistically significant.

Acknowledgments

We are very grateful to Dr. Aaron Mitchell and Dr. Frederick Lanni for their insightful suggestions and guidance during this project. We also thank Dr. Samantha King for productive discussions, particularly in regards to hyaluronic acid. We thank Dr. Haibing Teng from the Molecular Biosensor and Imaging Facility (MBIC) at Carnegie Mellon University for her assistance with confocal imaging. We thank Dr. Phil G. Campbell for providing A549 cells. This work was supported by NIH grant R01 AI139077-01A1 to N.L.H., the Eberly Family Career Development Professorship of Biological Science, an award from the Samuel and Emma Winters Foundation, as well as support from Carnegie Mellon University. The authors declare that there are no conflicts of interest.

Author Contributions

RAC contributed to the conception, design, experimentation, analysis and interpretation, and manuscript preparation; EE contributed with design and construction of bacterial clones, as well as data interpretation; OG contributed with *in vivo* experiments and data analysis, HY contributed with *in vivo* experiments, analysis and interpretation, and manuscript preparation; NLH contributed design, analysis and interpretation, and manuscript preparation.

Figure Captions

Fig 1. *vp1* enhances pneumococcal attachment to epithelial cells.

(A) A549 lung epithelial cells were exposed to the wild-type PMEN1 strain PN4595-T23, and isogenic *vp1* mutant and *vp1* complemented strains ($\Delta vp1$ and the $\Delta vp1:vp1$, respectively) for 1h at 37 °C. After washing the unbound bacteria, the remaining cell-bound bacteria were recovered and quantified on TSA plates. Visualization by a spot assay performed with 5 μ l of ten-fold dilutions (top panel). Quantification of the total number of bacteria bound to epithelial cells based on counts from TSA plates (bottom panel). (B) Representative image of wild-type and the $\Delta vp1$ strains bound to A549 cells. Bacterial cells were stained with BODYPI-FL vancomycin (green), actin was visualized with TRITC-phalloidin (red), and bacterial and host DNA were visualized with DAPI (blue). Data on A represents the mean $\pm$ S.E.M of at least six independent experiments. Statistical significance was calculated using unpaired one-way ANOVA analysis with Bonferroni correction, and multiple comparison between samples were performed. * $p < 0.0001$, ns = not significant with $p = 0.080$.

Fig 2. The *vp1* product positively regulates the expression of an operon critical to processing of hyaluronic acid.

(A) Microarray transcriptome analysis comparing the expression profiles of the wild-type PMEN1 strain PN4595-T23 and *vp1* derivative strain ($\Delta vp1$) on CDM supplemented with glucose. Data was analyzed for statistical significance using CyberT as per Student's T-test. The dashed blue lines represent 4-fold up- and down-regulation, respectively. The red circle highlights genes associated with hyaluronic acid metabolism. (B) Selected genomic regions with 3-fold difference in expression between the wild-type and the $\Delta vp1$ strains. Targets were

sorted by their genomic position on the genome. Operons are indicated by brackets. Color code illustrates expression ratio between the $\Delta vp1$ strain relative to wild-type strain. Gene IDs correspond to those in PMEN1 reference strain ATCC700669 (GenBank FM211187), and the predicted functions based on Pfam. Full set presented in S1 Table.

Fig 3. The *vp1* product promotes degradation of the hyaluronan polymer via its controls of the operon encoding the PTS-EII system.

(A) Schematic of the genomic region implicated in the metabolism of hyaluronic acid. Arrows correspond to predicted open reading frames. Black and white denote predicted operons. Grey corresponds to genes involved in HA metabolism, but not highly controlled by *vp1*. The IDs refer to gene names in the PMEN1 reference strain ATCC700669. (B and C). Measurements of breakdown of hyaluronan and isogenic deletion mutants for *vp1* ($\Delta vp1$), the hyaluronan acid locus PTS operon (Δ PTS-HAL), *vp1* and the PTS operon ($\Delta vp1$ - Δ PTS-HAL), *hysA* (Δhys), and *regR* ($\Delta regR$), and for a *vp1* complemented strains ($\Delta vp1:vp1$), and a PTS overexpressor in the $\Delta vp1$ background ($\Delta vp1$ -OE PTS-HAL). Both the pellets (B) and the supernatants (C) of bacteria grown in liquid cultures were collected and assessed for hyaluronidase activity. Data on B and C represents the mean $\pm$ S.E.M of at least six independent experiments. Statistical significance was calculated using unpaired one-way ANOVA analysis with Bonferroni correction, and multiple comparison between samples were performed. * $p < 0.05$, ** $p < 0.001$, ns = not significant relative to the wild-type strain. Note that on B, the complemented $\Delta vp1:vp1$ rescues the hyaluronidase deficiency of the $\Delta vp1$ mutant ($p < 0.0001$) and the overexpressor $\Delta vp1$ -OE PTS-HAL rescues the hyaluronidase activity of the mutants $\Delta vp1$ ($p < 0.0001$). Further, there is no significant difference between $\Delta vp1$ and Δ PTS-HAL, between $\Delta vp1$ and $\Delta vp1$ - Δ PTS-HAL.

679

680 **Fig 4. Pneumococcus attaches to host HA in a process controlled by VP1 and mediated**
 681 **by molecules involved in HA processing.**

682 (A) The wild-type PN4595-T23, $\Delta vp1$, ΔPTS -HAL, and $\Delta vp1$ -OE PTS-HAL strains were tested
 683 for attachment to epithelial A549 cells. Cell bound bacteria were enumerated by plating on
 684 TSA plates. (B) Representative image of strains attached to epithelial A549 cells. Bacterial
 685 cells were stained with BODYPI-FL vancomycin (green), actin was visualized with TRITC-
 686 phalloidin (red), and DNA was visualized with DAPI (blue). (C) Adherence measurements on
 687 A549 cells. *HAS1*-, *HAS2*-, and *HAS3*-knockdown, and the double-knockdown of *HAS2* and
 688 *HAS3* (*HAS2-3*) cells were exposed to wild-type and its derivative $\Delta vp1$, ΔPTS -HAL, and $\Delta vp1$ -
 689 OE PTS-HAL strains. siRNA treatment was administered 48 h before addition of bacteria, and
 690 adherent bacteria were enumerated by plating on TSA plates. Bound bacteria were
 691 enumerated by plating cells on TSA plates. (D) The wild-type PN4595-T23 and its derivative
 692 $\Delta vp1$, ΔPTS -HAL, and $\Delta vp1$ -OE PTS-HAL strains were assessed for attachment to hyaluronic
 693 acid-coated surfaces in FBS-supplemented DMEM media. The total cell-bound bacteria were
 694 recovered and quantified by growth on TSA plates. Data on A, C, and D represents the mean $\pm$
 695 S.E.M of at least four independent experiments. Statistical significance was calculated using
 696 unpaired one-way ANOVA (A and D) or two-ways ANOVA (C) analysis with Bonferroni
 697 correction, and multiple comparison between samples were performed. * $p < 0.05$, **** $p <$
 698 0.0001, ns = not significant. Note that the overexpressor $\Delta vp1$ -OE PTS-HAL rescues the
 699 attachment deficiency of the mutant $\Delta vp1$ ($p < 0.0001$) in D.

700

701 **Fig 5. HA processing region is required for pneumococcal colonization.**

CD1 mice were inoculated intranasally with 20 ml PBS containing approximately 5×10^5 CFU/mouse. At 2 hours (day 0, A), and 7-days (B) post inoculation, nasopharyngeal contents were collected and pneumococci were enumerated. Each column represents the mean of data from five mice. Error bars show the standard error of the mean. Significant differences in bacterial counts are seen comparing to the wild-type strain using two-way ANOVA and Tukey's multiple comparisons test. * $p < 0.0005$, ns = not significant. Note that at day 7, the complemented $\Delta vp1:vp1$ rescues the colonization deficiency of the $\Delta vp1$ mutant ($p = 0.003$). Similarly, the overexpressor OE PTS-HAL rescues the colonization deficiency of the mutants $\Delta vp1$ ($p < 0.0001$). The ΔPTS -HAL ($p = 0.0001$) and the double-mutant $\Delta vp1$ - ΔPTS -HAL ($p = 0.0001$) both are significantly different from the wild-type strain and the colonization deficiency in the $\Delta vp1$ strain is greater to the ΔPTS strain ($p = 0.0176$).

Fig 6. Working model for VP1 regulation and its role in cell-cell communication in *Streptococcus pneumoniae*.

The VP1 is processed and secreted into the extracellular milieu. *vp1* is highly upregulated in the presence of host cells and is induced by an upstream transcription factor, Rgg/Shp. In addition, previous work shows that levels of *vp1* respond to nutrients and to the master regulator CodY. VP1 induces changes in the expression of multiple operons, including two implicated in the processing and acquisition of hyaluronic acid. We propose that VP1 promotes attachment via its influence on the products of genes encoded in the PTS-HAL operon.

Fig S1. *vp1* enhances pneumococcal attachment to epithelial cells.

(A) A549 cells were inoculated with the wild-type PMEN1 strain PN4595-T23. After washing the unbound bacteria, the total cell-bound bacteria were recovered. For visualization, five microliters of ten-fold dilutions were used for spot assays on TSA plates (left panel). For quantification, the total number of bacteria was determined by enumeration on TSA plates (right panel). (B and C). Attachment assay were performed on CMEE cells (B) and NIH-3T3 cells (C) inoculated with the strain wild-type PN4595-T23, the *vp1* mutant and *vp1* overexpressor strain ($\Delta vp1$ and the $\Delta vp1::vp1$, respectively). As in A, cell-bound bacteria were recovered and enumerated on TSA plates (bottom panels). (D) A549 cells were inoculated with the wild-type TIGR4 strain and its derivative *vpo* mutant and *vpo* overexpressor strains (Δvpo and the $\Delta vpo::vpo$, respectively). The total cell-bound bacteria were recovered and enumerated as in A. (E) Representative orthogonal view image of A549 cells inoculated with wild-type PN4595-T23. Note the extracellular *foci* indicating surface binding of the bacteria. Bacterial cells were stained with BODYPI-FL vancomycin (green), actin was visualized with TRITC-phalloidin (red), and bacterial and human DNA was visualized with DAPI (blue). Data on A, B, C, and D represents the mean $\pm$ S.E.M of at least four independent experiments. Statistical significance was calculated using unpaired one-way ANOVA analysis with Bonferroni correction, and multiple comparison between samples were performed. * $p < 0.005$, ** $p < 0.001$, *** $p < 0.0001$, ns = not significant.

Fig S2. The *vp1* product promotes degradation of the hyaluronan polymer via its controls of the operon encoding the PTS-EII system in TIGR4.

(A) Schematic of the genomic region implicated in the metabolism of hyaluronic acid. Arrows correspond to predicted open reading frames in TIGR4. Black and white denote predicted operons; PTS-HAL has two additional open reading frames at the end of the predicted operon

(represented in white). Grey corresponds to genes involved in HA metabolism, but not highly controlled by *vp1*. The IDs refer to gene names in the TIGR4 genome. (B and C) Measurements of breakdown of hyaluronan by strain TIGR4 and isogenic mutants. Both the supernatants (B) and the pellets (C) of bacteria grown in liquid cultures were collected and assessed for hyaluronidase activity relative to the wild-type strain. Data on B and C represents the mean $\pm$ S.E.M of 8 independent experiments. Statistical significance was calculated using unpaired one-way ANOVA analysis with Bonferroni correction, and multiple comparison between samples were performed. * $p < 0.05$, ** $p < 0.05$, *** $p < 0.0001$ ns = not significant relative to the wild-type strain. Note that on C, the $\Delta vpo:vpo$ rescues the hyaluronidase deficiency of the Δvpo mutant ($p < 0.0001$) and the overexpressor Δvpo -OE PTS-HAL rescues the hyaluronidase activity of the mutants Δvpo ($p < 0.0001$). Further, Δvpo -OE PTS-HAL is also significantly different from the double-mutant Δvpo - Δ PTS-HAL ($p < 0.0001$).

Fig S3. Pneumococcus attaches to host HA in a process controlled by VP1 and mediated by molecules involved in hyaluronic acid processing.

(A) Spot assay to access attachment to host cells. The wild-type PN4595-T23, and its derivative strains $\Delta vp1$, $\Delta vp1:vp1$, Δ PTS-HAL, $\Delta vp1$ - Δ PTS-HAL, $\Delta vp1$ -OE PTS and $\Delta hysA$, were assessed for attachment to A549 cells using a spot assay. After washing the unbound bacteria, the total cell-bound bacteria were recovered and plated on TSA plates. (B) Quantification of binding, where the total number of the $\Delta vp1$ - Δ PTS, $\Delta regR$ and $\Delta hysA$, bound to epithelial cells was enumerated on TSA plates. (C) Measurement of the expression levels of hyaluronic acid synthases-encoding genes *HAS1*, *HAS2*, and *HAS3* in A549 cells at two time points where levels are compared to treatment with a scrambled control. (D) Measurement of the expression levels of hyaluronic acid synthases-encoding genes *HAS2* and *HAS3* after

treatment with specific siRNA. (E) Quantification of bacterial binding to A549 pre-incubated with varying concentration of HA. Data on B to E represents the mean $\pm$ S.E.M of at least four independent experiments. Statistical significance was calculated using unpaired one-way ANOVA (B, D, and E) analysis with Bonferroni correction, and multiple comparison between samples were performed. * $p < 0.005$, ** $p < 0.0001$, ns = not significant.

Fig S4. *vp1* mediates attachment to hyaluronic acid of *Streptococcus equis* via genes in the PTS-HAL operon.

(A) hyaluronic acid (HA) coating of 6 well plates under listed treatments, visualized by staining with Alcian Blue 8GX. (B) The PMEN1 strain PN4595-T23 (top panel) was assessed for attachment to hyaluronic acid-coated surfaces in FBS-supplemented DMEM media (A549 growth media) or Columbia broth (bacterial growth media). The total cell-bound bacteria were recovered and tested for growth on TSA plates.

REFERENCES

1. Troeger C, Blacker B, Khalil I. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory infections in 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Infect Dis.* 2018;18(11):1191–210.
2. Murdoch DR, Howie SRC. The global burden of lower respiratory infections: making progress, but we need to do better. *Lancet Infect Dis.* 2018;18(11):1162–3.
3. Weiser JN, Ferreira DM, Paton JC. *Streptococcus pneumoniae*: transmission, colonization and invasion. *Nat Rev Microbiol.* 2018 Jun;16(6):355–67.
4. Brown AO, Mann B, Gao G, Hankins JS, Humann J, Giardina J, et al. *Streptococcus pneumoniae* Translocates into the Myocardium and Forms Unique Microlesions That Disrupt Cardiac Function. *PLOS Pathog.* 2014 Sep 18;10(9):e1004383.
5. Loughran AJ, Orihuela CJ, Tuomanen EI. *Streptococcus pneumoniae*: Invasion and Inflammation. *Microbiol Spectr.* 2019;7(2).
6. Aggarwal SD, Eutsey R, West-Roberts J, Domenech A, Xu W, Abdullah IT, et al. Function of BriC peptide in the pneumococcal competence and virulence portfolio. *PLoS Pathog* [Internet]. 2018 Oct 11 [cited 2019 Jul 29];14(10). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6181422/>
7. Cuevas RA, Eutsey R, Kadam A, West-Roberts JA, Woolford CA, Mitchell AP, et al. A novel streptococcal cell-cell communication peptide promotes pneumococcal virulence and biofilm formation. *Mol Microbiol.* 2017 Aug;105(4):554–71.
8. Dawid S, Roche AM, Weiser JN. The blp bacteriocins of *Streptococcus pneumoniae* mediate intraspecies competition both in vitro and in vivo. *Infect Immun.* 2007 Jan;75(1):443–51.
9. de Saizieu A, Gardès C, Flint N, Wagner C, Kamber M, Mitchell TJ, et al. Microarray-based identification of a novel *Streptococcus pneumoniae* regulon controlled by an autoinduced peptide. *J Bacteriol.* 2000 Sep;182(17):4696–703.
10. Havarstein LS, Coomaraswamy G, Morrison DA. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proc Natl Acad Sci U S A.* 1995 Nov 21;92(24):11140–4.
11. Hoover SE, Perez AJ, Tsui H-CT, Sinha D, Smiley DL, DiMarchi RD, et al. A new quorum-sensing system (TprA/PhrA) for *Streptococcus pneumoniae* D39 that regulates a lantibiotic biosynthesis gene cluster. *Mol Microbiol.* 2015 Apr 13;
12. Junges R, Salvadori G, Shekhar S, Åmdal HA, Periselneris JN, Chen T, et al. A Quorum-Sensing System That Regulates *Streptococcus pneumoniae* Biofilm Formation and Surface Polysaccharide Production. *mSphere* [Internet]. 2017 Sep 13;2(5). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5597970/>

- 823 13. Kadam A, Eutsey RA, Rosch J, Miao X, Longwell M, Xu W, et al. Promiscuous signaling
824 by a regulatory system unique to the pandemic PMEN1 pneumococcal lineage. *PLOS*
825 *Pathog.* 2017 May 18;13(5):e1006339.
- 826 14. Lux T, Nuhn M, Hakenbeck R, Reichmann P. Diversity of Bacteriocins and Activity
827 Spectrum in *Streptococcus pneumoniae*. *J Bacteriol.* 2007 Nov;189(21):7741–51.
- 828 15. Motib Anfal, Guerreiro Antonio, Al-Bayati Firas, Piletska Elena, Manzoor Irfan, Shafeeq
829 Sulman, et al. Modulation of Quorum Sensing in a Gram-Positive Pathogen by Linear
830 Molecularly Imprinted Polymers with Anti-infective Properties. *Angew Chem.* 2017 Nov
831 30;129(52):16782–5.
- 832 16. Motib AS, Al-Bayati FAY, Manzoor I, Shafeeq S, Kadam A, Kuipers OP, et al. TprA/PhrA
833 Quorum Sensing System Has a Major Effect on Pneumococcal Survival in Respiratory
834 Tract and Blood, and Its Activity Is Controlled by CcpA and GlnR. *Front Cell Infect*
835 *Microbiol* [Internet]. 2019 [cited 2019 Sep 20];9. Available from:
836 <https://www.frontiersin.org/articles/10.3389/fcimb.2019.00326/full>
- 837 17. Tomasz A. Control of the Competent State in *Pneumococcus* by a Hormone-Like Cell
838 Product: An Example for a New Type of Regulatory Mechanism in Bacteria. *Nature.*
839 1965 Oct 9;208(5006):155–9.
- 840 18. Zhi X, Abdullah IT, Gazioglu O, Manzoor I, Shafeeq S, Kuipers OP, et al. Rgg-Shp
841 regulators are important for pneumococcal colonization and invasion through their effect
842 on mannose utilization and capsule synthesis. *Sci Rep.* 2018 Apr 23;8(1):6369.
- 843 19. Havarstein LS, Diep DB, Nes IF. A family of bacteriocin ABC transporters carry out
844 proteolytic processing of their substrates concomitant with export. *Mol Microbiol.* 1995
845 Apr 1;16(2):229–40.
- 846 20. Aprianto R, Slager J, Holsappel S, Veening J-W. High-resolution analysis of the
847 pneumococcal transcriptome under a wide range of infection-relevant conditions. *Nucleic*
848 *Acids Res.* 2018 02;46(19):9990–10006.
- 849 21. Hall-Stoodley L, Hu FZ, Gieseke A, Nistico L, Nguyen D, Hayes J, et al. Direct Detection
850 of Bacterial Biofilms on the Middle-Ear Mucosa of Children With Chronic Otitis Media.
851 *JAMA J Am Med Assoc.* 2006 Jul 12;296(2):202–11.
- 852 22. Hoa M, Syamal M, Sachdeva L, Berk R, Coticchia J. Demonstration of nasopharyngeal
853 and middle ear mucosal biofilms in an animal model of acute otitis media. *Ann Otol*
854 *Rhinol Laryngol.* 2009 Apr;118(4):292–8.
- 855 23. Post JC, Hiller NL, Nistico L, Stoodley P, Ehrlich GD. The role of biofilms in
856 otolaryngologic infections: update 2007. *Curr Opin Otolaryngol Head Neck Surg.* 2007
857 Oct;15(5):347–51.
- 858 24. Sanderson AR, Leid JG, Hunsaker D. Bacterial biofilms on the sinus mucosa of human
859 subjects with chronic rhinosinusitis. *The Laryngoscope.* 2006 Jul;116(7):1121–6.

- 860 25. Chao Y, Marks LR, Pettigrew MM, Hakansson AP. Streptococcus pneumoniae biofilm
861 formation and dispersion during colonization and disease. Front Cell Infect Microbiol.
862 2015;4(January):194.
- 863 26. Marks LR, Davidson BA, Knight PR, Hakansson AP. Interkingdom Signaling Induces
864 Streptococcus pneumoniae Biofilm Dispersion and Transition from Asymptomatic
865 Colonization to Disease. mBio. 2013;4(4):e00438-13.
- 866 27. Chao Y, Marks LR, Pettigrew MM, Hakansson AP. Streptococcus pneumoniae biofilm
867 formation and dispersion during colonization and disease. Front Cell Infect Microbiol.
868 2014;4:194.
- 869 28. Croucher NJ, Harris SR, Barquist L, Parkhill J, Bentley SD. A high-resolution view of
870 genome-wide pneumococcal transformation. PLoS Pathog. 2012;8(6):e1002745.
- 871 29. Marks LR, Parameswaran GI, Hakansson AP. Pneumococcal interactions with epithelial
872 cells are crucial for optimal biofilm formation and colonization in vitro and in vivo. Infect
873 Immun. 2012 Aug;80(8):2744–60.
- 874 30. Tonnaer EL, Mylanus EA, Mulder JJ, Curfs JH. Detection of bacteria in healthy middle
875 ears during cochlear implantation. Arch Otolaryngol Head Neck Surg. 2009
876 Mar;135(3):232–7.
- 877 31. Trappetti C, Gualdi L, Meola LD, Jain P, Korir CC, Edmonds P, et al. The impact of the
878 competence quorum sensing system on Streptococcus pneumoniae biofilms varies
879 depending on the experimental model. BMC Microbiol. 2011 Apr 14;11(1):75.
- 880 32. Vidal JE, Ludewick HP, Kunkel RM, Zähler D, Klugman KP. The LuxS-Dependent
881 Quorum-Sensing System Regulates Early Biofilm Formation by Streptococcus
882 pneumoniae Strain D39. Infect Immun. 2011 Oct;79(10):4050–60.
- 883 33. Couchman JR, Pataki CA. An Introduction to Proteoglycans and Their Localization. J
884 Histochem Cytochem. 2012 Dec;60(12):885–97.
- 885 34. Iozzo RV, Schaefer L. Proteoglycan form and function: A comprehensive nomenclature
886 of proteoglycans. Matrix Biol J Int Soc Matrix Biol. 2015 Mar;42:11–55.
- 887 35. Weigel PH, DeAngelis PL. Hyaluronan synthases: a decade-plus of novel
888 glycosyltransferases. J Biol Chem. 2007 Dec 21;282(51):36777–81.
- 889 36. Pomin VH, Mulloy B. Glycosaminoglycans and Proteoglycans. Pharmaceuticals
890 [Internet]. 2018 Feb 27 [cited 2019 Sep 3];11(1). Available from:
891 <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5874723/>
- 892 37. Taylor KR, Gallo RL. Glycosaminoglycans and their proteoglycans: host-associated
893 molecular patterns for initiation and modulation of inflammation. FASEB J Off Publ Fed
894 Am Soc Exp Biol. 2006 Jan;20(1):9–22.
- 895 38. Andre GO, Converso TR, Politano WR, Ferraz LFC, Ribeiro ML, Leite LCC, et al. Role of
896 Streptococcus pneumoniae Proteins in Evasion of Complement-Mediated Immunity.
897 Front Microbiol. 2017;8:224.

- 898 39. Frolet C, Beniazza M, Roux L, Gallet B, Noirclerc-Savoye M, Vernet T, et al. New
899 adhesin functions of surface-exposed pneumococcal proteins. *BMC Microbiol.* 2010 Jul
900 12;10:190.
- 901 40. García B, Merayo-Llodes J, Rodríguez D, Alcalde I, García-Suárez O, Alfonso JF, et al.
902 Different Use of Cell Surface Glycosaminoglycans As Adherence Receptors to Corneal
903 Cells by Gram Positive and Gram Negative Pathogens. *Front Cell Infect Microbiol.*
904 2016;6:173.
- 905 41. Hammerschmidt S. Adherence molecules of pathogenic pneumococci. *Curr Opin*
906 *Microbiol.* 2006 Feb;9(1):12–20.
- 907 42. Birkenhauer E, Neethirajan S, Weese JS. Collagen and hyaluronan at wound sites
908 influence early polymicrobial biofilm adhesive events. *BMC Microbiol.* 2014 Jul
909 16;14:191.
- 910 43. Rajas O, Quirós LM, Ortega M, Vazquez-Espinosa E, Merayo-Llodes J, Vazquez F, et al.
911 Glycosaminoglycans are involved in bacterial adherence to lung cells. *BMC Infect Dis.*
912 2017 02;17(1):319.
- 913 44. Bhaskar KR, O’Sullivan DD, Opaskar-Hincman H, Reid LM, Coles SJ. Density gradient
914 analysis of secretions produced in vitro by human and canine airway mucosa:
915 identification of lipids and proteoglycans in such secretions. *Exp Lung Res.*
916 1986;10(4):401–22.
- 917 45. Monzon ME, Casalino-Matsuda SM, Forteza RM. Identification of glycosaminoglycans in
918 human airway secretions. *Am J Respir Cell Mol Biol.* 2006 Feb;34(2):135–41.
- 919 46. Jiang D, Liang J, Noble PW. Regulation of non-infectious lung injury, inflammation, and
920 repair by the extracellular matrix glycosaminoglycan hyaluronan. *Anat Rec Hoboken NJ*
921 2007. 2010 Jun;293(6):982–5.
- 922 47. Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. *Annu Rev Cell Dev*
923 *Biol.* 2007;23:435–61.
- 924 48. Lennon FE, Singleton PA. Role of hyaluronan and hyaluronan-binding proteins in lung
925 pathobiology. *Am J Physiol Lung Cell Mol Physiol.* 2011 Aug;301(2):L137-147.
- 926 49. Kujawa MJ, Caplan AI. Hyaluronic acid bonded to cell-culture surfaces stimulates
927 chondrogenesis in stage 24 limb mesenchyme cell cultures. *Dev Biol.* 1986
928 Apr;114(2):504–18.
- 929 50. Nusgens B-V. [Hyaluronic acid and extracellular matrix: a primitive molecule?]. *Ann*
930 *Dermatol Venereol.* 2010 Apr;137 Suppl 1:S3-8.
- 931 51. Solis MA, Chen Y-H, Wong TY, Bittencourt VZ, Lin Y-C, Huang LLH. Hyaluronan
932 regulates cell behavior: a potential niche matrix for stem cells. *Biochem Res Int.*
933 2012;2012:346972.

- 934 52. Bidossi A, Mulas L, Decorosi F, Colomba L, Ricci S, Pozzi G, et al. A functional
935 genomics approach to establish the complement of carbohydrate transporters in
936 *Streptococcus pneumoniae*. PloS One. 2012;7(3):e33320.
- 937 53. Oiki S, Mikami B, Maruyama Y, Murata K, Hashimoto W. A bacterial ABC transporter
938 enables import of mammalian host glycosaminoglycans. Sci Rep. 2017 21;7(1):1069.
- 939 54. Chapuy-Regaud S, Ogunniyi AD, Diallo N, Huet Y, Desnottes J-F, Paton JC, et al. RegR,
940 a global LacI/GalR family regulator, modulates virulence and competence in
941 *Streptococcus pneumoniae*. Infect Immun. 2003 May;71(5):2615–25.
- 942 55. Maruyama Y, Nakamichi Y, Itoh T, Mikami B, Hashimoto W, Murata K. Substrate
943 specificity of streptococcal unsaturated glucuronyl hydrolases for sulfated
944 glycosaminoglycan. J Biol Chem. 2009 Jul 3;284(27):18059–69.
- 945 56. Marion C, Stewart JM, Tazi MF, Burnaugh AM, Linke CM, Woodiga SA, et al.
946 *Streptococcus pneumoniae* can utilize multiple sources of hyaluronic acid for growth.
947 Infect Immun. 2012 Apr;80(4):1390–8.
- 948 57. Yadav MK, Chae S-W, Park K, Song J-J. Hyaluronic acid derived from other streptococci
949 supports *Streptococcus pneumoniae* in vitro biofilm formation. BioMed Res Int.
950 2013;2013:690217.
- 951 58. Hendriksen WT, Bootsma HJ, Estevão S, Hoogenboezem T, de Jong A, de Groot R, et
952 al. CodY of *Streptococcus pneumoniae*: link between nutritional gene regulation and
953 colonization. J Bacteriol. 2008 Jan;190(2):590–601.
- 954 59. Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, van der Linden M, et al. Rapid
955 pneumococcal evolution in response to clinical interventions. Science. 2011 Jan
956 28;331(6016):430–4.
- 957 60. Hiller NL, Eutsey RA, Powell E, Earl JP, Janto B, Martin DP, et al. Differences in
958 Genotype and Virulence among Four Multidrug-Resistant *Streptococcus pneumoniae*
959 Isolates Belonging to the PMEN1 Clone. PLoS ONE. 2011 Dec 19;6(12):e28850.
- 960 61. Nesin M, Ramirez M, Tomasz A. Capsular transformation of a multidrug-resistant
961 *Streptococcus pneumoniae* in vivo. J Infect Dis. 1998 Mar;177(3):707–713.
- 962 62. Wyres KL, Lamberts LM, Croucher NJ, McGee L, von Gottberg A, Liñares J, et al.
963 Pneumococcal capsular switching: a historical perspective. J Infect Dis. 2013 Feb
964 1;207(3):439–49.
- 965 63. Tettelin H, Nelson KE, Paulsen IT, Eisen JA, Read TD, Peterson S, et al. Complete
966 genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science. 2001 Jul
967 20;293(5529):498–506.
- 968 64. Brittan JL, Buckeridge TJ, Finn A, Kadioglu A, Jenkinson HF. Pneumococcal
969 neuraminidase A: an essential upper airway colonization factor for *Streptococcus*
970 *pneumoniae*. Mol Oral Microbiol. 2012 Aug;27(4):270–83.

- 971 65. Kjos M, Aprianto R, Fernandes VE, Andrew PW, van Strijp JAG, Nijland R, et al. Bright
972 fluorescent *Streptococcus pneumoniae* for live-cell imaging of host-pathogen
973 interactions. *J Bacteriol.* 2015 Mar;197(5):807–18.
- 974 66. Balis JU, Bumgarner SD, Paciga JE, Paterson JF, Shelley SA. Synthesis of lung
975 surfactant-associated glycoproteins by A549 cells: description of an in vitro model for
976 human type II cell dysfunction. *Exp Lung Res.* 1984;6(3–4):197–213.
- 977 67. Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G. A continuous tumor-cell line
978 from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int J*
979 *Cancer.* 1976 Jan 15;17(1):62–70.
- 980 68. Nardone LL, Andrews SB. Cell line A549 as a model of the type II pneumocyte.
981 Phospholipid biosynthesis from native and organometallic precursors. *Biochim Biophys*
982 *Acta.* 1979 May 25;573(2):276–95.
- 983 69. Berger JT, Voynow JA, Peters KW, Rose MC. Respiratory carcinoma cell lines. MUC
984 genes and glycoconjugates. *Am J Respir Cell Mol Biol.* 1999 Mar;20(3):500–10.
- 985 70. Carterson AJ, Höner zu Bentrup K, Ott CM, Clarke MS, Pierson DL, Vanderburg CR, et
986 al. A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue
987 culture model for *Pseudomonas aeruginosa* pathogenesis. *Infect Immun.* 2005
988 Feb;73(2):1129–40.
- 989 71. Raffel FK, Szelestey BR, Beatty WL, Mason KM. The *Haemophilus influenzae* Sap
990 transporter mediates bacterium-epithelial cell homeostasis. *Infect Immun.*
991 2013;81(1):43–54.
- 992 72. Cook LC, Federle MJ. Peptide pheromone signaling in *Streptococcus* and *Enterococcus*.
993 *FEMS Microbiol Rev.* 2014 May;38(3):473–92.
- 994 73. Martin R, Larsen AH, Corey RA, Midtgaard SR, Frielinghaus H, Schaffitzel C, et al.
995 Structure and Dynamics of the Central Lipid Pool and Proteins of the Bacterial Holo-
996 Translocon. *Biophys J.* 2019 May 21;116(10):1931–40.
- 997 74. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. The Phyre2 web portal for
998 protein modeling, prediction and analysis. *Nat Protoc.* 2015 Jun;10(6).
- 999 75. Dong W, Lu W, McKeehan WL, Luo Y, Ye S. Structural basis of heparan sulfate-specific
000 degradation by heparinase III. *Protein Cell.* 2012 Dec;3(12):950–61.
- 001 76. Ma K, Cao Q, Luo S, Wang Z, Liu G, Lu C, et al. cas9 Enhances Bacterial Virulence by
002 Repressing the *regR* Transcriptional Regulator in *Streptococcus agalactiae*. *Infect*
003 *Immun.* 2018;86(3).
- 004 77. Reese MG. Application of a time-delay neural network to promoter annotation in the
005 *Drosophila melanogaster* genome. *Comput Chem.* 2001 Dec;26(1):51–6.
- 006 78. Solovyev V, Salamov A. Automatic Annotation of Microbial Genomes and Metagenomic
007 Sequences. In *Metagenomics and its Applications in Agriculture, Biomedicine and*
008 *Environmental Studies.* Nova Science Publisher; 2011. 61–78 p.

- 009 79. Takao A. Cloning and expression of hyaluronate lyase genes of *Streptococcus*
010 *intermedius* and *Streptococcus constellatus* subsp. *constellatus*(1). *FEMS Microbiol Lett.*
011 2003 Feb 14;219(1):143–50.
- 012 80. Sung CK, Li H, Claverys JP, Morrison DA. An *rpsL* Cassette, Janus, for Gene
013 Replacement through Negative Selection in *Streptococcus pneumoniae*. *Appl Environ*
014 *Microbiol.* 2001 Nov;67(11):5190–6.
- 015 81. Wang T, Cui Y, Jin J, Guo J, Wang G, Yin X, et al. Translating mRNAs strongly correlate
016 to proteins in a multivariate manner and their translation ratios are phenotype specific.
017 *Nucleic Acids Res.* 2013 May;41(9):4743–54.
- 018 82. Chow G, Tauler J, Mulshine JL. Cytokines and growth factors stimulate hyaluronan
019 production: role of hyaluronan in epithelial to mesenchymal-like transition in non-small
020 cell lung cancer. *J Biomed Biotechnol.* 2010;2010:485468.
- 021 83. Glanville DG, Han L, Maule AF, Woodacre A, Thanki D, Abdullah IT, et al. RitR is an
022 archetype for a novel family of redox sensors in the streptococci that has evolved from
023 two-component response regulators and is required for pneumococcal colonization.
024 *PLoS Pathog.* 2018;14(5):e1007052.
- 025 84. Kahya HF, Andrew PW, Yesilkaya H. Deacetylation of sialic acid by esterases
026 potentiates pneumococcal neuraminidase activity for mucin utilization, colonization and
027 virulence. *PLOS Pathog.* 2017 Mar 3;13(3):e1006263.
- 028 85. Bogaert D, de Groot R, Hermans P. *Streptococcus pneumoniae* colonisation: the key to
029 pneumococcal disease. *Lancet Infect Dis.* 2004 Mar;4(3):144–54.
- 030 86. Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of *Streptococcus pneumoniae*
031 virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol.* 2008
032 Apr;6(4):288–301.
- 033 87. Hendriksen WT, Bootsma HJ, Estevão S, Hoogenboezem T, de Jong A, de Groot R, et
034 al. CodY of *Streptococcus pneumoniae*: link between nutritional gene regulation and
035 colonization. *J Bacteriol.* 2008 Jan;190(2):590–601.
- 036 88. Lysenko ES, Lijek RS, Brown SP, Weiser JN. Within-host competition drives selection for
037 the capsule virulence determinant of *Streptococcus pneumoniae*. *Curr Biol CB.* 2010 Jul
038 13;20(13):1222–6.
- 039 89. Bergmann S, Hammerschmidt S. Versatility of pneumococcal surface proteins.
040 *Microbiology.* 2006 Feb 1;152(2):295–303.
- 041 90. Paterson GK, Orihuela CJ. Pneumococcal microbial surface components recognizing
042 adhesive matrix molecules targeting of the extracellular matrix. *Mol Microbiol.* 2010 Jul
043 1;77(1):1–5.
- 044 91. Jensch I, Gámez G, Rothe M, Ebert S, Fulde M, Somplatzki D, et al. PavB is a surface-
045 exposed adhesin of *Streptococcus pneumoniae* contributing to nasopharyngeal
046 colonization and airways infections. *Mol Microbiol.* 2010 Jul 1;77(1):22–43.

92. Kanwal S, Jensch I, Palm GJ, Brönstrup M, Rohde M, Kohler TP, et al. Mapping the recognition domains of pneumococcal fibronectin-binding proteins PavA and PavB demonstrates a common pattern of molecular interactions with fibronectin type III repeats. *Mol Microbiol*. 2017 Sep;105(6):839–59.
93. Pracht D, Elm C, Gerber J, Bergmann S, Rohde M, Seiler M, et al. PavA of *Streptococcus pneumoniae* modulates adherence, invasion, and meningeal inflammation. *Infect Immun*. 2005 May;73(5):2680–9.
94. Antikainen J, Kuparinen V, Lähteenmäki K, Korhonen TK. Enolases from Gram-positive bacterial pathogens and commensal lactobacilli share functional similarity in virulence-associated traits. *FEMS Immunol Med Microbiol*. 2007 Dec;51(3):526–34.
95. Bergmann S, Rohde M, Chhatwal GS, Hammerschmidt S. α -Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Mol Microbiol*. 2001 Jun;40(6):1273–87.
96. Papasergi S, Garibaldi M, Tuscano G, Signorino G, Ricci S, Peppoloni S, et al. Plasminogen- and fibronectin-binding protein B is involved in the adherence of *Streptococcus pneumoniae* to human epithelial cells. *J Biol Chem*. 2010 Mar 5;285(10):7517–24.
97. Yamaguchi M, Terao Y, Mori Y, Hamada S, Kawabata S. PfbA, a novel plasmin- and fibronectin-binding protein of *Streptococcus pneumoniae*, contributes to fibronectin-dependent adhesion and antiphagocytosis. *J Biol Chem*. 2008 Dec 26;283(52):36272–9.
98. Voss S, Hallström T, Saleh M, Burchhardt G, Pribyl T, Singh B, et al. The choline-binding protein PspC of *Streptococcus pneumoniae* interacts with the C-terminal heparin-binding domain of vitronectin. *J Biol Chem*. 2013 May 31;288(22):15614–27.
99. Shivshankar P, Sanchez C, Rose LF, Orihuela CJ. The *Streptococcus pneumoniae* adhesin PsrP binds to Keratin 10 on lung cells. *Mol Microbiol*. 2009 Aug;73(4):663–79.
100. King SJ, Hippe KR, Weiser JN. Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by *Streptococcus pneumoniae*. *Mol Microbiol*. 2006 Feb;59(3):961–74.
101. Limoli DH, Sladek JA, Fuller LA, Singh AK, King SJ. BgaA acts as an adhesin to mediate attachment of some pneumococcal strains to human epithelial cells. *Microbiol Read Engl*. 2011 Aug;157(Pt 8):2369–81.
102. Singh AK, Pluvinaige B, Higgins MA, Dalia AB, Woodiga SA, Flynn M, et al. Unravelling the multiple functions of the architecturally intricate *Streptococcus pneumoniae* β -galactosidase, BgaA. *PLoS Pathog*. 2014 Sep;10(9):e1004364.
103. Warner JB, Lolkema JS. CcpA-dependent carbon catabolite repression in bacteria. *Microbiol Mol Biol Rev MMBR*. 2003 Dec;67(4):475–90.
104. Carvalho SM, Kloosterman TG, Kuipers OP, Neves AR. CcpA Ensures Optimal Metabolic Fitness of *Streptococcus pneumoniae*. *PLoS ONE*. 2011 Oct 21;6(10):e26707.

105. Corradetti B, Taraballi F, Martinez JO, Minardi S, Basu N, Bauza G, et al. Hyaluronic acid coatings as a simple and efficient approach to improve MSC homing toward the site of inflammation. *Sci Rep*. 2017 11;7(1):7991.
106. Khademhosseini A, Suh KY, Yang JM, Eng G, Yeh J, Levenberg S, et al. Layer-by-layer deposition of hyaluronic acid and poly-L-lysine for patterned cell co-cultures. *Biomaterials*. 2004 Aug;25(17):3583–92.
107. Aoki K, Matsumoto S, Hirayama Y, Wada T, Ozeki Y, Niki M, et al. Extracellular mycobacterial DNA-binding protein 1 participates in mycobacterium-lung epithelial cell interaction through hyaluronic acid. *J Biol Chem*. 2004 Sep 17;279(38):39798–806.
108. Aaberge IS, Eng J, Lermak G, Løvik M. Virulence of *Streptococcus pneumoniae* in mice: a standardized method for preparation and frozen storage of the experimental bacterial inoculum. *Microb Pathog*. 1995 Feb;18(2):141–52.
109. Huang H. Design and Characterization of Artificial Transcriptional Terminators. Massachusetts Institute of Technology; 2008.
110. Kadam A, Janto B, Eutsey R, Earl JP, Powell E, Dahlgren ME, et al. *Streptococcus pneumoniae* Supragenome Hybridization Arrays for Profiling of Genetic Content and Gene Expression. *Curr Protoc Microbiol*. 2015;36:9D.4.1-9D.4.20.
111. Kayala MA, Baldi P. Cyber-T web server: differential analysis of high-throughput data. *Nucleic Acids Res*. 2012 Jul;40(Web Server issue):W553-559.
112. Horta CCR, Magalhães B de F, Oliveira-Mendes BBR, do Carmo AO, Duarte CG, Felicori LF, et al. Molecular, Immunological, and Biological Characterization of *Tityus serrulatus* Venom Hyaluronidase: New Insights into Its Role in Envenomation. *PLoS Negl Trop Dis* [Internet]. 2014 Feb 13 [cited 2019 Sep 20];8(2). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3923731/>

Fig 1. *vp1* enhances pneumococcal attachment to epithelial cells.

Fig 2. The *vp1* product positively regulates the expression of an operon critical to processing of hyaluronic acid.

Fig 3. The *vp1* product promotes degradation of the hyaluronan polymer via its controls of the operon encoding the PTS-EII system.

Fig 4. Pneumococcus attaches to host HA in a process controlled by VP1 and mediated by molecules involved in HA processing.

Fig 5. HA processing region is required for pneumococcal colonization.

Fig 6. Working model for VP1 regulation and its role in cell-cell communication in *Streptococcus pneumoniae*.

Fig S1. *vp1* enhances pneumococcal attachment to epithelial cells.

Fig S2. The *vp1* product promotes degradation of the hyaluronan polymer via its controls of the operon encoding the PTS-EII system in TIGR4.

Fig S3. Pneumococcus attaches to host HA in a process controlled by VP1 and mediated by molecules involved in hyaluronic acid processing.

Fig S4. *vp1* mediates attachment to hyaluronic acid of *Streptococcus equis* via genes in the PTS-HAL operon.