Published in final edited form as:

Int J Pediatr Otorhinolaryngol. 2009 October ; 73(10): 1417–1422. doi:10.1016/j.ijporl.2009.07.007.

Interaction of phase variation, host and pressure/gas composition: Pneumococcal gene expression of PsaA, SpxB, Ply and LytA in simulated middle ear environments

Ha-Sheng Li-Korotky^{a,b,c,*}, Chia-Yee Lo^a, Fan-Rui Zeng^a, David Lo^a, and Juliane M. Banks^a

^a Division of Pediatric Otolaryngology, Children's Hospital of Pittsburgh; Pittsburgh, Pennsylvania, United States

^b Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States

^c Department of Communication Science and Disorders, University of Pittsburgh School of Health and Rehabilitation Sciences, Pittsburgh, Pennsylvania, United States

Abstract

Objective—*Streptococcus pneumoniae*, a leading cause of otitis media (OM), undergoes spontaneous intra-strain variations in colony morphology. Transparent (T) variants are more efficient in colonizing the nasopharynx while opaque (O) variants exhibit greater virulence during systemic infections. This study was intended to delineate the underlying molecular mechanisms by which the predominant *S. pneumoniae* variant efficiently infects the middle ear (ME) mucosa.

Methods—Human ME epithelial cells were preconditioned for 24 hrs under one of the three gas/pressure conditions designed to simulate those for 1) normal ME (NME), 2) ME with Eustachian tube obstruction (ETO) and 3) ME with tympanostomy tube placement (TT), and then were incubated with $\sim 10^7$ CFU/mL of either T or O variants of *S. pneumoniae* (6A) for 3 hrs. Relative expression levels of genes encoding virulence factors, PsaA (surface adhesion), SpxB (pyruvate oxidase), Ply (pneumolysin), and LytA (autolysin), were assessed separately in epithelium-attached and supernatant bacteria 3 hrs post infection using real-time PCR.

Results—Basal levels of the virulence molecules in inocula were comparable between two variants. However, relative expression levels of the gene transcripts were significantly induced in epithelium-attached T variants 3 hrs after infection. Comparing with NME and TT conditions, ETO environment produced the largest effect on the differential expression of the virulence genes in the infected ME epithelial cells between T (induced) and O (suppressed) phenotypic pneumococci.

Conclusions—T variant is a predominant phenotype responsible for the pathogenesis of pneumococcal OM.

Corresponding author: Ha-Sheng Li-Korotky, M.D., Ph.D., Division of Pediatric, Otolaryngology, Children's Hospital of Pittsburgh, 5121 Rangos Research Center, 530 45th Street, Pittsburgh, Pennsylvania 15201, United States. Tel. (412) 692-6664; Fax: (412) 692-5075; Ha-Sheng.Li@chp.edu (H.S. Li-Korotky).

Partial results were presented at 9th International Symposium on Recent Advances in Otitis Media, June 3–7, 2007, St. Petersburg, Florida, United States.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Otitis media; *Streptococcus pneumoniae*; Phase variation; Simulated middle ear environment; Human middle ear epithelial cell line; Virulence expression

1. Introduction

Streptococcus pneumoniae (SP) is a leading cause of acute otitis media (OM). With the emergence and dissemination of antibiotic resistant SP strains, coupled to changing patterns of virulence due to pneumococcal great capability of adaptation, and the inadequacy of available vaccines that were developed from a limited number of pneumococcal serotypes, medical management has become increasingly complex and costly [1–4].

Variability and adaptability are characteristics for SP to survive and prosper within different microniches [5,6]. SP strains highly adapt to and asymptomatically colonize the mucosal surface of the nasopharynx, and its binding to host middle ear mucosa (MEM) is a critical step for the pathogenesis of OM. The mechanisms that control SP colonization and subsequent infection to the MEM remain poorly understood.

Clinical SP isolates are highly heterogeneous populations. More than 90 SP serotypes have been identified based on the unique expressions of specific cell-surface molecules, pneumococcal capsular polysaccharides (PCPs) [7]. In addition to inter-strain differences in PCPs, SP is both a phenotypically and genetically heterogonous species [8,9]. One manifestation of this variability is the previously characterized intra-strain change in colony morphology visualized as differences in opacity [10–12]. Past studies documented reversible phase variations at frequencies of 10^{-3} to 10^{-6} among at least three discernible phenotypes, transparent (T), intermediate and opaque (O) variants [10]. Most clinical isolates of SP are mixed populations of the T and O variants.

In general, T variants have increased epithelial adherence attributable to increased amount of cell-surface adhesions and decreased levels of PCPs [13–15]. This increased surface adherence correlated with the selection of T variants during carriage in an infant rat model of colonization [10]. In addition, the T phenotype is predominant during natural carriage in humans, suggesting that the colony opacity determines the ability of SP to colonize its natural host [12]. On the other hand, the O variants have increased PCP levels that correlate with increased resistance to opsonophagocytosis and an ability to cause sepsis in a murine model [14,16] and invasive infections in humans [17]. Predominant selection of the SP variant and differential expression of the virulence genes for that variant during the pathogenesis of acute OM, such as *Eustachian* tube obstruction (ETO), and during the treatment of this disease with the placement of a tympanostomy tube (TT) remain largely unexplored.

Our recent study showed that T variants had a higher growth rate and greater epithelial adherence and killing than did O variants under the simulated ME conditions of ETO and TT, suggesting that the T variants are more highly adapted to various ME environments than the O variants [18]. Here, we attempted to delineate the underlying changes in SP virulence gene expression in an *in vitro* simulated otitis model by comparison between T and O variants of SP. In this study, we focused on four previously characterized SP genes, including PsaA (surface adhesion), SpxB (pyruvate oxidase), Ply (pneumolysin), and LytA (autolysin). The four pneumococcal molecules encode candidate virulence factors and are likely to be associated with SP phenotypic variations and expressed during ME infection. Our study established unique patterns of basal and adaptive expression of those virulence

genes in two SP phenotypic variants (T and O) during early infection of the human ME epithelial cells.

2. Materials and methods

2.1. Study design

Human middle ear epithelial cells (HMEECs) were preconditioned for 24 hrs to one of the three simulated ME environments: A) normal middle ear (NME): approximately physiological ME gas composition (8% O₂, 6% CO₂, 86% N₂) and ambient pressure; B) *Eustachian* tube obstruction (ETO): physiological ME gas composition and subambient pressure (−250 mm H₂O); and C) tympanostomy tube (TT): approximately ambient gas composition (21% O₂, 5% CO₂, and 74% N₂) and pressure. Cells were exposed to T or O variants of the same SP strain (6A, ~107 CFU/ml) and incubated for 1 hr and 3 hrs under the three simulated ME environments. bacterial inocula, epithelium-attached and supernatant bacteria were collected at 0, 1 and 3 hrs and immediately frozen at −80°C. The experiment in triplicate was repeated for three times in order to generate reproducible results.

2.2. Bacterial culture

Two phenotypic variants, opaque (O) and transparent (T) of SP 6A, that were passage one of the original Weiser strains (originally discovered and characterized by Dr. Jeffrey N. Weiser, University of Pennsylvania) [10] and kindly provided by Dr. Thomas F. DeMaria (Ohio State University) were used. Bacteria from frozen stocks were streaked on tryptic soy agar plates (Becton Dickinson, Cockeysville, MD) pretreated by spread of 100 µl of catalase (5000 U) (Worthington Biochemical Co., Freehold, NJ) (TSA+C plate) and incubated overnight at 37°C and 7% CO₂. A single typical colony of each variant was selected and restreaked on a chocolate agar plate and incubated for 16 hrs at 37°C and 7% CO₂. A broth culture containing colonies from the whole plate was then grown in Todd-Hewitt broth (Becton Dickinson) supplemented with 0.5% yeast extract (Difco laboratory, Detroit, MI) (TH+Y broth) at 37°C and 7% CO₂ without shaking. The number of viable bacteria was determined by serial dilutions and plate counts. The log phase of exponential growth for both SP variants reached an equal level at 3 hrs when the bacterial inocula were collected for use in the experiments.

2.3. Middle ear epithelial cell culture

The Human Middle Ear Epithelial Cell Line (HMEEC) was previously established using a retrovirus containing the E6/E7 genes of human papilloma virus type 16 [19] and generously provided by Dr. David Lim (House Ear Institute, Los Angeles, CA). HMEEC cells were seeded onto 12-well plates at a density of 3.5×10^5 cells/well and then placed in a humidified 5% CO₂ incubator at 37°C. The medium was composed of Dulbecco's Modified Eagle Media (DMEM) (Invitrogen Corporation, Carlsbad, CA) and Bronchial Epithelial Basal Medium (BEGM) (Clonetics, Walkersville, MD), supplemented with bovine pituitary extract (52 µg/ml), hydrocortisone (0.5 µg/ml), hEGF (0.5 ng/ml), epinephrine (0.5 µg/ml), transferrin (10 µg/ml), insulin (5 µg/ml), triiodothyronine (6.5 ng/ml), retinoic acid (0.1 ng/ml), gentamicin (50 µg/ml) and amphotericin-B (50 ng/ml).

2.4. Exposure to simulated ME conditions

HMEEC cultures were placed in a humidified modular incubator chamber (Billups-Rothenberg, Del Mar, CA) that was then placed in an incubator with 7% CO₂ at 37°C. The chamber gas composition, either ambient (21% O₂, 5% CO₂, and 74% N₂) or ME (8% O₂, 6% CO₂, and 86% N₂) was supplied as pressure-regulated tank gas (Certified Standard Spec cylinders, Airgas Great Lakes Inc., Royal Oak, MI) and monitored for composition by an O₂

analyzer (Analytical Industries, Inc., Pomona, CA). The pressure of the incubator chamber was monitored and controlled by a custom-written software program (GasContr, VEE Pro 7.0 from Agilent Technologies, Santa Clara, CA). Twelve-well plates with monolayers of 80–90% confluence were preconditioned to the three simulated ME conditions for 24 hrs before bacterial challenge.

2.5. SP challenge

After 24 hrs exposure to the selected gas/pressure, monolayers were washed 3 times with Dulbecco's Phosphate-Buffered Saline (Invitrogen) and culture medium without antibiotics was added. Cells were treated with either 25 μ l PBS or 25 μ l PBS containing 10^7 CFU/ml of T or O variants, returned to the incubator chambers at the selected gas/pressures, and incubated with a supply of 7% CO₂ at 37°C for 1 and 3 hrs. At the end of each time point, monolayers were washed 3 times and detached from the surface by adding 100 μ l of 0.25% trypsin/0.02% EDTA. Cells from the SP-infected monolayers and bacteria/cell debris from the supernatants were collected by centrifugation and stored at –80°C for gene expression assays.

2.6. RNA extraction

Bacterial inocula and bacteria/cell pellets were individually homogenized using a TissueLyzer (Qiagen, Valencia, CA). Total RNA was extracted with RNAprotect Bacteria Reagent and RNeasy Mini Kit (Qiagen) following manufacturer's instructions. RNA quality and quantity were evaluated by gel electrophoresis on a 1.5% agarose/Tris-Borate-EDTA (TBE) gel using ethidium bromide staining and 260/280 absorbance (ABS) ratio in a Beckman DU 600 Spectrophotometer (Beckman, Fullerton, CA, USA).

2.7. Reverse transcription and real-time PCR

We focused on four known and previously characterized genes coding for SP virulence factors that are likely to be expressed during SP infection of the ME, including PsaA (surface adhesion), SpxB (pyruvate oxidase), Ply (pneumolysin), and LytA (autolysin). Gene-specific primers (Table) were designed using OLIGO Primer Analysis Software (v6.3, Molecular Biology Insights, Inc., Cascade, CO). Predicted amplicon sequences were examined against GenBank database and PCR products were size-fragmented on 2.5% agarose/Tris-Acetate-EDTA (TAE) gel. The reverse transcription reaction was set-up with 250 ng of DNA-free total RNA, random primers and SuperScript III (Invitrogen). The reaction was incubated at 25°C for 10 min, 50°C for 50 min and 85°C for 5 min in a 2700 thermocycler (Applied Biosystems Inc., Foster City, CA). The real-time PCR reaction was set-up with SYBR Green PCR reagents (Applied Biosystems), including 5 μ l of 10X SYBR PCR Buffer, 6 μ l of 25 mM MgCl₂, 4 μ l of each dNTPs (blended with 2.5 mM dATP, dGTP and dCTP, and 5 mM dUTP), 2.5 μ l of each gene-specific primer (5 μ M), 0.5 μ l of AmpErase UNG (0.5 U), 0.25 μ l of AmpliTaq Gold (1.25 U) and 5 μ l of cDNA in a final volume of 50 μ l. The conditions for the real-time PCR were 50°C for 2 min, 95°C for 12 min, and 40 cycles at 95°C for 15 sec, and 60°C for 1 min in an ABI PRISM 7900 Sequence Detection system (Applied Biosystems).

2.8. Data collection, analysis and normalization

The 7900 Sequence Detection Software (Applied Biosystems) was used for instrument control, automated data collection and data analysis. Relative quantification (fold difference) of the expression levels of each transcript was calculated using the $2^{-\Delta\Delta C_t}$ method [20] with modifications. The log cDNA copy number of a target gene that was automatically normalized to the ROX internal passive reference (log Δ Rn) was plotted as a function of

cycle number. The cycle number at which the signal crossed the mid, exponential-linear portion of the log ΔRn -cycle function was defined as the cycle threshold (Ct).

Our focus in this study was the effects of the pathogen-host interaction and ME environment on virulence expression between T and O variants 3 hrs post infection. Therefore, data normalization included three steps. 1) Normalization of 16S rRNA expression: The 16S expression data from pneumococcal infected epithelial cells and supernatants were first normalized to the 16S expression level (in Ct) of each inoculum that was comparable between T (Ct = 10.27) and O variant (Ct = 10.34) ($p = .92$), $\Delta Ct =$

$Ct_{16S \text{ rRNA of the infected specimen}} - Ct_{16S \text{ rRNA of the inoculum}}$, and then, the 16S expression data 3-hr post infection were normalized to that of the 1-hr post infection, $\Delta \Delta Ct =$

$(Ct_{16S \text{ rRNA of the infected specimen}} - Ct_{16S \text{ rRNA of the inoculum}})_{1 \text{ hr}} -$

$(Ct_{16S \text{ rRNA of the infected specimen}} - Ct_{16S \text{ rRNA of the inoculum}})_{3 \text{ hr}}$. 2) Normalization of the virulence gene expression: The ΔCt represents the Ct of the virulence gene (target) that was normalized to that of bacterial 16S rRNA ($\Delta Ct = Ct_{\text{Target}} - Ct_{16S \text{ rRNA}}$) of the same specimen. 3) Normalization of the 3-hr expression: Relative quantification (fold difference) of the mRNA expression levels of the virulence gene (target) was calculated using a modified $2(\Delta \Delta Ct)$ method, where $\Delta \Delta Ct = (Ct_{\text{Target}} - Ct_{16S \text{ rRNA}})_{1 \text{ hr}} - (Ct_{\text{Target}} - Ct_{16S \text{ rRNA}})_{3 \text{ hr}}$. This modified calculation reflects a fold change as up- or down-regulation of the virulence genes between 1 hr and 3 hrs after bacterial challenge.

2.9. Statistical analyses

Two-way between subjects ANOVA was performed to analyze the levels of gene expression (dependent variable) as a function of independent variables including phenotype (T and O variants) and the simulated ME environments (NME, ETO, and TT) using SPSS software package (SPSS Inc., Chicago, IL, USA). *Post hoc* analyses of the simple main effects and simple comparisons, and marginal comparisons were performed depending on the significance of the phenotype-environment interactions. Independent-samples T test was performed between the two variants when it was appropriate.

3. Results

3.1. Basal expression of virulence genes in T and O inocula

Basal expression levels of four selected virulence genes, PsaA, SpxB, Ply, and LytA, were measured in the inoculum of each variant. Results from the independent-samples *t* test showed that there was no significant difference in gene expression levels between two phenotypes ($p \geq 0.068$). Results showed that each gene was relatively abundant expressed and comparable between T and O variants (Fig. 1).

3.2. Differential expression of virulence genes in epithelium-attached bacteria

The pattern of differences on expression levels of the four genes 3 hrs after the infection of epithelial cells among the simulated ME environments were not significantly different between T variants and O variants ($p \geq 0.06$). T variants had significantly higher expression levels for PsaA and SpxB molecules under each ME condition in both epithelium-attached bacteria than did O variants ($p \leq 0.001$, partial $\eta^2 \geq 0.475$, effect size) (Figs. 2 and 3). Mixed results were observed in both Ply and LytA expressions and the significant differences were observed in epithelium-attached bacteria under the ETO condition for Ply and LytA ($p \leq 0.001$, partial $\eta^2 \geq 0.49$) and under the TT condition for LytA ($p = 0.022$, partial $\eta^2 = 0.258$) (Figs. 4 and 5). By comparison with NME and TT conditions, ETO environment produced the largest effect sizes ($p \leq 0.001$, partial $\eta^2 \geq 0.49$) on differential expression of each virulence gene between T and O variants. Results showed the inductions of virulence expression for all four genes in epithelium-bound T variants that were exposed to all

simulated ME environments, in which ETO condition significantly aggravated molecular activities of SP genes in T variants and suppressed expression in O variants.

3.3. Differential expression of virulence genes in supernatant bacteria

The pattern of differences on the expression levels of the four genes 3 hrs after infection of the supernatants among the simulated ME environments were significantly different between T and O variants ($p \leq 0.041$, partial $\eta^2 = 0.298$). Differential expression among the simulated ME conditions was significantly different for PsaA in O variants ($p = 0.001$, partial $\eta^2 = 0.556$) (Fig. 2); SpxB in T ($p = 0.004$, partial $\eta^2 = 0.466$) and O variants ($p < 0.0001$, partial $\eta^2 = 0.777$) (Fig. 3); Ply in O variants ($p = 0.036$, partial $\eta^2 = 0.309$) (Fig. 4); and LytA in T variants ($p = 0.017$, partial $\eta^2 = 0.365$) (Fig. 5).

In general, molecular activities were significantly induced in T variants by comparison with that in O variants for PsaA, SpxB, and Ply factors ($p \leq 0.033$, partial $\eta^2 \geq 0.228$) (Figs. 2–4) under all simulated ME conditions and for LytA factor ($p < 0.0001$, partial $\eta^2 = 0.64$) under NME condition (Fig. 5).

4. Discussion

The relatively abundant expression levels of PsaA, SpxB, Ply and LytA factors in the inocula were comparable between the two phenotypic variants, suggesting that the basal levels of these genes are not sole indicators of the virulence during interactions of pathogen, host, and environment. In general, molecular activities of these virulence genes were up-regulated in epithelium-attached T variants under all simulated ME environments 3 hrs post-infection. However, by comparison with NME and TT conditions, ETO environment produced the largest effect on the differential expression of each virulence gene between T and O variants. Results suggest that the pathological ETO condition may modulate molecular activities for all four genes in epithelium-attached bacteria, up-regulating T virulence expression and down-regulating O virulence expression. The data were consistent with our recent findings showing an enhanced adhesion and killing in T-variant infected ME epithelial cells under the simulated ETO environment [18]. Results from the enhanced virulence expressions of the supernatant T variants further confirm that the T phenotype is predominantly selected during the pathogenesis of OM.

SP surface adhesin A (PsaA) is a SP 37-kDa protein that belongs to a group of proteins designated the lipoprotein receptor I antigen family and is a highly conserved, species-common, and immunogenic surface lipoprotein [21,22]. The PsaA gene is expressed in all 90 serotypes of SP [23] and is also present in the viridans group streptococcal species such as *S. mitis*, *S. oralis*, and *S. anginosus*, suggesting the ubiquity of this protein [22]. Our results showed that PsaA is one of the most abundantly expressed genes in both SP variants. Even though the two variants in inocula had similar basal levels of the expression, this gene was differentially regulated during the interaction between pathogen and host epithelial cells. The molecular activity of the PsaA was significantly altered in epithelium-bound and free-floating bacteria, induced in T variants and suppressed in O variants, under all simulated conditions. Our results suggest that T-variants are highly adapted to the ever-changing ME microenvironments by comparison with O variants. Interestingly, the TT condition also significantly stimulated the PsaA gene activity in epithelium-attached T variants, indicating that the ventilation tube may facilitate an adherence and colonization of the T variants to the ME epithelial cells. Our finding that the induction of the PsaA molecular activity in epithelium-bound T variants was independent of microenvironments is similar to a recent study in a pneumococci-infected murine model that showed a similar PsaA gene expression between niches (nasopharynx, lungs and bloodstream) at 72 hrs after infection [24]. However, among other conditions, ETO condition produced the largest effect

on differential expression of virulence molecules in infected epithelial cells and supernatants, indicating that ETO has a potential role in epithelium-associated adherence of T variants and killing of O variants.

SpxB encodes a member of the family of bacterial pyruvate oxidases which decarboxylates pyruvate to acetyl phosphate plus H_2O_2 and CO_2 . The enzyme is required for survival during exposure to high levels of endogenous and exogenous H_2O_2 , suggesting that SpxB contributes to H_2O_2 -resistant energy source and helps to maintain SP viability during oxidative stress [25]. SpxB is required for resistance to the toxic by-product of its own activity [26]. A recent study showed that the gene responsible for H_2O_2 production (SpxB) confers a survival advantage in colonization [27]. Increased SpxB activity also alters colony shape by mediating cell death [28]. Increased H_2O_2 levels were observed in infected ME fluid in animal models of acute OM [29].

Interestingly, our recent study demonstrated distinct phase-specific morphologies in T and O variants under all simulated ME conditions and media that did not show significant phase switching during the experiment [18]. The current study showed persistently enhanced levels of SpxB expression in both epithelium-attached and supernatant T variants across the exposure environments, indicating that the T phenotypic pneumococcus is highly adapted to ever-changing ME conditions and associated stress, and has a survival advantage in those conditions by comparison with the O variant. These results showing the increased SpxB gene expression in T-variants and reduced expression in O variants are consistent with previous reports at the protein levels [30]. Our study also showed that SpxB was significantly induced in T-variant infected epithelial cells under ETO and TT conditions, indicated by effect sizes, suggesting that pathogen-host interaction and pathological ME environments facilitate T-variant virulence.

Pneumolysin (Ply) is yet another SP virulence factor that penetrates the physical defenses of the host [31,32]. Ply is a 53-kDa protein produced by all clinical isolates of the pathogen [33,34]. Unlike other SP antigens, this molecule is not surface exposed. Our study showed that Ply gene was consistently induced in T variants of both epithelium-attached and free-floating states under the three simulated ME conditions. Ply, a thiol/sulfhydryl-activated cytolysin, is able to lyse cholesterol containing membranes and generate multiple conductance pores in the membrane of nucleated cells [35], indicating a perturbation effect of Ply on ME epithelial cells, ion transporters and associated effusion. The augmented Ply virulence in T variants in this study indicates that T-phenotypic SP not only has higher capability of adherence but also is more cytotoxic to the ME epithelial cells than O variants.

LytA, an N-acetyl muramoyl-L-alanine amidase, is one of the best-characterized autolysins functioning in the separation of daughter cells during cell division [36] and is required for cell lysis [37]. In this study, LytA gene were significantly induced only in epithelium-attached T variants under pathological ME conditions (ETO and TT). Although the differential expression of LytA was associated with colony morphology in this study, defined mutants in the LytA gene has no significant contribution to nasopharyngeal colonization in neonatal rat model of pneumococcal carriage [38]. Nevertheless, SP deficient in Ply or LytA has reduced virulence in meningitis [39].

5. Conclusions

During the interaction of SP and host epithelial cells, molecular activities of PsaA (surface adhesion), SpxB (pyruvate oxidase), Ply (pneumolysin), and LytA (autolysin) were induced in epithelium-attached T variants under all simulated ME environments, in which ETO condition produced the largest effect on the differential expression of pneumococcal

virulence factors between T and O variants. Our study established unique molecular activity patterns that may underlie the opaque/transparent intra-phasic transformation of the phenotypic morphologies and associated virulence during the pathogenesis of OM. T variant is a predominant phenotype responsible for the pathogenesis of pneumococcal otitis media.

Acknowledgments

This work was supported by Public Health Service grant NIDCD-DC007511 (H.-S. Li-Korotky), funds from the Lester A. Hamburg Endowed Fellowship in Pediatric Otolaryngology and the Eberly Family Endowed Chair in Pediatric Otolaryngology, and Summer Internships (F. Zen and D. Lo) from the Children's Hospital of Pittsburgh. Authors thank Dr. William J. Doyle for critical review, Dr. Jeffrey N. Weiser (University of Pennsylvania School of Medicine, Philadelphia, PA) who discovered and originally characterized and opaque and transparent variants of *S. pneumoniae* 6A and Dr. Thomas F. DeMaria (Ohio State University Medical Center, Columbus, OH) who kindly provided those variants. The Human Middle Ear Epithelial Cell Line (HMEEC) was generously provided by Dr. David Lim (House Ear Institute, Los Angeles, CA).

References

1. Jedrzejewski MJ. Pneumococcal virulence factors: structure and function. *Microbiol Mol Biol Rev.* 2001; 65:187–207. [PubMed: 11381099]
2. Di Guilmi AM, Dessen A. New approaches towards the identification of antibiotic and vaccine targets in *Streptococcus pneumoniae*. *EMBO Rep.* 2002; 3:728–734. [PubMed: 12151330]
3. Bogaert D, Hermans PW, Adrian PV, Rumke HC, de Groot R. Pneumococcal vaccines: an update on current strategies. *Vaccine.* 2004; 22:2209–2220. [PubMed: 15149779]
4. Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol.* 2008; 6:288–301. [PubMed: 18340341]
5. Hava DL, LeMieux J, Camilli A. From nose to lung: the regulation behind *Streptococcus pneumoniae* virulence factors. *Mol Microbiol.* 2003; 50:1103–1110. [PubMed: 14622402]
6. Orihuela CJ, Gao G, Francis KP, Yu J, Tuomanen EI. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. *J Infect Dis.* 2004; 190:1661–1669. [PubMed: 15478073]
7. Claverys JP, Prudhomme M, Mortier-Barriere I, Martin B. Adaptation to the environment: *Streptococcus pneumoniae*, a paradigm for recombination-mediated genetic plasticity? *Mol Microbiol.* 2000; 35:251–259. [PubMed: 10652087]
8. Dowson CG, Barcus V, King S, Pickerill P, Whatmore A, Yeo M. Horizontal gene transfer and the evolution of resistance and virulence determinants in *Streptococcus*. *Soc Appl Bacteriol Symp Ser.* 1997; 26:42S–51S. [PubMed: 9436316]
9. Hakenbeck R, Balmelle N, Weber B, Gardes C, Keck W, de Saizieu A. Mosaic genes and mosaic chromosomes: intra- and interspecies genomic variation of *Streptococcus pneumoniae*. *Infect Immun.* 2001; 69:2477–2486. [PubMed: 11254610]
10. Weiser JN, Austrian R, Sreenivasan PK, Masure HR. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. *Infect Immun.* 1994; 62:2582–2589. [PubMed: 8188381]
11. Saluja SK, Weiser JN. The genetic basis of colony opacity in *Streptococcus pneumoniae*: evidence for the effect of box elements on the frequency of phenotypic variation. *Mol Microbiol.* 1995; 16:215–227. [PubMed: 7565084]
12. Weiser JN. Phase variation in colony opacity by *Streptococcus pneumoniae*. *Microb Drug Resist.* 1998; 4:129–135. [PubMed: 9651000]
13. Cundell DR, Pearce BJ, Sandros J, Naughton AM, Masure HR. Peptide permeases from *Streptococcus pneumoniae* affect adherence to eucaryotic cells. *Infect Immun.* 1995; 63:2493–2498. [PubMed: 7790061]
14. Kim JO, Weiser JN. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. *J Infect Dis.* 1998; 177:368–377. [PubMed: 9466523]

15. Gould JM, Weiser JN. The inhibitory effect of C-reactive protein on bacterial phosphorylcholine platelet-activating factor receptor-mediated adherence is blocked by surfactant. *J Infect Dis.* 2002; 186:361–371. [PubMed: 12134232]
16. Kim JO, Romero-Steiner S, Sorensen UB, Blom J, Carvalho M, Barnard S, Carlone G, Weiser JN. Relationship between cell surface carbohydrates and intrastrain variation on opsonophagocytosis of *Streptococcus pneumoniae*. *Infect Immun.* 1999; 67:2327–2333. [PubMed: 10225891]
17. Serrano I, Melo-Cristino J, Ramirez M. Heterogeneity of pneumococcal phase variants in invasive human infections. *BMC Microbiol.* 2006; 6:1–6. [PubMed: 16401340]
18. Li-Korotky HS, Banks JM, Lo CY, Zeng FR, Stolz DB, Swarts JD, Doyle WJ. Interaction of pneumococcal phase variation and middle ear pressure/gas composition: An in vitro model of simulated otitis media. *Microb Pathog.* 2008; 45:201–206. [PubMed: 18619763]
19. Chun YM, Moon SK, Lee HY, Webster P, Brackmann DE, Rhim JS, Lim DJ. immortalization of normal adult human middle ear epithelial cells using a retrovirus containing the E6/E7 genes of human papillomavirus type 16. *Ann Otol Rhinol Laryngol.* 2002; 111:507–517. [PubMed: 12090706]
20. Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, Reed MW. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal Biochem.* 2000; 285:194–204. [PubMed: 11017702]
21. Sampson JS, Furlow Z, Whitney AM, Williams D, Facklam R, Carlone GM. Limited diversity of *Streptococcus pneumoniae* psaA among pneumococcal vaccine serotypes. *Infect Immun.* 1997; 65:1967–1971. [PubMed: 9125591]
22. Jado I, Fenoll A, Casal J, Perez A. Identification of the psaA gene, coding for pneumococcal surface adhesin A, in viridans group streptococci other than *Streptococcus pneumoniae*. *Clin Diagn Lab Immunol.* 2001; 8:895–898. [PubMed: 11527799]
23. Morrison KE, Lake D, Crook J, Carlone GM, Ades E, Facklam R, Sampson JS. Confirmation of psaA in all 90 serotypes of *Streptococcus pneumoniae* by PCR and potential of this assay for identification and diagnosis. *J Clin Microbiol.* 2000; 38:434–437. [PubMed: 10618136]
24. LeMessurier KS, Ogunniyi AD, Paton JC. Differential expression of key pneumococcal virulence genes *in vivo*. *Microbiology.* 2006; 152:305–311. [PubMed: 16436418]
25. Spellerberg B, Cundell DR, Sandros J, Pearce BJ, Idanpaan-Heikkila I, Rosenow C, Masure HR. Pyruvate oxidase, as a determinant of virulence in *Streptococcus pneumoniae*. *Mol Microbiol.* 1996; 19:803–813. [PubMed: 8820650]
26. Pericone CD, Park S, Imlay JA, Weiser JN. Factors contributing to hydrogen peroxide resistance in *Streptococcus pneumoniae* include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction. *J Bacteriol.* 2003; 185:6815–6825. [PubMed: 14617646]
27. Regev-Yochay G, Trzcinski K, Thompson CM, Lipsitch M, Malley R. SpxB is a suicide gene of *Streptococcus pneumoniae* and confers a selective advantage in an in vivo competitive colonization model. *J Bacteriol.* 2007; 189:6532–6539. [PubMed: 17631628]
28. Belanger AE, Clague MJ, Glass JI, Leblanc DJ. Pyruvate oxidase is a determinant of Avery's rough morphology. *J Bacteriol.* 2004; 186:8164–8171. [PubMed: 15576764]
29. Takoudes TG, Haddad J Jr. Hydrogen peroxide in acute otitis media in guinea pigs. *Laryngoscope.* 1997; 107:206–210. [PubMed: 9023244]
30. Overweg K, Pericone CD, Verhoef GG, Weiser JN, Meiring HD, De Jong AP, De Groot R, Hermans PW. Differential protein expression in phenotypic variants of *Streptococcus pneumoniae*. *Infect Immun.* 2000; 68:4604–4610. [PubMed: 10899862]
31. Sato K, Quartey MK, Liebler CL, Le CT, Giebink GS. Roles of autolysin and pneumolysin in middle ear inflammation caused by a type 3 *Streptococcus pneumoniae* strain in the chinchilla otitis media model. *Infect Immun.* 1996; 64:1140–1145. [PubMed: 8606070]
32. Cockeran R, Anderson R, Feldman C. Pneumolysin in the immunopathogenesis and treatment of pneumococcal disease. *Expert Rev Anti Infect Ther.* 2003; 1:231–239. [PubMed: 15482118]
33. Paton JC, Berry AM, Lock RA, Hansman D, Manning PA. Cloning and expression in *Escherichia coli* of the *Streptococcus pneumoniae* gene encoding pneumolysin. *Infect Immun.* 1986; 54:50–55. [PubMed: 3019892]

34. Paton JC, Andrew PW, Boulnois GJ, Mitchell TJ. Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: the role of pneumococcal proteins. *Ann Rev Microbiol.* 1993; 47:89–115. [PubMed: 7903033]
35. El-Rachkidy RG, Davies NW, Andrew PW. Pneumolysin generates multiple conductance pores in the membrane of nucleated cells. *Biochem Biophys Res Commun.* 2008; 368:786–792. [PubMed: 18261465]
36. Ronda C, Garcia JL, Garcia E, Sanchez-Puelles JM, Lopez R. Biological role of the pneumococcal amidase. Cloning of the *lytA* gene in *Streptococcus pneumoniae*. *Eur J Biochem.* 1987; 164:621–624. [PubMed: 3569279]
37. Lopez R, Garcia E, Garcia P, Garcia JL. The pneumococcal cell wall degrading enzymes: a modular design to create new lysins? *Microb Drug Resistance.* 1997; 3:199–211.
38. Weiser JN, Markiewicz Z, Tuomanen EI, Wani JH. Relationship between phase variation in colony morphology, intrastrain variation in cell wall physiology, and nasopharyngeal colonization by *Streptococcus pneumoniae*. *Infect Immun.* 1996; 64:2240–2245. [PubMed: 8675333]
39. Hirst RA, Gosai B, Rutman A, Guerin CJ, Nicotera P, Andrew PW, O'Callaghan C. *Streptococcus pneumoniae* deficient in pneumolysin or autolysin has reduced virulence in meningitis. *J Infect Dis.* 2008; 197:744–751. [PubMed: 18260758]

Fig. 1. Relative basal gene expression encoding four pneumococcal virulence factors in T or O inocula. The ΔC_t (mean $\pm$ SD) represents the cycle threshold (C_t) of the target gene that was normalized to that of the bacterial 16S rRNA of the same variant.

Fig. 2. Relative gene expression levels (fold change, mean $\pm$ SD) encoding PsaA from epithelia-attached and supernatant variants at 3 hrs after challenge under the three simulated ME conditions. Data at 3 hrs were normalized to that at 1 hr.

Fig. 3. Relative gene expression levels (fold change, mean $\pm$ SD) encoding SpxB from epithelia-attached and supernatant variants at 3 hrs after challenge under the three simulated ME conditions. Data at 3 hrs were normalized to that at 1 hr.

Fig. 4. Relative gene expression levels (fold change, mean $\pm$ SD) encoding Ply from epithelia-attached and supernatant variants at 3 hrs after challenge under the three simulated ME conditions. Data at 3 hrs were normalized to that at 1 hr.

Fig. 5. Relative gene expression levels (fold change, mean $\pm$ SD) encoding LytA from epithelia-attached and supernatant variants at 3 hrs after challenge under the three simulated ME conditions. Data at 3 hrs were normalized to that at 1 hr.

Table

Gene-specific primers encoding pneumococcal virulence factors.

Gene	Acc#	5'-Sequence	3'-Sequence
16S rRNA	NC_008533	CGCGAAGAACCTTACCA	ACCCAACATCTCACGACACGA
PsaA	AF248229	CGCCAAAGACCCTAACAAT	CTCCTTCGCTGGTTACAATGA
SpxB	L39074	TTGATGGACGCTTTGGCTGAA	TGAGTCGCACCTGGACCACCT
LytA	AJ419978	GCAACCATATAGGCAAGTCCA	CCCAACGTCCCAGGCACCATT
Ply	M17717	CAGCTACCCGATGAGTTTGTT	TGCTCCAGGATAGAGGCGACT