Pneumococcal Virulence Gene Expression and Host Cytokine Profiles during Pathogenesis of Invasive Disease

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Pneumococcal disease continues to account for significant morbidity and mortality worldwide. For the development of novel prophylactic and therapeutic strategies against the disease spectrum, a complete understanding of pneumococcal behavior in vivo is necessary. We evaluated the expression patterns of the proven and putative virulence factor genes adcR, cbpA, cbpD, cbpG, cpsA, nanA, pcpA, piaA, ply, psaA, pspA, and spxB after intranasal infection of CD1 mice with serotype 2, 4, and 6A pneumococci by real-time reverse transcription-PCR. Simultaneous gene expression patterns of selected host immunomodulatory molecules, CCL2, CCL5, CD54, CXCL3, interleukin-6, and tumor necrosis factor alpha, were also investigated. We show that pneumococcal virulence genes are differentially expressed in vivo, with some genes demonstrating niche- and serotype-specific differential expression. The in vivo expression patterns could not be attributed to in vitro differences in expression of the genes in transparent and opaque variants of the three strains. The host molecules were significantly upregulated, especially in the lungs, blood, and brains of mice. The pneumococcal-gene expression patterns support their ascribed roles in pathogenesis, providing insight into which protein combinations might be more appropriate as vaccine antigens against invasive disease. This is the first simultaneous comparison of bacterial- and host gene expression in the same animal during pathogenesis. The strategy provides a platform for prospective evaluation of interaction kinetics between invading pneumococci and human patients in culture-positive cases and should be feasible in other infection models.

Streptococcus pneumoniae (the pneumococcus) continues to be responsible for significant mortality and morbidity worldwide, causing a broad spectrum of diseases, including pneumonia, meningitis, bacteremia, and otitis media (40, 64). In developing countries, up to 1 million children under 5 years of age die each year from pneumonia, of which S. pneumoniae is the single most common cause. Indeed, pneumonia is responsible for 20 to 25% of all deaths in this age group in those countries (77, 79). Even in developed countries, where effective antimicrobial therapy is readily accessible, morbidity and mortality are substantial. In these countries, deaths from pneumococcal disease occur primarily among the elderly, in whom bacteremic pneumonia is associated with case-fatality rates of 10 to 20% and pneumococcal bacteremia with a case-fatality rate of up to 60% (79). Furthermore, the less serious but highly prevalent infections, such as otitis media and sinusitis, have a significant impact on health care costs in developed countries. In the United States, estimates for acute otitis media are between $1.4 billion and $4.1 billion per year (11).

The continuing problems of pneumococcal disease are partly attributable to the rate at which the organism is acquiring resistance to multiple antimicrobials and the rapid global spread of highly resistant clones (63). The problem is exacerbated by the shortcomings associated with the current capsule-based vaccines, including high cost, serotype-specific protection, limited serotype coverage, and the likelihood of replacement carriage and disease caused by nonvaccine serotypes. Consequently, concerted global efforts are currently focused on developing alternative pneumococcal vaccine strategies that address these shortcomings without compromising efficacy. One of these approaches involves the development of vaccines based on pneumococcal proteins that contribute to pathogenesis and are common to all serotypes (8, 9, 48, 51, 59).

Asymptomatic colonization of the upper respiratory tract almost invariably precedes disease (19, 40, 64). However, the factors and/or events that trigger the progression from nasopharyngeal colonization to invasive pneumococcal disease (IPD) in certain individuals are poorly understood. This transition involves a major alteration in the microenvironment to which the pneumococcus is exposed, which requires alteration in the transcriptional profiles of complex sets of bacterial and host genes. In recent years, a systematic examination of pneumococcal-gene expression patterns in distinct environmental niches has gained momentum (32, 47, 49, 54, 55). However, progress has been hampered by technical difficulties associated with isolating sufficient quantities of pure and intact bacterial RNA from infected host tissues to perform accurate, quantitative mRNA analyses. Furthermore, much information has relied on discrete studies of either pneumococcal-gene expression or host innate immune responses during the pathogenesis of disease. Consequently, there is a paucity of data on concomitant host innate immune responses to pneumococcal infection, which could shed light directly on factors necessary for bacterial clearance or progression to disease.

Previous mouse intranasal (i.n.) challenge experiments in our laboratory with the virulent pneumococcal strains D39...
(serotype 2), WCH43 (serotype 4), and WCH16 (serotype 6A) indicated that D39 and WCH43 were more virulent than WCH16. Nevertheless, WCH16 and WCH43 had a greater propensity to translocate to the brains of infected mice within 72 h than D39 (unpublished observations). These distinct pathogenicity profiles are reminiscent of findings by other workers (52) and make the three strains attractive for gene expression analysis during disease progression. In this work, we evaluated the gene expression patterns of proven and putative pneumococcal virulence factors after i.n. infection of CD1 mice with the three strains. We reasoned that the relative expression levels of genes in discrete niches should support their ascribed roles in pathogenesis, which in turn would give us insight into which proteins might be more appropriate as vaccine antigens against carriage and/or IPD. Furthermore, we hypothesized that the level of the host innate immune response would correlate with the pneumococcal load and disease progression. The genes evaluated were those encoding choline binding proteins (cbpA [pspC], cbpD, cbpG, pcpA, and pspA), the pneumococcal cytotoxin pneumolysin gene (ply), the first gene of the capsule polysaccharide biosynthesis locus (cpsA), one of the three known pneumococcal neuraminidase genes (nanA), ion transporter genes (psaA and piaA), a putative Zn$^{2+}$-dependent regulator gene (adcR), and the pyruvate oxido-gase gene (spxB). Each of the encoded proteins have proven or ascribed roles in pneumococcal pathogenesis, including colonization of the nasopharynx, pneumonia, sepsis, and/or meningitis (1, 2, 5, 13, 14, 17, 18, 20, 22, 23, 35, 37, 38, 39, 41, 45, 47, 53, 57, 58, 64, 66, 67, 68, 69, 70, 72, 75, 80, 81). We also investigated gene expression patterns of selected host molecules known to modulate immune responses to pneumococcal infection. They were the chemokines CCL2 (MCP-1) (78), CCL5 (56), and CXCL2 (MIP-2) (18, 31); the inflammatory cytokines interleukin-6 (IL-6) (3, 7, 15) and tumor necrosis factor alpha (TNF-α) (3, 28, 43, 46, 61); and intracellular adhesion molecule 1 (ICAM-1, or CD54) (44).

We present data showing that pneumococcal virulence genes are differentially expressed in vivo and that some genes demonstrate niche- and strain-specific differential expression. There was significant upregulation of the host molecules, especially in the lungs, blood, and brains of mice, impacting on the ability of the host to control the infection. To our knowledge, this is the first investigation in which expression levels of pneumococcal and host response genes have been simultaneously compared in the same animal during progression from nasopharyngeal colonization to invasion of the lungs, blood, and brain.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The pneumococcal strains used in this study were D39, a virulent type 2 strain (NCTC 7466), and virulent type 4 (WCH43) and type 6A (WCH16) clinical (blood) isolates, from the Women’s and Children’s Hospital, North Adelaide, Australia. Serotype-specific capsule production was confirmed by Quellung reaction, as described previously (6). Opaque-phase variants of the three strains, selected on Todd-Hewitt broth supplemented with 1% yeast extract (THY)-catalase plates (76), were used in all animal experiments. Before infection, the bacteria were grown statically at 37°C in THY broth to an optical density at 600 nm (OD$_{600}$) of 0.25 (equivalent to approximately 1 × 10$^7$ to 2 × 10$^8$ CFU/ml). The bacteria grown in this manner also served as a source of in vitro-derived RNA.

**Mice.** Outbred 5- to 6-week-old female CD1 (Swiss) mice were used in all experiments. The Ethics Committee of the University of Adelaide approved all animal experiments.

**In vivo challenge of mice for gene expression analyses.** For D39 and WCH43 challenges, groups of 14 to 15 mice were used. For WCH16 challenge, two separate challenge experiments, each comprising 12 mice, were performed. The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal; Rhone-Merieux) at a dose of 66 μg per g of body weight and challenged i.n. with 25 μl of bacterial suspension containing approximately 1 × 10$^7$ to 2 × 10$^8$ CFU in phosphate-buffered saline. The challenge dose was confirmed retrospectively by serial dilution and plating of the inocula on blood agar. At 72 h postchallenge, the mice were sacrificed by CO$_2$ asphyxiation, and nasal wash, nasal tissue, lung, blood, and brain samples were processed as described previously (32, 49). A 40-μl aliquot of each sample was serially diluted in phosphate-buffered saline and plated on blood agar to enumerate pneumococci present in each niche and to determine the presence, if any, of contaminating microflora. Blood plates were incubated at 37°C in 5% air, 5% CO$_2$ overnight. Samples were then stored at −80°C until further processing was done. In addition, a 400-μl aliquot of blood and homogenized nasal, lung, and brain tissue was also harvested from each mouse for extraction of eukaryotic RNA.

**Extraction of total RNA from bacteria and host tissues.** RNA was isolated and purified from in vitro- and in vivo-derived bacterial pellets and host tissues with acid-phenol–chloroform–isoamyl alcohol (125:25:1; pH 4.5; Ambion), essentially as described previously (32). The purity of each bacterial-RNA preparation was confirmed by one-step reverse transcription (RT)-PCR, with or without reverse transcriptase, using 16S-RNA-specific primers, and the products were visualized after electrophoresis on a 2% Tris-borate-EDTA-agarose gel. In all cases, a PCR product was seen only in the presence of reverse transcriptase. The purity of eukaryotic-RNA samples was ascertained using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers in the RT-PCR in the presence of reverse transcriptase. A single PCR product was obtained, ruling out contaminating DNA. For bacterial RNA, samples from individual mice were selected based on the number of CFU recovered from specific niches and also on the absence or minimal presence of contaminating bacteria. Where indicated, bacterial-RNA samples obtained from four or five mice from a specific niche were pooled. The RNA was then purified further using a Qiagen RNeasy minikit. RNA obtained from lung and brain homogenates was further enriched for prokaryotic RNA using the MicroBEnrich kit (Ambion). The amount of RNA recovered following purification/enrichment was determined by OD$_{260/280}$ measurements.

**Linear amplification of total RNA.** Bacterial-RNA samples derived in vivo (and eukaryotic RNA samples where necessary) were linearly amplified using an advanced RNA linear-amplification kit, SenseAMP Plus (Genisphere), described previously (32). To obtain sufficient quantities of bacterial RNA for real-time RT-PCR analysis, two rounds of amplification were performed for all samples. We had previously determined that one and two rounds of RNA amplification had high integrity and a high correlation coefficient compared to unamplified RNA (unpublished observations).

**Real-time relative qRT-PCR.** The abundances of $adcr$, $cbpA$, $cbpD$, $cbpG$, $cpsC$, $nanA$, $pcaP$, $piaA$, $ply$, $psaA$, $psaB$, and $spxB$ mRNAs present in amplified RNA recovered from pneumococci harvested from all niches were measured by real-time relative quantitative RT-PCR (qRT-PCR). The abundances of the selected host CCL2, CCL5, CD54, CXCL2, IL-6, and TNF-α mRNA species in the respective niches were also quantitated. Gene-specific LUX fluorogenic primer sets labeled with JOE were designed using D-LUX primer designer software (Invitrogen), employing primers specific for 16S rRNA (for pneumococcal RNA) and GAPDH (for eukaryotic RNA) as internal controls for data normalization (Table 1). Real-time RT-PCR was performed on a Rotorgene RG-2000 (Corbett Research, Mortlake, NSW, Australia) using a Superscript III One-Step RT-PCR System (Invitrogen) according to the manufacturer’s instructions. Quantitative differences for each transcript were determined using the $2^{-ΔΔC_{t}}$ method (34). Expression data for each bacterial gene were analyzed relative to the level of the respective transcript in pneumococci grown in THY broth to an OD$_{600}$ of 0.25 and are presented as a relative increase/decrease between niches.

**Statistical analysis.** Differences in median survival times between groups were analyzed by the Mann-Whitney U test (two tailed). Differences in the relative expression levels of genes between niches were analyzed by Student’s t test. All analyses were performed using Graphpad Prism version 4.03. A P value of <0.05 was considered significant.
RESULTS

Differences in the pathogenicity characteristics of challenge strains. In previous mouse i.n. virulence studies using D39 (serotype 2), WCH43 (serotype 4), and WCH16 (serotype 6A), we observed that D39 and WCH43 were more virulent than WCH16. However, WCH16 and WCH43 had a greater propensity to translocate to the brains of infected mice within 72 h than D39 (unpublished observations). The distinct pathogenicity characteristics displayed by the three strains were attractive for comparative gene expression analysis during the progression of disease from the nasopharynx to the lungs, blood, and brains of mice. Indeed, bacterial counts obtained from mice infected with the three strains 72 h postinfection showed that they colonized the nasopharynx at similar levels of approximately 10⁵ CFU per mouse (Fig. 1A, B, and C). However, WCH16 appeared to be unable to proliferate in the lungs and blood of the infected mice to the same...
degree as D39 and WCH43 yet translocated to the brain as well as WCH43 (Fig. 1C).

Gene expression profiles of opaque and transparent variants. Earlier studies had indicated that the pneumococcus undergoes spontaneous phase variation between an opaque and a transparent colony phenotype (30, 76). It was suggested that this phenomenon might provide insight into the interaction of the pneumococcus with its host, with the opaque phenotype being significantly more virulent than the transparent phenotype (10, 76). Indeed, pilot i.n. challenge experiments in our laboratory using opaque and transparent variants of D39 and WCH16 indicated that the median survival times for mice challenged with the transparent variants were significantly longer than for mice challenged with their respective opaque counterparts (not shown). Therefore, the in vitro expression levels of the proven and putative pneumococcal virulence genes $adcR$, $cbpA$, $cbpD$, $cbpG$, $cpsA$, $nanA$, $pcpA$, $piaA$, $ply$, $psaA$, $pspA$, and $spxB$ in transparent and opaque variants of the three strains were measured by real-time qRT-PCR. We observed that expression of $pcpA$ was significantly higher in the transparent phenotype in D39 (Fig. 2A), while the expression levels of $psaA$ and $pcpA$ were significantly higher in the transparent phenotype in WCH43 (Fig. 2B). Interestingly, in WCH16, expression of $cbpA$ was significantly higher in the transparent phenotype, while the expression levels of $nanA$ and $ply$ were significantly higher in the opaque phenotype (Fig. 2C). The expression levels of the other genes were not significantly different in the two phenotypic variants. To ensure maximal recovery of bacteria from in vivo niches, opaque variants of all strains were used in all animal experiments.

Differential expression of virulence-associated genes. The relative expression levels of the virulence factors in various in vivo niches were evaluated at 72 h after challenge. This time point was chosen because we had observed in previous mouse i.n. challenge studies using D39 and WCH43 that the maximum recovery of virulent pneumococci was obtained from various in vivo niches, beyond which the mice succumbed rapidly to infection (unpublished data). We also observed that, 72 h after challenge, bacteria plated on THY-catalase agar were predominantly of the transparent phenotype in the nasal wash (and in brain samples from WCH43 infection). However, the bacteria were predominantly of the opaque phenotype in lung and blood samples (data not shown), in agreement with previous reports (10, 30, 51, 64, 76).

A total measure of mRNA quantities for each virulence gene investigated in vitro and in vivo indicated that for D39 and WCH43, $cpsA$, $ply$, $psaA$, and $pspA$ were the most abundant while $nanA$, $piaA$, $cbpD$, and $pcpA$ were the least abundant (data not shown). However, for WCH16, $cpsA$, $adcR$, $psaA$, and $pspA$ were the most abundant while $cbpA$ and $nanA$ were the least abundant (data not shown). A comparison of the mRNA levels of the virulence genes in various niches relative to levels in vitro indicated differential expression for the three strains studied. For D39 and WCH43, data from a typical experiment comprising pooled RNA from four or five mice are shown in Fig. 3 and 4. Notably, sufficient RNA was obtained from two individual mice not included in the pooled mRNA, allowing analysis of variations in the relative in vivo expression of the virulence genes between individual mice (see Fig. S1 and S2 and Tables S1 and S2 in the supplemental material). However, for WCH16, insufficient RNA quantities were obtained from the lungs and blood of individual mice due to very low numbers of bacteria harvested from those niches (Fig. 1C). Therefore, data from two separate experiments, comprising
FIG. 2. Comparison of virulence gene mRNA concentrations between opaque and transparent variants of pneumococci grown in vitro. (A) D39. (B) WCH43. (C) WCH16. Real-time RT-PCR data for each gene were normalized against those obtained for the 16S rRNA control. The mRNA concentration was calculated as $2^{-\Delta C_T}$ for each virulence gene, where $\Delta C_T$ represents the threshold cycle ($C_T$) value of the gene subtracted from the $C_T$ value of the 16S rRNA control. Values are mean ± standard error of the mean [mRNA] opaque/transparent ratios for each gene transcript from two replicate experiments. The data were analyzed using Student’s $t$ test (two tailed). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 
pooled RNA from four or five mice in each experiment, are shown. The relative differences in virulence gene expression between niches are presented in Tables 2, 3, and 4. It is worth noting that, while there were some variations in the relative in vivo expression levels of the virulence genes between individual mice, in general, the differential expression patterns of each gene in various niches were comparable. For analytical purposes, data for experiments involving pooled pneumococcal RNA from four or five mice are discussed further.

**Gene expression profiles show a requirement for carriage.** We observed that the mRNA expression levels of most genes (except cbpG and spxB expression for D39 and cbpG, piaA, and spxB expression for WCH43) were significantly higher in the nasopharynx than in the lungs and blood, except for psaA expression in the blood, where the difference did not reach statistical significance (Fig. 3). For WCH43, the mRNA expression levels of all genes were significantly higher in the nasopharynx than in the lungs, blood, and brain, except for cbpD expression in the lungs and piaA expression in the blood, where the differences did not reach statistical significance (Fig. 4).

In the WCH16 (serotype 6A) infection experiments, the virulence genes were also differentially expressed in various niches. However, the expression patterns were distinct from those observed for D39 and WCH43. In the first of the two WCH16 experiments, the relative expression levels of ply, expression levels of all D39 genes were significantly higher in the nasopharynx than in the lungs and blood, except for psaA expression in the blood, where the difference did not reach statistical significance (Fig. 3). For WCH43, the mRNA expression levels of all genes were significantly higher in the nasopharynx than in the lungs, blood, and brain, except for cbpD expression in the lungs and piaA expression in the blood, where the differences did not reach statistical significance (Fig. 4).

In the WCH16 (serotype 6A) infection experiments, the virulence genes were also differentially expressed in various niches. However, the expression patterns were distinct from those observed for D39 and WCH43. In the first of the two WCH16 experiments, the relative expression levels of ply,
Nevertheless, in general terms, data from the two WCH16 strains harvested from all in vivo niches were significantly higher in the lungs and brain than in the naive mice challenged with D39 (Fig. 3). However, pspA expression was lower in the lungs and blood of mice challenged with WCH16. In addition, the expression levels of nanA were higher in the nasopharynx and lungs than in vitro for D39 and in all the in vivo niches for WCH43, but its expression levels were quite similar in vitro and in vivo for WCH16. Furthermore, in the brain, the relative mRNA concentration of spxB was low for WCH43 but was very high in the same niche for WCH16. Finally, it was interesting that cbpG expression was downregulated in all in vivo niches for D39 and WCH43 but upregulated in all in vivo niches for WCH16.

Host innate immune responses correlate with infection in discrete niches. The mRNA levels for genes encoding host factors known to modulate immune responses to pneumococcal infection were measured 72 h after challenge with the three strains. In the nasal tissue, mRNA expression levels of CCL2, CD54, and CXCL2 were significantly higher in D39-infected mice than in naive mice (Fig. 6A). A similar result was obtained with WCH43-infected mice, but in addition, the mRNA expression level of CCL5 was also significantly higher in infected nasal tissue (Fig. 6A). However, in the nasal tissues of WCH16-infected mice, there was massive upregulation of the mRNAs of all host genes studied (Fig. 6A).

In the lungs, the mRNA levels of all host genes studied were significantly higher in D39-infected mice than in the lungs of naive mice (Fig. 6B). Again, a similar result was obtained in WCH43-infected mice. However, expression of CCL5 and CD54 was not upregulated in the lungs of WCH43-infected mice (Fig. 6B). Interestingly, while there was significant upregulation of CCL2, CCL5, CXCL2, IL-6, and TNF-α mRNAs in the lungs of WCH16-infected mice, CD54 mRNA expres-

### TABLE 3. Differential expression of WCH43 virulence genes in various niches

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nose vs. lungs</th>
<th>Nose vs. blood</th>
<th>Lungs vs. brain</th>
<th>Blood vs. brain</th>
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<tbody>
<tr>
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<td>+7.3d</td>
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<td>cbpD</td>
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<td>+11.9d</td>
<td>−1.4</td>
<td>+4.9</td>
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<tr>
<td>cbpG</td>
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<td>+31.1d</td>
<td>+2.1b</td>
<td>+7.9</td>
</tr>
<tr>
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<td>+9.8e</td>
<td>+3.4d</td>
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</tr>
<tr>
<td>nanA</td>
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<td>+15.9d</td>
<td>+4.2</td>
<td>+2.6</td>
</tr>
<tr>
<td>pspA</td>
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<td>+17.4d</td>
<td>+13.6d</td>
<td>+2.8</td>
</tr>
<tr>
<td>piaA</td>
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<td>+5.2d</td>
<td>+3.0b</td>
<td>+1.4</td>
</tr>
<tr>
<td>ply</td>
<td>+16.1b</td>
<td>+294.1d</td>
<td>+10.3d</td>
<td>+18.2d</td>
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<tr>
<td>psaA</td>
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<tr>
<td>spxB</td>
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<td>+34.3b</td>
<td>+7.9b</td>
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</tbody>
</table>

*a The values represent the relative amounts of mRNA in the first niche compared to the second. Data were analyzed using Student’s t test (two-tailed).

*b P < 0.05.

c P < 0.01.

d P < 0.005.

### TABLE 4. Differential expression of WCH16 virulence genes in various niches

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nose vs. lungs</th>
<th>Nose vs. blood</th>
<th>Lungs vs. brain</th>
<th>Blood vs. brain</th>
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<tbody>
<tr>
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<td>+2.0</td>
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<td>+25.7d</td>
<td>+37.5b</td>
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*a The values represent the relative amounts of mRNA in the first niche compared to the second. Data were analyzed using Student’s t test (two-tailed).

*b P < 0.05.

c P < 0.01.

d P < 0.005.
sion was significantly downregulated compared to levels in the lungs of naïve mice (Fig. 6B). In the blood, the mRNA expression levels of all host genes studied were significantly higher in D39- and WCH43-infected mice than in naïve mice. However, in WCH16-infected mice, the level of CD54 expression was similar to that seen in naïve mice (Fig. 6C). Finally, there was significant upregulation of all host mRNA genes studied in the brain tissues of WCH43- and WCH16-infected mice (Fig. 6D).

DISCUSSION

The pathogenesis of pneumococcal disease is a complex process involving a series of interactions between pathogen and host. During disease progression, the pneumococcus must adapt to a range of environments, such as the nasopharyngeal and lung mucosa, blood, and cerebrospinal fluid, and optimal survival in distinct host niches requires differential expression of virulence factors. Several well-characterized pneumococcal virulence factors are known to contribute to pathogenesis (1, 2, 5, 13, 14, 17, 18, 20, 22, 23, 35, 37, 38, 39, 41, 45, 47, 53, 57, 58, 64, 66, 67, 68, 69, 70, 72, 75, 80, 81). In recent years, direct molecular evidence for pneumococcal-gene expression patterns in the distinct environmental niches has emerged (32, 47, 49, 54, 55), but it is limited to discrete examination of either pneumococcal-gene expression or host innate immune responses. In this work, we investigated virulence gene expression in three pneumococcal strains and simultaneous host cytokine responses in a murine model. Our objective was to gain new insight into pneumococcal behavior in vivo and to dissect bacterial and host factors necessary for clearance or progression to disease.

We observed that the pneumococcal virulence genes examined are differentially expressed in various in vivo niches. This is not altogether surprising and presumably reflects the relative contributions of these factors at different stages of the pathogenic process. The niche-specific differences were not attributable to phase variation, which had only minor effects on the relative [mRNA] for these genes between transparent and opaque phenotypes. The expression levels of the vast majority of the genes were relatively high in the nasopharynx for the three strains, implying a requirement for carriage. This study thus provides direct molecular evidence supporting the hy-

![Fig. 5. WCH16 virulence gene mRNA concentrations in various in vivo niches relative to concentrations in vitro. Real-time RT-PCR data for each gene were normalized against those obtained for the 16S rRNA control. Quantitative differences for each transcript were determined using the $2^{-\Delta \Delta C_T}$ method (34). The data are means ± standard deviations of triplicate reactions for each gene transcript. Data for pooled pneumococcal RNA from four or five mice from two independent experiments (A and B) are presented.](image-url)
pothesis advanced by others (10, 22, 23, 53) that many pneumococcal virulence factors have dual roles in the establishment and maintenance of carriage, as well as involvement in certain stages of invasive disease.

It is striking that while in vivo expression patterns for the majority of the genes were similar across the three strains, some genes demonstrated strain- and niche-specific differential expression. For example, the relative expression levels of cbpA were higher in WCH43 and WCH16 harvested from all in vivo niches than that obtained in vitro but lower in the lungs and blood of mice challenged with D39. This finding provides molecular evidence that explains the discordance in the literature regarding the effects of cbpA mutation on the virulence of different pneumococcal strains (6, 29, 51, 53). However, upregulation of cbpA expression in the brains of WCH16-infected mice is consistent with its putative role in adherence to, and invasion of, the brain microvascular endothelium (53, 60, 64, 81). Interestingly, pspA expression was opposite to that obtained for cbpA expression, being upregulated in the lungs and blood of D39-infected mice but downregulated in the same niches for WCH43-infected mice. This agrees with the suggestion that the virulence properties of CbpA and PspA may complement each other in the host (12). Upregulation of pspA expression in the lungs and blood of D39-infected mice correlates with attenuation of an isogenic pspA mutant in pneumonia and bacteremic infection models (6, 51). Downregulation of pspA in WCH43 recovered from the lungs and blood could indicate that PspA does not contribute as significantly to pneumonia and sepsis in this serotype and might explain why PspA immunization does not offer effective protection against serotype 4 strains in mice (24, 65).

The differential expression of nanA in D39 and WCH43 in vivo, but not in WCH16, implies a role for NanA in pathogenesis in various serotypes and suggests that its expression is probably not essential for virulence in WCH16. Furthermore, spxB was highly upregulated in the brain for WCH16 but downregulated in the same niche for WCH43. Downregulation of spxB in the brain has also been demonstrated in another serotype 4 strain, albeit in a rabbit model (54). Expression levels of genes, such as adeR, cbpD, cpsA, pcpA, and psaA, were consistently high in various in vivo niches, implying pleiotropic effects on pathogenesis. Indeed, this has been demonstrated for the capsule (1, 2, 35, 41, 45) and PsaA (5, 19, 39). Expression of piaA was generally downregulated in the lungs, blood, and brain, implying functional redundancy with similar iron acquisition proteins. Upregulation of cbpG expression in all in vivo niches only for WCH16 suggests that CbpG could be important for pathogenesis in this strain, where it may have a dual role on mucosal surfaces and in the blood (17).

Based on the comprehensive assessment of in vivo gene expression patterns for the three strains, such information could be used in lieu of mutagenesis studies to assess niche-specific roles of novel virulence genes during pathogenesis, particularly for strains that are difficult to transform. In such instances, including an invariantly expressed gene (such as cpsA or psaA) would validate the fidelity of the assay. For
pneumococcal protein vaccine development, the strain- and niche-specific expression data provide an additional rationale for multiple protein combinations to obtain broader coverage. This proposition is exemplified by for multiple protein combinations to obtain broader coverage.

With regard to the host innate immune response, all the tested host molecules were significantly upregulated, especially in the lungs, blood, and brain tissues of mice infected by the three strains. The response most likely corresponds to the intense inflammation usually observed during severe pneumococcal pneumonia in humans, which often leads to tissue injury, shock, and death. The expression of TNF-α is important for surviving pneumococcal infection but may also cause inflammatory host injury under certain conditions. TNF-α is capable of recruiting inflammatory cells to the site of infection, directly and via upregulation of adhesion molecules and by stimulating the release of cytokines that are directly chemotactic for inflammatory cells. IL-6 can activate monocytes and can synergize with TNF-α to increase the respiratory burst of neutrophils. Another study highlighted the involvement of high levels of IL-6 in the lethality of pneumococcal infection. Upregulation of TNF-α and IL-6 in the blood of D39- and WCH43-infected mice may be partly due to upregulation of Ply in the blood, as reported previously. Indeed, Ply has been shown to activate murine macrophages, resulting in the secretion of TNF-α and IL-6, proposed to occur through Toll-like receptor 4 signaling. Ply also stimulates expression of TNF-α and IL-1 from monocytes, as well as that of CD54, an important component of the leukocyte-trafficking system. Surprisingly, CD54 expression was significantly downregulated in the lungs of WCH16-infected mice and was not significantly upregulated in the blood. While the reason for this observation is not immediately clear, we speculate that it could be due, in part, to lower expression of ply in the lungs and blood of WCH16-infected mice. Transcription of pspA increased in the nasopharynges of infected mice, consistent with the role of PspA in protecting pneumococci from being killed by apolactoferrin at mucosal sites. In contrast, pspA expression in D39 was lower in the lungs than in the nasopharynx. The absence of PspA was previously suggested to limit the chemokine response in the lungs, and deletion of pspA had minimal impact on the chemokine responses of Detroit-562 cells to S. pneumoniae. This suggests that PspA may not play a significant role in generating a CXC chemokine response from nasopharyngeal epithelial cells. However, in the current study, CCL2, CCL5, and CXCL2 chemokines were highly expressed in the lungs of mice infected with D39, implying that other factors could be responsible for these responses.

Our results also showed that the chemokine response was higher in the lungs and blood than in nasal tissue. It was previously shown that CbpA may suppress CXC chemokine production by nasopharyngeal epithelial cells, thereby promoting colonization by preventing the neutrophil recruitment required for bacterial clearance. CbpA has also been shown to elicit cytokine and ICAM-1 expression from human alveolar epithelial cells. In the lungs, an early, sharp host innate immune response was shown to be vital in overcoming pneumococcal infection. Thus, CbpA-mediated attenuation of this response may prevent clearance, leading to a sustained inflammation that aids pneumococcal entry into the blood. While PspA and CbpA have significant and differential effects on chemokine responses of respiratory epithelial cells in vitro, effects on other cell types could influence the overall host response in a given niche. Moreover, other proinflammatory factors, such as lipoteichoic acid and Ply, are likely to contribute. Indeed, it was shown that Ply, PspA, and CbpA (PspC) all contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice. The involvement of proinflammatory cytokines, CXC chemokines, and cell adhesion molecules in pneumococcal meningitis is well established.

For WCH16-infected mice, a significant finding was that the tested host molecules were massively upregulated in the nasopharynx and brain. This could explain why WCH16 is not as virulent as D39 and WCH43. Indeed, none of the WCH16-infected mice died before the 72-h end point of the experiments. These results correlate with that reported in another study using different challenge and mouse strains, that distinct cytokine profiles characterize pneumococcal carriage, self-resolving pneumonia, and lethal disease. In that work, the authors proposed that activation of the immune system resulted in nonlethal disease for a serotype 6B strain (as seen for WCH16), whereas evasion of the immune system resulted in detrimental disease for serotypes 3 and 14, as observed for D39 and WCH43.

The results of this work open new vistas in the study of pneumococcal gene expression and host response during pathogenesis of invasive disease. Future studies examining global pneumococcal and host gene transcriptional profiles, and at earlier time points (e.g., 24 h and 48 h), will provide a more comprehensive analysis of pneumococcal and host gene expression kinetics. It will also be important to correlate mRNA transcript levels in bacteria and host tissues with protein levels in such experiments. Such studies will also necessarily include a dissection of the bacterial transcription factors that regulate gene expression in response to different host environmental cues and the host signals that trigger the expression of those factors. Analysis of the key events that control these interactions will increase our understanding of pneumococcal pathogenesis, as well as aid in the design of appropriate tools to control the inflammatory response.

In conclusion, the relative expression levels of pneumococcal virulence genes in discrete niches support their ascribed roles in pathogenesis and provide insight into which proteins and combinations thereof might be more appropriate as vaccine antigens against carriage or IPD. There was significant host innate immune response, impacting on the ability of the host to control the infection. One previous study employed microarray technology for simultaneous analysis of host-pathogen interactions in a localized murine granulomatous pouch infection model using a virulent Escherichia coli strain. Other studies have used in vivo expression technology, signature-tagged mutagenesis, differential fluorescence induction, and subtrac-
tive hybridization to measure bacterial-gene expression in vivo (reviewed in reference 21). Our approach is unique, providing a platform to directly examine the kinetics of pneumococcal- and host gene expression from asymptomatic colonization to the establishment of invasive disease. To our knowledge, this is the first comprehensive investigation in which the expression levels of bacterial and host response genes have been simulta-
neously compared in the same animal during pathogenesis using a model that mimics human disease. The strategy could be directly applied to prospective evaluation of the interactions between invading pneumococci and human patients in culture-
positive cases and should be broadly applicable to the analysis of in vivo molecular interactions between other bacterial pathogens and their respective hosts in appropriate infection models. Such studies would be pertinent to the development of improved prophylactic and therapeutic interventions.

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