

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, CXCL2, 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 vac-

cines, 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

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(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 capsular polysaccharide biosynthesis locus (*cpsA*), one of the three known pneumococcal neuraminidase genes (*nanA*), ion transporter genes (*psaA* and *piaA*), a putative Zn²⁺-dependent regulator gene (*adcR*), and the pyruvate oxidase 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 (OD₆₀₀) of 0.25 (equivalent to approximately 1×10^8 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. 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 μ g per g of body weight and challenged i.n. with 25 μ l of bacterial suspension containing approximately 1×10^7 to 2×10^7 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 CO₂ asphyxiation, and nasal wash, nasal tissue, lung, blood, and brain samples were processed as described previously (32, 49). A 40- μ l 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 95% air, 5% CO₂ overnight. Samples were then stored at -80°C until further processing was done. In addition, a 400- μ l 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 (125:24:1; 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 rRNA-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), as 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*, *cpsA*, *nanA*, *pcpA*, *piaA*, *ply*, *psaA*, *pspA*, 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^{- $\Delta\Delta$ CT} 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 OD₆₀₀ 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.

TABLE 1. Oligonucleotide primers used in this study

Primer ^a	Sequence (5'→3')	Accession no.
Prokaryotic genes		
16S F (JOE)	CGGCCTAACTACGTGCCAGCAGC(JOE)G	NC_003098
16S R	GCTCGCTTACGCCAATAAAT	
<i>adcR</i> F (JOE)	CGGGTAACAAATTCAGAATTGGCC(JOE)G	NP_346586
<i>adcR</i> R	TTCCAACATCCCTTCCTTGACC	
<i>cbpA</i> F (JOE) R6 ^b	CGGCGATAGTGAGGAGCGAGGC(JOE)G	NP_359586
<i>cbpA</i> R R6 ^b	TCGCTACGAACACTTGAAACG	
<i>cbpA</i> F (JOE) TIGR4 ^c	CGCATTGGGAAGTGTGGTTCATG(JOE)G	NP_346601
<i>cbpA</i> R TIGR4 ^c	TGTTCTGCCTGACTTTCATTTCG	
<i>cbpA</i> 6B F ^d	TGCTAAGTTGAAGGAAGCTGTTG	AAF13460
<i>cbpA</i> 6B R (JOE) ^d	CGAGGTAGCTCTCCAGAACTCCT(JOE)G	
<i>cbpD</i> F (JOE)	CGAGACAGTGGCGCATGTATTCT(JOE)G	NP_346612
<i>cbpD</i> R	TTTCCATAAGCTGCCGGAATTT	
<i>cbpG</i> F (JOE)	CGCATAGAAGGGAGGGGAAGTG(JOE)G	NP_344916
<i>cbpG</i> R	ATCCGCTTCTTCCCATAGTCA	
<i>cpsA</i> F (JOE)	CGACACATGGTCCTATTAGTTCGGTGT(JOE)G	NP_357908
<i>cpsA</i> R	GTGGCGTTGTGGTCAAGAGG	
<i>nanA</i> F (JOE)	CGGAGAGCGCCTCAAGATATTACTC(JOE)G	NP_359129
<i>nanA</i> R	GCCCATTCGGAAGTACAATTCC	
<i>pcpA</i> F (JOE)	CGACTACGATAGCCATCGGGTGTAGT(JOE)G	NP_346554
<i>pcpA</i> R	CAATGGCTACTGGTTGGGTTACAG	
<i>piaA</i> F (JOE)	CGATTTTCAAAGTTAAGTCCATCAAAT(JOE)G	NP_358528
<i>piaA</i> R	GCAAATTACGGTGTAAAGTGCTGA	
<i>ply</i> F (JOE)	CGGACCCCAAATAGAAATCGTC(JOE)G	NP_359331
<i>ply</i> R	TAGAGAGTGTACCGGGCTTGC	
<i>psaA</i> F (JOE)	CGTCTTATACGAACCACTTCTGAAGA(JOE)G	NP_346089
<i>psaA</i> R	GTAACCAAGCATTGCCACCTG	
<i>pspA</i> F (JOE) R6 ^b	CGGCCAACAAAGCTACAGACAAAGC(JOE)G	NP_357715
<i>pspA</i> R R6 ^b	TTTGCTCTTCTTCGCGTTTCTT	
<i>pspA</i> F (JOE) TIGR4 ^c	CGGTGAACCTGTAGCCATAGCAC(JOE)G	NP_344663
<i>pspA</i> R TIGR4 ^c	GGCGACAGGATGGGCTAAAG	
<i>pspA</i> 6A F ^e	TTCCGTGCTCCTCTTCAATCTG	AAF27701
<i>pspA</i> 6A R (JOE) ^e	CGGTGTATTGTCCAGCTTGTCTAC(JOE)G	
<i>spxB</i> F (JOE)	CGGTATTTGACAGGTTCTGTCTAC(JOE)G	NP_345231
<i>spxB</i> R	GCAAATGGGAAGTTTGAACCAA	
Eukaryotic genes		
GAPDH F (JOE)	CGGAGCAAACGGGTCATCATCTC(JOE)G	NM_008084
GAPDH R	CTCGTGGTTCACACCCATCAC	
CCL2 F (JOE)	CGGGCTTTGAATGTGAAGTTGACC(JOE)G	NM_011333
CCL2 R	AGTGCTTGAGGTGGTTGTGGAA	
CCL5 F (JOE)	CACGACGTGCTCCAATCTTGACATCG(JOE)G	NM_013653
CCL5 R	CTCTGGGTTGGCACACACTTG	
CD54 F (JOE)	CGGCGGGGAGGAGATACTGAGC(JOE)G	NM_010493
CD54 R	ACCGTGAATGTGATCTCCTTGG	
CXCL2 F (JOE)	CGTGGTCTTCAGGCATTGACAG(JOE)G	NM_009140
CXCL2 R	CCACCAACCACCAGGCTACAG	
IL-6 F (JOE)	CGGACCCTTCCTACTTCACAAGTC(JOE)G	NM_031168
IL-6 R	CAGGTCTGTTGGGAGTGGTATCC	
TNF- α F (JOE)	CGAGTGGGCTACAGGTTGTCTACT(JOE)G	NM_013693
TNF- α R	CTATGGCCCAGACCCTCACAC	

^a Pneumococcal- and mouse gene-specific LUX fluorogenic primer sets labeled with JOE were designed using D-LUX primer design software (Invitrogen).

^b Primer sequence derived from the *S. pneumoniae* R6 genome as deposited in The Institute for Genomic Research (TIGR) Comprehensive Microbial Resource.

^c Primer sequence derived from the *S. pneumoniae* TIGR4 genome as deposited in The Institute for Genomic Research (TIGR) Comprehensive Microbial Resource.

^d Primer sequence derived from *S. pneumoniae* serotype 6B (strain BG9163) sequence as deposited in the National Center for Biotechnology Information database.

^e Primer sequence derived from *S. pneumoniae* serotype 6A (strain DBL6A) sequence as deposited in the National Center for Biotechnology Information database.

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

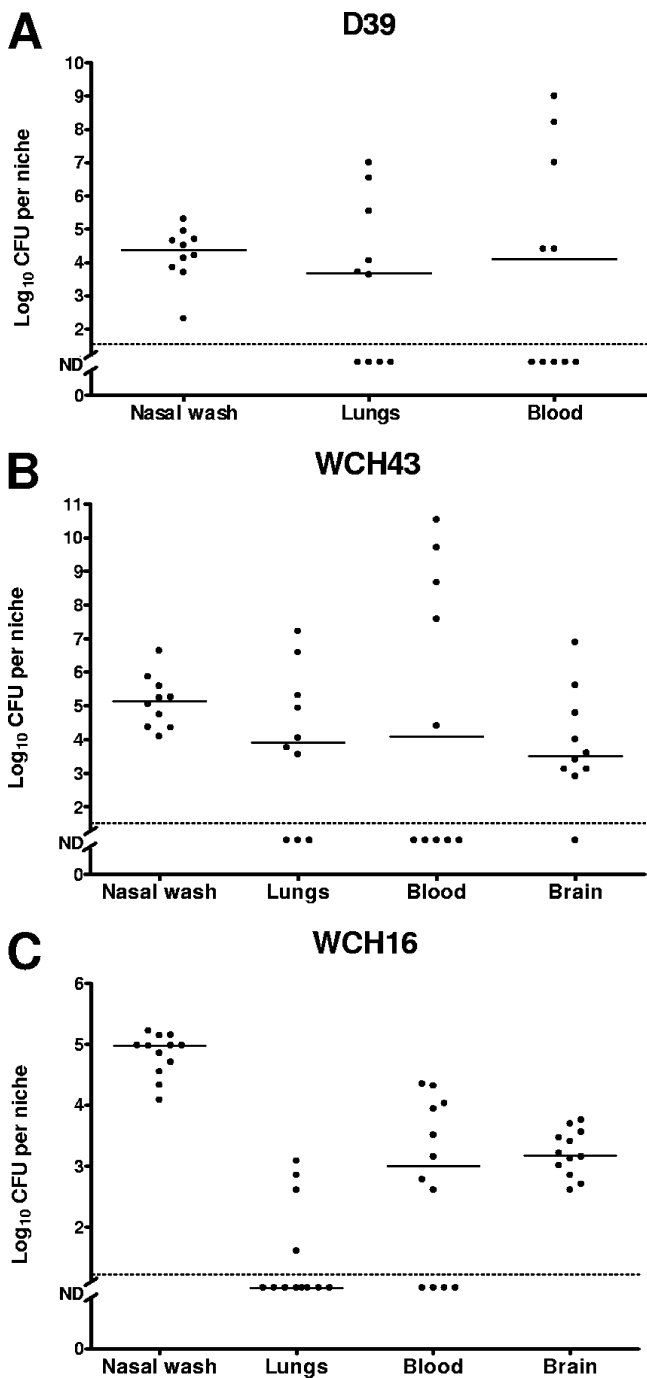


FIG. 1. Bacterial recovery from various niches of CD1 mice 72 h after i.n. challenge. The data are for D39 (A), WCH43 (B), and WCH16 (C). For D39 and WCH43, CFU counts were from 10 mice; for WCH16, CFU counts were for 12 mice. The horizontal lines indicate the median level of CFU recovered from each niche for the indicated strains. The limit of detection, indicated by dashed lines, was 40 CFU for nasal-wash and blood samples and 80 CFU for lung and brain samples. ND, not detected.

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 un-

dergoes 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

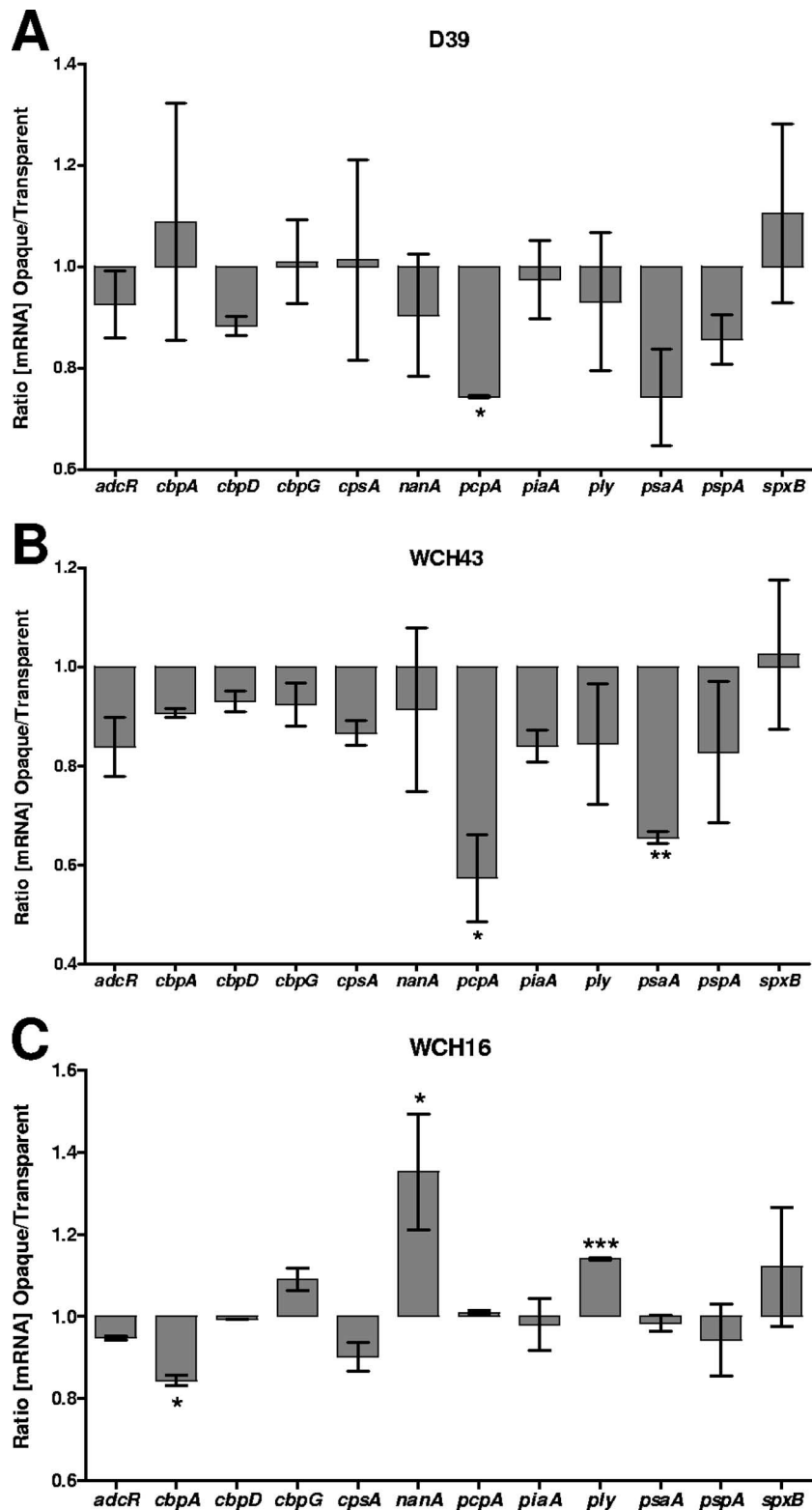


FIG. 2. Comparison of virulence gene mRNA concentrations between opaque and transparent variants of pneumococci grown in vitro. (A) D39. (B) WCH43. (C) WCH16. Real-time RT-PCR data for each gene were normalized against those obtained for the 16S rRNA control. The mRNA concentration was calculated as $2^{-\Delta C_T}$ for each virulence gene, where ΔC_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 \pm 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). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

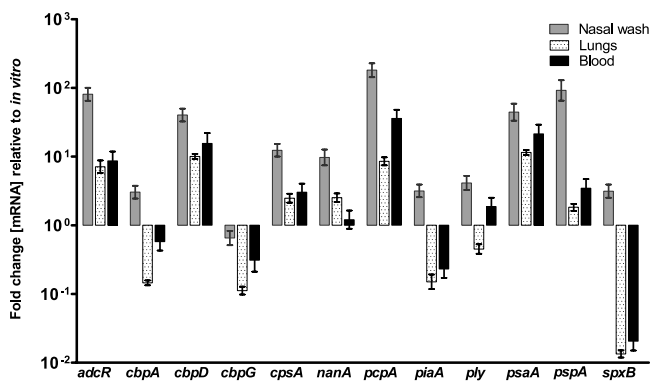


FIG. 3. D39 challenge; D39 virulence gene mRNA concentrations in various in vivo niches relative to concentrations in vitro. Real-time RT-PCR data for each gene were normalized against those obtained for the 16S rRNA control. Quantitative differences for each transcript were determined using the $2^{-\Delta\Delta CT}$ method (34). The data are means \pm standard deviations of triplicate reactions for each gene transcript. Data for pooled pneumococcal RNA from four or five mice are presented.

TABLE 2. Differential expression of D39 virulence genes in various niches

Gene	Expression ^a		
	Nose vs. lungs	Nose vs. blood	Lungs vs. blood
<i>adcR</i>	+11.5 ^d	+10.6 ^d	-1.1
<i>cbpA</i>	+21.1 ^d	+5.9 ^c	-3.6 ^c
<i>cbpD</i>	+4.1 ^d	+2.9 ^b	-1.4
<i>cbpG</i>	+5.9 ^c	+2.4 ^b	-2.5 ^c
<i>cpsA</i>	+5.1 ^d	+4.6 ^c	-1.1
<i>nanA</i>	+3.9 ^d	+9.1 ^d	+2.4 ^c
<i>pcpA</i>	+21.4 ^d	+5.7 ^c	-3.8 ^c
<i>piaA</i>	+21.3 ^d	+15.5 ^d	-1.4
<i>ply</i>	+9.3 ^d	+2.5 ^b	-3.7 ^c
<i>psaA</i>	+3.9 ^c	+2.4	-1.7 ^b
<i>pspA</i>	+50.9 ^d	+30.1 ^d	-1.7 ^c
<i>spxB</i>	+237.2 ^d	+172.5 ^d	-1.4

^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.

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 those seen in vitro. Furthermore, the mRNA

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*,

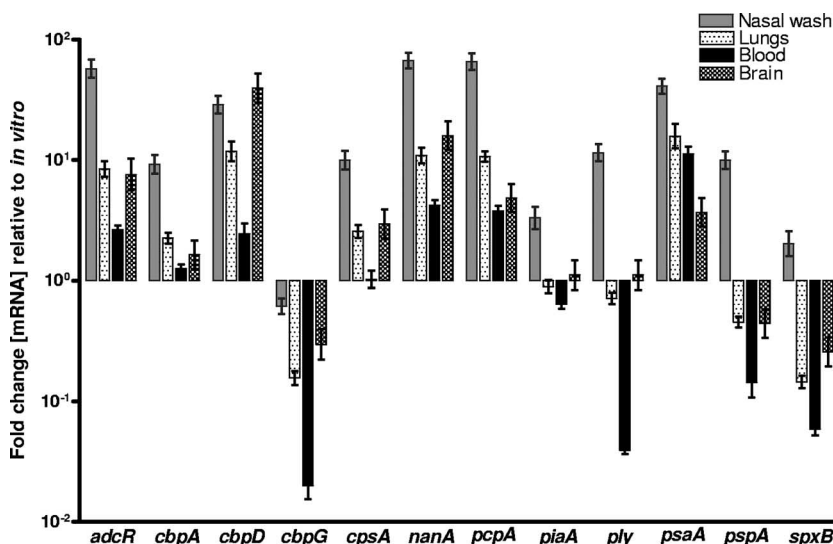


FIG. 4. WCH43 challenge; WCH43 virulence gene mRNA concentrations in various in vivo niches relative to concentrations in vitro. Real-time RT-PCR data for each gene were normalized against those obtained for the 16S rRNA control. Quantitative differences for each transcript were determined using the $2^{-\Delta\Delta CT}$ method (34). The data are means \pm standard deviations of triplicate reactions for each gene transcript. Data for pooled pneumococcal RNA from four or five mice are presented.

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

Gene	Expression ^a					
	Nose vs. lungs	Nose vs. blood	Nose vs. brain	Lungs vs. blood	Lungs vs. brain	Blood vs. brain
<i>adcR</i>	+6.8 ^d	+21.7 ^d	+7.6 ^c	+3.2 ^d	+1.1	-2.9 ^b
<i>cbpA</i>	+4.1 ^d	+7.3 ^d	+5.6 ^d	+1.8 ^c	+1.4	-1.3
<i>cbpD</i>	+2.5 ^c	+11.9 ^d	-1.4	+4.9 ^c	-3.4 ^c	-16.3 ^d
<i>cbpG</i>	+3.9 ^d	+31.1 ^d	+2.1 ^b	+7.9 ^d	-1.9	-14.9 ^d
<i>cpsA</i>	+3.9 ^d	+9.8 ^d	+3.4 ^d	+2.5 ^c	-1.2	-2.9 ^c
<i>nanA</i>	+6.1 ^d	+15.9 ^d	+4.2 ^c	+2.6 ^d	-1.5	-3.8 ^d
<i>pcpA</i>	+6.2 ^d	+17.4 ^d	+13.6 ^d	+2.8 ^d	+2.2 ^b	-1.3
<i>piaA</i>	+3.7 ^c	+5.2 ^d	+3.0 ^b	+1.4 ^b	-1.3	-1.7 ^b
<i>ply</i>	+16.1 ^d	+294.1 ^d	+10.3 ^d	+18.2 ^d	-1.6	-28.3 ^d
<i>psaA</i>	+2.6 ^c	+3.7 ^d	+11.2 ^d	+1.4 ^b	+4.3 ^d	+3.1 ^c
<i>pspA</i>	+22.0 ^d	+69.6 ^d	+22.6 ^d	+3.2 ^c	-1.0	-3.1 ^c
<i>spxB</i>	+14.0 ^d	+34.3 ^d	+7.9 ^d	+2.5 ^c	-1.8 ^b	-4.4 ^d

^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.

pspA, and *spxB* were significantly higher in the nasopharynx than in other niches, but the expression levels of *cbpA*, *cbpG*, *cpsA*, and *pcpA* were significantly higher only in the nasopharynx than in the blood. Indeed, expression levels of *cbpA* and *cpsA* were significantly higher in the lungs than in the nasopharynx, while the expression levels of *adcR*, *cbpG*, and *pcpA* were significantly higher in the lungs and brain than in the nasopharynx (Fig. 5A). However, gene expression patterns in the second WCH16 experiment were largely dissimilar to those of the first experiment (Fig. 5B). These discrepancies could be attributed to generally low numbers of bacteria harvested from the lungs and blood of mice infected with WCH16 (Fig. 1C). Nevertheless, in general terms, data from the two WCH16 experiments indicate consistent trends in gene expression for *cbpA*, *cbpG*, *cpsA*, *pcpA*, *piaA*, *ply*, *pspA*, and *spxB* (Fig. 5).

Strain- and niche-specific differential expression of virulence factors. We observed that there was a high level of

similarity in the patterns of in vivo expression of the majority of the virulence factors for D39 and WCH43. However, certain genes demonstrated strain- and niche-specific differential expression. For instance, while the relative expression levels of *cbpA* were higher in WCH43 and WCH16 harvested from all in vivo niches than that obtained in vitro (Fig. 4 and 5A), its relative expression levels were lower in the lungs and blood of mice challenged with D39 (Fig. 3). However, *pspA* expression was lower in the lungs and blood of mice challenged with WCH43. 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 naïve 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 naïve 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 4. Differential expression of WCH16 virulence genes in various niches

Gene	Expression ^a											
	Nose vs. lungs		Nose vs. blood		Nose vs. brain		Lungs vs. blood		Lungs vs. brain		Blood vs. brain	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
<i>adcR</i>	-10.2 ^d	+2.2 ^c	-1.7 ^d	-23.1 ^d	-15.1 ^d	+17.0 ^d	+6.0 ^d	-45.2 ^d	-0.7 ^d	+8.7 ^d	-8.8 ^d	+392.5 ^d
<i>cbpA</i>	-2.0 ^b	+1.7	+27.7 ^c	-4888.2 ^d	+1.0	+563.5 ^d	+56.8 ^c	-4943.2 ^d	+2.1 ^b	+327.4 ^d	-27.8 ^d	+5019.3 ^c
<i>cbpD</i>	-5.6 ^d	+3.8 ^d	-1.1 ^d	-14.6 ^d	-23.4 ^d	+3.1 ^c	+4.9 ^d	-55.8 ^d	-4.2 ^d	-1.2	-20.8 ^c	+45.5 ^d
<i>cbpG</i>	-5.0 ^d	-3.3 ^c	+6.0 ^d	-551.3 ^d	-20.0 ^d	-260.2 ^d	+29.8 ^d	-167.7 ^d	-4.0 ^d	-79.2 ^d	-119.4 ^c	+2.1 ^c
<i>cpsA</i>	+2.0 ^c	-2.0	+69.7 ^c	-2.1 ^c	+1.7 ^b	-4.17 ^c	+142.7 ^d	+1.0	+3.6 ^d	+210 ^b	-40.0 ^d	-2.0 ^b
<i>nanA</i>	+1.9	+1.0	+2.0	-1.1 ^d	-10.1 ^d	+1.0 ^d	+1.0	-1.1 ^d	-12.2 ^d	+1.0 ^d	-12.2 ^c	+2.1 ^d
<i>pcpA</i>	-9.4 ^d	+2.6 ^d	+3.8 ^d	-1323.4 ^d	-27.9 ^d	-57.6 ^d	+36.0 ^d	-3444.3 ^d	-3.0 ^d	-149.8 ^d	-107.1 ^c	+23.0 ^d
<i>piaA</i>	+3.0 ^c	+3.5 ^b	-1.10	+16.3 ^c	+1.0	-1.30	-3.5 ^c	+4.7 ^c	-3.0 ^c	-4.76 ^c	+1.1	-22.2 ^c
<i>ply</i>	+2.7 ^b	+1.8	+3.7 ^c	+2.0	+2.2 ^c	+1.0	+1.4 ^b	+1.0	+1.2	-1.9 ^b	-1.7 ^b	-2.0 ^b
<i>psaA</i>	-1.5	-3.0 ^b	-1.79	-3.7 ^c	+2.0 ^c	+705.2 ^c	-1.2	-1.2	+3.2 ^c	-1.9 ^b	+3.6 ^b	+2598 ^d
<i>pspA</i>	+2.6 ^c	-1.9 ^b	+1.8 ^b	-1.1	+6.0 ^c	-3.23 ^b	-1.5 ^b	+1.7 ^b	+2.3 ^c	-1.7	+3.3 ^d	-2.9 ^b
<i>spxB</i>	+8.5 ^c	+8.2 ^b	+25.7 ^d	+37.5 ^b	+1.2	-222.2 ^b	+3.0	+4.6 ^b	-7.1 ^d	-1428.6 ^b	-22.2 ^d	-6666.7 ^b

^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.

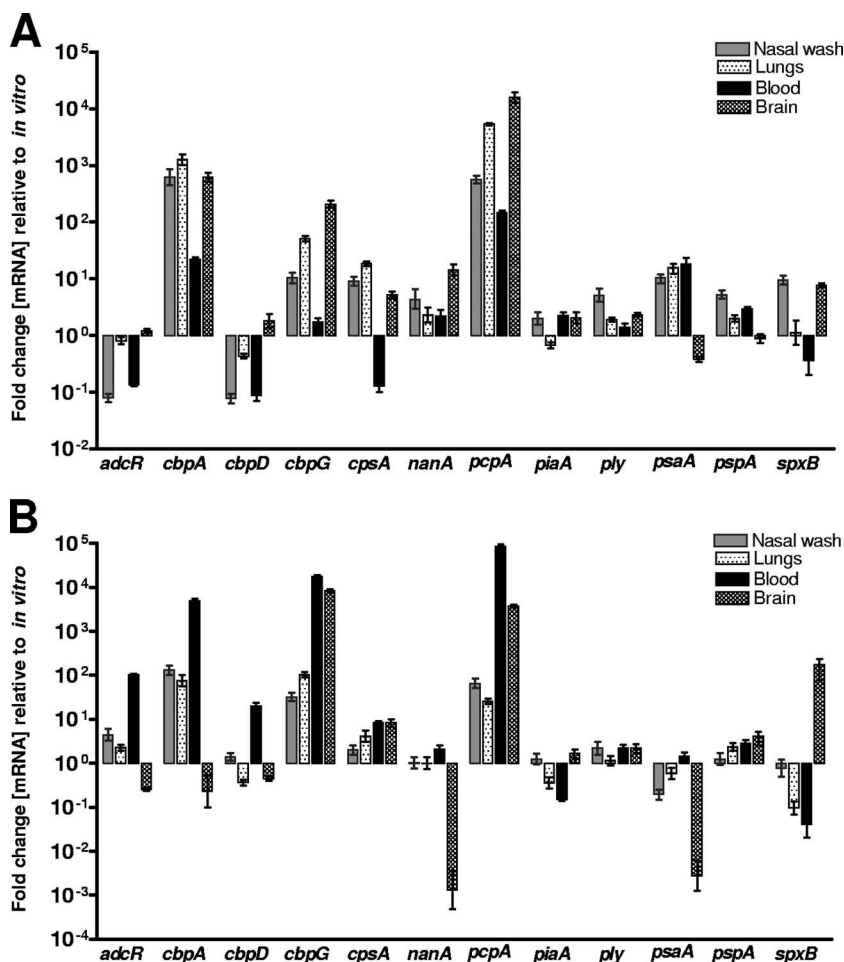


FIG. 5. WCH16 virulence gene mRNA concentrations in various in vivo niches relative to concentrations in vitro. Real-time RT-PCR data for each gene were normalized against those obtained for the 16S rRNA control. Quantitative differences for each transcript were determined using the $2^{-\Delta\Delta CT}$ method (34). The data are means \pm 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.

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-

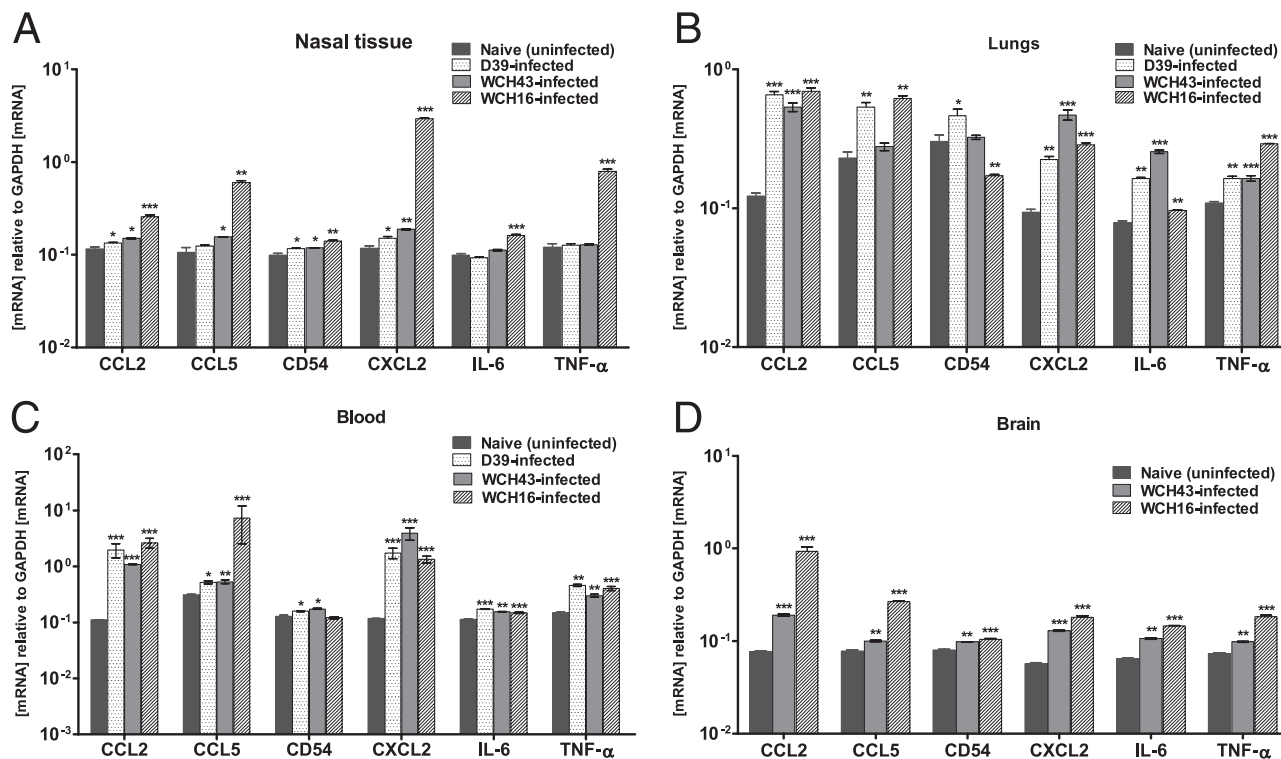


FIG. 6. Host mRNA responses to *S. pneumoniae* D39, WCH43, and WCH16 at 72 h postinfection. Relative mRNA concentrations for each gene in the nasopharynxes (A), lungs (B), blood (C), and brains (D) of mice are shown. Real-time RT-PCR data for each gene were normalized against those obtained for the GAPDH mRNA control. The data are means \pm standard deviations of triplicate reactions for each gene transcript. Data for pooled RNA from four or five mice are presented. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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- and WCH43-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 *adcR*, *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 *cbpA*, *pspA*, *ply*, and *psaA* expression profiles, which parallel their documented roles and protective efficacies in colonization and invasive disease, either alone or in combination (8, 9, 12, 48, 50, 59).

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 (4, 15, 16, 26, 71, 73). The expression of TNF- α is important for surviving pneumococcal infection but may also cause inflammatory host injury under certain conditions (61). TNF- α is capable of recruiting inflammatory cells to the site of infection, directly and via upregulation of adhesion molecules (46) and by stimulating the release of cytokines that are directly chemotactic for inflammatory cells (28). IL-6 can activate monocytes (7) and can synergize with TNF- α to increase the respiratory burst of neutrophils (43). Another study highlighted the involvement of high levels of IL-6 in the lethality of pneumococcal infection (15). 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 (3). Indeed, Ply has been shown to activate murine macrophages (27), resulting in the secretion of TNF- α and IL-6, proposed to occur through Toll-like receptor 4 signaling (36). Ply also stimulates expression of TNF- α and IL-1 from monocytes (25), as well as that of CD54, an important component of the leukocyte-traffic system (71). 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 nasopharynxes of infected mice, consistent with the role of PspA in protecting pneumococci from being killed by apolactoferrin at mucosal sites (69). In contrast, *pspA* expression in D39 was lower in the lungs than in the nasopharynx or blood. 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* (18). 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 (18). CbpA has also been shown to elicit cytokine and ICAM-1 expression from human alveolar epithelial cells (44). In the lungs, an early, sharp host innate

immune response was shown to be vital in overcoming pneumococcal infection (15). 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 (62, 80). The involvement of proinflammatory cytokines, CXC chemokines, and cell adhesion molecules in pneumococcal meningitis is well established (31, 60, 74, 82). Thus, it is not surprising that these molecules were significantly upregulated in the brain tissues of WCH43- and WCH16-infected mice, likely in response to *cbpA* expression (53, 60, 64, 81).

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 (33) 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 (42). 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 simultaneously 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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