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Analysis of the In Vitro Transcriptional Response of Human Pharyngeal Epithelial Cells to Adherent *Streptococcus pneumoniae*: Evidence for a Distinct Response to Encapsulated Strains▽†

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Infection of the human host by *Streptococcus pneumoniae* begins with colonization of the nasopharynx, which is mediated by the adherence of bacteria to the respiratory epithelium. Several studies have indicated an important role for the pneumococcal capsule in this process. Here, we used microarrays to characterize the in vitro transcriptional response of human pharyngeal epithelial Detroit 562 cells to the adherence of serotype 2 encapsulated strain D39, serotype 19F encapsulated strain G54, serotype 4 encapsulated strain TIGR4, and their nonencapsulated derivatives (Δ*cps*). In total, 322 genes were found to be upregulated in response to adherent pneumococci. Twenty-two genes were commonly induced, including those encoding several cytokines (e.g., interleukin 1β [IL-1β] and IL-6), chemokines (e.g., IL-8 and CXCL1/2), and transcriptional regulators (e.g., FOS), consistent with an innate immune response mediated by Toll-like receptor signaling. Interestingly, 85% of genes were induced specifically by one or more encapsulated strains, suggestive of a capsule-dependent response. Importantly, purified capsular polysaccharides alone had no effect. Over a third of these loci encoded products predicted to be involved in transcriptional regulation and signal transduction, in particular mitogen-activated protein kinase signaling pathways. Real-time PCR of a subset of 10 genes confirmed the microarray data and showed a time-dependent upregulation of, especially, innate immunity genes. The downregulation of epithelial genes was most pronounced upon adherence of D39Δ*cps*, as 68% of the 161 genes identified were repressed only by this nonencapsulated strain. In conclusion, we identified a subset of host genes specifically induced by encapsulated strains during in vitro adherence and have demonstrated the complexity of interactions occurring during the initial stages of pneumococcal infection.

The gram-positive bacterium *Streptococcus pneumoniae* is a major cause of mucosal infections, such as otitis media, and of more severe invasive diseases like pneumonia and septicemia. Children, elderly people, and immunocompromised patients in particular are affected by this pathogen. Colonization of the nasopharynx, mediated by the adherence of the bacterium to epithelial cells of the upper respiratory tract, is common and usually asymptomatic. It is, however, also the critical first step for infection of the host by *S. pneumoniae* (7). In addition, nasopharyngeal carriage of *S. pneumoniae* has been proposed to serve as a main source for transmission of this pathogen within the community (16). Neither the host nor the bacterial factors required for adherence and colonization have been completely characterized yet.

S. pneumoniae expresses several proteins described as being important for its virulence, such as pneumolysin and choline-binding proteins (reviewed in reference 35). Of these, CbpA (also known as PspC) and CbpG have been shown to function as an adhesin for eukaryotic cells (34, 45). Another major virulence factor is the polysaccharide capsule, of which at least 90 different serotypes are known (19). Pneumococci can occur in different colony variants known as opaque- and transparent-phase variants, characterized by high and low levels of capsule production, respectively. Generally, transparent-phase variants display enhanced adherence and colonization abilities compared to those of opaque variants (24, 53). Apparently, reduced capsule expression (down to 20% of wild-type [WT] levels) benefits the initial adherence and colonization of pneumococci, possibly by allowing better exposure of surface proteins important for adherence (32). In line with this, pneumococci in close contact with murine or cultured epithelial cells appeared to contain substantially lower levels of capsule than bacteria more distant from cells (17). Interestingly, a recent study showed that, despite its inhibitory effect on adherence, the presence of capsule does confer an advantage during colonization: only encapsulated strains are able to transit efficiently from the luminal mucus to the epithelial surface (38). Finally, there is an absolute requirement for capsule in systemic infections, with various degrees of severity depending on the capsular serotype (9, 49). Once inside the host, the capsule is considered to protect *S. pneumoniae* against host immunity, preventing, for instance, complement-mediated opsonophagocytosis (1).

The innate immune system provides a critical nonspecific first line of defense against microorganisms and is essential for the proper development of subsequent adaptive immune responses. Key elements of innate immunity are the so-called pattern recognition receptors, among which the membrane-

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bound Toll-like receptors (TLRs) play a central role in the response to microbes (22). TLRs recognize conserved microbial structures referred to as pathogen-associated molecular patterns, such as those occurring in bacterial lipoteichoic acid, lipopolysaccharide, and DNA (unmethylated CpG motifs). The involvement of both TLR2 and TLR4 in the host response to pneumococci, mediated by recognition of pneumococcal lipoteichoic acid (TLR2) and pneumolysin (TLR4), has been demonstrated using various in vitro and in vivo infection models (18, 33, 48, 50, 52). Recent studies also indicated a role for TLR9 in host defense against pneumococcal infection, particularly at the early stages (2, 36). The central signaling adaptor myeloid differentiation factor-88 mediates signal transduction from most of the TLRs upon ligand binding (22) and has been shown to be crucial for the host response to pneumococcal infection (3, 9, 23, 26). The subsequent activation of the nuclear factor-κB (NF-κB) pathway leads to the production of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and IL-6.

DNA microarray technology is particularly suitable for examining the host response on a global scale, as several thousand genes can be surveyed at once. Thus far, only a few studies have taken this approach to identify host factors involved in pneumococcal colonization and subsequent disease. Rogers et al. have analyzed the transcriptional response of a human monocytic cell line to pneumococci, focusing on pneumolysin-dependent and -independent gene expression (44). They identified 40 genes that were responsive independent of pneumolysin and 142 pneumolysin-responsive genes, including those encoding IL-8, MIP-1β, lysozyme, and caspases 4 and 6 (44). The examination of gene expression profiles for the middle ears of rats during early and late stages of *S. pneumoniae*-induced otitis media identified transient expression of cytokines and persistent upregulation of several transcription factors, including c-Jun and c-*myc* (11, 30). Similarly, transcriptional profiling of the cortex and hippocampus of rats during acute pneumococcal meningitis identified 598 differentially regulated genes, associated mainly with growth control, signal transduction, cell death/survival, the cytoskeleton, and innate and adaptive immunity (12). Finally, a microarray-based screen for host genes with increased expression during colonization of the murine nasal mucosa identified siderocalin, a component of innate immunity involved in iron sequestering (37).

The first interaction between the pneumococcus and the host in colonization and subsequent infection occurs during the adherence of the bacterium to the respiratory epithelial cell surface. To gain molecular insight into the response of the epithelium during this interaction, we used microarrays to characterize the transcriptional profile of the human pharyngeal epithelial cell line Detroit 562 after 2 h of adherence of *S. pneumoniae* WT strains D39 (serotype 2), G54 (serotype 19F), and TIGR4 (serotype 4). Considering the crucial role of pneumococcal capsule in this process, we simultaneously analyzed the responses to the three corresponding isogenic capsule locus deletion mutants (D39Δ*cps*, G54Δ*cps*, and TIGR4Δ*cps*), as well as to purified type 2 capsular polysaccharides. This approach allowed us to identify epithelial genes that were responsive in a capsule-dependent and -independent fashion. Finally, we used real-time PCR to follow the expression of a small selection of genes over time.

MATERIALS AND METHODS

Cell culture. The human pharyngeal epithelial cell line Detroit 562 (ATCC CCL-138) was grown and maintained at 37°C in a 5% CO_2 atmosphere in RPMI 1640 without phenol red (Invitrogen, The Netherlands) supplemented with 1 mM sodium pyruvate and 10% (vol/vol) fetal calf serum (FCS).

Pneumococcal strains, growth conditions, and pneumococcal polysaccharides. The *S. pneumoniae* strains used in this study were the WT isolates D39 (serotype 2; NCTC 7466), G54 (serotype 19F [13]), and TIGR4 (51) and their isogenic capsule locus (*cps*) deletion mutants D39Δ*cps*, G54Δ*cps*, and TIGR4Δ*cps*, constructed as follows. A Δ*cps* PCR fragment, consisting of a kanamycin resistance cassette flanked by *dexA* and *aliA* sequences, was amplified from *S. pneumoniae* strain D39(FP22) with primer pair FI4 and PE21 as described previously (41). This PCR product was introduced into *S. pneumoniae* by transformation, and kanamycin-resistant colonies, in which the entire capsule gene cluster was replaced with the kanamycin resistance gene, were verified by PCR. For adherence assays, bacteria were grown to mid-exponential phase in Todd-Hewitt broth supplemented with 5 g/liter yeast extract (THY) and stored in 1-ml aliquots in THY containing 15% glycerol at −80°C. The number of CFU per milliliter (CFU/ml) was determined by plating serial 10-fold dilutions of test aliquots on Columbia blood agar plates. Before each assay, bacteria were thawed rapidly, washed once with RPMI 1640 medium without phenol red containing 1% FCS, and resuspended to the required CFU/ml in the same medium.

Purified pneumococcal polysaccharide type 2 powder was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and suspended in distilled water for use at a final concentration of 20 μg/ml.

Exposure of cells to *S. pneumoniae*. The adherence of pneumococci to epithelial cells was assayed essentially as described previously, with a few modifications (25). Briefly, monolayers of Detroit 562 cells in tissue culture dishes (60 mm; samples for microarray analysis) or 6-well plates (35 mm; samples for time curve) were washed twice with phosphate-buffered saline (PBS), after which bacteria were added at a multiplicity of infection of 20:1 (bacteria/cells). Uninfected control cells were incubated with RPMI 1640 medium with 1% FCS only (mock infected). After a predetermined incubation period (2 h for microarray analysis; 30 min, 2 h, and 4 h for time curve), nonadherent bacteria were removed by three washes with PBS. For quantification of the adherence, epithelial cells were subsequently detached by treatment with 25% trypsin, 1 mM EDTA in PBS and lysed by the addition of ice-cold 0.025% Triton X-100 in PBS. Serial 10-fold dilutions were plated on blood agar plates to count the number of adherent bacteria, and the results were corrected mathematically to account for small differences in count in the initial inoculum.

RNA isolation. After washing, Detroit 562 cells were lysed directly in the tissue culture dish by the addition of RLT buffer (QIAGEN Benelux B.V.), collected with a rubber policeman, and transferred to a microcentrifuge tube. Subsequently, total RNA was isolated using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Total RNA from independent triplicate experiments was isolated. The quality and integrity of the purified RNA were verified by running all samples on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Microarray hybridization. RNA was labeled using the recommended protocols supplied by the manufacturer (Affymetrix, Santa Clara, CA). Briefly, double-stranded cDNA was synthesized from 6 μg of total RNA using a T7-oligo(dT) primer. The cDNA was purified and converted to cRNA in an in vitro transcription reaction in the presence of biotinylated nucleotides. Subsequently, the biotinylated cRNA was purified and quantified by spectrophotometric methods. The cRNA yield was adjusted for carryover of unlabeled total RNA. Twenty micrograms of cRNA was fragmented for 35 min at 94°C and hybridized to Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays for 16 h. After hybridization, the GeneChips were washed and stained using a GeneChip Fluidics station 450 (Affymetrix) and scanned with an Affymetrix GeneChip scanner.

Microarray data analysis. The Affymetrix expression data were analyzed using the software package Gene Spring, version 7.3 (Agilent). All experiments (infected and mock-infected cells) were performed in triplicate to allow the assessment of in-group variation. First, the expression data obtained from infected cells were normalized to the median of the uninfected control samples. An initial selection of genes was made based on a 1.5-fold change in expression relative to that of uninfected control cells. Subsequently, an error model based on replicate measurements was defined in Gene Spring, and the significance of differential expression was established using analysis of variance and the false-discovery rate method of Benjamini and Hochberg (5) to correct for multiple testing.

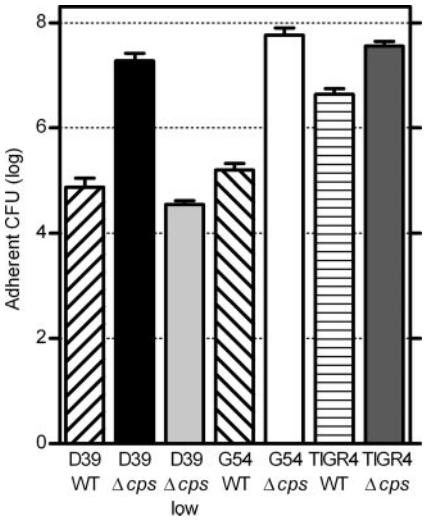

FIG. 1. In vitro adherence of *S. pneumoniae*. Adherence of WT *S. pneumoniae* strains D39, G54, and TIGR4 and their isogenic nonencapsulated derivatives (Δ*cps*) to Detroit 562 human pharyngeal epithelial cells. Data were obtained from three independent experiments and are presented as the means ± standard errors of the means. D39 Δ*cps* low, low-dose D39Δ*cps*.

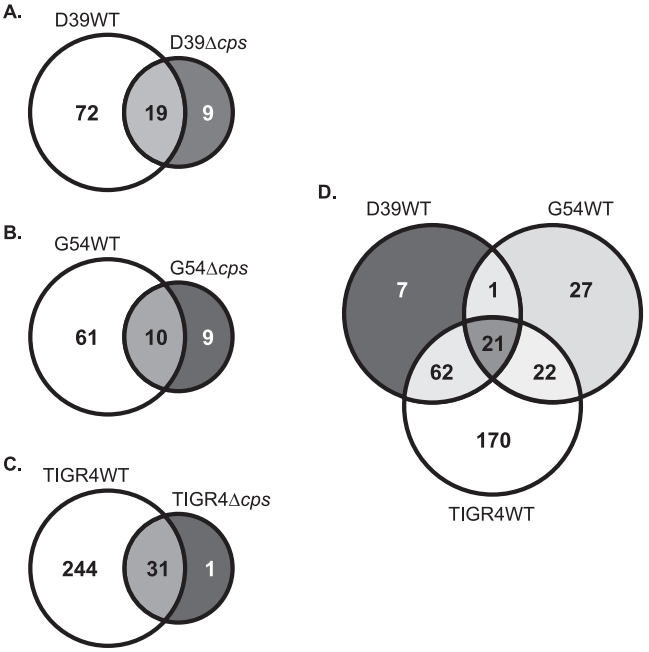

FIG. 2. Distribution of genes upregulated in epithelial cells in response to adherent pneumococci. (A) Distribution of epithelial genes induced by adherent cells of serotype 2 encapsulated WT D39 and its nonencapsulated derivative D39Δ*cps*. (B) Distribution of epithelial genes induced by adherent cells of serotype 19F encapsulated WT G54 and its nonencapsulated derivative G54Δ*cps*. (C) Distribution of epithelial genes induced by adherent cells of serotype 4 encapsulated WT TIGR4 and its nonencapsulated derivative TIGR4Δ*cps*. (D) Distribution of epithelial genes induced by the three WT *S. pneumoniae* strains. The number of genes in each area within the Venn diagrams is indicated.

The transcriptional profiling of Detroit cells in response to the adherence of *S. pneumoniae* strains was performed in three separate experiments. First, the responses to adherent D39, D39Δ*cps*, and G54Δ*cps* cells were analyzed. Second, the gene expression data of epithelial cells after the adherence of WT *S. pneumoniae* strain G54 were collected and normalized to the results for a separate set of uninfected control cells (obtained at the same time). The last experiment consisted of exposing cells to strain TIGR4, TIGR4Δ*cps*, low-dose D39Δ*cps*, and purified type 2 capsular polysaccharides, with uninfected control cells used for normalization. In all cases, genes were considered significantly up- or downregulated when P was <0.05 and the average severalfold change was ≥ 2.0 or ≤ 0.5, respectively.

The gene annotation and functional classification were based on information from the Gene Ontology (4) or KEGG (20) databases. Significant overlap of specific sets of upregulated or downregulated genes with KEGG pathways was assessed using GeneSpring.

Real-time PCR. Real-time quantitative PCR was used to validate selected data from the microarray experiments and to follow the expression of a subset of genes over time. Total RNA (2 μg) was reverse transcribed using a high-capacity cDNA reverse transcription kit according to the instructions provided by the manufacturer (Applied Biosystems, Foster City, CA). Duplicate quantitative PCR assays were performed on 1 μl of 10×-diluted cDNA using TaqMan gene expression assays (Applied Biosystems) of several target genes, encoding ADRB2 (Hs00240532_s1), CXCL2 (Hs00601975_m1), DUSP5 (Hs00244839_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1), MAP3K8 (Hs00178297_m1), MOAP1 (Hs00377893_g1), NF-κBIA (Hs00153283_m1), TNFAIP3 (Hs00234712_m1), TRIB1 (Hs00179769_m1), and 18S (Hs99999901_s1). All assays were done in 10-μl reaction mixtures containing TaqMan universal PCR master mix, 20× TaqMan gene expression assay mix, and cDNA on an Applied Biosystems 7500 FAST real-time PCR system according to the manufacturer's instructions. The relative quantitation method (threshold cycle [$\Delta\Delta C_T$]) (31) was used to evaluate the quantitative variation in gene expression between a particular experimental condition and control (uninfected) cells relative to each gene examined. The *GAPDH* amplicon was used as the endogenous control for the normalization of data.

IL-6 enzyme-linked immunosorbent assay. The concentrations of IL-6 in Detroit 562 culture supernatants were measured by using a commercial enzyme-linked immunosorbent assay kit (PeliKine compact; Sanquin, Amsterdam, The Netherlands) according to the instructions of the manufacturer. The levels of IL-6 were calculated based on standards provided with the kit and expressed as the severalfold increase relative to the levels measured in culture supernatants from uninfected control cells.

Cytotoxicity assay. As an indicator of cell viability during pneumococcal exposure, the cytosolic lactate dehydrogenase (LDH) released was quantified in cell culture supernatants using a CytoTox96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) as recommended by the manufacturer.

Microarray data accession number. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under GEO Series accession number GSE8527.

RESULTS

Transcriptional response of epithelial cells exposed to *S. pneumoniae*. To be able to distinguish between a capsule-dependent and a capsule-independent host response to pneumococcal adherence, we constructed an isogenic capsule locus deletion mutant of the WT, serotype 2 encapsulated *S. pneumoniae* strain D39. Consistent with the results of earlier studies showing a negative influence of capsule expression on the adherence and colonization ability of *S. pneumoniae* (17, 24, 53), the nonencapsulated derivative D39Δ*cps* displayed significantly higher in vitro adherence to epithelial cells than the WT D39 strain (Fig. 1). Further, pneumococcal adherence was found to increase in both a dose- and time-dependent manner (data not shown).

Next, we used microarray analysis to characterize the transcriptional response of the human pharyngeal epithelial cells after 2 h of adherence of D39 and D39Δ*cps*. During this time frame, no adverse effect of pneumococcal exposure on cell

TABLE 1. Common response genes in pharyngeal epithelial Detroit 562 cells during pneumococcal adherence[a]

Gene function and product	Description or molecular function	Fold change[b] in expression in response to:					
		WT D39	D39Δ*cps*	WT G54	G54Δ*cps*	WT TIGR4	TIGR4Δ*cps*
Immune response							
IL-1β	Interleukin 1β	3.5	2.4	–	2.2	2.3	1.5
IL-6	Interleukin 6	4.7	2.7	1.5	2.5	10.5	2.8
IL-8	Interleukin 8	9.6	5.8	3.1	5.8	8.4	3.8
CCL20	Chemokine (C-C motif) ligand 20	–	5.7	–	4.8	5.0	2.6
CXCL1	Chemokine (C-X-C motif) ligand 1	3.9	3.3	1.7	3.5	2.8	2.0
CXCL2	Chemokine (C-X-C motif) ligand 2	6.4	4.6	3.6	5.6	9.1	4.6
CXCL3	Chemokine (C-X-C motif) ligand 3	4.5	2.9	2.0	3.8	5.6	2.7
TNF-αIP3	TNF-α-induced protein 3; inhibits NF-κB activation	4.6	3.9	4.1	4.0	2.9	2.2
NF-κBIA	Inhibits NF-κB by sequestering it in cytoplasm	2.1	2.1	2.5	2.2	2.6	2.3
NF-κBIZ	Molecule possessing ankyrin repeats induced by LPS	2.7	2.6	2.6	2.5	2.5	2.1
FOS	Forms transcription factor AP-1 with JUN family members	13.0[c]	2.8	4.2	3.2	42.5	5.9
GEM	GTP-binding mitogen-induced protein	3.5	–	2.7	–	6.8	2.8
PTGS2	Prostaglandin-endoperoxide synthase	4.3	2.5	1.8	2.3	4.3	2.8
Signal transduction							
HBEGF	Heparin-binding EGF-like growth factor	5.5	2.1	2.0	2.0	6.3	2.0
GDF15	Growth differentiation factor 15	4.2	2.3	–	2.0	6.0	2.0
MAP3K8	MAPK kinase kinase 8	3.4	1.8	2.3	1.6	4.4	2.1
Regulation of transcription							
EGR1	Early growth response 1	6.6	3.3	2.0	2.9	11.8	2.5
ZC3H12A	Zinc finger CCCH-type containing 12A	3.4	2.7	1.8	2.7	3.0	2.0
MYNN	Myoneurin	2.3	–	2.0	–	4.5	2.3
BHLHB2	Basic helix-loop-helix domain containing, class B, 2	2.3	–	2.3	–	5.6	2.7
Antiapoptosis							
BIRC3	Baculoviral IAP repeat-containing 3	2.8	2.3	–	2.3	2.2	1.8
IER3	Immediate early response 3, induced by TNF	4.2	3.0	2.3	2.9	5.1	3.1

[a] Genes are classified as common if they show a change of ≥2-fold in response to all genetic strain backgrounds and at least four out of six conditions.
[b] Average change (*n*-fold) from three independent experiments; $P < 0.05$. –, not induced (<1.5-fold change).
[c] $P = 0.15$.

viability was evident, since no substantial LDH release was detected, while the level of adherence was at or near maximum (data not shown). By selecting gene probes that showed at least a twofold increase in expression compared to the expression in uninfected cells, a total of 136 probes representing 100 genes were found to be upregulated in Detroit cells in response to the two adherent *S. pneumoniae* strains (Fig. 2A and see Table S1 in the supplemental material). Of these, 19 were found to be induced in response to both WT D39 and D39Δ*cps*, whereas the expression of the vast majority (72) appeared to be upregulated upon adherence of the WT strain only (Fig. 2A), indicating a capsule-dependent set of host response genes. To determine whether this gene set is truly induced by the presence of encapsulated strains, or whether the induction is merely the result of suppression of the expression of those particular genes by the higher number of adherent nonencapsulated bacteria, we used an additional, lower dose of D39Δ*cps*, giving adherence levels equivalent to those of its encapsulated WT parent strain (Fig. 1). Subsequent microarray analysis of the transcriptional response of the Detroit cells to low-dose D39Δ*cps* identified only four genes that were upregulated compared to their transcription in uninfected control cells, none of which were identified under the previous two conditions (see Table S1 in the supplemental material). This clearly shows that the observed difference in response is not due to downregulation of gene expression by higher numbers of adherent nonencapsulated bacteria.

To examine if this particular expression profile was unique for the serotype 2 strain or was a more general capsule-dependent response, we included two additional, clinical isolates and their isogenic capsule mutants (Δ*cps*) in our analyses: serotype 19F encapsulated G54 and TIGR4 of serotype 4. Again, the nonencapsulated derivatives showed greater levels of adherence than their WTs in our in vitro assay (Fig. 1). Similar to our observations with D39, microarray analysis showed that the majority of the genes upregulated by adherent WT and Δ*cps* bacteria were induced only in response to the encapsulated strains (Fig. 2B and C and see Table S1 in the supplemental material). Strikingly, the presence of the serotype 4 strain triggered a much stronger response than the other two strains: almost three times as many genes were found to be upregulated. Even so, a considerable overlap was observed between the three sets of capsule-dependent response genes (Fig. 2D).

TABLE 2. Functional categorization of pharyngeal epithelial Detroit 562 genes induced in response to adherent encapsulated pneumococci

Global classification	No. of genes induced in response to strain(s):							
	D39	G54	TIGR4	All 3	D39 and G54	D39 and TIGR4	G54 and TIGR4	Total
Apoptosis	0	0	5	1	0	4	2	12
Cell cycle, proliferation, and differentiation	0	3	19	1	0	3	1	27
Cytoskeleton organization and biogenesis	0	0	5	0	0	0	0	5
DNA metabolism	0	0	1	0	0	0	0	1
Extracellular matrix and adhesion	1	3	9	0	0	1	0	14
Immune response	1	0	4	7	0	5	1	18
Metabolism	1	3	6	0	0	1	2	14
Protein metabolism	0	1	1	0	0	1	0	5
Regulation of transcription	1	1	31	5	1	15	3	57
RNA metabolism	0	1	6	0	0	1	0	8
Signal transduction	2	5	18	5	0	20	5	55
Stress response	0	0	3	0	0	1	0	4
Transport and binding proteins	0	1	13	0	0	1	1	16
Unknown function	1	9	49	2	0	9	7	77
Total	7	27	170	21	1	62	22	311

Evidently, the transcriptional response of pharyngeal epithelial cells to adherent pneumococci is greatly affected by the capsule expression of the bacteria. To find out if this response requires the context of bacterial cells, we analyzed the expression pattern of Detroit cells after a 2-h incubation with purified type 2 capsular polysaccharides. Only four genes showed an increase in expression of twofold or more: *IGFBP5*, encoding insulin-like growth factor binding protein 5; *CRISP3*, coding for cysteine-rich secretory protein 3; and two hypothetical open reading frames (see Table S1 in the supplemental material). Although none of these loci were induced by the serotype 2 encapsulated strain D39, one of the hypothetical open reading frames was upregulated in response to TIGR4 and TIGR4Δ*cps*, and the other three genes were also identified using the low-dose D39Δ*cps*.

Capsule-independent and capsule-dependent transcriptional responses. Twenty-two genes were identified that can be considered part of the common host response to pneumococcal infection, as they were induced by all genetic strain backgrounds, in most cases regardless of capsule (Table 1). The majority of these commonly induced genes encode proteins known to play a role in the immune response, such as several cytokines (IL-1β and IL-6), chemokines (IL-8, CCL20, and CXCL1-3), and transcriptional regulators (NF-κBIA and FOS). Other commonly induced genes included *EGR1*, involved in transcriptional regulation of cell growth and differentiation, and *BIRC3* and *IER3*, whose products are proposed to be involved in the inhibition of apoptosis.

Over 300 genes were found to be induced specifically upon the adherence of one or more of the WT strains D39, G54, and TIGR4. These genes can be considered part of either a capsule-dependent (i.e., induced by multiple encapsulated strains tested) or capsular serotype-dependent (i.e., upregulated specifically by exposure to one) host response. Genes upregulated in response to adherent encapsulated pneumococci were distributed among a variety of functional categories, but genes predicted to encode proteins involved in signal transduction and the regulation of transcription clearly predominated (Table 2 and see Table S1 in the supplemental material). No function could be assigned to approximately 25% of the transcripts (Table 2).

Notably, a significant number of genes that were upregulated in response to all encapsulated strains encode proteins that are part of the so-called MAPK signaling pathways (Table 3), which influence a variety of cellular processes, such as cell growth, differentiation, and apoptosis, in response to a wide range of stimuli (42). For example, *ADRB2* and *EGFR* encode receptors that can trigger the MAPK cascade upon the binding of appropriate ligands, and several kinase- and phosphate-encoding genes were identified that are involved in the subsequent transduction of signals from the cell membrane to the nucleus (Table 3). Several of the other signal transduction molecules identified have been described as playing a role in the immune response. For example, the gene encoding the IL-1 receptor-associated kinase 2 (IRAK2) was induced specifically by strain D39; genes encoding two cytokines belonging to the TNF ligand family (TNFSF18 and TNFSF9) and annexin 1 (ANXA1) were induced by TIGR4; the cytokine gene *LIF* and a suppressor of cytokine signaling (*SOCS1*) were induced by both D39 and TIGR4; and the gene coding for the GTP-binding protein GEM was upregulated in response to all three WT strains.

Close to 60 of the genes induced specifically in response to the encapsulated strains could be classified as transcription factors. These are predicted to be involved in various biological processes and include immunoregulatory genes (*JUN* and Fos gene family members *FOSL1*, *FOSB*, *PRDM1*, and *CEBPB*) and genes encoding proteins involved in the regulation of cell growth and differentiation (seven members of the Krüppel-like factor family). Several transcription factors were particularly strongly induced by the presence of TIGR4 and/or D39: *ATF3*, identified as a negative regulator of TLR4 (14), and two other members of the early growth response family (*EGR2* and *EGR3*). Other genes of interest included several apoptosis-related genes and several genes encoding extracellular matrix receptors (ITGAV, ITGB2, and SDFR1), as well as a receptor ligand (PLAU; urokinase plasminogen activator) which was

TABLE 3. Capsule-dependent response genes in the MAPK signaling pathways in pharyngeal epithelial Detroit 562 cells during pneumococcal adherence

Locus	Description and/or molecular function	Fold change[a] in expression in response to:					
		WT D39	D39Δ*cps*	WT G54	G54Δ*cps*	WT TIGR4	TIGR4Δ*cps*
ADRB2	β2-Adrenergic receptor; initiates activation of MAPKs ERK1 and ERK2	2.3	–	–	–	3.8	–
EGFR	Epidermal growth factor receptor	–	–	3.0	–	2.4	–
DUSP1	Dual-specificity phosphatase 1; inactivation of ERK2 by dephosphorylation	2.7	–	1.6	–	12.1	2.0
DUSP2	Inactivation of ERK1 and ERK2	2.3	–	–	–	4.9	–
DUSP4	Inactivation of ERK1, ERK2, and JNK	2.7	–	1.6	–	3.5	1.7
DUSP5	Inactivation of ERK1	3.8	–	2.2	–	7.0	1.7
DUSP6	Inactivation of ERK2	4.4	1.7	2.1	1.9	6.4	1.9
GADD45AB	Activation of MAPKKK activity (MEKK4 kinase)	2.4	–	–	–	2.7	–
MAPKAPK2	MAPK-activated protein kinase	–	–	2.2	–	–	–
MAP3K8	Induces production of NF-κB	3.4	1.8	2.3	1.6	4.4	2.1
NR4A1	Nuclear receptor subfamily 4, group A, member 1	2.2	–	1.6	–	5.1	–
TRIB1	MAPKK1; activation of ERK1 and ERK2	2.2	–	1.6	–	6.7	1.8

[a] Average change (*n*-fold) from three independent experiments; $P < 0.05$. –, not induced (<1.5-fold change).

also found to be upregulated at 48 h in a rat otitis media model (11).

Remarkably, more than half of the upregulated genes appeared to be capsular serotype specific, i.e., specifically induced by TIGR4 (Fig. 2D). In addition to the transcription factors and signal transduction proteins mentioned above, the predominant functional classes represented in this gene set were transporters (e.g., four solute carrier family proteins) and cell proliferation (e.g., epithelial membrane protein EMP1) (Table 2).

To validate our microarray data, relative transcript levels were determined by quantitative real-time PCR for a selection of common and capsule-dependent host response genes, namely, the innate immune genes *IL-6*, *IL-8*, *CXCL2*, *NF-κBIA*, and *TNF-αIP3*; the MAPK pathway genes *MAP3K8*, *DUSP5*, *ADRB2*, and *TRIB*; and the apoptosis-related gene *MOAP1*. Overall, the expression ratios obtained by microarray and real-time PCR analyses were concordant for all six conditions examined ($R^2 = 0.84$; Fig. 3).

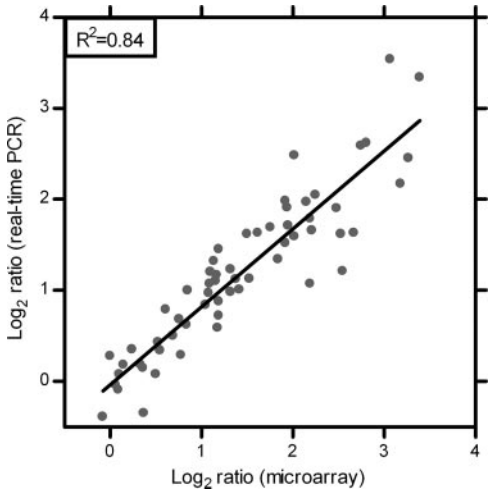

FIG. 3. Validation of microarray data by real-time PCR. Ratios of transcript abundances in infected versus uninfected cells obtained by microarray analysis (*x* axis) or real-time PCR (*y* axis).

To examine if changes observed in gene expression corresponded with changes in protein expression, we measured IL-6 in supernatants of Detroit cells after pneumococcal adherence. In accordance with the gene expression data, the protein levels of IL-6 started to increase after 2 h of exposure of epithelial cells to all *S. pneumoniae* strains (data not shown).

Expression of selected genes over time. The global transcriptional profiling described above focused on a 2-h time period of adherence. Based on the lists generated by these analyses, we selected a subset of five common innate immunity genes (see above) and followed their expression over time (30 min, 2 h, and 4 h) by performing real-time PCR upon the adherence of the WT D39 and TIGR4 strains and their nonencapsulated derivatives (Fig. 4A). In addition, we examined the expression of five capsule-dependent genes at the different time points (Fig. 4B). No difference in expression between uninfected control cells and infected cells was observed for the control 18S rRNA gene under any condition (data not shown). The expression of the common response genes was upregulated upon adherence in a time-dependent manner: there was no significant increase in expression compared to the expression in uninfected control cells at the earliest time point (30 min), while the highest expression levels were achieved after 4 h of adherence (Fig. 4A). As expected, the response of these innate immunity genes to adherent encapsulated and nonencapsulated bacteria was comparable. The same was true for one of the genes of the MAPK signaling pathway, *MAP3K8* (Fig. 4B), in agreement with the microarray results (Table 1). The other three MAPK genes showed a capsule-dependent expression pattern, particularly at the 2-h time point and in response to the TIGR4 strain (Fig. 4B). However, both the difference in induction between encapsulated and nonencapsulated strains and the overall severalfold change of gene expression were less pronounced than originally determined by the microarray analysis. Furthermore, no upregulation of the apoptosis-modulating gene *MOAP1* could be demonstrated by real-time PCR

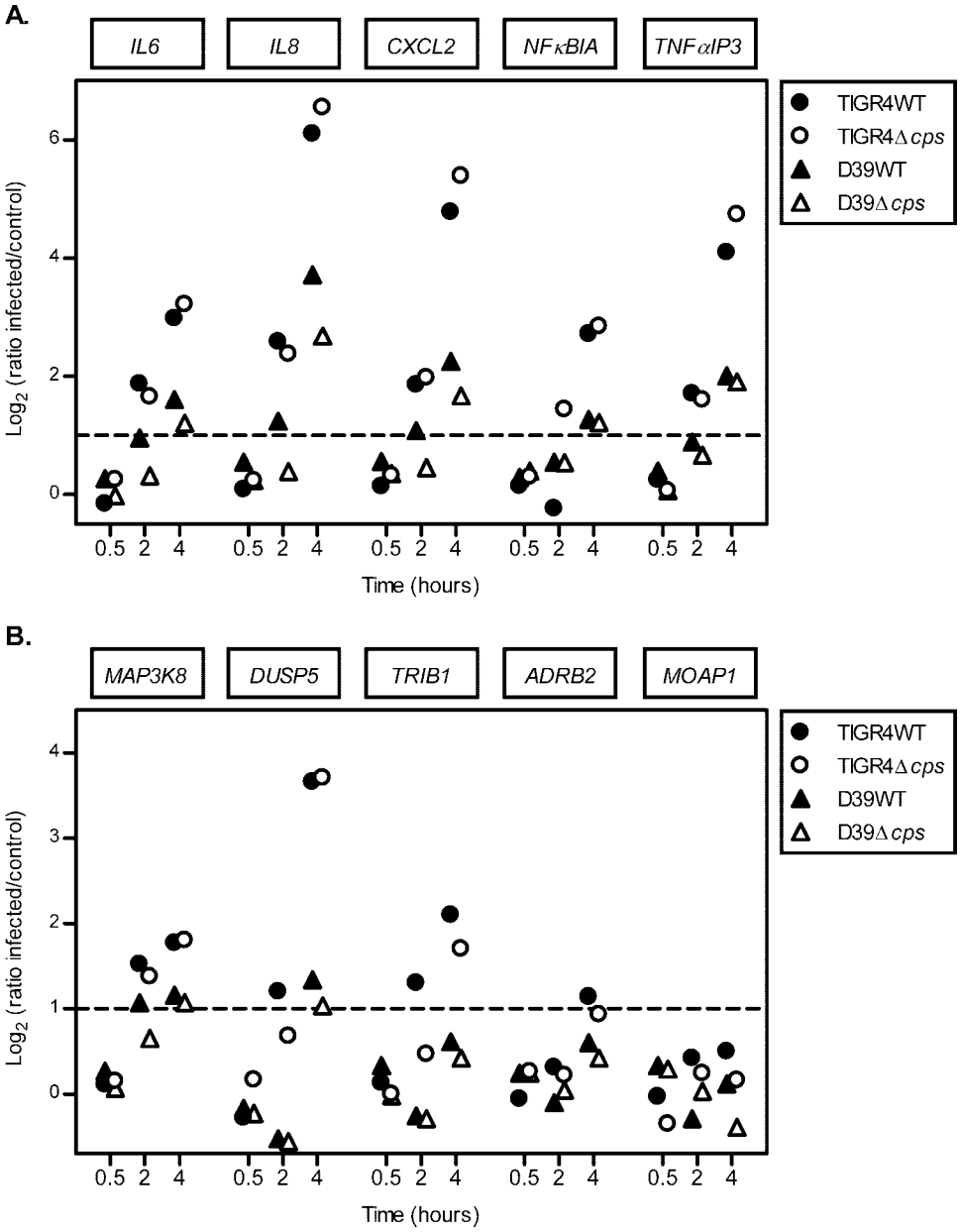

FIG. 4. Real-time PCR analysis of gene expression over time. Expression of specific genes (indicated at the top of each graph) was measured by real-time PCR after predetermined time points of adherence (*x* axis). Log_2 ratios of infected cells/control cells are the averages of the results of two separate experiments. The dashed lines indicate a twofold change in expression relative to expression in uninfected control cells.

under any condition (Fig. 4B), while it appeared to be induced twofold by WT TIGR4 in our microarray analysis (see Table S1 in the supplemental material). For all genes examined, the presence of TIGR4 and TIGR4Δ*cps* resulted in the highest levels of expression changes, particularly at the latest time point. Interestingly, measurement of LDH release indicated that cell viability was also affected by the TIGR4 strains after 4 h, while LDH release by cells infected with the D39 strains showed levels similar to those of the uninfected control cells (Fig. 5). Finally, we investigated the influence of low-dose D39Δ*cps* and purified type 2 capsular polysaccharides (20 and 50 μg/ml) on epithelial gene expression, but no upregulation was observed for any of the 10 genes examined (data not shown).

Genes downregulated in response to *S. pneumoniae*. The expression of 142 genes (represented by 150 gene probes) was found to be decreased at least twofold upon the adherence of WT and D39Δ*cps* (Fig. 6 and see Table S2 in the supplemental material). These repressed genes belonged mainly to metabolism, regulation of transcription, cell cycle, and, most predominantly, unknown functional categories (Table 4). Contrary to our observations made with upregulated genes, the vast majority (111) of repressed genes were downregulated only in response to the nonencapsulated strain D39Δ*cps*. In most

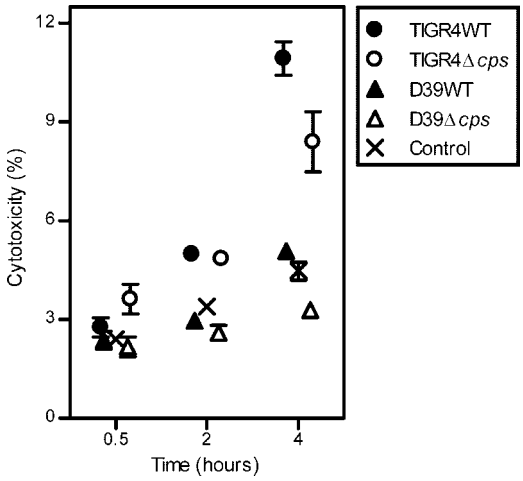

FIG. 5. Cytotoxicity of epithelial cells upon adherence of pneumococci. Pneumococcal strains were allowed to adhere to Detroit 562 cells for time periods indicated on the *x* axis, after which cytotoxicity was assessed by LDH release. Error bars represent the standard errors of the means.

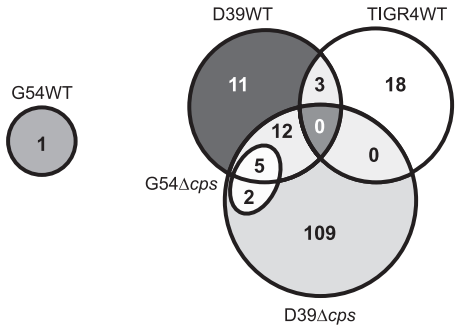

FIG. 6. Distribution of genes downregulated in pharyngeal epithelial Detroit 562 cells. Distribution of epithelial genes repressed by adherent cells of WT strains D39, G54, and TIGR4 and isogenic nonencapsulated derivatives D39Δ*cps* and G54Δ*cps*. The number of genes in each area within the Venn diagrams is indicated. The ellipse represents genes repressed in response to G54Δ*cps*. No genes were found to be repressed in response to TIGR4Δ*cps*.

cases, these genes showed a similar trend in response to WT strain D39, albeit to a lesser extent (e.g., 1.6- to 1.9-fold downregulation [see Table S2 in the supplemental material]). Interestingly, this response appeared to be specific for the D39 genetic background: only eight genes were found to be repressed by adherent cells of WT G54 or its nonencapsulated derivative G54Δ*cps*, no genes were downregulated in response to TIGR4Δ*cps*, and 21 genes were downregulated in response to WT TIGR4 (Fig. 6; Table 4). Eight genes were downregulated in response to different strain backgrounds, among which were *KRT4*, encoding cytoskeletal protein keratin 4; *CLDN8*, encoding membrane protein claudin 8 involved in cell-cell adhesion, and the antiapoptotic gene *API5*.

DISCUSSION

The interplay between host and pathogen during the infectious process is complex and dynamic, requiring multiple factors from either side during different stages of infection. For the pneumococcus, colonization of the human nasopharynx is a prerequisite for infection (7). Colonization is initiated by the adherence of *S. pneumoniae* to the host surface, influenced by several outer-surface components, one of which is the pneumococcal capsule. For the host, the response tends to be focused on clearing the pathogen, mediated by the activation of innate and adaptive defense mechanisms. A few studies have attempted to characterize the host response to pneumococci, using both in vitro and in vivo models of infection (11, 12, 30, 37, 44). In this study, we examined the global transcriptional response of human epithelial cells to pneumococcal adherence in vitro, in particular in relation to the pneumococcal capsule.

A total of 322 genes were found to be upregulated in the human pharyngeal epithelial Detroit 562 cell line in response to adherent cells of WT and nonencapsulated *S. pneumoniae* strains. In all cases, the adherence of the encapsulated strains triggered a much greater transcriptional response than the adherence of their isogenic nonencapsulated derivatives. This difference in gene induction does not appear to be associated with the number of adherent bacteria, as all WT strains adhered approximately one to two orders of magnitude less than their nonencapsulated mutants. Moreover, the results obtained with the lower dose of D39Δ*cps* showed that this differential gene expression is indeed the consequence of the upregulation of the genes in question in response to the encapsulated strain and not downregulation by the greater number of nonencapsulated bacteria attached to the cell surface. Detroit 562 genes induced by all pneumococcal strains tested included those encoding several cytokines, chemokines, and transcriptional regulators. Such transcriptional profiles are consistent with a general innate immune response to bacteria mediated by TLR signaling, i.e., a stereotyped gene expression program (8). For *S. pneumoniae*, the activation of TLR2, TLR4, and TLR9 by, respectively, pneumococcal cell wall components, pneumolysin, and, most likely, CpG DNA has been reported (2, 18, 33, 36, 48, 50). Since both WT strains and their Δ*cps* derivatives contain these TLR ligands, stimulation of the TLRs by all strains used in our experimental setup is likely to occur, resulting in the common induction of, for instance, IL-6 gene expression and protein levels. Similar expression profiles have been reported in other studies using various cell types and in vivo models, underscoring the generality of this response. In respiratory epithelial cells, the expression of IL-8 and CXCL2/3 (MIP-2ab) was shown to be increased in response to D39 (15, 43). In a human monocytic cell line, the expression of IL-8, but not IL-1β, was found to be induced after 3 h of exposure to pneumococci (44), while IL-1β and IL-6 production was observed in monocytes after 24 h of exposure (10). In vivo, IL-1β and MIP-2ab were induced during early pneumococcal otitis media in rats (11), while AP-1 family transcription factors were induced both early and late during otitis media (11, 30).

A large set of genes was found to be upregulated specifically upon the adherence of the WT strains, suggesting a capsule-dependent host response. For the most part, distinct gene sets were induced by serotype 2-encapsulated strain D39, serotype 19F-encapsulated strain G54, and serotype 4-encapsulated strain TIGR4, indicating that the observed response is largely

TABLE 4. Functional categorization of pharyngeal epithelial Detroit 562 genes repressed in response to adherent pneumococci

Global classification	No. of genes repressed in response to strain(s):					
	D39	D39Δ*cps*	TIGR4	D39 and D39Δ*cps*	D39 and TIGR4	Total
Apoptosis	0	1	0	0	1	2
Cell cycle, proliferation, and differentiation	2	11	2	1	0	16
Cytoskeleton organization and biogenesis	0	4	0	2	0	6
DNA metabolism	0	4	1	0	0	5
Extracellular matrix and adhesion	0	5	0	3	0	8
Immune response	0	1	0	0	0	1
Metabolism	2	12	0	0	1	15
Nuclear export	0	1	0	0	1	2
Protein metabolism	0	4	2	1	0	7
Regulation of transcription	2	12	6	2	0	22
RNA metabolism	2	3	1	0	0	6
Signal transduction	1	8	0	0	0	9
Stress response	0	3	1	1	0	5
Transport and binding proteins	0	3	1	1	0	5
Unknown function	2	39	4	6	0	51
Total	11	111	18	17	3	160

capsular serotype specific. The polysaccharide structures of capsular serotypes 2, 4, and 19F are very diverse (6), potentially explaining the limited overlap in the induced gene sets. The incubation of Detroit cells with purified type 2 polysaccharides hardly triggered a transcriptional response, indicating that the capsule is best recognized in the context of (live) bacterial cells. Interestingly, the adherence of the TIGR4 strain had the most pronounced effect on epithelial gene expression, suggesting that the strain genetic background is also of importance during this interaction. The recognition of pneumococcal capsule by the host is not mediated by TLRs. In mice, it has been described as depending on another pattern recognition receptor, namely, the C-type lectin receptor (CLR) SIGN-R1 (homologue of DC-SIGN), expressed by mouse splenic and peritoneal macrophages (21). For immune activation to occur upon antigen binding by CLRs, simultaneous signaling through TLRs by appropriate ligands is believed to be required (27). SIGN-R1 has been shown to be essential for host resistance to both systemic and pulmonary infections with *S. pneumoniae* strains of different serotypes (21, 28, 29). However, the expression of a homologue of this receptor on human epithelial cells has not been reported yet, and the exact role for this or other lectin-type receptors in mediating the capsule-dependent response observed in our experimental setup remains to be determined.

The encapsulated strains appeared to particularly induce several components of MAPK signaling pathways. The MAPKs play pivotal roles in various cellular processes, including cell growth, inflammation, and apoptosis (42). Three major subfamilies of MAPKs have been identified: extracellular signal-regulated kinases (ERK1/2), p38 MAPKs, and c-Jun kinases (JNKs). Several studies have demonstrated activation of both the JNK and p38 MAPK pathways in response to pneumococci in bronchial and lung epithelial cells, as well as endothelial cells (39, 40, 47). Our results are consistent with preferential activation of the JNK and p38 MAPK pathways. In human endothelial cells, the activation of MAPK pathways by nonencapsulated pneumococci was shown to induce programmed cell death or apoptosis, executed by caspases 6 and 9 (40). Similarly, nonencapsulated *S. pneumoniae* induced caspase-6-dependent apoptosis of lung epithelial cells (46). In both cases, encapsulated strains mainly induced cell death in the absence of DNA fragmentation, indicative of necrosis rather than apoptosis. When examining the transcriptional response of monocytes to pneumococci, Rogers et al. observed no significant effect on cell viability but did report the upregulation of caspases 4 and 6 (44). Here, we found induction of the expression of several putative apoptosis-related genes, some of which have been described as inducing apoptosis in a caspase-dependent manner (e.g., *MOAP1* and *EGLN3*), although the caspases themselves were not identified. Furthermore, no loss of cell viability was apparent during the 2-h time period of adherence used for the microarray analysis, although extended (4-h) adherence of the TIGR4 strain did result in increased LDH release. For both endothelial and lung epithelial cells, caspase-dependent apoptosis was observed only after 16 h of pneumococcal infection. The possibility cannot, therefore, be excluded that prolonged adherence will lead to apoptotic or necrotic cell death of the pharyngeal epithelial cells in our setup as well. Apoptosis can benefit both the pathogen, contributing to its virulence by inducing tissue injury, and the host, enhancing bacterial killing and controlling the inflammatory response (as opposed to necrosis). The induction of necrotic rather than apoptotic cell death by encapsulated strains could potentially be mediated by the expression of some of the genes identified as capsule (or serotype) dependent in this study, but further experiments are needed to verify this hypothesis.

A total of 161 genes were found to be downregulated in the epithelial cells. This response appeared to be specific for the D39 genetic background, as very few genes were found to be repressed by WT or nonencapsulated G54 or TIGR4. Moreover, this did not appear to be mediated by the capsule, as the expression of the majority of genes was decreased upon the adherence of the nonencapsulated D39 derivative only. Possibly the absence of capsule allows greater access to other D39 surface components involved in eliciting this particular host

response, an effect likely to be enhanced by the greater number of adherent cells of D39Δ*cps* than of adherent cells of WT D39.

Adherence to and subsequent colonization of the nasopharyngeal epithelium are indispensable for pneumococcal infection. By analyzing the in vitro transcriptional response of human pharyngeal epithelial cells to pneumococcal adherence, we obtained evidence for a subset of host genes specifically induced by encapsulated *S. pneumoniae* strains. In vivo, the situation is likely to be complicated by the presence of multiple bacterial species at the epithelial surface. For example, a synergistic increase in the production of proinflammatory cytokines was observed in response to costimulation with *S. pneumoniae* and *Haemophilus influenzae*, in vitro by pharyngeal and lung epithelial cells (IL-8), and in vivo in the murine nasopharynx (MIP-2) (43). Future studies, for instance using more extended periods of adherence or exposure to mixed bacterial infections, will be required to determine exactly the role of the pneumococcal capsule in triggering the host response. Furthermore, our data suggest a central role for the TLR, lectin-type receptor, and MAPK pathways in defining the host response to pneumococci, emphasizing the complexity of the host-pathogen interaction even at the earliest stages of the infectious cycle.

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