Characterization of In Vitro Biofilm-Associated Pneumococcal Phase Variants of a Clinically Relevant Serotype 3 Clone

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An increasing proportion of children with acute otitis media due to Streptococcus pneumoniae have serotype 3 infections since licensure of the seven-valent pneumococcal conjugate vaccine (PCV7) (23, 24). For example, the proportion of cases of AOM due to a serotype 3 clone of the sequence type 180 complex by the multilocus sequencing typing scheme (www.mlst.net) (14, 24) increased from 3% (5/182) in 1999 to 11% (9/82) in 2002 (P < 0.01) (24). Akin to the incidence of AOM, the incidence of serotype 3 invasive disease is also increasing among children with invasive disease, despite an overall decline in the numbers of invasive infections (9).

The capsule polysaccharide is a key virulence factor of S. pneumoniae, and strains with at least 20% of the parental amount of capsule can colonize the nasopharynx (21). Phase variants, which differ in the amount of capsule present, contribute to the pneumococcus’s ability to adapt to the environment and evade the host response. The opaque variants have more capsule present than the transparent variants (20). Two subpopulations of phase variants are present in the nasopharynx. On the nasal mucosa surface, transparent phase variants predominate; however, within the nasal mucosa, the majority of the strains are opaque phase variants (7).

Human and animal models clearly demonstrate that otitis media with effusion and recurrent otitis media are biofilm diseases (13, 17). The bacteria, including S. pneumoniae, cannot usually be detected in the biofilms by culture; instead, PCR, fluorescence in situ hybridization, and immunostaining are often used to identify the pathogen (13, 17). Due to the difficulty associated with the culture of bacteria from in vivo biofilms, in vitro biofilm models have been used to study the characteristics of the bacteria in this environment (1, 33). By using an in vitro serotype 3 biofilm model, capsule production was shown to decrease over time (1). While the presence of phase variants was not determined in that study, Waite et al. demonstrated that acapsular variants in an in vitro serotype 3 biofilm increased over time (33).

Acapsular strains of serotype 3 have been found to have mutations in the first gene of the capsular operon, cps3DSUM (12). The capsular operon contains only two type-specific genes: cps3D and cps3S. The cps3D gene encodes an enzyme linked immunosorbent assays. Using real-time reverse transcription-PCR, we determined that capsule enzyme-linked immunosorbent assays. Using real-time reverse transcription-PCR, we determined that capsule expression in the phase variants was likely regulated at multiple levels. These in vitro phase variation data, which underscore the plasticity of the pneumococcus, need to be confirmed with in vivo analyses of the middle ear mucosa during otitis media.

Among cases of acute otitis media (AOM) due to Streptococcus pneumoniae, serotype replacement is occurring since licensure of the seven-valent pneumococcal conjugate vaccine (PCV7) (23, 24). For example, the proportion of cases of AOM due to a serotype 3 clone 3 infections since licensure of the seven-valent pneumococcal conjugate vaccine. These serotype 3 strains are genetically related by molecular subtyping. During otitis media with effusion and recurrent otitis media, biofilms commonly develop. Pneumococcal in vitro biofilms are comprised of phase variants that differ in colony morphology. By using a representative strain of the mucoid serotype 3 clone, rough phase variants with a diverse array of mutations were detected in biofilms formed in vitro. Most phase variants had mutations in the cps3D gene, the first gene of the capsular operon. Eleven had single nucleotide polymorphisms (SNPs) in the cps3D gene, one had a SNP in the −10 promoter, and three had large deletions in the cps3D gene. Reversion to the mucoid phenotype was associated with reversion of the mutation in the cps3D gene. Unlike the phase variants detected in the nasopharynx, which have at least 20% of the parental amount of capsule, the in vitro biofilm-associated phase variants had ≤12% of the parental amount of capsule, as determined by capsule enzyme-linked immunosorbent assays. Using real-time reverse transcription-PCR, we determined that capsule expression in the phase variants was likely regulated at multiple levels. These in vitro phase variation data, which underscore the plasticity of the pneumococcus, need to be confirmed with in vivo analyses of the middle ear mucosa during otitis media.
TABLE 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Base pairs</th>
<th>GenBank accession no.</th>
<th>Primer sequence (5'-3')</th>
<th>Annealing temp (°C)</th>
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<tr>
<td>Before cps3D</td>
<td>850–873</td>
<td>U15171</td>
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<td>U15171</td>
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<td>50</td>
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<tr>
<td>After cps3D</td>
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<td>U15171</td>
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<td>50</td>
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<tr>
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<td>U15171</td>
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<tr>
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<td>U15171</td>
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<tr>
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<td>U15171</td>
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MATERIALS AND METHODS

Strain description. A representative of the serotype 3 clone that is associated with a higher proportion of cases of acute otitis media since licensure of PCV7 (24) was used for these studies.

Biofilms. S. pneumoniae strains were grown in Todd-Hewitt broth (THB; Fisher, Pittsburgh, PA) or on 5% sheep blood agar (SBA; Fisher) at 34°C with 5% CO₂. Nine different plate-based biofilms were generated. Each filter (0.2 μm/25 mm; Sterilite) was seeded with 10⁶ colonies of a representative of the serotype 3 clone and incubated on 5% SBA plates overnight at 34°C. Each biofilm was vigorously “washed” daily by the addition of phosphate-buffered saline (PBS) and scraping of the filter against the agar in an effort to remove adventitiously associated cells (16). The biofilm was placed on a new 5% SBA plate after each wash. After 4 to 7 days, the biofilms were vortexted and the bacteria were grown overnight on 5% SBA. The capsular operon’s promoter, cps3D, and cps3S genes were sequenced for a sample of 46 serotype 3 rough phase variants; and the sequences were compared to those of the genes of the mucoid serotype 3 clone.

DNA. DNA was extracted by suspending bacterial colonies in 500 μl of PBS and boiling for 20 min. PCR was performed with an aliquot of the supernatant. The primers are listed in Table 1. A 30-μl reaction mixture containing 1.5 mM MgCl₂, 0.33 μM each primer, 25 μM each deoxynucleoside triphosphate, 0.5 U of the thermostable DNA Taq polymerase mix, 3 μl of 10× buffer, and 2 μl of DNA template was used. PCR was performed in a 9700 thermal cycler (Perkin-Elmer, Boston, MA). The PCR products were sequenced by using a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and were run on a 3730 DNA sequencer (Applied Biosystems). The cps3D gene was amplified with published primers (33) and internal primer sequences (Table 1). For the two rough phase variants without a detectable mutation in the promoter, the cellular transcriptase, were also performed for each strain. The 16S rRNA gene was used as an internal control for data normalization. Primer-probe sets were selected by using Primer Express software (Applied Biosystems). Reverse transcription was performed with 800 ng of RNA in a 100-μl reaction volume by using a high-capacity cDNA archive kit (Applied Biosystems), according to the manufacturer’s protocol. To quantify the mRNA, the cDNA templates were diluted 10-fold in 1× PCR buffer and used in subsequent experiments. Quantitative real-time PCR was performed with TaqMan universal master mix (Applied Biosystems) on an ABI Prism 7900HT instrument with the following conditions: 95°C for 12 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers were used at concentrations of 250 nM and the probes were used at concentrations of 100 nM in a 25-μl reaction mixture.

The results were calculated by using the comparative critical threshold (Ct) method (User Bulletin No. 2 [http://docs.appliedbiosystems.com/pebidocs/04038359.pdf]; Applied Biosystems), in which the amount of target is normalized to relative to that of a reference (that of the serotype 3 clone), which consisted of an internal calibrator target RNA (16S rRNA). The cps3S primers amplified positions 2613 to 2636. The 16S rRNA primers amplified positions 2613 to 2636, and the probe targeted the sequence from positions 2613 to 2636. The 16S rRNA primers amplified positions 1186 to 1258 of the genome of TIGR4, and the probe targeted the sequence from positions 1208 to 1229. For each strain, three replicates were performed with duplicate and independent RNA samples. The change (n-fold) in the level of expression of the cps3DSUM transcript was relative to the level of expression of the serotype 3 clone. Statistical analyses were performed by performing an unpaired Student t test. Significant differences (P < 0.05) in the change in Ct for each rough phase variant compared to that for the serotype 3 clone were determined by using the unpaired t test.

Capsule determination. A competitive inhibition enzyme-linked immunosorbent assay (ELISA) technique was used to determine the quantities of capsular polysaccharide (10) by using 5-ml cultures grown to an optical density at 600 nm.
of 0.5 in THB and stored at −80°C. One hundred microliters of 1 µg/ml type 3 polysaccharide from ATCC was added to each well of a Costar 96-well plate (Fisher), and the plate was incubated overnight at 4°C. Unless otherwise noted, each additional incubation step was performed in an incubator at room temperature for 2 h. The plate was blocked with 200 µl blocking buffer (1% bovine serum albumin in PBS (154 mM NaCl, 2.22 mM Na2HPO4, 1.06 mM KH2PO4, pH 7.4)). Except after the blocking step, the plate was washed three times with 0.05% Tween 20 in PBS (pH 7.4) between each step. The bacterial cultures were heat killed for 20 min at 65°C and centrifuged (14,000 × g) for 20 min. The pellets were resuspended in 500 µl blocking buffer. Fifty microliters of serial dilutions of bacteria was first added to the plate, followed by the addition of 50 µl of a 1:20 dilution of serotype 3 mouse immunoglobulin M monoclonal antibody (monoclonal antibody Hyp3M6, provided by the laboratory of Moon Nahm, University of Alabama, Birmingham). After incubation, 100 µl of a 1:3,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma, St. Louis, MO) in blocking buffer was added and the mixture was incubated for 1.5 h. The plates were developed with p-nitrophenyl phosphate (Sigma), and the optical density at 405 nm was read. The concentration that produced 50% inhibition was determined from an interpolated standard curve for the serotype 3 polysaccharide (Hyp3M6). The optical density at 405 nm was read. The concentration that produced 50% inhibition was determined from an interpolated standard curve for the serotype 3 polysaccharide (Hyp3M6).

### RESULTS

Genotype and capsule production of serotype 3 rough phase variants. We detected 1011 to 1012 colonies per biofilm; overall, 24% (2471/10,363) of the biofilms visualized had a rough morphology after 4 to 7 days of biofilm maturation. Among 46 rough serotype 3 phase variants characterized from nine different biofilms, 15 genotypically unique rough phase variants were detected. Eleven had single nucleotide polymorphisms (SNPs) in the cps3D-coding region, one had an SNP in the putative −10 promoter, and three had large deletions in cps3D.

The deletion in one of the last group of rough phase variants extended into the 5′ end of the cps3S gene (Table 2). Among the rough phase variants with point mutations, both transitions and inversions were detected. For two rough phase variants, no mutations were reproducibly detected in the cps3D, cps3S, pgm, or galU gene compared to the sequences of the genes of the serotype 3 clone. Three rough phase variants with missense mutations had the most capsule, generating from 3 to 12% of the parental amount of capsule (Table 2). All rough phase variants had ≤12% of the amount of capsule present in the serotype 3 clone (180.4 ± 8.6 µg/ml).

Control of capsule production among seven rough phase variants. To determine whether capsule production was regulated during transcription, the cps3DSUM transcript levels of seven rough phase variants were compared to the transcript level of the serotype 3 clone. The rough phase variants included two strains with missense mutations (I297T and V111), a strain with a nonsense mutation (Y353stop), a strain with a large deletion (200 to 261 residues), a strain with a −10 promoter down-mutation (CATAAT instead of TATAAT), and two rough phase variants with unknown mutations. The amount of steady-state 16S rRNA levels varied less than twofold among each of the rough phase variants and the serotype 3 clone. The amounts of the steady-state cps3DSUM transcript levels of the serotype 3 rough phase variants with unknown, missense, and nonsense mutations were significantly greater than that of the serotype 3 clone. In contrast, the serotype 3 clone had a >20-fold higher level of expression of the cps3DSUM transcript compared to that of the rough phase variant with the promoter mutation (Fig. 1).

Reversion frequencies of seven rough phase variants. Daily subculturing of both the V111 and the I297T rough phase variants resulted in occasional spontaneous reversion to the mucoid phenotype. Sequencing of the cps3D promoter region...

![FIG. 1. Expression of cps3DSUM. Data are expressed as the fold difference for each serotype 3 rough phase variant compared to the value for the serotype 3 clone. The phase variant with a deletion was missing 200 to 261 residues of the cps3D gene. Statistical analyses were done by the unpaired Student t test, which was used to determine the change in Cq for each rough phase variant compared to that of the serotype 3 clone; P was <0.05 for all comparisons.](http://jcm.asm.org/...
and the coding sequences of the mucoid isolates revealed that the SNPs associated with the rough phenotype had reverted back to the wild type. Moreover, capsule ELISA of a reverted strain for each type of rough phase variant demonstrated that the reverted strain had >97% the parental amount of capsule. The reversion frequency for the V111 rough phase variant was $3.6 \times 10^{-5}$.

**DISCUSSION**

Biofilms are present in the middle ear during recurrent otitis media and otitis media with effusion (13, 17). The serotype 3 strain that was used to form biofilms in this study was a representative strain of the serotype 3 clone that is associated with an increase in the proportion of cases of AOM in the PCV7 era (24). In this study, we found that nearly one-fourth of the cases of AOM in the PCV7 era were caused by serotype 3 variants which generated the SNPs associated with the rough phenotype. The in vitro data indicate that biofilm-associated rough phase variants have a vast array of mutations within the cps3D gene and can regulate capsule production at multiple levels. Remarkably, these in vitro biofilm-associated phase variants do not have sufficient amounts of capsule to colonize the nasopharynx. Taken together, these data underscore the plasticity of the pneumococcus and its ability to rapidly adapt to the host environment.

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**REFERENCES**