Identifying Mutator Phenotypes among Fluoroquinolone-Resistant Strains of *Streptococcus pneumoniae* Using Fluctuation Analysis

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The occurrence of mutator phenotypes among laboratory-generated and clinical levofloxacin-resistant strains of *Streptococcus pneumoniae* was determined using fluctuation analysis. The in vitro selection for levofloxacin-resistant mutants of strain D39, each with point mutations in both *gyrA* and *parC* or *parE*, was not associated with a significant change in the mutation rate. Two of eight clinical isolates resistant to levofloxacin (MIC, >8 μg/ml) had estimated mutation rates of 1.2 × 10⁻⁷ and 9.4 × 10⁻⁹ mutations per cell division, indicating potential mutator phenotypes, compared to strain D39, which had an estimated mutation rate of 1.4 × 10⁻⁹ mutations per cell division. The levofloxacin-resistant isolates with the highest mutation rates showed evidence of dysfunctional mismatch repair and contained missense mutations in *mut* genes at otherwise highly conserved sites. The association of hypermutability in levofloxacin-resistant *S. pneumoniae* clinical isolates with mutations in DNA mismatch repair genes provides further evidence that mismatch repair mutants may have a selective advantage in the setting of antibiotic pressure, facilitating the development of further antibiotic resistance.

Resistance of *Streptococcus pneumoniae* to penicillin and other commonly used agents has led to increasing dependence on newer classes of antimicrobials such as fluoroquinolones for serious infections, including pneumonia (7, 15). Although fluoroquinolone resistance in pneumococci is still relatively uncommon, surveillance studies have noted increasing resistance concomitant with increasing use of fluoroquinolones (3, 8). Fluoroquinolones directly inhibit DNA synthesis by targeting two tetrameric enzymes involved in DNA replication, topoisomerase IV, encoded by the genes *parC* and *parE*, and DNA gyrase, encoded by *gyrA* and *gyrB* (9). Fluoroquinolone resistance most commonly develops as a result of spontaneous point mutations in specific regions within these genes, referred to as quinolone resistance-determining regions (QRDRs).

Reduced susceptibility to fluoroquinolones occurs in a stepwise manner (10). Spontaneous mutations usually occur first in either *parC* or *gyrA*, depending on the selecting fluoroquinolone, and arise at frequencies of 10⁻⁶ to 10⁻⁹ (30). First-step mutants usually do not have detectable antibiotic resistance, but if progeny of first-step mutants acquire a second mutation in the QRDR of the other target enzyme, strains with reduced fluoroquinolone sensitivity will occur. Furthermore, previous studies have suggested that first-step mutants may acquire a second mutation at a higher rate (5).

Antibiotic selection is known to enrich bacterial cultures for “mutator phenotypes,” subpopulations of bacteria with higher mutation rates (16). Since most mutations are deleterious, mutator alleles are likely to have negative effects on fitness. However, in an evolving clonal population, a deleterious mutator can rise to high frequency (hitchhike) in association withadaptive mutations (6). Mismatch repair mutants are the most commonly observed mutants in bacterial populations under selective conditions (32). The *hexA-hexB* mismatch repair genes in *S. pneumoniae*, equivalent to the *mutS-mutL* genes in *Escherichia coli*, play a major role in preventing spontaneous mutations during bacterial replication. Pneumococci defective in *hexA* have been reported to display a mutator phenotype with a 10-fold increase in mutation frequency (20). Moreover, *hexA* mutant strains of *S. pneumoniae* were selected preferentially over the wild-type ancestor during exposure to low cefotaxime concentrations, demonstrating a selective advantage of the hypermutable strain during antibiotic exposure (20).

We hypothesized that *S. pneumoniae* with a mutator phenotype could be identified among populations that had undergone selection for antibiotic resistance. We focused on fluoroquinolone resistance, since the two-step selection process might be facilitated by a mutator phenotype.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** D39, a serotype 2 strain that was isolated prior to the use of antibiotics, was used as a negative, nonmutator control. A Hex-deficient mutant, which has the D39 background (35), was used as a putative hypermutator control for the assay. Laboratory-generated levofloxacin-resistant isolates were produced by sequential passages of the D39 strain on selective media with 1 μg/ml levofloxacin followed by 4 μg/ml levofloxacin. The mutants were characterized with respect to mutations in the QRDRs of genes *parC*, *parE*, *gyrA*, and *gyrB* by methods previously described (23). Clinical isolate susceptibilities were determined by the disk diffusion method, according to the National Committee for Clinical Laboratory Standards (19). Strains with zone diameters of ≤13 mm, corresponding to a MIC of ≥8 μg/ml, were considered to be levofloxacin resistant. The MICs of levofloxacin for resistant isolates...
as well as laboratory strains were confirmed by use of the Etest (AB Biodisk), performed according to the manufacturer's recommendations.

**Fluctuation analysis.** The fluctuation assay consists of determining the distribution of mutant numbers across multiple parallel cultures. The mutation rate is determined by analyzing that distribution. The probable number of mutational events per culture (m) that gave rise to the distribution of mutants observed is estimated. m can be converted to mutation rate, \( \mu \), by dividing it by some function of the number of cells at risk (28).

For the assay, 10 parallel cultures per strain were first inoculated with a small number (~10^3) of bacterial cells. To do this, frozen starter cultures were inoculated into liquid THY medium (Todd-Hewitt broth plus yeast extract), allowed to grow to steady state at 37°C, and diluted 100-fold into prewarmed medium to achieve an initial homogeneous population. Cultures were again grown to steady state and then subcultured into prewarmed THY medium in 24-well microtiter plates, diluting by 1/10^4 for a total volume of 500 μl per well. The initial inoculum \( (N_0) \) was confirmed by plating an appropriate dilution of representative cultures on nonselective medium. Cultures were incubated in candle jars at 37°C to achieve 10 to 12 generations (7 to 8 h for a strain with doubling time of ~40 min).

The average final number of cells \( (N_f) \) per strain was determined by plating an appropriate dilution of two cultures on nonselective medium. The number of optochin-resistant mutants in each culture was determined by plating the entire appropriate dilution of two cultures on nonselective medium. The number of events per culture \( (\mu) \) was determined by analyzing that distribution. The probable number of mutational events \( (m) \) per strain was then estimated. The mutation rate \( \mu \) was calculated as the number of events per cell achieved in 40 min.

**Calculation and statistical comparison of mutation rates from the fluctuation test data** were carried out using a computer program written in the Sniegowski laboratory (31, 33). The program makes an initial estimate of the rate based on either the P(0) method of Luria and Delbrück or the median method of Lea and Coulson, and then it refines this initial estimate by searching around it for the rate that best fits the full set of observed data based on the Luria-Delbrück distribution-generating algorithm of Ma et al. (11, 13, 14). Confidence limits on the mutation rate estimate obtained in this way were determined using the standard theory for the variance of a maximum likelihood estimate, which agrees closely with a method proposed by Stewart (34). The program FT and descriptive files for its use are available for download at http://www.bio.upenn.edu/faculty/sniegowski/#software.

**Transformation experiments.** Quantification of mismatch repair was carried out as previously described (1). Briefly, transformation efficiency was assessed among the isolates using donor \( S. pneumoniae \) DNA carrying a marker incorporated into low efficiency (novobiocin) and compared to another marker incorporated into high efficiency (streptomycin). An increased ratio of novobiocin to streptomycin-resistant transformants is characteristic of Hex-deficient mismatch repair mutants. Transformation frequencies were expressed as the mean of three determinations for each marker.

**Sequence analysis of mut genes.** Genomic DNA from isolates 2530 and 1237, with increased average mutation rates, was used to amplify the entire \( mutL \) (hexB), \( mutS \) (hexA), \( mutK \), and \( mutY \) genes using primers based on the TIGR4 strain whole-genome sequence (cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?database=sp). Products generated following amplification with Vent (exo +) DNA polymerase (New England Biolabs, Beverly, MA) were used to obtain DNA sequence. Sequences were also generated for isolates 0455 and 3386, without increased average mutation rates, for comparison. Gene sequences among the four isolates were compared to current database entries, searchable \( S. pneumoniae \) genomes (http://www.sanger.ac.uk/Projects/Microbes), and previously published \( hexA \) and \( hexB \) sequence variants (18). All reported mutations were confirmed using independently derived amplification products.

**Nucleotide sequence accession number.** Sequences from mutator isolates 2530 and 1237 have been deposited in GenBank under accession no. EF452716 to -23.

## RESULTS

We tested indirectly for the involvement of mutator phenotypes in the development of high-level fluoroquinolone resistance by assaying fluoroquinolone-resistant clinical isolates and in vitro-produced resistant mutants of \( S. pneumoniae \) for elevated mutation rates. We measured and compared mutation rates in resistant and sensitive isolates by assaying for spontaneous resistance to optochin, the consequence of a non-ORDR point mutation, using fluctuation analysis (13, 24).

First, we tested whether the ORDR point mutations seen in fluoroquinolone-resistant pneumococci alter mutation rates. The two-step in vitro selection for spontaneous resistance to the “respiratory” fluoroquinolone levofloxacin in strain D39 was not associated with a significant change in its mutation rate. This was the case for three independently derived high-level fluoroquinolone-resistant mutants of D39 (D39-2, -3, and -19), each with point mutations in both \( gyrA \) and \( parC \) or \( parE \), encompassing the commonly observed mutations conferring resistance to fluoroquinolones in clinical isolates (Table 1).

This result showed that these changes in DNA gyrase (\( gyrA \)) and topoisomerase IV (\( parC \), or \( parE \)) do not themselves alter mutation rates in \( S. pneumoniae \).

We then assayed mutation rates in eight clinical isolates with resistance to the fluoroquinolone levofloxacin (MIC, ≥8 μg/ml). Among this group, the average mutation rate varied widely between \( 1.2 \times 10^{-8} \) and \( 2.7 \times 10^{-8} \) mutations per cell.

### TABLE 1. Strain characteristics

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Levofoxacin MIC (μg/ml)</th>
<th>Mutation</th>
<th>Mutation rate</th>
<th>95% Confidence limit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>( \text{parC} )</td>
<td>( \text{grA} )</td>
<td>( \text{parE} )</td>
</tr>
<tr>
<td>In vitro</td>
<td>Flex-deficient mutant</td>
<td>2</td>
<td>0.5</td>
<td>S81F</td>
<td>D45N</td>
</tr>
<tr>
<td>D39-2</td>
<td></td>
<td>2</td>
<td>16</td>
<td>S79Y</td>
<td>S81F</td>
</tr>
<tr>
<td>D39-3</td>
<td></td>
<td>2</td>
<td>&gt;32</td>
<td>S81F</td>
<td>1.6 × 10^{-8}</td>
</tr>
<tr>
<td>D39-19</td>
<td></td>
<td>2</td>
<td>16</td>
<td>S81Y</td>
<td>9.2 × 10^{-9}</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td>2</td>
<td>0.5</td>
<td>S81F</td>
<td>1.4 × 10^{-8}</td>
</tr>
<tr>
<td>2530</td>
<td></td>
<td>35B</td>
<td>8</td>
<td>D83N</td>
<td>S81F</td>
</tr>
<tr>
<td>1237</td>
<td></td>
<td>6B</td>
<td>8</td>
<td>S79F</td>
<td>S81Y</td>
</tr>
<tr>
<td>455</td>
<td></td>
<td>9N</td>
<td>&gt;32</td>
<td>S79Y</td>
<td>S81F</td>
</tr>
<tr>
<td>3386</td>
<td></td>
<td>34</td>
<td>&gt;32</td>
<td>S79F</td>
<td>S81F</td>
</tr>
</tbody>
</table>
division. Again there was no association between the specific sequence changes in gyrA or parC and the frequency of spontaneous mutations. For two isolates, the average mutation rates were significantly greater than that of the control strain D39 (Fig. 1). This suggested that two of the eight FQ-resistant isolates, 2530 and 1237, with average mutation rates of 1.2 × 10^{-7} and 9.1 × 10^{-8}, respectively, may have mutator phenotypes. These were also compared to eight recent fluoroquinolone-sensitive clinical isolates for which the mean average mutation rate was 2.0 × 10^{-8} (range, 1.0 × 10^{-8} to 4.3 × 10^{-8}).

To further characterize these two isolates with higher average mutation rates, we determined whether strains 2530 and 1237 were affected in mismatch repair function to account for their hypermutability. Both strains 2530 and 1237 showed increased transformation frequency with a selectable chromosomal marker that is taken up at low efficiency in the D39 nonmutator control due to its proficient DNA mismatch repair function (Table 2). The increased ratio of novobiocin-resistant to streptomycin-resistant transformants of >1.0 in isolates 2530 and 1237 was typical of S. pneumoniae defective mismatch repair systems (1).

Because the phenotype of isolates 2530 and 1237 resembled that of a Hex-deficient mutant (average mutation rate of 6.2 × 10^{-8}), we analyzed their mutL (hexB) and mutS (hexA) genes for alterations that could account for their higher mutation rates (Table 2). In addition, sequences from two other well-characterized genes that contribute to mismatch repair, mutX and mutY, were determined. These four mut genes from strains 2530 and 1237 were sequenced and compared to two of the other isolates from this study without higher average mutation rates, 455 and 3386, as well as sequences in current databases, including 25 entries each for mutL and -S and 12 entries each for mutX and -Y. Mutations encoding missense changes were found in mutL, a highly conserved gene, for both hypermutable isolates and not in other strains or database entries (Table 2) (18). In addition, isolate 2530 contained mutations that would encode novel missense changes in each of the other Mut enzymes with multiple such changes within MutS.

**DISCUSSION**

We postulated that the first step in selection for fluoroquinolone resistance enriches mutator phenotypes in S. pneumoniae populations and accelerates the development of high-level resistance through second-step mutations. Even small increases in mutation rates (weak mutators) have been shown to have a measurable effect on the evolution of fluoroquinolone resistance in vitro (22). A single round of antibiotic selection can be sufficient to enrich mutator cells to majority frequency in a bacterial population (16); as a result, the population of cells can then more rapidly produce additional mutants that confer higher-level resistance or resistance to other antibiotics.

Using a fluctuation assay, we screened a sample of levofloxacin-resistant and -sensitive S. pneumoniae isolates for the presence of mutator phenotypes and found evidence for hypermutability among two clinical levofloxacin-resistant isolates. Fluctuation analysis was used because mutation rates, which measure the risk of mutation per cell division, are more accurate and reproducible than mutation frequencies, which measure the proportion of mutant colonies to the total number of organisms plated (5, 13). Analyses of mutation frequencies, although more amenable to use with large numbers of strains, are inherently less reliable because of natural variation, as documented by the classic 1943 study of Luria and Delbrück (13). Our approach, therefore, was to use a more rigorous test that accounts for this variability even though this precluded the analysis of large numbers of isolates. Since few mutators were identified among the 16 strains tested, our data are not sufficient to determine whether mutators occur more commonly among fluoroquinolone-resistant isolates. However, the identification of mutators among antibiotic-resistant pneumococci offers a mechanism for the initial development of such strains.

We would not necessarily expect that all resistant isolates would be mutators. Since S. pneumoniae is naturally competent for DNA uptake, the acquisition of resistance in a mutator strain could lead to spread of altered sequences among the population of nonmutators. A recent survey of fluoroquinolone-resistant isolates in the United States reported that

<table>
<thead>
<tr>
<th>Strain</th>
<th>Novr/Strr transformant ratio</th>
<th>MutL</th>
<th>MutS</th>
<th>MutX</th>
<th>MutY</th>
</tr>
</thead>
<tbody>
<tr>
<td>2530</td>
<td>1.2</td>
<td>V216A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1237</td>
<td>1.1</td>
<td>F272Y</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Novr, novobiocin resistant (20 μg/ml); Strr, streptomycin resistant (100 μg/ml).

b Deduced amino acid changes based on DNA sequence analysis are indicated by the position relative to the predicted initiation codon.
these are predominantly genetically diverse strains (26). A further source of mutated ORDRs is through horizontal transfer of DNA from other streptococci that coexist in the same environment (2). In addition, there is a fitness cost to maintaining the mutator phenotype. Once resistance is achieved in the setting of antibiotic pressure, the mutator phenotype may eventually be selected against and lost.

Bacterial mutators may have a selective advantage in a changing environment where they can more easily adapt. Numerous laboratory experiments in E. coli have demonstrated that mutator phenotypes can rise to high frequency in bacterial cultures subjected to selective pressure (32). In the lungs of cystic fibrosis patients, where prolonged antibiotic exposure and other selective pressures are increased, hypermutable strains of several bacterial species, including Pseudomonas aeruginosa, Staphylococcus aureus, Haemophilus influenzae, and S. pneumoniae, have been observed (4, 21, 25, 27). It has also been proposed that pathogenic bacterial isolates are more likely to be mutators (12, 21).

Mutator phenotypes are caused by mutational defects in DNA replication and repair loci that elevate mutation rates genome-wide. Minor alterations in Mut enzymes may be associated with a strong mutator phenotype (31). Missense changes identified in Mut enzymes of the two isolates with increased average mutation rates in this study could account for their hypermutability and defective mismatch repair. The mismatch repair function of S. pneumoniae has generally been attributed to MutL or -S, although other Mut enzymes likely contribute to this function. The N-glycosylase endonuclease protein MutY is deficient in repair of A/G mismatches such as those that occur when 7,8-dihydro-8-oxoguanine (8-OxoG) is generated from guanine during oxidative stress (29). In this regard, mutations noted in QRDRs in isolates 2530 and 1237 involve guanine (G→A in gyrA and parC). We have previously described the role of aerobic growth and endogenous hydrogen peroxide production on the high rate of spontaneous mutation characteristic of this species (24). Likewise, mutX encodes a dGTP pyrophosphohydrolase that when absent confers a mutant phenotype in S. pneumoniae (17). The data presented here, however, are not sufficient to define which of these mutations is responsible for the increased average mutation rate in isolates 2530 or 1237 or whether these mutations occur as a result rather than cause of their hypermutability.

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