Novel Expansions of the Gene Encoding Dihydropteroate Synthase in Trimethoprim-Sulfamethoxazole-Resistant Streptococcus pneumoniae

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A study of eight sulfonamide-resistant clinical isolates of Streptococcus pneumoniae revealed chromosomal mutations within the gene encoding dihydropteroate synthase that play a role in conferring resistance to sulfamethoxazole. The presence of the sfd mutation, found previously only in a laboratory mutant, was shown to occur in three of the wild-type clinical isolates. The duplication of Ser64, the other previously defined mutation in the dihydropteroate synthase gene of S. pneumoniae, was observed in only one of the isolates characterized. We report two previously unidentified amino acid alterations, namely, a duplication of Arg28 and Pro46 and an insertion of an arginine residue between Gly60 and Ser61 in trimethoprim-sulfamethoxazole-resistant strains. The significance of these mutations was confirmed by site-directed mutagenesis and by the transformation of a susceptible strain of S. pneumoniae to sulfamethoxazole resistance. Two resistant isolates did not contain any mutations within the gene encoding dihydropteroate synthase. The results presented suggest the independent generation of resistant mutations among South African clinical isolates. It is also proposed that the mechanism of sulfonamide resistance in S. pneumoniae involves the expansion of a specific region within dihydropteroate synthase, which probably forms part of the sulfonamide binding site.

The sulfonamide class of drugs has played an important role in the treatment of pneumococcal diseases. On the recommendation of the World Health Organization, trimethoprim in combination with sulfamethoxazole (co-trimoxazole) has been widely administered for the treatment of respiratory tract infections in children (29). In recent years, high rates of resistance to co-trimoxazole have been reported worldwide, especially in Spain, Portugal, Hungary, and South Africa (8, 10, 11, 18, 32). In South Africa, co-trimoxazole resistance among systemic isolates increased from 32% in 1985 to 44% in 1991, while co-trimoxazole resistance in association with multiple antibiotic resistance increased from 3.8% in 1985 to 14.8% in 1991 (10).

Despite the fact that trimethoprim-sulfamethoxazole resistance is widespread in pneumococci, there is little information on the molecular basis of resistance to this agent. Our group (1) has recently identified the mutations in the dihydrofolate reductase gene that confer resistance to trimethoprim. We have now investigated the mechanism of resistance to sulfamethoxazole in Streptococcus pneumoniae.

The target for sulfonamide action is dihydropteroate synthase (DHPS), which catalyzes the condensation of para-aminobenzoic acid with 7,8-dihydro-6-hydroxymethylpterine-phosphosphate to form 7,8-dihydropteroate (28). The dihydropteroate is subsequently converted to tetrahydrofolate, an essential metabolite for the synthesis of purines, thymidine, glycine, methionine, pantothenic acid, and N-formylmethionyl tRNA. Sulfonamides are structural analogues of para-aminobenzoic acid and therefore competitively inhibit DHPS by acting as alternative substrates (4, 25, 28).

In most gram-negative bacteria, sulfonamide resistance is largely plasmid borne and due to the acquisition of alternative drug-resistant variants of DHPS. Two such plasmid genes, sfd and sulII have been characterized and sequenced (24, 30). Chromosomal mutations in the dhps gene that confer resistance to sulfonamides have been identified in a number of bacteria. In Escherichia coli, a single change of Phe28 to Leu in DHPS has been demonstrated to be responsible for sulfonamide resistance (5). Horizontal transfer has been implicated in the acquisition of a 6-bp insert in the gene encoding DHPS in Neisseria meningitidis (7), while in Staphylococcus aureus as many as 14 mutations are thought to be involved in conferring resistance to sulfonamides (9). Studies by Lopez and coworkers (14) on the dhps gene of a sulfonamide-resistant S. pneumoniae strain initially revealed a 6-bp repeat that duplicated amino acids 66 and 67, in an area distinct from that observed in N. meningitidis. Recent studies by Maskell and coworkers (19) have demonstrated that sulfonamide resistance in S. pneumoniae may be caused by the presence of 3- or 6-bp duplications within the gene in the area encoding Arg64 to Tyr67, close to, but distinct from, the mutations observed by Lopez et al. (14).

In this study, we attempted to better understand the mechanisms involved in conferring sulfonamide resistance in S. pneumoniae by characterization of the mutations with the gene encoding DHPS.

MATERIALS AND METHODS

Bacterial strains and plasmds. S. pneumoniae strains used in this study are listed in Table 1. The cloning vector pGEM-TZf(+) (Promega, Madison, Wis.), allowing blue-white screening of transformants, was used. The S. pneumoniae recipient strain CP1015, derived from Rx, a strain that is only partly related to R6, was used in transformation experiments. S. pneumoniae R6, ATCC 49619, and CP1015 were used as susceptible controls when determining MICs.

Susceptibility testing. MICs were determined by using the agar dilution method described by the National Committee for Clinical Laboratory Standards (20). For the susceptibility testing of sulfamethoxazole (Sigma Chemical Company, St. Louis, Mo.), Mueller-Hinton agar (Difco, Detroit, Mich.) supple-
menced with 5% lysed defibrinated horse blood was used. Plates were incubated at 37°C in 5% CO₂ for 48 h.

**DNA extraction.** Chromosomal DNA was extracted based on the method described by Paton et al. (23). Cultures were harvested from blood agar plates and resuspended in 90 μl of a suspension buffer (10 mM Tris-HCl, 0.14 M NaCl, 0.1 M sodium citrate, 1 mM EDTA). Thereafter, sodium deoxycholate (1%) was added to a final concentration of 0.1%, and the suspension was left to stand at room temperature for 10 min. The aqueous phase was extracted twice with TE (10 mM Tris-HCl, 1 mM EDTA)-saturated phenol and once with chloroform. The DNA was recovered by adding 2.5 volumes of ice-cold ethanol and incubating at −70°C for 30 min. After centrifugation, the pellet obtained was washed with 70% ethanol, resuspended in 50 to 100 μl of distilled water containing 20 μg of RNase A per ml and stored at −20°C.

**PCR.** The PCR primer sequences were based on the published sequence of the DHPS gene of *S. pneumoniae* R6 (14). The following sets of primers were used to amplify the DHPS genes: F1, 5′-ATGTCGAAATAGCAATC-3′ (position 1 to 21); F2, 5′-GATCCTTTTGGCACGCT-3′ (position 61 to 78); F3, 5′-GATACGGGGGGAGAATCG-3′ (position 50 to 101); F4, 5′-CTGGATTGCAGCCGAGATAAAT-3′ (position 572 to 593); R1, 5′-TGGAACAGGGCTGATGATT-3′ (position 208 to 225); R2, 5′-AGGCACGAGGACCTCTG-3′ (position 291 to 312); R3, 5′-TGAGTGGCGCGGCATACGTG-3′ (position 410 to 431); R4, 5′-GCGATCTTTTGAGGCCAATA-3′ (position 596 to 615); and R5, 5′-CCGATGTAGTCCATACATTGTGG-3′ (position 967 to 988)

**DNA amplification.** DNA amplification was performed in 50-μl volumes containing a 1 μM concentration of each primer, 1.5 mM MgCl₂, a 0.2 mM concentration of each deoxynucleoside triphosphate, 1 U of Taq polymerase, and 2 μl of PCR product in a buffer provided by the manufacturer. Amplification reactions were performed with an Omegene thermocycler (Hybird, Middlesex, United Kingdom) using the following program: denaturation at 95°C for 5 min, followed by 32 cycles at 95°C with an Omnigene thermocycler (Hybaid, Middlesex, United Kingdom) using the following program: denaturation at 95°C for 5 min, followed by 32 cycles at 95°C for 1 min, primer-specific annealing temperatures for 1 min, 72°C for 2 min, and a final extension at 72°C for 7 min. An annealing temperature of 58°C was used for PCRs with primer pairs F1-R3 and F5-R6. 54°C reactions were used for F2-R5, and F3-R2 were annealed at 62°C.

**Sequence analysis.** Sequence analysis was carried out with both manual sequencing according to the chain termination sequencing method of Sanger and colleagues (27) and with an automated sequencer. Single-stranded PCR products were prepared for manual sequencing by using streptavidin magnetic particles (Boehringer, Mannheim, Germany). Briefly, a standard amplification reaction (100-μl mixture) was performed with a 5’ biotinylated forward primer and an unlabelled reverse primer. Nonincorporated deoxynucleoside triphosphates and primers were removed by precipitation with polyethylene glycol as described previously (2), and the pellet was resuspended in 40 μl of TE buffer. Single-stranded DNA bound to the magnetic beads was prepared according to the manufacturer’s recommendations. The unlabelled DNA was salt precipitated from the supernatant and resuspended in 12 μl of water. Sequence analysis was carried out by using the Sequenase, version 2.0, sequencing kit (United States Biochemicals, Cleveland, Ohio) according to the manufacturer’s recommendations, with 3 μl of single-stranded DNA per dyeoxy chain termination sequencing reaction.

**Cloning PCr products.** The amplified PCR products of the DHPS gene were prepared according to the method of Sambrook et al. (26). Blunt end ligations were then performed into a Smal-digested expression vector pGEM-TZf+ (Promega). The ligations were carried out in 12-μl reaction volumes with 0.1 μg of small-restricted vector, 0.3 μg of PCR product, 8% polyethylene glycol 6000, 4.34 mM ATP, 5 U of Smal, and 2 U of T4 DNA ligase (United States Biochemicals) in a ligation buffer provided by the manufacturer. The mixture was then subjected to an overnight incubation between 10 and 30°C for 30 s according to the method described by Lund et al. (17). Competent *E. coli* JM109 cells were electrotransformed by using the purified ligated DNA as described previously (6). Transformants were screened for the lack of α-complementation on Luria-Bertani agar containing 50 μg of ampicillin per ml, 20 μl of 50-mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) stock solution and 100 μl of a 100 mM IPTG (isopropyl-β-D-thiogalactopyranoside) stock solution.

**Transformations.** The sulfonamide-susceptible *S. pneumoniae* recipient strain CP0105 was grown at 37°C to an A₅₅₀ of 0.4 in competence and transformation medium containing 10 g of Casitone (Difco), 5 g of tryptone (Difco), 4 g of yeast extract, and 5 g of NaCl per liter supplemented with 0.2% glucose and 17 mM KHP₀₄. Glycerol was added to a final concentration of 15% before cells were stored at −70°C. Competent cells were produced by diluting thawed cells to a density of 1:100 in a complete transformation medium, pH 7 competence and transformation medium supplemented with final concentrations of 1 mM CaCl₂ and 0.4% bovine serum albumin. After incubation at 37°C for 2 h (A₅₅₀ = 0.05) the pH of the suspension was raised to 7.5 with 1 M NaOH and then incubated for a further 20 min. For transformations, 1 μg of the cloned PCR product per ml of cells was incubated for 30 min at 30°C, followed by 2 h of incubation at 37°C. Cells were then diluted 10-fold before being plated onto a selection medium containing 128 or 256 μg of sulfamethoxazole per ml.

The plates were incubated for 48 h at 37°C in 5% CO₂. DNA-free controls were also tested in order to confirm that any growth observed was the result of transformation and not mutation. Cells were also plated onto an antibiotic-free medium for the determination of viable counts. The MICs of transformants were determined as described above.

**Site-directed mutagenesis.** Mutagenesis was performed using the Muta-Gene kit (Bio-Rad, Hercules, Calif.) according to the manufacturer’s instructions. Mutagenic primers were produced to contain mutations at Glu₄₅-Gly, Leu₁₁₅-Phe, His₁₂₅→Asp, Phe₁₅₆→Val, Gly₁₅₇→Asn, Thr₁₇₀→.
Lys, Glu25-Gly, Leu115-Phe, His123-Asp, Phe156-Val, Gly157-Asn, Glu163-Lys, Thr170-Lys, Gly175-Asp, Leu283-Leu, Ala284-Cys, Glu285-Glu, Lys319-Arg, Ser, Leu320-Val, and Leu321-Gln. These primers were designed to be 28-mers and showed 100% identity to the S. pneumoniae R6 DNA sequence on either side of the mutagenic amino acid. The presence of the mutagenic base in the synthesized DNA was confirmed by sequence analysis before transformation was attempted. Transformants were selected on antibiotic plates, containing sulfamethoxazole concentrations of 8, 16, 32, 128, and 256 μg/ml, as described above. The insertion mutations from the resistant isolates, shown in Table 1, were confirmed to play a role in sulfamethoxazole resistance. These insertion mutants were used as the controls to test the efficacy of the transformation system.

DHFR analysis. The dihydrofolate reductase (DHFR) fragments encompassing the published S. pneumoniae (1) mutation was generated with primers F1 5′-GGAGAATTCTGCAAATTACGGTCTCCTGTG and R1 5′-TTCCTTTTCCCTCTTT CTTCCTG-3′, based on the S. pneumoniae DHFR gene from the EMBL database (accession no. Z29773). Standard PCRs were performed as described above. The cycling parameters were denaturation at 93°C for 3 min, followed by 32 cycles of 93°C for 1 min, 53°C for 1 min, and 72°C for 2 min, and a final extension of 72°C for 2 min. Sequence analysis of the DHFR gene was performed by using the 5′-biotinylated primer F1 as described above.

Nucleotide sequence accession numbers. The DNA sequences containing the novel mutations identified in this study have been assigned accession no. AJ132956 (DNA sequence of isolate 13) and AJ132957 (DNA sequence of isolate 42) in the EMBL database.

RESULTS

Sequence analysis of pneumococcal DHPS genes in sulfamethoxazole-resistant and -susceptible strains. Eight sulfamethoxazole-resistant and five sulfamethoxazole-susceptible isolates, including S. pneumoniae ATCC 49619, for which there is a broad range of MICs, were randomly chosen from a collection of clinical isolates from hospitals around South Africa (Table 1). Sequence analysis of sulA revealed a number of polymorphic changes in both susceptible and resistant isolates when compared to sulA of R6. A section of the deduced DNA sequence containing the mutations observed in the resistant strains is presented in Fig. 1. In isolates 8, 45, and 111, we observed the presence of the sulA mutation, the (13), the duplication of Ile66 and Glu67. Strain 104 contained a duplication of an existing serine residue at position 61 (19). Sequence analysis of isolate 13 showed that it contained an insertion of an arginine residue between Glu60 and Ser61, while isolate 42 was found to contain a duplication of Arg58 and Pro59. These mutations are being reported for the first time. The above-mentioned changes were absent in all the susceptible isolates studied.

We noted with interest that the sulfamethoxazole-resistant isolates 11 and 85 showed no changes in this area between Arg58 and Glu67. There was considerable sequence diversity elsewhere in the gene, resulting in amino acid changes among the resistant strains not seen in susceptible isolates. Comparison of the nucleotide sequence of the sulfonamide-resistant isolates revealed other amino acid changes at Glu45-Gly, Leu115-Phe, His123-Asp, Phe156-Val, Gly157-Asn, Glu163-Lys, Thr170-Lys, Gly175-Asp, Leu283-Leu, Ala284-Cys, Glu285-Glu, Lys319-Arg, Ser, Leu320-Val, and Leu321-Gln. Of these changes only Lys319-Asn and Asn321-Ser occur exclusively in isolates 11 and 85, respectively.

Transformation to sulfamethoxazole resistance. Whole DNA and PCR products from the sulfonamide-resistant isolates were used for transformation of recipient S. pneumoniae CP1015. Whole DNA from each isolate was capable of transforming CP1015 to sulfamethoxazole resistance. PCR products of primer pairs F2-R5 and F3-R2 from isolates 111, 8, 45, 13, 104, and 42 transformed CP1015 to sulfamethoxazole resistance (Table 2). The products with DNA from strains 11 and 85 did not transform CP1015 to resistance. The MICs for the transformants obtained by using whole DNA or PCR products were within 1 dilution of donor DNA (Table 2), except for the strain for which the MIC was the highest (MIC for strain 111, 4,096 μg/ml). In this instance, whole DNA transformed CP1015 to full resistance while the PCR product transformed CP1015 to a level of resistance at which the MIC was 1,024 μg/ml. The higher MICs observed for the recipient strain than for the donor strains may be due to the higher background MIC for CP1015, which was determined to be 64 μg/liter (19). In the DNA-free controls no spontaneous resistant mutations were detected.

Site-directed mutagenesis. By using site-directed mutagenesis, it was determined that the amino acid substitutions observed, Glu45-Gly, Leu115-Phe, His123-Asp, Phe156-Val, Gly157-Asn, Glu163-Lys, Thr170-Lys, Gly175-Asp, Leu283-Leu, Ala284-Cys, Glu285-Glu, Lys319-Arg, Ser, Leu320-Val, and Leu321-Gln, did not confer resistance to sulfamethoxazole. Of these changes only Lys319-Asn and Asn321-Ser occur exclusively in isolates 11 and 85, respectively. The amino acid substitutions that occur at, or close to, conserved areas may, however, be particularly important, not so much in conferring resistance but rather in that they may contribute to the resistant phenotype observed by affecting the tertiary conformation of the SulA protein.

DHFR characterization. Sequence analysis of the DHFR gene of the resistant isolates revealed the presence of the Iso195-Leu substitution previously reported to be responsible for trimethoprim resistance in S. pneumoniae (1). Isolates 11 and 85 did not, however, contain this mutation, and neither did any of the susceptible isolates.

DISCUSSION

Two separate duplications within sulA, the gene encoding DHPS, have previously been shown to confer sulfonamide resistance on S. pneumoniae. The 6-bp repeat described by Lopez et al. (14), which results in the duplication of Ile66 and Glu67, and the duplication of Ser61 observed by Maskell et al. (19) were found among the isolates in this study. An insertion of arginine between Gly60 and Ser61 described by Lopez et al. (14), which results in the duplication of Ile66 and Glu67, and the duplication of Ser61 observed by Maskell et al. (19) were found among the isolates in this study. An insertion of arginine between Gly60 and Ser61 described by Lopez et al. (14), which results in the duplication of Ile66 and Glu67, and the duplication of Ser61 observed by Maskell et al. (19) were found among the isolates in this study. An insertion of arginine between Gly60 and Ser61 described by Lopez et al. (14), which results in the duplication of Ile66 and Glu67, and the duplication of Ser61 observed by Maskell et al. (19) were found among the isolates in this study. An insertion of arginine between Gly60 and Ser61 described by Lopez et al. (14), which results in the duplication of Ile66 and Glu67, and the duplication of Ser61 observed by Maskell et al. (19) were found among the isolates in this study.
The existence of more than one mechanism of resistance in a single bacterium has been alluded to by a number of researchers. At least two different types of mutations can contribute to rendering \textit{E. coli} resistant to sulfonamides (22). Some mutations produce altered enzymes that differ structurally from the wild-type strain in that they do not combine as readily with sulfonamide. Other mutants have enzymes that resemble those of the wild-type strains, but it is thought that these mutants have different permeability characteristics that reduce sulfonamide uptake into the cells. The permeability of sulfonamides or its analogues have to date not been investigated in \textit{S. pneumoniae}, so this mechanism of resistance remains a possible contributing factor to sulfonamide resistance in this species.

Other possible mechanisms that may confer resistance to sulfonamides had been alluded to after observations made in other bacteria. In \textit{S. aureus} some resistant strains were observed to display an overproduction of para-aminobenzoic acid (13).

The presence of an efflux mechanism for the active transport of sulfonamides has been alluded to in \textit{E. coli}. The \textit{sur} gene sequence identified in sulfathiazole-resistant \textit{E. coli} was found to match the \textit{E. coli bcr} gene, which is responsible for conferring bicyclomycin resistance when it is overproduced (3).
product of sur (bcr) is similar to that of the family of proton motive force-dependent drug-H\textsuperscript{+} antiporters, and it may be that sur (bcr) functions in an analogous manner effluxing paraaminobenzoic acid or another intermediate in folate biosynthesis, in addition to sulfathiazole from the cell (21). These observations have yet to be investigated in pneumococci and hence cannot be ruled out as having some role in conferring resistance to sulfonamides.

The operon dedicated to folate synthesis in S. pneumoniae consists of four genes, namely, sulA, sulB, sulC, and sulD (15). The functions of SulB, SulC, and SulD have been determined. It appears that SulC has cyclohydrolase activity and catalyzes the first step in the folate biosynthetic pathway (12). SulD is a bifunctional protein, which catalyzes two successive steps in folate biosynthesis (16), and sulB encodes dihydrofolate synthetase, which catalyzes the last step of the folate pathway (12). Mutations in these genes could play a role in conferring resistance although no evidence has as yet been discovered. Efforts are currently being made to sequence sulB, sulC, and sulD. The absence of mutations in sulA in sulfonamide-resistant isolates of S. pneumoniae could, however, imply the presence of mutations in any of the other genes making up the operon. Mutations in these genes may allow the pneumococcus to synthesize folate by a different pathway in the presence of sulfonamides.

The isolates studied are all co-trimoxazole resistant, implying that resistance to both components occurs and may be attributable to any combination of the resistance mechanisms. Besides a permeability change, which may affect the action of both trimethoprim and sulfamethoxazole, a single mutation...
TABLE 2. MICs determined for sulfamethoxazole-resistant transformantsa

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<th>Donor strain</th>
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a Transforms were selected on antibiotic plates containing 16 and 32 μg of sulfamethoxazole per ml. MICs were determined for two to four transformants, according to the method outlined in the experimental section. The MICs of the transformants achieved were dependent on the size of the fragment used and the degree of homology between the PCR product and the recipient strain. The mechanism for sulfonamide resistance in *S. pneumoniae* appears to involve the expansion of the region that probably forms the sulfonamide binding site, therefore leading to alterations in the structural conformation of the site. The presence of distinct resistance-mediating alterations identified in this study lends support for the independent generation of resistant alleles that contribute to the dissemination of sulfonamide resistance among clinical isolates of *S. pneumoniae*. Sulfonamide resistance does, however, appear to be more complex, and the results presented in this work further substantiate the possibility that additional mechanisms are involved in conferring sulfonamide resistance on *S. pneumoniae*.

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REFERENCES

AUTHORS’ CORRECTIONS

Mechanism of Sulfonamide Resistance in Clinical Isolates of Streptococcus pneumoniae

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Nucleotide sequence accession numbers. sulA sequences of J93/196, PN93/917, PN93/1802, R12, J93/155, PN94/720, J94/76, and P48 have been deposited in GenBank under accession no. AF245691 through AF245698, respectively.

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