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Mutations in the Dihydrofolate Reductase Gene of Trimethoprim-Resistant Isolates of *Streptococcus pneumoniae*

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Streptococcus pneumoniae **isolates resistant to several antimicrobial agent classes including trimethoprim-sulfamethoxazole have been reported with increasing frequency throughout the world. The MICs of trimethoprim, sulfamethoxazole, and trimethoprim-sulfamethoxazole (1:19) for 259 clinical isolates from South Africa were determined, and 166 of these 259 (64%) isolates were resistant to trimethoprim-sulfamethoxazole (MICs ≥20 mg/liter). Trimethoprim resistance was found to be more strongly correlated with trimethoprim-sulfamethoxazole resistance (correlation coefficient, 0.744) than was sulfamethoxazole resistance (correlation coefficient, 0.441). The dihydrofolate reductase genes from 11 trimethoprim-resistant (MICs, 64 to 512 μg/ml) clinical isolates of *Streptococcus pneumoniae* were amplified by PCR, and the nucleotide sequences were determined. Two main groups of mutations to the dihydrofolate reductase gene were found. Both groups shared six amino acid changes (Glu20-Asp, Pro70-Ser, Gln81-His, Asp92-Ala, Ile100-Leu, and Leu135-Phe). The first group included two extra changes (Lys60-Gln and Pro111-Ser), and the second group was characterized by six additional amino acid changes (Glu14-Asp, Ile74-Leu, Gln91-His, Glu94-Asp, Phe147-Ser, and Ala149-Thr). Chromosomal DNA from resistant isolates and cloned PCR products of the genes encoding resistant dihydrofolate reductases were capable of transforming a susceptible strain of *S. pneumoniae* to trimethoprim resistance. The inhibitor profiles of recombinant dihydrofolate reductase from resistant and susceptible isolates revealed that the dihydrofolate reductase from trimethoprim-resistant isolates was 50-fold more resistant (50% inhibitory doses [ID_{50}s], 3.9 to 7.3 μM) than that from susceptible strains (ID_{50}s, 0.15 μM). Site-directed mutagenesis experiments revealed that one mutation, Ile100-Leu, resulted in a 50-fold increase in the ID_{50} of trimethoprim. The resistant dihydrofolate reductases were characterized by highly conserved redundant changes in the nucleotide sequence, suggesting that the genes encoding resistant dihydrofolate reductases may have evolved as a result of inter- or intraspecies recombination by transformation.**

Trimethoprim is an antimicrobial agent used extensively in combination with sulfamethoxazole (co-trimoxazole) for the treatment of urinary, enteric, and respiratory infections in developing countries. Trimethoprim selectively inhibits the bacterial dihydrofolate reductase (DHFR), thus preventing the reduction of dihydrofolate to tetrahydrofolate (8). Despite the trend toward trimethoprim monotherapy in place of therapy with trimethoprim-sulfamethoxazole combinations for the treatment of urinary tract infections, *Streptococcus pneumoniae* has been shown to be moderately susceptible to trimethoprim, and it has been suggested that infections caused by this organism require the synergy induced by sulfamethoxazole in the trimethoprim-sulfamethoxazole combination for effective therapy (7). Attempts to develop a DHFR inhibitor (epiroprim) with activity superior to that of trimethoprim against *S. pneumoniae* and other gram-positive organisms have been successful in vitro against trimethoprim-susceptible organisms; however, its efficacy is limited against trimethoprim-resistant organisms (33).

Soon after the introduction of co-trimoxazole in the late 1960s, the first isolate of co-trimoxazole-resistant *S. pneumoniae* was isolated (25). Since then resistance has steadily increased, with the incidence ranging from 1 to 5% in Italy, Sweden, and Switzerland and up to 15 to 80% in Spain, the United States, and South Africa (28). Co-trimoxazole resistance is often associated with multiply resistant strains and is strongly correlated with the upsurge in resistance to penicillin (28). In Spain, an area associated with a high prevalence of endemic penicillin-resistant pneumococci, 26% of the co-trimoxazole-resistant strains from clinical specimens and 45% from carriers were associated with penicillin resistance (40). Among isolates from child carriers of resistant *S. pneumoniae* strains in South Africa, 42.5% of penicillin-resistant isolates were also resistant to co-trimoxazole (29, 30).

A variety of mechanisms of resistance to trimethoprim have been identified in clinically important bacteria. The most frequently encountered mechanism is the production of an additional DHFR enzyme which is less sensitive than the chromosomal enzyme to inhibition by trimethoprim (2). This mechanism is highly prevalent in enterobacteria (24), and to date, 16 of these enzymes have been characterized; they have predominantly been found as gene cassettes within plasmids or other mobile genetic elements (26, 41). These enzymes are unusual in that they share less than 50% amino acid identity with the DHFR enzyme of the host and the DHFR enzymes of other bacteria (26).

In staphylococci, plasmid-mediated high-level trimethoprim resistance is dominated by the ubiquitous Tn*4003*-mediated S1 DHFR (9, 42). The origin of this extrachromosomal enzyme differs from those of enzymes found in enterobacteria in that the resistant enzyme is, with the exception of three amino acid substitutions, identical to the DHFR of *Staphylococcus epidermidis*. It has therefore been suggested that this enzyme is a mutated form of the original *S. epidermidis* chromosomal DHFR that has been mobilized across the species barrier into

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other staphylococcal species (10). More recently, a second trimethoprim-resistant DHFR, the S2 DHFR, has been isolated from an isolate of *Staphylococcus haemolyticus*. The similarity between the active-site residues of this enzyme and those of other staphylococcal enzymes suggests that it may have origins similar to that of the S1 DHFR (12). In *Staphylococcus aureus*, low to intermediate levels of trimethoprim resistance have been determined by a Phe98-Tyr mutation to the chromosomal DHFR (11). An identical change occurs within the S1 DHFR and the chromosomal DHFR of *S. epidermidis* (10).

In some clinical isolates of *Escherichia coli* and *Haemophilus influenzae* trimethoprim resistance has been shown to result from alterations in the host chromosomal DHFR (14, 50). These changes include both regulatory mutations resulting in the overexpression of DHFR and mutations to the host chromosomal DHFR which result in a decreased affinity of the DHFR for trimethoprim (15, 16, 20, 21). In *E. coli* the most significant changes in the structural gene are from a glycine at position 30 to a tryptophan at position 30 and a glutamine-for-glutamic acid substitution at position 158. The amino acid change at position 30, which occurs within the active site of the DHFR, is thought to relate to the increase in the K_i value of the enzyme for trimethoprim (21). In *H. influenzae*, the alterations in either the C-terminal or the middle section of the chromosomal DHFR may cause resistance, probably as a result of a change in the secondary structure and the subsequent loss of trimethoprim binding (16). A combination of increased production and an altered DHFR is deemed necessary to produce high levels of resistance (21). There are no published reports on the mechanisms of resistance to trimethoprim in *S. pneumoniae*; however, biochemical assays of DHFRs from spontaneous laboratory mutants resistant to the antifolate compound methotrexate were shown to either overproduce dihydrofolate reductase or produce a modified chromosomal enzyme (45).

In a collection of 259 clinical isolates of *S. pneumoniae* from South Africa, we determined the incidence of resistance to trimethoprim and sulfamethoxazole individually and in combination as well as the MICs of other clinically significant antimicrobial agents. We selected 16 isolates for which the trimethoprim MICs covered a broad range, the DHFR gene for each isolate was amplified by PCR, and the nucleotide sequence was determined. This paper reports a relationship between the MIC of trimethoprim-sulfamethoxazole combinations in relation to those of the individual drugs and provides evidence that a single amino acid change to the DHFR gene is associated with trimethoprim resistance in pneumococci.

MATERIALS AND METHODS

Bacterial strains. Two hundred fifty-nine isolates were randomly selected from a collection of clinical isolates of *S. pneumoniae* which were collected from 1985 to 1995 from hospitals around South Africa. *S. pneumoniae* R6 and ATCC 49619 were used as susceptible controls for susceptibility testing, and *S. pneumoniae* R6 was used in the transformation experiments.

Susceptibility testing. The MICs were determined by the agar dilution method according to the guidelines set forth by the National Committee for Clinical Laboratory Standards (38). For susceptibility testing with trimethoprim, sulfamethoxazole, and trimethoprim-sulfamethoxazole (1:19), Mueller-Hinton agar (Difco, Detroit, Mich.) supplemented with 5% lysed defibrinated horse blood was used. Susceptibility testing with erythromycin, clindamycin, penicillin, tetracycline, and chloramphenicol was performed on Mueller-Hinton agar supplemented with 5% horse blood. All antimicrobial agents except trimethoprim lactate were supplied by Sigma Chemical Company (St. Louis, Mo.); trimethoprim lactate was supplied by (Wellcome, London, United Kingdom). Exponential-phase cultures grown in broth containing serum were diluted with saline so that the turbidity matched that of a 0.5 McFarland standard and were further diluted 10-fold before an inoculum was placed onto the agar surface with a multipoint inoculator (Mast Laboratories, Derbyshire, United Kingdom). The plates were incubated aerobically at 37°C for 20 h.

PCR. Samples for PCR were prepared by boiling approximately 10^9 CFU of *S. pneumoniae* in 0.5 ml of distilled H_2O for 5 min. Primers for amplifying the DHFR gene were based on the sequence for a resistant *S. pneumoniae* DHFR gene from the EMBL database (accession no. Z74777). The DHFR genes from trimethoprim-resistant and -susceptible isolates were amplified with primers 5′-GACAACATAGATAAATCTGTAGG-3′ and 5′-CACAGAAAAGCCGTG AATAAACC-3′. DNA amplification for DNA sequencing was performed in 100-μl volumes with 2 U of *Taq* DNA polymerase (Promega, Madison, Wis.), 400 nM primer, 1 mM $MgCl_2$, 0.1 mM (each) deoxynucleoside triphosphate, and 1 μl of DNA in the buffer provided by the manufacturer. The PCR products used in the cloning and mutagenesis experiments were amplified with 2.5 U of DNA polymerase with proofreading activity (*Pwo*; Boehringer Mannheim, Mannheim, Germany), 400 nM primer, 2 mM $MgSO_4$, 0.2 mM (each) deoxynucleoside triphosphate, and 1 μl of DNA in the buffer provided by the manufacturer. Amplification was performed with an OmniGene (Hybaid, Middlesex, United Kingdom) thermocycler on the following cycle: denaturation at 94°C for 3 min, followed by 32 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 30 s and a final extension at 72°C for 2 min.

Nucleotide sequencing and analysis. Double-stranded PCR products were extracted with chloroform and were precipitated with polyethylene glycol to remove contaminating nucleotides and primers, as described previously (4). Sequencing of heat-denatured templates was based on the chain termination method (43) with the Sequenase, version 2.0, DNA Sequencing Kit (United States Biochemicals, Cleveland, Ohio) and [^{35}S]dATP (Amersham) label. The PCR primers and the internal primers 5′-GGAAGCATGACTAAGAAAATC G-3′ and 5′-GGCGTCGCTTGCTTCCA-3′, based on the nucleotide sequence of the trimethoprim-susceptible DHFR of *S. pneumoniae* ATCC 49619 (EMBL accession no. Z74778), were used in the sequencing experiments. The PCR products were sequenced on a single strand, and for each primer, both dGTP and dITP labelling reactions were performed.

Transformation. *S. pneumoniae* R6 was grown in C medium supplemented with yeast extract (C+y medium) (52). Five milliliters of an overnight culture was inoculated into 100 ml of C+y medium and was grown at 37°C. At optical densities at 650 nm of between 0.01 and 0.5, aliquots of cells were collected and the efficiency of transformation of the cells to streptomycin resistance in the presence of DNA from a streptomycin-resistant pneumococcus was determined. Cells from the aliquot which had the highest transformation efficiency were stored at −70°C in 15% glycerol for further transformation experiments. Transformations were performed by incubating the thawed cells with 1 μg of donor DNA per ml at 30°C for 30 min. The cells were allowed to express resistance for 60 min at 37°C before they were plated onto selection medium (Mueller-Hinton agar with 5 μg of trimethoprim per ml supplemented with 5% lysed horse blood). The pneumococcal chromosomal DNA used in the transformation experiments was prepared as described previously (4). For the transformation of wild-type strains, cells were grown to an optical density at 650 nm of 0.2 in brain heart infusion broth (Difco) supplemented with 5% horse serum. One milliliter of culture was incubated at 30°C for 30 min with 1 μg donor DNA per ml and 1 μg of competence-stimulating peptide (23). The cells were allowed to express resistance for 60 min at 37°C before they were plated on selection medium.

Cloning of PCR products. The PCR-amplified DHFR genes were blunt end ligated into a *Hinc*II (Promega)-restricted expression vector, pGEM3Zf(+) (Promega). The ligation reactions were performed in 20-μl volumes with 0.1 μg of restricted vector, 1 μg of target DNA, 25 mM Tris-Cl (pH 7.5), 7.5 mM $MgCl_2$, 25 mM NaCl, 2.5 mM dithiothreitol, 10 μg of bovine serum albumin per ml, 0.5 mM dATP, 5% polyethylene glycol 6000, 10 U of *Hinc*II, and 10 U of T4 DNA ligase (United States Biochemicals). The ligation mixture was cycled between 10 and 37°C for 4 h in a Perkin-Elmer Cetus (Norwalk, Conn.) thermocycler. *E. coli* JM109 cells were electrotransformed with the ligation mixture, as described previously (17). Transformants were selected on Mueller-Hinton agar with 10 μg of trimethoprim per ml, 20 μg of ampicillin per ml, and 80 μg of methicillin per ml. The cloned PCR products were sequenced to determine the orientation of the insert and to ensure sequence integrity.

Pulsed-field gel electrophoresis. *S. pneumoniae* DNA was prepared in agarose blocks by a previously described method (32), with the following modifications. Agarose blocks were prepared from a suspension of cells grown on blood agar and diluted to turbidity equivalent to that of a 1.0 McFarland standard. The gel blocks were equilibrated in 1× restriction buffer and incubated with 25 U of *Sma*I (Boehringer Mannheim) restriction enzyme at 30°C for 4 h. Macrorestricted fragments were electrophoresed for 18 h at 200 V with a 2- to 16-s ramp with a Gene Navigator system (Pharmacia LKB, Uppsala, Sweden) with the following ramp times: 2 s for 3 h, 6 s for 3 h, 9 s for 4 h, 12 s for 4 h, and 16 s for 4 h. The relatedness of the isolates was determined according to the criteria described previously (51).

Site-directed mutagenesis. Site-directed mutagenesis was performed by a PCR-based megaprimer method described previously (47).

Preparation of DHFR. Sixty milliliters of bacterial culture were grown overnight in Luria-Bertani broth on a shaking platform at 37°C. The bacteria were harvested by centrifugation (3,800 × *g* at 4°C for 10 min). All further manipulations were carried out at 4°C. The bacterial pellet was washed with 30 ml of buffer A (50 mM sodium phosphate buffer [pH 7.4] with 10 mM β-mercaptoethanol and 1 mM EDTA) and was finally resuspended in 1 ml of buffer A. The suspension was sonicated three times for 30 s each time at 1-min intervals with

TABLE 1. Incidence of resistance to various antimicrobial agents, their association with co-trimoxazole resistance, and the MICs of each agent at which 50 and 90% of isolates are inhibited

Antimicrobial agent	No. (%) of resistant isolates	No. (%) of isolates with resistance associated with Tmp-Smx resistance	MIC (μg/ml)[a]	
			50%	90%
Tmp-Smx (1:19)[b]	166 (64.1)	166 (100)	2/38	8/152
Penicillin	111 (42.9)	100 (90.1)	0.06	1
Chloramphenicol	75 (29.0)	70 (93.3)	2	16
Tetracycline	148 (57.1)	124 (83.8)	8	32
Erythromycin	68 (26.3)	57 (83.8)	0.25	16
Clindamycin	54 (20.8)	40 (74.1)	0.25	64
Trimethoprim	166 (64.1)	156 (94.0)	8	256
Sulfamethoxazole	172 (66.4)	144 (83.7)	128	1,024

[a] 50% and 90%, MICs at which 50 and 90% of isolates are inhibited, respectively.
[b] Tmp-Smx, trimethoprim-sulfamethoxazole.

a MSE Soniprep 150 apparatus and was then clarified by centrifugation at 30,000 × *g* for 15 min and stored at −20°C. Estimation of the protein concentrations in crude lysates was determined by the method of Waddel (53).

DHFR assays. DHFR assays were performed by the method of Osborn and Huennekens (39) with an ATI Unicam UV2 spectrophotometer with heated cuvette carriage (37°C). The reaction was performed in 1-ml quartz cuvettes containing 40 mM sodium phosphate buffer (pH 6.0), 10 mM β-mercaptoethanol, 0.08 mM NADPH, enzyme, and distilled water to 950 μl. A blank, which did not contain NADPH, and the sample were placed in the spectrophotometer and were allowed to equilibrate for 2 min. Any decrease in absorbance measured at this stage was taken as dihydrofolate-independent NADPH oxidase activity and was deducted from the final reading. Dihydrofolate was then added to both cuvettes, and the decrease in absorbance was measured for 2 min or until a zero reading was recorded.

Nucleotide sequence accession numbers. The nucleotide sequence data reported here have been assigned the indicated EMBL accession numbers: isolate 56, Z84380; isolate 30, Z84378; isolates 21, 46, 47, 48, 39, and 61, Z84381; and isolate 92, Z84379. The sequences of the open reading frames of the DHFR genes from isolates 17, 45, and 55 were identical to the previously submitted nucleotide sequence for a trimethoprim-resistant DHFR (accession no. Z74777).

RESULTS

Susceptibility tests. The MICs of trimethoprim-sulfamethoxazole (1:19) and the other antimicrobial agents tested for the 259 clinical isolates of *S. pneumoniae* from South Africa are presented in Table 1. Breakpoints for resistance to the various antimicrobial agents were as follows: trimethoprim-sulfamethoxazole, ≥1/19 μg/ml; penicillin, ≥0.125 μg/ml; chloramphenicol, ≥8 μg/ml; tetracycline, ≥4 μg/ml; erythromycin, ≥0.5 μg/ml; clindamycin, ≥0.5 μg/ml; trimethoprim, ≥8 μg/ml; and sulfamethoxazole, ≥128 μg/ml. The drug to which resistance was most frequently encountered was trimethoprim-sulfamethoxazole (64.1%), followed by tetracycline (57.1%), penicillin (42.9%), chloramphenicol (29%), erythromycin (26.3%), and clindamycin (20.8%). These isolates form part of a reference collection, and the prevalence of resistance is not necessarily reflective of that among community-acquired isolates in South Africa. Coresistance was a major feature associated with trimethoprim-sulfamethoxazole resistance. Resistance to penicillin and chloramphenicol was associated with trimethoprim-sulfamethoxazole resistance in more than 90% of the isolates, resistance to tetracycline and erythromycin was associated with trimethoprim-sulfamethoxazole resistance in more than 80% of the isolates, and resistance to clindamycin was associated with trimethoprim-sulfamethoxazole resistance in 74% of the isolates. The patterns of multidrug resistance are presented in Table 2.

The relationship between resistance to trimethoprim-sulfamethoxazole in combination and the individual components was determined for the 259 clinical isolates. The MICs of trimethoprim-sulfamethoxazole (1:19) were correlated with the MICs of the individual components, and the trimethoprim MICs were found to be more strongly correlated to trimethoprim-sulfamethoxazole MICs (correlation coefficient, 0.744) than the sulfamethoxazole MICs (correlation coefficient, 0.441). There were also more isolates that were resistant to sulfamethoxazole (MICs, ≥128 μg/ml) but susceptible to trimethoprim-sulfamethoxazole (MICs, ≤1/19 μg/ml) (28 of 166 isolates) than isolates that were resistant to trimethoprim (MICs, ≥8 μg/ml) but susceptible to trimethoprim-sulfamethoxazole (10 of 166).

TABLE 2. Patterns of multidrug resistance associated with resistance to trimethoprim-sulfamethoxazole

Resistance associated with Tmp-Smx[a]	No. (%) of isolates (n = 166)
Tmp-Smx Pen Chl Tet Cly Erm	11 (6.6)
Tmp-Smx Pen Chl Tet Erm	10 (6.0)
Tmp-Smx Pen Tet Cly Erm	18 (10.8)
Tmp-Smx Pen Chl Tet	35 (21.1)
Tmp-Smx Pen Chl Erm	1 (0.6)
Tmp-Smx Pen Cly Erm	1 (0.6)
Tmp-Smx Pen Tet Erm	8 (4.8)
Tmp-Smx Chl Tet Cly	1 (0.6)
Tmp-Smx Chl Tet Erm	3 (1.8)
Tmp-Smx Pen Chl	1 (0.6)
Tmp-Smx Pen Tet	11 (6.6)
Tmp-Smx Chl Tet	8 (4.8)
Tmp-Smx Tet Erm	4 (2.4)
Tmp-Smx Cly Erm	1 (0.6)
Tmp-Smx Pen	12 (7.2)
Tmp-Smx Tet	15 (9.0)
Tmp-Smx	26 (15.6)

[a] Smx, sulfamethoxazole; Tmp, trimethoprim; Pen, penicillin; Chl, chloramphenicol; Tet, tetracycline; Cly, clindamycin; Erm, erythromycin.

Nucleotide sequences of DHFRs. Eleven trimethoprim-resistant clinical isolates for which the MICs covered a broad range were selected for further study. In addition to these, seven trimethoprim-susceptible strains of *S. pneumoniae*, including strains R6, ATCC 49619, and five clinical isolates of different serotypes, were used as controls for the PCR and sequencing experiments. The numbers, sources, and serotype of the clinical isolates and the MICs of trimethoprim, sulfamethoxazole, and trimethoprim-sulfamethoxazole (1:19) for the clinical isolates are presented in Table 3. The DHFR genes from these isolates were amplified by PCR, and the nucleotide sequences were determined.

The variability within the DHFR genes of the trimethoprim-susceptible laboratory strains and unrelated clinical isolates of different serotypes of *S. pneumoniae* was low (up to 0.12% different). The nucleotide sequence of *S. pneumoniae* ATCC 49619 was identical to that which appears in the EMBL database (accession no. Z74778). The sequence of *S. pneumoniae* R6 differed from this sequence by a single nucleotide, guanine-46-thymine, which caused the replacement of valine-16 with a similar amino acid, leucine. This mutation was not present in any of the trimethoprim-susceptible or -resistant clinical isolates. The number of nucleotide differences between the *S. pneumoniae* ATCC 49619 control strain and the trimethoprim-susceptible clinical isolates ranged from two to six and translated into one or two amino acid substitutions. One of these mutations, Asp92-Ala occurred in all the trimethoprim-susceptible and -resistant clinical isolates. Three other amino acid substitutions occurred in the trimethoprim-susceptible isolates:

TABLE 3. Properties of trimethoprim-resistant pneumococcal isolates[a]

Isolate no.	DHFR	Type	Hospital	Serotype	MIC (mg/liter)		
					Tmp	Smx	Tmp-Smx
17	P1	Nasal swab	Morningside	6A	512	512	16/304
45	P1	Nasal swab	Baragwanath	6A	256	512	8/152
55	P1	CSF	Baragwanath	6A	256	512	4/76
56	P1	Blood culture	Red Cross	19F	128	256	4/76
30	P3	Blood culture	Red Cross	14	64	256	4/76
21	P2	Gastric juice	Tshepong	23F	512	256	8/152
46	P2	Blood culture	Baragwanath	23F	256	512	8/152
47	P2	Blood culture	Baragwanath	14	128	512	8/152
48	P2	Nasal swab	Johannesburg	23F	512	512	8/152
39	P2	Sputum	East Rand	23F	128	128	4/76
61	P2	CSF	Baragwanath	23F	64	64	2/38
92	Tmp^s	CSF	Baragwanath	1	8	64	0.5/9.5
113	Tmp^s	Blood culture	Hilbrow	6B	1	16	0.125/2.375
120	Tmp^s	Blood culture	Baragwanath	8	1	8	0.125/2.375
119	Tmp^s	Blood culture	Red Cross	23F	1	8	0.125/2.375
124	Tmp^s	Nasal swab	Johannesburg	23F	1	4	0.125/2.375
R6	Tmp^s				2	16	0.25/4.75
ATCC 49619	Tmp^s				4	16	0.25/4.75

[a] Smx, sulfamethoxazole; Tmp, trimethoprim; CSF, cerebrospinal fluid.

Glu20-Asp, which occurred in most of the resistant isolates, and Ile65-Val and Pro111-Ala, which were unique.

The nucleotide sequences of the resistant isolates were dominated by two main groups of mutations. Group 1 included isolates 17, 45, 55, and 56, and group 2 included isolates 21, 46, 47, 48, 39, and 61. The nucleotide sequences along the length of the DHFR genes from isolates of the two resistant groups differed from that of the *S. pneumoniae* ATCC 49619 strain by between 6 and 7% (30 to 36 nucleotide substitutions). About two-thirds of these mutations were redundant, with 8 and 12 of these mutations translating into amino acid substitutions in groups 1 and 2, respectively (Fig. 1). The isolates from both groups shared six amino acid substitutions (Glu20-Asp, Pro70-Ser, Gln81-His, Asp92-Ala, Ile100-Leu, and Leu135-Phe). The first group included two extra amino acid substitutions (Lys60-Gln and Pro111-Ser), and the second group was characterized by six additional amino acid substitutions (Glu14-Asp, Ile74-Leu, Gln91-His, Glu94-Asp, Phe147-Ser, and Ala149-Thr). Isolate 56 differed from the first group in that the Glu20-Asp substitution at the beginning of the gene was absent, and an additional Glu141-Asp was present. The DHFR sequence of isolate 30 was more homologous to the sequences of the first group of enzymes than to those of the second group and differed from the sequences of the first group by the absence of 12 nucleotide substitutions and the presence of an additional 13 substitutions. These translated into the absence of the Pro70-Ser, Gln81-His, and Pro111-Ser amino acid substitutions and the presence of additional Ala77-Val and Val78-Ala substitutions (Fig. 1).

The first 90 nucleotides upstream of the genes encoding trimethoprim-resistant and -susceptible DHFRs were sequenced. With the exception of the second group of DHFR genes from resistant isolates, the upstream sequence was found to be identical in all the clinical isolates and laboratory strains. One point mutation (guanine to adenine) was located 15 bp upstream from the start of the open reading frame in the second group of DHFR genes from resistant isolates. This mutation did not appear to form part of an upstream regulatory region, and its involvement in potentially increasing DHFR production in the host is unknown.

Genetic relatedness of *S. pneumoniae* isolates. The pulsed-field gel electrophoresis patterns were obtained for the *Sma*I-restricted DNAs from all the isolates except trimethoprim-susceptible isolates 92 and 120 whose DHFR genes were sequenced (data not shown). The profiles of the trimethoprim-susceptible isolates were unrelated to each other and to those of any of the resistant isolates. Of the four isolates which harbored the first type of trimethoprim-resistant DHFR, two of the isolates, 45 isolates and 55, had an indistinguishable profile and are possibly related to isolate 21, which is of the same serotype (serotype 6A). Despite possessing nearly identical DHFR genes, isolate 56 was completely unrelated to this group in terms of serotype and restriction profile. Five of six of the isolates harboring the second type of trimethoprim-resistant DHFR were of serotype 23F. Of these, the pulsed-field gel electrophoresis profiles of isolates 21, 46, and 39 were indistinguishable and were closely related to the profiles of isolates 48 and 61. Isolate 47 possessed a DHFR gene whose sequence was identical to the other group 2 isolates, but isolate 47 was genetically unrelated.

Transformation. Chromosomal DNAs from isolates 45 and 21, which were representative of the two DHFR groups, and cloned PCR products of the DHFR genes from these isolates were capable of transforming recipient strain R6 to trimethoprim resistance. One transformed colony from each experiment was selected, the DHFR gene from the trimethoprim-resistant transformant was amplified by PCR, and the nucleotide sequence was determined.

The sequence of the DHFR gene from the strain which was transformed with chromosomal DNA from isolate 21 (group 2) was identical to that of the DHFR gene from the original parent strain. The sequence of the DHFR gene from R6 transformed with the cloned PCR product (group 2) was, with the exception of the missing 3′-terminal redundant nucleotide substitution, identical to that of the donor DHFR gene. The DHFR genes from the strains transformed with chromosomal DNA and the cloned DHFR PCR product from isolate 45 (group 1) exhibited two different truncated DHFR genes, indicative of the heterologous recombination crossover point. The sequence of the DHFR gene from the strain transformed

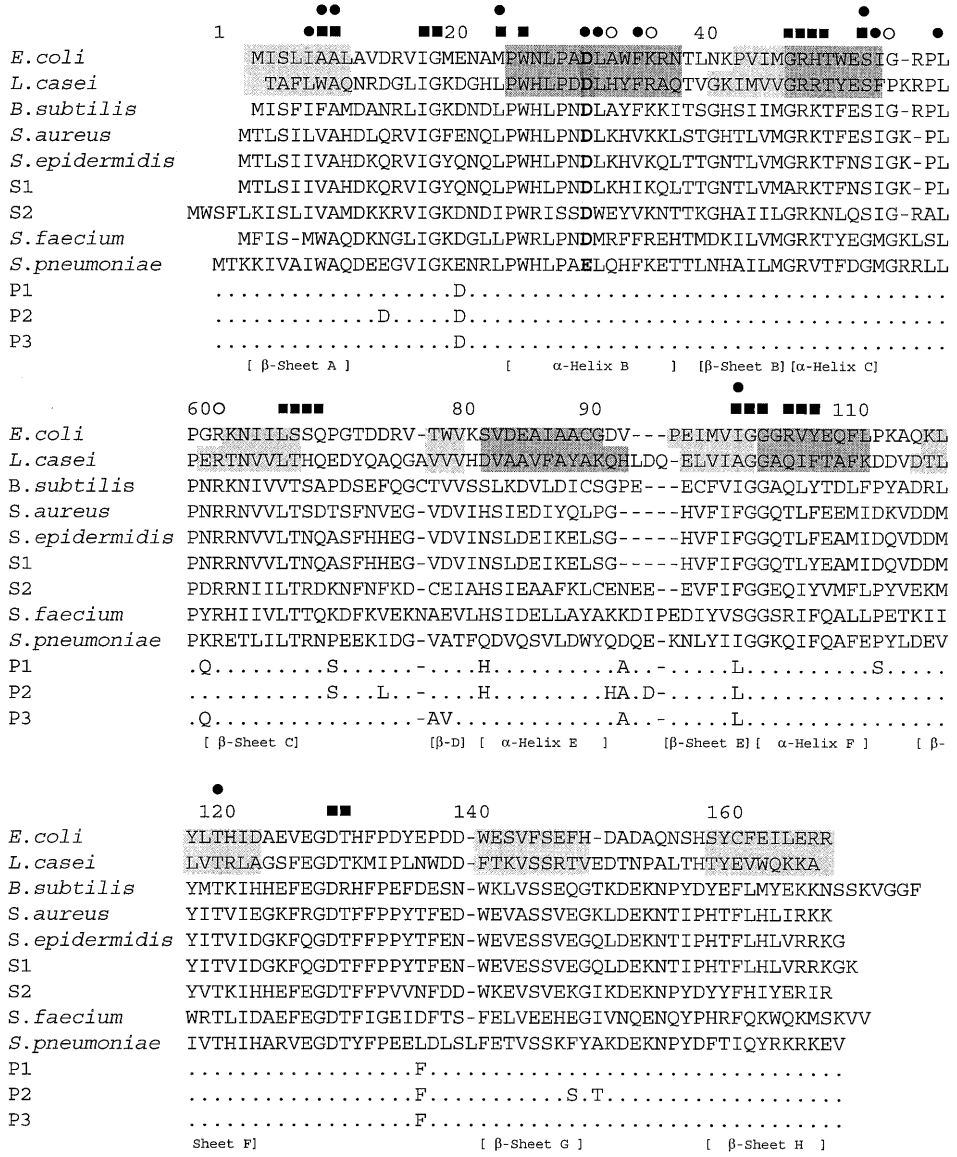

FIG. 1. Comparison of the amino acid sequences of the trimethoprim-susceptible DHFRs of *E. coli* (48), *L. casei* (35), *B. subtilis* (27), *S. aureus* (13), *S. epidermidis* (11), *S. faecium* (22), and *S. pneumoniae* (ATCC 49619); the plasmid-mediated S1 and S2 trimethoprim-resistant DHFRs of staphylococci (12, 42); and the trimethoprim-resistant pneumococcal DHFRs from this study (P1, isolates 17, 45, and 55; P2, isolates 21, 46, 47, and 39; P3, isolate 30). Amino acids are numbered according to the numbering for the *S. pneumoniae* DHFR. Positions involved in binding trimethoprim and methotrexate (●), methotrexate alone (○), and NADPH cofactor (■) (6, 19, 36, 37) are indicated above the sequences. Features of secondary structure (β sheets, light shading; α helices, dark shading) of the *E. coli* and *L. casei* DHFRs are marked below the sequences (6, 19). The unusual Asp30-Glu substitution in *S. pneumoniae* is highlighted in boldface.

with chromosomal DNA was identical to that of the donor DNA up to nucleotide 345, but from nucleotide 345, it was identical to that of the original R6 host DHFR gene. The missing mutations included a Leu135-Phe substitution and the remaining redundant nucleotide changes downstream. The sequence of the DHFR gene from the R6 strain transformed with the PCR product was identical to that of the donor DNA from nucleotide 225 to the end of the gene. The redundant nucleotide substitutions and the amino acid substitutions Glu20-Asp, Lys60-Gln, Pro70-Ser, and Ile74-Leu were absent from the 5′ end of the donor DHFR. The MICs of trimethoprim for all of the resistant transformants were 32 μg/ml, including the transformants harboring the incomplete donor DHFR genes. This suggests that in the group 1 resistant enzymes, only the gene region from nucleotides 225 to 345 is associated with trimethoprim resistance.

The MIC of trimethoprim for the resistant transformants (32 μg/ml) was 3 to 5 dilutions higher than those for the trimethoprim-susceptible controls (1 to 4 μg/ml). However, the MICs of trimethoprim for the resistant transformants were still 1 to 4 dilutions lower than those for the original resistant isolates (64 to 512 μg/ml). To determine whether the nature of the R6 host may play a role in suppressing the expression of the resistant DHFR genes, two trimethoprim-susceptible clinical isolates (isolates 113 and 124) which were serotypes 6B and 23F, respectively, were transformed with cloned PCR products of the two resistant DHFR groups. The trimethoprim MICs for these transformed clinical isolates were 32 μg/ml, which sug-

gests that in the resistant donors, other factors may be necessary to produce the high MICs which occur for the clinical isolates. Resistance to sulfamethoxazole or trimethoprim-sulfamethoxazole (1:19) did not cotransfer with trimethoprim resistance to the recipient strains. Sulfonamide resistance appeared to have no impact on the level of trimethoprim resistance, since the trimethoprim MICs for four different strains resistant to sulfonamides but susceptible to trimethoprim and which were transformed to trimethoprim resistance remained 32 μg/ml.

Inhibitor kinetics. As a result of the strong T7 and SP6 promoters flanking the pGEM3Zf(+) multiple cloning site, the specific activity of DHFR for the strains harboring the cloned DHFR PCR products was 1,000-fold higher than that for the *E. coli* JM109 host. The DHFR activity for the trimethoprim-susceptible *S. pneumoniae* ATCC 49619 DHFR clone could be reduced to 50% (50% inhibitory dose [ID_{50}]) by the addition of 0.15 μM trimethoprim. This explains the reduced susceptibility of *S. pneumoniae* to trimethoprim in comparison to those of other susceptible clinical bacteria (*E. coli* ID_{50}, 0.007 μM). The ID_{50}s of trimethoprim for the cloned DHFRs representative of resistance groups 1 and 2 were 3.9 and 7.3 μM, respectively. This represented a 26- to 50-fold increase in resistance to trimethoprim compared to that for the trimethoprim-susceptible control. This increase in resistance correlates well with the increase in resistance observed in the trimethoprim-resistant transformants.

Site-directed mutagenesis. An analysis of the amino acid substitutions in the genes encoding resistant DHFR is presented in Fig. 1. Since trimethoprim resistance was expressed in the truncated genes produced in the transformation experiments, it appears that the amino acid substitutions from the truncated regions are not involved or play a minor role in trimethoprim resistance. It is therefore likely that only the transforming region consisting of amino acid substitutions from Gln81-His to Ile100-Leu is involved in conferring trimethoprim resistance. Of the five amino acid substitutions found in this region, only two occurred in all the resistant isolates. Of the two substitutions, Asp92-Ala was also found in the trimethoprim-susceptible clinical isolates, whereas the Ile100-Leu substitution occurred only in resistant strains. Since this amino acid substitution occurs within a predicted substrate and cofactor binding site, it was thought that this substitution is responsible for resistance. To verify the potential role of the Ile100-Leu substitution in trimethoprim resistance, the mutation was introduced into the *S. pneumoniae* ATCC 49619 DHFR gene. The cloned PCR products containing this mutation expressed a DHFR for which the trimethoprim ID_{50} was 7.4 μM, a value indicating 50 times greater resistance than that for the trimethoprim-susceptible chromosomal enzyme. The inhibitor profile of the mutant was identical to that of the group 2 resistant enzymes, and the mutant enzyme was almost twice as resistant as the group 1 enzymes. The mutant DHFR gene could transform R6 to trimethoprim resistance so that the MIC for the strain was 32 μg/ml. This suggests that only a single Ile100-Leu mutation is necessary for resistance in *S. pneumoniae.*

DISCUSSION

Even though *S. pneumoniae* is only moderately susceptible to trimethoprim, the strong correlation between trimethoprim and co-trimoxazole resistance suggests that trimethoprim resistance is an essential component of co-trimoxazole resistance. Very few (6%) of the trimethoprim-resistant isolates were sensitive to co-trimoxazole. This in vitro evidence provides impetus to the argument that even in the case of *S. pneumoniae*, there is little difference in the efficacy between the use of trimethoprim as an agent on its own and the use of trimethoprim in combination with sulfamethoxazole, and despite the obvious synergy between the two agents, trimethoprim therapy alone at a higher dose is as effective and causes fewer side effects than co-trimoxazole therapy. It is possible, however, that the shift to using trimethoprim as an agent on its own may lead to an increase in the incidence of trimethoprim-resistant but sulfamethoxazole-susceptible organisms. This is possible, especially since resistance to the two agents is rarely cotransferred by transformation.

From the model for trimethoprim, methotrexate, dihydrofolate, and NADPH cofactor binding for the *E. coli* and *Lactobacillus casei* DHFRs (6, 19, 36, 37) and the alignment of the amino acid sequences of trimethoprim-susceptible chromosomal DHFR genes from *E. coli* (48), *L. casei* (35), *Bacillus subtilis* (27), *S. aureus* (13), *S. epidermidis* (10), *Streptococcus faecium* (22), and *Neisseria gonorrhoeae* (5), an interesting feature of both the resistant and susceptible DHFRs from *S. pneumoniae* is that position 30 is occupied by a glutamic acid instead of the conserved aspartic acid residue (Fig. 1). With the exception of the trimethoprim-susceptible DHFR of *Lactococcus lactis* (GenBank accession no. X60681), a glutamic acid-30 residue at the equivalent position is normally associated with enzymes which are resistant to extremely high levels of trimethoprim, such as those of vertebrate origin (3) and the highly resistant plasmid-borne trimethoprim-resistant DHFRs associated with the enterobacteria, such as the type I-like DHFRs (*dfrI*, *dfrIb*, *dfrV*, *dfrVI*, and *dfrVII* [26]) and the type XII DHFR (44). The amino acid at the position corresponding to Glu-30 forms strong hydrogen bonds with trimethoprim and dihydrofolate in sensitive bacterial enzymes, and the substitution with glutamic acid in the vertebrate enzymes is thought to make a major contribution toward trimethoprim resistance. This could in part explain the moderate susceptibility of the *S. pneumoniae* DHFR to trimethoprim.

Most of the mutations to trimethoprim-resistant chromosomal DHFRs which have been described in isolates of *E. coli* (21, 49), *S. aureus* (11), and *H. influenzae* (16) do not show any homology with each other with respect to the position of the amino acid substitutions involved in conferring trimethoprim resistance. Of the mutations observed in *S. pneumoniae*, it is thought that only the Ile100-Leu mutation is involved in conferring trimethoprim resistance, since not only is this the only *S. pneumoniae* mutation to occur within a putative trimethoprim binding site (Fig. 1) but this mutation on its own was also able to confer levels of resistance equivalent to those of the DHFRs from clinical isolates which harbored other mutations. The other mutations were homologous to gene regions which form loop structures between regions of secondary structure and residues which were not involved in trimethoprim binding; this suggests they are unlikely to play a role in enzyme function, and thus in trimethoprim resistance. The Ile100 of the *S. pneumoniae* DHFR is homologous to Ile94 of *E. coli* DHFR, which makes van der Waals contact with the pyrimidine ring of trimethoprim (36, 37). By replacing isoleucine with a similar amino acid, leucine, the affinity of trimethoprim for the active site may be reduced without affecting dihydrofolate binding. In a study of trimethoprim-resistant DHFRs from *H. influenzae*, one clinical isolate (R1047) harbored an identical Ile-Leu substitution in a position equivalent to that in the resistant *S. pneumoniae* DHFRs in part of a gene region capable of transforming a sensitive *H. influenzae* DHFR to trimethoprim resistance (16). The requirement of a single mutation for the development of resistance to trimethoprim is

compatible with the rapid emergence of resistance to this agent in *S. pneumoniae.*

The ID_{50}s for the trimethoprim-resistant chromosomal DHFRs from pneumococci (3.9 to 7.3 μM) are similar to those for chromosomal mutant DHFRs from *S. aureus* (11), plasmid-mediated resistant DHFRs from gram-positive bacteria such as S1 and S2 (10, 12), and the plasmid-mediated DHFRs which confer intermediate resistance in *E. coli* (26). The discrepancy between the trimethoprim MICs for the transformants (MICs, 32 μg/ml) and those for the original donor isolates (MICs, 64 to 512 μg/ml) suggests that factors over and above the production of the modified chromosomal DHFR are responsible for the high levels of resistance in these isolates. In *S. aureus* it was shown that for clinical isolates which expressed a modified chromosomal DHFR at normal levels, trimethoprim MICs were 16 to 64 μg/ml (11), which are similar to the MICs obtained for the pneumococcus transformants. Furthermore, for the *S. aureus* isolates which showed an approximate 10-fold increase in DHFR expression, trimethoprim MICs were significantly higher (≥512 μg/ml) (11). Although no significant nucleotide changes were identified immediately upstream of the DHFR genes from trimethoprim-resistant pneumococcal isolates, overproduction of DHFR has not been ruled out, since it is possible that the regulatory regions for DHFR expression in pneumococci are located further upstream and were therefore not incorporated into the resistant transformants. Other factors contributing to resistance may include permeability barriers, pump efflux mechanisms, or differences in host folate metabolism.

The levels of sequence diversity within the resistant DHFR genes were significantly higher (6 to 7%) than the levels of diversity found within DHFR genes of trimethoprim-susceptible populations of *S. pneumoniae* (0 to 0.12%). It is therefore unlikely that the resistant DHFRs have evolved through recombination within *S. pneumoniae* isolates. Furthermore, since a single Ile100-Leu mutation appears to be essential for resistance, the process by which the resistant DHFR genes have evolved is highly degenerate and is characteristic of recombination with DNA from other sources. A comparison of the amino acid sequence of DHFR genes from a number of species (Fig. 1) indicates that despite the low amino acid sequence identity between the DHFRs of different bacteria, the residues involved in substrate and cofactor binding are highly conserved (6, 19, 42). For the DHFRs for which a crystal structure is available (*E. coli* and *L. casei*), it was shown that most of the diversity in the amino acid sequence occurs in the loop structures which connect the conserved elements (α helix and β sheet) of the secondary structure (6). In closely related DHFRs such as those from *S. aureus* and *S. epidermidis*, more than half of the amino acid changes occur in the loops which connect the regions of secondary structure (Fig. 1). Similarly, most of the diversity occurs in the corresponding regions of the closely related trimethoprim-resistant DHFRs (1). Since sequence identity is low between DHFR genes of different species, it is most likely that the donor DNA involved in heterologous recombination to form the trimethoprim-resistant DHFRs in *S. pneumoniae* originated from closely related streptococcal species, since most of the changes are not involved in resistance but appear to be a product of a redundant recombination process. Furthermore, the pattern of nucleotide changes within the trimethoprim-resistant DHFRs resembles the patterns of nucleotide changes resulting in resistant penicillin-binding protein (PBP) genes in *S. pneumoniae*, in which large numbers of redundant nucleotide substitutions occur in distinct and often highly conserved patterns (18, 31, 34, 46). It is therefore likely that a similar pattern of heterologous recombination that has been suggested to have taken place in the formation of the resistant PBP genes (18, 31) is responsible for the evolution of resistant DHFRs. Since, like PBP genes, DHFR genes are considered essential housekeeping genes in all bacteria, it is likely that other streptococcal species whose gene sequences share close homology with *S. pneumoniae* gene sequences may provide the necessary gene pool from which interspecies recombination can take place. However, since no sequence data for the DHFR genes of other streptococci are available, it is difficult to predict the exact origins of the resistant DHFR genes. From the transformation experiments, it appears that the horizontal spread of trimethoprim-resistant DHFRs may be enhanced by intraspecies recombination, and since the resistant DHFRs can be transformed into a host unchanged, it is difficult to predict the extent of the roles played by inter- and intraspecies recombination in the spread of trimethoprim resistance in *S. pneumoniae.*

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