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Multiple Mutations Modulate the Function of Dihydrofolate Reductase in Trimethoprim-Resistant *Streptococcus pneumoniae*

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Trimethoprim resistance in *Streptococcus pneumoniae* can be conferred by a single amino acid substitution (I100-L) in dihydrofolate reductase (DHFR), but resistant clinical isolates usually carry multiple DHFR mutations. DHFR genes from five trimethoprim-resistant isolates from the United Kingdom were compared to susceptible isolates and used to transform a susceptible control strain (CP1015). All trimethoprim-resistant isolates and transformants contained the I100-L mutation. The properties of DHFRs from transformants with different combinations of mutations were compared. In a transformant with only the I100-L mutation (R12/T2) and a D92-A mutation also found in the DHFRs of susceptible isolates, the enzyme was much more resistant to trimethoprim inhibition (50% inhibitory concentration [IC_{50}], 4.2 μM) than was the DHFR from strain CP1015 (IC_{50}, 0.09 μM). However, K_m values indicated a lower affinity for the enzyme's natural substrates (K_m for dihydrofolate [DHF], 3.1 μM for CP1015 and 27.5 μM for R12/T2) and a twofold decrease in the specificity constant. In transformants with additional mutations in the C-terminal portion of the enzyme, K_m values for DHF were reduced (9.2 to 15.2 μM), indicating compensation for the lower affinity generated by I100-L. Additional mutations in the N-terminal portion of the enzyme were associated with up to threefold-increased resistance to trimethoprim (IC_{50} of up to 13.7 μM). It is postulated that carriage of the mutation M53-I—which, like I100-L, corresponds to a trimethoprim binding site in the *Escherichia coli* DHFR—is responsible for this increase. This study demonstrates that although the I100-L mutation alone may give rise to trimethoprim resistance, additional mutations serve to enhance resistance and modulate the effects of existing mutations on the affinity of DHFR for its natural substrates.

The broad-spectrum diaminopyrimidine chemotherapeutic agent trimethoprim has now been in use for over 3 decades, most often in combination with sulfamethoxazole as cotrimoxazole. The combination has proven to be both effective and inexpensive, with synergistic activity against a wide range of respiratory, intestinal, and urinary tract pathogens.

Susceptibility of *Streptococcus pneumoniae* to trimethoprim alone is rarely determined, but cotrimoxazole resistance was first reported in 1972 (11) and has now increased dramatically in many regions of the world (12). In South Africa, 64% of *S. pneumoniae* isolates are resistant (cotrimoxazole MIC, ≥20 mg/liter) (1); in Spain, the level of cotrimoxazole-resistant isolates from children with otitis media was found to have risen from 64% in 1992 to 81% in 1996 (7). A survey of patients with community-acquired lower respiratory tract infections from whom *S. pneumoniae* was isolated during 1994 and 1995 (9) showed that the overall rate of cotrimoxazole resistance among isolates from the United Kingdom was 8.4% (range, 1.3 to 26%). However, according to the most recent survey by this group (1996 to 1997), the rate of cotrimoxazole resistance among United Kingdom isolates has increased to 12.6%, while the rate of resistance in the Republic of Ireland is now 16.1% (8).

Resistance of *S. pneumoniae* to trimethoprim has been found to be due to alteration of the chromosomal dihydrofolate reductase (DHFR) gene. Although there are multiple differences between resistant and susceptible genes, it has been reported that a single amino acid mutation in the DHFR gene, resulting in the replacement of isoleucine 100 with leucine (I100-L), is sufficient to confer resistance (1, 15; P. G. Hartman, C. Broger, P. Caspers, G. Dale, H. Langen, H. Locher, M. Ott, D. Roeder, D. Stuber, R. L. Then, and B. Wipf, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C108, 1997).

In this study, the DHFR gene sequences of five trimethoprim-resistant and four susceptible United Kingdom isolates of *S. pneumoniae* were determined. Transformation of the susceptible strain CP1015 with the amplified coding sequence from resistant isolates was used as a means of determining which of several mutations was likely to be significant. The DHFRs produced by trimethoprim-resistant CP1015 transformants contained a range of amino acid substitutions, and their kinetics (K_m values for dihydrofolate [DHF] and NADPH) and susceptibilities to inhibition by trimethoprim were compared.

MATERIALS AND METHODS

Bacterial strains. The sources, serotypes, and susceptibilities to sulfamethoxazole and trimethoprim of the clinical isolates used in this study are listed in Table 1. All isolates were from clinical samples collected in the United Kingdom.

Susceptibility testing. Sulfamethoxazole and trimethoprim lactate were obtained from the Sigma-Aldrich Company Ltd. (Dorset, United Kingdom). The

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TABLE 1. Sources and characteristics of trimethoprim-resistant and -susceptible *S. pneumoniae* isolates

Isolate	Source[a]	Serotype[b]	MIC (mg/liter)	
			Sulfamethoxazole	Trimethoprim
Pn94/720[c]	CSF	23F	512	256
Pn93/1293[d]	Blood culture	6B	512	256
Pn93/1802[e]	Blood culture	9V	256	256
P48	Unknown	ND	1,024	256
R12	Sputum	ND	256	512
Pn93/1791	Blood culture	19F	16	4
Pn93/917	CSF	9V	32	8
Pn93/908	CSF	14	32	4
Pn94/258	Blood culture	23F	32	4
CP1015		—	64	4
ATCC 49619		—	64	4

[a] CSF, cerebrospinal fluid.
[b] ND, not done; —, nonencapsulated.
[c] "Spanish" 23F multiresistant clone.
[d] "Spanish" 6B multiresistant clone.
[e] "French/Spanish" 9V multiresistant clone.

methods used to determine the susceptibilities of isolates and control strains to sulfamethoxazole and trimethoprim were as described previously (13).

DNA extraction. Extraction of DNA from isolates and control strains was performed by methods described previously (13) or by boiling a suspension of approximately 10^9 CFU in 250 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) for 5 min.

PCR. DHFR gene sequences were amplified with primers (Gibco BRL/Life Technologies Ltd., Paisley, United Kingdom) produced according to the sequence for the DHFR gene of a trimethoprim-resistant strain of *S. pneumoniae* (EMBL accession no. Z74777). The DHFR genes from trimethoprim-resistant and -susceptible isolates were amplified with the following primers: DHFR 1 (forward), 5′-TGTAAGCTATTCCAAACCAG-3′ (positions 76 to 95); DHFR 2 (reverse), 5′-CTACGTTCCATTAGACTTCC-3′ (positions 646 to 665). DNA amplification was performed as previously described (13).

Nucleotide sequence determination. PCR products were purified with the QIAquick PCR purification kit (Qiagen Ltd., Crawley, United Kingdom). Each sequencing reaction was carried out at on least two independently amplified PCR products. Sequencing reactions were performed by the *Taq* Dye-Deoxy termination method (PE-Applied Biosystems), and products were separated on an ABI 377 DNA sequencer. The primers used were DHFR 1 and 2 together with DHFR 3 (5′-GACAACATAGATAAATCTGTAGG-3′ [positions 27 to 49]) and DHFR 4 (5′-GGCGTCGCTTGCTTCCA-3′ [positions 310 to 325]). Analysis of sequences was performed with the ABI Prism Sequence Navigator program (version 1.0.1 [Perkin-Elmer]).

Transformation. The trimethoprim-susceptible recipient strain CP1015 was used as previously described (13). DHFR coding sequences of resistant isolates were amplified with primers DHFR 1 and 2, and 10-μl volumes of PCR products were used to transform CP1015. Following the final 2-h incubation step at 37°C, 50-μl volumes of suspension were transferred to Iso-Sensitest agar plates (Oxoid, Basingstoke, United Kingdom) containing 5% lysed horse blood and trimethoprim at 16 and 128 mg/liter and to antibiotic-free medium for the determination of viable counts. As previously described, a DNA-free control was included in each experiment to monitor any spontaneous mutation to trimethoprim resistance. Up to six transformants were selected at the highest trimethoprim concentration for each experiment. Single colonies of transformants were subcultured on 3 successive days onto Iso-Sensitest agar containing 16 and 128 mg of trimethoprim per liter. Resistance of transformants was confirmed by agar dilution susceptibility tests, as described previously (13), and the transformants were then stored at −70°C (Microbank Storage System; Prolab Diagnostics, Wirral, United Kingdom). The DHFR genes of selected transformants were amplified by PCR (DHFR primers 1, 2, and 3) and sequenced as previously described (13).

Enzyme extraction. Partially purified enzyme extracts were prepared in the absence of trimethoprim from CP1015, ATCC 49619, and selected trimethoprim-resistant CP1015 transformants by methods based on those described by Baccanari et al. (2). Growth from 18-h cultures on brain heart infusion agar (Oxoid) supplemented with 5% horse blood was used to inoculate 6 liters of brain heart infusion (Oxoid) that had been prewarmed to 37°C. After 6 h of incubation on a shaking platform at 37°C, late-log-phase growth was harvested by centrifugation at 3,800 × *g* for 20 min at 4°C in an RC5C Plus centrifuge (Sorvall [United Kingdom] Ltd., Stevenage, United Kingdom). The deposit was resuspended in a solution of 20 mM phosphate buffer (pH 7), 2 mM dithiothreitol, and 1 mM EDTA and centrifuged again, and the deposit was stored overnight at −70°C. The deposit was thawed, and the cells were broken by passage three times through a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) at a pressure of 14,000 lb/in^2. The pressate was then centrifuged at 100,000 × *g* for 30 min in a Beckman L7–65 ultracentrifuge (Beckman-RIIC Ltd., High Wycombe, United Kingdom). The supernatant fluid was removed, made 45% saturated with ammonium sulfate, and stirred at 4°C for 1 h. The precipitate formed was deposited by centrifugation at 15,000 × *g* for 20 min and discarded. The supernatant was then made 90% saturated with ammonium sulfate and stirred at 4°C for 1 h. The precipitate formed was deposited by centrifugation at 15,000 × *g* for 20 min, redissolved in approximately 10 ml of buffer, and dialyzed against the same buffer overnight at 4°C. The extract was stored at 4°C until required.

DHFR assay. Dihydrofolic acid (Sigma-Aldrich) was suspended in a solution of 5 mM HCl and 50 mM 2-mercaptoethanol (3), adjusted to a final concentration of 25 mM according to absorption at 282 nm at pH 7 and 284 nm at pH 13 (6), and stored in 50-μl volumes at −70°C. Aliquots were thawed for use and diluted with 0.1 M imidazole-HCl buffer (pH 7) and 2-mercaptoethanol to a working concentration of 2.5 mM DHF in 0.6 M 2-mercaptoethanol. Solutions of NADPH (Sigma-Aldrich) were made in 0.1 M imidazole-HCl buffer (pH 7) to a concentration of 2.5 mM, confirmed by reading the absorbance of the diluted solution at 340 nm (6). Stock NADPH was stored in aliquots of 500 μl at −70°C. Trimethoprim lactate (Sigma-Aldrich) solutions were prepared in 0.1 M imidazole-HCl buffer (pH 7) to concentrations of 10 and 100 μM and stored at −70°C.

Determinations of K_m values for each substrate (DHF and NADPH) and 50% inhibitory concentration (IC_{50}) values for trimethoprim were based on the methods of Baccanari et al. (2). A Pye Unicam PU8700 series spectrophotometer (Philips Analytical, Cambridge, United Kingdom) with a heated cuvette carriage and an inbuilt program for the integrated determination of initial reaction rates was used. Reactions were performed in semi-micro plastic cuvettes in a final volume of 1 ml of imidazole-HCl buffer (pH 7) at 30°C. For determinations of K_m values for each substrate, the concentration of the other substrate was held at a constant 150 μM. Nonspecific NADPH-oxidase activity was determined for each enzyme preparation by determining rates for each NADPH concentration in the absence of DHF, and these values were then deducted. Initial rates for each concentration of NADPH and DHF were used to determine K_m and V_{max} values according to Michaelis-Menten kinetics by using the Enzfitter program (version 1.05 [EGA]; Elsevier-Biosoft). The standard mixture for the determination of IC_{50} values was 100 μM NADPH, 50 μM DHF, 24 mM 2-mercaptoethanol, 0.1 M imidazole-HCl buffer (pH 7), trimethoprim, and enzyme extract in a final volume of 1 ml. IC_{50} values were determined by plotting initial reaction rates against each trimethoprim concentration using the Cricketgraph software program (version 1.3.2) in order to establish the inhibitor concentration that reduced the initial uninhibited rate by 50%.

Nucleotide sequence accession numbers. Sequences of the DHFR genes of P48, Pn93/1293, Pn93/1791, Pn93/1802, Pn93/720, Pn93/908, Pn93/258, R12, Pn93/917, and the spontaneous trimethoprim-resistant mutant have been deposited in the GenBank database under accession no. AF288411 through AF288420, respectively.

RESULTS

DHFR nucleotide and amino acid sequences. DHFR sequences for five trimethoprim-resistant and four trimethoprim-susceptible isolates of *S. pneumoniae* were compared with those of *S. pneumoniae* ATCC 496l9 (EMBL Z74778) and CP1015 (Table 2). Three of the resistant isolates—Pn94/720, Pn93/1293, and Pn93/1802—were United Kingdom representatives of multiresistant strains having international spread (10).

The DHFR coding sequence, consisting of 504 nucleotides encoding 168 amino acids, in trimethoprim-susceptible isolates showed a divergence of 3 to 7 nucleotides (0.6 to 1.4%) compared to the ATCC 49619 sequence, resulting in 2 to 3 differences in amino acid sequence (1.2 to 1.8%). DHFR coding sequences of trimethoprim-resistant isolates showed a 37- to

TABLE 2. Amino acid differences in DHFR sequences of trimethoprim-resistant and -susceptible isolates

Isolate	Amino acid at position[a]:																						MIC (mg/liter)
	14*	16	20*	26	53	60*	70*	74*	78	81*	83	86	91*	92*	94*	100*	111	120	133	135*	147	149*	164	
ATCC 49619	E	V	E	H	M	K	P	I	A	Q	V	V	Q	D	E	I	P	H	E	L	F	A	K	4
CP1015	—	L	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4
Pn93/1791	—	—	—	—	—	—	—	—	—	—	—	I	—	—	—	—	—	—	K	—	—	—	—	4
Pn93/917	—	—	D	—	—	—	—	—	—	—	—	—	—	A	—	—	A	—	—	—	—	—	—	8
Pn93/908	—	—	D	—	—	—	—	—	—	—	—	—	—	A	—	—	A	—	—	—	—	—	—	4
Pn94/258	—	—	—	—	—	—	—	—	—	—	—	—	—	A	—	—	A	—	—	—	—	—	—	4
Pn93/720	D	—	D	—	—	—	S	L	—	H	—	—	H	A	D	L	—	—	—	F	S	T	E	256
Pn93/1293	—	—	D	Y	—	—	S	—	T	Y	—	—	—	S	D	L	—	—	—	F	S	S	—	256
Pn93/1802	—	—	D	Y	I	Q	S	—	T	—	—	—	—	A	—	L	—	Q	—	F	S	T	—	256
P48	—	—	D	—	—	—	S	—	—	H	I	—	—	A	D	L	—	Q	—	F	S	T	—	256
R12	—	—	D	Y	I	Q	S	—	T	—	—	—	—	A	—	L	—	Q	—	F	S	T	—	512

[a] *, positions at which amino acids are altered in all trimethoprim-resistant isolates reported by Hartman et al. (37th ICAAC), Adrian and Klugman (1), Pikis et al. (15), and the present study; —, amino acid is the same as in ATCC 49619.

38-nucleotide divergence (7.3 to 7.5%) from ATCC 49619, resulting in 11 to 13 differences (6.5 to 7.7%) in amino acid sequence (Table 2). The DHFR coding sequence of ATCC 49619 is identical to that of CP1015 except that a leucine at position 16 in CP1015 is a valine in ATCC 49619.

Comparisons of sequences 72 nucleotides upstream of the DHFR coding region revealed that, with a few exceptions, the sequences of trimethoprim-resistant and -susceptible DHFRs were identical to that of ATCC 49619. The exceptions were as follows: in Pn93/720 and Pn93/1293, a guanine-to-adenine mutation was found 15 bp upstream from the start of the coding sequence, and in Pn93/1802 and R12, the nucleotide 31 bp upstream was guanine instead of adenine.

CP1015 transformants. The amplified DHFR coding sequences from all five trimethoprim-resistant isolates were used to transform the DHFR gene of *S. pneumoniae* CP1015. The DHFR genes of the resulting resistant transformants were sequenced to determine which of the mutations had been acquired. Transformation rates were generally around 10^{-5}.

The trimethoprim susceptibility of all transformants was within 1 dilution of that of the donor. While some transformants had acquired all of the variant amino acids of the donor, others had taken up only part of the donor sequence (Table 3), making it possible to compare the properties of DHFRs containing a range of different amino acid substitutions. All transformants contained the I100-L substitution and a D92-A or D92-S substitution; the D92-A mutation was also found in three of four trimethoprim-susceptible isolates. One transformant of R12 (T2) contained only the D92-A and I100-L mutations and required 128 mg of trimethoprim per liter for inhibition of growth, 1 dilution lower than that of the donor isolate.

Spontaneous mutation to trimethoprim resistance. On several occasions during transformation experiments, a small number of colonies appeared on trimethoprim selective media following incubation in the absence of donor PCR product (negative control). These spontaneous mutants were found to require 64 mg of trimethoprim per liter for inhibition, and the DHFR coding sequence was identical to that of CP1015 with the exception of an L31-F mutation.

DHFR kinetics. K_m values for DHF and NADPH were determined for the DHFRs of *S. pneumoniae* ATCC 49619, CP1015, and a selection of transformants (Table 3). DHF/NADPH K_m values for the ATCC 49619 and CP1015 DHFRs were similar, at 3.3/17.7 and 3.1/18 μM, respectively. The DHF and NADPH K_m values for *S. pneumoniae* ATCC 49619 are comparable to those reported by others: 2.83 and 8.31 μM, respectively (Hartman et al., 37th ICAAC). The DHFR of the transformant carrying only two mutations (R12/T2) had a K_m for DHF of 27.7 μM (8.9-fold higher than that of CP1015), and the K_m for NADPH was 35.1 μM (twofold higher than that of CP1015). For the transformed DHFR enzymes, K_m values ranged from 9.2 to 27.7 μM for DHF (2.9- to 8.9-fold higher than that of CP1015) and 20.3 to 50.6 μM for NADPH (1.1- to

TABLE 3. Amino acid sequence changes and DHFR characteristics of selected CP1015 transformants

Isolate	Amino acid at position[a]:																		MIC (mg/liter)	IC_{50} (μM)	K_m (μM)[b]		Specificity constant[c]
	14	16	20	26	53*	60	70	74	78	81	91	92	94	100*†	120	135	147	149			DHF	NADPH	
ATCC 49619		V																	4	0.05	3.9 (0.83)	17.8 (4.4)	0.024
CP1015	E	L	E	H	M	K	P	I	A	Q	Q	D	E	I	H	L	F	A	4	0.09	3.1 (0.48)	18.2 (3.2)	0.014
720/T1	D	V	D				S	L		H	H	A	D	L		F	S	T	512	2.9	9.2 (3.3)	30.7 (7.9)	0.09
720/T2		V	D				S	L		H	H	A	D	L					256	1.4	12.6 (3.3)	21.9 (5.2)	0.04
1802/T1		V	D	Y	I	Q	S		T			A		L	Q	F	S	T	512	13.7	14.3 (2.3)	29.0 (4.4)	0.026
1802/T2		V	D	Y	I	Q	S		T			A		L					512	10.1	24.1 (8.6)	52.7 (23.7)	0.007
R12/T1									T			A		L	Q	F	S	T	256	2.1	15.2 (2.8)	20.3 (4.7)	0.016
R12/T2												A		L					128	4.2	27.7 (9.7)	35.1 (11.4)	0.0063

[a] *, trimethoprim binding site in the DHFR of *E. coli* K-12; †, NADPH binding site in the DHFR of *E. coli* K-12 (as reported by Dale et al. [4, 5]).
[b] Values in parentheses are standard errors.
[c] V_{max}/K_m (μM).

2.8-fold higher than that of CP1015). K_m values for the DHFRs of transformants with additional mutations were in most cases lower than that of R12/T2. No DHFR activity was detected in CP1015 containing the spontaneous L31-F mutation.

Inhibition by trimethoprim. Trimethoprim IC_{50} values for the DHFRs of *S. pneumoniae* ATCC 49619 and CP1015 were 0.05 and 0.09 μM, respectively (Table 3), values which are comparable to the 0.15 and 0.17 μM reported for the DHFR of ATCC 49619 by others (1; Hartman et al., 37th ICAAC). Values for the IC_{50} of transformed DHFR enzymes ranged from 1.4 to 13.7 μM, 15- to 152-fold higher than that of the *S. pneumoniae* CP1015 recipient. The highest IC_{50} values were observed in transformants with multiple mutations in addition to I100-L.

DISCUSSION

Previous studies have demonstrated that the mutation I100-L is critical to the generation of trimethoprim resistance (1, 15; Hartman et al., 37th ICAAC). The results presented here are consistent with the requirement for this mutation but also demonstrate that further changes have a considerable impact on the function of the enzyme. In transformant R12/T2, which has acquired only I100-L and D92-A (D92-A is also present in many susceptible isolates), the MIC of trimethoprim is raised from 4 to 128 mg/liter and the trimethoprim IC_{50} is increased from 0.09 to 4.2 μM. However, the specificity constant (V_{max}/K_m), a measure of enzyme efficiency at low substrate concentrations, is decreased, and the affinity of the enzyme for its natural substrates is considerably reduced, with the K_m for DHF nearly ninefold higher, suggesting that trimethoprim resistance is gained at a cost to enzyme function. Kinetic properties of other transformants indicate that further mutations in the enzyme can partially compensate for this change (Table 3). For example, comparisons of 1802/T1 with 1802/T2 and R12/T1 with R12/T2 define one or more of the changes H120-Q, L135-F, F147-S, and A149-T as responsible for a decrease in the K_m for both DHF and NADPH, with a consequent increase in the specificity constant of the enzyme.

Comparisons of transformants 1802/T2 with R12/T2 and 1802/T1 with R12/T1 demonstrate that further mutations in the N-terminal half of the enzyme increase the concentration of trimethoprim required to inhibit the enzyme two- to sixfold (there is also an increase in MIC, although it is unclear whether this is significant). The two transformants, 1802/T1 and 1802/T2, in which mutations include M53-I, an additional site associated with trimethoprim binding in *Escherichia coli*, showed a substantial increase in the trimethoprim concentrations required for inhibition of DHFR. It can be postulated by comparison to 720/T1 and 720/T2 that one or more of the mutations at positions 26, 53, and 60 are significant to this change. Two further lines of evidence indicate that the mutation M53-I is likely to be important in trimethoprim resistance. Firstly, the amino acids corresponding to positions 53 and 100 have both been defined as trimethoprim binding sites in the *E. coli* K-12 DHFR (4, 5). Secondly, among the *S. pneumoniae* isolates studied by Hartman et al. (37th ICAAC), isolate 747, with mutation M53-I, was three- to fourfold-more resistant than isolates that contained a mutation only at position 26 or 60.

Recent investigations of the mechanism of trimethoprim resistance in *S. pneumoniae* have shown numerous different nucleotide and amino acid mutations in the DHFR genes of resistant isolates (1, 15; Hartman et al., 37th ICAAC). In the previous and present studies, there were 26 points at which amino acid substitutions were found to occur in the DHFR coding sequences of trimethoprim-resistant isolates, and of these, 12 were common to all four studies (Table 2). Overall levels of nucleotide divergence from the reference strain were similar in the studies, at between 6 and 7.5% for resistant isolates and under 1.5% for susceptible isolates. Like Adrian and Klugman (1), we found a single point mutation 15 bp upstream from the start of the open reading frame in two isolates, Pn94/720 and Pn93/1293. A second point mutation was observed 31 bp upstream in both Pn93/1802 and R12. The significance of these mutations is unknown; one transformant of Pn93/1802/T1 did contain the −31 mutation, but the MIC of trimethoprim was the same as that for the transformant lacking the mutation (Pn93/1802/T2).

Spontaneous mutants were obtained on DNA-free control plates in several transformation experiments; the L31-F mutation was found in all cases and resulted in an intermediate level of resistance (MIC, 64 mg/liter). Substitutions at L31 are not cited in the literature for the *S. pneumoniae* DHFR or for those of other, closely related organisms. However, this is a trimethoprim binding site for the DHFR of *E. coli* K-12 (4, 5). The substitutions F31-S and F31-W are also associated with the DHFRs of naturally occurring methotrexate-resistant mammalian cells (14). Since we were unable to isolate any detectable DHFR activity from the spontaneous L31-F mutant, it is possible that the resultant enzyme is relatively unstable in *S. pneumoniae*.

In conclusion, at least some of the additional mutations found in trimethoprim-resistant clinical isolates can further decrease the susceptibility to inhibition and/or partially compensate for the reduced affinity for DHF associated with I100-L. Given the range of different mutations observed among isolates reported here and in other studies, it is likely that there are alternative routes to achieving these changes.

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