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Adaptation to Sulfonamide Resistance in *Neisseria meningitidis* May Have Required Compensatory Changes To Retain Enzyme Function: Kinetic Analysis of Dihydropteroate Synthases from *N. meningitidis* Expressed in a Knockout Mutant of *Escherichia coli*

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Previously, the effects of three point mutations (at amino acid positions 31, 84, and 194) in the gene coding for a sulfonamide-resistant dihydropteroate synthase of *Neisseria meningitidis* were analyzed by site-directed mutagenesis. Changes at positions 31 and 194 abolished the phenotypic expression of sulfonamide resistance, while a change at position 84 appeared to be neutral. These studies are here extended to correlate the alterations in phenotype with effects on enzyme kinetics by expressing the cloned meningococcal genes in an *Escherichia coli* strain that had its *dhps* gene partially deleted and replaced by a resistance determinant. The most dramatic effects were produced by mutations at position 31. A change from the Leu found in the resistant isolate to a Phe (the residue found in sensitive isolates) led to a 10-fold decrease in the K_m and a concomitant drop in the K_i. Changes at position 194 also affected both the K_m and K_i but not to the same extent as mutations at position 31. Changing position 84 altered the K_m only slightly but significantly. This latter change was interpreted as a compensatory change modulating the function of the enzyme. In another type of resistance gene, 2 amino acid residues, proposed to be an insertion, were deleted, resulting in a sensitive enzyme. However, the resulting K_m was raised 10-fold, suggesting that compensatory changes have accumulated in this type of resistance determinant as well. This resistance gene differs by as much as 10% from the sensitive isolates, which makes identification of important mutations difficult.

The enzyme dihydropteroate synthase (DHPS) catalyzes the reaction between dihydropteridine pyrophosphate and *p*-aminobenzoate (PABA) as a part of the biosynthetic pathway leading to tetrahydrofolate (THF) (4, 23), which acts as a cofactor in the biosynthesis of purines, pyrimidines, and amino acids. Sulfonamides are structural analogs of PABA and act as antimetabolites by competing with PABA for the active site of DHPS (4).

The massive use of sulfonamides for both prevention and treatment of meningococcal disease (9, 19) led to the isolation of resistant strains of *Neisseria meningitidis* as early as 1937 (8), only a couple of years after the discovery of this class of drugs. Resistance has been shown to be mediated by altered forms of the chromosomal *dhps* gene (10, 21). Two types of resistant DHPSs have so far been identified by nucleotide sequence comparisons. One type can be considered to be encoded by a meningococcal wild-type gene that has mutated to mediate resistance, while the gene encoding the other type differs substantially (10%) from the corresponding genes in sulfonamide-susceptible strains of *N. meningitidis*. It was thus concluded that the latter type of resistance determinant has spread by horizontal transfer from another bacterium (10, 21). Both types of resistance determinants have been analyzed by site-directed mutagenesis, and differences from the wild-type enzymes responsible for resistance have been identified (10).

In order to determine that these changes lead to differences in the properties of the DHPS enzyme, determinations of K_m and K_i values in cell extracts were needed. One problem with this approach was the background activity of the *Escherichia coli* DHPS enzyme. To eliminate that problem, a mutant lacking DHPS activity was constructed. The lack of chromosomal background activity made it possible to compare the resistance determinants and the in vitro-mutated variants by measuring their activities directly from crude cell extracts.

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MATERIALS AND METHODS

Bacteria and media. The *E. coli* strains C600 (*thi-1 thr-1 leuB6 lacY1 tonA21 supE44*) and MM383 [*thyA polA12*(Ts) *rha lac*, streptomycin resistant] (17) were grown on LB medium (2), twofold-concentrated YT broth (18), and Iso-Sensitest medium (Oxoid Ltd., Basingstoke, United Kingdom) and supplemented with ampicillin (50 μg/ml) and kanamycin (40 μg/ml) when necessary. Growth media were supplemented with thymidine (200 μg/ml) when *E. coli* MM383 was grown. Transformations were done as described previously (25), except with *E. coli* MM383 and pCF100 (temperature-sensitive replicon), for which the growth temperature was 30°C and the heat shock step was done at 37°C.

DNA techniques. DNA was blotted to nitrocellulose membranes (Millipore) as previously described (25). The 0.33-kb *Bam*HI-*Pst*I fragment of the *dhps* gene of *E. coli* and the 1.25-kb kanamycin resistance (Km^r) gene cartridge (Pharmacia, Uppsala, Sweden) were used as probes. The entire *dhps* gene, PCR amplified with primers EC2 and EC3 as described previously (10), was used to probe the Kohara miniset phage collection (13), kindly provided by Carin Karlsson, Department of Microbiology, Uppsala University, Uppsala, Sweden. The probes were labeled with [α-^{32}P]dCTP from New England Nuclear by the random priming method. All nonradioactive ingredients and minicolumns were from Pharmacia. Restriction enzymes were from Boehringer, Mannheim, Germany. T4 DNA ligase was purchased from New England BioLabs, Inc., Beverly, Mass. The Wizard minipreparation procedure (Promega) was used for plasmid preparations. Standard procedures (25) were used for ligations, restriction enzyme digestions, and agarose gel electrophoresis. The *dhps* gene in the sulfonamide-resistant meningococcus 5M, isolated in Slovenia (14), was PCR amplified with primers NM1 and NM2 and DNA sequenced as described previously (10). The mutagenesis procedure of Vandeyar et al. (30) was followed as described before (10).

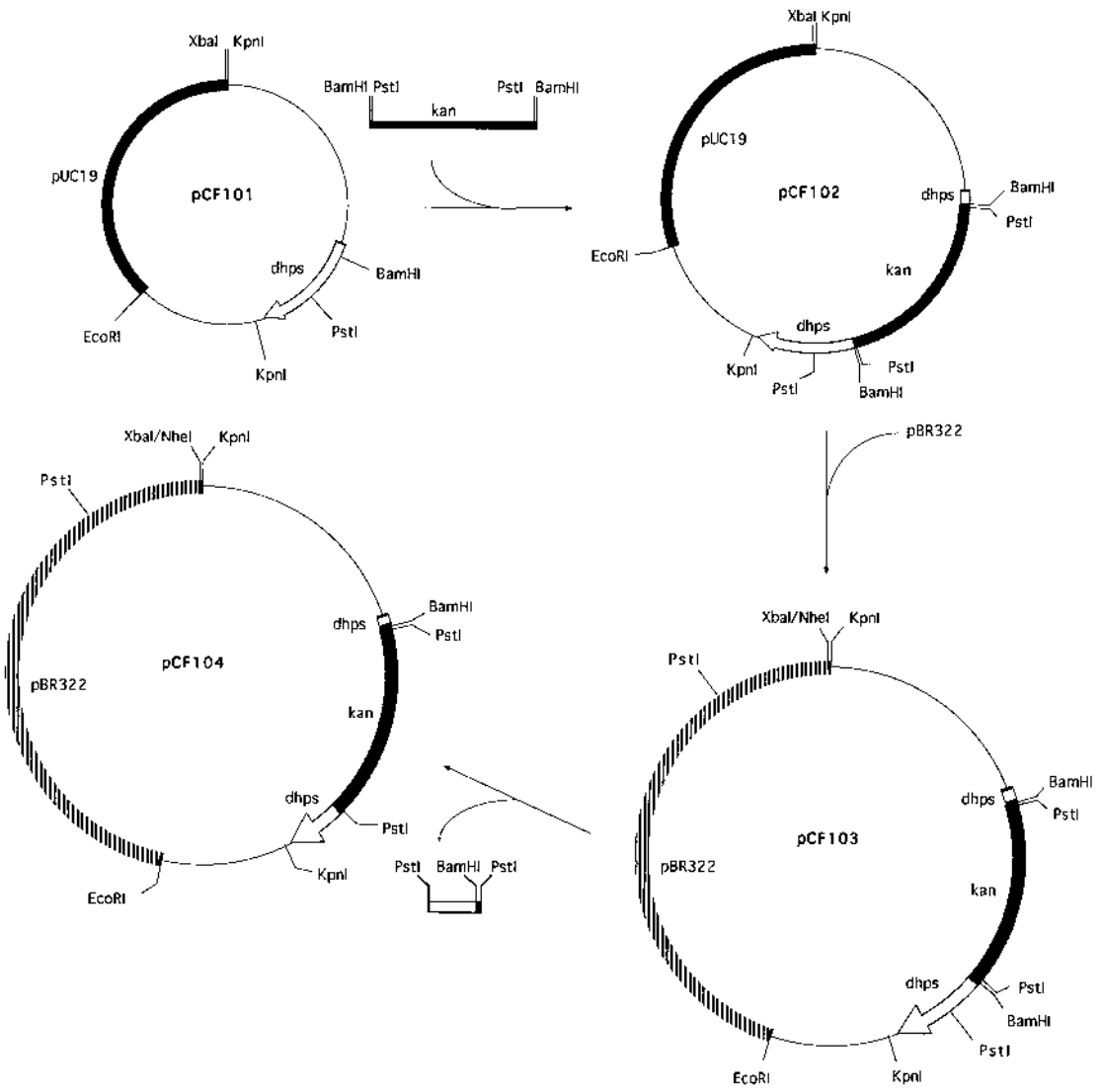

FIG. 1. Construction of plasmid pCF104 as described in detail in Materials and Methods.

Construction of plasmid pCF104. To obtain a part of the *E. coli* chromosome surrounding the *folP* gene of *E. coli*, the relevant clones in the Kohara clone library (13) were found by hybridization with a probe generated by PCR with primers EC2 and EC3 (10) and covering the entire *folP* gene. Two overlapping lambda phage clones, 18H7 (520) and 14F11 (521), were found to contain the region covering the *folP* gene. The *folP* gene was localized by hybridization to an *Eco*RI fragment of about 8 kb shared by both lambda clones (data not shown). This fragment was digested with *Kpn*I, and the 2.3-kb *Kpn*I fragment known to harbor the *E. coli folP* gene (6) was ligated, together with the adjoining 0.8-kb *Kpn*I-*Eco*RI fragment (5), into pUC19, forming pCF101 (Fig. 1). A kanamycin resistance gene cartridge (Pharmacia) was inserted in the *Bam*HI site in the 5′ portion of the coding region of the *folP* gene, generating plasmid pCF102 (Fig. 1). The entire insert in plasmid pCF102 was removed with the enzymes *Eco*RI and *Xba*I (in the pUC19 polylinker) and ligated into pBR322 digested with *Eco*RI and *Nhe*I (Fig. 1). The resulting plasmid, pCF103, was digested with *Pst*I and religated, to yield pCF104 (Fig. 1), which lacks 330 bp of the coding region, to ensure complete inactivation of the *folP* gene. Verification of all plasmid constructions was done by restriction enzyme analysis.

Replacement of the chromosomal *folP* gene with the inactivated variant of pCF104. Inactivation of the chromosomal *folP* gene was done essentially as described by Russel and Model (24). Plasmid pCF104 was introduced in *E. coli* MM383, which has a temperature-sensitive DNA polymerase I. Since pBR322 is dependent on DNA polymerase I activity for its replication, selecting for colonies that grow at 42°C on plates containing kanamycin makes it possible to obtain clones where the plasmid has integrated into the chromosome by homologous recombination (Fig. 2A). This recombination results in a duplication of the *folP* gene, where one copy is the wild type and the other carries the kanamycin resistance cassette and is thus inactivated. The resulting clone, designed from MM383::pCF104, expresses ampicillin resistance due to the presence of the *bla* gene of pBR322 (Fig. 2). Subsequent growth of cells with an integrated plasmid at 30°C allows segregation of the plasmid from the chromosome. It has been proposed that it is lethal for the cell to have a ColE1-type plasmid integrated into the chromosome and at the same time express DNA polymerase I activity, because this results in excessive initiation of replication of the bacterial chromosome from the plasmid origin (31). Segregation of the integrated plasmid results either in a plasmid with an inactivated *folP* gene or in a plasmid with the wild-type gene. Cells with a wild-type gene in the segregated plasmid carry an inactivated form of the *folP* allele on the chromosome (Fig. 2B). After growth at 30°C, the cells were lysogenized with P1 (cml clr, 100) by standard methods (16) and lysates were used to infect *E. coli* MM383 with or without pCF100 (Table 1), which is a temperature-sensitive plasmid construct of pSC101 with a functional *sul2* gene (22) that can supply DHPS activity to the cell. Selection with kanamycin at 30°C resulted in a number of recipients resistant to kanamycin but sensitive to ampicillin (Ap^s). Cells carrying the complementing plasmid pCF100 showed normal growth rates. However, a surprising result was that recipients without pCF100 could grow, albeit slowly, in rich media in the absence of an intact *folP* gene.

Determination of DHPS activity. Cells were grown in LB growth medium (2) to late logarithmic phase, and a cell extract was prepared by passage through a pressure cell (X-press) as previously described (29). Enzyme activity was measured by the incorporation of radioactivity from ^{14}C-labeled PABA into DHP. The enzyme assays were done in a total volume of 0.2 ml containing 10 mM

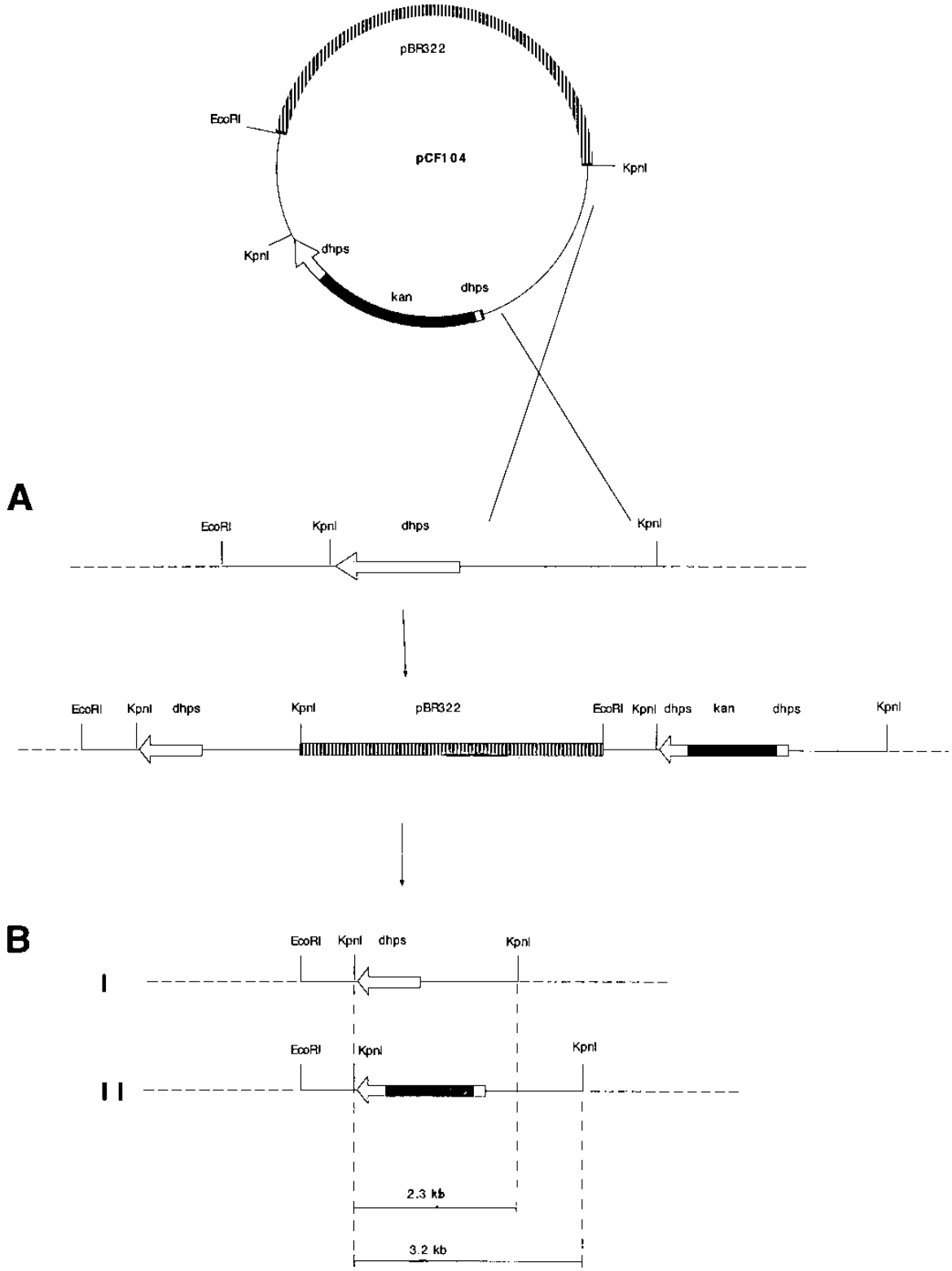

FIG. 2. Plasmid pCF104's integration into and excision from the chromosome of *E. coli* MM383 as described in detail in Materials and Methods.

Tris-HCl (pH 8.4), 5 mM dithiothreitol, and 5 mM $MgCl_2$ plus PABA in concentrations ranging from 0.5 to 25 μM, and the other substrate, dihydropteridin pyrophosphate, was added in excess to produce a pseudo-first-order reaction with respect to PABA. Sulfathiazole, when added, was used in concentrations ranging from 10 to 0.75 mM, depending on sulfonamide susceptibility. The pteridine substrate was reduced to its 7,8-dihydro form and phosphorylated by the methods of Blakley (3) and Shiota et al. (28). The enzyme extracts, depending on their clonal source, were diluted 2 to 120 times in 0.1 M phosphate buffer

TABLE 1. Enzyme kinetic parameters of DHPSs with single or multiple changes by site-directed mutagenesis[a]

Strain	Amino acid[b] at position:			Mean K_m ± SD (μM)	Mean K_i ± SD (μM)	MIC (mM STZ)[c]
	31	84	194			
3976	Leu	Ser	Cys	2.9 ± 0.4	218 ± 50	≥0.5
3976	***Phe***	Ser	Cys	0.5 ± 0.1	0.6 ± 0.2	<0.02
3976	Leu	***Pro***	Cys	6.9 ± 0.4	686 ± 75	≥0.5
3976	Leu	Ser	***Gly***	0.6 ± 0.2	5 ± 0.5	0.12
3976	***Phe***	***Pro***	Cys	0.3 ± 0.1	0.5 ± 0.1	
3976	***Phe***	Ser	***Gly***	0.4 ± 0.2	0.3 ± 0.1	
3976	Leu	***Pro***	***Gly***	1.4 ± 0.3	33 ± 9	
3976[d]	***Phe***	***Pro***	***Gly***	0.5 ± 0.1	0.4 ± 0.1	
5M	Leu	Pro	Gly	2.4 ± 0.1	15 ± 6	
5M[d]	***Phe***	Pro	Gly	0.4 ± 0.2	0.4 ± 0.1	
BT054	Phe	Pro	Gly	0.3 ± 0.1	0.1 ± 0.05	
BT490	Phe	Pro	Gly	5.4 ± 1.7	0.7 ± 0.2	

[a] The DHPS from the sulfonamide-resistant strain 3976 was changed by site-directed mutagenesis as shown here. All constructs were cloned in C600Δ*folP*::Km^r. K_m and K_i values for PABA and sulfathiazole were determined after placing the data in Lineweaver-Burk (15) and Eadie-Hofstee (7, 11) plots with the program CA-CricketGraph for the Macintosh.

[b] Amino acids in italic type are changes from those of the sulfonamide-resistant strain 3976 or from strain 5M.

[c] Results are from Fermér et al. (10). STZ, sulfathiazole.

[d] The configuration found in the susceptible enzymes of BT490 and BT054.

(pH 7.0)–0.01 M dithiothreitol. Reaction mixtures without dihydropteridin pyrophosphate were preincubated for 2 min at 37°C, and the enzymatic reactions were performed at the same temperature for 5 min after addition of the missing substrate. The DHPS activity was heat inactivated at 100°C for 2 min, and 100-μl samples of the mixtures were then spotted onto Whatman 3MM chromatography paper. The enzymatic product was separated chromatographically with 0.1 M phosphate buffer as described previously (23).

Determination of enzyme kinetic parameters. K_m and K_i values for PABA and sulfathiazole were determined after placing the data in Lineweaver-Burk (15) and Eadie-Hofstee (7, 11) plots with the program CA-CricketGraph (Computer Associates, San Diego, Calif.) for a Macintosh computer. Standard deviations were calculated from at least four independent K_m or K_i values. For the original resistance determinant of strain 3976 and the mutant variant changed at position 84, six measurements each were taken to verify the small but reproducible difference in K_m values (Table 1).

RESULTS

To be able to measure the activities of cloned *folP* analogs from *N. meningitidis* and study their effects on the host *E. coli* strain, a knockout mutant of *folP* was constructed as outlined in Materials and Methods. The same method has also been used to knock out the two genes coding for subsequent enzymes in the folic acid biosynthesis of *E. coli*, i.e., folylpolyglutamate synthetase-dihydrofolate (DHF) synthetase (*folC* gene [20]) and DHF reductase (DHFR) (*folA* gene [12]). With the inactivation of the *folC* gene, a complementing gene had to be present on a plasmid to allow growth of the knockout mutant even in rich growth media. The FolC product was thus found to be essential for growth of *E. coli*. It was therefore surprising that the transductant of *E. coli* MM383 lacking DHPS activity could indeed grow in the absence of complementing plasmid in rich media.

To make sure that this ability to grow was not peculiar to the thymidine-requiring strain MM383, a P1 lysate from this slow-growing mutant was used to infect *E. coli* C600, and again, slowly growing Km^r and Ap^s recipients were obtained. This mutant strain, called C600Δ*folP*::Km^r, was further analyzed in order to confirm the loss of DHPS activity. Three methods were used to provide evidence for gene inactivation in C600Δ*folP*::Km^r. First, PCR analysis was done with primers EC1 and EC2, which flank the *folP* coding sequence (10). The expected amplification products of about 1.8 kb, which corresponds to the insertion of a 1,250-bp kanamycin resistance gene and the deletion of 330 bp of the *folP* coding region (Fig. 1), were obtained from both the mutant strain and the plasmid used for the inactivation experiment, pCF104 (Fig. 1). Second, Southern blot analysis was done on chromosomal DNA digested with *Kpn*I (Fig. 3). The 330-bp *Bam*HI-*Pst*I fragment of the *folP* gene (Fig. 1) and the kanamycin resistance gene cartridge were used as probes. Figure 2B shows the sizes of the chromosomal *Kpn*I fragments carrying the wild-type or the mutated form of *folP* obtained after plasmid excision (Materials and Methods). The 330-bp *Bam*HI-*Pst*I fragment showed hybridization only to the 2.3-kb fragment of *E. coli* C600 (Fig. 3), and the kanamycin resistance determinant hybridized as expected only to the deletion mutant C600*folP*::Km^r (Fig. 3). Third, although growth was slow, it was possible to make cell extracts to show that the putative *folP* mutants lacked detectable levels of DHPS activity. These data clearly support the inactivation of the chromosomal *folP* gene in C600Δ*folP*::Km^r.

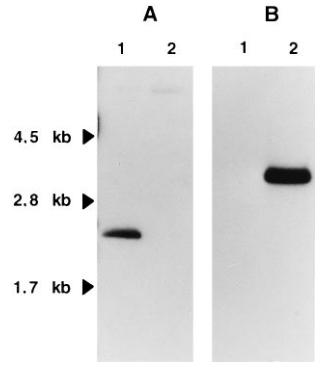

FIG. 3. Chromosomal DNA from *E. coli* C600 (1) and the deletion mutant C600Δ*folP*::Km^r (2) were digested with *Kpn*I and run on a 0.7% agarose gel. The restriction fragments were transferred to nitrocellulose membranes placed on both sides of the gel, resulting in two identical filters. The filter in panel A was probed with the 330-bp *Bam*HI-*Pst*I fragment from the *E. coli folP* gene. This fragment is deleted in the DHPS-negative mutant (Fig. 1 and 2), and it as expected hybridized only to *E. coli* C600 (lane 1). The filter in panel B was probed with the kanamycin resistance cartridge that had been inserted into the partially deleted *folP* gene of C600Δ*folP*::Km^r (Fig. 1 and 2), which hybridized only to a fragment of the expected size from this mutant (lane 2).

Analysis of in vitro-mutated DHPS enzymes by determinations of the kinetic parameters K_m and K_i. Two types of sulfonamide resistance determinants have been identified in *N. meningitidis* (10, 21). The *dhps* gene in strain 3976 is a type that could be considered a meningococcal gene altered by point mutations to express sulfonamide resistance. The sulfonamide-resistant DHPS enzyme in 3976 differs from the corresponding enzyme of susceptible isolates at three positions conserved in all known DHPSs, of which two (Leu-31 and Cys-194 [Fig. 4]) were shown by MIC determinations to affect resistance (10). The third (Ser-84) did not have any obvious effect on the resistance phenotype. Plasmid clones carrying the original 3976 gene and the three variants with individually changed amino acids were transferred to the deletion mutant C600Δ*folP*::Km^r, which led to normal growth in all cases, which means that each construct produces a functional DHPS.

Cell extracts were prepared for each clone, and DHPS activities were measured as described in Materials and Methods. The enzyme reactions exhibited pseudo-first-order kinetics, since the second substrate, dihydropteridine pyrophosphate, was added in excess, and the pseudo-first-order kinetics was confirmed when plotting the data in Lineweaver-Burk (15) and

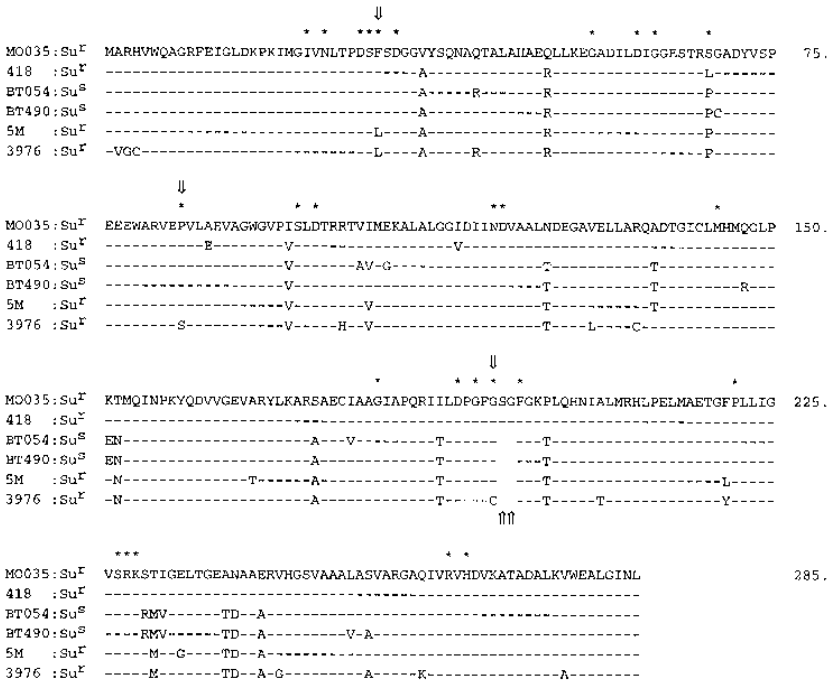

FIG. 4. Alignment of the polypeptides encoded by the two types of resistance genes and the *dhps* genes in the sulfonamide-susceptible (Su^s) meningococcal strains BT490 and BT054 (21). Hyphens indicate positions identical to those in the DHPS of MO035. Positions where the amino acid sequences of the other meningococci differ from that of MO035 are indicated. Amino acids conserved in all known DHPS polypeptides are indicated by asterisks. Positions changed by mutagenesis in the enzyme of strain 3976 are indicated with arrows pointing down, and positions deleted in the enzymes of strains 418 and MO035 are indicated with arrows pointing up. Su^r, sulfonamide resistance.

Eadie-Hofstee (7, 11) plots. K_m values for PABA were calculated directly from the plots, while K_i values were calculated from the apparent K_m values (Tables 1 and 2).

These data, shown in Table 1, correlate well with the earlier MIC determinations also included in Table 1. Just as the alteration at position 31 has the strongest effect on the MIC, it also produces the strongest effect on both the K_m and the K_i. The alteration at position 194 has a considerable effect on both enzyme kinetics and the MIC. The most pronounced effect of the alteration of Ser-84 in the resistance gene to Pro-84 found in the sensitive strain was an increase in the K_m value (Table 1). This difference in the K_m, although slight, was repeatedly found in a number of independent determinations. Ser-84 could therefore be seen as a mutation compensating for the two other mutations mediating sulfonamide resistance. To further evaluate the effect on mutations at the different positions, constructs were made to include two of the alterations in all possible combinations.

TABLE 2. Changes in enzyme kinetic parameters of DHPS after removal of two amino acids by site-directed mutagenesis[a]

Strain	Ser-Gly insertion	Mean K_m ± SD (μM)	Mean K_i ± SD (μM)	MIC (mM)[b]
418	+	3.8 ± 0.8	141 ± 13	≥0.5
418	−	33 ± 1.3	30 ± 13	<0.02
MO035	+	2.4 ± 0.3	120 ± 19	≥0.5
MO035	−	38 ± 6	12 ± 1	<0.02

[a] The DHPSs from the sulfonamide-resistant strains MO035 and 418 were changed by site-directed mutagenesis to remove the proposed Ser-Gly insertion. All constructs were cloned in C600Δ*folP*::Km^r. K_m and K_i values for PABA and sulfathiazole were determined after placing the data in Lineweaver-Burk (15) and Eadie-Hofstee (7, 11) plots with the program CA-CricketGraph for the Macintosh.

[b] Results are from Fermér et al. (10).

The enzyme kinetic determinations showed that a phenylalanine at position 31 has the most pronounced effect, regardless of which amino acid residues are present at the other positions, resulting in Michaelis-Menten constants of wild-type levels (Table 1). Cys-194 in the resistance determinant affects resistance only when it is in combination with Leu-31 (Table 1). Changing all three amino acids did not alter the result much in comparison with the result from changing two amino acids, except when Leu was at position 31, again stressing the importance of this individual change. Indeed, all constructs with Phe at position 31 have K_m and K_i values not significantly different from those of the DHPS from the sulfonamide-sensitive isolate BT054. Among other clinical isolates with sequences similar to that of strain 3976, isolate 5M, originally isolated in Slovenia (14), was the most relevant for this study. This isolate has Leu-31 but has wild-type amino acids at positions 84 and 194. The mutagenesis of the sulfonamide-resistant DHPS of 5M further confirms the importance of Phe-31. Mutagenesis of Leu-31 to Phe at this position in 5M alone resulted in a dramatic change from resistance to susceptibility in one step (Table 1).

The other type of sulfonamide resistance determinant, represented here by the *dhps* genes in MO035 and 418, has, in addition to substantial sequence divergence, two extra codons in a conserved section of the enzyme (Fig. 4), and their deletion results in sulfonamide-susceptible clones when the genes are transformed into *E. coli* DH5α (Table 2) (10). Their susceptibility is also in concordance with the decrease in the K_i values. However, the deletion does not result in the same drop in K_m values seen with the 3976 DHPS; instead, a 10-fold

increase in K_m values is obtained (Table 2). It thus seems unlikely that the insertion of the two extra codons is the only alteration leading to resistance. We have earlier proposed that Ser-195 and Gly-196 are the results of replication errors (10), and if this is true, compensatory mutations, as yet unidentified, have most likely accumulated in these enzymes.

One surprising result of our study was that the DHPS enzyme of the sulfonamide-susceptible meningococcus BT490 had a K_m value 10 times higher than the other sulfonamide-susceptible strain, BT054, and in the same range as the values of the resistant strains (Table 1). This may be a reflection of the natural variability of the genome of *N. meningitidis*, a variability evident in comparisons of the DNA sequences of different susceptible isolates (21). The higher K_m values of the resistant enzymes probably do not result in any great selective disadvantage, since the susceptible DHPS of BT490 has been maintained without any obvious advantages.

DISCUSSION

Sulfonamides act as competitive inhibitors of DHPS (4), an enzyme catalyzing the formation of dihydropteroic acid, a precursor to folic acid (23), in bacteria and in some lower eucaryotes. The presence of this enzyme only in microorganisms makes it a potential drug target for new classes of inhibitors. The genetic background of sulfonamide resistance in *Neisseria meningitidis*, selected for by the massive use of the drug both in the prevention and in the treatment of meningococcal disease (9, 19), has been analyzed by DNA sequencing (21) and by site-directed mutagenesis (10), and two types of resistance determinants have so far been identified. One, represented by strain 3976, shows high similarity to the *dhps* genes in susceptible isolates, and the other type, represented by MO035, diverges substantially (10%) at the nucleotide level and has been proposed to have been acquired by horizontal transfer from another bacterium.

Three highly conserved amino acid residues differ between the DHPSs of 3976 and susceptible isolates (Fig. 4), two of which (Leu-31 and Cys-194) influenced resistance (Table 1) (10). The third position, Pro-84, does not have any obvious effect on resistance, as judged from the MICs, but the K_m determinations showed that this divergence from the consensus results in a decrease in the K_m value. The construct with two alterations (Leu-31 and Cys-194) gives a higher K_m than the construct with all three mutations. The alteration Ser-84 could therefore be seen to compensate for the negative effects introduced by the two other alterations. The change of Leu-31 in a resistant isolate to Phe-31 resulted in a decrease in both the K_i and the K_m down to wild-type levels (Table 1). The other amino acid residue affecting resistance, Cys-194, does not mediate resistance alone but will in tandem with Leu-31 produce a highly resistant enzyme. These three alterations together, Leu-31, Ser-84, and Cys-194, result in a DHPS enzyme well adapted to the sulfonamide pressure that has acted on it for decades with a high K_i value for sulfathiazole and a relatively low K_m for PABA (Table 1). In fact, one sulfonamide-susceptible meningococcus, BT490, even had a K_m value slightly higher than those of the resistant strains (Tables 1 and 2). These data could be compared to the corresponding data obtained from a sulfonamide-resistant mutant of *E. coli* K-12 (6). The laboratory selection resulted in a DHPS enzyme with a K_i value comparable to that of 3976 but with a K_m value 300 times that of the wild type, compared to the sevenfold rise in the K_m of 3976. Again, the situation with the meningococcal isolate 5M parallels more closely those of the *E. coli* mutants, since in this isolate Phe-31 is the only alteration associated with sulfonamide resistance. In this situation the effects on the kinetic properties of the enzyme are not as dramatic as those of the *E. coli* mutants. It is too early to tell whether this means that the meningococcal DHPS in itself is more adaptable to the mutational changes or if there are also compensatory mutations in the 5M sequence, because any compensatory mutations could be hidden in the background of sequence divergence seen among different isolates of meningococci (21).

The gene encoding the other resistance determinant, represented here by the enzymes of MO035 and 418, not only diverges substantially from the corresponding genes in susceptible isolates but also contains an insertion of 6 bp, two codons, in the central and well-conserved part of *dhps* (10). Removal of these codons, considered the result of a replication error, leads to the loss of sulfonamide resistance (MICs in Table 2), but unlike that of the 3976 DHPS, the K_m value of the resulting enzyme is about 10 times that of the original resistant enzyme (Table 2) and about 80 times that of the wild type (Table 1). It is unlikely that the original *dhps* gene from which the resistance determinant evolved has such an elevated K_m. It is more probable that the resistance phenotype caused by the insertion of the two extra codons in a segment highly conserved among DHPSs was followed by a decrease in function and stability. However, the resistance determinant was nevertheless maintained in the meningococcal population as a result of the heavy selection pressure of sulfonamides, and it is reasonable to suggest that changes later accumulated to compensate for the 6-bp insertion in this class of sulfonamide resistance determinants.

The meningococcal isolates analyzed here may thus be live examples of evolutionary events, where an original mutation leading to resistance was selected by the earlier heavy use of sulfonamides. Later, compensating mutations modulated the effects on the enzyme properties to such an extent that today the disadvantage of carrying the resistance determinants is diminished, as we see here by the effects on the kinetic properties of the enzyme. A simulation of the evolution of a resistance gene has been recently presented by Schrag and Perrot (27), in whose work the effects of mutations leading to streptomycin resistance in *E. coli* were analyzed. In essence, the original streptomycin resistance led to considerably slower growth compared to that of normal wild-type *E. coli*. However, the appearance of faster-growing but still resistant variants led to the conclusion that other mutations could compensate for the deleterious effects of the original resistance mutation.

The *folP* gene in *E. coli* was knocked out by the method of Russel and Model (24) in order to facilitate analyses of cloned DHPS enzymes without any disturbance from the chromosomal enzyme. The same approach was used earlier in order to inactivate the two other *E. coli* genes involved in the biosynthesis of folic acid, *folC* (20) and *folA* (12). Deletion of *folA*, encoding DHFR, results in cells auxotrophic for all folate end products and with a doubling time increased by a factor of 2.5 in rich media. The DHFR deficiency requires that the cells not express any thymidylate synthase activity, and this may be explained by the accumulation of DHF and/or the depletion of tetrahydrofolate from one-carbon metabolism (12). However, the deletion of *folC*, which encodes an enzyme catalyzing two distinct activities, those of folylpolyglutamate synthetase and DHF synthetase, was feasible only if both activities were present in complement on a plasmid. It has not been determined which of the activities is the essential one (20). DHF synthetase adds L-glutamate to DHP, forming DHF, which is subsequently reduced to THF by DHFR. Besides the need for THF in the formation of a number of compounds of one-carbon metabolism, THF is needed for the initiation of protein

synthesis. However, Baumstark et al. stressed that protein synthesis could take place without formylmethionyl-tRNA (1) and that growth without DHF synthetase activity could therefore be expected on a rich medium. The nonviability of the *folC* mutant might therefore be explained by the lack of folylpolyglutamate synthetase activity. It is known that the polyglutamic forms of many folate derivates are the ones that are biologically active (26). This agrees with our results from the *E. coli* mutant lacking DHPS activity. This deletion mutant grows, although very poorly, on rich media.

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