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Genetic Characterization of Trimethoprim Resistance in *Haemophilus influenzae*

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We previously demonstrated that trimethoprim (Tmp) resistance in *Haemophilus influenzae* is mediated by chromosomally encoded dihydrofolate reductase (DHFR) with a modified primary structure and distinct kinetic properties. To gain insight into the relationship of the DHFR structure and the level of Tmp resistance that it confers on the host bacterium, we cloned and characterized the *folH* genes of one Tmp-susceptible and two Tmp-resistant *H. influenzae* strains. Differences were observed between Tmp-susceptible and Tmp-resistant isolates both in the promoter region and in the coding sequences. The effect of differences between *H. influenzae folH* genes on Tmp susceptibility was investigated in *Escherichia coli*. Various *folH* gene hybrids were constructed, and their influence on Tmp susceptibility was determined. Resistance in *E. coli* mediated by *folH* from *H. influenzae* strain R1047 was associated with alterations in the promoter and the central part of *folH*. In contrast, the *E. coli* Tmp resistance phenotype associated with the *folH* gene of *H. influenzae* R1042 was characterized by alterations in one or more of three amino acid residues at the C-terminal part of the protein. These data indicate that Tmp resistance is not only related to alterations in the promoter region of the *folH* gene and the Tmp binding domains at the N-terminal and central part of DHFR. Alterations in the C-terminal part may also cause Tmp resistance, probably as a result of a change in secondary structure and the subsequent loss of Tmp binding affinity.

Trimethoprim (Tmp), 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine, is a synthetic compound with antibacterial and antiparasitic activities. This drug interferes with folate metabolism by inhibiting the activity of dihydrofolate reductase (DHFR) (8, 22). Tmp is often used in combination with sulfamethoxazole to treat a wide variety of aerobic gram-positive and gram-negative infections (11). Tmp-resistant microorganisms were reported shortly after the introduction of Tmp in the 1960s. Several mechanisms for Tmp resistance have been described (for a review, see reference 9). The major cause of Tmp resistance is the production of an altered (plasmid-encoded) DHFR that lacks the capacity to bind Tmp (1, 4, 23–25). Overproduction of the normal (chromosomally encoded) DHFR is an additional mechanism of resistance (3, 7, 14, 23).

We previously demonstrated that Tmp resistance in *Haemophilus influenzae* is due to chromosomally mediated overproduction of the modified DHFR (5). However, the possibility of alterations in permeability barriers or efflux pumps could not be excluded. Subsequent purification of DHFR from Tmp-susceptible and Tmp-resistant *H. influenzae* isolates indicated that the mechanism of resistance was due to a Tmp-resistant DHFR with modified structural and kinetic properties (6).

In the study described in this report we investigated the effects of differences between *H. influenzae folH* genes on Tmp susceptibility in *Escherichia coli*. The *folH* genes of three *H. influenzae* strains displaying different levels of susceptibility to Tmp were cloned and characterized by DNA sequencing. We also analyzed the promoter region and the N-terminal part of the DHFRs from nine other genetically distinct *H. influenzae* isolates which varied in their Tmp susceptibilities. The effects of the various alterations in the *folH* gene on the Tmp susceptibility of the host were investigated in *E. coli* by mutagenesis experiments with *H. influenzae folH* gene hybrids.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The *H. influenzae* isolates used in the study are listed in Table 1. These strains were grown on *Haemophilus* test medium (HTM) agar plates (Oxoid) supplemented with HTM supplement SR158 (Oxoid) and thymidine phosphorylase (Sigma, Bornem, Belgium). *E. coli* DH5α (18), HB101 (18), and M1183 were also used in the study. Strain M1183 [*malT lda* (P_r;CI857) *malPQ endA thi hsdR*] was kindly provided by L. M. Schouls, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. The *E. coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) broth and LB agar (Difco Laboratories, Detroit, Mich.) unless stated otherwise.

Antibiotic susceptibility testing. The MIC of Tmp was determined by the agar dilution method as described previously (11a). The *E. coli* recombinants containing *H. influenzae folH* genes and gene hybrids (see below) were grown overnight at 37°C in LB broth containing 100 μg of ampicillin (Sigma) per ml (18). The recombinants (10^5 CFU in 10 μl) were spotted onto Iso-Sensitest agar plates (Oxoid, Unipath Ltd., Basingstoke, United Kingdom) containing Tmp at graded concentrations of 0.12 to 512 μg/ml (Sigma). The MIC was defined as the lowest concentration which completely inhibited visible growth in comparison with that on a Tmp-free agar plate. Susceptibility testing of all strains and recombinants was performed fourfold.

Cloning of the *folH* genes from various *H. influenzae* strains. The *folH* gene-containing DNA of *H. influenzae* R1047 present in recombinant pRGS7 (5, 6) was used to generate subclones in the M13 plasmids pTZ18 and pTZ19 (Sigma). Recombinant clone pRGS12 contained a 1.5-kb *Eco*RI-*Xba*I DNA insert. The DNA insert contained the entire *folH* gene because Tmp resistance was conferred to *E. coli* HB101 by this recombinant plasmid.

The *folH* genes from another nine Tmp-susceptible and -resistant *H. influenzae* strains from Spain were cloned. Chromosomal *H. influenzae* DNA was purified (18), and the *folH* genes were amplified by PCR. Two oligonucleotide primers (Applied Biosystems, Gouda, The Netherlands) were used for DNA amplification by PCR: 5′-GACGGATCCCAAGCCTGAATTAATTGGCTC (oligo-1;

TABLE 1. Description of *H. influenzae* isolates

Strain	Type	Geographic origin	Isolation site	Year of isolation	Tmp MIC (μg/ml)
R906[a]	nt[b]	NA[c]	Laboratory isolate		0.5
R1042[a]	nt	England	Sputum	1981	128
R1047[a]	nt	England	Eye	1981	32
1201[a]	b	Spain	Blood	1987	32
1209[a]	nt	Spain	Pleural fluid	1988	64
1217[a]	nt	Spain	Blood	1988	0.25
1226	nt	Spain	Ear	1988	32
1270	nt	Spain	Ear	1989	16
1276[a]	nt	Spain	Ear	1989	16
1288[a]	nt	Spain	Conjuctiva	1989	64
1289[a]	b	Spain	Pleural fluid	1989	32
1297[a]	nt	Spain	Sputum	1989	0.12
1303[a]	f	Spain	Cerebrospinal fluid	1989	16
1307[a]	f	Spain	Nasopharynx	1989	8

[a] *folH* genes were cloned and (partial) sequence analysis was performed.
[b] nt, nontypeable.
[c] NA, not applicable.

corresponding to a region upstream of the putative DHFR promoter with a *Bam*HI restriction site introduced at bases 4 to 9) and 5′-CTAGAATTCAGTT GCAGTTTTGCGTCATAAT (oligo-2; complementary to a region 3′ to the termination codon with an *Eco*RI restriction site introduced at bases 4 to 9). The reaction mixtures contained 50 ng of chromosomal DNA, 50 pmol of each oligonucleotide, and 1 U of *Taq* DNA polymerase in 100 μl of PCR buffer (Perkin-Elmer Cetus, Gouda, The Netherlands). PCR amplification was performed as described previously (17). Forty cycles of PCR (2 min at 95°C, 2 min at 56°C, and 2 min at 72°C) were performed, and polymerization was continued for a further 7 min at 72°C at the end of the last cycle. PCR products were analyzed by agarose gel electrophoresis. The amplified DNA was digested with *Xba*I and *Eco*RI, cloned into pTZ18 and pTZ19, and transformed into *E. coli* HB101. The DNA cloning procedures were performed as described by Sambrook et al. (18).

DNA sequencing. Nucleotide sequence analysis of the *folH* genes of the *H. influenzae* strains was performed by the dideoxy nucleotide chain termination method (19). T7 DNA polymerase (Promega, Leiden, The Netherlands) was used in combination with the Sequenase kit of U.S. Biochemicals (Cleveland, Ohio). DNA sequencing with double-stranded plasmid templates was performed as recommended by the manufacturer. *Taq* DNA polymerase (Promega) was used to overcome band compressions. Universal M13 primers (17-mer) were used to initiate the sequence analysis. On the basis of the DNA sequence information, internal primers (20-mer) were synthesized (Applied Biosystems) to proceed with sequencing. Comparisons of different nucleotide and amino acid sequences were performed with the FastA program (13).

DHFR domain exchange experiments. The *Eco*RI-*Xba*I restriction fragments containing the *folH* genes of *H. influenzae* R1047 (*folH1*), R906 (*folH2*), and R1042 (*folH3*) were cloned into the M13 plasmid pTZ18 and plasmid pPLc245, containing the heat-inducible p_L promoter (15). The pTZ18 and the pPLc245 recombinant were transformed into *E. coli* DH5α and M1183. Induction of the p_L promoter was performed at 42°C (18). The restriction sites *Eco*RI, *Sty*I, *Mun*I, and *Xba*I were used to construct gene hybrids by exchanging *Eco*RI-*Sty*I, *Sty*I-*Mun*I, or *Mun*I-*Xba*I *folH* gene-containing fragments. DNA cloning was carried out as described by Sambrook et al. (18). The gene hybrids were checked by restriction fragment length polymorphism analysis. For this purpose, the restriction enzymes *Alu*I, *Vsp*I, and *Mbo*I were used.

Genetic typing of the *H. influenzae* strains. To verify that the Tmp-resistant *H. influenzae* strains isolated in different geographic areas of Spain were not clonal, we characterized the strains by pulsed-field gel electrophoresis as described previously (12).

Nucleotide sequence accession numbers. The nucleotide sequences of the DHFR genes and the flanking regions of strains R1047 (*folH1*), R906 (*folH2*), and R1042 (*folH3*) are present in the EMBL, GenBank, and DDBJ databases under the accession numbers X84205, X84206, and X84207, respectively.

RESULTS

Genetic characterization of the *folH* genes from *H. influenzae* R1047, R1042, and R906. The 1.5-kb *Eco*RI-*Xba*I insert present in recombinant plasmid pRGS12 was sequenced. The first 383 nucleotides 5′ of the *Eco*RI site were identical to the pGEM3 sequence. The remaining 1,144-bp sequence contained an open reading frame (Fig. 1). Within this open reading frame, a protein-coding sequence started at nucleotide 634 and ended at nucleotide 1113. We designated this gene *folH1*, encoding the DHFR of *H. influenzae* R1047, for which the Tmp MIC is 32 μg/ml. The start codon is preceded by a potential ribosomal binding site (AGGG) similar to that of *E. coli* and by motifs with a high degree of homology to the canonical *E. coli* −10 (TATAAT) and −35 (TTGACA) promoters (Fig. 1) (20).

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GATCACTATCAAATAAACCTTGTTGATCAGTTAATAAATAAAGTTGTTCCGCTTGAACAA    60
GAATAGCGACTAATGCAGATAAATTGTCATTATCGCCAACTTTAATTTCTGCGGTTGCCA   120
CCGCATCATTTTCATTAATCACAGGAATAATATGGTTATCTAAAAGTGCATGTAAAGTAT   180
CTCGCGCATTTAAAAAACGTTCGCGATCTTCAATATCAGCACGAGTTAATAAAAGTTGTC   240
CGATGTGAATATCATAAATAGCAAATAATTTTTCCCAAGCCTGAATTAATTGGCTCTGAC   300
CAACTGCTGCAAGCAGTTGCTTTGAAGCAATAGTGGGTGGTAATTGAGGATGATTTAAAT   360
AATGGCGACCCGCAGCAATGGCACCAGAAGTCACGATCACTATACGAAATCCATCATTAT   420
GCAGTTGTGCAATTTGACGAACGATTTCCATCATGTGCGGTGAATTTAGTTTTGGTGAAC   480
CCTGTGTTAGAGTACTTGTACCAAATTTCACTACGATTGTTTTCTTGTTCATAGTCGTTT   540
CCAAAGTTCTTAAAATTGACATTAACGTTATCACTAAATCAATGACAAATCACTTAATAT   600
CAGGTATAGTAACGGCAAATTTTAGGGGGACTTATGACATTTAGTTTAATTGTAGCGACG   660
                                 M  T  F  S  L  I  V  A  T
ACATTAAATAGTGTAATTGGTAAAGATAACCAAATTCCTTGGCACTTGCCTGCAGATTTA   720
 T  L  N  S  V  I  G  K  D  N  Q  I  P  W  H  L  P  A  D  L
GCTTGGTTTCGTCAGAACACCACTGGTAAACCTGTCATTATGGGGCGTAAAACCTTTGAA   780
 A  W  F  R  Q  N  T  T  G  K  P  V  I  M  G  R  K  T  F  E
AGTATTGGTCGTGCACTACCTAAACGTACCAATATCGTACTTTCTCGCCAGCTTTTTGAA   840
 S  I  G  R  A  L  P  K  R  T  N  I  V  L  S  R  Q  L  F  E
CACGAAGGTGTGATATGGAAAGATAGCTTTGAAAGTGCGGTCAATTTTGTCAGAGATTTT   900
 H  E  G  V  I  W  K  D  S  F  E  S  A  V  N  F  V  R  D  F
GATGAAATTATGTTGCTTGGTGGGGGAGAGTTATTCAAACAATATTTACCCAAAGCAGAT   960
 D  E  I  M  L  L  G  G  G  E  L  F  K  Q  Y  L  P  K  A  D
AAGTTATACCTTACTCAAATTCAAACAGAACTAGATGGTGATACTTTTTTCCCTCAATTG  1020
 K  L  Y  L  T  Q  I  Q  T  E  L  D  G  D  T  F  F  P  Q  L
AATTGGGAGGAGTGGGAAATTGAATTTGATGAATATCGTAAGGCGGATGAACAAAATCGC  1080
 N  W  E  E  W  E  I  E  F  D  E  Y  R  K  A  D  E  Q  N  R
TATGATTGCCGATTTTTAATTCTTACCCGAAAATAAATCAAATAAATGAAACAATACTTC  1140
 Y  D  C  R  F  L  I  L  T  R  K
TAGA 1144
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FIG. 1. Nucleotide sequence of the *folH* gene of *H. influenzae* R1047. The amino acid sequence of DHFR is depicted. The −35 and −10 promoter regions are underlined.

The PCR-amplified *folH* genes and flanking DNA sequences of *H. influenzae* R906 and R1042 were also analyzed by DNA sequencing. Computer analysis of the sequence revealed a DHFR-coding sequence of 480 bp. We designated the gene of Tmp-susceptible strain R906 *folH2*, while we identified that of the high-level Tmp-resistant strain R1042 (MIC, 128 μg/ml) *folH3*. Comparison of the *folH* genes of *H. influenzae* R1047, R906, and R1042 indicated that they had approximately 95% homology at the nucleotide level. The GC contents were 37.0% for *folH1* (strain R1047), 36.8% for *folH2* (strain R906), and 37.0% for *folH3* (strain R1042). The GC content of each of the three *folH* genes and flanking sequences was approximately 35%. The derived amino acid sequences of the *folH* genes were >91% identical.

Genetic relatedness of the Spanish *H. influenzae* isolates. Before examining parts of the *folH* nucleotide sequences of the Tmp-resistant Spanish isolates, we investigated the genetic heterogeneity of the strains. Only two of the strains, strains 1201 and 1289, had an identical pulsed-field pattern after digestion with *Sma*I (Fig. 2). Both of these strains were type b. The pattern seen with strain 1217 was nearly identical to that observed with the type b strains, except that the lowest-molecular-weight band was a doublet. The overall high degree of

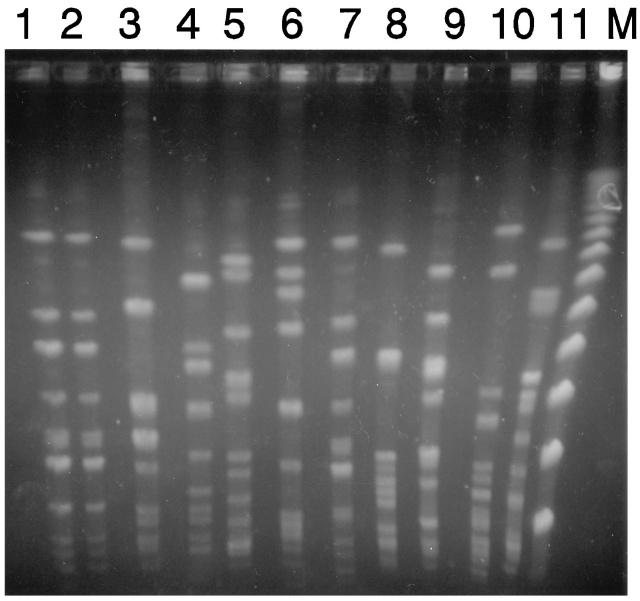

FIG. 2. Pulsed-field gel electrophoresis of *Sma*I-digested DNA from *H. influenzae* strains isolated in Spain. The characteristics of the strains are summarized in Table 1. Lanes: 1, 1201; 2, 1289; 3, 1307; 4, 1303; 5, 1288; 6, 1209; 7, 1217; 8, 1226; 9, 1270; 10, 1276; 11, 1297; M, molecular size markers consisting of bacteriophage λ concatemers.

DNA polymorphism among the Spanish isolates indicates that these isolates are genetically heterogeneous strains and therefore are epidemiologically nonrelated.

Characterization of the *folH* gene sequences from various Spanish *H. influenzae* isolates. Since the first 70 amino acids of DHFR are thought to be involved in Tmp binding (16), we analyzed the *folH* genes of nine additional *H. influenzae* strains that vary in their levels of Tmp susceptibility. The Tmp MICs for these strains ranged from 0.12 to 64 μg/ml. The deduced DHFR amino acid sequences did not display a consistent substitution in regions that are thought to be involved in Tmp binding (Fig. 3). An N→ S substitution at position 13 was the only consistent substitution in the DHFR of Tmp-resistant *H. influenzae* strains in comparison with the amino acid sequences

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strain  MIC          DNA sequence upstream of folH
        μg/ml

                -35                  -10
                ____                 ____
R1047    32  ATGACAAATCACTTAATATCGGTATAGTAACGGCAAATTTTAGGGGGACTT
R1042   128  ....A.....................................A..
1209     64  ..........................................
1288     64  ....A.....................................A..
1201     32  T...A.....C..................T.............A..
1289     32  ....A.....................................A..
1276     16  ..........................................
1303     16  ..........................................
1307      8  ..........................................

R906   0.50  ....A.....................................A..
1217   0.25  ....A.....................................A..
1297   0.12  ....A.....................................A..
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FIG. 4. Nucleotide sequence of the promoter region of *folH* genes of Tmp-susceptible and -resistant *H. influenzae* strains. The Tmp MICs are depicted.

of the Tmp-susceptible strains. In general, as the Tmp susceptibility of the strain decreased, the total number of amino acid differences in comparison with the amino acid sequences of Tmp-susceptible strains increased.

Since overproduction of the wild-type DHFR is a mechanism of Tmp resistance in *E. coli*, which is associated with mutations in the promoter region (3, 7, 23), we determined the nucleotide sequence upstream of the putative *folH* start codon (Fig. 4). This region in strain R1042, which appeared to overproduce a modified DHFR sixfold compared with the level of production by strain R906 (6), was identical to those in the three Tmp-susceptible strains (strains R906, 1217, and 1297) and to those in two Tmp-resistant strains (strains 1288 and 1289) (Fig. 4). These data indicate that overproduction of DHFR in *H. influenzae* R1042 compared with the level of production in strain R906 cannot be explained by differences in the promoter region.

DHFR production by *H. influenzae* R1047 was demonstrated to be 11.5 times greater than that by R906 (6). The DNA sequence containing the promoter region demonstrated two nucleotide differences compared with the nucleotide sequence of strain R906. Furthermore, the sequence was identical to those of four Spanish isolates that were invariably resistant to Tmp. These data suggest that the promoter sequence of R1047 may be responsible for DHFR overproduction and the subsequent Tmp resistance phenotype of the strain.

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strain    MIC          N-Terminal first 90 amino acids of DHFR
          (μg/ml)

                    1         2         3         4         5         6         7         8         9
          123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890

R1047      32   MTFSLIVATTLNSVIGKDNQIPWHLPADLAWFRQNTTGKPVIMGRKTFESIGRALPKRTNIVLSRQLFEHEGVIWKDSFESAVNFVRDFD
R1042     128   ..................M.............................P.............P.K....V..N.L...........
1209       64   ..................M.............................P..............................
1288       64   ..................M.............................P..............D..V....L...........
1201       32   ..................M.............................P.............P......V......R..D......
1289       32   ..................M.............................P.............P......V....L.R.........
1276       16   ..................M.............................P..............................D......
1303       16   ..................M.............................P..............................
1307        8   ..................M.............................P..............................

R906     0.50   ............N.....M.............................P.A............................
1217     0.25   ............N.....M.............................P..................V....L..........
1297     0.12   ............N.....M.............................P..................V..G.L..........


                    1         1         1         1         1         1         1
                    0         1         2         3         4         5         6
          123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890

R1047      32   EIMLLGGGELFKQYLPKADKLYLTQIQTELDGDTFFPQLNWEEWEIEFDEYRKADEQNRYDCRFLILTRK
R1042     128   ....I.....................................K......H...........S......

R906     0.50   ....I.....................................................................
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FIG. 3. Comparison of the N-terminal 90 amino acids of the deduced DHFRs of *H. influenzae* strains with various Tmp susceptibilities. The entire DHFR sequences of strains R1047 (*folH1*), R906 (*folH2*), and R1042 (*folH3*) are depicted. The Tmp MICs are also depicted.

Identification of the *folH* gene differences responsible for Tmp resistance in *H. influenzae*. The effects of the various *folH* gene alterations on the Tmp susceptibility or resistance phenotype were investigated. The MICs for three *E. coli* recombinants containing the *folH* genes of *H. influenzae* R1047 (*folH1*), R906 (*folH2*), and R1042 (*folH3*), respectively, were determined. The presence of the *folH* genes in *E. coli* invariably gave an increase in resistance to Tmp in comparison with the resistance of the wild-type strain (Fig. 5A). Furthermore, the measured MICs for the *E. coli* recombinants were significantly higher than those for the corresponding *H. influenzae* strains. This may be explained by the presence of the *folH* genes on a multicopy plasmid, causing an increase in the level of expression in *E. coli*. The association between the level of DHFR expression and the level of Tmp resistance demonstrated previously (3, 5, 7, 14, 23) was confirmed, because the presence of the heat-inducible p_L promoter influenced the level of Tmp resistance. An increase in the level of *folH2* and *folH3* gene expression by heat induction of p_L resulted in a twofold increase in the level of Tmp resistance of the recombinants. Additionally, the p_L promoter did not affect the level of expression of the *folH1* gene, presumably because of the greater distance between p_L and the structural gene (633 bp) in comparison with the distance in the homologs *folH2* and *folH3* (171 bp). Alterations in the structural *folH* genes included differences in 5 amino acid residues between strains R906 and R1047 and differences in 10 amino acid residues between strains R906 and R1042 (Fig. 6). Comparison of the three DHFR proteins demonstrated only two conserved amino acid substitutions, present in *folH2* and *folH3* at positions 74 (I↔V) and 79 (F↔L), respectively, whereas all other substitutions resulted in a change in the net protein charge (Fig. 6). In order to investigate the influence of the various alterations in the structural *folH* gene and its promoter sequence, we constructed various *folH* hybrid genes in *E. coli* and determined the MICs. The restriction enzymes *Eco*RI, *Xba*I, *Sty*I, and *Mun*I were applied to exchange *Sty*I-*Xba*I, *Sty*I-*Mun*I, and *Mun*I-*Xba*I fragments between the different *folH* genes (Fig. 6). The gene hybrids were verified by restriction fragment length polymorphism analysis. For this purpose, the restriction enzymes *Alu*I, *Vsp*I, and *Mbo*I were used. *Alu*I was able to discriminate the internal *Sty*I-*Mun*I fragments, and *Vsp*I and *Mbo*I sites were exclusively present in the *Mun*I-*Xba*I fragments of *folH2* and *folH3*, respectively. Exchange of the *Sty*I-*Xba*I fragments clearly affected the level of Tmp resistance of *folH2* and *folH3* (Fig. 5B). Since Tmp resistance was entirely transferred after *Sty*I-*Xba*I exchange, we conclude that at least one determinant(s) involved in this type of Tmp resistance is located downstream of the *Sty*I restriction site of *folH3*.

To identify the domain(s) responsible for the Tmp resistance of *folH3* strains, the *Sty*I-*Mun*I and *Mun*I-*Xba*I fragments of *folH2* and *folH3* were exchanged. The results are presented in Fig. 5C and D. Only the alterations present in the C-terminal part of *folH3* DHFR were involved in the resistance phenotype, indicating that the three amino acid substitutions at positions 135 (E↔K), 142 (R↔H), and 154 (F↔V) in the DHFR of *H. influenzae* R1042 are responsible for Tmp resistance.

The Tmp resistance phenotypes of the *folH1*-containing strains were also investigated by domain substitution experiments. Transfer of the *Sty*I-*Mun*I fragments of *folH1* to *folH2* resulted in an increase in Tmp resistance (MIC, 8 to 128 μg/ml), whereas the *folH1* recombinant containing the *Sty*I-*Mun*I domain of *folH2* showed a decrease in resistance (MIC, 512 to 64 μg/ml) (Fig. 5C). Since the alterations present in *folH1* strains, which led to three amino acid substitutions at positions 54 (P↔A), 56 (A↔P), and 95 (I↔L), are all located

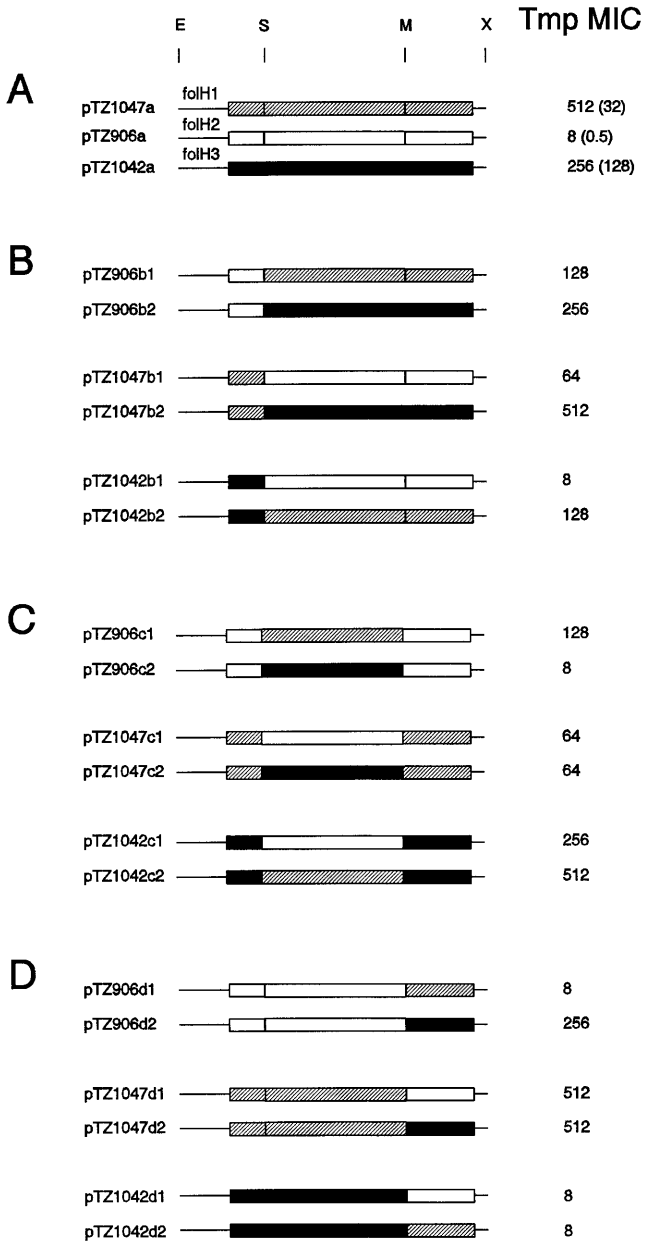

FIG. 5. Tmp MICs of *H. influenzae* R906, R1047, and R1042 and the various *E. coli* recombinants containing recombinant *folH* genes and hybrids constructed by DHFR domain exchange mutagenesis. All recombinant plasmids were constructed in pTZ18. The *folH1*, *folH2*, and *folH3* gene(s) and gene parts are depicted by shaded, open, and black bars, respectively. The restriction sites *Eco*RI (E), *Sty*I (S), *Mun*I (M), and *Xba*I (X) are depicted. Numbers in parentheses are the Tmp MICs (in micrograms per milliliter) for the *H. influenzae* strains. MIC determinations were done fourfold. The MICs were repeatedly similar. The MICs for the *E. coli* parent strains DH5α and M1183 were <0.5 μg/ml.

at the *Sty*I-*Mun*I part of the gene, we conclude that the central domain of DHFR encoded by *folH1* is responsible for Tmp resistance. In addition, exchange of the *Sty*I-*Mun*I fragments between *folH1* and *folH3* strains also demonstrated the presence of Tmp resistance determinants in the central domain of *folH1*. Transfer of the *Sty*I-*Mun*I fragments of *folH1* to *folH3* strains resulted in a twofold increase in Tmp resistance (MIC, 256 to 512 μg/ml), whereas the *folH1* recombinant containing

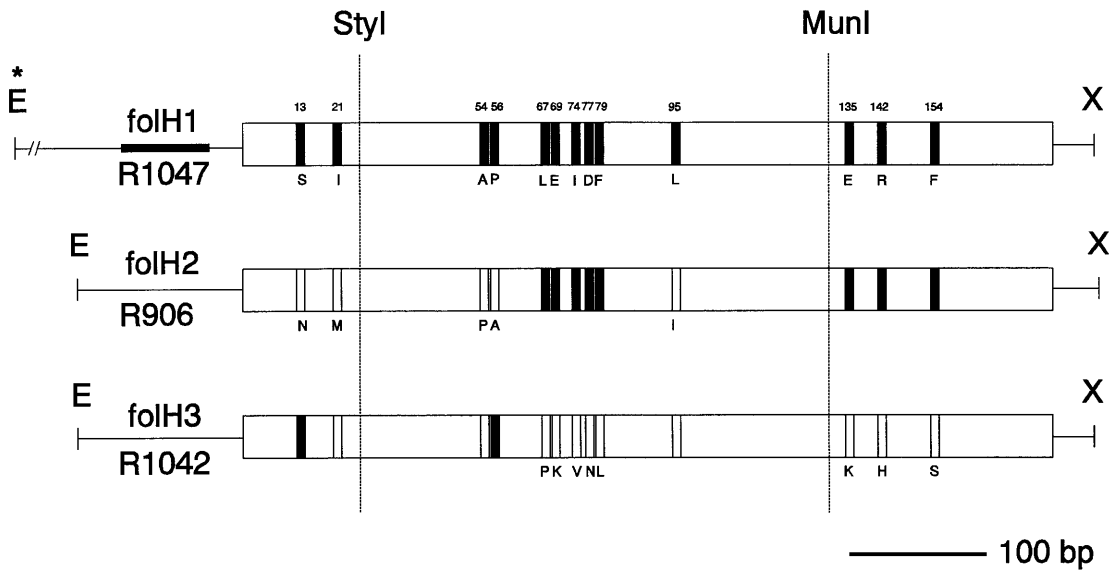

FIG. 6. Physical map of the *Eco*RI (E)-*Xba*I (X) restriction fragments containing the *folH* genes of *H. influenzae* R906, R1047, and R1042. The amino acid differences and their positions are depicted. The black bar in the promoter region of *folH1* represents 14-bp differences in comparison with the homologous DNAs in *folH2* and *folH3*. *, *Eco*RI restriction site present in the plasmid vector pTZ18.

the *Sty*I-*Mun*I domain of *folH2* showed a decrease in resistance (MIC, 512 to 64 μg/ml) (Fig. 5C). Thus, exchange of the central domain of *folH1* and *folH3* clearly indicated the importance of this domain in the Tmp resistance of the *folH1* recombinant.

To investigate the involvement of the R1047 promoter in Tmp resistance, the *Eco*RI-*Sty*I fragments of the *folH* genes, containing the promoter region and the 5′ part of the gene, were exchanged (Fig. 5B). Substitution of the *Eco*RI-*Sty*I fragment of *folH1* to *folH2* resulted in an increase in Tmp resistance (MIC, 8 to 64 μg/ml). On the other hand, substitution of the *Eco*RI-*Sty*I fragment of *folH1* by *folH2* resulted in a decrease in Tmp resistance (MIC, 512 to 128 μg/ml). Since two amino acid substitutions are present at positions 13 (N↔S) and 21 (M↔I), we conclude that the promoter or the substitutions at positions 13 and 21, or both the promoter and the substitutions are involved in the Tmp resistance of the *folH1* recombinant.

DISCUSSION

In the present study, the *folH* genes and flanking sequences of three *H. influenzae* strains that vary in their levels of Tmp resistance were characterized. The base compositions of the *folH2* gene of the Tmp-susceptible strain R906 and the *folH1* and *folH3* genes of Tmp-resistant strains R1047 and R1042, respectively, were similar to that of the *H. influenzae* chromosome: G+C contents of 37.0% (*folH1*), 36.8% (*folH2*), and 37.0% (*folH3*), in comparison with 39% for the entire chromosome (10). This observation suggests that Tmp resistance in *H. influenzae* is due to alterations that occur in the species-specific *folH* genes rather than the horizontal transfer of resistance genes from other bacterial species.

Comparison of the translated products of the three *folH* genes revealed more than 95% sequence identity. Computer analysis of the deduced DHFR amino acid sequences demonstrated significant homology with other prokaryotic and eukaryotic DHFRs. The DHFR protein of *H. influenzae* R1047 encoded by *folH1* had 48% identity and 51.3% similarity in an overlap of 158 amino acids with the DHFR of *E. coli* K-12 (21). The homologies of the deduced protein with other prokaryotic Tmp-resistant and -susceptible DHFRs varied between 34.9 and 49.1%. Homology with eukaryotic DHFRs was significantly less and was mainly restricted to the first 68 amino acids.

Since overproduction of the wild-type DHFR is a mechanism of Tmp resistance in *E. coli*, which is associated with mutations in the promoter region, the 52-bp nucleotide sequence upstream of the putative *folH* start codon was determined in various *H. influenzae* strains. Since this region in the Tmp-resistant strain R1042, which appeared to overproduce a modified DHFR by sixfold compared with the level of production by Tmp-susceptible strain R906 (6), was identical to the *folH* promoter region of strain R906, we conclude that overproduction of DHFR is presumably caused by unknown regulatory mechanisms affecting the expression of DHFR.

The DHFR production of *H. influenzae* R1047 was demonstrated to be 11.5 times greater than that of strain R906 (6). We observed two nucleotide differences among the *folH* promoter regions of these strains. Furthermore, the R1047 *folH* promoter sequence was identical to the homologous sequences in four Spanish isolates that were invariably resistant to Tmp. These data suggest that the promoter sequence of R1047 may be (partly) responsible for DHFR overproduction and the subsequent Tmp resistance phenotype of the strain.

The effects of the various *H. influenzae folH* gene alterations on the Tmp susceptibility or resistance phenotype were investigated in *E. coli*. Various *folH* gene hybrids were constructed, and the influence on Tmp susceptibility was determined. We demonstrated that one or more of three amino acid substitutions in the C-terminal part of *folH3* (strain R1042; MIC, 128 μg/ml) were associated with the expression of Tmp resistance in *E. coli*. The Tmp resistance of *folH1* (strain R1047; MIC, 32 μg/ml) was associated with amino acid substitutions in the central part of DHFR. In addition, promoter activity or amino acid substitutions in the N-terminal part of *folH1*, or both, were also involved in Tmp resistance. Interestingly, the binding domains of inhibitory components such as Tmp are present at the N-terminal and central parts of the protein (2). The C-terminal part of DHFR has never been shown to harbor binding do-

mains. A possible explanation for the importance of alterations in the C-terminal part of DHFR may be the contributional effects of this region on the maintenance of the secondary structure of the enzyme. Alterations in this region may lead to a change in structure, which causes a loss of or a decrease in the level of Tmp binding and subsequently results in a Tmp resistance phenotype.

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