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Characterization of the Gene for the Chromosomal Dihydrofolate Reductase (DHFR) of *Staphylococcus epidermidis* ATCC 14990: the Origin of the Trimethoprim-Resistant S1 DHFR from *Staphylococcus aureus*?

GLENN E. DALE, CLEMENS BROGER, PETER G. HARTMAN, HANNO LANGEN, MALCOLM G. P. PAGE, RUDOLF L. THEN, AND DIETRICH STÜBER*

Departments of Gene Technologies, Infectious Diseases, and Computational and Structural Chemistry, Pharmaceutical Research, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland

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The gene for the chromosomally encoded dihydrofolate reductase (DHFR) of *Staphylococcus epidermidis* ATCC 14990 has been cloned and characterized. The structural gene encodes a polypeptide of 161 amino acid residues with a calculated molecular weight of 18,417. This trimethoprim-sensitive (Tmp^s) DHFR, SeDHFR, differs in only three amino acids (Val-31→Ile, Gly-43→Ala, and Phe-98→Tyr) from the trimethoprim-resistant (Tmp^r) S1 DHFR encoded by transposon Tn*4003*. Since in addition the *S. epidermidis* gene also forms part of an operon with *thyE* and open reading frame 140 as in Tn*4003*, the chromosomally located gene encoding the Tmp^s SeDHFR is likely to be the molecular origin of the plasmid-located gene encoding the Tmp^r S1 DHFR. Site-directed mutagenesis and kinetic analysis of the purified enzymes suggest that a single Phe→Tyr change at position 98 is the major determinant of trimethoprim resistance.

Dihydrofolate reductase (DHFR) (5,6,7,8-tetrahydrofolate: NADP oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F). DHFR is a key enzyme in the tetrahydrofolate pathway, as it is necessary for maintaining intracellular pools of H_4F and its derivatives, which are essential cofactors in the biosynthesis of purines, thymidylate, and several amino acids. As a result, it is the target enzyme of a group of antifolate drugs, such as methotrexate, trimethoprim (TMP), and pyrimethamine, that are used as antitumor and antimicrobial agents (7).

Antimicrobial resistance, in both hospitals and the community, is a clinically important and increasing problem (14). Of particular interest are the methicillin-resistant *Staphylococcus aureus* strains, which also carry resistances to other antibiotics such as erythromycin, tetracycline, streptomycin, gentamicin, and sulfonamides (30). These and other multiresistant staphylococci are a major cause of hospital-acquired infections such as bacteremias, pneumonias, and surgical wound infections (14). TMP is a very potent, selective inhibitor of DHFR and is highly active in vitro against most staphylococci. Although TMP alone is not the drug of choice to treat staphylococcal infections, it has been used in combination with sulfamethoxazole to successfully treat patients infected with multiresistant staphylococci (15, 39).

Recently, a plasmid-encoded TMP-resistant (Tmp^r) DHFR (S1 DHFR) from a clinical isolate of *S. aureus* has been described (10). Despite the fact that this strain was isolated in Zürich, Switzerland, and that the plasmid differed from those found elsewhere (25, 32), the sequence of the DHFR gene was found to be identical to that of a DHFR gene from an Australian isolate (32). This surprisingly high level of conservation, which is quite different from the large variation observed among Tmp^r DHFRs in gram-negative species, was explained by the worldwide spread of transposon Tn*4003*, in which this gene is located (32).

We recently cloned and characterized the gene for the chromosomal TMP-sensitive (Tmp^s) DHFR from *S. aureus* ATCC 25923 (SaDHFR) (18). Despite the high degree of homology between the S1 DHFR and SaDHFR, the chromosomal gene was not located in tandem with the gene encoding thymidylate synthase, as is the case for the plasmid-encoded Tmp^r DHFR. Therefore, we concluded that the S1 DHFR did not originate from the *S. aureus* chromosome (18). In this study we report the cloning and characterization of the gene, designated *dfrC*, for the chromosomal Tmp^s DHFR from *Staphylococcus epidermidis* (SeDHFR) and its expression in *Escherichia coli*. We have also identified the amino acid mainly responsible for conferring resistance to TMP by site-directed mutagenesis and kinetic analysis of the purified proteins. We believe that the gene for this enzyme is the molecular origin of Tmp^r DHFR located on the transposon Tn*4003* in staphylococci. Thus, our data provide evidence that the direction of transfer of genetic elements in nature can be from coagulase-negative staphylococci to *S. aureus.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. *S. epidermidis* ATCC 14990 has been described previously (11). *E. coli* Tg1 (33) was used for the preparation of single-stranded DNA and for the construction of an M13mp18 bacteriophage library. The cloning vector pUC18 and the DNA of bacteriophage M13mp18 were supplied by Boehringer Mannheim, Mannheim, Germany. The expression plasmids pS1 and pS1(RNA-Is) have previously been described (16, 18). For expression of DHFR in *E. coli*, strain M15(pREP4-groESL) was used (17). *E. coli* strains were transformed as described previously (33).

Enzymes and chemicals. Restriction enzymes, calf intestinal phosphatase, Klenow fragment of DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were obtained from Boehringer Mannheim and used under conditions recommended by the manufacturer. Lysozyme was from Pharmacia, and lysostaphin was from Sigma. [α-^{32}P]dATP, [α-^{32}P]dCTP, and α-^{35}S-dATP were purchased from Amersham (Amersham, United Kingdom).

Isolation of DNA. The isolation of recombinant M13mp18 phages and of the DNA of these phages has been described previously (33). Plasmid DNA was isolated by using the Qiagen system as recommended by the manufacturer

* Corresponding author. Phone: 0041-61-6887297. Fax: 0041-61-6881448.

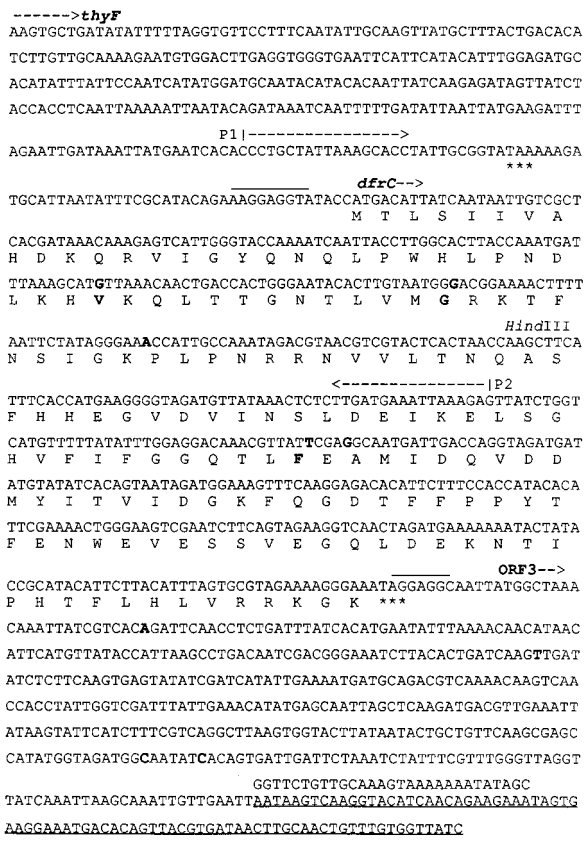

FIG. 1. Nucleotide and deduced amino acid sequences of the *dfrC* gene from *S. epidermidis* ATCC 14990. The *thyF* gene for thymidylate synthetase and ORF3 are indicated. The solid lines indicate the putative Shine-Dalgarno sequences of *dfrC* and ORF3. P1 and P2 indicate the primers used for isolating the *thy-dfr* fragment. Nucleotide and amino acid differences from the corresponding region of Tn*4003* (32) are in bold. Nucleotides 1227 to 1308, which differ completely from the corresponding region of Tn*4003*, are underlined; above these nucleotides the sequence of IS*257*R1, present in Tn*4003*, is given. Asterisks indicate the stop codons for thymidylate synthetase and SeDHFR.

(Diagen, Düsseldorf, Germany). Isolation of chromosomal DNA from *S. epidermidis* ATCC 14990 was carried out by the alkaline lysis method (5) by disrupting the cell wall with lysostaphin (0.1 mg/ml) and lysozyme (1 mg/ml). The DNA was sheared by being drawn through a needle (0.8 by 135 mm) three times and then was analyzed on an agarose gel.

PCR. PCR were performed in a thermal cycler (Biometra, Göttingen, Germany). The genomic PCR was performed as described previously (24) by using as a template 0.1 μg of chromosomal DNA and by using as primers the oligonucleotides P1 and P2 (Fig. 1). For the preparation of a radiolabelled probe we followed the same protocol except that plasmid DNA was substituted for genomic DNA, that the dATP and dCTP concentrations were 1/100 of the normal concentration, and that 25 μCi each of [α-^{32}P]dATP and [α-^{32}P]dCTP were added to the reaction mixture. All PCR fragments were analyzed and isolated from 6% polyacrylamide gels (33).

DNA hybridizations. DNA digested with restriction enzymes was separated in 0.7% agarose gels and then transferred to Zeta-Probe membranes by alkali transfer and cross-linked to the membrane (37) for subsequent hybridization by a standard protocol (33). M13mp18 bacteriophages and pUC18 libraries carrying fragments of the expected size were screened according to the method of Church and Gilbert (13) on replica filters. All hybridizations were carried out with the [α-^{32}P]dATP- and [α-^{32}P]dCTP-labelled DNA probe described above. Hybridization was detected by autoradiography at −80°C on Kodak XAR film with intensifying screens.

Determination of nucleotide sequences. DNA restriction fragments were subcloned in M13mp18, M13mp19, or pUC18 vectors. Nucleotide sequences were determined by the dideoxy chain termination method (35).

Oligonucleotide-directed mutagenesis. Mutageneses were performed by using the oligonucleotide-directed in vitro mutagenesis system of Amersham according to the protocol of the manufacturer. A 600-bp *Eco*RI-*Bam*HI fragment from pS1(RNA-Is) carrying the S1 DHFR coding region was subcloned into the corresponding sites of bacteriophage M13mp18 double-stranded DNA. The single-stranded DNA of the resulting phage, M13mp18(S1-RNA-Is), was used as a template for the mutagenesis reactions together with one of the following mutagenic primers (site of mutation underlined): Ile-31→Val (5′-GAT TTA AAG CAT GTT AAA CAA CTG ACC), Ala-43→Gly (5′-CTT GTA ATG GGA CGG AAA AC), or Tyr-98→Phe (5′-GGA CAA ACG TTA TTC GAA GCA ATG ATT G). Mutations were compounded by subsequent mutagenesis or by using the internal *Hin*dIII site. Mutant double-stranded M13mp18(S1-RNA-Is) DNA was isolated, and the *Eco*RI-*Bam*HI fragment was transferred into the expression vector pDS56/RBSII,NcoI (38). Finally, each mutation was confirmed by nucleotide sequence determination of the mutagenized DHFR gene in its final construct.

Expression and purification of SeDHFR and its muteins. Single colonies of *E. coli* M15(pREP4-groESL) (17) harboring expression plasmids were grown overnight in 10 ml of Luria-Bertani medium with ampicillin (100 μg/ml) and kanamycin (25 μg/ml) and inoculated into 1 liter of the same medium. The cells were grown at 37°C to an A_{600} of 0.9, and gene expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (38). After 2 h, the cells were harvested by centrifugation and were resuspended in 20 ml of buffer A (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 8], 20% glycerol, 0.15 M NaCl, 1 mM $MgSO_4$, 2 mM dithiothreitol, and 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]). The suspension was passed through a French press, and the resulting homogenate (25 ml) was centrifuged for 30 min at 20,000 rpm (Kontron rotor A8.8). The supernatant was passed through a Q-Sepharose fast-flow column (1 by 10 cm; Pharmacia LKB Biotechnology Inc.) equilibrated with 40 mM Tris-HCl, pH 7.5. The DHFR activity eluted in the flowthrough and the 200 mM NaCl wash solution. The fractions were applied to a Blue-Sepharose CL-6B column (1 by 10 cm; Pharmacia LKB Biotechnology Inc.) equilibrated with 40 mM Tris-HCl, pH 7.5. The column was washed with the same buffer containing 200 mM KCl. The DHFR was eluted with 2 mM folate in 40 mM Tris-HCl (pH 7.5)–200 mM KCl. The protein solution was concentrated 10× with a Centriprep-10 (Amicon, Inc.) and applied to a Superdex 200 column (1.6 by 60 cm; Pharmacia LKB Biotechnology Inc.) equilibrated with phosphate-buffered saline. The fractions containing DHFR were pooled. S1 DHFR and all the muteins containing an alanine at position 43 were obtained as approximately 80% homogeneous preparations. Even after size-exclusion chromatography multiple bands could be seen on Coomassie blue-stained gels. The remaining proteins were purified to near homogeneity.

Enzyme assays. The DHFR assays have been described previously (4). The concentration of dihydrofolate was measured spectrophotometrically at 282 nm by using a molar extinction coefficient of 2.8×10^4 M^{-1} cm^{-1} at pH 7.4 (2). The concentration of NADPH was determined spectrophotometrically at 340 nm by using a molar extinction coefficient of 6.2×10^3 M^{-1} cm^{-1} (2). DHFR activities were determined by measuring the decrease in A_{340} occurring as NADPH and dihydrofolate were converted to products with a Kontron Uvikon 860 spectrophotometer. A unit of DHFR activity was defined as the conversion of 1 μmol of dihydrofolate per min.

Homology modelling of SeDHFR. The amino acid sequence of the *S. epidermidis* DHFR was compared with the sequences of all those DHFRs for which the three-dimensional structure had been determined by X-ray crystallography (human [31], chicken [26], *E. coli* [8, 12, 21, 26, 27], and *Lactobacillus casei* [8]). The *E. coli* sequence was found to match most closely the *S. epidermidis* sequence. The alignment of the two sequences showed 36% amino acid identity. This level of identity has been shown to be sufficient to imply close similarity of two three-dimensional structures (34). A three-dimensional model of the *S. epidermidis* DHFR was built by using the structure of *E. coli* DHFR (12), entry 7DFR of the Brookhaven protein databank (6), as a template. Going from *E. coli* to *S. epidermidis*, a single amino acid insertion and one deletion of a tripeptide had to be accommodated. Both changes are located at the surface of the enzymes and are not close to substrate or cofactor. Because of the tripeptide deletion, helix III, formed by residues V-78 to C-85 in the *E. coli* enzyme, was slightly shifted in the *S. epidermidis* enzyme; otherwise, all secondary structure elements were transferred from the *E. coli* structure. The ternary complex including folate and NADPH was modelled. Model building and energy optimization were performed by using the program Moloc (22, 29).

Other methods. Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% polyacrylamide gels and visualized by Coomassie brilliant blue staining. Computer-aided sequence comparisons were performed on a VAX by using Genetics Computer Group software of the University of Wisconsin (20).

Nucleotide sequence accession number. The sequence data reported here have been submitted to the GenBank/EMBL database (accession number Z48233).

RESULTS

Cloning of the *dfrC* gene. In an attempt to clone the chromosomal origin of the S1 DHFR gene we designed the PCR primers P1 and P2, specific for the *thyE* and *dfrA* genes of Tn*4003* (Fig. 1), and screened various species of Tmp^s staph-

ylococci, namely, *S. epidermidis*, *S. hominis*, and *S. haemolyticus*. Only *S. epidermidis* (both Tmp^s and Tmp^r) strains were positive for the PCR (data not shown). The PCR fragment from the reaction using genomic DNA of *S. epidermidis* ATCC 14990 as a template was isolated, treated with kinase, and subcloned into a dephosphorylated M13mp18 *Hin*cII-digested plasmid. Sequencing of this fragment confirmed that we had isolated the *thy-dfr* fragment from the *S. epidermidis* chromosome. The PCR fragment differed from the corresponding fragment of Tn*4003* by 4 bp. Because of this high degree of sequence homology we chose to use the *dfrA* gene of pS1 (18) as the probe for Southern analysis of *Hin*dIII-digested chromosomal DNA from *S. epidermidis* ATCC 14990. Since *dfrA* and the corresponding region from the *S. epidermidis* chromosome contain an internal *Hin*dIII site (Fig. 1), we chose to clone the 5′ and 3′ regions of the gene as separate *Hin*dIII fragments. In order to clone the 5′ end of the *S. epidermidis* DHFR, a 221-bp *Eco*RI-*Hin*dIII fragment from pS1 (18) was isolated, labelled with [α-^{32}P]dATP and [α-^{32}P]dCTP, and used as the probe for Southern analysis of the *Hin*dIII-digested chromosomal DNA. Only one band with an approximate size of 1 kb was detected by the probe. Accordingly, genomic *Hin*dIII fragments of about 1 kb were isolated and cloned into M13mp18. The resulting bacteriophage library was screened by using the same *Eco*RI-*Hin*dIII radiolabelled probe, and both the double-stranded and the single-stranded DNAs of putative positive clones were isolated. For cloning of the 3′ end of the *S. epidermidis* DHFR a 346-bp *Hin*dIII-*Bam*HI fragment from pS1 was isolated and labelled with [α-^{32}P]dATP and [α-^{32}P]dCTP and then used as the probe for Southern analysis of the *Hin*dIII-digested chromosomal DNA. Only one band, with an approximate size of 4.2 kb, was detected. The genomic fragments of this size were cloned into the vector pUC18, and positive clones were identified by colony hybridization using the same *Hin*dIII-*Bam*HI fragment as a probe. The DNAs of putative positive clones were isolated, and both strands of the DNAs were sequenced by the dideoxynucleotide chain termination method (35).

Sequence analysis. A DNA sequence of 1,308 nucleotides from the genome of *S. epidermidis* was determined (Fig. 1). The sequence contains three open reading frames (ORFs) of 483 (*dfrC*, bp 377 to 819), 292 (*thyF*, bp 1 to 292), and 477 nucleotides (ORF3, bp 832 to 1308). Both *dfrC* and ORF3 are preceded by a typical Shine-Dalgarno sequence, but there is no typical transcription termination signal 3′ to any of the ORFs. Within the first 1,226 nucleotides the DNA sequence differs from the corresponding DNA sequence from Tn*4003* by 9 nucleotides, 4 of which result in amino acid changes, while the remaining 5 are silent mutations. Nucleotides 1227 to 1308 of the *S. epidermidis* sequence differ completely from the corresponding region of Tn*4003* because of the absence of the insertion element IS*257*R1, which is present in the transposon. The amino acid sequence of the predicted protein, SeDHFR, encoded by the *dfrC* gene, differs from that of the S1 DHFR by three amino acids (Fig. 2), all of which are predicted to be either in the active site or in contact with the cofactor NADPH (Fig. 3). The *thyF* gene is identical to the *thyE* gene of Tn*4003*. However, ORF3 differs by four nucleotides from ORF140, resulting in the change of one amino acid (Ala-41→Val). SeDHFR is thus most closely related to the S1 DHFR (32), with 98% amino acid identity, and also to the chromosomally encoded enzyme of *S. aureus* (18), with 82% amino acid identity, but it is less similar to the chromosomally encoded enzymes of *Bacillus subtilis* (23) and *E. coli* (36), with 40 and 36% amino acid identities, respectively.

Mutagenesis, expression, and purification of SeDHFR. Recently, we have constructed plasmid pS1(RNA-Is) to optimize the gene encoding S1 DHFR for expression in *E. coli* (16). Since S1 DHFR and SeDHFR differ by only three amino acids, we mutated the gene for S1 DHFR of pS1(RNA-Is) to generate a plasmid for high-level expression of SeDHFR. Furthermore, we constructed six additional plasmids allowing the expression of the muteins with all possible combinations of amino acid exchanges between S1 DHFR and SeDHFR (Table 1). S1 DHFR, SeDHFR, and their muteins were produced in *E. coli* cells simultaneously overproducing the *E. coli* chaperonins GroEL and GroES (Fig. 4), and the enzyme could be purified in three steps to near homogeneity (Fig. 4). In the Q-Sepharose step the DNA was removed from the cellular extract, and then the DHFR was specifically eluted from the Blue-Sepharose with 2 mM folate and 200 mM KCl in order to remove most of the contaminating proteins. Finally, folate and the remaining contaminating proteins were removed in the Superdex 200 step.

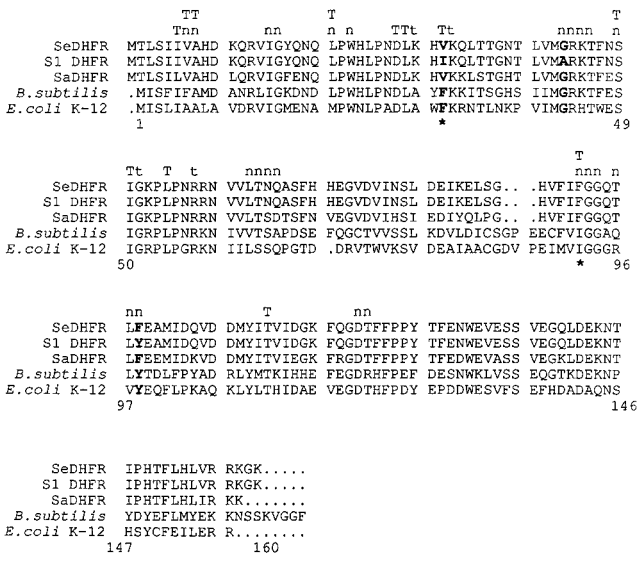

FIG. 2. Compilation of the amino acid sequences of SeDHFR, SaDHFR (18), S1 DHFR (32), and the chromosomal DHFRs from *B. subtilis* (23) and *E. coli* K-12 (36). Positions involved in the binding of TMP (T), methotrexate (T and t), and/or the cofactor NADPH (n), which were taken from studies with the *E. coli* K-12 enzyme (8, 21, 26, 27), are indicated. Amino acid differences between SeDHFR and S1 DHFR are in bold. Amino acid residues marked with an asterisk are identical in all known DHFRs from diverse species except for the staphylococcal enzymes. The numbering of the amino acids is based on that of SeDHFR, with the Met at its N-terminal end being at position 0.

Kinetic properties of enzymes. Some kinetic parameters of the purified wild-type and mutant *S. epidermidis* DHFRs are presented in Table 1. The Michaelis constants (K_m) of NADPH were decreased approximately fivefold for all proteins containing a Gly residue at position 43. Interestingly, a substantial difference in solubility was also associated with this residue, a protein containing Gly being much more soluble than one containing Ala (data not shown). The K_m of the substrate were decreased approximately fivefold for enzymes with a Tyr located at position 98, and they were increased twofold for enzymes with an Ile at position 31. The combination of a Tyr at position 98 and an Ile at position 31 brought the K_m for H_2F back towards the wild-type level. Accurate comparisons of the rates for substrate-to-product conversion (k_{cat}) could not be obtained because of difficulties in obtaining purified S1 DHFR and mutants with an Ala residue at position 43. However, the k_{cat} for SeDHFR and Se(F98Y) were determined to be 13.0

FIG. 3. Molecular model of SeDHFR with NADPH (yellow) and folate (red)/TMP (green). Views of the active site, the C-alpha trace of the enzyme, and the side chains of residues 27, 31, 43, 57, 92, and 98 of SeDHFR (blue) are shown. Mutated residues in S1 DHFR are superimposed (white).

sec^{-1} and 10.6 sec^{-1}, respectively. Thus, the catalytic efficiency (k_{cat}/K_m) is almost fivefold greater for the mutant enzyme. There is a dramatic difference in TMP-binding affinities (K_i) of the enzymes. A Tyr at position 98 renders the enzymes highly resistant to TMP, while substitutions at other positions caused minor changes in the kinetic parameters (Table 1).

DISCUSSION

Recently, we described a plasmid-encoded Tmp^r DHFR from a clinical isolate of *S. aureus* (10), and even though the plasmid differed from those described elsewhere (25, 32), the *dfr* gene was found to be identical in sequence to the *dfrA* gene from an Australian isolate (32). This surprisingly high level of sequence conservation was explained by the worldwide spread of transposon Tn*4003*, in which the gene is located (32). The origin of the S1 DHFR, however, remained obscure. It has been proposed that it may have evolved from the chromosome of a gram-positive organism, probably a soil *Bacillus* sp. (32), carrying the precursor operon *thyE-dfrA*-ORF140. It was also speculated that it may have evolved from the chromosome of *S. aureus* by mutation and mobilization (9). Recently, we cloned and characterized the gene for the chromosomal DHFR of *S. aureus* (18), and despite the high degree of homology (82% on the amino acid level) between the two DHFRs, we concluded that the S1 DHFR did not originate from the *S. aureus* chromosome because the gene did not form part of an operon with *thyE*. However, because of the high degree of homology between S1 DHFR and SaDHFR and the fact that both enzymes, unlike all other known DHFRs, have an unusual alteration of residues 31 and 92 in the active site (Fig. 2 and 3), we were prompted to screen several staphylococcal species for the chromosomal origin of the S1 DHFR. Since in a PCR screening assay based on the DNA sequences of *dfrA* and *thyE* only *S. epidermidis* (both Tmp^s and Tmp^r) strains were positive, we cloned and characterized the chromosomal gene (*dfrC*) for the Tmp^s SeDHFR from *S. epidermidis* ATCC 14990.

The sequences of the genes for SeDHFR and S1 DHFR, as well as their flanking regions, are nearly identical. Therefore, the DHFR genes belong to identical operons. However, the *S.*

TABLE 1. Enzyme kinetic and inhibition properties for purified SeDHFR and its muteins

Recombinant DHFR	K_m of H_2F (μM)[a]	K_m of NADPH (μM)[a]	K_i of TMP (nM)	IC_{50}[b] of TMP (μM)
SeDHFR	5.1 ± 0.3	2.5 ± 0.3	5.0	0.029
Se(V-31→I)	13.5 ± 0.4	1.8 ± 0.3	8.8	0.036
Se(G-43→A)	4.9 ± 0.6	11.4 ± 0.3	16.0	0.065
Se(F-98→Y)	0.86 ± 0.08	2.2 ± 0.2	302.5	1.57
Se(V-31→I,G-43→A)	9.3 ± 0.7	15.6 ± 1.3	37.6	0.093
Se(V-31→I,F-98→Y)	1.9 ± 0.3	2.5 ± 0.6	345	4.2
Se(G-43→A,F-98→Y)	0.86 ± 0.05	12.1 ± 0.6	480	6.2
S1 DHFR	6.6 ± 0.5	12.4 ± 1.0	652	9.8

[a] Values are means ± standard errors.
[b] IC_{50}, 50% inhibitory concentration.

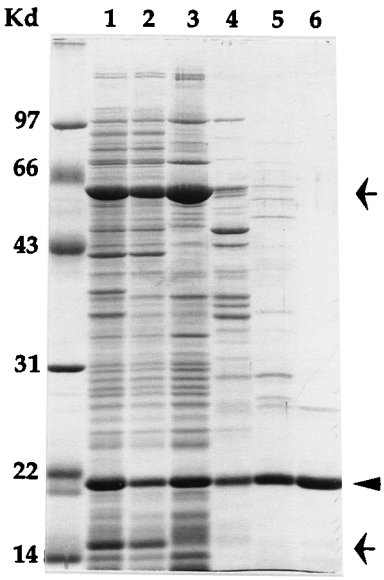

FIG. 4. Purification of SeDHFR. Results of SDS-PAGE analysis of total cellular extract (lane 1), soluble protein extract after cell disruption (lane 2), flowthrough from Q-Sepharose (lane 3), eluate from Q-Sepharose (lane 4), eluate from Blue-Sepharose (lane 5), and peak fraction from the Superdex 200 column (lane 6) are shown. The position of SeDHFR is indicated by the triangle; GroEL and GroES are indicated by arrows. Molecular masses of marker proteins (1 μg of each protein) are indicated on the left.

epidermidis operon does not contain the insertion element IS*257*R1, which is present on transposon Tn*4003*. Consequently, the single SeDHFR gene that we have characterized is located in the *S. epidermidis* chromosome and is not part of transposon Tn*4003*. Because of the high degree of sequence identity between the two operons, we believe that the gene for S1 DHFR originated from the *S. epidermidis* chromosome. Possibly the gene was mobilized by a mechanism similar to that proposed for the evolutionary development of Tn*4003* (32). It is widely accepted that members of the genus *Staphylococcus* do not live in genetic isolation and that coagulase-negative staphylococci may be important as intermediaries and reservoirs for DNA sequences that eventually appear in *S. aureus* (3, 25). In fact, Skurray and coworkers have previously shown that highly Tmp^r strains of *S. epidermidis* which carry sequences homologous to *dfrA* also produce a DHFR with properties similar to those of the *S. aureus* S1 enzyme (25). Our finding that *dfrA* most likely originated from the *S. epidermidis* chromosome supports the role of coagulase-negative staphylococci in the dissemination of resistance elements to *S. aureus*. SeDHFR and the S1 DHFR differ by three amino acids, all of which are predicted to be in or near the active site of the enzyme (Fig. 3). It is intriguing that in SeDHFR, as in SaDHFR and S1 DHFR, amino acids at position 31s and 92 are exchanged compared with all other known DHFRs. Position 31 is occupied by a small hydrophobic residue, and position 92 is occupied by a phenylalanine (Fig. 2 and 3). Perhaps this alternation is characteristic of all chromosomally encoded DHFRs from the genus *Staphylococcus*, as this exchange is also observed in a DHFR from *S. haemolyticus* (unpublished results). Both of these residues have been shown to make strong hydrophobic contacts with the diaminopyrimidine ring of TMP in the structure of the *E. coli* DHFR (26). Surprisingly, mutation of residue 31 from Val to Ile has little effect on the binding of TMP, as the K_i is essentially unaffected, although it has an effect on the binding of dihydrofolate, as the K_m for H_2F is increased about threefold.

As the residue in position 43 can make contact with the adenine moiety of NADPH, the approximately fivefold increase of the K_m for NADPH produced by the Gly-to-Ala mutation is not surprising. The Gly-43 residue is conserved in all bacterial chromosomal DHFRs sequenced to date, and it is not predicted to be involved in the binding of TMP or folate; thus, it was rather unexpected that a mutation here would have an influence on the binding of TMP, albeit minimal (Table 1). It is interesting to note that a Gly at this position is also associated with an increased solubility of the enzyme overproduced in *E. coli*, with or without the co-overproduction of the chaperonins GroES and GroEL. This residue is located at the start of a conserved α-helix, and thus an Ala, being less flexible than a Gly, could disrupt the normal folding process, leading to increased aggregation (28, 40).

From the kinetic data for the purified proteins (Table 1) it is clear that the major resistance determinant is the Phe-98→Tyr substitution resulting in a greater than 60-fold increase in the K_i for TMP. This increase in resistance was unexpected because the chromosomal enzymes from both *E. coli* (36) and *B. subtilis* (23) have Tyr residues at this position. The corresponding residue in *E. coli* (Tyr-100) provides an aromatic-aromatic interaction with both the nicotinamide and the pteridine rings (1) and has even been suggested to make a hydrogen bond with the N-4 nitrogen of TMP (19). Our model of a TMP complex of SeDHFR carrying the mutation Phe-98→Tyr shows that steric effects of the additional phenolic group can be ruled out as a reason for the increased K_i of TMP. A Phe-98→Tyr mutation in the chromosomal enzyme of *S. aureus* leads to a similar increase of K_i for TMP (unpublished results). We have sequenced various clinical isolates of *S. aureus* resistant to TMP and found that all chromosomally encoded resistant DH FRs have the same Phe-98→Tyr substitution (unpublished results). Perhaps it is the combination of the unusual exchange of residues 92 and 31 and the mutation of Phe-98 to Tyr which distinguishes the effect of the Tyr on TMP binding for S1 DHFR from that for the *E. coli* enzyme.

In summary, we have cloned and characterized the gene encoding DHFR from *S. epidermidis* ATCC 14990. Comparison of the sequence of this gene and that of the *dfrA* gene encoded by Tn*4003* strongly suggests that the TMP-resistant DHFR found in *S. aureus* originated from the chromosome of *S. epidermidis*, confirming the theory that the transfer of genetic elements in nature is also from coagulase-negative staphylococci to *S. aureus*. Finally, we have identified the residue mainly responsible for conferring TMP resistance to staphylococcal DHFRs and we believe that further understanding of the mechanism of TMP resistance will aid us in our endeavor to develop novel antifolate compounds.

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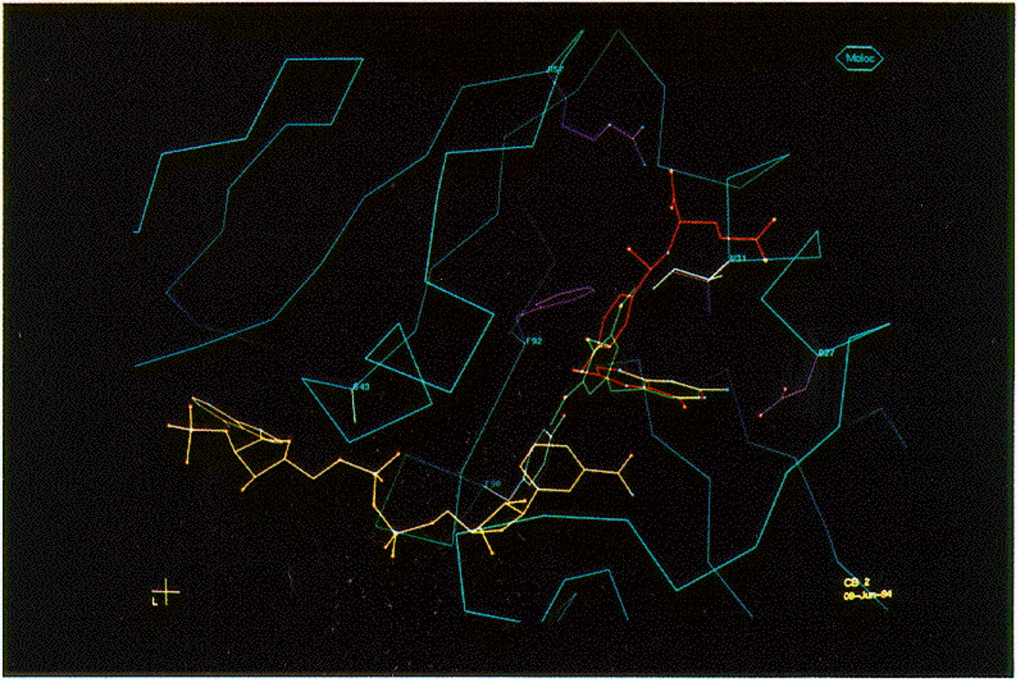
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