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Cloning and Characterization of a Novel, Plasmid-Encoded Trimethoprim-Resistant Dihydrofolate Reductase from *Staphylococcus haemolyticus* MUR313

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In recent years resistance to the antibacterial agent trimethoprim (Tmp) has become more widespread, and several trimethoprim-resistant (Tmp^r) dihydrofolate reductases (DHFRs) have been described from gram-negative bacteria. In staphylococci, only one Tmp^r DHFR has been described, the type S1 DHFR, which is encoded by the *dfrA* gene found on transposon Tn*4003*. In order to investigate the coincidence of high-level Tmp resistance and the presence of *dfrA*, we analyzed the DNAs from various Tmp^r staphylococci for the presence of *dfrA* sequences by PCR with primers specific for the *thyE-dfrA* genes from Tn*4003*. We found that 30 of 33 isolates highly resistant to Tmp (MICs, ≥512 μg/ml) contained *dfrA* sequences, whereas among the Tmp^r (MICs, ≤256 μg/ml) and Tmp^s isolates only the *Staphylococcus epidermidis* isolates (both Tmp^r and Tmp^s) seemed to contain the *dfrA* gene. Furthermore, we have cloned and characterized a novel, plasmid-encoded Tmp^r DHFR from *Staphylococcus haemolyticus* MUR313. The *dfrD* gene of plasmid pABU17 is preceded by two putative Shine-Dalgarno sequences potentially allowing for the start of translation at two triplets separated by nine nucleotides. The predicted protein of 166 amino acids, designated S2DHFR, encoded by the longer open reading frame was overproduced in *Escherichia coli*, purified, and characterized. The molecular size of the recombinant S2DHFR was determined by ion spray mass spectrometry to be 19,821.2 ± 2 Da, which is in agreement with the theoretical value of 19,822 Da. In addition, the recombinant S2DHFR was shown to exhibit DHFR activity and to be highly resistant to Tmp.

Antimicrobial resistance in both hospitals and the community is a clinically important and increasing problem (10, 21). Of particular interest is methicillin-resistant *Staphylococcus aureus* (MRSA), which invariably carries genes for resistance to other antibiotics such as erythromycin, tetracycline, streptomycin, gentamicin, and sulfonamides (27). These and other multiresistant staphylococci are a major cause of hospital-acquired infections such as bacteremias, pneumonias, and surgical wound infections (10). Although trimethoprim (Tmp) alone is not the drug of choice for the treatment of staphylococcal infections, it has been used in combination with sulfamethoxazole to successfully treat patients infected with multiresistant staphylococci (22, 33). Tmp is a very selective potent inhibitor of dihydrofolate reductase (DHFR) and is active in vitro against most staphylococci, but resistance to Tmp is an ever growing problem (9).

The widespread resistance of staphylococci to Tmp was first reported in the 1980s (2, 23) and was found to be due to the plasmid-encoded production of the Tmp^r type S1 DHFR (S1DHFR) encoded by the *dfrA* gene located on transposon Tn*4003* (8, 11, 28). In recent studies of Tmp resistance among coagulase-negative staphylococci (7, 17, 18), it was shown that a specific probe for the *dfrA* gene hybridized with all Tmp^r *Staphylococcus epidermidis* isolates. However, none of the *Staphylococcus haemolyticus* strains tested were shown to hybridize with the S1DHFR probe (17). Recently, Burdeska (7) isolated a small plasmid (pABU17, 3.8 kb) from *S. haemolyticus* MUR313 which, when it was transformed into the Tmp^s strain *S. aureus* 113, resulted in a high-level Tmp-resistant strain (MIC, >1,024 μg/ml).

Using a PCR approach to study the molecular epidemiology of the gene encoding the S1DHFR among staphylococci, we isolated total DNAs from both Tmp^r and Tmp^s strains of *S. aureus*, *S. epidermidis*, *Staphylococcus hominis*, and *S. haemolyticus* from various geographical locations and analyzed their DNAs for sequences similar to those from the *thyE-dfrA* operon. Here we report on the results of that PCR analysis of diverse staphylococci. In addition, we report on the cloning, expression in *Escherichia coli*, and purification of a novel, plasmid-encoded Tmp^r DHFR, designated the S2DHFR, encoded by the gene *dfrD* located on the plasmid pABU17 found in the highly Tmp^r strain *S. haemolyticus* MUR313.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The staphylococcal clinical isolates used in the present study have been described previously (7, 34). The highly Tmp^r strain *S. haemolyticus* MUR313 (MIC, >1,024 μg/ml) was isolated in Murcia, Spain, in 1987 by Martin Luengo. For the expression of DHFR in *E. coli*, strain M15(pREP4) was used (32). *E. coli* strains were transformed as described previously (29). The cloning vector pUC18 was supplied by Boehringer Mannheim, Mannheim, Germany. Plasmid pABU17 (7) and the expression plasmid pDS56/RBSII,NcoI were described previously (32).

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

Isolation of DNA. Plasmid DNA was isolated with the Qiagen system by following the protocols supplied by the manufacturer (Qiagen, Basel, Switzerland). Isolation of plasmid DNA from *S. haemolyticus* MUR313 was carried out

TABLE 1. PCR analysis of staphylococci for the presence of *dfrA* sequences

Origin of strain[a]	No. of strain analyzed	No. of strains containing *dfrA* sequences/total no. of strains tested[b]							
		Tmp MIC, ≥ 512 μg/ml				Tmp MIC, 0.25–256 μg/ml			
		A	B	C	D	A	B	C	D[c]
Europe	35	5/5	5/5	7/8	1/1	0/10			6/6
South America	18	2/2	4/4	1/1		0/6		0/1	4/4
North America	7	3/3			1/1				3/3
Others	5	0/2		1/1		0/2			

[a] Clinical isolates collected between 1983 and 1989.
[b] A, *S. aureus*; B, *S. hominis*; C, *S. haemolyticus*; D, *S. epidermidis*.
[c] For all *S. epidermidis* strain tested, MICs were ≤8 μg/ml.

by the alkaline lysis method (5) by first disrupting the cell wall with lysostaphin (0.1 mg/ml) and lysozyme (1 mg/ml) and then by using the Qiagen system.

PCR. PCRs were performed in a thermal cycler (Biometra, Göttingen, Germany). The PCR profile used for determining the existence of *dfrA* sequences in the total DNA extracts was an initial denaturation step for 3 min at 95°C; this was followed by 25 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, with 100 ng of DNA used as the template and by using as specific primers the oligonucleotides P1 (5′-CCCTGCTATTAAAGCACC-3′) and P2 (5′-CATGACCAGATA ACTC-3′) (12). DNAs from *S. aureus* 157/4696 (8) and ATCC 25923 were used as positive and negative controls, respectively. The PCR used in the subcloning of *dfrD* (primers P3 and P4; see Fig. 1) followed a standard PCR amplification protocol (19). All PCR fragments were isolated from 6% polyacrylamide gels (29).

Determination of nucleotide sequences. DNA restriction fragments were subcloned in pUC18 vectors. Nucleotide sequences were determined by the dideoxy chain termination method (30).

Expression and purification of S2DHFR. The coding region of *dfrD* was amplified from pABU17 by PCR with the oligonucleotides P3 and P4 (Fig. 1). The DNA fragment was phosphorylated at the 5′ ends, digested with *Bam*HI, and then inserted into the filled-in *Nco*I site and the *Bam*HI site of the expression plasmid pDS56/RBSII,NcoI (32). In the resulting plasmid, named pS2, the DHFR gene is under the control of the regulatable promoter-operator element N25OPSN25OP29 and an efficient ribosomal binding site (32). Since N25OPSN25OP29 contains two *lac* operators, transcription from this element is blocked in the presence of the *lac* repressor, which is encoded by plasmid pREP4, and is induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture medium. For the expression of S2DHFR, *E. coli* M15 cells containing plasmids pS2 and pREP4 were grown to an A_{600} of 0.9 in 1 liter of super medium (29) in the presence of 100 μg of ampicillin per ml and 25 μg of kanamycin per ml, and then the production of the recombinant S2DHFR was induced by the addition of IPTG (32). After 3 h, the cells (15 g from 3 liters of culture medium) were harvested by centrifugation and were resuspended in 50 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 (DTT), 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 was centrifuged for 30 min at 20,000 rpm (Kontron rotor A8.8). Essentially no DHFR activity was detected in the supernatants; therefore, the pellet was resuspended in 25 ml of 8 M urea–40 mM Tris-HCl (pH 7.5). The suspension was centrifuged at 54,000 × *g* (Heraeus Sepatech HFA28.1) for 30 min. The supernatant was diluted 10-fold with 20 mM Tris-HCl–6 M urea–50 mM DTT (pH 7.5) to reduce the ionic strength. Aliquots of 10 ml were applied to a 6-ml Resource Q column (Pharmacia) equilibrated with 20 mM Tris-HCl–6 M urea–50 mM DTT (pH 7.5). S2DHFR was eluted with a linear gradient from 0 to 1 M NaCl at a flow rate of 3 ml/min. Fractions containing the S2DHFR from a total of 12 runs (150 ml) were combined and concentrated by a factor of 10 with a Centriprep-10 apparatus (Amicon, Danvers, Mass.) and were applied in two portions to a column (2.6 × 60 cm) of Superdex 200 (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 8)–6 M guanidine-HCl. The fractions containing DHFR were pooled (48 ml) and were again concentrated 10-fold with a Centriprep-10 apparatus. The solution was dialyzed overnight at 4°C against 1 liter of phosphate-buffered saline–5 mM DTT. Finally, the dialysate was centrifuged at 54,000 × *g* (Heraeus Sepatech HFA28.1) for 20 min.

Ion spray mass spectrometry. Mass spectral analysis was performed on a Sciex API-III spectrometer. Prior to the analysis, the S2DHFR was desalted on a Poros R/H reversed-phase high-pressure liquid chromatography column (0.21 by 10 cm; Perseptive Biosystems, Boston, Mass.).

Enzyme assays. The DHFR assay has been described previously (4). Dihydrofolate (DHF) was prepared from folic acid, and its concentration was measured spectrophotometrically at 282 nm by using the molar extinction coefficient of 2.8 × 10^4 M^{-1} cm^{-1} at pH 7.4 (1). The concentration of NADPH was determined spectrophotometrically at 340 nm by using the molar extinction coefficient of 6.2 × 10^4 M^{-1} cm^{-1} (1). The DHFR activities were determined by measuring the decrease in A_{340}, which occurred as NADPH was oxidized and DHF was reduced to tetrahydrofolate, by using a Kontron Uvikon 860 spectrophotometer. One unit of DHFR activity was taken as the conversion of 1 μmol of dihydrofolate per min.

Other methods. Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% polyacrylamide gels and were visualized by Coomassie brilliant blue staining. Computer-aided sequence comparisons were performed on a VAX by using Genetics Computers Group software of the University of Wisconsin (15).

Nucleotide sequence accession number. The EMBL Data Library accession number of the DNA sequence for *dfrD* is Z50141.

RESULTS

Dissemination of S1DHFR. On the basis of the DNA sequence of the S1DHFR encoded by Tn*4003*, we designed the PCR primers P1 and P2 specific for the 3′ end of the *thyE* gene and the 5′ end of the *dfrA* gene, respectively (12). We analyzed by PCR 33 clinical isolates of staphylococci for which Tmp MICs were 512 or 1,024 μg/ml and 32 clinical isolates for which Tmp MICs were between 0.25 and 256 μg/ml for sequences similar to *dfrA* encoded by Tn*4003* (Table 1). The isolates were from a variety of geographic locations. Among the highly Tmp^r clinical isolates (MICs, 512 or 1024 μg/ml), 30 of the 33 strains tested produced a fragment of the expected size (262 bp). However, among the 32 clinical isolates for which MICs were lower, only the 13 *S. epidermidis* (both Tmp^r and Tmp^s) strains produced a fragment of the expected size (12). From the three highly resistant strains which failed to produce a fragment of the expected size, two were *S. aureus*, ATU5 and ATU6, both of which were isolated in Colombo, Sri Lanka, in 1985; the third strain was *S. haemolyticus* MUR313 (MIC, >1,024 μg/ml), which was isolated in Murcia, Spain, in 1987.

Cloning and sequence analysis of the *dfrD* gene. *S. haemolyticus* MUR313, which contains the 3.8-kb plasmid pABU17 (7), was negative in the PCR analysis. Since, however, pABU17 (Fig. 1) was previously shown to confer high-level Tmp resistance when it was transformed into *S. aureus* (7), we characterized the novel, plasmid-encoded Tmp resistance gene. Plasmid pABU17 was digested with *Eco*RI and *Bam*HI, and the restriction products were separated on a 6% polyacrylamide gel. Four restriction fragments were isolated and subcloned into pUC18; two *Eco*RI fragments of approximately 2 kb and 100 bp and two *Eco*RI-*Bam*HI fragments of approximately 850 and 870 bp. By using pUC18-specific primers, each construct was sequenced and analyzed for the presence of DHFR-related sequences. Figure 1 shows the sequence of 778 nucleotides, obtained from a region encompassing part of the 2-kb *Eco*RI fragment, the complete 100-bp *Eco*RI fragment, and part of the 870-bp *Eco*RI-*Bam*HI fragment. This DNA sequence contains an open reading frame of 498 nucleotides (*dfrD*). The *dfrD* gene is preceded by two putative Shine-Dalgarno sequences potentially allowing for the start of trans-

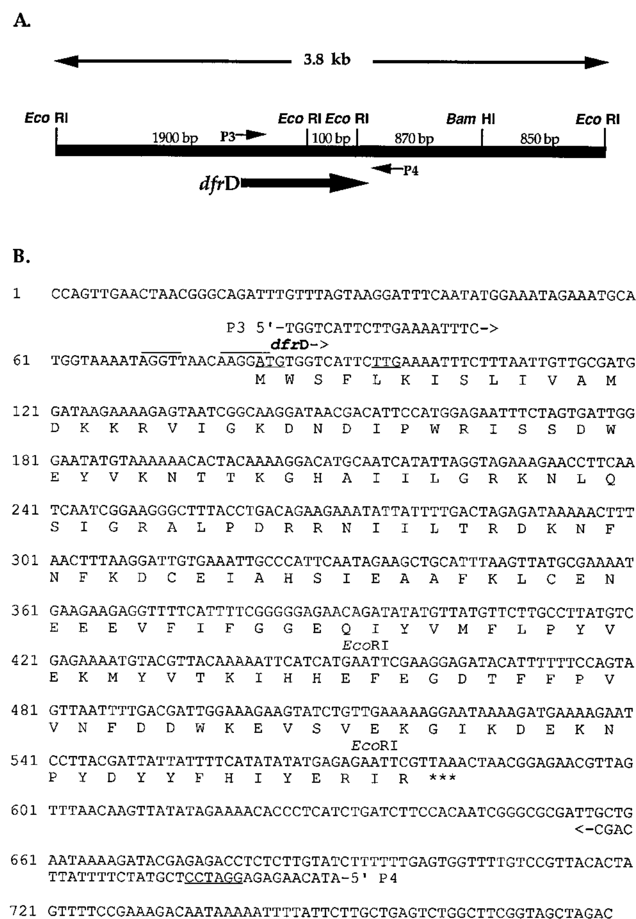

FIG. 1. (A) Diagram of the 3.8-kb plasmid pABU17 from *S. haemolyticus* MUR313 displaying relevant restriction sites, the PCR primers P3 and P4, and the location of the *dfrD* gene. (B) Nucleotide and deduced amino acid sequences of the *dfrD* gene. The solid lines indicate the two putative Shine-Dalgarno sequences; the putative start sites for translation are underlined. P3 and P4 indicate the oligonucleotides used to subclone the *dfrD* gene into an expression plasmid; the *Bam*HI site is underlined. Asterisks indicate the position of the stop codon.

lation at the ATG triplet at positions 82 to 84 or at the in-frame TTG triplet at positions 94 to 96. The predicted protein (166 amino acids) encoded by the longer open reading frame (start of translation at the ATG triplet) was named S2DHFR. Its amino acid sequence is most closely related to the chromosomal DHFR from *Bacillus subtilis* with approximately 50% amino acid identity. S2DHFR is somewhat less similar to the chromosomally encoded enzymes of *S. aureus*, *S. epidermidis*, and *E. coli* K-12, with 41, 39, and 35% amino acid identities, respectively, and it exhibits a 40% identity with the S1DHFR encoded by Tn*4003* (Fig. 2). The S2DHFR contains two structural features which are present only in those enzymes isolated from staphylococci (8, 12, 14, 28), namely, a small hydrophobic residue (Val in the chromosomal enzymes, Ile in the plasmid-encoded S1DHFR) at position 35, a position occupied by a Phe residue in the *B. subtilis* and *E. coli* chromosomal DHFRs, and at position 99 a Phe residue, which is a small hydrophobic residue in the chromosomal DHFRs from *B. subtilis* and *E. coli* (Fig. 2). Therefore, S2DHFR seems to be structurally more closely related to the staphylococcal enzymes.

Enhanced expression, purification, and analysis of S2DHFR. The expression of S2DHFR in *E. coli* was accomplished by integrating its gene into the pDS expression system (32). Plasmid pS2 contains the S2DHFR-coding region which was amplified from pABU17 by PCR with primers P3 and P4 (Fig. 1). The 3′ PCR primer (P4) included a *Bam*HI site following the termination codon of *dfrD*; the 5′ PCR primer (P3) included the first codon downstream of the ATG triplet at positions 82 to 84 and is placed in pS2 just downstream of a Shine-Dalgarno sequence; this is followed by an ATG triplet. The authenticity of the plasmid was confirmed by DNA sequence determination of the entire insert (both strands). *E. coli* M15(pREP4) cells containing plasmid pS2 overexpressed S2DHFR (Fig. 3). The S2DHFR could be purified in two chromatography steps to near homogeneity (Fig. 3). Since most of the recombinant S2DHFR was produced in inclusion bodies, 8 M urea was necessary to solubilize the enzyme. Consequently, the ion-exchange column and the sizing column were run in the presence of 6 M urea and 6 M guanidine-HCl, respectively. After the sizing step about 5 mg of S2DHFR was obtained. In the final dialysis step to refold the enzyme, more than 95% of the protein precipitated. However, the refolded enzyme was active, and its molecular size of 19,821.2 ± 2 Da measured by ion spray mass spectrometry was in good agreement with the calculated value of 19,822.8 Da. Some kinetic parameters of the purified S2DHFR are presented in Table 2. The Michaelis constant (K_m) values for NADPH and DHF are similar to those determined for the other *S. aureus* enzymes (12), whereas the 50% inhibitory concentration of Tmp for S2DHFR is more than 10-fold higher than that for S1DHFR.

DISCUSSION

Since widespread Tmp resistance among staphylococci was first reported (2, 23), a great deal of interest in understanding the mechanism of this resistance has arisen. After the initial observations, resistance was found to be due to the plasmid-driven production of a Tmp^r dihydrofolate reductase, the S1DHFR, encoded by the *dfrA* gene located on transposon Tn*4003* (8, 11, 28). Since the *dfrA* gene has been found by hybridization (7, 18) in *S. aureus* and *S. epidermidis*, a horizontal transfer in nature was suggested (18). This surprisingly high level of gene conservation, which is quite different from the large variations observed among Tmp^r DHFRs in gram-negative species, was explained by the worldwide spread of transposon Tn*4003* (8). However, in two independent studies an *S. aureus dfrA* probe failed to hybridize to several highly Tmp^r *S. haemolyticus* isolates (7, 17). Additionally, Burdeska (7) showed that a small plasmid (pABU17) from *S. haemolyticus* MUR313, which was negative in the hybridization experiments, could transfer high-level Tmp resistance to a recipient strain. The results of those studies suggested that there are other genes encoding Tmp^r DHFRs in staphylococci (3, 7, 17). We therefore used a PCR approach to determine the relationship between the presence of the *dfrA* gene and high-level Tmp resistance among both coagulase-positive and coagulase-negative staphylococci with the goal of identifying, cloning, and characterizing such genes.

We analyzed a total of 65 clinical isolates from various regions (7, 34) for DNA sequences homologous to those from *thyE-dfrA* from Tn*4003*. Our results indicate that none of the strains for which the MIC was ≤256 μg/ml (except *S. epidermidis* [12]) contains the *dfrA* gene as part of transposon Tn*4003* (Table 1). Consistent with that are the observations that these strains do not seem to contain a transferable plasmid-encoded Tmp resistance marker (7) and that at least the *S. aureus* strains in this MIC category contain mutated chromosomal DHFRs which are highly resistant to Tmp (unpublished se-

```
                         TT             T                          T
                       T nn      nn     n n     TTt Tt           nnn
       S2DHFR MWSFLKISLI VAMDKKRVIG KDNDIPWRIS SDWEYVKNTT KGHAIILGRK
   B.subtilis      MISFI FAMDANRLIG KDNDLPWHLP NDLAYFKKIT SGHSIIMGRK
       S1DHFR     MTLSII VAHDKQRVIG YQNQLPWHLP NDLKHIKQLT TGNTLVMARK
S.epidermidis     MTLSII VAHDKQRVIG YQNQLPWHLP NDLKHVKQLT TGNTLVMGRK
     S.aureus     MTLSIL VAHDLQRVIG FENQLPWHLP NDLKHVKKLS TGHTLVMGRK
       E.coli      MISLI AALAVDRVIG MENAMPWNLP ADLAWFKRNT LNKPVIMGRH
               1                                    *              49
                  Tt  T   t    nnnn                                n
       S2DHFR NLQSIGRALP DRRNIILTRD KNFNFKDCEI AHSIEAAFKL CENEEEVFIF
   B.subtilis TFESIGRPLP NRKNIVVTSA PDSEFQGCTV VSSLKDVLDI CSGPEECFVI
       S1DHFR TFNSIGKPLP NRRNVVLTNQ ASFHHEGVDV INSLDEIKEL SG   HVFIF
S.epidermidis TFNSIGKPLP NRRNVVLTNQ ASFHHEGVDV INSLDEIKEL SG   HVFIF
     S.aureus TFESIGKPLP NRRNVVLTSD TSFNVEGVDV IHSIEDIYQL PG   HVFIF
       E coli TWESIGRPLP GRKNIILSSQ PGTD.DRVTW VKSVDEAIAA CGDVPEIMVI
              50                                                   *
              nn nnn             T         nn
       S2DHFR GGEQIYVMFL PYVEKMYVTK IHHEFEGDTF FPVVNFDDWK EVSVEKGIKD
   B.subtilis GGAQLYTDLF PYADRLYMTK IHHEFEGDRH FPEFDESNWK LVSSEQGTKD
       S1DHFR GGQTLYEAMI DQVDDMYITV IDGKFQGDTF FPPYTFENWE VESSVEGQLD
S.epidermidis GGQTLFEAMI DQVDDMYITV IDGKFQGDTF FPPYTFENWE VESSVEGQLD
     S.aureus GGQTLFEEMI DKVDDMYITV IEGKFRGDTF FPPYTFEDWE VASSVEGKLD
       E.coli GGGRVYEQFL PKAQKLYLTH IDAEVEGDTH FPDYEPDDWE SVFSEFHDLD
              100                                                 149

       S2DHFR EKNPYDYYFH IYERIR
   B.subtilis EKNPYDYEFL MYEKKNSSKV GGF
       S1DHFR EKNTIPHTFL HLVRRKGK
S.epidermidis EKNTIPHTFL HLVRRKG
     S.aureus EKNTIPHTFL HLIRKK
       E.coli AQNSHSYCFE ILERR
              150             165
```

FIG. 2. Compilation of the amino acid sequences of S2DHFR, S1DHFR (28), and the chromosomal DHFRs from *S. aureus* (14) *S. epidermidis* (12), *B. subtilis* (20), and *E. coli* K-12 (31). The positions involved in the binding of trimethoprim (T), methotrexate (T and t), and the cofactor NADPH (n), taken from studies with the *E. coli* K-12 enzyme (6, 16, 25, 26, 31), are indicated. The amino acid residues at positions 35 and 99 are marked with an asterisk (see text). The amino acid residue marked in boldface is the tyrosine known to be the major contributor to Tmp resistance in *S. epidermidis* (12). The numbering of the amino acids is based on that of S2DHFR, with the Met at its N-terminal end being at position 0.

quencing data). On the contrary, 30 of the 33 analyzed strains for which the MIC was ≥512 μg/ml contained *dfrA* elements (Table 1). The finding that the same gene for resistance to Tmp is present in *S. aureus*, *S. epidermidis*, *S. hominis*, and *S. haemolyticus* indicates that these organisms share a common gene pool. The chromosomal DHFR gene of *S. epidermidis* is almost identical (differing by only eight nucleotides) to the *thyE-dfrA* region of Tn*4003* (12), underscoring the importance of *S. epidermidis* as a reservoir for DNA sequences that eventually appear in other staphylococcal species (3, 12, 24). Three of our isolates were negative in the PCR analysis; two were *S. aureus* ATU5 and ATU6, which were not further analyzed, and the third isolate was *S. haemolyticus* MUR313, from which we cloned the *dfrD* gene encoding the Tmp[r] S2DHFR.

To demonstrate that the *dfrD* gene encodes an active, Tmp-resistant DHFR we decided to produce S2DHFR in *E. coli*, to purify the recombinant enzyme, and to determine some of its kinetic parameters. Since we failed to produce the recombinant DHFR in a soluble and active form (with or without the cooverproduction of the chaperones GroES and GroEL [13]; data not shown) we had to purify S2DHFR from inclusion bodies. Upon denaturation and refolding of the enzyme, most of the purified protein precipitated. However, after removal of the insoluble protein sufficient amounts of soluble and active enzyme were obtained to determine the K_m for substrate and cofactor and the 50% inhibitory concentration of Tmp (Table

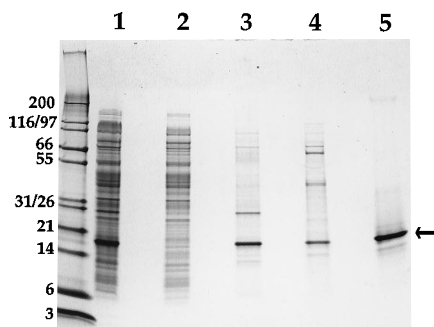

FIG. 3. Purification of S2DHFR and SDS-PAGE (10 to 20%) analysis of protein samples obtained during purification of S2DHFR. The samples shown correspond to the following purification steps: whole cells after solubilization with SDS-sample buffer (lane 1), soluble extract after cell disruption and centrifugation (lane 2), inclusion bodies solubilized with 6 M urea (lane 3), elution fraction from the Resource Q column (lane 4), and peak fraction from the Superdex 200 column prior to dialysis (lane 5). The position of S2DHFR is indicated with an arrow. The molecular masses (in kilodaltons) of the marker proteins (1 μg of each protein) are indicated on the left.

TABLE 2. Enzyme kinetic and inhibition properties of purified recombinant staphylococcal DHFRs

DHFR	K_m (μM)		IC_{50} of Tmp (μM)[a]
	DHF	NADPH	
S2DHFR	5.1	1.7	127
S1DHFR[b]	6.6	12.4	9.8
SeDHFR[b]	5.1	2.5	0.029

[a] IC_{50}, 50% inhibitory concentration.
[b] Data for S1DHFR and SeDHFR were taken from Dale et al. (12).

2). The refolded recombinant S2DHFR is highly resistant to Tmp and shows K_m values for substrate and cofactor which are comparable to those for the Tmp-resistant S1DHFR and the Tmp-susceptible SeDHFR (the chromosomally encoded DHFR of *S. epidermidis* ATCC 14990). Since the enzyme was isolated from inclusion bodies, denatured, and finally refolded, the enzyme kinetics might not be accurate. Nevertheless, the data demonstrate that the *dfrD* gene encodes the novel Tmp-resistant DHFR responsible for the high-level Tmp resistance of *S. haemolyticus* MUR313.

Comparisons between S2DHFR and the other DHFRs show that the active site of S2DHFR is very similar to those known from staphylococci (8, 12, 14, 28). Like the other staphylococcal enzymes, S2DHFR has a small hydrophobic amino acid (Val) at position 35, which is occupied by a Phe residues in chromosomal DHFRs from other organisms (Fig. 2). The valine residue is proposed to make strong hydrophobic contacts with the pyrimidine ring of Tmp (26). At the far interior of the active site and making van der Waals contacts with the pyrimidine ring of Tmp (26), the staphylococcal enzymes contain a phenylalanine (residue 99), whereas this position is occupied by a small hydrophobic amino acid in other DHFRs (Fig. 2). Since the active sites of S2DHFR and S1DHFR seem to be rather similar and results from mutagenesis experiments of S1DHFR clearly implicated the presence of a Tyr residue at position 98 as being the major determinant for Tmp resistance (12), one could speculate that the tyrosine residue of S2DHFR at the corresponding position (residue 105) will also be the major determinant for resistance of this enzyme. The molecular origin of *dfrD* is unknown, but because of the striking similarities between the active site of S2DHFR and those of the other staphylococcal enzymes, it is tempting to assume that it originates from a closely related staphylococcal species.

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