Cloning and Characterization of a Novel Trimethoprim-Resistant Dihydrofolate Reductase from a Nosocomial Isolate of *Staphylococcus aureus* CM.S2 (IMCJ1454)

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A novel gene, *dfrG*, encoding a trimethoprim (TMP)-resistant dihydrofolate reductase (DHFR, designated S3DHFR) was cloned from a clinical isolate of methicillin-resistant *Staphylococcus aureus*. *Escherichia coli* expressing *dfrG* was highly resistant to TMP. Recombinant S3DHFR exhibited DHFR activity that was not inhibited by TMP.

Trimethoprim (TMP) is a potent inhibitor of bacterial dihydrofolate reductase (DHFR) and is effective in vitro against methicillin-resistant *Staphylococcus aureus* (MRSA). In combination with sulfamethoxazole, TMP has been used successfully to treat patients infected with MRSA and is effective at eradicating carriage (10, 16). Resistance of *S. aureus* to TMP was first reported in the 1980s (12) and was found to be due to plasmid-mediated production of an additional DHFR that was less sensitive to TMP than intrinsic DHFR (*S. aureus* DHFR [SaDHFR]) encoded by the *dfrB* gene on the chromosome (1, 12). Plasmid-mediated production of an additional TMP-resistant DHFR is one of the most common mechanisms of resistance to TMP in bacterial organisms. At least 14 different types of TMP-resistant DHFRs in gram-negative bacteria have been reported (10); however, only a limited number of TMP-resistant DHFRs in gram-positive bacteria have been reported (10).

A total of 43 clinical isolates of MRSA from Chiang Mai, Thailand, and 244 clinical isolates of MRSA from Tokyo, Japan, were analyzed in this study. All isolates were positive for *dfrB* by PCR and also positive for *femB* encoding coagulase and for *mecA* associated with methicillin resistance. All isolates from Chiang Mai, Thailand, were resistant to TMP, whereas all those from Tokyo, Japan, except one, *S. aureus* IMCJ934, were sensitive to TMP (Table 1). Crude extracts prepared from a TMP-resistant isolate from Chiang Mai, *S. aureus* CM.S2 (IMCJ1454), showed DHFR activity, and K_m values of the extract for DHF and NADPH were similar to those of crude extracts from TMP-sensitive strain ATCC 25923 (Table 2); however, the 50% inhibitory concentration (IC₅₀) of TMP for

the crude extract of strain CM.S2 was more than 15,000-fold greater than that of ATCC 25923.

HindIII-digested fragments of the *S. aureus* CM.S2 genome were cloned, transformed into *Escherichia coli* DH5 α cells, and selected on agar medium containing TMP (8 μ g/ml). The resultant plasmid, named pSA1, had a 3.5-kb insert containing a complete open reading frame (ORF) surrounded by truncated ORFs (data not shown). The complete ORF consisted of 498 bp encoding a putative protein of 165 amino acids with similarities to TMP-resistant DHFR from *Staphylococcus haemolyticus* (79% identity) (7), *Bacillus anthracis* (67% identity) (2), and *Bacillus cereus* (65% identity) (15) (Fig. 1). The deduced

TABLE 1. MICs of trimethoprim in *S. aureus* and *E. coli* strains

Strain	MIC of TMP (μ g/ml)	Characteristic(s) or genotype
<i>S. aureus</i> CM.S2 (IMCJ1454)	>512	Clinical isolate from Chiang Mai, Thailand, in 2003
<i>S. aureus</i> IMCJ934	>512	Clinical isolate from Tokyo, Japan, in 2001
<i>S. aureus</i> ATCC 29213	4	Quality control strain for antimicrobial susceptibility testing
<i>E. coli</i> DH5 α (pSA1)	>512	Transformant harboring a 3.5-kb BamHI fragment with <i>dfrG</i> ligated to pHSG398
<i>E. coli</i> DH5 α (pHSG398)	<2	Transformant harboring pHSG398
<i>E. coli</i> DH5 α (pT7dfrG)	>512	Transformant harboring PCR-amplified <i>dfrG</i> ligated to pCART7/NT
<i>E. coli</i> DH5 α (pT7dfrB)	128	Transformant harboring PCR-amplified intrinsic <i>dfrB</i> ligated to pCART7/NT
<i>E. coli</i> DH5 α (pCART7/NT)	<2	Transformant harboring pCART7/NT
<i>E. coli</i> DH5 α	<2	<i>supE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1</i>

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TABLE 2. Enzyme kinetic and inhibitory properties of staphylococcal DHFRs

DHFR	Origin	K_m (μ M)		IC ₅₀ of TMP (μ M)
		DHF	NADPH	
Crude enzyme	<i>S. aureus</i> CM.S2 (IMCJ1454)	5.83 $\pm$ 2.09	15.17 $\pm$ 1.73	214
	<i>S. aureus</i> ATCC 25923	3.16 $\pm$ 1.99	14.78 $\pm$ 2.73	0.013
TMP-resistant DHFRs	<i>S. aureus</i> CM.S2 (IMCJ1454)	2.68 $\pm$ 1.09	2.38 $\pm$ 1.97	254
	<i>S. haemolyticus</i> MUR313	5.1	1.7	127
	<i>S. aureus</i>	6.6	12.4	9.8
TMP-sensitive DHFRs	<i>S. aureus</i> CM.S2 (IMCJ1454)	3.01 $\pm$ 1.40	2.97 $\pm$ 0.57	0.014
	<i>S. aureus</i> ATCC 25923			0.012

^a Data from references 6, 7, and 8.

protein is somewhat less similar to the intrinsic TMP-sensitive DHFRs from *S. aureus* (SaDHFR) (8), *S. epidermidis* (SeDHFR) (6), and *E. coli* K-12 (17), with 41%, 40%, and 40% similarity, respectively (Fig. 1). This complete ORF was named *dfrG*, and the deduced protein was designated S3DHFR. Amino acid sequence alignment of DHFRs suggests that residues involved in the binding of TMP and NADPH in other DHFRs are conserved in S3DHFR (Fig. 1). An ORF downstream of *dfrG*,

designated *orfUI*, was located in the opposite direction of *dfrG* and consists of 1,950 bp encoding 650 amino acids, although the deduced amino acid sequence did not show any significant homology to sequences of other previously reported proteins. An ORF upstream of *dfrG* consisted of 582 nucleotides and was identical to the 3'-flanking region of the *SAV0404* gene encoding a hypothetical protein (11). *dfrG* and *orfUI* were flanked by a 28-bp inverted repeat and a 7-bp direct repeat,

FIG. 1. Multiple-sequence alignment of the amino acid sequence of S3DHFR from *S. aureus* CM.S2 (IMCJ1454) isolate with those of DHFRs from other bacteria. The amino acid sequence of S3DHFR was compared with that of type S1 from *S. aureus*, S2 from *S. haemolyticus* MUR313, and the chromosomal DHFRs from *B. anthracis* Ames, *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *Bacillus subtilis* Marburg, *E. faecalis* V583, a methotrexate-resistant mutant of *E. faecium* strain A, *Streptococcus pneumoniae* ATCC 49619, *Staphylococcus epidermidis* ATCC 14900 (SeDHFR), *S. aureus* ATCC 25923 (SaDHFR), and *E. coli* K-12. Sequence comparison was performed by aligning the proteins with the ClustalW program (<http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html>). Amino acid positions involved in the binding of trimethoprim (T) and NADPH cofactor (N) are according to studies of the *E. coli* K-12 enzyme (3, 9, 13, 14). Identical residues are indicated by white letters on black background. Gaps introduced to maximize alignment are indicated by dashes.

FIG. 2. Dendrogram of S3DHFR and DHFR from a variety of organisms. The dendrogram was created by the ClustalW program. Branch lengths correspond to the number of amino acid exchanges of the DHFR proteins (accession number and species given in parentheses) of types I (X00926, from *E. coli*), Ib (I40985, from *E. coli*), IV (A60935, from *E. coli*), V (X12868, from enterobacterial plasmid pLMO150), VI (Z86002, from *Proteus mirabilis*), VII (X58425, from *E. coli*), VIII (U10186, from *E. coli*), IX (A49788, from *E. coli*), X (AY123253, from *Klebsiella pneumoniae*), XII (I41043, from *E. coli*), E1 (AF028812, from *E. faecalis*), S1 (X13290, from *S. aureus*), and S2 (Z50141, from *S. haemolyticus* MUR313) and the chromosomal DHFRs of *B. anthracis* Ames (AE017031), *B. cereus* ATCC 14579 (AE017005), *B. cereus* ATCC 10987 (AE017271), *B. subtilis* Marburg (L77246), *Enterobacter aerogenes* (M26022), *E. coli* K-12 (P00379), *E. faecalis* V583 (AE016951), *E. faecium* mutant strain A (741860A), *Haemophilus influenzae* R1047 (X84205), *Salmonella enterica* serovar Paratyphi ATCC 9150 (CP000026), *S. aureus* ATCC 25923 (SaDHFR; Z16422), *S. epidermidis* ATCC 14900 (SeDHFR; Z48233), *Streptococcus pneumoniae* ATCC 49619 (Z74778), and *Vibrio vulnificus* YJ016 (BA000037).

indicating that the region is in an insertion sequence (IS). The DNA sequence, ranging from 275 bp upstream of *dfrG* to the 363 bp of the 5'-terminal region of *dfrG*, was identical to that previously reported for plasmid pMG1 in *Enterococcus faecium* (18). The *dfrG* gene may have been acquired from *E. faecium* via IS-mediated recombination. The ancestral origin of S3DHFR, however, remains unknown; S3DHFR showed little similarity to and considerable phylogenetic distance from intrinsic DHFR of *E. faecium* (Fig. 2).

The MICs of TMP in *E. coli* transformants harboring pSA1 or pT7dfrG carrying *dfrG* were significantly increased than those in control strains (Table 1), indicating that *dfrG* is responsible for TMP resistance. An *E. coli* transformant harboring pT7dfrB carrying *dfrB* also showed increased MIC, but it was not as high as those of *E. coli* strains expressing *dfrG*. *dfrB* is believed to encode a TMP-sensitive DHFR of *S. aureus* because it was found in all *S. aureus* strains, regardless of TMP susceptibility. Similar results were reported for *dfrE* encoding

Enterococcus faecalis DHFR (4). The increased MIC for TMP in *E. coli* carrying *dfrB* may be explained by the multicopy effects of high expression of the housekeeping protein DHFR.

For functional analysis of S3DHFR and DHFR from *S. aureus* CM.S2 (SaDHFR_{CM.S2}), overexpression and purification of these recombinant DHFRs were achieved. Overexpression of S3DHFR or SaDHFR_{CM.S2} was accomplished by integration of the respective coding regions downstream of the His-tagged coding region of the pCR/T7NT expression vector and transformation into the *E. coli* strain BL21-AI. Recombinant protein in soluble extracts was purified by affinity chromatography to determine enzymatic activities. The K_m values of recombinant S3DHFR for DHF and NADPH were $2.68 \pm 1.09 \mu\text{M}$ and $2.38 \pm 1.97 \mu\text{M}$, respectively (Table 2). The K_m values of DHF and NADPH for S3DHFR did not differ from those of SaDHFR_{CM.S2}, but the IC_{50} values of TMP for these DHFRs differed significantly. The IC_{50} of TMP for S3DHFR was more than 8,000-fold greater than IC_{50} values for TMP-

sensitive SaDHFR and SaDHFR_{CM.S2}, indicating that S3DHFR and SaDHFR_{CM.S2} are indeed DHFRs but that only S3DHFR plays a critical role in TMP resistance. The K_m values of crude extracts for NADPH were sixfold greater than those of recombinant S3DHFR (Table 2). Crude extracts may contain other factor(s) that bind to NADPH.

Detection of *dhfrG* was performed by PCR on isolates from Chiang Mai, Thailand, and Tokyo, Japan. All Chiang Mai isolates were resistant to TMP and contained *dhfrG*, whereas all Tokyo isolates but one were sensitive to TMP and did not contain *dhfrG* (data not shown). The single Tokyo isolate IMCJ934 was resistant to TMP and contained *dhfrG* (Table 1).

Pulsed-field gel electrophoresis (PFGE) analysis revealed 13 patterns of SmaI digestion in the 43 MRSA isolates from Chiang Mai, Thailand (data not shown). Cluster analysis showed that 12 of the 13 PFGE patterns formed a cluster (>75% similarity). The PFGE pattern of *S. aureus* CM.S2 genomic DNA was identical to that of 18 MRSA isolates. These results suggest that clonal expansion of MRSA carrying *dhfrG* occurred at the hospital in Chiang Mai. The TMP-resistant isolate from Tokyo, Japan, IMCJ934, showed the same PFGE pattern as that of one of the Chiang Mai isolates, *S. aureus* CM.S2 (data not shown).

dhfrG was detected by Southern blotting on fragments of SmaI-digested genomic DNA, but it was not detected on plasmids (data not shown). Conjugal transfer of TMP resistance from *S. aureus* CM.S2 to recipient strains *S. aureus* IMCJ565RFP^r or IMCJ644RFP^r was unsuccessful, suggesting that *dhfrG* is located on the chromosome and not on a plasmid of these clinical isolates. It remains to be determined whether *dhfrG* can be transferred by phages or mobile elements.

A single amino acid substitution (Phe to Tyr) at codon 98 of SaDHFR was reported to be associated with TMP resistance in *S. aureus* (5). Therefore, approximately 390 bp of internal DNA sequence of *dhfrB* encoding SaDHFR was determined. When *S. aureus* ATCC 29213 was used as a control (5), all isolates from Chiang Mai, Thailand, exhibited three silent mutations: CAT to CAC in codon 77 and TTT to TTC in codons 91 and 118. All isolates from Tokyo, Japan, contained four silent mutations: AAA to AAG in codon 30, CAT to CAC in codon 77, and TTT to TTC in codons 91 and 118. These results indicate that these mutational changes are not associated with TMP resistance in the isolates from Chiang Mai or Tokyo. Other possible mechanisms of TMP resistance, such as overexpression of intrinsic DHFR, efflux, or impermeability, may be involved.

The CM.S2 strain was the dominant clone from Chiang Mai, Thailand. MRSA surveillance is being carried out in the hospital from which these isolates were obtained. *S. aureus* CM.S2 is resistant to clindamycin, erythromycin, gentamicin, and tetracycline and is less sensitive to arbekacin. Fosfomycin, linezolid, and vancomycin are effective in vitro; quinupristin-dalfopristin and daptomycin were not available for testing. Results of this surveillance will be reported in the future.

Our data strongly suggest that the TMP resistance-associated gene *dhfrG* is prevalent in Thailand, and an isolate harboring this gene was found in Japan. This gene may spread world-

wide, and measures against this, such as gene monitoring and adequate use of TMP, should be established.

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