ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1994, p. 857–860
0066-4804/94/$04.00+0
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Vol. 38, No. 4

Mutations in the *tetA*(B) gene That Cause a Change in Substrate Specificity of the Tetracycline Efflux Pump

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Received 20 September 1993/Returned for modification 10 December 1993/Accepted 5 January 1994

The *tetA*(B) gene from transposon Tn*10* fails to mediate resistance to the novel tetracycline analog 9-(dimethylglycylamido)minocycline (DMG-Mino) (P. E. Sum, V. J. Lee, R. T. Testa, J. J. Hlavka, G. A. Ellestad, J. D. Bloom, Y. Gluzman, and F. P. Tally, J. Med. Chem. 37:184–188, 1994; R. T. Testa, P. Petersen, N. V. Jacobus, P. E. Sum, V. J. Lee, and F. P. Tally, Antimicrob. Agents Chemother. 37:2270–2277, 1993). Mutations in either of two codons of *tetA*(B) that resulted in increased resistance to DMG-Mino also caused diminished resistance to tetracycline, identifying amino acid residues critical for the recognition of tetracycline.

A prevalent mechanism of tetracycline resistance among bacterial pathogens is the reduction in the accumulation of tetracycline, which is actively expelled by a membrane-associated protein (3, 12). The most prevalent of these tetracycline efflux proteins among bacterial pathogens is encoded by the *tetA*(B) gene from transposon Tn*10* among gram-negative bacteria and the *tet*(K) gene from *Staphylococcus aureus* among gram-positive bacteria (4, 16). The energy for expulsion of tetracycline against a concentration gradient is derived by coupling the efflux of the cation-drug complex with the influx of a proton (9, 24, 25, 28). The complete nucleotide sequence of several related tetracycline efflux genes from gram-negative bacteria reveals that they all encode very hydrophobic proteins containing 12 membrane-spanning alpha helices (1a, 2, 5, 8, 11, 15, 19, 20). Biochemical (5, 22) and genetic (1) evidence supports this two-dimensional structure. Several genetic studies have revealed the importance of residues connecting transmembrane helices 2 to 3 (13, 25–27) and 10 to 11 (13) as well as the importance of the charged aspartate residue at position 287, which is within a transmembrane region (21, 23). However, no previous studies have identified the residues that play a role in the recognition of tetracycline.

The fact that the TetA(B) protein confers minocycline resistance uniquely (14) left open the possibility that related efflux pumps from enteric bacteria exhibit different substrate specificities. However, more recent studies have indicated that the TetA(B) protein exhibits no substrate specificity for minocycline compared with other tetracycline efflux pumps derived from enteric bacteria but, rather, that the TetA(B) pump is a superior efflux pump for both tetracycline and minocycline (7). A novel tetracycline analog, 9-(dimethylglycylamido)minocycline (17) (DMG-Mino; illustrated in Fig. 1), which is active against TetA(B)- or TetK-containing strains (18), provides a new tool that can be used to study substrate specificity. We found that a single mutation in codon 231 or codon 308 of the TetA(B) protein results in resistance to DMG-Mino, which was at the expense of resistance to tetracycline.

Mutagenesis. Plasmid pCBSal, which contains the *tetA*(B) gene (7), and pGG57, which contains the *tetK* gene (7), were transformed into strain LE30 (6). This strain contains the *mutD* allele, which greatly enhances the frequency of transitions and transversions caused by the loss of the DNA polymerase proofreading function (6). Cells were transformed by the $CaCl_2$ heat shock method (10), with the modification that cells were grown in M9 minimal medium (10) to ensure the greatest chance that the *mutD* allele would not be mutagenized. (The mutation frequency of strain LE30 is enhanced by several log units in rich medium [6]). Transformants containing plasmids pCBSal or pGG57 (Table 1) were selected by growing the plasmids on LB medium containing 50 μg of ampicillin per ml. Plasmid preparations (10) were made from each of nine plates which contained 2,000 to 5,000 transformants of LE30(pCBSal) per plate and from each of nine plates

CH_3 OH $N(CH_3)_2$ OH $CONH_2$ OH O HO O H O

Tetracycline

$N(CH_3)_2$ $N(CH_3)_2$ OH $CONH_2$ OH O HO O H O

Minocycline

$(CH_3)_2N$ O N H $N(CH_3)_2$ $N(CH_3)_2$ OH $CONH_2$ OH O HO O H O

9-(dimethylglycylamido)minocycline (DMG-Mino)

FIG. 1. Chemical structures of tetracycline, minocycline, and DMG-Mino.

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TABLE 1. Characteristics of the parent plasmids used in the study

Plasmid	Replicon	Selective marker	*tet* gene	Source
pCBSal	pBR322	Ampicillin	*tetA*(B)	7
pGG57	pBR322	Ampicillin	*tetK*	7
pGG9	pACYC184	Chloramphenicol	*tetA*(B)	This work
pGG76	pACYC184	Chloramphenicol	*tetK*	7

containing LE30(pGG57). Thus, each pool contained a plasmid that was independently mutagenized.

Mutated plasmids were transformed at a high frequency, as determined by the number of ampicillin-resistant transformants, into strain MC1061 by electroporation (Bio-Rad Gene Pulsar Apparatus, according to the manufacturer's specification). DMG-Mino-resistant strains were selected by inoculating LB agar medium containing 3 μg of DMG-Mino per ml and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), which was included to induce the tetracycline efflux pump (7). Although no DMG-Mino-resistant transformants were detected from pools of plasmid pGG57 carrying *tetK*, DMG-Mino-resistant mutants were isolated following transformation with five of nine pools of mutagenized plasmid pCBSal carrying *tetA*(B), and one mutated transformant per pool was chosen for further study. The proportion of DMG-Mino-resistant transformants was approximately 10^{-6}, in comparison with an expected frequency for the *mutD* strain of 10^{-4} per gene (6) or in comparison with the prevalence of auxotrophic mutants, which were detected at a frequency of 2% by streaking transformants onto M9 minimal plates and LB agar. Furthermore, no spontaneous DMG-Mino-resistant mutants have been observed (frequency $<1/10^9$) for either MC1061 carrying pCBSal, the transposon Tn*10*, or pGG57, suggesting that the appearance of DMG-Mino resistance is dependent on the mutagenesis procedure. Mutated plasmids of pCBSal were prepared following the growth of each independently isolated DMG-Mino-resistant clone and were designated pGC1, pGC2, pGC3, pGC4, and pGC5.

Transfer of mutated *tetA*(B) genes to an unmutagenized vector. To prove that mutations resulting in DMG-Mino resistance were located in the *tetA*(B) structural gene, the genes isolated from plasmids pGC1, pGC2, pGC3, pGC4, and pGC5 were cloned into the vector obtained from plasmid pGG76, which derives from pACYC184 and which encodes a chloramphenicol resistance marker (10). The *Sal*I-*Hin*dIII fragment containing the 1.3-kb fragment from plasmids pGC1, pGC2, pGC3, pGC4, and pGC5 was ligated into the large fragment generated by digesting pGG76 with *Sal*I and *Hin*dIII enzymes under standard conditions. The *Sal*I site is situated just prior to the *tetA*(B) structural gene. Restriction analysis showed that the desired 1.3-kb *tetA*(B)-containing fragment was inserted in place of the 1.8-kb fragment from plasmid pGG76. The wild-type *tetA*(B) allele was also cloned into the same vector to form plasmid pGG9.

Determination of nucleotide changes. DNA sequencing by the double-stranded modification of Sanger's technique (10) revealed the changes in particular amino acids of the TetA(B) protein compared with those in the wild type (15) that resulted in the ability to recognize DMG-Mino (Table 2). Four independently isolated mutants contained lesions localized within the same codon, codon 231, of *tetA*(B), and all four altered a tryptophan to a cysteine residue (Trp→Cys) by changing the codon from TGG to either TGT or TGC. The Trp→Cys alteration at position 231 resulted in the strongest DMG-Mino resistance (see below). One additional mutation within *tetA*(B), at codon 308 (Ser→Leu), resulted in a more modest resistance to DMG-Mino. The repeated isolation of mutations at codon 231, resulting in the strongest resistance to DMG-Mino, provides additional evidence that strong resistance may be constrained to a single codon. This is also consistent with the low frequency of DMG-Mino resistance. Furthermore, each lesion is a transversion, which is a rarer occurrence than the transition for the *mutD* strain (6). It appears, therefore, that resistance to DMG-Mino is a very rare genetic event, consistent with the fact that spontaneous mutations to DMG-Mino resistance in a Tn*10*-containing strain were not detected.

Characterization of resistance conferred by mutated TetA(B) proteins. Plasmid pGG9, which contained the wild-type *tetA*(B) structural gene in the pACYC184-based vector, was found to mediate the maximum MIC (275 μg of tetracycline per ml) when grown in the presence of 1.4 mM IPTG. This was identical to the maximum MIC mediated by plasmid pCBSal, which contains the *tetA*(B) gene but a different replicon (7). Thus, the maximum resistance to tetracycline was independent of the vector, although the concentration of IPTG required for maximal resistance was increased for plasmid pGG9, possibly because of its lower copy number.

Plasmids pGCR10, pGCR12, and pGCR15, described in Table 2, contained the mutated *tetA*(B) alleles transferred into the pACYC184-based vector. Strain MC1061 carrying each

TABLE 2. Plasmid derivatives containing mutations within *tetA*(B) mediating DMG-Mino resistance

Plasmid	Source	*tetA*(B) mutation	
		Codon 231	Codon 308
pGC1	pCBSal	TGG→TGT (Trp→Cys)	
pGC2	pCBSal	TGG→TGC (Trp→Cys)	
pGC3	pCBSal	TGG→TGC (Trp→Cys)	
pGC4	pCBSal	TGG→TGC (Trp→Cys)	
pGC5	pCBSal		TTG→TCG (Leu→Ser)
pGCR10	*tetA*(B)[a] from pGC1 cloned into pGG76	TGG→TGT (Trp→Cys)	
pGCR12	*tetA*(B) from pGC2 cloned into pGG76	TGG→TGC (Trp→Cys)	
pGCR13	*tetA*(B) from pGC3 cloned into pGG76	TGG→TGC (Trp→Cys)	
pGCR14	*tetA*(B) from pGC4 cloned into pGG76	TGG→TGC (Trp→Cys)	
pGCR15	*tetA*(B) from pGC5 cloned into pGG76		TTG→TCG (Leu→Ser)
pGCR16	pGCR12 and pGCR15[b]	TGG→TGC (Trp→Cys)	TGG→TCG (Leu→Ser)
pGCR17	pGCR12	TGC→TAC (Cys→Tyr)	

[a] A 1.2-kb *Sal*I-*Hin*dIII fragment of pGC1 cloned into a large *Sal*I-*Hin*dIII fragment of pGG76.

[b] A 0.4-kb *Bgl*I-HindIII fragment of pGCR15 cloned into a large *Bgl*I-*Hin*dIII fragment of pGCR12.

FIG. 2. Topology of the tetracycline efflux pump on the basis of the hydrophobicity plot. Amino acid residues are denoted by the single-letter code. The residues demarcated by the squares indicate the Trp-231 and Leu-308 codons involved in the recognition of tetracycline.

plasmid was tested for DMG-Mino resistance as well as resistance to tetracycline (Table 3) by inoculating the MC1061 strains onto LB agar at 37°C as described previously (7), except that the IPTG concentration was 1.4 mM. The concentrations tested were 1, 2, 4, 6, 8, 10, 25, and increasing intervals of 25 μg of tetracycline per ml or 0.5, 1, 1.5, 3, 5, 8, 10, 12, and 15 μg of DMG-Mino per ml. Resistance to DMG-Mino was markedly increased in strain MC1061 carrying the mutated plasmids compared with that in the wild type (Table 3). However, in each case the resistance to tetracycline was diminished. Thus, when a mutation caused a change in the protein so that DMG-Mino was better accommodated, there was a loss of ability to recognize tetracycline. This was particularly evident for resistance mediated by plasmids pGCR10 and pGCR12, which conferred the strongest level of resistance to DMG-Mino. A change in substrate specificity occurred, since these mutated proteins simultaneously became more resistant to DMG-Mino but were defective in conferring resistance to the original substrate, tetracycline.

Combining DMG-Mino-resistant alleles. If both residues 231 and 308 are involved in the common function of substrate recognition, then the combination of the two mutated alleles in the same gene might have a pronounced effect on the ability to recognize tetracycline. Indeed, this is the case. Plasmid pGCR16, containing both the Trp→Cys change at position 231 and the Leu→Ser change at position 308, mediated modestly more resistance to glycylcycline than the wild-type plasmid, but pGCR16 mediated resistance to only 8 μg of tetracycline per ml (Table 3). Therefore, when both side chains at positions 231 and 308 were modified, then the protein was severely crippled in its ability to recognize the original substrate.

TABLE 3. Resistance conferred by wild-type and modified TetA(B) proteins to *Escherichia coli* MC1061 carrying the indicated plasmid

Plasmid	*tetA*(B) genotype at codon:		MIC (μg/ml)	
	231	308	DMG-Mino	Tetracycline
None			0.5	1
pGG9 (wild type)	Trp	Leu	1.0	275
pGCR10	Cys[a]	Leu	15	100
pGCR12	Cys[b]	Leu	15	100
pGCR15	Trp	Ser	10	250
pGCR16	Cys	Ser	1.5	10
pGCR17	Tyr	Leu	1.5	250

[a] TGT codon.
[b] TGC codon.

Second-site suppressors of the Trp→Cys mutation at position 231. To identify other residues that are involved in the common function of recognizing tetracycline, mutants that might contain a second lesion in *tetA*(B) that could compensate for the original Trp→Cys change at position 231 were isolated, resulting in the restoration of strong resistance to tetracycline. To this end, we subjected plasmid pGCR12 to mutagenesis with the *mutD* strain as described above, but this time selecting for the ability to grow in the presence of 200 μg of tetracycline per ml. However, only mutations in the same codon were detected by this method. Twelve independent isolates were analyzed. Ten were true revertants back to tryptophan at position 231. The other two, represented by plasmid pGG17, were mutated so that residue 231 encoded tyrosine. As a result of regaining the capacity to mediate tetracycline resistance, there was loss of the ability to mediate resistance to DMG-Mino.

Residues important for substrate recognition are in the transmembrane domains. It is interesting that both of the amino acids that are altered as a result of mutations to DMG-Mino resistance reside within the assumed transmembrane regions of the protein rather than in the hydrophilic domains (Fig. 2). The side chains of amino acid residues within an alpha helix all point away from the helix. We propose that the side chains at positions 231 and 308 point into a channel or pore that is involved in the recognition of tetracycline. It is possible that the side chains at positions 231 and 308 interact, since they are positioned at approximately the same depth, within transmembrane domains 7 and 9, respectively, in the beta half of the Tet(B) protein (Fig. 2). The severe effect in the recognition of tetracycline when both alterations were in the same protein is consistent with the hypothesis that both residues 231 and 308 are important in the common function of substrate recognition.

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