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Expression of the *tetK* gene from *Staphylococcus aureus* in *Escherichia coli*: Comparison of Substrate Specificities of TetA(B), TetA(C), and TetK Efflux Proteins

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The *tetK* gene, which encodes a tetracycline efflux pump from *Staphylococcus aureus*, was expressed in *Escherichia coli* by using an inducible, low-level expression system. The *tetK* gene, as well as the *tetA*(B) gene from the transposon Tn*10* and the *tetA*(C) gene from plasmid pBR322, was subjected to the regulatory control of the *lac* repressor, and resistance to tetracycline was measured as a function of the isopropyl-β-D-thiogalactopyranoside concentration. The maximum resistance of the *E. coli* strain containing the *tetK* construct was comparable to the maximum resistance of the strain containing the *tetA*(C) construct but was less than the resistance of the strain containing the *tetA*(B) construct. Overexpression of the *tetK*, *tetA*(B), or *tetA*(C) genes was toxic. When expression was regulated so that resistance to tetracycline was comparable, then the TetA(B) and TetA(C) proteins conferred very similar levels of resistance to a variety of tetracycline derivatives. In contrast, the TetK protein was less capable of conferring resistance to the tetracycline derivatives minocycline, 6-deoxy-6-demethyltetracycline, and doxycycline. The implications for the recognition of various tetracycline substituents by the TetK protein are discussed.

Tetracycline resistance among both gram-positive and -negative bacterial pathogens is often mediated by an active efflux system (2, 7, 7a, 30, 31). In the study described here, genes of diverse resistance determinants were expressed in the same *Escherichia coli* host strain by using an inducible, low-level expression vector to compare the abilities of different efflux proteins to confer resistance to a variety of tetracyclines.

The biochemistry of efflux proteins has been most thoroughly defined in *E. coli* systems. The energy for expulsion of tetracycline against a concentration gradient is derived by coupling the efflux of the drug with the influx of a proton. Overall, the process is electroneutral, since tetracycline is expelled when chelated to a divalent cation (23, 47, 48). Each efflux system consists of a very hydrophobic membrane-associated protein (12, 27). The five related tetracycline efflux genes in *E. coli*, designated *tetA*(A) to *tetA*(E) (28), are tightly regulated by a repressor encoded by a neighboring *tetR* gene. The repressor binds to two operators within a common regulatory region (1, 3, 4, 17, 18, 22, 25, 36, 42–46). Although it is possible in some instances, such as in plasmid pBR322, to clone an efflux pump gene without its repressor gene, the constitutive expression of the efflux pump gene of transposon Tn*10* on a multicopy plasmid is lethal (9, 13, 34, 35), probably because of the dissipation of the proton gradient (13).

For gram-positive bacterial systems, the DNA sequence from a variety of sources (21, 24, 31, 33, 37, 38, 39, 40a) indicates that a very hydrophobic, membrane-associated protein is also responsible for the reduction in the accumulation of tetracycline. There are two related classes of efflux proteins among gram-positive bacteria which bear little homology to the efflux proteins of gram-negative bacteria: TetL from *Bacillus*, *Staphylococcus*, and *Streptococcus* species and TetK from *Staphylococcus aureus* (21, 28). Some cloned genes of the L class have been expressed in *E. coli*, resulting in tetracycline resistance (31, 39), whereas other cloned genes of the L type did not mediate tetracycline resistance in *E. coli* (11, 21). The *tetK* gene from *S. aureus* has not been reported to mediate resistance when cloned into *E. coli*. Expression may be prevented because of the secondary structure of the *tetK* mRNA that obscures the Shine-Dalgarno sequence and/or because of the TTG start codon of *tetK*, which is not well recognized by *E. coli* (24).

Because of potential lethality caused by overexpression of efflux pumps, an inducible, low-level expression vector was used to express the *tetK* gene in *E. coli*. The vector was also used to express two related efflux genes from *E. coli*, the *tetA*(C) gene from pBR322 and the *tetA*(B) gene from the transposon Tn*10*. Previous studies concerning the substrate specificities of different efflux proteins did not use an isogenic background or a system in which the expression of *tet* genes could be regulated (8, 32, 41). In the present study, differences in substrate specificity could be investigated by cloning each efflux gene in an isogenic background and adjusting the expression so that each gene mediated the equivalent resistance to tetracycline. Then, the resistance capabilities of each efflux system for other tetracyclines could be assessed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. *E. coli* MC1061 (*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK hsr hsm*$^+$ *strA*) (19) was the host for all experiments.

Plasmid pBR322 (6) was the source of *tetA*(C). Plasmid pCB258 (13) was the starting plasmid for developing the low-level regulatable expression system of *tet* genes. The construction of other plasmids is described below, and their structures are illustrated in Fig. 1. Plasmid transformations were done by standard heat shock procedures (29).

The M13 phage derivative MC71, which carries the 2.35-kb *Hin*dIII A fragment from plasmid pT181 containing

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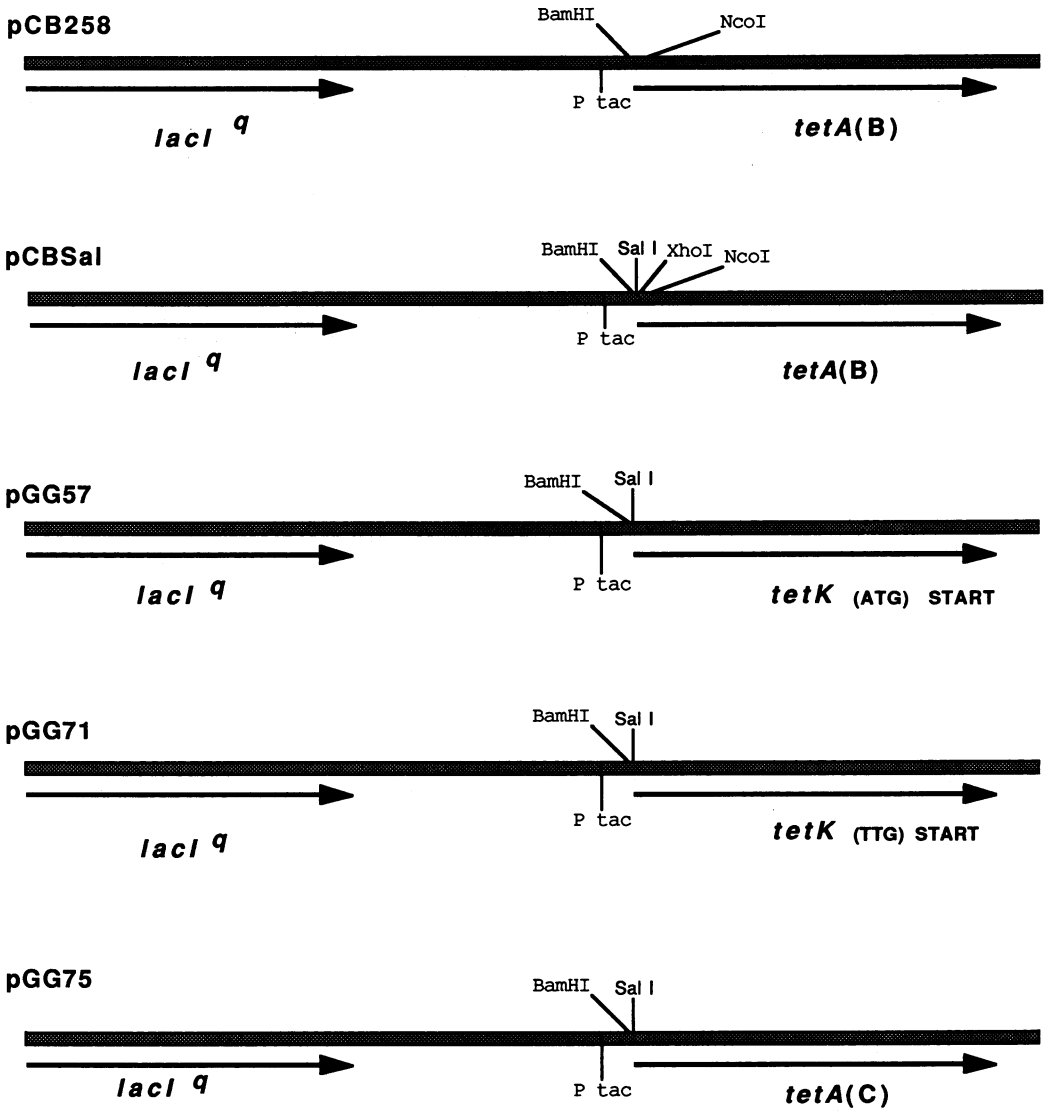

FIG. 1. Schematic representations of plasmids carrying tetracycline resistance genes. Transcription from the *tac* promoter is under the control of LacI and is induced by IPTG. The parent plasmid pCB258 (13) was deleted for the operator site of the tetracycline repressor, which is adjacent to the Shine-Dalgarno sequence of the *tetA*(B) gene from transposon Tn*10*. Substitutions within the ribosome-binding site resulted in convenient restriction sites, including the *SalI* restriction site immediately 5′ to the *tetA*(B) start codon, which facilitated cassette-type replacement of *tetA*(B) with other resistance genes. Plasmid pCBSal contains the *tetA*(B) structural gene from Tn*10*. The two plasmids pGG57 and pGG71 each have a 2.0-kb *SalI-HindIII* fragment containing the *tetK* structural gene; pGG57 has an ATG start codon, and pGG71 has the TTG start codon found in the native gene. Plasmid pGG75 contains the *tetA*(C) structural gene from pBR322, as well as an up mutation (described in the text).

tetK from *S. aureus* (24), was the generous gift of S. Projan and R. Novick.

P1 transduction (29) was used to isolate a derivative of MC1061 containing the transposon Tn*10* by using strain BAR304, a gift of Beth Rasmussen, as the donor.

DNA manipulations. Oligonucleotide-mediated site-directed mutagenesis reactions were performed as described by Kunkel et al. (26). DNA restriction digests, ligations, and fill-in reactions were done as described by Maniatis et al. (29). DNA sequence analysis to confirm the correct nature of all constructs was done by the method of Sanger et al. (40) by using Sequenase version 2.0 (U.S. Biochemicals). Polymerase chain reactions (PCRs) were performed as described by Innis et al. (20) with the addition of 2% dimethyl sulfoxide to the reactions. The oligomers used for PCR and site-directed mutagenesis are listed in Table 1.

Plasmid constructs. Plasmid pCBSal, which contains useful restriction sites preceding *tetA*(B) of transposon Tn*10* and deleted for part of the second operator site of the tetracycline repressor, was constructed by using a pair of synthesized complementary oligomers, SALTETB1 and SALTETB2, defined in Table 1. They were annealed to form a fragment, containing *Bam*HI and *Nco*I sticky ends, which was ligated into the large *Bam*HI-*Nco*I fragment of pCB258 (13).

Plasmid pGG71, a derivative of plasmid pCBSal that contained *tetK*, was constructed in two steps. First, *tetK* in the M13 bacteriophage MC71 was mutagenized with the

TABLE 1. Oligomers for genetic constructs

Oligomer	Sequence
SALTETB1	5′-GATCCATAGAGAAAGTCGACATGAACTCGAGTACAAAGATCGCATTGGTAATTACGTTACTCGATGC-3′
SALTETB2	5′-GTATCTCTTTCAGCTGTACTTGAGCTCATGTTTCTAGCGTAACCATTAATGCAATGAGCTACGGTAC-3′
PT1813	5′-CCTCAAGTAAAGAGGTCGACATGTTTAGTTTAG-3′
PT1814	5′-AAGAGGTCGACTTGTTTAGTTAAG-3′
PBRTET1	5′-TCAGGCACCGGTCGACATGAAATCTAACAATGCGC-3′
PBRTET3	5′-GTGCGCATAGAAATTGC-3′
TNTET8	5′-CATTAACGCATAAAGTGCTAGCAATACGCCAAAGTG-3′

oligomer PT1814 (defined in Table 1) by site-directed mutagenesis as described by Kunkel et al. (26) by introducing a *SalI* restriction site immediately preceding the TTG start codon. The RFI phage DNA containing the modified *tetK* gene was digested with *SalI-Hin*dIII and the 2.0-kb fragment was ligated into the large *SalI-Hin*dIII fragment of pCBSal. The resulting construct was designated pGG71.

Plasmid pGG57 is isogenic to plasmid pGG71, except that it contains an additional modification that changes the start codon of *tetK* from TTG to ATG. Plasmid pGG57 was constructed in the same way as pGG71 was, but with oligomer PT1813 (Table 1).

To construct plasmid pGG58, a *SalI* site was introduced 5′ to the ATG start codon within a fragment of *tetA*(C) of plasmid pBR322 by PCR with oligomers PBRTET1 and PBRTET3 (Table 1). The 200-bp amplified fragment was cloned, and the resulting plasmid was digested with *SalI-NheI* to generate a 149-bp fragment containing the 5′ end of *tetA*(C). A 1,196-bp fragment containing the 3′ end of *tetA*(C) was obtained by digesting pBR322 first with *AvaI*, filling in with the Klenow fragment, and then digesting with *NheI*. The two fragments of *tetA*(C) were ligated to the large fragment of pCBSal that had been digested with *Hin*dIII, filled in with the Klenow fragment, and then digested with *SalI*.

Selection for an up mutant of a strain carrying *tetA*(C) of pBR322. A spontaneous mutant was selected by growing MC1061(pGG58) on L agar containing 50 μg of ampicillin per ml and 100 μg of tetracycline per ml and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The tetracycline-resistant strain was found to carry a plasmid, designated pGG75, that contained an up mutation. DNA sequencing revealed a change of G to T seven nucleotides after the transcriptional start site of *tetA*(C) but no change in the *tetA*(C) structural gene.

MIC studies. *E. coli* MC1061 containing plasmid pCBSal, pGG57, pGG71, or pGG75, was tested for tetracycline resistance by diluting an overnight culture 1:50 into L broth (29) containing 50 μg of ampicillin per ml. Exponentially growing cells were diluted in 0.85% saline, and 200 to 500 cells were inoculated onto L agar containing 50 μg of ampicillin per ml (pH 7), IPTG at concentrations ranging from 0 to 2 mM, and 0 to 300 μg of tetracyclines (tetracycline, chlortetracycline, oxycycline, doxycycline, 6-demethyl-6-deoxytetracycline, or minocycline) per ml. The concentrations of tetracyclines tested were at 1-μg/ml increments between 1 and 10 μg/ml, 5-μg/ml increments between 10 and 20 μg/ml, 10-μg/ml increments between 20 and 100 μg/ml, and 25-μg/ml increments between 100 and 300 μg of tetracyclines per ml. Agar plates were incubated at 37°C for 18 h, and the MIC was determined as the dose of tetracyclines that prevented at least 90% of the cells from forming colonies.

To test for the resistance of preinduced cultures, cells were grown as described above, except that IPTG was included in liquid cultures at the same concentration as that in agar medium which contained tetracycline.

To test resistance to other tetracycline derivatives without preinduction (Table 2), the LB agar contained the indicated drug and 0.1 mM IPTG for strain MC1061(pCBSal), 0.5 mM IPTG for MC1061(pGG75), and 0.02 mM IPTG for MC1061(pGG57). For preinduction experiments (Table 2), broth and agar medium contained 0.4 mM IPTG for MC1061(pCBSal), 0.4 mM IPTG for MC1061(pGG75), and 0.03 mM IPTG for MC1061(pGG57). Experiments were repeated at least three times, and MICs were usually identical. The median MICs are reported for all experiments.

RESULTS

In order to compare different tetracycline resistance proteins, it was important to express each resistance gene in the same host strain. To this end, an inducible, low-level expression system was constructed that facilitated cassette-type substitution of tetracycline resistance genes (Fig. 1). By using this expression system, the maximal resistance capacity for efflux proteins could be determined, the abilities of pumps to efflux different tetracycline derivatives could be assessed, and the potential lethal effects of overexpression of efflux pumps (13) could be avoided.

Regulated expression of tetracycline resistance genes. To measure the expression of tetracycline resistance genes, the

TABLE 2. MICs for *E. coli* MC1061 expressing various resistance genes

Induction and drug	MIC (90% lethal dose [μg/ml]) for *E. coli* MC1061 expressing the following resistance gene:		
	tetA(B)[a]	*tetA*(C)[b]	*tetK*[c]
Without preinduction			
Tetracycline	125	100	125
Minocycline	8	6	3
Anhydrotetracycline	3	2	2
Oxytetracycline	>300	300	250
Doxycycline	40	20	7
Chlortetracycline	50	40	40
6-Demethyl-6-deoxytetracycline	20	20	7
Preinduction			
Tetracycline	275	175	175
Minocycline	30	10	5
Anhydrotetracycline	5	3	3
Doxycycline	40	40	20
Chlortetracycline	80	70	70
6-Demethyl-6-deoxytetracycline	30	30	9

[a] *tetA*(B) originating from transposon Tn*10* contained in plasmid pCBSal.
[b] *tetA*(C) originating from plasmid pBR322 contained in plasmid pGG75.
[c] *tetK* originating from *S. aureus* contained in plasmid pGG57.

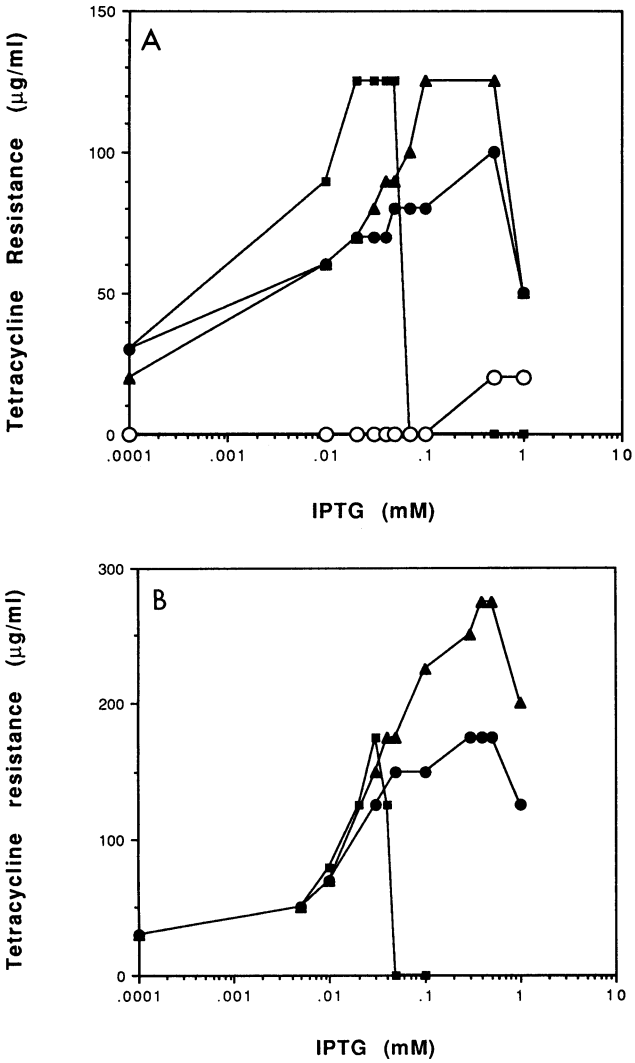

FIG. 2. Tetracycline resistance profile conferred by the plasmids pCBSal, pGG57, pGG75, and pGG71 in *E. coli* MC1061. Cells were tested for tetracycline resistance after they were grown in liquid medium, and the MIC (90% lethal dose) was determined as described in the text. (A) No preinduction. (B) Preinduction. Resistance determinant designations: ■, pGG57 (*tetK* with ATG start codon); ○, pGG71 (*tetK* with TTG start codon); ●, pGG75 [*tetA*(C)]; ▲, pCBSal [*tetA*(B)].

MIC of tetracycline for *E. coli* MC1061 containing plasmid pCBSal, pGG57, pGG71, or pGG75 was determined as a function of the inducer concentration. Cells were grown in the absence of IPTG and were then challenged with tetracycline in the presence of various IPTG concentrations (Fig. 2A). Strain MC1061(pGG57), containing the *tetK* gene from *S. aureus*, was very resistant to tetracycline; the maximum MIC for this strain in the presence of 0.02 mM IPTG was 125 μg/ml. However, a higher concentration of inducer did not lead to greater resistance. In fact, when the inducer concentration was increased above 0.07 mM, overexpression led to the loss of viability (Fig. 2A). Thus, when expression was carefully regulated, the *tetK* gene mediated strong resistance to tetracycline in *E. coli*, equivalent to the resistance mediated by the *tetA*(B) gene from transposon Tn*10*. However, strain MC1061(pGG71), which also contained the *tetK* gene, but with its native TTG start codon (Fig. 1), was only weakly tetracycline resistant (Fig. 2A). Therefore, the native TTG start codon prevented strong tetracycline resistance in MC1061(pGG71).

Whereas the maximum MIC for strain MC1061 carrying the *tetK* or *tetA*(B) gene was 125 μg of tetracycline per ml, the maximum MIC for strain MC1061(pGG58) containing the *tetA*(C) gene from plasmid pBR322 was only 20 μg of tetracycline per ml, even when 2 mM IPTG was added to the growth medium as an inducer (data not shown). Therefore, a mutant that carried plasmid pGG75, which was found to contain a lesion outside of the *tetA*(C) structural gene, as described in Materials and Methods, was selected. For MC1061(pGG75), which contained the wild-type *tetA*(C) gene from pBR322, the maximum MIC was 100 μg/ml (Fig. 2A). Thus, the capacity of the TetA(C) efflux protein was less than that of the TetA(B) or TetK efflux protein. When the concentration of IPTG exceeded the amount that induced maximum tetracycline resistance, then overexpression of *tet* genes on plasmids pCBSal or pGG75 resulted in cell toxicity, which was observed by a decreased growth rate (data not shown) and which is consistent with previous results for the *tetA*(B) of transposon Tn*10* (9, 13, 34, 35) and with our results with the *tetK* gene.

Figure 2A shows the resistance capabilities when the efflux systems were not induced before they were challenged with tetracycline, similar to the natural situation in which *tet* genes are turned off until cells encounter tetracycline. However, when cells simultaneously encountered the inducer and tetracycline, then inhibition of protein synthesis by tetracycline could have interfered with the induction of efflux proteins.

To eliminate the possibility that establishment of resistance affects MICs, cells were preinduced with IPTG prior to exposure to tetracycline. Then, the maximum resistance capacity mediated by each *tet* gene could be determined (Fig. 2B). The maximum MIC for strain MC1061(pGG75) containing *tetA*(C) was 175 μg of tetracycline per ml. As a comparison, the MIC of strain MC1061(pBR322), in which the *tetA*(C) gene is constitutively expressed, was 80 μg/ml, indicating that the *tetA*(C) gene was capable of mediating stronger resistance when it was optimally expressed by using this inducible system. Similarly, the maximum MIC for strain MC1061(pCBSal) containing *tetA*(B) was 275 μg of tetracycline per ml, whereas the MIC was 125 μg/ml for strain MC1061 carrying the transposon Tn*10* that was preinduced with 5 μg of tetracycline per ml. Again a twofold increase in the MIC was observed. The maximum MIC for strain MC1061(pGG57) containing the *tetK* gene was 175 μg/ml, which was comparable to that for MC1061(pGG75) [containing *tetA*(C)] but less than that for MC1061(pCBSal) [containing *tetA*(B)]. Therefore, the TetA(B) protein, which is encoded by transposon Tn*10*, conferred the strongest resistance to tetracycline in preinduced cells when establishment of resistance was not a factor.

Substrate specificities of tetracycline efflux pumps. Previous studies suggested that distinct classes of tetracycline efflux proteins vary in their ability to recognize and efflux different tetracycline analogs (5a, 8, 32, 40a). Among clinical isolates, the TetA(B) protein is the only efflux pump that is capable of conferring resistance to minocycline. Our strategy in reexamining the problem of substrate specificity was to use tetracycline resistance resulting from production of the TetA(B), TetA(C), or TetK protein in an isogenic host strain as the standard of comparison. Resistance to other tetracyclines could be measured by using the IPTG concentration

that induced maximum resistance to tetracycline. Alternatively, the isogenic host could be made equally resistant to tetracycline by adjusting the concentration of IPTG. If the same induction conditions were applied, any differences in resistance to other tetracycline derivatives could be attributed to the resistance protein and not to differences in gene expression or variables caused by strain differences.

When cells were not preinduced with IPTG, the TetA(B) and TetK proteins conferred equivalent levels of resistance to tetracycline (Table 2). Both proteins conferred strong resistance to tetracycline, oxycycline, and chlortetracycline, and both conferred weak resistance to anhydrotetracycline. However, the TetK protein was less capable of conferring resistance to minocycline, doxycycline, and 6-demethyl-6-deoxytetracycline. To underscore these differences, the resistance conferred by the TetK protein was compared with the resistance conferred by the TetA(C) protein. Again, the same weakness or incapacity of the TetK protein was detected. The TetK protein was less able to confer resistance to minocycline, doxycycline, and 6-demethyl-6-deoxytetracycline, even though the TetK protein conferred stronger resistance to tetracycline compared with the TetA(C) protein. In contrast, the ancestrally related proteins TetA(B) and TetA(C) exhibited similar capacities to confer resistance to other tetracyclines.

The resistances conferred by the Tet proteins were also compared in preinduced cells, and the same pattern was observed (Table 2). When IPTG was added to growing cultures prior to drug challenge, the TetK and TetA(C) proteins conferred equivalent levels of resistance to tetracycline, chlortetracycline, and anhydrotetracycline. However, the TetK protein was deficient in conferring resistance to minocycline, 6-demethyl-6-deoxytetracycline, and doxycycline. The TetA(B) protein conferred stronger resistance than the TetK protein to all tetracyclines, but particularly to doxycycline, 6-demethyl-6-deoxytetracycline, and minocycline. When the resistances mediated by the TetA(B) and TetA(C) proteins were compared, for no particular derivative was there a suggestion of a significant difference in substrate specificity, except possibly for minocycline.

It has been reported that the TetA(B) protein confers stronger resistance than the TetA(C) protein to minocycline (8, 32). To test whether this difference was attributable to a difference in substrate specificity, an effort was made to moderate the expression of the *tetA*(B) gene in strain MC1061(pCBSal) by reducing the IPTG concentration in agar medium to 0.04 mM IPTG. This partial induction of *tetA*(B) reduced the MIC to 100 μg of tetracycline per ml for cells that were not preinduced, which was comparable to the tetracycline resistance of the host strain expressing the *tetA*(C) gene (Table 2). When the *tetA*(B) gene was partially induced with 0.04 mM IPTG, the MIC was 7 μg of minocycline per ml for strain MC1061(pCBSal), which is close to the MIC of 6 μg minocycline per ml for the host expressing the *tetA*(C) gene without preinduction (Table 2). Similarly, for preinduced cells, when strain MC1061(pCBSal) was grown and tested in medium containing just 0.04 mM IPTG, the MIC was reduced to 175 μg of tetracycline per ml and 10 μg of minocycline per ml. These MICs are the same as those found for MC1061(pGG75) when the *tetA*(C) gene was fully preinduced (Table 2). In summary, there is little difference in the substrate specificities of the related TetA(B) and TetA(C) proteins, but there is a marked difference in the ability of the TetK protein to expel doxycycline, 6-demethyl-6-deoxytetracycline, and minocycline.

DISCUSSION

The resistance conferred by diverse tetracycline efflux proteins was compared in the same *E. coli* host strain by using a regulated, low-level expression system. The utility of the system in achieving optimal expression is suggested by the fact that a twofold increase in the maximum MIC of tetracycline was observed when strain MC1061(pCBSal) containing *tetA*(B) was compared with strain MC1061 containing the transposon Tn*10* determinant. Similarly, a twofold increase in MIC was observed for MC1061(pGG75) containing *tetA*(C) compared with the MIC for MC1061 (pBR322). Most important, the system was useful in expressing the *tetK* gene from *S. aureus* in *E. coli*, so that direct comparisons between the gram-positive and gram-negative efflux proteins could be made.

In constructing the expression system, several nucleotide changes were introduced into the parent plasmid pCB258, resulting in decreased expression of *tetA*(B). In the absence of IPTG, MC1061(pCB258) was more resistant than MC1061(pCBSal) to tetracycline and less IPTG was required to observe the toxicity caused by overexpression in MC1061(pCB258) (data not shown). The native *tetA*(B) gene is expressed at a low rate (16), despite a high rate of transcription of the gene (5). It is likely that translation of the native gene is inefficient, consistent with the fact that the Shine-Dalgarno sequence of the gene diverges from the consensus sequence. The changes in our expression system may have further diminished translation of the *tetA*(B) mRNA. In any case, the expression system provided a convenient means for constructing plasmids with different *tet* genes and reproducible means for inducing *tet* genes to their maximal capacities by modulating the IPTG concentration.

It was interesting that different concentrations of IPTG were required to induce the three resistance genes, even though the DNA sequences upstream of the start codons were identical. The copy number of the different plasmids pCBSal, pGG57, pGG58, and pGG75 within host strain MC1061 was approximately the same, as measured by agarose gel electrophoresis (data not shown). One possibility is that the genes are translated with different efficiencies and that the sequence downstream of the start codon plays a significant role in translation initiation. It has been suggested that the first 12 nucleotides of the structural gene can influence translational efficiencies (14). Sequences within the structural gene may play a more important role when a weak Shine-Dalgarno sequence precedes the structural gene.

In nature, *tet* genes are regulated, and the strongest resistance is observed when cells are preinduced with tetracyclines (7a, 32). In our expression system, maximum resistance also occurred following preinduction with the inducer IPTG (compare Fig. 2A and B). By determining the maximum level of resistance, it was possible to show that the TetK protein of *S. aureus* and the TetA(B) protein of transposon Tn*10* are equally capable of pumping out tetracycline in uninduced cells and that the TetA(C) protein of plasmid pBR322 is less capable of doing so. However, when cells were preinduced with IPTG, to eliminate the possibility that establishment of resistance contributed to the comparisons, the TetB protein clearly conferred the strongest resistance.

The TetA(B) protein may have a higher capacity for expelling tetracycline. Alternatively, more TetA(B) pumps may be present in MC1061 carrying the *tetA*(B) gene than in MC1061 carrying *tetA*(C) or *tetK*. The number of pumps is

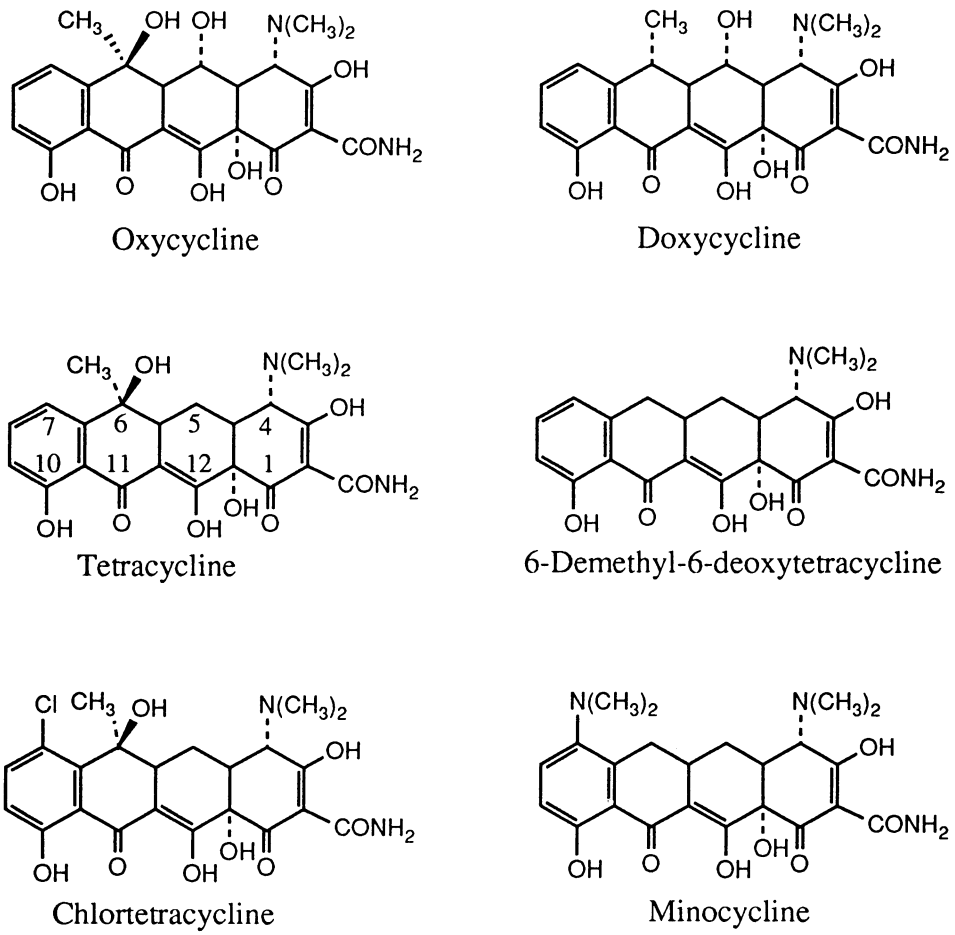

FIG. 3. Structures of six of the tetracyclines tested for their MICs (Table 2). The tetracyclines on the left were recognized equally by the TetK, TetA(B), and TetA(C) pumps. The TetK pump was deficient in effluxing the tetracyclines on the right.

limited by the toxicity associated with each pump. It is possible that the TetA(B) protein is better tolerated than the TetA(C) and TetK proteins, since all three proteins are toxic to the host if they are overexpressed. It is interesting that both the TetA(C) protein (10) and the TetK protein (15) have the capacity to act as potassium permeases, whereas the TetA(B) protein does not exhibit this capacity (10). It is possible that the uptake of potassium is a function related to the uptake of a proton, or hydronium ion, which energizes the active efflux of tetracycline. Perhaps the TetK and TetA(C) proteins are more leaky for cation uptake, contributing to their inefficiency in expelling tetracycline and/or contributing to their lethality.

It is interesting to compare these determinants for resistance to other tetracycline derivatives when their resistances to tetracycline were equivalent. We found that apparent substrate-specific differences between the TetA(B) and TetA(C) proteins (32) disappeared when expression of the two pumps was normalized in terms of tetracycline resistance. The TetA(B) protein uniquely confers clinically significant resistance to minocycline (32) because the TetA(B) protein is a better pump for all substrates. It does not have high specificity for minocycline comparison with the TetA(C) protein. The TetK protein, in contrast, is impaired in its capacity to efflux some tetracycline derivatives. It conferred high-level resistance to tetracycline, oxycycline, and chlortetracycline but markedly less resistance to minocycline, 6-demethyl-6-deoxytetracycline, and doxycycline (Table 2). On the basis of the fact that the same strain was the host for each efflux pump, we conclude that TetK is deficient in recognizing and/or transporting these tetracyclines. The common feature of these analogs is the lack of a hydroxyl substituent at the 6 position (Fig. 3). The TetK protein may require this substituent for maximal efflux of tetracyclines. The fact that minocycline is so effective against *S. aureus* strains that carry the *tetK* determinant (5a, 40a) is attributable to the inability of the TetK protein to pump the substrate out of the bacterial cytoplasm.

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