Characterization of a Novel Tetracycline Resistance That Functions Only in Aerobically Grown *Escherichia coli*

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A tetracycline resistance (*Tc^r) gene that was found originally on two *Bacteroides* plasmids (pBF4 and pCP1) confers tetracycline resistance on *Escherichia coli*, but only when it is grown aerobically. Using maxicells, we have identified a 44-kilodalton protein which is encoded by the region that carries the *Tc^r gene and which may be the *Tc^r gene product. Localization experiments indicate that this 44-kilodalton protein is cytoplasmic. To determine whether the tetracycline resistance gene is expressed under anaerobic conditions, we have constructed a protein fusion between the *Tc^r gene and *lacZ*. In strains of *E. coli* carrying the fusion, β -galactosidase activity was the same when the cells were grown under anaerobic conditions as when the cells were grown under aerobic conditions. This indicates that the tetracycline resistance gene product is made under anaerobic conditions but does not work. The failure of the *Tc^r protein to function under anaerobic conditions was not due to a requirement for function of the anaerobic electron transport system, because neither nitrate nor fumarate added to anaerobic media restored tetracycline resistance. Inhibition of the aerobic electron transport system with potassium cyanide did not prevent growth on tetracycline of cells containing the *Tc^r gene. A heme-deficient mutant, *E. coli* SHSP19, which carries the *Tc^r gene, was still resistant to tetracycline even when grown in heme-free medium. These results indicate that functioning of the *Tc^r gene product is not dependent on the aerobic electron transport system. Thus the requirement for aerobic conditions appears to reflect a requirement for oxygen. Spent medium from an *E. coli* strain carrying the *Tc^r gene, which was grown in medium containing tetracycline (50 μ g/ml), did not inhibit growth of a tetracycline-susceptible strain of *E. coli*. Thus, the *Tc^r gene product may be detoxifying tetracycline.

Guiney and co-workers (6) have found an unusual tetracycline resistance gene on the *Bacteroides* conjugal plasmid pBF4. The same gene has also been found on another *Bacteroides* plasmid, pCP1 (also called pBFTM10) (6, 27). This gene confers tetracycline resistance on *Escherichia coli*, but only when the cells are grown aerobically. Although this resistance gene was found on a *Bacteroides* plasmid, it does not confer tetracycline resistance on *Bacteroides* spp. (6).

Using Tn5 insertional mutagenesis, Mathews and Guiney (15) localized the tetracycline resistance gene on pCP1 to a 1-kilobase (kb) region within a 4.4-kb *Eco*RI restriction fragment that also contains a gene which codes for resistance to erythromycin and clindamycin. On pBF4, the tetracycline resistance gene lies on a slightly smaller 3.8-kb *Eco*RI fragment which also carries the erythromycin-clindamycin resistance gene (6, 29). The 3.8-kb *Eco*RI fragment of pBF4 is part of a transposable element, Tn4351 (21, 23). Similarly, the 4.4-kb *Eco*RI fragment on pBFTM10 is part of a closely related transposable element, Tn4400 (17). Smith and Gonda (24) have shown by heteroduplex and restriction mapping that these two transposons are more than 90% homologous. Guiney et al. (7) have shown that the tetracycline resistance gene on Tn4400 does not cross-hybridize on Southern blots with the tetracycline resistance genes found on pBR322, R100, and RK2. Also, the resistance genes on pBR322, R100, and RK2 confer resistance on *E. coli* regardless of whether it is grown aerobically or anaerobically. The tetracycline resistance gene from Tn4351 and Tn4400 has been designated *Tc^r to distinguish it from the tetracycline resistance genes (Tc^r) on pBR322, R100, and RK2.

Failure of the *Tc^r gene to confer resistance on anaerobi-

cally grown *E. coli* could occur because the *Tc^r gene is not transcribed or translated under anaerobic growth conditions. Alternatively, the *Tc^r gene product may be made in anaerobically grown cells but does not function. To determine which hypothesis is correct, we have constructed a *Tc^r-LacZ fusion and used this fusion to test the effect of anaerobiosis on gene expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A list of the strains and plasmids used in this study is given in Table 1. For routine culturing of cells, Luria broth (LB) or LB agar was used. Ampicillin and chloramphenicol were added to concentrations of 50 and 20 μ g/ml, respectively. Unless otherwise indicated, the concentration of tetracycline in broth or plates was 10 μ g/ml. For aerobic growth experiments, cells were inoculated into LB (5 or 10 ml) containing the antibiotics indicated and incubated at 37°C with shaking. In some experiments glucose (20 mM) was also added to LB (LB-glucose). For anaerobic growth experiments, LB (made in 0.1 M potassium phosphate buffer, pH 7.0) was first boiled to drive off oxygen and then dispensed in 10-ml portions into tubes under a stream of oxygen-free 90% nitrogen-10% carbon dioxide. Tubes were sealed with stoppers and autoclaved. Glucose (20 mM) and antibiotics were injected through the stoppers with syringes. Cultures were incubated at 37°C without shaking. In some experiments, cells were plated on 1% lactose-McConkey agar (Difco Laboratories, Detroit, Mich.).

DNA isolation and analysis. Plasmids were isolated from *E. coli* strains by the Ish-Horowitz modification of the method of Birnboim and Doly (14). Restriction digests and ligation with T4 DNA ligase were done as described by the manufacturer. Restriction digests were electrophoresed for 10 to

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Source or reference
<i>E. coli</i>		
Em24	RecA ⁻ Str ^r	J. Cronan; RecA Str ^r derivative of LE392 (14)
MBM7060	$\Delta(\arg F-lac)U169$ <i>trp</i> (Am)	T. Silhavy; derivative of MBM7014 (1)
MC1000	$\Delta lacX74$	T. Silhavy (1)
LCD12	$\Delta recA \Delta metE$ Tn10 Tc ^r	J. Cronan; derivative of R4445
MCS100	$\Delta recA \Delta metE$ Tc ^s	This study; Tc ^s derivative of LCD12 made Tc ^s by a modification of the fusaric acid selection method of Maloy and Nunn (13)
M2508	Lac ⁺ Δmel	20
SHSP19	HemA8	19
Plasmids		
pBR328	Ap ^r Cm ^r Tc ^r	4
pBR328 Δ Tc	Ap ^r Cm ^r Tc ^s	Tc ^s derivative of pBR328 obtained by digestion of pBR328 with a small 0.35-kb deletion within the <i>Bam</i> HI site (Colin Getty [22])
pNIL5	Su ^r *Tc ^r Cm ^r	3.8-kb <i>Eco</i> RI fragment of Tn4351 from pBF4 cloned into <i>Eco</i> RI site of RSF1010 (22)
pBS1	Ap ^r *Tc ^r	3.8-kb <i>Eco</i> RI fragment from pNIL5 cloned into <i>Eco</i> RI site of pBR328 Δ Tc; this study (Fig. 1)
pBS5	Ap ^r *Tc ^r	<i>Hind</i> III deletion of pBS1; this study (Fig. 1)
pBS9	Ap ^r *Tc ^s	<i>Cl</i> AI deletion of pBS1; this study (Fig. 1)
pBS2	Apr ^r *Tc ^r	3.8-kb <i>Eco</i> RI fragment from pNIL5 cloned into <i>Eco</i> RI site of pBR328 Δ Tc orientation is opposite of pBS1; this study (Fig. 1)
pBS4	Ap ^r *Tc ^s	<i>Hind</i> III deletion of pBS2; this study (Fig. 1)
pBS6	Ap ^r *Tc ^s	<i>Ava</i> I deletion of pBS2; this study (Fig. 1)
pBS10	Ap ^r *Tc ^s	<i>Cl</i> AI deletion of pBS2; this study (Fig. 1)
pMLB1034	Ap ^r * <i>lacZ</i>	1
pBS134	Ap ^r *Tc ^s LacZ ⁻	3.8-kb <i>Eco</i> RI fragment from pBS1 cloned into <i>Eco</i> RI site of pMLB1034; this study (Fig. 3)
pMLB1034H	Ap ^r * <i>lacZ</i>	<i>Hind</i> III linker site cloned into <i>Sma</i> I site of pMLB1034; this study (Fig. 3)
pBS134H9	Ap ^r *Tc ^s LacZ ⁺	0.9-kb <i>Eco</i> RI- <i>Hind</i> III fragment from pBS9 cloned into <i>Eco</i> RI- <i>Hind</i> III site of pMLB1034H; this study (Fig. 3)
pBS134H10	Ap ^r *Tc ^s LacZ ⁻	1.6-kb <i>Hind</i> III fragment from pBS10 cloned into <i>Hind</i> III site of pMLB1034H; this study (Fig. 3)

^a Antibiotic resistance abbreviations: Ap, ampicillin; Cm, chloramphenicol; Str, streptomycin; Su, sulfa; Tc, tetracycline.

15 h on 1% agarose slab gels with 4× GGB (4× GGB is 0.16 M Tris, 0.08 M sodium acetate, 8 mM EDTA [pH 8.3]). Gels were stained with ethidium bromide and photographed.

Construction of a *Tc^r-LacZ fusion. To obtain a *lacZ* fusion to the tetracycline resistance gene (*Tc^r) we used the *lacZ* fusion vector pMLB1034. The *lacZ* gene on pMLB1034 lacks a promoter, ribosome-binding site, and start codon (1); therefore it must be fused with another gene to confer the Lac⁺ phenotype. Each half of the *Tc^r gene was cloned upstream of the *lacZ* gene in the vector pMLB1034 (1, 28). Selection for spontaneous deletion mutants which produced Lac⁺ papillae was done on MacConkey lactose agar. β -Galactosidase activities of Lac⁺ clones were assayed as described by Miller (16). In one experiment, we attempted to detect the fusion proteins by electrophoresis on 8% sodium dodecyl sulfate-polyacrylamide gels (1, 10).

Transformation. Preparation of competent cells and transformation of the *E. coli* with plasmid DNA was performed as described by Lederberg and Cohen (11).

Determination of tetracycline resistance. For all experiments the inoculum was grown in medium, aerobic or anaerobic, which had the same composition as that used for the growth determination. Ampicillin (50 μ g/ml) was included to ensure plasmid maintenance. The MIC of tetracycline was determined by the tube dilution technique. Cells (0.05 ml) from overnight cultures were inoculated into 5 ml of LB, prepared aerobically or anaerobically, to which different concentrations of tetracycline had been added.

Tubes were then incubated at 37°C for 8 to 12 h. To test for tetracycline resistance in all other experiments, cultures were incubated at 37°C with shaking and the optical density (600 nm) was determined at 6- to 8-h intervals until there was no change in optical density or a maximum of 1.5 was reached.

Maxicells. The maxicell procedure was performed as described by Sancar et al. (18) with *E. coli* MCS100 as a host. Proteins from maxicells were separated by electrophoresis on 11% highly cross-linked sodium dodecyl sulfate-polyacrylamide gels as described by Hashimoto et al. (8).

The cell fractionation method of Tai and Kaplan (26) was used to localize the *Tc^r gene product in maxicells. The periplasmic, cytoplasmic, and membrane fractions were collected and electrophoresed on 11% highly cross-linked sodium dodecyl sulfate-polyacrylamide gels as described above.

All gels were stained with Coomassie brilliant blue, destained, dried, and then placed in a cassette with flashed X-ray film for 3 to 5 days. The molecular weight standards were 92,000, 66,200, 45,000, 31,000, 21,500, and 14,400 (Bio-Rad Laboratories, Richmond, Calif.).

Possible contribution of the electron transport system to tetracycline resistance. To determine whether the electron transport system was required for the functioning of the *Tc^r gene product, we altered growth conditions to affect the functioning of the electron transport chain. To test whether cells could become resistant to tetracycline under anaerobic

FIG. 1. Localization of the **Tc^r* gene within the 3.8-kb *EcoRI* fragment of Tn4351 (—) within pBR328ΔTc (—). Clones are grouped according to orientation of the *EcoRI* fragment. pBS4 and pBS5 were obtained by deleting a *HindIII* fragment from pBS2 and pBS1, respectively. pBS6 was derived by deleting an *AvaI* fragment from pBS2. pBS9 and pBS10 resulted from the deletion of a *Clal* fragment from pBS1 and pBS2, respectively. Antibiotic resistances for the clones are shown at the right.

conditions if electron acceptors were present, we grew cells in anaerobic LB-glucose, with or without added tetracycline, supplemented with nitrate (40 mM, pH 7) or fumarate (40 mM, pH 7) (9, 25). Growth was measured at 6-h intervals for 18 h.

In another set of experiments, we inhibited the aerobic electron transport chain by growing *E. coli* EM24, carrying pBS2 or pBR328, on LB-glucose which contained tetracycline and potassium cyanide (0.25 mM). We also tested the ability of the **Tc^r* gene product to confer resistance on a heme-deficient mutant, *E. coli* SPSH19 (*hema8*) (19). This strain was transformed with pBS2 or pBS134, both of which carry the **Tc^r* gene. Cells containing these plasmids were maintained on LB to which either δ -aminolevulinic acid (50 μ g/ml) or glucose (40 mM) had been added. In all experiments involving this strain, new glassware was used to minimize heme contamination. To test the level of tetracycline resistance, cells were grown on M9 minimal medium (14) supplemented with glucose (40 mM), Casamino Acids (20 mM; Difco), magnesium sulfate (1 mM), and thiamine (1 μ M). In some cases, tetracycline or δ -aminolevulinic acid (50 μ g/ml) or both were added to the medium.

Tetracycline detoxification assay. To determine whether tetracycline was being inactivated by cells containing the **Tc^r* gene, culture supernatant was obtained from cultures of *E. coli* EM24(pBS2) which had been grown on LB plus 50 μ g of tetracycline per ml. This supernatant was filtered through a 0.2- μ m membrane filter (Millipore Corp., Bedford, Mass.) and then tested for its ability to inhibit growth of tetracycline-susceptible (*Tc^s*) *E. coli* EM24. Filtered spent medium was diluted so that the final concentration of tetracycline

would be 40, 30, 20, or 10 μ g/ml, provided none of the antibiotic had been degraded. For the experiments reported here, the dilutions were made with M9 salts. However, in some experiments, spent medium from *E. coli* EM24 grown in LB was used instead of M9 salts, and the results were the same. The final volume of the dilutions was 4 ml. One milliliter of 5 $\times$ LB was added as a source of nutrients. Each tube was then inoculated with 0.05 ml of a fresh LB culture of *Tc^s* *E. coli* EM24. After 8 h of incubation at 37°C with shaking, the optical density (600 nm) was measured. As a control, filtered supernatant was obtained from *E. coli* EM24(pBR328) which had been grown on LB-tetracycline (50 μ g/ml). As a second control, to make sure tetracycline was not inactivated by long periods of aeration at 37°C, we added the same amount of tetracycline to LB, inoculated it with *Tc^s* *E. coli* EM24, and incubated it in parallel with the other cultures. Finally, to make sure that *E. coli* EM24 was not inhibited by some component of the filtered spent medium other than tetracycline, 4 ml of filtered spent medium from *E. coli* EM24 grown on LB was supplemented with 1 ml of 5 $\times$ LB and inoculated with *E. coli* EM24. Growth was normal.

RESULTS

Maxicell experiments. The 3.8-kb *EcoRI* fragment from Tn4351 which contained the **Tc^r* gene was cloned, in both orientations, into the *EcoRI* site of pBR328ΔTc, yielding pBS1 and pBS2. To further localize the gene we made a series of deletions. The resulting subclones and their tetracycline resistances are shown in Fig. 1. These deletions localized the gene to a 2.5-kb region of DNA. A 44-kilodalton (kDa) protein was seen only in maxicell extracts which contained pBS1, pBS2, and the other tetracycline resistant clones but not in *Tc^s* clones (Fig. 2). Thus the 44-kDa protein could be the **Tc^r* gene product.

It is interesting to note that in strains containing pBS1, pBS5, and pBS9, all of which have the 3.8-kb *EcoRI* fragment cloned in the same orientation, there was a marked decrease in the amount of β -lactamase being produced (Fig. 2). This decrease was seen repeatedly in separate experiments. Despite the lower level of β -lactamase, the MIC of ampicillin for these strains was the same as that for strains containing pBS2 and the other clones depicted in Fig. 1.

The 44-kDa protein that was associated with **Tc^r* expression was localized by the fractionation method of Tai and Kaplan (26). The proteins encoded by pBR328 were localized correctly during the maxicell procedure and subsequent fractionation; that is, β -lactamase was found in the periplas-

FIG. 2. Autoradiogram of cell extracts from maxicells containing the clones depicted in Fig. 1. Lanes 1 through 7 contain pBS1, pBS5, pBS9, pBS2, pBS4, pBS6, and pBS10, respectively. Migration distances (in kilodaltons) are indicated by numbers at the right. The upper arrow to the right of the gel indicates the position of the 44-kDa **Tc^r*-associated protein. The lower arrow to the right indicates the position of the *Ap^r* gene product.

FIG. 3. (A) Autoradiogram of maxicells containing pBR328. Lane 1 contains the maxicell extract from whole cells. The Tc^r gene product (top band), Ap^r gene product (middle band), and the Cm^r gene product (bottom band) are visible. Cells were fractionated into periplasmic (lane 2, Ap^r), cytoplasmic (lane 3, Cm^r), and membrane (lane 4, Tc^r) fractions. (B) Autoradiogram of maxicells containing pBS2. Lane 1 contains the maxicell extract from whole cells. The $*Tc^r$ -associated product (top band) and Ap^r gene product (from pBR328, second band) are visible. Cells were fractionated into periplasmic (lane 2, Ap^r), cytoplasmic (lane 3, $*Tc^r$), and membrane (lane 4) fractions.

mic fraction (Fig. 3A, lane 2), chloramphenicol transacetylase was found in the cytoplasmic fraction (Fig. 3A, lane 3), and the tetracycline resistance gene product was found in the membrane fraction (Fig. 3A, lane 4). When the same procedure was used to fractionate maxicells containing pBS2, the 44-kDa protein was found in the cytoplasmic fraction (Fig. 3B, lane 3). As expected, β -lactamase fractionated in the periplasmic fraction (Fig. 3B, lane 2).

Construction of a $*Tc^r$ -LacZ fusion. To determine whether the $*Tc^r$ gene is expressed under anaerobic conditions we wanted to obtain a fusion of the $*Tc^r$ gene with *lacZ*. To do this we cloned each half of the $*Tc^r$ gene in front of the *lacZ* gene in pMLB1034. The *EcoRI*-*HindIII* fragment from the $*Tc^r$ subclone pBS9 and the *HindIII* fragment from pBS10 (Fig. 1) were cloned into a derivative of the fusion vector that had a *HindIII* linker inserted into the *SmaI* site (pMLB1034H, Fig. 4); this yielded pBS134H9 and pBS134H10, respectively. The subclone pBS134H9 was Lac^+ and $*Tc^s$. The second subclone, pBS134H10, was Lac^- and $*Tc^s$. This result indicated that the pBS134H9 fusion was the $*Tc^r$ -LacZ fusion and that the direction of transcription of the $*Tc^r$ gene was toward the *HaeII* site (Fig. 4).

However, it was possible that there were two overlapping reading frames transcribed in opposite directions. If this were the case, the pBS134H10 construct could have been the correct one but was Lac^- because it was out of frame. To test this, pBS134H10 was plated on MacConkey lactose agar and incubated until Lac^+ papillae appeared. If pBS134H10 were Lac^- because it was out of frame, small deletions of a few nucleotides should put it back in frame and produce Lac^+ colonies. Two Lac^+ papillae were obtained. One fusion resulted from a deletion of 1.45 kb. The second fusion was unstable and was not studied further. Restriction analysis of the Lac^+ derivative of pBS134H10 showed that the deletion extended beyond the *HaeII* site which lies at one end of the $*Tc^r$ region (Fig. 4). This deletion extended past the site of a $*Tc^r$ *Tn5* insertion obtained by Mathews and

FIG. 4. Construction of the $*Tc^r$ -LacZ fusion. The *EcoRI*-*HindIII* region of pBS9 and the *HindIII* fragment of pBS10 were cloned into the multiple cloning site of pMLB1034H to yield pBS134H9 and pBS134H10, respectively. The *LacZ* phenotype is indicated below the plasmid name. The solid arrowhead under pBS134H10 indicates the endpoint of the deletion seen in a Lac^+ derivative of pBS134H10. The open arrow above pBS134H10 indicates the site of a *Tn5* insertion found by Mathews and Guiney (15) to be $*Tc^r$.

Guiney (15) (Fig. 4). These results indicate that the pBS134H10 construct is not a fusion with the $*Tc^r$ gene and that the pBS134H9 is the correct $*Tc^r$ -LacZ fusion.

We attempted to visualize the pBS134H9 fusion protein on Coomassie blue-stained gels, as described by Berman and Jackson (1). However, we were unable to detect the fusion protein (data not shown). This was probably due either to a low level of expression of the fusion protein or to its instability.

Expression of the $*Tc^r$ -LacZ fusion under aerobic and anaerobic conditions. β -Galactosidase activity of *E. coli* MBM7060, which contained the $*Tc^r$ -LacZ fusion plasmid pBS134H9, was the same regardless of whether cells were grown aerobically or anaerobically (Table 2). These results indicate that transcription and translation of the $*Tc^r$ gene occurs during both anaerobic and aerobic growth. Although the $*Tc^r$ gene was transcribed under anaerobic conditions, cells carrying pBS134 were not resistant to tetracycline under anaerobic conditions (Table 2). Thus, it appears that the $*Tc^r$ gene product does not confer resistance on anaero-

TABLE 2. Effect of aerobic and anaerobic growth conditions on tetracycline MIC and expression of the $*Tc^r$ gene product

Plasmid	Tetracycline MIC (μ g/ml)		β -Galactosidase activity (U) ^a	
	With O ₂	Without O ₂	With O ₂	Without O ₂
pBS134H9	3	3	91	103
pMLB1034H	3	3	12	11
pBS134	>200	3	NA	NA
M2508 ^b	3	3	1,098	935

^a All values are the means of triplicates from at least three different experiments. NA, Not applicable.

^b *E. coli* M2508 (wild-type *LacZ*) was grown in LB with isopropyl β -D-thiogalactoside.

bically grown cells because it does not function under anaerobic conditions.

Although it was shown by mapping that the Lac⁺ fusion obtained from pBS134H10 was probably to a gene other than the *Tc^r gene, the fusion joint was very close to the end of the *Tc^r gene. Because of this we also checked the β -galactosidase activity of this fusion in cells grown under aerobic and anaerobic conditions. The level of β -galactosidase activity was the same under anaerobic and aerobic growth conditions (data not shown).

Role of the electron transport system. One possible explanation for the failure of the *Tc^r gene product to confer resistance on anaerobically grown cells is that it requires a functioning electron transport system. To test this, we provided nitrate or fumarate as alternate electron acceptors during anaerobic growth. Neither nitrate nor fumarate reversed the tetracycline susceptibility of anaerobically grown cells (Table 3).

To determine whether the aerobic electron transport system had a role in resistance due to the *Tc^r gene, we used KCN (0.25 mM) to inhibit cytochrome *a₃*. We chose this concentration of KCN because it inhibited growth of *E. coli* on LB but not on LB-glucose (Table 3). This indicated that the electron transport chain had been poisoned but that cells were still able to grow by fermentation. Cells grown aerobically in medium containing KCN were still resistant to tetracycline (Table 3). From this result it appears that the aerobic electron transport system is not required for functioning of the *Tc^r gene product. To confirm this, we tested the ability of the *Tc^r gene to confer resistance on the heme-deficient mutant SHSP19 (*hema8*). This mutant is deficient in the first step of heme synthesis and cannot make heme or cytochromes unless supplied with δ -aminolevulinic acid (50 μ g/ml). *E. coli* SHSP19 grew to an optical density (600 nm) of 1.0 to 1.1 after 18 h of growth on minimal-glucose medium without added δ -aminolevulinic acid. *E. coli* SHSP19 grown in this medium was susceptible to tetracycline (10 μ g/ml). When *E. coli* SHSP19 containing pBS2 or pBS134 was grown under the same conditions in medium that contained tetracycline, it grew to an optical density of 0.6 to 0.7 after 18 h. This is comparable to the level of growth obtained when δ -aminolevulinic acid was added to the tetracycline-containing medium.

Role of aeration. Experiments showed that *E. coli* EM24

TABLE 3. Effect of inorganic electron acceptors and KCN on tetracycline resistance due to the *Tc^r gene

Growth condition	Additives ^a	Optical density (600 nm) ^b of <i>E. coli</i> EM24 carrying:		
		No plasmid	pBS2	pBR328
Anaerobic ^c	Glc	0.5	0.6	0.6
	Glc, TC	<0.05	<0.05	0.6
	Glc, TC, NO ₃	<0.05	<0.05	0.9
	Glc, TC, Fum	<0.05	<0.05	0.6
Aerobic ^d	KCN	<0.1	<0.1	<0.1
	KCN, Glc	0.7	0.7	0.6
	KCN, Glc, TC	<0.05	0.7	0.7

^a LB is the basal medium. The additives are: glucose, 20 mM (Glc); tetracycline, 10 μ g/ml (TC); nitrate, 40 mM (NO₃); fumarate, 40 mM (Fum); potassium cyanide, 0.25 mM (KCN).

^b Values are the means of duplicates from at least two different experiments.

^c Optical density shown is after 12 h of growth.

^d Optical density shown is after 14 h of growth with shaking (250 rpm).

TABLE 4. Detoxification of tetracycline by the *Tc^r gene product

Plasmid ^a	Optical density (600 nm) ^b with the following tetracycline concn ^c (μ g/ml):			
	40	30	20	10
pBS2	0.9	1.0	1.2	1.2
pBR328	<0.05	<0.05	<0.05	<0.05
None ^d	<0.05	<0.05	<0.05	<0.05

^a *E. coli* containing pBS2, pBR328, or no plasmid was grown on LB-tetracycline (50 μ g/ml) to generate spent medium which was then tested for ability to inhibit growth of *E. coli* EM24 (Tc^s). The MIC of tetracycline for *E. coli* EM24 is 3 μ g/ml.

^b Optical density of *E. coli* EM24 (Tc^s) after 8 h of growth on spent medium at 37°C with shaking (250 rpm).

^c Expected concentration of tetracycline in the spent medium if no detoxification occurred.

^d Spent medium from LB-tetracycline was inoculated with *E. coli* EM24 (Tc^s) and incubated in the same manner as the other tetracycline resistant cultures.

carrying pBS2 was only resistant to tetracycline when the cells were shaken vigorously. When the cells were inoculated into 5 ml of aerobically prepared LB or LB-glucose which contained tetracycline (10 μ g/ml) and incubated (37°C) under aerobic conditions without shaking, the optical density (600 nm) was less than 0.1 even after 24 h of incubation. Aeration at 250 rpm was required by cells containing the *Tc^r gene to obtain the high MICs shown in Table 2. Although *E. coli* EM24 carrying pBR328 grew somewhat more slowly in medium that was not shaken than in medium shaken at 250 rpm, the resistance level was not affected by the extent of aeration.

Although *E. coli* produces both catalase and superoxide dismutase, it was possible that due to the vigorous aeration required for growth, small amounts of reactive forms of oxygen present in the medium might be involved with resistance. To test this we grew *E. coli* EM24 carrying either pBS2 or pBR328 in LB and LB-glucose medium that contained tetracycline and catalase (bovine liver; 10,000 U) or superoxide dismutase (*E. coli* manganese; 1,000 U) (Sigma Chemical Co., St. Louis, Mo.). Neither enzyme affected growth of cells in medium containing tetracycline (data not shown).

Detoxification of tetracycline by the *Tc^r gene product. When cells containing the *Tc^r gene were grown in medium containing high concentrations of tetracycline (30 μ g/ml and higher), the medium acquired a brownish color and a black precipitate was observed when the cells were pelleted. This raised the question of whether the *Tc^r gene product acted by altering tetracycline. To test this, we took culture supernatant from cells grown on 50 μ g of tetracycline per ml, filter sterilized it, added nutrients, and then inoculated this medium with Tc^s *E. coli* EM24. *E. coli* EM24 (tetracycline MIC, 3 μ g/ml) was able to grow on the spent medium from cells containing the *Tc^r gene. When we did the same experiment with supernatant from cells carrying pBR328 instead of pBS2, we found that *E. coli* EM24 did not grow on the spent medium even when it had been diluted fivefold (Table 4). Tetracycline which had been incubated in uninoculated medium for 12 to 18 h was still toxic even when diluted fivefold.

DISCUSSION

Previously, Guiney et al. (6) had observed that the *Tc^r gene did not confer resistance on *E. coli* grown anaerobically in conditions under which energy was obtained by fermentation. We have shown that the cells are still susceptible to

tetracycline under anaerobic conditions when electron acceptors such as nitrate or fumarate are provided (Table 3). The results of our experiments with the *Tc^r-LacZ fusion indicate that the *Tc^r gene product is being made under anaerobic conditions at the same level as under aerobic conditions (Table 2). This indicates that the *Tc^r gene product only functions under aerobic conditions. The requirement for aerobic conditions appears to reflect a requirement for oxygen rather than an interaction between the *Tc^r gene product and the aerobic electron transport system. Support for this comes from the finding that resistance to tetracycline was not abolished by inhibiting electron transport with KCN (Table 3). Moreover, the *Tc^r gene conferred resistance on the heme-deficient mutant *E. coli* SHSP19 when it was grown under conditions that eliminated synthesis of cytochromes.

Results of the maxicell experiments show that a 44-kDa cytoplasmic protein is produced from the region which contains the *Tc^r gene. Although 44 kDa is within the size range predicted by the Tn5 localization of Mathews and Guiney (15), our maxicell experiments do not prove conclusively that this protein is the *Tc^r gene product. In other experiments, we have found that deletions beginning at the *Eco*RI site, which lies closest to the *Tc^r gene, and extending 0.5 kb toward the *Cla*I site have an effect on tetracycline resistance. Deletions in this region reduce the tetracycline MIC by twofold. They also reduce the amount of the 44-kDa protein seen in maxicells by a proportionate amount (B. Speer, unpublished data). These findings further support the identification of the 44-kDa protein as the *Tc^r gene product. Similar deletions made upstream of the LacZ fusion, pBS134H9, show a proportionate reduction in β -galactosidase activity (B. Speer, unpublished data). This finding further supports the conclusion that this fusion pBS134H9 is the desired *Tc^r-LacZ fusion.

The results of the spent-medium experiment (Table 4) indicate that tetracycline is being detoxified by the *Tc^r gene product. The fact that a change in the color of the medium is associated with detoxification of tetracycline could indicate that modification of tetracycline is the mechanism of resistance. This color change and the need for vigorous aeration could explain the requirement for oxygen. However, we cannot rule out the possibility that the cells are secreting a factor that binds tetracycline rather than modifies it. Further experiments are under way to determine whether detoxification of tetracycline is due to chemical modification by the *Tc^r gene product.

Tetracycline resistance genes have been found in both gram-negative and gram-positive bacteria, associated either with a plasmid or with the chromosome (3, 12). In all cases previously studied, with the possible exception of *tetM* and *tetN* in streptococci (2), resistance to tetracycline has been found to be due to a decrease in intracellular accumulation of the antibiotic. The mechanism is a tetracycline-specific efflux which is mediated by one or two cytoplasmic membrane proteins. At least one of these proteins is usually in the 32- to 36-kDa range (3, 5, 12). By contrast the *Tc^r gene product appears to be a cytoplasmic protein with an apparent molecular mass of 44 kDa. This resistance, conferred by the *Tc^r gene product, involves detoxification of tetracycline. Thus, the *Tc^r gene represents a novel type of tetracycline resistance.

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LITERATURE CITED

- Berman, M. L., and D. E. Jackson. 1984. Selection of *lac* gene fusions in vivo: *ompR-lacZ* fusions that define a functional domain of the *ompR* gene product. *J. Bacteriol.* **159**:750-756.
- Burdett, V. 1986. Streptococcal tetracycline resistance mediated at the level of protein synthesis. *J. Bacteriol.* **165**:564-569.
- Chopra, I., and T. G. B. Howe. 1978. Bacterial resistance to the tetracyclines. *Microbiol. Rev.* **42**:707-724.
- Covarrubias, L., L. Cervantes, A. Covarrubias, X. Soberon, I. Vichido, A. Blanco, Y. M. Kupersztock-Portnoy, and F. Bolivar. 1981. Construction and characterization of new cloning vehicles. V. Mobilization and coding properties of pBR322 and several deletion derivatives including pBR327 and pBR328. *Gene* **13**:25-35.
- Foster, T. J. 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiol. Rev.* **47**:361-409.
- Guiney, D. G., P. Hasegawa, and C. E. Davis. 1984. Expression in *E. coli* of cryptic tetracycline resistance genes from *Bacteroides* R plasmids. *Plasmid* **11**:248-252.
- Guiney, D. G., P. Hasegawa, and C. E. Davis. 1984. Homology between clindamycin resistance plasmids in *Bacteroides*. *Plasmid* **11**:268-271.
- Hashimoto, F., T. Horigome, M. Kanbayashi, K. Yoshida, and H. Sugano. 1983. An improved method for separation of low-molecular-weight polypeptides by electrophoresis in sodium dodecyl sulfate polyacrylamide gel. *Anal. Biochem.* **112**:192-199.
- Jones, H. M., and R. P. Gunsalus. 1985. Transcription of the *Escherichia coli* fumarate reductase genes (*frdABCD*) and their coordinate regulation by oxygen, nitrate, and fumarate. *J. Bacteriol.* **164**:1100-1109.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Lederberg, E. M., and S. M. Cohen. 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. *J. Bacteriol.* **119**:1072-1074.
- Lyon, B. R., and R. Skurray. 1987. Antimicrobial resistance of *Staphylococcus aureus*: genetic basis. *Microbiol. Rev.* **51**:88-134.
- Maloy, S. R., and W. D. Nunn. 1981. Selection for the loss of tetracycline resistance by *Escherichia coli*. *J. Bacteriol.* **145**:1110-1112.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Mathews, B. G., and D. G. Guiney. 1986. Characterization and mapping of regions encoding clindamycin resistance, tetracycline resistance, and a replicative fraction on the *Bacteroides fragilis* R plasmid pCP1. *J. Bacteriol.* **167**:517-521.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Robillard, N. J., F. P. Tally, and M. H. Malamy. 1985. Tn4400, a compound transposon isolated from *Bacteroides fragilis*, functions in *Escherichia coli*. *J. Bacteriol.* **164**:1248-1255.
- Sancar, A., A. M. Hack, and W. D. Rupp. 1979. Simple method for identification of plasmid-coded proteins. *J. Bacteriol.* **137**:692-693.
- Sasarman, A., M. Surdeanu, and T. Horodniceanu. 1968. Locus determining the synthesis of δ -aminolevulinic acid in *Escherichia coli* K-12. *J. Bacteriol.* **96**:1882-1884.
- Schmidt, R. 1968. Analysis of melibiose mutants deficient in α -galactosidase and thiomethylgalactoside permease II in *Escherichia coli* K-12. *J. Bacteriol.* **96**:462-471.
- Shoemaker, N. B., C. Getty, J. F. Gardner, and A. A. Salyers. 1986. Tn4351 transposes in *Bacteroides* spp. and mediates the integration of plasmid R751 into the *Bacteroides* chromosome. *J. Bacteriol.* **165**:929-936.

22. Shoemaker, N. B., C. Getty, E. P. Guthrie, and A. A. Salyers. 1986. Regions in *Bacteroides* plasmids pBFTM10 and pB8-51 that allow *Escherichia coli*-*Bacteroides* shuttle vectors to be mobilized by IncP plasmids and by a conjugative *Bacteroides* tetracycline resistance element. *J. Bacteriol.* **166**:959-965.
23. Shoemaker, N. B., E. P. Guthrie, A. A. Salyers, and J. F. Gardner. 1985. Evidence that the clindamycin-erythromycin resistance gene of *Bacteroides* plasmids pBF4 is on a transposable element. *J. Bacteriol.* **162**:626-632.
24. Smith, C. J., and M. A. Gonda. 1985. Comparison of the transposon-like structures encoding clindamycin resistance in *Bacteroides* R-plasmids. *Plasmid* **13**:182-192.
25. Spencer, M. E., and J. R. Guest. 1973. Isolation and properties of fumarate reductase mutants of *Escherichia coli*. *J. Bacteriol.* **114**:563-570.
26. Tai, S. H., and S. Kaplan. 1985. Intracellular localization of phospholipid transfer activity in *Rhodopseudomonas sphaeroides* and a possible role in biogenesis. *J. Bacteriol.* **164**:83-88.
27. Tally, F. P., D. R. Snyderman, M. J. Shimell, and M. H. Malmay. 1982. Characterization of pBFTM10, a clindamycin-erythromycin resistance transfer factor from *Bacteroides fragilis*. *J. Bacteriol.* **151**:686-691.
28. Weinstock, G. M., M. L. Berman, and T. J. Silhavy. 1983. Chimeric genetics with β -galactosidase, p. 27-64. In T. S. Papas, M. Rosenberg, and J. G. Chirikjian (ed.), *Gene amplification and analysis*. Elsevier/North-Holland Publishing Co. Inc., New York.
29. Welch, R. A., and F. L. Macrina. 1981. Physical characterization of *Bacteroides fragilis* R plasmid pBF4. *J. Bacteriol.* **145**:867-872.