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# A Tetracycline Efflux Gene on *Bacteroides* Transposon Tn*4400* Does Not Contribute to Tetracycline Resistance

BRENDA S. SPEER AND ABIGAIL A. SALYERS*

*Department of Microbiology, University of Illinois, Urbana, Illinois 61801*

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**Previously, we demonstrated that the *Bacteroides* transposon Tn*4351*, which confers tetracycline resistance only on aerobically grown *Escherichia coli*, carries a gene that codes for a tetracycline-inactivating enzyme (B. S. Speer and A. A. Salyers, J. Bacteriol. 170:1423–1429, 1988). However, Park et al. (B. H. Park, M. Hendricks, M. H. Malamy, F. P. Tally, and S. B. Levy, Antimicrob. Agents Chemother. 31:1739–1743, 1987) showed that *E. coli* carrying a closely related transposon, Tn*4400*, exhibits energy-dependent efflux of tetracycline as well as tetracycline-inactivating activity (B. H. Park and S. B. Levy, Antimicrob. Agents Chemother. 32:1797–1800, 1988). This result raised the question of whether efflux or inactivation or a combination of the two was necessary for resistance conferred by both transposons. We showed that cells carrying Tn*4351* did not exhibit the clear-cut efflux activity seen with cells carrying Tn*4400* but rather exhibited a tetracycline accumulation profile which could be explained solely on the basis of inactivation of tetracycline in the cytoplasm and rapid diffusion of altered tetracycline out of the cell. Additionally, we were able to clone the efflux and tetracycline-modifying genes of Tn*4400* separately. The region carrying the efflux gene spanned one of the two regions in which Tn*4400* differs from Tn*4351*. A clone containing the corresponding region of Tn*4351* did not exhibit efflux. Thus, it appears that Tn*4351* does not have the efflux gene and that efflux makes no contribution to the resistance conferred by Tn*4351*. The MIC for cells carrying the subclone from Tn*4400* that contained only the gene for tetracycline inactivation was the same as that for cells carrying both the inactivation and efflux genes. Cells carrying only the gene for tetracycline efflux were tetracycline sensitive. This was true even when the efflux gene was on a high-copy-number plasmid which increased the level of efflux to that associated with the Tc$^r$ gene on pBR328. These results indicate that efflux activity does not contribute significantly to the tetracycline resistance conferred by Tn*4400*.**

Two closely related *Bacteroides* transposons, Tn*4351* and Tn*4400*, both carry a tetracycline resistance gene that confers resistance on aerobically grown *Escherichia coli* but not on *Bacteroides* spp. (3, 6). Recently, we showed that the gene on Tn*4351* encodes a tetracycline-modifying enzyme (15). We have designated this resistance *Tc$^r$ to distinguish it from the efflux-type Tc$^r$ found in *E. coli* and from the chromosomal *Bacteroides* Tc$^r$. Since Tn*4351* and Tn*4400* are more than 90% homologous (14), we expected that the tetracycline resistance gene on Tn*4400* would also encode a tetracycline-modifying enzyme. However, Park et al. (9) reported that cells carrying Tn*4400* exhibited weak efflux of tetracycline and suggested that the efflux activity was responsible for resistance. On this basis, they classified the tetracycline resistance from Tn*4400* as a new class of efflux-type resistance, TetF (9). Subsequently, our group (16) and Park and Levy (10) showed that Tn*4400* also carries a gene for tetracycline modification similar to that found on Tn*4351*. This finding raised two questions. First, does the tetracycline resistance associated with Tn*4351* have an efflux component in addition to the modifying enzyme? Second, what contribution, if any, does efflux make toward tetracycline resistance?

We had shown previously that the tetracycline-modifying enzyme from Tn*4351* was a cytoplasmic protein (15). Thus, there should be some mechanism for exporting chemically altered tetracycline from the cytoplasm to the surrounding medium. It is possible that altered tetracycline simply diffuses out of the cell. However, the presence of both efflux and inactivation genes on Tn*4400* might be due to the need for an efflux pump to remove the altered tetracycline from the cell cytoplasm. Another possibility is that the efflux gene on Tn*4400* pumps unaltered tetracycline rather than altered tetracycline out of the cell and thereby contributes independently to tetracycline resistance. In either case, if efflux makes a significant contribution to resistance, Tn*4351* would be expected to exhibit efflux activity because it confers a higher level of resistance than Tn*4400* does. To determine what role, if any, efflux plays in the tetracycline resistance conferred by Tn*4351* and Tn*4400*, we have assayed cells carrying Tn*4351* for efflux activity. We have also localized the efflux gene on Tn*4400* to determine if it is distinct from the inactivation gene. Finally, we have attempted to clear up the confusion in the classification and nomenclature of the tetracycline resistance genes from Tn*4351* and Tn*4400*.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The plasmids used in this study are described in Table 1 or depicted in Fig. 1. Plasmids were transformed into *E. coli* EM24 or *E. coli* MCS100 (15). Cultures were grown at 37°C and 250 rpm in Luria broth or medium A (7) supplemented with 0.2% glucose and 0.4% Casamino Acids.

**DNA isolation and analysis of Tn*4400* clones.** Plasmids were isolated from *E. coli* strains by the Ish-Horowitz modification of the method of Birnboim and Doly (5). Restriction digests and ligation with T4 DNA ligase were done as directed by the manufacturer. Electrophoresis of restriction digests in 1% agarose were carried out in 1× GGB (15). Gels were stained with ethidium bromide and photographed. All of the pBR328 derivatives seemed to have a similar copy number as estimated from ethidium bromide gels (data not

* Corresponding author.

TABLE 1. Plasmids used in this study

| Plasmid | Description[a] | Source or reference |
|---|---|---|
| pMLB1034 | *'lacZ* Ap$^r$ | 1 |
| pBR328 | Tc$^r$ | 5 |
| pFD160 | Ap$^r$ | 13 |
| Tn*4351*-containing plasmids | | |
| pBS1 | *Tc$^r$ Ap$^r$ | 15 |
| pBS10 | *Tc$^s$ Ap$^r$ | 15 |
| pBS9 | *Tc$^s$ Ap$^r$ | 15 |
| pBS134 | *Tc$^r$ Ap$^r$ | Insertion of the *Eco*RI fragment from Tn*4351* (15) |
| pBSP7 | *Tc$^r$ Ap$^r$ | Spontaneous deletion which forms a translational fusion between a gene upstream of *Tc$^r$ and *'lacZ*; made as described by Berman and Jackson (1) (this study) |
| Tn*4400*-containing plasmids | | |
| pBBS1 | *Tc$^r$ Ap$^r$ | Insertion of the *Eco*RI fragment from pDP1 (Tn*4400*) containing Tc$^r$ (this study) |
| pBBS5 | *Tc$^r$ Ap$^r$ | *Hin*dIII deletion made in pBBS1 (this study) |
| pBBS9 | *Tc$^s$ Ap$^r$ | *Cla*I deletion made in pBBS1 (this study) |
| pBBS134 | *Tc$^r$ Ap$^r$ | Insertion of the *Eco*RI fragment from pBBS1 (this study) |
| pBBP37 | *Tc$^r$ Ap$^r$ | Spontaneous deletion mutant in Tn*4400* obtained as described for pBSP7 above (this study) |
| pBBP24 | *Tc$^s$ Ap$^r$ | Spontaneous deletion mutant in Tn*4400* obtained as described for pBSP7 above (this study) |
| pBBP17 | *Tc$^s$ Ap$^r$ | Spontaneous deletion mutant in Tn*4400* obtained as described for pBSP7 above (this study) |
| pBBP817 | *Tc$^s$ Ap$^r$ | *Hin*dIII deletion in pBBS134P17 (this study) of the *Bam*HI and *Sal*I sites of pBR328 (this study) |
| pBEL42 | *Tc$^s$ Ap$^r$ | *Eco*RI-*Bam*HI region from pBBS134P24 inserted into the *Bam*HI and *Sma*I sites of pFD160 (this study) |

[a] Ap, Ampicillin, Tc, tetracycline.

shown). Similar findings were observed when the copy number for the pUC derivatives was estimated.

**Construction of *lacZ* translational fusions.** While searching for genes other than the *Tc$^r$ and Em$^r$ genes on Tn*4351* or Tn*4400*, we obtained some *lacZ* fusions which were useful because they provided deletions that were not easily obtainable otherwise. Translational (protein) fusions to *'lacZ* were made by the method of Berman and Jackson (1). The *Eco*RI fragments from Tn*4351* or Tn*4400* (Fig. 1) were cloned into the fusion vector pMLB1034 in the orientation shown in Fig. 2. These clones were Lac$^-$. *E. coli* MBM7060 containing these clones was plated as a confluent streak on MacConkey lactose medium. Spontaneous deletions which created a Lac$^+$ in-frame translational fusion between *'lacZ* and a gene transcribed in the direction indicated in Fig. 2 by the arrow labeled P7 were obtained by picking Lac$^+$ papillae which appeared after several days on a background of Lac$^-$ cells. The fusions were mapped by restriction digestion, and β-galactosidase activity was assayed for as described by Miller (8). One fusion in which approximately 0.2 kilobases (kb) of the region upstream of the *Tc$^r$ gene was deleted (pBSP7) was obtained with the DNA from Tn*4351*. A similar fusion was obtained with the DNA from Tn*4400*. This fusion had a deletion of approximately 0.2 kb of the region upstream of the *Tc$^r$ gene (pBBP37). Two larger fusions with deletions of the entire *Tc$^r$ gene were also obtained. One of these is shown in Fig. 2 (pBBP24). The fusion junction of the second fusion (pBBSP17) is indicated in Fig. 2 by the solid triangle.

**Tetracycline accumulation assays.** Cells were assayed for the accumulation of [7-$^3$H]tetracycline (0.76 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) as previously described (7). *E. coli* with or without Tc$^r$ plasmids was grown in medium A to an $A_{530}$ of 0.3 to 0.5 and harvested by centrifugation. Cells were washed twice in 10 mM Tris hydrochloride (pH 8.0) and suspended to an optical density at 350 nm of 3 to 5 (10-fold) in 50 mM $KPO_4$–1 mM $MgSO_4$ (pH 6.0). Cells were energized by the addition of glucose to a final concentration of 1% and deenergized by the addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 100 μM. The reaction vessel was preincubated at 37°C in a shaking water bath for 5 to 10 min. Assays were initiated by the addition of [$^3$H]tetracycline to a final concentration of 5 μM. Samples (100 μl) were taken at timed intervals, diluted 100-fold with 10 ml of cold 0.1 M LiCl–0.1 M $KPO_4$ (pH 6.6), filtered through mixed-ester cellulose GN-6 membrane filters (0.45-μm-pore size; Gelman Sciences, Inc., Ann Arbor, Mich.), and washed with 4 ml of the same buffer. Filters were solubilized in aqueous liquid scintillation fluor (Amersham Corp., Arlington Heights, Ill.), and radioactivity was measured by scintillation counting.

**Accumulation of altered tetracycline.** To determine if chemically altered tetracycline, i.e., the product resulting from the action of the tetracycline-modifying enzyme (*Tc$^r$ protein) on tetracycline, entered and exited the cell by diffusion, we measured the accumulation of altered tetracycline as described above. Altered tetracycline was obtained by growing *E. coli* carrying pBS2 in 10 ml of Luria broth to which 0.21 mg of [$^3$H]tetracycline had been added. After 12 h of growth at 37°C and 250 rpm, the cells were pelleted and the supernatant was put on ice. The reaction was terminated after all of the tetracycline had been converted to altered tetracycline by removal of the cells by centrifugation. The procedures used to ascertain that all of the tetracycline had been converted to altered tetracycline have been described previously (16). To initiate the accumulation assay, we added 2 ml of spent medium containing 0.047 mM $^3$H-altered tetracycline (0.071 mCi) to 8 ml of a twice-washed cell suspension. This gave a final concentration of $^3$H-altered tetracycline of 5 μM, assuming that no tetracycline was lost during incubation and centrifugation and assuming that the molecular weight of altered tetracycline was similar to that of tetracycline. Accumulation was assayed as described for

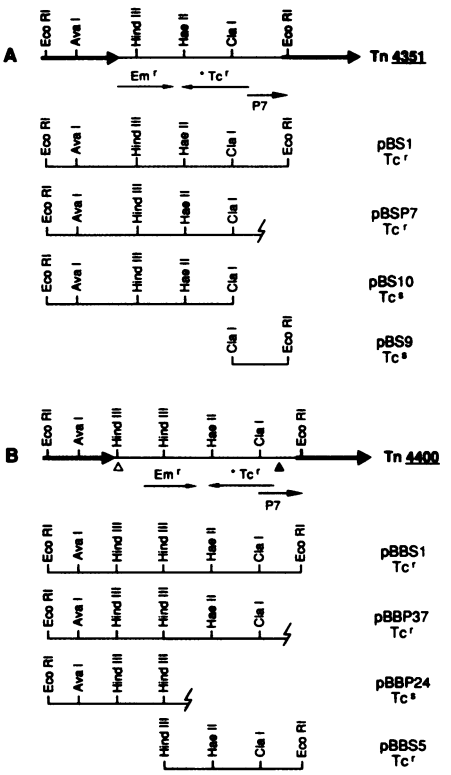


FIG. 1. Maps of Tn*4351* (A) and Tn*4400* (B). The *Eco*RI fragments from both transposons were cloned into the *Eco*RI site of pBR328ΔTc (5) or pMLB1034 (1). Solid horizontal arrows show the direction of transcription and the extent of the *Tc$^r$ and Em$^r$ genes. The broken arrows show the direction of transcription and approximate location of a protein of unknown function (P7; see Fig. 2). Subsequent deletions were made by restriction digestion (pBS10, pBS9, and pBBS5) or spontaneous deletion (pBSP7, pBBP37, and pBBP24). Antibiotic resistances of the clones are shown to the right. The solid triangle below Tn*4400* indicates a region in which a 50- to 100-kb deletion is located in Tn*4400*. The open triangle indicates the inserted region in Tn*4400* that is not present in Tn*4351*.

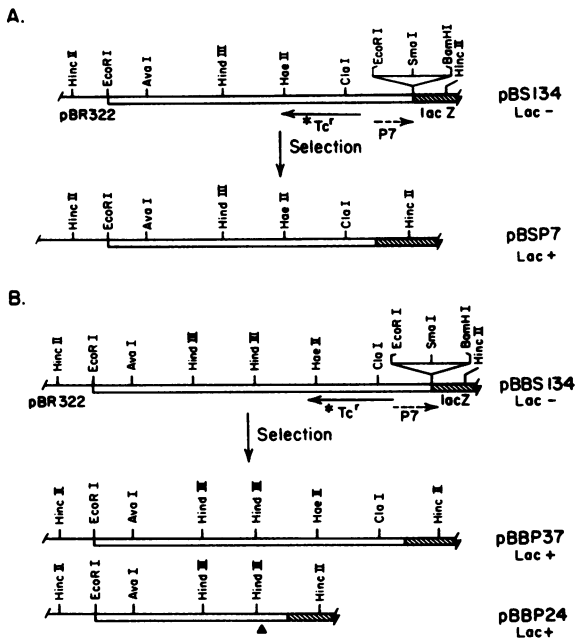


FIG. 2. Maps of clones from spontaneous deletions which created fusions to *'lacZ*. The *Eco*RI fragments from Tn*4351* (A) and Tn*4400* (B) were cloned into the *'lacZ* fusion vector pMLB1034. *lac*$^+$ deletions which created fusions to the *'lacZ* gene were selected for on MacConkey lactose medium. Subsequent fusions to a gene upstream of *Tc$^r$ are designated pBSP7 for Tn*4351* and pBBP37 for Tn*4400*. A fusion to a gene which lies downstream of the *Tc$^r$ gene is designated pBBP24. The solid triangle below pBBP24 indicates the extent of deletion in pBBP17. The horizontal solid arrows indicate the location and direction of transcription of the *Tc$^r$ gene. The broken arrows indicate the direction of transcription of the protein (P7) located by the translational fusion.

tetracycline and was allowed to continue for 70 min. After 70 min, the cells were pelleted and resuspended in the same volume of the original buffer and samples were again taken at intervals to determine if $^3$H-altered tetracycline was being bound by the cells. Radioactivity trapped by filters alone was subtracted prior to determination of the concentration accumulated.

**Maxicell extracts from Tn*4400* and Tn*4351*.** The maxicell procedure of Sancar et al. (11) was performed with *E. coli* MCS100 as the host. Proteins were separated by electrophoresis on 11% high cross-linked sodium dodecyl sulfate-polyacrylamide gels as described by Hashimoto et al. (4).

**Determination of tetracycline resistance conferred by Tn*4351*, Tn*4400*, and subclones.** The MIC of tetracycline was determined aerobically at 37°C with aeration at 250 rpm by the tube dilution method (15). For clones and cells which appeared to be Tc$^r$, increments in the tetracycline concentration of 1 μg/ml were used in the range from 1 to 10 μg/ml. For higher MICs, increments of 50 μg/ml were used to establish the approximate MIC and increments of 5 μg/ml in the appropriate concentration range were used to obtain a more accurate MIC. All MICs represent averages of triplicate determinations from at least three separate trials. All of the pBR328 derivatives appeared to have a similar copy number, as estimated from electrophoresed gels (data not shown). Similar findings were obtained with the pUC derivatives.

## RESULTS

**Accumulation of tetracycline by cells carrying clones from Tn*4351*.** Efflux assays of cells carrying pBS1 (Tn*4351*) showed that their accumulation pattern (Fig. 3B) was clearly different from that seen with tetracycline-sensitive cells (Fig. 3A, open squares). However, cells carrying pBS1 did not exhibit a clear inflection point after the addition of CCCP like that in cells carrying pBR328 (efflux-type resistance) (Fig. 3A, open circles). Rather, the accumulation was linear during the time course of the experiment and appeared to be unaffected by CCCP. The slight deviations from linearity seen in Fig. 3B were within the experimental error and were not associated with CCCP addition in repeated experiments.

One interpretation of the accumulation pattern seen with cells containing Tn*4351* is that it indicates the existence of efflux because the levels of accumulation were consistently lower than those exhibited by sensitive cells. If so, the efflux was weak, because no clear increase in the intracellular concentration of label was seen after the addition of CCCP. However, the same sort of linear pattern would be expected if tetracycline were altered inside the cell and the altered tetracycline diffused rapidly out of the cell. In this case, the

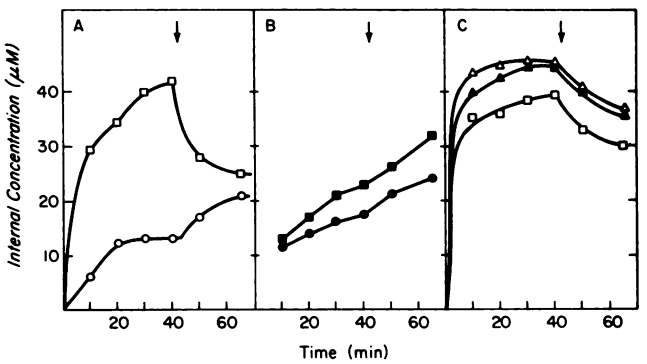


FIG. 3. Tetracycline accumulation by *E. coli* carrying various tetracycline resistance clones from Tn*4351*. (A) Tc$^s$ (□) and pBR328 Tc$^r$ (○); (B) pBS1 *Tc$^r$ (■) and pBSP7 *Tc$^r$ (●); (C) pBS9 Tc$^s$ (△), pBS10 Tc$^s$ (▲), and Tc$^s$ (□). Samples were taken at 10-min intervals over a 65-min time period. Immediately after the 40-min sample was taken, CCCP was added to a final concentration of 50 μM (arrows). Each experiment was run with one control from panel A along with the experimental sample. All experiments were performed as duplicates and repeated at least three times.

cells would not accumulate the high levels of label seen with sensitive cells because label in the form of altered tetracycline would be constantly being lost from the cell. Also, the process would be energy independent, and the uptake of label in the form of unaltered tetracycline would continue to occur even after the addition of CCCP because the conversion of tetracycline to altered tetracycline would keep the concentration of tetracycline inside the cell low. Since tetracycline uptake has an energy-independent component, keeping the intracellular concentration of unaltered tetracycline low would continue to drive tetracycline entry into the cell even after cessation of active transport of tetracycline by the addition of CCCP. A third possibility is that both efflux and diffusion of altered tetracycline occurred and that the rapid inactivation of tetracycline to a diffusible form obscured efflux.

Tetracycline-modifying activity associated with Tn*4351* was much higher than that associated with Tn*4400*. Since an efflux-type accumulation profile could be seen with Tn*4400* (see below), it was possible that lowering or eliminating the tetracycline-modifying activity of pBS1 would reveal efflux. Accordingly, we tested derivatives of Tn*4351* which had reduced inactivating activity or lacked inactivating activity entirely. In the course of experiments in which we used *lacZ* translational fusions to locate genes on Tn*4351*, we had obtained a fusion to a gene that was divergently transcribed from the *Tc$^r$ gene (pBSP7). Although the function of the resulting protein and the starting point of the gene are unknown, the deletion generated by this fusion had an interesting property. It reduced the MIC for pBS134 (Tn*4351*) by over 50% (Table 2). Also, expression of the *Tc$^r$ gene, as indicated by the intensity of the protein band in maxicell extracts, was lowered by at least fourfold (data not shown). Since this lower level of expression was closer to that seen with Tn*4400*, we tested pBSP7 for efflux. The profile was the same as that seen with pBS134 (Fig. 3B, solid circles). Thus, a lower level of tetracycline alteration did not affect the accumulation profile. We also tested two subclones, pBS9 and pBS10, which lacked an intact *Tc$^r$ gene and thus had no tetracycline-modifying activity. The accumulation profiles for these clones were identical to the accumulation profile for sensitive cells (Fig. 3C).

TABLE 2. MICs of tetracycline for plasmids containing clones from either Tn*4351* and Tn*4400*

| Transposon | Plasmid | Phenotype | MIC (μg/ml) |
|---|---|---|---|
| Tn*4351* | pBS1 | *Tc$^r$ Efx$^-$ | >250 |
| | pBS9 | *Tc$^s$ Efx$^-$ | 3 |
| | pBS10 | *Tc$^s$ Efx$^-$ | 3 |
| Tn*4400* | pBBS1 | *Tc$^r$ Efx$^+$ | 30 |
| | pBBS5 | *Tc$^r$ Efx$^-$ | 30 |
| | pBBS9 | *Tc$^s$ Efx$^-$ | 3 |
| Tn*4351* | pBS134 | *Tc$^r$ Efx$^-$ | >250 |
| | pBSP7 | *Tc$^r$ Efx$^-$ | 100 |
| Tn*4400* | pBBS134 | *Tc$^r$ Efx$^+$ | 25 |
| | pBBP37 | *Tc$^r$ Efx$^+$ | 25 |
| | pBBP24 | *Tc$^s$ Efx$^+$ | 3 |
| | pBEL42 | *Tc$^s$ Efx$^+$ | 3 |

**Accumulation of altered tetracycline.** To determine if chemically altered tetracycline diffused across the cell membrane, we measured the uptake of altered tetracycline by *E. coli* which contained no plasmid, pBR328, or pBS1. Cells containing the resistance genes were tested along with sensitive cells in case one of the resistance genes affected the partitioning of altered tetracycline. The results for cells carrying pBS1 are shown in Fig. 4. The accumulation of altered tetracycline by these cells reached an equilibrium within 20 to 30 min. Moreover, there was no effect on uptake when CCCP was added. After 70 min, cells were pelleted, suspended in transport buffer, and assayed for the loss of accumulated altered tetracycline. Once cells were suspended in transport buffer, the amount of altered tetracycline inside the cells rapidly decreased until a new equilibrum was reached (Fig. 4). Similar results (not shown) were obtained with sensitive cells and cells carrying pBR328. The rapid initial accumulation, the reversibility of this accumulation, and the energy independence of accumulation suggested that altered tetracycline readily diffuses into and out of the cell.

**Location of the efflux gene on Tn*4400*.** Although the results shown in Fig. 3 indicated that Tn*4351* had no efflux gene, we could not completely rule out efflux on the basis of these

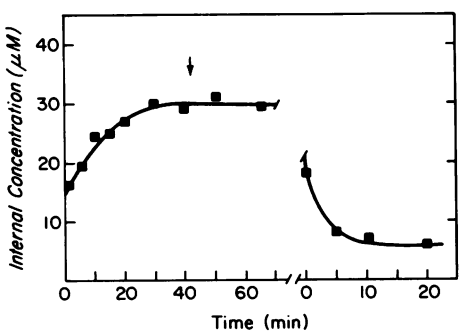


FIG. 4. Accumulation and release of $^3$H-altered tetracycline by *E. coli* carrying pBS1 *Tc$^r$. Cells were allowed to accumulate altered tetracycline for a total of 70 min and were then pelleted and suspended in transport buffer. Samples were taken at 5-min intervals for the first 30 min and then at 10-min intervals once equilibrium was reached. CCCP was added to the initial reaction mixture after 40 min (arrow).

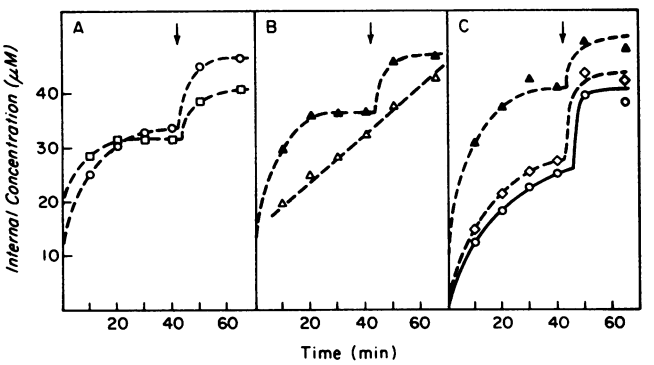


FIG. 5. Tetracycline accumulation by *E. coli* carrying various tetracycline resistance clones from Tn*4400*. (A) pBBS1 *Tc$^r$ (□) and pBBP37 *Tc$^r$ (○); (B) pBBP24 Tc$^s$ (▲) and pBBS5 *Tc$^r$ (△); (C) pBR328 Tc$^r$ (○), pBBP24 *Tc$^s$ (▲), and pBEL42 *Tc$^s$ (◇). Assays were performed as described in the legend to Fig. 3.

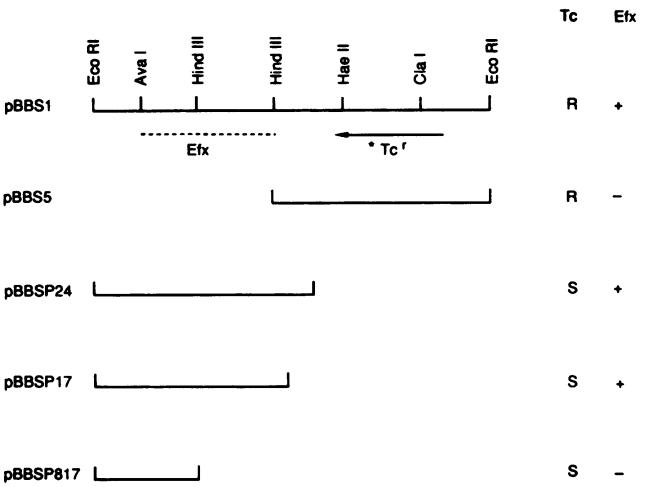


FIG. 6. Localization of the efflux gene from Tn*4400*. The solid lines under pBBS1 indicate the portion of the transposon that is contained within each clone. Tetracycline resistance (R) or sensitivity (S) and efflux (Efx) capability (+ or −) are indicated to the right.

data. In the case of pBSP7, the tetracycline-inactivating activity might not have been reduced enough to allow efflux to be seen clearly. In the case of the two modification-negative subclones, pBS9 and pBS10, a promoter or a part of the efflux gene might have been lost. To resolve these questions, we localized the efflux gene on Tn*4400*.

Efflux could be seen clearly with cells carrying the region from Tn*4400* which is analogous to the region in pBS1 (Fig. 5). A 0.2-kb deletion in Tn*4400* (pBBP37) which corresponded to the 0.2-kb deletion in pBSP7 still resulted in efflux (Fig. 5A). Thus, the efflux gene was not in the region upstream of the *Tc$^r$ gene. The accumulation profile of a clone which contained only the *Tc$^r$ region of Tn*4400* (pBBS5) was identical to that seen with Tn*4351* (Fig. 3B and 5B). This result indicated that efflux was not a second activity of the *Tc$^r$ gene. Also, it demonstrated that even the low inactivating activity of Tn*4400* could produce this type of linear profile. A clone (pBBP24) which contained part of the Em$^r$ gene but none of the *Tc$^r$ gene exhibited efflux. This result demonstrated that the efflux gene was separate from the *Tc$^r$ gene.

To localize the efflux gene further, we used a deletion derivative (pBBP17) which was similar to pBBP24 except that an additional 400 base pairs was deleted (Fig. 2B, solid arrows). pBBP17, like pBBP24, exhibited efflux activity. However, when we deleted the internal *Hin*dIII fragment from pBBP17 to yield pBBP817, a Tc$^s$ profile was seen (data not shown). Figure 6 shows a summary of the localization of the efflux gene on Tn*4400*.

**Contribution of the efflux gene to resistance.** When MICs were determined for the efflux and tetracycline-modifying genes of Tn*4400*, we found that the MIC for cells carrying the efflux gene (pBBP24) was equal to that for Tc$^s$ cells, whereas the MIC for cells carrying the tetracycline-modifying gene (pBBS5) was equal to the MIC for the entire *Eco*RI fragment (Table 2). These results indicated that the gene on Tn*4400* which mediated tetracycline efflux made no contribution to tetracycline resistance.

**Strength of the Tc$^s$ efflux activity.** We were unsuccessful in detecting the efflux protein in maxicells or in the coupled in vitro transcription-translation system. One explanation for these results and for the Tc$^s$ phenotype of cells carrying the efflux gene could be that expression of the protein was low and, consequently, the efflux activity was too weak to confer resistance. Accordingly, we attempted to increase the production of the efflux protein. To do this, we cloned the *Eco*RI-*Bam*HI region of pBBP24 into pFD160 (a pUC19 derivative) downstream from the *lac* promoter (Table 1). We chose pFD160 rather than pUC18 because pFD160 can be mobilized into *Bacteroides* recipients. If increased production of the efflux protein had conferred resistance on the cells, we planned to test the expression in *Bacteroides* spp.

We were only able to obtain the insert into pFD160 in one orientation. However, this construct had higher efflux activity than the original clone did. The higher efflux activity was probably due to the higher copy number of the pUC vector rather than to the presence of the *lac* promoter because the addition of isopropyl-β-D-thiogalactopyranoside did not affect efflux activity. Regardless of the reason for the higher efflux activity, the activity of this construct was now comparable to that mediated by the Tc$^r$ gene on pBR328 (Fig. 5C). Despite the higher efflux activity, the tetracycline MIC for cells carrying clone pBEL42 was indistinguishable from that for Tc$^s$ cells (Table 2). We were still unable to detect a protein associated with the cloned region in maxicell extracts (data not shown).

## DISCUSSION

Our findings demonstrate that Tn*4351*, unlike Tn*4400*, does not exhibit efflux activity. Using conditions which produced an unambiguous efflux pattern with *E. coli* carrying Tn*4400* sequences, we were unable to detect unambiguous tetracycline efflux by *E. coli* carrying Tn*4351* sequences, even when subclones with no or reduced tetracycline-modifying activity were used. Although the accumulation of tetracycline by cells carrying Tn*4351* was lower than that for sensitive cells, it was not affected by CCCP, as was the accumulation by cells carrying Tn*4400* or pBR328. The reason for the lower accumulation of label by cells carrying Tn*4351* appears to be that once tetracycline is modified, it diffuses rapidly out of the cell. The findings of Park and Levy (10) also support the diffusion of altered tetracycline across the cell membrane. Thus, the combination of tetracycline uptake and loss of altered tetracycline by diffusion produces a linear accumulation that levels off after all the tetracycline

has been converted to altered tetracycline. The fact that the *Tc$^r$ gene from Tn*4400*, when cloned away from the efflux gene, produces the same type of linear profile lends further support to this hypothesis.

Further evidence that there is no efflux gene on Tn*4351* comes from the finding that the efflux gene on Tn*4400* is located in a region upstream of the Em$^r$ gene. Although Tn*4351* and Tn*4400* are over 90% homologous (14), there are two regions in which differences have been detected. One of these is a 0.5-kb insertion in the region of Tn*4400* in which the efflux gene is located (14). The location of the efflux gene in this region of Tn*4400*, together with our finding that the analogous region of Tn*4351* (on pBS10) was clearly negative for efflux (Fig. 3C), demonstrates that Tn*4351* does not contain an active efflux gene. The precise ends of the efflux gene on Tn*4400* have not been determined. However, it is known that the insertion element and Em$^r$ gene of Tn*4400*, which flank the 0.5-kb insertion, are highly homologous to the same regions on Tn*4351* (14). Thus, it is possible that the 0.5-kb insertion contains the entire efflux gene. Alternatively, Tn*4351* may have resulted from a deletion in Tn*4400* which removed only part of the gene. Clearly, the gene spans the 0.5-kb insertion because deletion of this region abolished efflux activity (Fig. 6).

Our findings also demonstrate that the efflux gene on Tn*4400* does not make any detectable contribution to the resistance conferred by this transposon. The efflux gene alone does not confer resistance, because the MIC for *E. coli* carrying the efflux gene but not the tetracycline-modifying gene from Tn*4400* was identical to that for the Tc$^s$ wild type. The efflux gene did not appear to act synergistically with the tetracycline-modifying gene. The MIC for *E. coli* carrying the tetracycline-modifying gene but not the efflux gene was identical to the MIC for *E. coli* carrying all of Tn*4400*. Also, the MIC for Tn*4351*, which does not carry the efflux gene, was higher than the MIC for Tn*4400*. This difference between Tn*4351* and Tn*4400* can be explained by the higher tetracycline-modifying activity of Tn*4351*.

Our finding that the efflux portion of Tn*4400* failed to confer tetracycline resistance when cloned away from the *Tc$^r$ portion of the transposon was surprising. It is possible that the pump was not strong enough to reduce intracellular tetracycline concentrations to a level at which some ribosomes were still functional. However, we were able to increase the Tn*4400* efflux activity to equal the activity associated with pBR328 by cloning the efflux region into a high-copy-number plasmid (Fig. 5C). Despite this, cells carrying the clone from Tn*4400* were still sensitive to tetracycline. Our finding raises the question of how efflux proteins encoded by pBR328 or Tn*10* actually function in the cell.

It is not clear whether the difference in tetracycline-modifying activity between Tn*4351* and Tn*4400* is due to a difference in the structural gene for the modifying enzyme or to a difference in the upstream region. The latter possibility seems more likely because deletions in the region upstream of the *Tc$^r$ gene in Tn*4351* reduced the MIC to a level close to the MIC associated with Tn*4400*. Also, the upstream region of Tn*4351* is known to contain a small region that is not present in Tn*4400*. This region could be near the *Tc$^r$ promoter. Our *lacZ* fusion experiments showed that both Tn*4351* and Tn*4400* have a divergently transcribed gene upstream of the *Tc$^r$ gene. However, it is not clear whether this gene has any function in tetracycline resistance. Previous work revealed no evidence for regulation of expression of the *Tc$^r$ gene (15).

Since the tetracycline resistance gene on Tn*4351* and Tn*4400* does not cross-hybridize with any known tetracycline resistance gene and because it has a different mechanism from that of other described resistance genes, it clearly belongs in a separate class. We suggest designating this resistance class X. We feel this is justified because in the current classification system of tetracycline resistances the classes are grouped by mechanism. Our designation would separate the classes of tetracycline resistance into three groups: efflux (A to E, G, K, and L), ribosomal protection (M to O), and chemical modification (X). [Therefore, the designation of the structural gene being uniform, the *Tc$^r$ gene designation would be *tetA(X)*.] The class F designation suggested by Park et al. (9) seems inappropriate because it was based on the assumption that the efflux activity found on Tn*4400* was responsible for resistance. It might be more appropriate to reserve this classification for the recently cloned *Bacteroides* Tc$^r$ gene (12), which appears to have an efflux mechanism (2; M. Nickolich and A. Salyers, unpublished data).

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