Energy-Dependent Efflux Mediated by Class L (TetL) Tetracycline Resistance Determinant from Streptococci

LAURA M. McMURRY,^{1*} BRITT H. PARK,¹ VICKERS BURDETT,² AND STUART B. LEVY^{1,3}

Departments of Molecular Biology and Microbiology¹ and Medicine,³ Tufts University School of Medicine, Boston, Massachusetts 02111, and Department of Microbiology, Duke University, Durham, North Carolina 27710²

Received 7 May 1987/Accepted 24 July 1987

The class L (TetL) tetracycline resistance determinant from streptococci specified resistance and an energy-dependent decreased accumulation of tetracycline in both *Streptococcus faecalis* and *Escherichia coli*. Using *E. coli*, we showed that the reduced uptake resulted from active efflux. The streptococcal class M determinant, known to render the protein synthesis machinery of *S. faecalis* resistant to tetracycline inhibition, did not alter tetracycline transport in either host.

Tetracycline resistance (Tc^r) determinants are widespread among gram-negative and gram-positive bacteria, both anaerobes and aerobes (10). The original source of these determinants is unknown, but recent studies have shown that previously identified determinants appear to have moved into new hosts. Of particular note, determinants with strong homology to class M (TetM), initially found among streptococci (3), have now been identified among newly appearing Tc^r strains of *Mycoplasma* (17), *Ureaplasma* (16), *Campylobacter* (19), *Gardnerella* (15), *Neisseria* (14), and *Clostridium* (7) spp. The wide dispersal of this determinant can probably be attributed in part to its existence on transposon Tn916 originally described in *Streptococcus faecalis* (5). Two other streptococcal determinants, class L (TetL) and class N (TetN), have also been discovered (3).

The mechanism of resistance specified by the class M determinant in *S. faecalis* occurs at the level of the target of tetracycline, namely, the protein synthesis machinery within the cell (2). The class L determinant, on the other hand, does not prevent the inhibition of protein synthesis in *S. faecalis* but rather decreases tetracycline uptake (2). The purpose of the present study was to determine the mechanism for this decrease in both *S. faecalis* and *Escherichia coli* as hosts.

The naturally occurring plasmids pMV158 (class L) and pAM211 (class M) (2) were used in *S. faecalis*. In *E. coli*, we used pVB-A15 (a hybrid of pMV158 with the *E. coli* cloning vector pVH2124 [3]) and pJ13 (a 5-kilobase *Hinc*II region of the chromosomal class M determinant from *Streptococcus agalactiae* B109 containing the entire Tc^r determinant cloned into the *E. coli* vector pACYC177 [3, 8]). These plasmids were introduced into *E. coli* SK1592 (3) by transformation.

The levels of resistance to tetracycline and minocycline expressed aerobically and anaerobically in *S. faecalis* and *E. coli* were assayed at 37°C by the gradient plate method on Penassay medium (Difco Laboratories) (4). In both genera, the class M determinant specified resistance to both tetracycline and minocycline, while the class L determinant, as previously reported for streptococci (2), expressed only resistance to tetracycline. In *S. faecalis*, Tc^r mediated by class L on pMV158 varied (MIC, 50 to 100 µg/ml) in different experiments and that specified by class M on pAM211 was

FIG. 1. Tetracycline accumulation in tetracycline-susceptible and -resistant streptococci. *S. faecalis* ATCC 9790r with or without the class L determinant on plasmid pMV158 (3) was grown in KTY (3) medium (with 1 µM tetracycline for the Tc^r derivative) to an A₅₉₀ of 0.4 to 0.8. Cells were washed twice with 50 mM sodium-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.4) and suspended in KTY medium without glucose to an A₅₉₀ of 2.0. The cells were energized by adding glucose (1%) and incubating at 37°C for 15 min. The cells were deenergized by adding CCCP (250 µM). Uptake was initiated by the addition at time zero of [³H]tetracycline (500 Ci/mol; New England Nuclear Corp., Boston, Mass.) (1.2 µM). At intervals, 100-µl samples were diluted into 6 ml of cold buffered saline, filtered through 0.45-µm-pore-size Gelman GN-6 Metricel membrane filters, and washed with 3 ml of buffered saline. The filters were dried at 37°C, and radioactivity was determined in Betafluor (National Diagnostics, Somerville, N.J.). ■, Susceptible cells (CCCP added at arrow); □, susceptible cells (CCCP added prior to tetracycline); ●, resistant cells (CCCP added at arrow); ○, resistant cells (CCCP added prior to tetracycline).

* Corresponding author.

FIG. 2. Tetracycline accumulation in tetracycline-susceptible and -resistant *E. coli* bearing Tc^r determinants from streptococci. *E. coli* SK1592 with and without plasmid pVB · A15 (class L) or pJI3 (class M) was grown in minimal medium A (12) with 0.5% glycerol, 0.1% Casamino Acids (Difco), and 2 μ M tetracycline for resistant cells at 37°C to an A_{530} of 0.8. The cells were assayed for uptake of [3 H]tetracycline at 4 μ M tetracycline in pH 6.0 buffer with lactate by filtration (Fig. 1; as described in reference 11, except that no chloramphenicol was used). ○, DNP added before tetracycline; ●, DNP (2 mM) added at the arrow.

10 to 30% lower than that for pMV158 in the same experiment. Approximately 10 to 25% as much Tc^r was expressed by the determinants in *E. coli* as compared with levels expressed in *S. faecalis* (even though *S. faecalis* was intrinsically severalfold more susceptible than *E. coli* was to tetracycline). These results could relate to inefficiency of the resistance mechanism in the foreign *E. coli* host or to

decreased gene expression. It should be noted that the cloned determinants are on multicopy plasmids in *E. coli*, whereas they are in low-copy-number plasmids in streptococci. Under anaerobic growth conditions, cells were resistant to 25 to 70% lower levels of tetracycline.

In previous studies, active uptake of tetracycline was seen in susceptible *S. faecalis* cells and in those bearing the class M determinant (2). A decreased uptake was noted for cells bearing the class L determinant (2); however, the cause of the reduced uptake was not demonstrated. We studied the effect of destruction of membrane proton motive force by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on the tetracycline accumulation of susceptible *S. faecalis* cells and of those bearing the class L determinant. We found that susceptible cells actively accumulated tetracycline, whereas cells bearing the class L determinant took up less tetracycline than did susceptible cells (Fig. 1) as previously reported (2). However, when class L-containing

FIG. 3. Changes in tetracycline accumulation following energization of starved *E. coli*. *E. coli* DL-54, an *uncA* mutant of ML308-225 (18), and its class L transformant DL-54(pVB · A15) were grown in (minimal) medium A with 0.2% glucose and 2 μ g of thiamine hydrochloride per ml (plus 2 μ M tetracycline for resistant cells) and starved for energy substrates in medium A without glucose in the presence of 5 mM DNP for 4 h at 37°C at an A_{530} of 3. The cells were washed four times in medium A to remove DNP, and uptake of [3 H]tetracycline (added at time zero to 2.5 μ M) was measured in this medium (37°C, A_{530} of 2). The steady-state accumulation of tetracycline in starved cells is represented as 100% (— —). For an unexplained reason, the absolute uptake in starved susceptible cells was about twice that of starved resistant cells. Glucose (0.2%) was added at the arrow. Samples of 50 μ l were diluted into 10 ml of medium A containing 0.1 M lithium chloride at 20°C, filtered (see legend to Fig. 1), and washed with 4 ml of medium A plus lithium chloride. Radioactivity on the filters was determined as described in the legend to Fig. 1.

TABLE 1. Effect of class L or M Tc^r determinants on Tc^r expressed by class A or B in *E. coli*

Plasmid ^a	Tc^r determinant class	MIC (μ g/ml) ^b
pVB · A15	L	23 $\pm$ 3
pJI3	M	10 $\pm$ 2
RK231	A	62 $\pm$ 5
pIP15	A	44 $\pm$ 6
R100-1	B	101 $\pm$ 15
R222	B	100 $\pm$ 15
RK231 + pVB · A15	A + L	57 $\pm$ 9
pIP15 + pJI3	A + M	85 $\pm$ 11 ^c
R100-1 + pVB · A15	B + L	110 $\pm$ 15
R222 + pVB · A15	B + L	107 $\pm$ 14
R222 + pJI3	B + M	178 $\pm$ 7 ^c

^a All plasmids were in *E. coli* SK1592. Sources or references for plasmids are: pVB · A15 and pJI3 (3, 8), pIP15 and R222 (13), RK231 (6), and R100-1 (9; M. Malamy).

^b MICs were determined by the gradient plate method (4). Each value is the mean $\pm$ standard deviation of three or more determinations.

^c MIC is significantly higher than the sum of the MICs for the two plasmids tested alone.

cells were similarly deenergized with CCCP, they accumulated more tetracycline than when energized (Fig. 1); this finding demonstrated that the reduced tetracycline uptake was energy dependent.

The transport of tetracycline in the presence or absence of class L or class M determinant was similarly examined in *E. coli*. Again, an active uptake was found in the susceptible cells and in those bearing the class M determinant. Again, cells bearing the class L determinant showed a greatly decreased accumulation of tetracycline, and this reduced uptake was reversed by dinitrophenol (DNP), an inhibitor similar in action to CCCP (Fig. 2).

The class L-mediated lowering of tetracycline accumulation was most likely explained by an active efflux of the drug, by analogy with the mechanisms of Tc^r (12) specified by classes A to D in *E. coli*. However, an active prevention of drug entry was an alternative possibility. To clarify the actual mechanism, we depleted class L-containing *E. coli* cells of endogenous energy reserves by incubation with DNP (1). We then loaded the starved cells with tetracycline and determined whether energization with glucose would cause loss of tetracycline (i.e., efflux). Strain DL-54 (*uncA*) (18), with and without pVB · A15, was used as a host, since it was more easily starved than was SK1592. Upon the addition of glucose to starved DL-54 cells preincubated with [3H]tetracycline, the amount of tetracycline increased as expected, consonant with the development of active tetracycline uptake (Fig. 3). In contrast, the amount of tetracycline in resistant cells decreased after glucose energization (Fig. 3). This result indicates that the class L resistance determinant indeed specified an active efflux mechanism. Efflux was also seen if the resistant cells were energized with lactate (data not shown). Since in an *unc* mutant (missing the membrane-bound ATPase involved in oxidative phosphorylation) lactate should create a proton motive force across the membrane without formation of ATP (1), the class L-mediated active efflux may depend upon proton motive force, as is true for the class A to D Tc^r determinants (12).

We examined the effect of the two streptococcal determinants L and M on the expression of two common Tc^r determinants (class A and class B) in *E. coli*. Interaction between the class A or class B determinant (encoding active efflux) and the class L determinant produced additive or less than additive Tc^r (Table 1). On the other hand, interaction between the class A or class B determinant and the class M determinant (encoding a nonefflux mechanism) gave rise to resistance levels greater than the sum (Table 1), consistent with a synergy between two different kinds of resistance mechanisms. We should note, however, that synergy has been seen between different determinants, both from gram-negative cells and both apparently involving active efflux; a greater than additive resistance level occurred when the class F determinant from *Bacteroides fragilis* was present in the same cell as any of the class A to E determinants (unpublished observations).

These studies demonstrated that the class L streptococcal Tc^r determinant expressed resistance in a gram-negative *E. coli* host via a mechanism similar to that used in the original gram-positive parent. The resistance levels for both class L and class M were considerably less in *E. coli* than in *S. faecalis*. The class L determinant was shown to be responsible for an energy-dependent efflux of the drug.

We thank Denis Desjarlais for help in some of these experiments. This work was supported by grant MV73 from the American

Cancer Society and Public Health Service grants A116756 (to S.B.L.) and A115619 (to V.B.) from the National Institutes of Health.

LITERATURE CITED

- Berger, E. A. 1973. Different mechanisms of energy coupling for the active transport of proline and glutamine in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 70:1514-1518.
- Burdett, V. 1986. Streptococcal tetracycline resistance mediated at the level of protein synthesis. J. Bacteriol. 165:564-569.
- Burdett, V., J. Inamine, and S. Rajagopalan. 1982. Heterogeneity of tetracycline resistance determinants in *Streptococcus*. J. Bacteriol. 149:995-1004.
- Curiale, M. S., and S. B. Levy. 1982. Two complementation groups mediate tetracycline resistance determined by *Tn10*. J. Bacteriol. 151:209-215.
- Franke, A. E., and D. B. Clewell. 1981. Evidence for a chromosome-borne resistance transposon (*Tn916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. 145:494-502.
- Guiney, D. G., and E. Jakobson. 1983. Location and nucleotide sequence of the transfer origin of the broad host range plasmid RK2. Proc. Natl. Acad. Sci. USA 80:3595-3598.
- Hächler, H., F. H. Kayser, and B. Berger-Bächi. 1987. Homology of a transferable tetracycline resistance determinant of *Clostridium difficile* with *Streptococcus (Enterococcus) faecalis* transposon *Tn916*. Antimicrob. Agents Chemother. 31:1033-1038.
- Inamine, J., and V. Burdett. 1982. Genetic and physical characterization of two chromosomal resistance determinants of *Streptococcus agalactiae*, p. 170-173. In D. Schlessinger (ed.), Microbiology—1982. American Society for Microbiology, Washington, D.C.
- Jacob, A. E., J. A. Shapiro, L. Yamamoto, D. I. Smith, S. N. Cohen, and D. Berg. 1977. Plasmids studied in *Escherichia coli* and other enteric bacteria, p. 616. In A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Levy, S. B. 1984. Resistance to the tetracyclines, p. 191-204. In L. E. Bryan (ed.), Antimicrobial drug resistance. Academic Press, Inc., Orlando, Fla.
- McMurry, L. M., J. C. Cullinane, and S. B. Levy. 1982. Transport of the lipophilic analog minocycline differs from that of tetracycline in susceptible and resistant *Escherichia coli* strains. Antimicrob. Agents Chemother. 22:791-799.
- McMurry, L. M., R. E. Petrucci, Jr., and S. B. Levy. 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 77:3974-3977.
- Méndez, B. S., C. Tachibana, and S. B. Levy. 1980. Heterogeneity of tetracycline resistance determinants. Plasmid 3:99-108.
- Morse, S. A., S. R. Johnson, J. W. Biddle, and M. C. Roberts. 1986. High-level tetracycline resistance in *Neisseria gonorrhoeae* is result of acquisition of streptococcal *tetM* determinant. Antimicrob. Agents Chemother. 30:664-670.
- Roberts, M. C., S. L. Hillier, J. Hale, K. K. Holmes, and G. E. Kenny. 1986. Tetracycline resistance and *tetM* in pathogenic urogenital bacteria. Antimicrob. Agents Chemother. 30:810-812.
- Roberts, M. C., and G. E. Kenny. 1986. Dissemination of the *tetM* tetracycline resistance determinant to *Ureaplasma urealyticum*. Antimicrob. Agents Chemother. 29:350-352.
- Roberts, M. C., L. A. Koutsy, K. K. Holmes, D. L. LeBlanc, and G. E. Kenny. 1985. Tetracycline-resistant *Mycoplasma hominis* strains contain streptococcal *tetM* sequences. Antimicrob. Agents Chemother. 28:141-143.
- Simoni, R. D., and M. K. Shallenberger. 1972. Coupling of energy to active transport of amino acids in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 69:2663-2667.
- Taylor, D. E., K. Hiratsuka, H. Ray, and E. K. Manavathu. 1987. Characterization and expression of a cloned tetracycline resistance determinant from *Campylobacter jejuni* plasmid pUA466. J. Bacteriol. 169:2984-2989.