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Active Efflux of Chloramphenicol in Susceptible *Escherichia coli* Strains and in Multiple-Antibiotic-Resistant (Mar) Mutants

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The multiple-antibiotic resistance (*mar*) locus (min 34) regulates a resistance to chloramphenicol in *Escherichia coli* that does not involve acetyltransferase. Transport studies showed that wild-type cells had an apparent endogenous active efflux of chloramphenicol which depended on the proton motive force. This efflux was not altered by a 39-kb chromosomal deletion which included the *mar* locus. Nevertheless, mutations at the *mar* locus led to a stronger net chloramphenicol efflux. Therefore, a gene encoding the putative efflux system cannot be at the *mar* locus but may be positively influenced by that locus.

The *marRAB* operon at min 34 (1636.7 kb) on the *Escherichia coli* chromosome leads to multiple antibiotic resistance when the putative operator or the negative transcriptional self-regulator *marR* gene is mutated (6). One of the antibiotics affected is chloramphenicol (6, 12), a bacteriostatic drug which inhibits protein synthesis.

Mar-related chloramphenicol resistance does not involve inactivation by chloramphenicol acetyltransferase (12). We therefore performed transport studies to determine whether Mar mutants might accumulate less drug. In the course of these studies, we discovered that wild-type *E. coli* cells appear to be able to actively cause chloramphenicol efflux. Efflux was more effective in Mar mutants, resulting in lower drug accumulation.

MATERIALS AND METHODS

Strains. Table 1 lists some *E. coli* strains used in this study. Others are ML308-225 (18), its *unc* derivative DL-54 (25), Mar mutants AG106 and AG111 (12), and the standard wild-type K-12 strain MG1655 (14). Strains LM249, LM313.5, and LM314.5 (Table 1) were created by P1 transduction from AG1025, which bears *marA*::Tn*5* (6).

Chloramphenicol accumulation in cells. Accumulation of chloramphenicol by cells was examined by using radiolabeled [*dichloroacetyl*-1,2-^{14}C]chloramphenicol (43 mCi/mmol; New England Nuclear). At concentrations above 10 μM, unlabeled chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) was added to reduce the specific activity. Counting efficiency was 90%. Results are expressed as picomoles of chloramphenicol per A_{530} unit, where 1 A_{530} unit represents the number of cells in 1 ml when the A_{530} is equal to 1. This corresponds to 7.7 × 10^8 AG100 cells, about 0.3 mg of protein, and 1.1 μl in internal volume (18). Four methods were used. In method 1, used unless otherwise specified, cells were grown to logarithmic phase in LB broth (containing, per liter, 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl) at 30°C, washed twice in 50 mM potassium phosphate (pH 6.0) at room temperature, and resuspended in the same buffer to A_{530} = 10. [^{14}C]chloramphenicol was added to 1.0 to 1.3 μM unless otherwise noted. Accumulation by cells at 30°C was measured by diluting 50 μl of cell-labeling suspension into 10 ml of 100 mM LiCl–50 mM potassium phosphate (pH 6.0) and immediately collecting cells on Gelman metricel mixed-cellulose ester membrane filters (pore size, 0.45 μm) and determining the radioactivity on the filters. Binding of radiolabel to filters in the absence of cells was subtracted. In method 2 (13, 21), cells were incubated either in 50 mM potassium phosphate (pH 6.0) at 30°C (A_{530} = 10) or in LB with 0.1% glucose at 37°C (A_{530} = 4). Then 50 μl of cell suspension was placed on top of 200 μl of silicone oil above 20 μl of formic acid in a 1.5-ml Eppendorf tube. The oil was a mixture of 15 parts by volume of "200 fluid 1.0 cs blend, specific gravity = 0.816" and 85 parts of "blend specific gravity = 1.05" (both from William F. Nye, Inc., New Bedford, Mass.). During immediate centrifugation at 15,000 × *g* for 1 min, the cells sedimented and the labeling medium floated above the oil layer. The tube was frozen at −80°C for 30 min, the bottom of the tube was cut off, and radioactivity in the formic acid was determined. In method 3, drug accumulation occurred as in method 2. Radioactivity in 50 μl of the cell suspension was determined both before and after cells had been removed by a 1-min centrifugation, and the radioactivity associated with the cell pellet was calculated by subtraction. In method 4 (1), cells growing at 37°C in nutrient broth (containing, per liter, 13 g of Difco nutrient broth, 5 g of yeast extract, 5 g of NaCl, and 1 g of glucose) in logarithmic phase (A_{530} near 1) were incubated with 31 μM [^{14}C]chloramphenicol (specific activity, 1.54 mCi/mmol). Uptake was measured by the subtraction method.

Chloramphenicol accumulation in membrane vesicles. We made everted membrane vesicles from ML308-225, AG106, and AG111 cells by using a French pressure cell as described previously (18), using cells grown at 37°C in minimal medium A (18). The integrity of the vesicles was affirmed by their ability either to actively concentrate ^{45}Ca by using NADH (in AG106 and AG111 cells) (18) or to quench acridine orange fluorescence (as a result of creation of proton gradient) on addition of lactate (for ML308-225 cells) (20). Transport of radiolabeled chloramphenicol (2 to 10 μM) into vesicles was measured in 50 mM potassium phosphate (pH 7.5) with Mg^{2+} (0 to 5 mM) with a variety of energy sources (lactate, ATP, NADH, phenazine methosulfate-ascorbate). Samples were diluted into 10 ml

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TABLE 1. Susceptibility levels of *E. coli* strains and their uptake of chloramphenicol before and after addition of glucose

Strain	Characteristics	MIC[a] (μg/ml) of:			Cm uptake[b] (pmol/A_{530} unit)		
		Tc	Cm	Nal	−Glucose	+Glucose	+/− ratio
AG100	Wild type (6)	2.5	4.6	ND[c]	18.5 ± 5.5	7.9 ± 1.9	0.44 ± 0.07
CH164	AG100 with 39-kbp deletion from 33.6 to 34.3 min including *mar* locus; *zdd*-230::Tn*9* (6)	ND	ND	ND			
LCHM164	Cm[s] derivative of CH164 isolated by ampicillin selection; Tn*9* presumably no longer present	ND	ND	ND	20.3 ± 2.7	9.8 ± 0.6	0.48 ± 0.03
Tc2.5-1	Mar strain selected on 2.5 μg of Tc per ml (8)	ND	ND	ND	18.4 ± 3.1	3.8 ± 1	0.20 ± 0.03
LM249	Tc2.5-1 *marA*::Tn*5*	ND	ND	ND	19.5	7.5	0.39
LM313.1	Mar strain of AG100 selected on 15 μg of Cm per ml and then on 80 μg of Cm per ml	19	>160	11.6	5.8 ± 2.3	0.36 ± 0.26	0.077 ± 0.074
LM313.5	LM313.1 *marA*::Tn*5*	4.1	22	2.7	13.2	5.5	0.42
LM314.1	Mar strain selected on 65 μg of Tc per ml; derived from Tc2.5-1	52	76	15	11.4	0.97	0.085
LM314.5	LM314.1 *marA*::Tn*5*	4.0	5.7	3.1	20.6	7.8	0.38

[a] MIC of Tc (tetracycline), Cm (chloramphenicol), and Nal (nalidixic acid) was determined by the gradient plate method (8) on LB agar at 30°C.

[b] External chloramphenicol concentration was 1.3 μM. Accumulation of chloramphenicol after 20 min was measured in cells lacking glucose in the medium (−Glucose). Glucose was then added, and accumulation was measured 10 min later (+Glucose). The ratio was calculated. Values are means ± standard deviations. The number of experiments for each value was 6 for AG100, 2 for LCHM164, 4 for Tc2.5-1, 1 for LM249, 2 for LM313.1, 1 for LM313.5, 1 for LM314.1, and 1 for LM314.5.

[c] ND, not determined for this table; see also reference 8.

of 100 mM LiCl–50 mM potassium phosphate (pH 7.5) at room temperature and filtered as for cells.

RESULTS AND DISCUSSION

Active efflux of chloramphenicol in wild-type cells. Accumulation in AG100 cells was measured by method 1. Cells were energized with 0.2% glucose added 20 min before addition of radiolabeled chloramphenicol. Accumulation of the drug reached a plateau by 8 min (Fig. 1, curve A). Subsequent addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which destroys the proton motive force (24), caused nearly a doubling in the concentration of cell-associated chloramphenicol (curve A). In a second experiment, cells were initially incubated with chloramphenicol in the absence of glucose for 20 min. Glucose was then added, causing some of the drug which had initially been accumulated to rapidly leave (Fig. 1, curve B). Subsequent CCCP addition allowed reentry of drug (curve B). These results suggested that susceptible strain AG100 cells could actively cause chloramphenicol efflux.

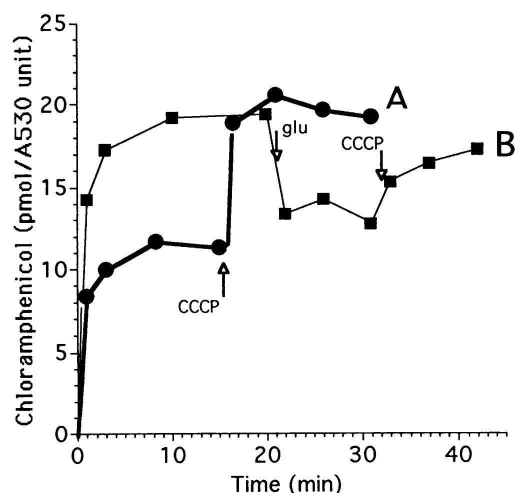

FIG. 1. Accumulation of chloramphenicol by energized and deenergized AG100 cells. At t = 0, chloramphenicol was added to 1.0 μM. In curve A, glucose was added 20 min prior to chloramphenicol; CCCP (100 μM) was added at t = 15.5 min. In curve B, initial accumulation took place in the absence of glucose; glucose was added at t = 21 min and CCCP was added at t = 32 min.

Earlier work with *E. coli* (1) had shown chloramphenicol to be actively taken up rather than expelled. In attempts to resolve this conflict with our data, we used three additional methods not involving filtration to measure incorporation of [^{14}C]chloramphenicol (see Materials and Methods). For both methods 2 and 3, chloramphenicol accumulation decreased when cells were energized by glucose (in phosphate buffer) and increased when CCCP was added (in LB medium). Therefore, an active efflux was found with both methods. We then tried method 4, which was used in earlier work on *E. coli* that reported a 140-fold concentration of chloramphenicol by energized cells at an external chloramphenicol concentration of 31 μM (1). We found that energized cells accumulated approximately 65 pmol per A_{530} unit (1.1 μl), which represents a theoretical concentration of only twofold. Furthermore, cells deenergized by 50 μM CCCP accumulated more drug, not less (approximately 550 pmol/A_{530} unit). These results were consistent with an active efflux. As noted below, some of this accumulation may represent binding to ribosomes. Finally, the effect of pH on accumulation in AG100 cells was measured by using method 1 at 1.0 μM external chloramphenicol. As the pH was increased (from 6.0 through 7.0 to 7.7), accumulation in the absence of glucose decreased a total of 20%. The ratio of accumulation in the presence of glucose to that in its absence decreased only 20% as the pH rose from 6.0 to 7.7. These results show that the pH of the assay was not critical.

Therefore, by all four methods, *E. coli* K-12 strain AG100 manifested an apparent active efflux of chloramphenicol without a strong pH dependence. No active uptake was seen. Active efflux was also seen (by adding glucose as in Fig. 2) for the non-K-12 strain ML308-225 (data not shown), for its

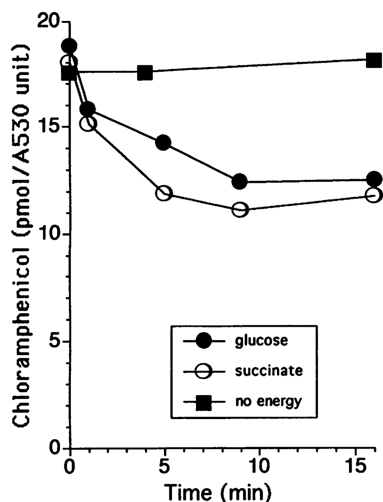

FIG. 2. Active efflux of chloramphenicol in DL-54 *unc* cells energized by glucose or succinate. Accumulation of chloramphenicol (1.3 μM external concentration) in the absence of energy substrate was measured after 20 min and plotted as $t = 0$. At 1 min later, 0.2% glucose, 10 mM sodium succinate, or nothing was added at $t = 0$.

derivative DL-54 (see below), and for standard wild-type K-12 strain MG1655 (data not shown).

Energetics of efflux. The energetics of the efflux were studied by using non-K-12 *unc* strain DL-54, possessing a faulty F_0/F_1-ATPase unable to synthesize ATP via electron transport driven by succinate (24, 25). As expected, DL-54 did not grow in minimal medium with succinate.

For transport studies, cells were grown in LB broth with 0.5% glycerol and 5 mM sodium succinate and uptake was measured by method 1. DL-54 showed active efflux of chloramphenicol following addition of glucose (Fig. 2). Active efflux of similar magnitude was also seen with 10 mM succinate (Fig. 2). Therefore the efflux is probably energized by proton motive force without ATP involvement.

Everted membrane vesicles provide a means to characterize proton motive force-dependent efflux systems in vitro (18). Such vesicles use energy substrates to concentrate substances which intact cells expel. Vesicles from wild-type cells and Mar mutants were prepared and assayed as described in Materials and Methods. Vesicles were able to energize with proton motive force substrates. However, no active uptake of [^{14}C]chloramphenicol was seen in any of the everted vesicle preparations. Such a negative result could occur if the preparative or assay conditions were inadequate to show chloramphenicol transport.

No saturation of chloramphenicol uptake. Proton motive force comprises two components, $\Delta\Psi$ (electrical potential) and ΔpH (chemical potential). Chloramphenicol is an uncharged, nonpolar molecule. It would not be expected to be excluded from *E. coli* cells purely by passive equilibration with $\Delta\Psi$ (as an anion) or with ΔpH (as a base). We therefore suspected that a specific transport system was involved. One characteristic of such a system should be saturability. We looked for saturation of the active efflux at increasing external concentrations of chloramphenicol.

Since 1 A_{530} unit is equivalent to a cellular volume of 0.0011 ml, cells would take up 1.4 pmol/A_{530} unit simply by equilibration with 1.3 μM external chloramphenicol. In fact, deenergized wild-type AG100 cells took up 18.5 pmol/A_{530} unit (Table 1), considerably more. Our first observation upon performing saturation experiments was that this energy-independent component was saturable as chloramphenicol concentrations were increased from 0.075 to 9 μM. From a Scatchard plot of picomoles per A_{530} unit per external concentration (micromolar) versus picomoles per A_{530} unit, half saturation in strain LCHM164 (a derivative of AG100 [see below]) was observed at 1.1 μM (one determination). About 57 pmol of chloramphenicol/A_{530} unit was maximally bound. For Mar mutant Tc2.5-1, similar values of 1.8 μM and 48 pmol/A_{530} unit were found (one determination). The maximum values corresponded to 38,000 to 45,000 molecules of chloramphenicol per cell. *E. coli* cells grown under conditions used here are reported to have approximately 70,000 ribosomes per cell (4), each capable of binding one chloramphenicol molecule (9, 13). Our half saturation value of 1 to 2 μM agrees with the K_d of 2.3 μM found for chloramphenicol binding to isolated ribosomes (15) and 3 μM for binding to intact cells (13). Therefore, this saturable accumulation we see in the absence of glucose might represent binding to ribosomes.

Next we looked for saturation of the active efflux component in AG100. Saturation would be represented by a decrease in the ability of glucose-energized cells to keep out chloramphenicol as the external drug concentration rose. Cells at an A_{530} of 30 were required to obtain sufficient radioactivity on the filters. The specific activity was 1.12 mCi/mmol. As the external chloramphenicol concentration increased from 10 to 230 μM, the ratio of uptake with glucose to uptake in its absence did not decrease (data not shown). This finding meant that no saturation of the active component in cells was detected up to the highest concentration tested.

Active efflux of chloramphenicol in Mar mutants. We examined the transport of chloramphenicol in several Mar mutants of AG100. These mutants had been selected on either tetracycline or chloramphenicol as noted (Table 1). The high selection levels precluded their being simply *ompF* mutants (8). They were defined as Mar mutants because, besides being resistant to multiple antibiotics, they had enhanced transcription of mRNA which hybridized with a *marORAB* probe (17) and because in all cases introduction of *marA*::Tn*5* by P1 transduction resulted in loss of most resistance (Table 1; data not shown). Strain Tc2.5-1 also displayed an outer membrane protein profile on polyacrylamide gel electrophoresis which was characteristic of Mar strains (8).

In Mar mutants, energization by glucose was more effective than it had been in the wild-type parent AG100: the ratio of chloramphenicol uptake at equilibrium in the presence of glucose to uptake in its absence was lower (Table 1, strains Tc2.5-1, LM313.1, and LM314.1). Therefore, more drug appeared to be kept out in the Mar mutants. The *marORAB* operon was responsible for this effect, because inactivation with *marA*::Tn*5* restored the ratio to the wild-type value (Table 1, strains LM249, LM313.5, and LM314.5).

Attempts were made to compare the rate of efflux of chloramphenicol during the first 80 s after glucose addition for Mar mutants and their *marA*::Tn*5* derivatives or the wild type. A very small increase in rate (1.2- to 1.8-fold) was seen for Mar mutants, but the increase did not appear to be proportional to the level of Mar resistance. The kinetic assay was not precise enough for more detailed studies.

To determine whether sequences within or near the *mar* locus were required for efflux in wild-type cells, we used strain LCHM164, which was identical to wild-type AG100 except that 39 kb including the *mar* locus had been deleted. The ratio of uptake with glucose to uptake without glucose was the same as for the wild type (Table 1). Therefore, wild-type efflux could occur in the absence of the *mar* locus region.

Concluding remarks. Our experiments show an energy-dependent loss of chloramphenicol from wild-type cells which is enhanced in Mar mutants. We have interpreted these results in terms of an active efflux system which is stronger in Mar mutants and thereby contributes to their enhanced chloramphenicol resistance. We cannot exclude (although consider less likely) an alternative explanation, i.e., that cellular energization lowers the affinity of ribosomes for the drug.

An earlier worker (1) had seen much greater uptake of chloramphenicol in energized *E. coli* cells than we did and reported an active uptake, not efflux. We were not able to repeat these earlier observations. Using a variety of methods (including the one cited in reference 1), we consistently observed only an energy-dependent decrease of chloramphenicol accumulation. Other workers, using a silicone oil method with cells growing in nutrient broth, found that at 15 μM chloramphenicol, the amount of drug associated with cells not attributable to ribosomes was concentrated only by a factor of 2 (13), similar to our results. Energy dependence of transport was not investigated in that study. In *Haemophilus* cells an active uptake of chloramphenicol has been found (5).

The active efflux of chloramphenicol in susceptible *E. coli* cells described here may depend on proton motive force and is possibly carrier mediated, although no saturability or cell-free assay system could be shown. That the efflux was almost equally effective at pH 7.7 and pH 6.0 may indicate an electrogenic mechanism.

The gene for the putative chloramphenicol transporter of wild-type cells cannot be located within the 39-kb deletion encompassing the *mar* locus. However, overexpression of the *marRAB* operon in Mar mutants correlated with a strengthened net efflux. This effect could result from a direct or indirect effect of a *mar* product on the putative transporter of wild-type cells or could involve a second *mar*-specific transporter. Candidates for another chromosomal gene(s) involved in the chloramphenicol efflux might be *cmlA* (min 19) (23) and *cmlC* (min 72) (2). When mutated, both probably lead to nondegradative, nonribosomal chloramphenicol resistance, but they have not been further characterized. The effect of *ram*, a multiresistance gene from *Klebsiella pneumoniae*, on active efflux of chloramphenicol has recently been observed by one of us (12a). The sequences of certain plasmid-mediated or amplifiable, nondegradative chloramphenicol resistance determinants suggest that they encode membrane-embedded transport proteins (3, 10, 11, 26) which may expel chloramphenicol. Chromosomally encoded systems in susceptible *E. coli* which actively expel other toxic substances have also been described (7, 16, 19, 22). Whether chloramphenicol and other substances are the natural substrates for the chromosomal systems remains unknown.

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REFERENCES

1. **Abdel-Sayed, S.** 1987. Transport of chloramphenicol into sensitive strains of *Escherichia coli* and *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **19:**7–20.
2. **Baughman, G. A., and S. R. Fahnestock.** 1979. Chloramphenicol resistance mutation in *Escherichia coli* which maps in a major ribosomal protein gene cluster. J. Bacteriol. **137:**1315–1323.
3. **Bissonnette, L., S. Champetier, J.-P. Buisson, and P. H. Roy.** 1991. Characterization of the nonenzymatic chloramphenicol resistance (*cmlA*) gene of the In*4* integron of Tn*1696*: similarity of the product to transmembrane transport proteins. J. Bacteriol. **173:**4493–4502.
4. **Bremer, H., and P. P. Dennis.** 1987. Modulation of chemical composition and other parameters of the cell by growth rate, p. 1527–1542. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
5. **Burns, J. L., and A. L. Smith.** 1987. Chloramphenicol accumulation by *Haemophilus influenzae*. Antimicrob. Agents Chemother. **31:**686–690.
6. **Cohen, S. P., H. Hächler, and S. B. Levy.** 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J. Bacteriol. **175:**1484–1492.
7. **Cohen, S. P., D. C. Hooper, J. S. Wolfson, K. A. Souza, L. M. McMurry, and S. B. Levy.** 1988. Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. Antimicrob. Agents Chemother. **32:**1187–1191.
8. **Cohen, S. P., L. M. McMurry, D. C. Hooper, J. S. Wolfson, and S. B. Levy.** 1989. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob. Agents Chemother. **33:**1318–1325.
9. **Das, H. K., A. Goldstein, and L. C. Kanner.** 1966. Inhibition by chloramphenicol of the growth of nascent protein chains in *Escherichia coli*. Mol. Pharmacol. **2:**158–170.
10. **Desomer, J., D. Vereecke, M. Crespi, and M. VanMontagu.** 1992. The plasmid-encoded chloramphenicol-resistance protein of *Rhodococcus fascians* is homologous to the transmembrane tetracycline efflux proteins. Mol. Microbiol. **6:**2377–2385.
11. **Dittrich, W., M. Betzler, and H. Schrempf.** 1991. An amplifiable and deletable chloramphenicol-resistance determinant of *Streptomyces lividans* 1326 encodes a putative transmembrane protein. Mol. Microbiol. **5:**2789–2797.

11a. **George, A. M., R. M. Hall, A. J. Phelps, R. G. Forage, and H. W. Stokes.** Unpublished results.

12. **George, A. M., and S. B. Levy.** 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. J. Bacteriol. **155:**531–540.
13. **Hurwitz, C., and C. B. Braun.** 1967. Measurement of binding of chloramphenicol by intact cells. J. Bacteriol. **93:**1671–1676.
14. **Jensen, F. K.** 1993. The *Escherichia coli* K-12 "wild types" W3110 and MG1655 have an *rph* frameshift mutation that leads to pyrimidine starvation due to low *pyrE* expression levels. J. Bacteriol. **175:**3401–3407.
15. **Lessard, J. L., and S. Pestka.** 1972. Studies on the formation of transfer ribonucleic acid-ribosome complexes. XXIII. Chloramphenicol, aminoacyl-oligonucleotides, and *Escherichia coli* ribosomes. J. Biol. Chem. **247:**6909–6912.
16. **Lomovskaya, O., and K. Lewis.** 1992. *emr*, an *Escherichia coli* locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89:**8938–8942.
17. **Maneewannakul, K., E. Nulty, and L. M. McMurry.** 1993. Unpublished results.
18. **McMurry, L., 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.
19. **McMurry, L. M., D. A. Aronson, and S. B. Levy.** 1983. Susceptible *Escherichia coli* cells can actively excrete tetracyclines. Antimicrob. Agents Chemother. **24:**544–551.
20. **McMurry, L. M., M. Stephan, and S. B. Levy.** 1992. Decreased function of the class B tetracycline efflux protein Tet with mutations at aspartate 15, a putative intramembrane residue. J. Bacteriol. **174:**6294–6297.
21. **Prokesch, R. C., and W. L. Hand.** 1982. Antibiotic entry into

human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **21**:373–380.

22. **Purewal, A. S., I. G. Jones, and M. Midgley.** 1990. Cloning of the ethidium efflux gene from *Escherichia coli*. FEMS Microbiol. Lett. **68**:73–76.
23. **Reeve, E. C. R.** 1968. Genetic analysis of some mutations causing resistance to tetracyclines in *Escherichia coli* K-12. Genet. Res. **11**:303–309.
24. **Rosen, B. P., and E. R. Kashket.** 1978. Energetics of active transport, p. 559–620. *In* B. P. Rosen (ed.), Bacterial transport. Marcel Dekker, Inc., New York.
25. **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.
26. **Stokes, H. W., and R. M. Hall.** 1991. Sequence analysis of the inducible chloramphenicol resistance determinant in the Tn*1696* integron suggests regulation by translational attenuation. Plasmid **26**:10–19.