Proc. Natl. Acad. Sci. USA
Vol. 77, No. 7, pp. 3974–3977, July 1980
Biochemistry

Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*

(everted membrane vesicles/tetracycline transport/transposon Tn10/plasmids)

LAURA MCMURRY, RICHARD E. PETRUCCI, JR., AND STUART B. LEVY*

Department of Molecular Biology and Microbiology and Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts 02111

Communicated by Boris Magasanik, April 21, 1980

ABSTRACT **Tetracycline resistance encoded by four genetically different determinants residing on plasmids in *Escherichia coli* was shown to be associated in each case with an energy-dependent decrease in accumulation of the antibiotic in whole cells in which resistance had been induced. The different class determinants examined were those on plasmids RP1 (class A), R222 (class B), R144 (class C), and RA1 (class D). This decrease in accumulation was attributable to an active efflux, because everted (inside-out) membrane vesicles made from tetracycline-induced *E. coli* cells containing any one of the four plasmids were shown to concentrate tetracycline by an active influx. This active uptake was not seen in inside-out vesicles from sensitive cells or uninduced R222-containing cells. In vesicles from induced R222-containing cells, the efflux appeared to be carrier-mediated with a K_m of about 6 μM. These results demonstrate that active export of tetracycline is a common component of the mechanism for tetracycline resistance encoded by different plasmid-borne determinants in bacteria.**

The tetracyclines are bacteriostatic antibiotics used to treat a broad spectrum of microbial disease agents in humans, animals, and plants (1). They act by inhibiting protein synthesis, specifically by preventing aminoacyl tRNA from binding to the A site on the ribosome during peptide elongation (2–4). Bacterial resistance to tetracycline is widespread and is caused by at least four different resistance determinants (5) carried on plasmids in the host bacterial cell. In most cases, resistance is inducible by subinhibitory amounts of tetracycline, and for some plasmids it can reach 200 times the resistance of plasmidless cells. The most common tetracycline resistance determinant in *Escherichia coli* appears to be that borne on transposon Tn10.†

Plasmid-mediated tetracycline resistance does not lead to inactivation of the tetracycline molecule (6–8). Rather, resistance is associated with a decrease in tetracycline accumulation (8–11). A tetracycline-inducible inner membrane protein of molecular weight about 37,000, TET, is associated with Tn10-encoded resistance (8, 12), and a repressor activity of TET synthesis has been found (13). A TET protein of similar size encoded by other plasmid-borne resistance determinants has been described (8, 12, 14–16).

In previous work we showed two transport systems for tetracycline in sensitive *E. coli* K-12 cells, only one of which was sensitive to energy inhibitors (17). Both were altered by the tetracycline resistance plasmid R222 (11). The energy-dependent component of uptake in sensitive cells was replaced in resistant cells by a non-energy-requiring uptake of a lesser rate. In addition, the energy-independent uptake system in sensitive cells was decreased to $\frac{1}{3}$–$\frac{1}{5}$ (11). This decrease appeared to be at least partly reversed by energy inhibitors (11), which suggested that energy was required for the lowered tetracycline accumulation. Using everted membrane vesicles, we now demonstrate that resistant cells containing any one of four genetically different plasmid-borne tetracycline resistance determinants (5) possess an active efflux system for this antibiotic.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The prototroph *E. coli* strain ML308-225 (*lacI*$^-$ *lacY*$^+$ *lacZ*$^-$) (18) (obtained from P. D. Bragg) was used for these studies. Plasmids R222 (resistant to chloramphenicol, sulfonamides, streptomycin, and tetracycline), RP1 (resistant to ampicillin, kanamycin, and tetracycline), RA1 (resistant to tetracycline), and R144 (resistant to kanamycin and tetracycline) were introduced into this strain by conjugation with the appropriate plasmid-containing C600 strain used by Méndez *et al.* (5). The resulting strains we designate, respectively, as D1-209, D15-12, D7-3, and D2-29.

Media and Chemicals. Medium A (19) used for growing cells contained (g/liter) K_2HPO_4, 7; KH_2PO_4, 3; trisodium citrate, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0. It was adjusted to pH 7.0 and supplemented with 0.1% casamino acids (Difco) and 0.2% glucose or 0.5% glycerol. Tetracycline hydrochloride, D(−)-lactic acid (lithium salt), ATP (from equine muscle, disodium salt), phenazine methosulfate (PMS), and L-ascorbic acid (sodium salt) were purchased from Sigma. Lithium chloride was purchased from Fisher. 2,4-Dinitrophenol (DNP) came from General Biochemicals (Chagrin Falls, OH).

Radiochemicals and Efficiencies of Counting. [7-^{3}H]-Tetracycline (0.6–1 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was obtained as the powdered free base from New England Nuclear. A portion was freshly dissolved in a small volume of methanol and diluted into water each week to avoid contaminating breakdown products, which accumulate in solution with time (8).

L-[^{3}H]Proline (5 Ci/mmol), $^{45}CaCl_2$ (16.7 mCi/mg), [^{14}C]-dextran (1.1 μCi/mg, average molecular weight 20,000), and ^3H_2O (1.31 μCi/ml) were obtained from the same source. The counting efficiencies of tritium in aqueous samples in Ultrafluor (National Diagnostics, Somerville, NJ) and on filters in Liquifluor (New England Nuclear) were 30% and 25%, respectively.

Preparation of Everted Membrane Vesicles. Cells were grown in medium A with glucose or glycerol. Plasmid-con-

Abbreviations: DNP, 2,4-dinitrophenol; PMS, phenazine methosulfate.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* To whom reprint requests should be addressed.

† Tachibana, C., Marshall, B., Schluederberg, S. & Levy, S. B. (1980) American Society for Microbiology, Miami Beach, FL, May 11–16, p. 115 (abstr.).

taining cells were grown with 4 μM (1.9 μg/ml) tetracycline to induce high-level resistance when required. We showed by replica plating that >95% of uninduced cells (grown without tetracycline) retained the plasmid at the time of cell harvest. Cells were washed once in 100 mM KPO_4/10 mM NaEDTA, pH 6.6, at 4°C and resuspended in the same buffer. They were lysed by using a French pressure cell at 5000 pounds/inch^2 (34 MPa) as described (20) and unlysed cells were removed by centrifuging at 27,000 × *g* for 10 min. Vesicles were sedimented at 150,000 × *g* for 1 hr, resuspended gently either in 5 mM Tris·HCl, pH 7.2/70 mM KCl/0.25 mM dithiothreitol/50% (vol/vol) glycerol, or in 50 mM KPO_4, pH 6.6, by using a glass rod and adding small increments of suspending buffer. The vesicles were then frozen at approximately 8 mg of protein per ml in dry ice/ethanol and stored at −70°C. Protein determinations were made by the method of Lowry (21), using bovine serum albumin as a standard.

Tetracycline Uptake by Cells and Vesicles. *Whole cells.* Cells were grown overnight at 37°C in medium A containing 0.2% glucose (4 μM tetracycline was added for the plasmid-containing strains to allow induction). In the morning the cells were inoculated into the same medium (again containing 4 μM tetracycline for plasmid-containing cells) at an OD_{530} of 0.1, grown to OD_{530} = 0.8 (late logarithmic phase), centrifuged at 4°C, washed once in 10 mM Tris·HCl, pH 8.0 at 4°C, and resuspended to OD_{530} = 4 (about 2 × 10^9 cells per ml) in 50 mM KPO_4/10 mM $MgSO_4$, pH 6.6, and samples were put into glass tubes in a water bath shaker. After 5–10 min of preincubation at 31°C with or without 1 mM DNP, [^{3}H]tetracycline was added to 5 μM and 5 μCi/ml. At various times, 50-μl samples were removed, mixed with 10 ml of 0.1 M LiCl/0.1 M KPO_4, pH 6.6 at 20°C, filtered through a Gelman GN-6 Metricel (mixed esters of cellulose) 0.45-μm-pore membrane filter, washed with 4 ml of the same buffer, and dried, and radioactivity was measured in Liquifluor in a scintillation counter. Radioactivity trapped by the filters alone was subtracted.

Vesicles. Vesicles were thawed and diluted to 0.7 mg of protein per ml in 50 mM KPO_4/10 mM $MgSO_4$ at the specified pH at 4°C and assayed as for whole cells. The LiCl steps were at the same specified pH. The energy source was added just prior to putting the vesicles at 31°C, and the [^{3}H]tetracycline was added after 5 min at 31°C. For the determination of the effect of pH on tetracycline uptake, 50 mM Tris–maleate/10 mM $MgSO_4$ buffer was used to allow a pH range from 5.7 to 9.5. The KPO_4 in the LiCl steps was replaced by 50 mM Tris–maleate at the same pH as the uptake buffer. Using vesicles made from cells in which protein was labeled during growth with [^{3}H]proline, we found that ≥90% of the vesicle protein was retained by the filters. In calculations, no correction was made for this ≤10% loss.

Volumes of Cells and Vesicles. Cell volume was determined on a cell pellet of known OD_{530} units by using [^{14}C]dextran to identify excluded water (22) [OD units = OD_{530} × volume (ml) centrifuged to obtain pellet]. Total water was determined by weighing the pellet before and after drying. The internal pellet volume thus measured included periplasmic space. The internal cell volume was found to be 1.1 μl per OD_{530} unit (300 μg protein). To determine vesicle volume we also used methods described by Rottenberg (22). Vesicles were incubated under assay conditions (50 mM KPO_4/10 mM $MgSO_4$, pH 6.6, 31°C) for 15 min with [^{14}C]dextran and in some cases ^3H_2O; vesicles were sedimented at 85,000 × *g* for 2 hr at 15°C, and the pellet was dissolved overnight at 37°C in 1% sodium dodecyl sulfate for determination of protein and radioactivity. In two determinations total pellet water was determined (before dissolving in sodium dodecyl sulfate) by weighing before and after drying to constant weight at 55°C. In the other three determinations total pellet water was found from the ^3H_2O content. The average internal volume measured on five different vesicle preparations from glucose-grown cells was 8.4 ± 3.7 (SD) μl/mg of protein.

Uptake of Calcium and Proline by Vesicles. Uptake of ^{45}Ca was measured at 31°C as described (20) at a vesicle protein concentration of 0.2 mg/ml. Gelman GN-6 0.45-μm-pore filters were used. Vesicles were preincubated for 15 min at 31°C prior to addition of energy source, and ^{45}Ca was added 1–2 min afterwards. The uptake of [^{3}H]proline was determined at pH 6.6 by the same procedures used for [^{3}H]tetracycline uptake, using vesicle protein at 0.7 mg/ml, 10 μM total proline, and radioactivity of 5 mCi/ml.

Minimal Inhibitory Concentrations of Tetracycline. Concentrations were determined as described (17) for preinduced cells grown in medium A with glucose.

RESULTS AND DISCUSSION

We chose for this work the *E. coli* strain ML308-225, which has been used in transport studies involving membrane vesicles (23). We first examined tetracycline accumulation in this non-K-12 strain and in its R222 plasmid-bearing derivative in the phosphate/magnesium buffer to be used for vesicle assays. In these experiments, DNP was used as an energy inhibitor. DNP primarily acts to collapse the proton gradient across the cell membrane (24). Such a gradient, resulting from electron transport or ATP hydrolysis via the membrane-bound ATPase, provides energy in the form of a protonmotive force for a number of transport processes as well as for oxidative phosphorylation (24–26). We found, as noted before (17), an energy-dependent and an energy-independent uptake in sensitive cells (Fig. 1). As before (11), the R222-containing cells had lost the energy-dependent uptake, while they showed a DNP-sensitive decrease of the energy-independent uptake. Exogenously added glucose (10 mM) or D-lactate (20 mM) did not change the uptake values of these whole cells (not shown); endogenous energy supplies were evidently adequate. Tetracycline accumulation by cells in the absence of energy was higher than would be expected on the basis of diffusion alone. This finding may be due to binding of the drug to cellular components.

One explanation for the behavior of resistant cells in Fig. 1 is that these cells possessed an energy-dependent efflux for tetracycline. Certain energy-dependent efflux systems in *E. coli*

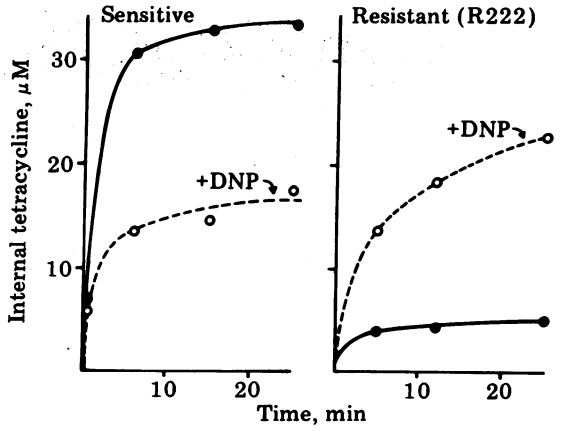

FIG. 1. Tetracycline uptake by *E. coli* ML308-225 (sensitive) and by R222-containing induced (resistant) cells with (○) and without (●) 1 mM DNP. Cells were grown overnight in medium A containing glucose and uptake was measured in the absence of added energy source.

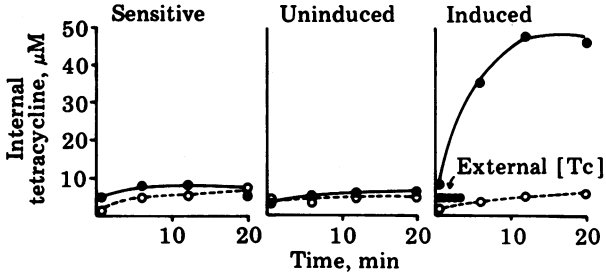

FIG. 2. Tetracycline (Tc) uptake by everted membrane vesicles made from sensitive ML308-225 cells and from uninduced and induced R222-containing cells. ○, No energy; ●, D-lactate. Cells were grown in glycerol and vesicles were frozen in 5 mM Tris·HCl, pH 7.2/70 mM KCl/0.25 mM dithiothreitol/50% glycerol. The assay was done at pH 6.6.

have proven amenable to study *in vitro* by using membrane vesicles prepared by lysis of cells with a French pressure cell (20, 27). The membranes in these vesicles have an orientation opposite to that *in vivo*, so that an active efflux *in vivo* becomes an active uptake *in vitro*. We therefore prepared everted membrane vesicles from sensitive and resistant cells to look for active uptake of tetracycline.

To show that our vesicles were indeed everted, we measured calcium and proline uptakes. Calcium, but not proline, should be taken up actively by everted vesicles in the presence of electron transport substrates, such as NADH, which cause formation of a protonmotive force (28, 29). Vesicles made from glycerol-grown cells and frozen in the buffer containing glycerol (see *Materials and Methods*) showed uptake of 210 ± 30 (SD for three preparations) nmol of calcium per mg of vesicle protein in 10 min with 5 mM NADH as energy source and 20 ± 3.5 nmol/mg with 20 mM D-lactate. With 20 mM sodium ascorbate/0.1 mM PMS, uptake was about 40% that with NADH. These numbers, except for the lower lactate values (which may be due to a difference in strain or in growth conditions for cells), are like those reported by others for similarly prepared everted vesicles (27). Four preparations grown in glucose and frozen in 50 mM KPO_4, pH 6.6, showed an average uptake of 110 ± 70 nmol/mg with NADH, 5.7 ± 5.5 nmol/mg with D-lactate, and 40 ± 30 nmol/mg with ascorbate/PMS.

Further confirmation that the vesicles were everted was the virtual absence of active proline uptake. For four vesicle preparations (two glycerol-grown, two glucose-grown), the average uptake by 20 min was 0.038 ± 0.023 nmol/mg with D-lactate and 0.027 ± 0.021 nmol/mg without D-lactate. The latter value corresponds to an internal proline concentration consistent with simple equilibration with the external 10 μM proline. That our assay for proline uptake was functioning was shown with right-side-out membrane vesicles. When we prepared such vesicles from succinate-grown cells by using EDTA and lysozyme (23) and assayed them as described here, we found the proline was concentrated about 60-fold (assuming a volume of 5 μl/mg of protein).

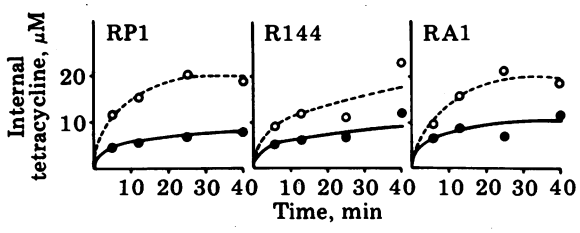

FIG. 3. Tetracycline uptake by *E. coli* ML308-225 cells containing plasmid RP1, R144, or RA1, with (○) or without (●) 1 mM DNP. All cells were grown in glucose and induced with 4 μM tetracycline.

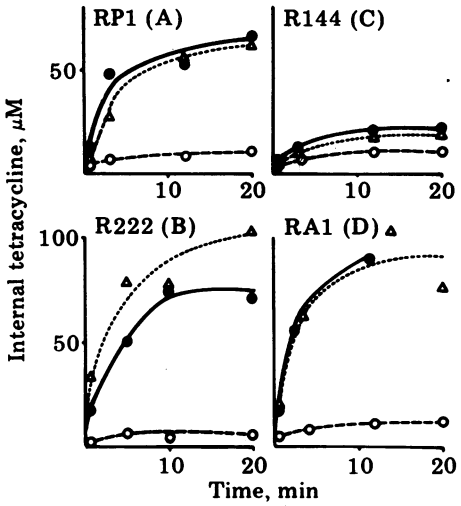

FIG. 4. Uptake of tetracycline by everted membrane vesicles from ML308-225 cells containing plasmid RP1, R222, R144, or RA1 (types in parentheses). ○, No energy; ●, 20 mM D-lactate; △, 5 mM ATP. Cells were grown in glucose and vesicles were frozen in 50 mM KPO_4, pH 6.6. The assay was done at pH 7.5.

We examined tetracycline uptake in everted vesicles from sensitive, uninduced, and induced resistant cells. Neither the vesicles from sensitive cells nor those from uninduced R222-containing cells had any active uptake (stimulated by 20 mM D-lactate). The small uptake that did occur corresponded approximately to equilibration with external (5 μM) tetracycline (Fig. 2). Vesicles from *induced* R222-containing cells, however, showed an extensive uptake of tetracycline supported by D-lactate (Fig. 2; see also Fig. 4) and ATP (see Fig. 4) and completely inhibited by DNP (not shown). The internal concentration at steady state was 10- to 20-fold greater than the external 5 μM. Sodium ascorbate (20 mM) followed by PMS (0.1 mM), added 30 sec prior to [^{3}H]tetracycline, energized uptake in the induced vesicles as well as did D-lactate. NADH (5 mM) was about 10% as effective as lactate. When the membrane-bound ATPase occurs on the outer surface of a vesicle membrane, rather than on the inner surface as is the case *in vivo*, it becomes accessible to externally added (impermeant) ATP molecules, and the polarity of the protonmotive force induced by ATP is reversed (29, 30). That ATP stimulated tetracycline uptake supported the idea that the energy-dependent movement of tetracycline across the vesicle membrane was opposite in direction to that across the membrane of whole cells. Because efflux was demonstrated *in vitro* by using membrane vesicles, it would appear that the resistant cells *in vivo* need only the energized membrane (protonmotive force or a derivative thereof), and not cytoplasmic factors, to drive tetracycline out of the cell against a concentration gradient.

We determined the optima in pH and magnesium concentration for this *in vitro* efflux system. Lactate-dependent tetracycline accumulation by 20 min was maximal at pH 7.7; little if any accumulation occurred below pH 6.2 or above pH 9.5. Magnesium was needed for uptake. No lactate-stimulated uptake of tetracycline occurred in the absence of $MgSO_4$ at either pH 6.6 or 7.5 in 50 mM KPO_4; the uptake at pH 6.6 had a broad maximum from 3 to 10 mM $MgSO_4$.

We next examined the saturation kinetics of this efflux system. The rate of lactate-dependent uptake between 15 sec and 3 min after addition of [^{3}H]tetracycline was measured in vesicles from glucose-grown cells at pH 7.5 as a function of external tetracycline concentration. Using Lineweaver–Burk plots (31), a K_m of 6 ± 2 μM and a V_{max} ranging from 0.1 to 1 nmol/min

Table 1. Minimal inhibitory concentrations (MICs) for tetracycline compared to uptake of tetracycline in everted vesicles

Strain	Plasmid introduced	MIC, μM	Tetracycline uptake, μM in vesicles*
ML308-225	None	1.0	<2
D2-29	R144	85	11
D15-12	RP1	125	45
D7-3	RA1	225	83
D1-209	R222	245	120

* The D-lactate-dependent concentration of tetracycline after 20 min of uptake in 50 mM KPO_4/10 mM $MgSO_4$ at pH 7.5 was determined by averaging values for two preparations of vesicles from glucose-grown ML308-225 cells containing the plasmids shown. External tetracycline in the vesicle assay was 5 μM.

per mg of protein was found, based on three determinations using two vesicle preparations. A lactate-dependent uptake between 0 and 15 sec occurred at a rate too fast to measure accurately but appeared to be similarly saturable. The saturability of the lactate-dependent efflux of tetracycline suggests that this transport is carrier-mediated.

As mentioned previously, four different classes of tetracycline resistance determinants, distinguishable by DNA·DNA hybridization, have been found among naturally occurring plasmids (5). These classes differ in their level of resistance to tetracycline and its analogs. The determinant on R222 represents class B. We chose one representative plasmid bearing each of the other three classes to see whether these determinants also coded for an active tetracycline efflux. The plasmids were RP1 (class A), R144 (class C), and RA1 (class D). Each of these plasmids was introduced separately into the ML308-225 strain by conjugation. When the cells were induced by growing in 4 μM tetracycline, all three hosts showed a decrease of tetracycline uptake that was counteracted by DNP (Fig. 3). Vesicles from induced glucose-grown cells in all cases demonstrated an energy-dependent uptake of tetracycline with lactate and ATP (Fig. 4). The magnitude of this uptake appeared to be related to the resistance level encoded by the plasmid (Table 1).

These studies demonstrate that an energy-dependent efflux of the antibiotic tetracycline is coded for by each of four different tetracycline resistance determinants, whose DNA did not crosshybridize in DNA·DNA hybridization experiments. Class A, B, and C determinants examined in the minicell system (12) encode inducible TET proteins of similar but probably not exactly the same molecular weight (unpublished). The efflux systems are also inducible and are manifest in membrane vesicles. The inner membrane TET protein from R222 could be, therefore, its saturable carrier. Alternatively, but less likely, a plasmid-encoded product could cause a chromosomally derived system to export tetracycline.

That tetracycline resistance plasmids of all four classes code for efflux of tetracycline strongly suggests that efflux is part of the resistance mechanism. Nevertheless, in a comparison of sensitive and resistant cells, the tetracycline accumulation in the resistant cell does not appear to be reduced enough, whether via active efflux or via lowered uptake rate or a combination, to account entirely for the degree of resistance conferred by the plasmids (ref. 8; compare Fig. 1 to Table 1). Uncovering of mechanisms in addition to those involving transport may be needed before understanding of tetracycline resistance is complete.

We appreciate the work of Brooke Hadwen and Stanley J. Miller III in some of these experiments. The work in this paper was supported by American Cancer Society Grant MV-73C.

1. Goodman, L. S., Goodman, A. G. & Koelle, G. B. (1975) *The Pharmacological Basis of Therapeutics* (Macmillan, New York).
2. Suarez, G. & Nathans, D. (1965) *Biochem. Biophys. Res. Commun.* **18**, 743–750.
3. Hierowski, M. (1965) *Proc. Natl. Acad. Sci. USA* **53**, 594–599.
4. Sarkar, S. & Thach, R. E. (1968) *Proc. Natl. Acad. Sci. USA* **60**, 1479–1486.
5. Méndez, B., Tachibana, C. & Levy, S. B. (1980) *Plasmid* **3**, 99–108.
6. DeZeeuw, J. R. (1968) *J. Bacteriol.* **95**, 498–506.
7. Sompolinsky, D., Krawitz, T., Zaidenzaig, Y. & Abramova, N. (1970) *J. Gen. Microbiol.* **62**, 341–349.
8. Levy, S. B., McMurry, L., Onigman, P. & Saunders, R. M. (1977) in *Topics in Infectious Diseases*, eds. Drews, J. & Högenauer, G. (Springer, Vienna), Vol. 5, pp. 181–206.
9. Franklin, T. J. & Godfrey, A. (1965) *Biochem. J.* **94**, 54–60.
10. Izaki, K., Kiuchi, K. & Arima, K. (1966) *J. Bacteriol.* **91**, 628–633.
11. Levy, S. B. & McMurry, L. (1978) *Nature (London)* **275**, 90–92.
12. Levy, S. B. & McMurry, L. (1974) *Biochem. Biophys. Res. Commun.* **56**, 1060–1068.
13. Yang, H.-L., Zubay, G. & Levy, S. B. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1509–1512.
14. Tait, R. C. & Boyer, H. W. (1978) *Cell* **13**, 73–81.
15. Gayda, R. C., Tausde, J. H., Knigge, K. M. & Markovitz, A. (1979) *Plasmid* **2**, 417–425.
16. Wojdani, A., Avtalion, R. R. & Sompolinsky, D. (1976) *Antimicrob. Agents Chemother.* **9**, 526–534.
17. McMurry, L. & Levy, S. B. (1978) *Antimicrob. Agents Chemother.* **14**, 201–209.
18. Winkler, H. H. & Wilson, T. H. (1966) *J. Biol. Chem.* **241**, 2200–2211.
19. Davis, B. D. & Mingioli, E. S. (1950) *J. Bacteriol.* **60**, 17–28.
20. Rosen, B. P. & Tsuchiya, T. (1979) *Methods Enzymol.* **56**, 233–241.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
22. Rottenberg, H. (1979) *Methods Enzymol.* **55**, 547–569.
23. Kaback, H. R. (1971) *Methods Enzymol.* **22**, 99–120.
24. Harold, F. M. (1972) *Bacteriol. Rev.* **36**, 172–230.
25. Harold, F. M. (1977) in *Current Topics in Bioenergetics*, ed. Sanadi, D. R. (Academic, New York), Vol. 6, pp. 83–151.
26. Rosen, B. P. & Kashket, E. R. (1978) in *Bacterial Transport*, ed. Rosen, B. P. (Dekker, New York), pp. 559–620.
27. Tsuchiya, T. & Rosen, B. P. (1975) *J. Biol. Chem.* **250**, 7687–7692.
28. Rosen, B. P. & McClees, J. S. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 5042–5046.
29. Adler, L. W., Ichikawa, T., Hasan, S. M., Tsuchiya, T. & Rosen, B. P. (1977) *J. Supramol. Struct.* **7**, 15–27.
30. Adler, L. W. & Rosen, B. P. (1977) *J. Bacteriol.* **129**, 959–966.
31. Whitaker, J. R. (1972) *Principles of Enzymology for the Food Sciences* (Dekker, New York).