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Role of an Energy-Dependent Efflux Pump in Plasmid pNE24-Mediated Resistance to 14- and 15-Membered Macrolides in *Staphylococcus epidermidis*

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We have elucidated a new mechanism for bacterial resistance to the 14-membered macrolides oleandomycin and erythromycin and the 15-membered macrolide azithromycin. Plasmid pNE24, previously isolated from a clinical specimen of *Staphylococcus epidermidis*, was characterized as causing resistance to 14-membered but not 16-membered macrolides by a mechanism suggested to involve reduced antibiotic permeation of bacterial cells (B. C. Lampson, W. von David, and J. T. Parisi, Antimicrob. Agents Chemother. 30:653–658, 1986). Our recent investigations have demonstrated that *S. epidermidis* 958-2 containing plasmid pNE24 also contains an energy-dependent macrolide efflux pump which maintains intracellular antibiotic concentrations below those required for binding to ribosomes. Thus, when strain 958-2 was pretreated with the inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), macrolide accumulated at the same rate and to the same extent as in CCCP-treated or untreated control cells lacking plasmid pNE24 (strain 958-1). In contrast, macrolide did not accumulate in energy-competent strain 958-2 but did accumulate to levels equal to those of ribosomes immediately following CCCP addition. Furthermore, intracellular macrolide was excreted and bacteria resumed growth when CCCP but not macrolide was removed from the growth medium. As expected, the 16-membered macrolide niddamycin accumulated to the same level in energy-competent strains 958-1 and 958-2 at the same rapid rate. Macrolide incubated with lysates prepared from both strains or recovered from cells of strain 958-2 was unmodified and bound to ribosomes from strains 958-1 and 958-2 with identical affinities and kinetics, thus precluding a role for ribosome or drug alteration in the resistance mechanism. We conclude that the presence of plasmid pNE24 results in specific energy-dependent efflux of 14- and 15-membered macrolides.

Bacterial strains containing novel mechanisms of resistance to antibiotics are continually being selected in the clinical environment. The most frequent form of resistance to macrolide antibiotics is combined macrolides-lincosamides-streptogramin B resistance (12). In this case, the mechanism involves dimethylation of adenine 2058 in 28S rRNA by a specific methylase, resulting in ribosomes which no longer bind macrolides-lincosamides-streptogramin B antibiotics (19, 26). The *erm* genes that code for specific methylases are located in the chromosome or on transmissible plasmids and can be constitutively or inducibly expressed (11, 31). Three other forms of resistance to macrolides are also known, i.e., hydrolysis of the macrolide ring by esterases (1), phosphorylation (33), and glycosylation (17) at the 2′ position. We are aware of only two reports indicating that macrolide resistance might occur by a mechanism involving reduced antibiotic entry into cells. One case involves a clinical isolate of *Staphylococcus epidermidis* containing plasmid pNE24 (20), and the other concerns macrolide producers (13).

The mechanism by which macrolides gain entry into bacterial cells is intimately related to potential resistance mechanisms. Recently reported experimental data indicate that the neutral but not the protonated form of basic macrolides is capable of entering bacterial cells by passive diffusion (3, 10, 25). This inference is supported by the following facts. (i) There is no evidence for a membrane carrier for macrolides or involvement of chemical energy (10, 21) or the membrane proton motive force (PMF) (7) in passage of macrolides across the cell membrane of bacteria. (ii) Restriction of the entrance of the protonated form can be thermodynamically explained by strong ionic hydrogen bonding of water (22) and the recent demonstration that the free energy of transfer between water and organic solvent is directly related to the entropy of solvation of a solute (32). (iii) The potency of basic but not neutral macrolides increases as the extracellular pH is increased (10, 23), with a concurrent increase in the macrolide uptake rate (10). Our own recent experimental and theoretical analyses indicate that the calculated permeability coefficient of the neutral macrolide form, based on equations derived from model (30) or natural membranes (9), and the measured velocity of initial net flux of macrolides into *Bacillus subtilis* cells are compatible with passive diffusion of the neutral form through a biological membrane with a mass selectivity coefficient, *sm* (28), of −4.6 relative to methanol. Our value of $sm = -4.6$ is within the range of other biological membranes (28).

S. epidermidis containing plasmid pNE24 was isolated and characterized as causing resistance to 14-membered but not 16-membered macrolides by a mechanism which appeared to involve reducing the permeability of cells to the antibiotic (20). However, on the basis of the considerations mentioned above, we considered it unlikely that significant bacterial resistance to macrolides could arise by a mechanism involving reduced permeation. In this report, we show that *S. epidermidis* 958-2 containing plasmid pNE24 also contains an energy-dependent macrolide efflux pump which maintains intracellular antibiotic concentrations below those required for binding to ribosomes.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. epidermidis* 958-1 and its transformant containing plasmid pNE24 (strain 958-2) were obtained from J. Parisi (University of Missouri, Columbia). Bacteria were grown at 37°C in Todd-Hewitt medium (Difco Laboratories).

Macrolide uptake studies. Bacteria were harvested during logarithmic growth and resuspended in 1/10 of the original volume in Todd-Hewitt medium. Concentrated cells were preincubated for 10 min with or without 0.1 mM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), followed by incubation at 37°C for 30 to 70 min with [^{3}H]erythromycin, [^{14}C]erythromycin, [^{14}C]azithromycin, or [^{14}C]niddamycin (0.5 to 4 μg/ml) with constant shaking. In several studies, CCCP was added 30 to 40 min after macrolide. Triplicate samples (200 μl) were removed from incubation at the times indicated, and cells were separated from the medium by centrifugation (13,000 × *g* for 1 min) through silicone oil (6). Cell pellets were processed as described previously for quantitation of radioactivity in a Packard Tri-Carb 300 scintillation counter (6). The value at time zero is the amount of macrolide associated with cells incubated with macrolide at 4°C, and this value was subtracted from all datum points. The actual value at 0.5 to 1 μg/ml was 1.4 ± 0.5 pmol/μl of cell volume or 0.8 ± 0.3 pmol/μl of the total cell pellet (n = 8). Intracellular volumes were determined by incubating cells with [^{3}H]$_2$O or [^{14}C]inulin (Amersham CFA.399) for 10 to 15 min, followed by centrifugation through silicone oil, and subtracting the inulin value as the contribution of extracellular space. A value of 2.64×10^{-9} μl per cell was determined by this method. The concentration of macrolide within cells was calculated on the basis of the specific activity of the radiolabeled macrolide, the amount cell associated, and the measured intracellular volume. The number of molecules of macrolide per cell was calculated from these data by using Avogadro's number (6.02×10^{23} molecules per gram-molecular weight of compound).

One set of experiments was conducted to measure the macrolide accumulation rate over a shorter period (0 to 90 s). Since the processing time of centrifugation was 1 min, cells were rapidly chilled in an ice water bath to stop further macrolide influx (10). Control experiments showed that cells incubated at 4°C for 20 min accumulated no macrolide over the zero time point (incubation on ice followed by immediated processing). The initial rates of macrolide influx into CCCP-pretreated strains 958-1 and 958-2 were examined by this method.

Effects of CCCP removal on cell growth and intracellular macrolide levels. Cells growing at 37°C were incubated with 0.1 mM CCCP (i), 1 μg of macrolide per ml plus 0.1 mM CCCP (ii), 1 μg of macrolide per ml (iii), or no addition (iv) for 30 min. The cells were washed twice by centrifugation (10,000 × *g* for 5 min) with an equal volume of medium and suspended in fresh medium at 37°C. The wash and suspension media for (ii) and (iii) contained 1 μg of radiolabeled macrolide per ml to ensure that no macrolide would be lost from the cells during washing. Optical density readings (A_{600}) and CFU per milliliter were measured concurrently to monitor growth and allow calculation of intracellular macrolide concentrations based on a value of 2.64×10^{-9} μl per cell (determined with [^{3}H]$_2$O; see above).

Radiolabeled macrolide was used when determining intracellular levels. Triplicate cell samples (1 ml) were removed from (ii) and (iii) before the wash (30-min point) and after the wash (2-h point) for determination of intracellular macrolide levels as follows. Cells were trapped on a glass fiber filter (Whatman GF/F) and washed three times with 0.9% NaCl. Filters were placed in scintillation vials containing 0.5 ml of NCS tissue solubilizer (Amersham Corp.) and heated at 50°C for 30 min. After cooling, the solution was neutralized with 20 μl of glacial acetic acid and dissolved in 10 ml of Insta Gel (Packard Instrument Co.) for radioactivity determination.

Macrolide efflux following removal of CCCP was also examined in concentrated cells. Cells were incubated in Todd-Hewitt medium at 37°C with 1 μg of [^{14}C]erythromycin per ml for 5 min in the presence or absence of 0.1 mM CCCP. Cells were then washed by centrifugation in medium containing 1 μg of [^{14}C]erythromycin per ml but lacking CCCP and suspended in medium containing 1 μg of [^{14}C]erythromycin per ml. After 15 min of incubation, the cells were again challenged with CCCP. The amount of cell-associated macrolide was determined by the silicone oil method.

Analysis of macrolide binding to ribosomes. Bacteria were harvested during logarithmic growth, washed with 50 mM sodium phosphate buffer (pH 7.5) containing 5 mM $MgCl_2$, and lysed on a Braun homogenizer with glass beads. The glass beads were removed by centrifugation, and the lysate was centrifuged (30,000 × *g* for 30 min), yielding the S30 fraction. The interaction of increasing concentrations of [^{14}C]erythromycin with ribosomes in the S30 extract was measured by trapping the ribosome-macrolide complex on nitrocellulose filters (24) as previously described (14, 15). Binding affinity was calculated by Scatchard analysis (24). Competition for [^{14}C]erythromycin binding to ribosomes was conducted by using 0.1 μM [^{14}C]erythromycin and 0.02 μM ribosomes with a 50% inhibitory concentration for unlabeled erythromycin of 0.1 μM. The rate of dissociation of the macrolide-ribosome complex was measured following addition of a 100-fold excess of unlabeled macrolide, with subsequent monitoring of the amount of macrolide that remained bound. Dissociation rate constants were calculated from these data as previously described (14, 15).

MIC measurement. MIC was determined by microtiter broth dilution by using cells in logarithmic growth and a final inoculum of 2×10^5 CFU/ml. The antibiotic concentration that yielded no visible growth after 18 h at 37°C was defined as the MIC.

Assay for macrolide degradation. The possibility of macrolide degradation was investigated with whole cells and S30 extracts. [^{14}C]erythromycin (4 μg) was added to 0.5 ml of S30 containing 4.5 mg of protein per ml and ATP and GTP at 1 mM each. Following incubation at 37°C for 1 h, 10 ml of saturated Na_2CO_3 was added and the samples were extracted twice with 2 ml of hexane-ethyl acetate (1:1). The amount of erythromycin in the organic phase was determined by scintillation counting, and samples were dried under N_2. Methanol (50 μl) was added, and samples were spotted on Silica Gel 60 thin-layer chromatography plates. Plates were developed in chloroform-methanol-concentrated ammonium hydroxide (9:1:0.1). The position of radioactive material was determined by using a radioactivity scanner (Radiomatics Instruments Inc.). Macrolide recovered from incubation with extracts was also tested for the ability to bind to ribosomes. Strain 958-2 was incubated in Todd-Hewitt medium with 0.5 μg of [^{14}C]erythromycin per ml at 37°C for 20 min. Cells and cell-associated macrolide were recovered on glass fiber filters (GF/F), and macrolide was extracted and analyzed as described above.

Chemicals. Erythromycin A, its derivatives, azithromycin, niddamycin, and [*N-methyl*-^{3}H]erythromycin A (20 mCi/mmol), [*N-methyl*-^{14}C]erythromycin A (105 mCi/mmol),

TABLE 1. MICs for *S. epidermidis* 958-1 and 958-2

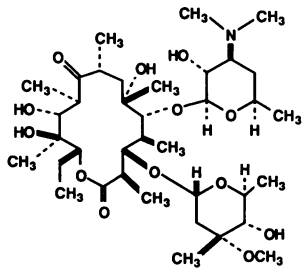
Strain	MIC (μg/ml)[a]					
	Erythromycin	Azithromycin	Compound A	Compound B	Niddamycin	Chalcomycin
958-1	0.04	0.04	6.25	6.25	0.78	0.78
958-2	25 (625)	25 (625)	25 (4)	50 (8)	0.78 (1)	0.78 (1)

[a] The values in parentheses are MIC ratios (958-2/958-1).

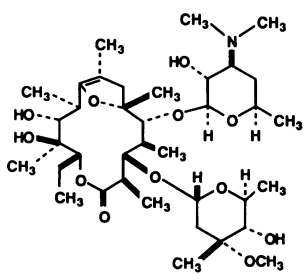
[*N-methyl*-^{14}C]azithromycin (7 mCi/mmol), and [*N-methyl*-^{14}C]niddamycin (5 mCi/mmol) were prepared at Abbott Laboratories. Preparation of compounds A (18) and B (L. A. Freiberg, C. M. Edwards, D. J. Bacino, L. Seif, P. A. Lartey, and D. Wittern, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1029, 1989) has already been described. Verapamil and CCCP were obtained from Sigma Chemical Co.

RESULTS

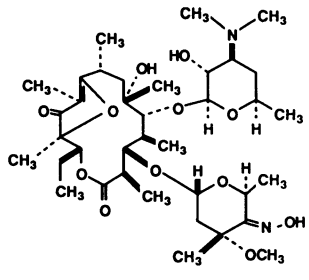
MICs for strains 958-1 and 958-2. The initial report (20) showed that strain 958-2, containing plasmid pNE24, was resistant to erythromycin but susceptible to the 16-membered macrolide tylosin. We obtained similar data (Table 1) showing resistance to erythromycin and susceptibility to the 16-membered macrolides niddamycin and chalcomycin. Strain 958-2 was also resistant to the 15-membered macrolide azithromycin. We tested several erythromycin derivatives for activity against strains 958-1 and 958-2 to see whether modification of erythromycin could improve its potency against strain 958-2. Two derivatives, compounds A and B (Fig. 1), which were 150-fold less active against strain 958-1, had the same (compound A) or only 2-fold less (compound B) activity against strain 958-2 compared with the parent molecule, erythromycin. Thus, the ratios of MICs (958-2/958-1) were 625 for erythromycin and azithromycin but only 4 and 8 for compounds A and B, respectively (Table 1). Verapamil (500 μg/ml) had no effect on the MICs of any of the macrolides tested (see Discussion).



ERYTHROMYCIN



Cmpd A



Cmpd B

FIG. 1. Structures of erythromycin and two derivatives. Cmpd, Compound.

Macrolide interaction with ribosomes. Previous data indicated that ribosomes from strain 958-2, containing plasmid pNE24, did not bind as high a level of erythromycin as did ribosomes from plasmid-free strain 958-1 (20); however, the affinity and kinetics of erythromycin interaction with ribosomes were not reported. We thus felt it was important to examine the interaction of erythromycin with ribosomes from strains 958-1 and 958-2 in more detail to evaluate the possible role of a mechanism which restricts macrolide access to ribosomes in the resistance phenotype of strain 958-2. We devised a method to examine the macrolide-ribosome interaction in the presence of cytoplasmic factors in the S30 fraction. A maximum of 200 μg of S30 protein could be used in each 0.5-ml binding reaction, since nitrocellulose filters became saturated above 200 μg of protein and trapping of the ribosome-macrolide complex became less efficient.

Ribosomes from strains 958-1 and 958-2 were present in the S30 extracts in similar amounts (Fig. 2A) and bound erythromycin with identical affinities (K_d, 1.0×10^{-8} and 1.1×10^{-8} M, respectively; Fig. 2B). In addition, the dissociation rate constants were identical at 37°C (0.044 and 0.047 min^{-1}, respectively; Fig. 2C), as the association rate constants must therefore be. Ribosomes recovered from strains 958-1 and 958-2 were in the range of 20,000 to 30,000 per cell. This value was determined by using the known 1:1 association of erythromycin with ribosomes at saturation binding and the cell number used to prepare S30 extracts.

The two erythromycin derivatives (compounds A and B) with reduced potency against strain 958-1 (Table 1) were tested for the ability to compete for erythromycin binding to ribosomes from strains 958-1 and 958-2. The two erythromycin derivatives both had a 50% inhibitory concentration of 12 μM when they competed for [^{14}C]erythromycin with either 958-1 or 958-2 ribosomes. The 50% inhibitory concentration of unlabeled erythromycin was 0.1 μM under the same reaction conditions (0.1 μM [^{14}C]erythromycin and 0.02 μM ribosomes).

Lack of erythromycin modification. Greater than 95% of the [^{14}C]erythromycin was recovered following incubation with S30 extracts prepared from strains 958-1 and 958-2. This recovered material migrated as a single component with the same R_f value (0.51) as starting material by thin-layer chromatographic analysis and was fully active in ribosome binding (data not shown). Almost identical results were observed with macrolide recovered from strain 958-2 (95% recovery from cells, greater than 90% migration as erythromycin on thin-layer chromatography, and activity in binding to ribosomes).

Uptake of macrolides by strains 958-1 and 958-2. A previous report showed a marked decrease in uptake of erythro-

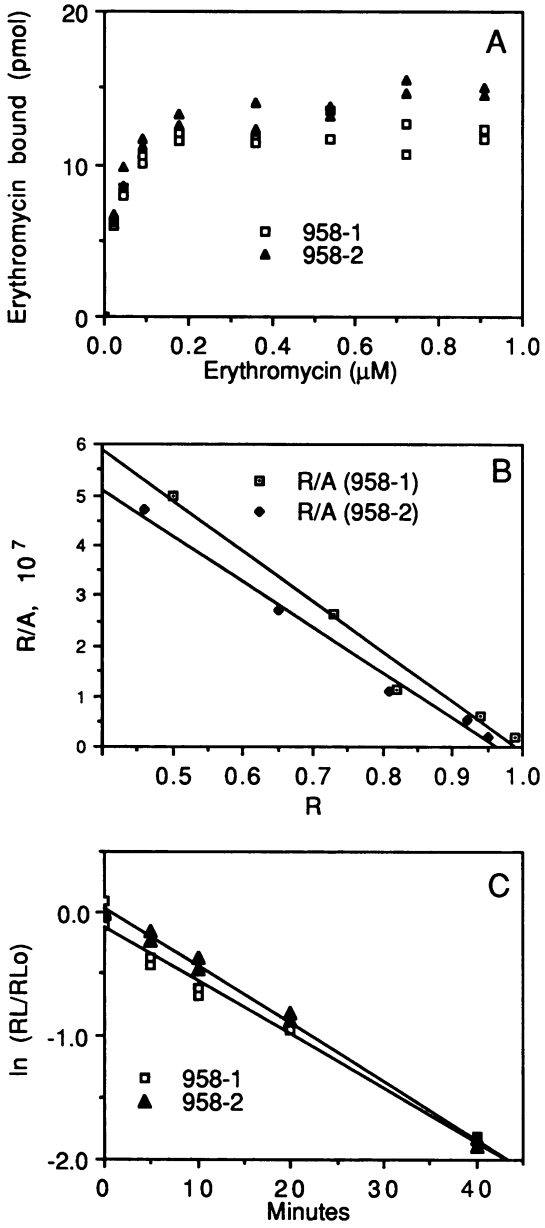


FIG. 2. Binding of erythromycin to ribosomes from strains 958-1 and 958-2. S30 extracts were prepared by mechanical breakage and differential centrifugation. (A) Increasing amounts of [^{14}C]erythromycin were incubated with 200 μg of S30 protein in 0.5 ml of ribosome-binding buffer for 30 min at 37°C. The ribosome-erythromycin complex was collected in duplicate on nitrocellulose filters which were assayed for radioactivity by scintillation counting. (B) Scatchard analysis of binding affinity using data from panel A. R, Fraction of ribosomes bound; R/A, fraction of ribosomes bound/free drug. (C) Ribosomes were incubated with 0.3 μM [^{14}C]erythromycin for 30 min at 37°C, followed by addition of unlabeled erythromycin to 30 μM. Erythromycin remaining bound to ribosomes was monitored in duplicate by filter binding. Data were plotted as ln [(*RL*)/(*RLo*)] = $-k_{-1}t$, where *RLo* is the concentration of the erythromycin-ribosome complex at time zero, *RL* is the concentration at time *t* after addition of unlabeled erythromycin, and k_{-1} is the dissociation rate constant.

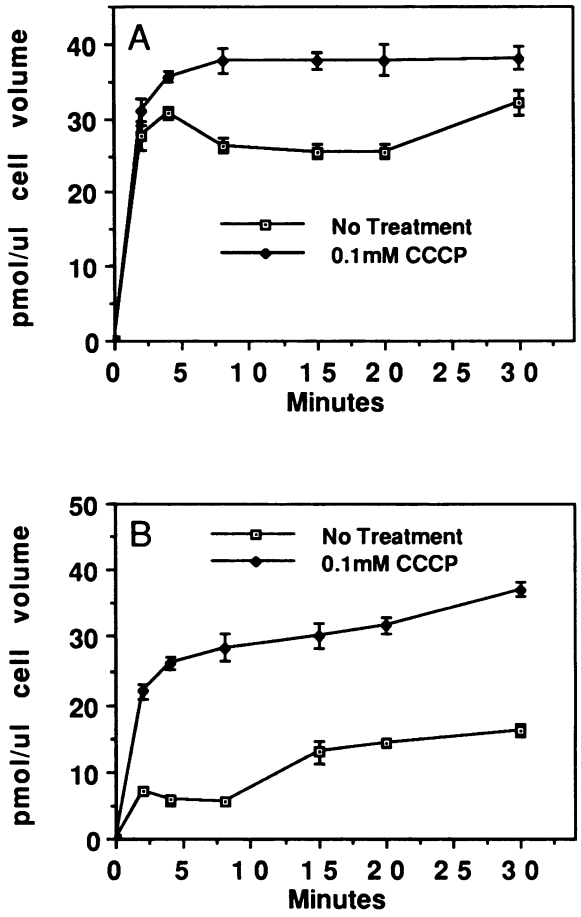


FIG. 3. Kinetics of erythromycin accumulation in strains 958-1 and 958-2 following pretreatment with CCCP. Cells (3×10^9/ml) were incubated with 1.0 μg of [^{3}H]erythromycin per ml at 37°C, and the amount of macrolide accumulated was determined following centrifugation through silicone oil. CCCP (0.1 mM) was added 10 min before macrolide when indicated. (A) Strain 958-1. (B) Strain 958-2. Results are presented as means ± standard deviations (n = 3).

mycin by strain 958-2 compared with plasmid-free strain 958-1 (20). However, neither the intracellular concentrations nor the rates of macrolide accumulation were measured. We thus measured these aspects of macrolide interaction.

Erythromycin rapidly accumulated in susceptible strain 958-1, reaching a level of 30 pmol/μl of intracellular volume (47,700 molecules per cell) and concentrating 23-fold within the first 5 min because of binding to ribosomes (Fig. 3A). The intracellular level decreased slightly over the next 5 min to 25 pmol/μl of intracellular volume (39,700 molecules per cell). In contrast, a much lower level of erythromycin associated with strain 958-2, stabilizing at 5 pmol/μl of cell volume (7,950 molecules per cell) after 5 to 10 min (Fig. 3B). This low-level binding was temperature dependent and occurred over the first 5 min of incubation (data not shown). A slow increase occurred between 10 and 30 min, most likely because of deterioration of cellular energy metabolism under conditions of concentration. Similar data were obtained with azithromycin (data not shown). These data are slightly different from those previously reported (20), in which strain 958-2 accumulated erythromycin to over 50% of the level of 958-1 in 30 min at a similar extracellular macrolide concentration (1.4 μM).

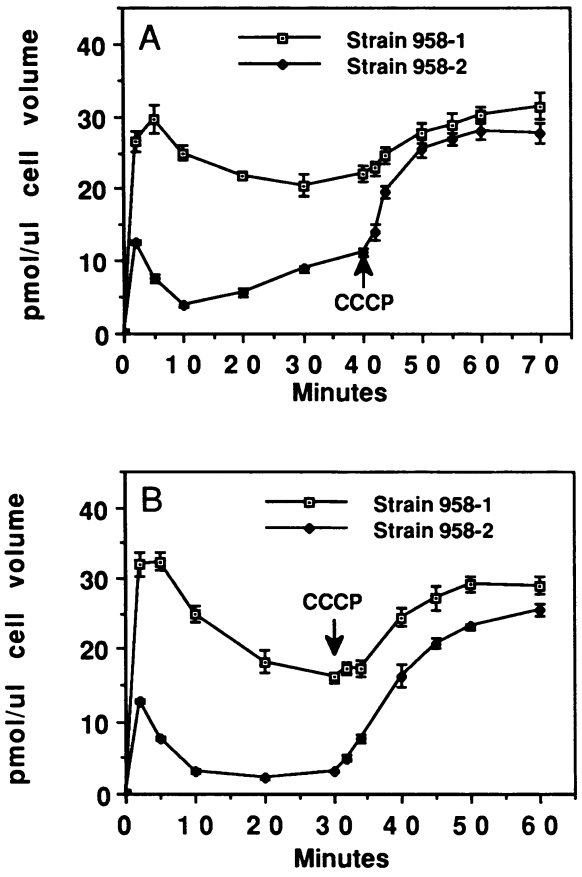


FIG. 4. Effect of CCCP on kinetics of macrolide accumulation in strains 958-1 and 958-2 following macrolide pretreatment. The procedures used are described in the legend to Fig. 3. Radiolabeled erythromycin or azithromycin (1.0 and 0.5 μg/ml, respectively) was added at time zero. The arrows indicate the times of CCCP addition. (A) Erythromycin. (B) Azithromycin addition to strains 958-1 and 958-2. Results are presented as means ± standard deviations (n = 3).

Strain 958-2, when pretreated with CCCP, accumulated erythromycin with similar kinetics and to the same level as did control or CCCP-treated 958-1 (32 to 38 pmol/μl of intracellular volume, 51,000 to 60,400 molecules per cell; Fig. 3; see below). CCCP pretreatment had a slight but reproducible effect on erythromycin accumulation in strain 958-1 (Fig. 3A). Erythromycin and azithromycin also accumulated rapidly and to similar levels following CCCP treatment of 958-2 cells preexposed to macrolide (Fig. 4A and B).

We also investigated the kinetics of erythromycin influx during the 0- to 90-s period following macrolide addition to CCCP-treated strains 958-1 and 958-2 (Fig. 5). The initial rates of accumulation, calculated by linear regression analysis (r = 0.98) using the 0-, 15-, and 30-s points, were identical at 37 pmol/μl of cell volume per min.

Since the MICs of the 16-membered macrolides niddamycin and chalcomycin were identical for strains 958-1 and 958-2 (Table 1), we predicted that identical rapid uptake of [^{14}C]niddamycin would occur in both strains in the absence of CCCP. Niddamycin accumulated at the same rapid rate and to the same extent when energy-competent cells of strains 958-1 and 958-2 were tested (Fig. 6). A slight decrease in macrolide level also occurred during the second 5 min of macrolide exposure (see Discussion), with the level stabilizing at 30 pmol/μl of intracellular volume (47,700 molecules per cell).

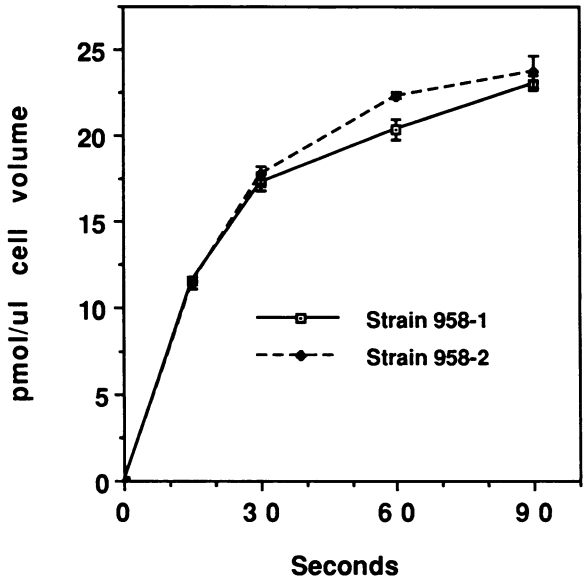


FIG. 5. Initial rate of erythromycin accumulation in CCCP-treated strains 958-1 and 958-2. Cells were pretreated for 10 min with 0.1 mM CCCP, and the kinetics of [^{14}C]erythromycin (0.5 μg/ml) accumulation was measured. Uptake was stopped by rapid chilling in ice water, and samples were processed by centrifugation through silicone oil. Results are presented as means ± standard deviations (n = 3).

Recovery of strain 958-2 from the effects of CCCP and macrolide treatment. The effects of drug addition and removal on the growth of strain 958-2 were examined (Fig. 7). Addition of 1 μg of erythromycin per ml had no effect on the

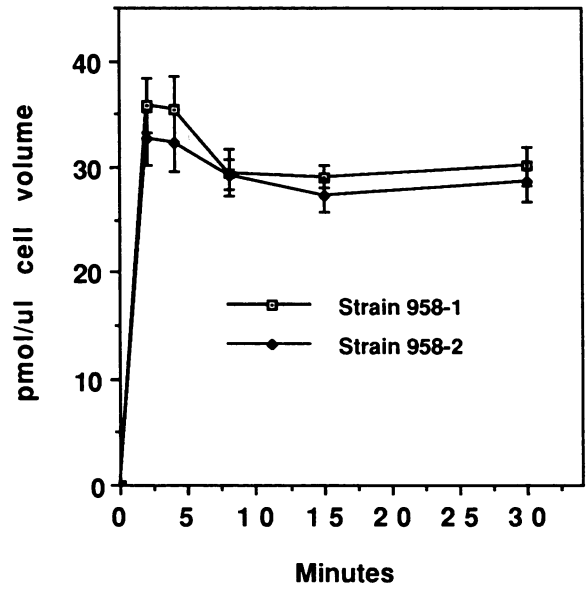


FIG. 6. Niddamycin accumulation in strains 958-1 and 958-2. [^{14}C]niddamycin (4 μg/ml) was added to cells incubated at 37°C, and macrolide accumulation was monitored as described in the legend to Fig. 3. Results are presented as means ± standard deviations (n = 3).

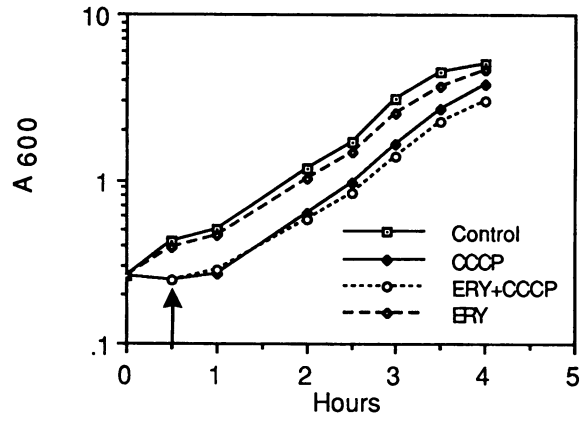


FIG. 7. Recovery of strain 958-2 from treatment with CCCP and erythromycin (ERY). Cells growing at 37°C were incubated for 30 min with (i) 0.1 mM CCCP; (ii) 1 μg of [^{14}C]erythromycin per ml plus 0.1 mM CCCP; (iii) 1 μg of [^{14}C]erythromycin per ml; or (iv) no addition. The cells were then washed twice by centrifugation with an equal volume of medium and suspended in fresh medium at 37°C. The wash and suspension media for ii and iii contained 1 μg of [^{14}C]erythromycin per ml. Growth was monitored by A_{600}. Triplicate cell samples (1 ml) were removed from ii and iii before the wash (30-min point) and after the wash (2-h point) for determination of intracellular macrolide levels (see text). The arrow indicates the time at which cells were washed.

growth of strain 958-2. In contrast, addition of CCCP rapidly stopped bacterial growth and the cells recovered immediately upon removal. Cells also recovered rapidly from simultaneous treatment with CCCP and macrolide when only CCCP was removed. Radiolabeled erythromycin was used to measure the amount of cell-associated macrolide during the experiment. CCCP caused macrolide accumulation (8.2 ± 1.9 pmol/μl of cell volume before CCCP treatment and 27.6 ± 0.9 pmol/μl of cell volume 30 min after CCCP treatment), and growth ceased. Bacterial growth resumed and macrolide levels decreased to pre-CCCP levels (4.9 ± 0.7 pmol/μl of cell volume) 60 min after CCCP, but not macrolide, removal. Centrifugation and washing had minimal effect on macrolide levels in the absence of CCCP treatment (data not shown). Identical growth recovery was observed when the same experiment was conducted with the 15-membered macrolide azithromycin (data not shown). During these experiments, macrolide was included in the wash medium to ensure that no macrolide would be lost from cells during centrifugation.

Macrolide entrance and efflux were also investigated with concentrated cells of strain 958-2. Control cells (not treated with CCCP) and cells treated with CCCP were incubated with 1 μg of [^{14}C]erythromycin per ml for 5 min. The cells were then washed by centrifugation with medium which contained radiolabeled macrolide at 1 μg/ml to ensure that no intracellular macrolide would be lost during centrifugation. The centrifugation process caused a slight increase in macrolide influx into control cells, which rapidly decreased to control levels following suspension (data not shown). Macrolide accumulated in CCCP-treated cells (5.8 ± 0.5 pmol/μl of cell volume before CCCP addition and 28.5 ± 1.2 pmol/μl of cell volume 5 min after CCCP addition), and the level decreased (11.2 ± 0.7 pmol/μl of cell volume) 15 min after removal of CCCP but not macrolide. Macrolide also accumulated (35.2 ± 1.9 pmol/μl of cell volume) after a second challenge with CCCP (30 min after the first challenge), demonstrating that cells were still metabolically active at that time.

DISCUSSION

Our data show that the membrane PMF is involved in the macrolide resistance phenotype of *S. epidermidis* 958-2. Macrolide did not accumulate in strain 958-2 unless the PMF was dissipated with CCCP, and restoration of the PMF by removal of CCCP resulted in macrolide efflux from the bacterial cells. In contrast, macrolide accumulated rapidly in strain 958-1 to levels which were consistent with the number of ribosomes, and dissipation of the PMF with CCCP had less dramatic effects on the kinetics and level of accumulation. We conclude that strain 958-2, containing plasmid pNE24, also contains an energy-dependent macrolide efflux pump which maintains intracellular antibiotic concentrations below those required for binding to ribosomes on the basis of data and the results discussed below.

However, inasmuch as previously published data on strain 958-2 (20) left open the possibility that ribosome modification, intracellular macrolide degradation, and/or reduced membrane permeability to 14-membered macrolides may participate in the resistance mechanism, we sought to exclude these as causes of the 958-2 resistance phenotype. Ribosomes from both strains bound erythromycin with identical kinetics and affinities when assayed in an S30 extract containing cytoplasmic components. Furthermore, treatment of strain 958-2 with CCCP immediately allowed macrolide to enter and bind to ribosomes, demonstrating that ribosomes in strain 958-2 are capable of binding 14- and 15-membered macrolides in vivo. These data rule out changes in the ribosomes and inhibition of macrolide binding to ribosomes by cytoplasmic factors as the resistance mechanism in strain 958-2. Macrolide recovered from incubation with S30 extracts was unmodified and fully able to bind to ribosomes. Incubation was conducted under conditions which allow macrolide cleavage by esterase (4), and we have detected such cleavage by using extracts of an esterase-producing strain (unpublished data). Furthermore, macrolide recovered from cells of strain 958-2 was intact and capable of binding to ribosomes. These data rule out macrolide modification or degradation as the resistance mechanism. The initial rates of macrolide uptake in CCCP-treated strains 958-1 and 958-2 were identical (37 pmol/μl of cell volume per min) when measured during the first 30 s. These data rule out a role for reduced permeability into strain 958-2 as the resistance mechanism, as was observed for quinolone resistance (8).

We consistently observed a slight effect of CCCP treatment on the kinetics of macrolide accumulation in strain 958-1. Pretreatment with CCCP resulted in slightly higher macrolide accumulation levels, while non-CCCP-treated cells showed a rapid initial increase, followed by a slight decline; subsequent addition of CCCP reversed this initial decline in the macrolide level. Similar results were obtained for erythromycin, azithromycin, and the 16-membered macrolide niddamycin, the latter being fully active against strain 958-2. We cannot exclude the existence of a weak macrolide efflux pump in strain 958-1 or the possibility that plasmid pNE24 contributes to the functional capacity of such a putative pump. However, we and others (21) have observed a similar phenomenon in the interaction of erythromycin with macrolide-susceptible *B. subtilis* (unpublished data). Preliminary data indicate that this phenomenon is related to a minor effect of CCCP on the accessibility of macrolide to

ribosomes, possibly through an effect on polysomes (unpublished data), which are known to restrict macrolide access to the 50S subunit (29).

Since ribosomes from strain 958-2 bind macrolide normally, the PMF must maintain the intracellular macrolide concentration below that required for ribosome binding. Macrolide levels rapidly increased in strain 958-2 following addition of CCCP, and following removal of CCCP but not macrolide, macrolide levels dropped and growth resumed. Restoration of the PMF following removal of CCCP must, therefore, have resulted in energy-dependent efflux of macrolide to levels below those required for binding to ribosomes. Macrolide dissociation from ribosomes may be the rate-limiting step in recovery, since the rate constant we measured at 37°C yielded a $t_{1/2}$ of 15 min and the energy-dependent macrolide efflux is fast enough to compete against the driving passive diffusion force of up to 10 to 20 μg of macrolide per ml (see below).

The erythromycin MICs for strains 958-1 and 958-2 were 0.04 and 25 μg/ml, respectively. Since the velocity of passive diffusion is proportional to the concentration ($v = PA(C_{out} - C_{in})$, where v = net flux, P = permeability coefficient, A = area, and C = concentration), the rate of macrolide entry at 25 μg/ml would be 618-fold faster, or about 47,000 molecules per cell per s on the basis of our measured influx rate of 37 pmol/μl of intracellular volume per min (Fig. 5) at an external concentration of 0.5 μg/ml. Thus, the efflux pump appears able to remove several thousand macrolide molecules per s in energy-competent strain 958-2 to maintain macrolide levels below those required for binding to ribosomes. The association of about 7,900 macrolide molecules with energy-competent 958-2 cells may reflect the steady-state macrolide association with the efflux pump and, thus, the number of sites involved per cell; however, further work is required to substantiate this hypothesis. Since cells continue to grow under these conditions, the macrolide cannot be intracellular, as it would have then bound to ribosomes and inhibited growth. This level of binding does not represent temperature-independent, rapid binding to cells like that which occurs with aminoglycosides (28), as it is temperature dependent and increases over 5 to 10 min of incubation.

The retention of potency equal to that of erythromycin against strain 958-2 by the two erythromycin derivatives with poor ribosome-binding affinity indicates that the energy-dependent macrolide efflux pump has a better affinity for erythromycin than for either of the two analogs. Since these two derivatives have 150-fold poorer activity against strain 958-1 (MICs, 6.5 μg/ml) because of reduced ribosome-binding affinity (120-fold difference between the 50% inhibitory concentrations), one would predict that they would also have reduced activity, relative to that of erythromycin, against strain 958-2. However, both of these derivatives had virtually the same potency as erythromycin against strain 958-2. We conclude that the intracellular concentration of these derivatives at their MICs for strain 958-2 is higher than the concentration of erythromycin, which has the same MIC but better affinity for the ribosomes. The simplest explanation for these data is that the efflux pump has poor affinity for these erythromycin derivatives. In view of the recent report (16) that both erythromycin and verapamil can apparently compete with the multiple drug resistance determinant (efflux pump) of certain cancer cells, we tested verapamil for similar interference with the macrolide efflux pump present in strain 958-2. No effects were observed.

In conclusion, our data demonstrate the existence of an energy-dependent efflux pump in *S. epidermidis* containing plasmid pNE24 which recognizes specific structural features present in 14-membered macrolides and the 15-membered macrolide azithromycin. A new membrane protein is present in strain 958-2 (20), and it may represent a component of this efflux system. Interestingly, three clinical isolates of *S. epidermidis* with a phenotype similar to that of 958-2 were previously isolated in Japan (23). This raises many questions as to the prevalence, species distribution, and origins of this novel form of resistance to macrolides. Inasmuch as macrolides-lincosamides-streptogramin B resistance is believed to have spread from macrolide producers to pathogenic bacteria (2), one may consider whether or not the same could be true for the mechanism we discovered in *S. epidermidis*. In particular, three tylosin resistance determinants were cloned from *Streptomyces fradiae* (4), one of which (*tlrA*) codes for macrolides-lincosamides-streptogramin B resistance (5, 34). The mechanism coded for by *tlrB* and *tlrC* is unknown. The recent suggestion (13) that resistance in some macrolide producers involves lack of permeability to specific macrolides is of particular interest. As shown by our present report, it is possible to mistake impermeability for operation of an effective efflux pump. We are currently investigating this possibility.

LITERATURE CITED

1. **Arthur, M., A. Andremont, and P. Courvalin.** 1987. Distribution of erythromycin esterase and rRNA methylase in members of the family *Enterobacteriaceae* highly resistant to erythromycin. Antimicrob. Agents Chemother. **31**:404–409.
2. **Arthur, M., A. Brisson-Noel, and P. Courvalin.** 1988. Origin and evolution of genes specifying resistance to macrolides, lincosamides, and streptogramin antibiotics: data and hypotheses. J. Antimicrob. Chemother. **20**:783–802.
3. **Barre, J., M. Fournet, R. Zini, L. Deforges, J. Duval, and J. Tillement.** 1986. *In vitro* [^{3}H] erythromycin binding to *Staphylococcus aureus*. Biochem. Pharmacol. **35**:1001–1004.
4. **Barthelemy, P. D., D. Autissior, G. Gerband, and P. Courvalin.** 1984. Enzymatic hydrolysis of erythromycin by a strain of *Escherichia coli*. A new mechanism of resistance. J. Antibiot. **37**:1692–1696.
5. **Birmingham, V. A., K. L. Cox, J. L. Larson, S. E. Fishman, C. L. Hershberger, and E. T. Seno.** 1986. Cloning and expression of a tylosin resistance gene from a tylosin-producing strain of *Streptomyces fradiae*. Mol. Gen. Genet. **204**:532–539.
6. **Capobianco, J. O., C. C. Doran, and R. C. Goldman.** 1989. Mechanism of mupirocin transport into sensitive and resistant bacteria. Antimicrob. Agents Chemother. **33**:156–163.
7. **Capobianco, J. O., and R. C. Goldman.** 1990. Erythromycin and azithromycin transport into *Haemophilus influenzae* ATCC 19418 under conditions of depressed proton motive force ($\Delta\mu H$). Antimicrob. Agents Chemother. **34**:1787–1791.
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. **Collander, R.** 1954. The permeability of Nitella cells to non-electrolytes. Physiol. Plant. **7**:420–445.
10. **Dette, G. A., H. Knothe, and S. Kaula.** 1987. Modifying effects of pH and temperature on (^{14}C)erythromycin uptake into *Staphylococcus aureus*—relation to antimicrobial activity. Zentralbl. Bakteriol. Mikrobiol. Hyg. A **265**:393–403.
11. **Dubnau, D.** 1984. Translational attenuation: the regulation of bacterial resistance to the macrolide-lincosamide-streptogramin B antibiotics. CRC Crit. Rev. Biochem. **16**:103–132.
12. **Duval, J.** 1985. Evolution and epidemiology of MLS resistance. J. Antimicrob. Chemother. **16**(Suppl. A):137–149.
13. **Fierro, J. F., C. Hardisson, and J. A. Salas.** 1987. Involvement of cell impermeability in resistance to macrolides in some producer *Streptomyces*. J. Antibiot. **41**:142–144.

14. **Goldman, R. C., S. W. Fesik, and C. Doran.** 1990. Role of protonated and neutral forms of macrolides in binding to ribosomes from gram-positive and gram-negative bacteria. Antimicrob. Agents Chemother. **34**:426–431.
15. **Goldman, R. C., and S. K. Kadam.** 1989. Binding of novel macrolide structures to macrolides-lincosamides-streptogramin B-resistant ribosomes inhibits protein synthesis and bacterial growth. Antimicrob. Agents Chemother. **33**:1058–1066.
16. **Hofsli, E., and J. Nissen-Meyer.** 1989. Effect of erythromycin and tumor necrosis factor on the drug resistance of multidrug-resistant cells: reversal of drug resistance by erythromycin. Int. J. Cancer **43**:520–525.
17. **Kuo, M.-S., D. G. Chirby, A. D. Argoudelis, J. I. Cialdella, J. H. Coats, and V. P. Marshall.** 1989. Microbial glycosylation of erythromycin A. Antimicrob. Agents Chemother. **33**:2089–2091.
18. **Kurath, P., P. H. Jones, R. S. Egan, and T. J. Perun.** 1971. Acid degradation of erythromycin A and erythromycin B. Separatum Experimentia **27**:362.
19. **Lai, C. J., and B. Weisblum.** 1971. Altered methylation of ribosomal RNA in an erythromycin-resistant strain of *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA **68**:856–860.
20. **Lampson, B. C., W. von David, and J. T. Parisi.** 1986. Novel mechanism for plasmid-mediated erythromycin resistance by pNE24 from *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. **30**:653–658.
21. **Mao, J. C., and M. Putterman.** 1968. Accumulation in gram-positive and gram-negative bacteria as a mechanism of resistance to erythromycin. J. Bacteriol. **95**:1111–1117.
22. **Meot-Ner (Mautner), M.** 1984. The ionic hydrogen bond and ion solvation. 2. Solvation of onium ions by one to seven H_2O molecules. Relation between monomolecular, specific, and bulk hydration. J. Am. Chem. Soc. **106**:1265–1272.
23. **Omura, S., S. Namiki, M. Shibata, T. Mura, and J. Sawada.** 1970. Studies on the antibiotics from *Streptomyces spinichromogenes* var. kujimyceticus. V. Some antimicrobial characteristics of kujimycin A and kujimycin B against macrolide resistant staphylococci. J. Antibiot. **9**:448–460.
24. **Pestka, S.** 1974. Binding of [^{14}C]erythromycin to *Escherichia coli* ribosomes. Antimicrob. Agents Chemother. **6**:474–478.
25. **Sabath, L. D., V. Lorian, D. Gerstein, P. Loder, and M. Finland.** 1968. Enhancing effect of alkalinization of the medium on the activity of erythromycin against gram-negative bacteria. Appl. Microbiol. **16**:1288–1292.
26. **Skinner, R., E. Cundliffe, and F. J. Schmidt.** 1983. Site of action of a ribosomal RNA methylase responsible for resistance to erythromycin and other antibiotics. J. Biol. Chem. **258**:12702–12706.
27. **Stein, W. D.** 1981. Permeability for lipophilic molecules, p. 1–28. *In* S. L. Bonting and J. J. De Pont (ed.), Membrane transport. Elsevier/North Holland Publishing Co., Amsterdam.
28. **Taber, H. W., J. P. Mueller, P. F. Miller, and A. S. Arrow.** 1987. Bacterial uptake of aminoglycoside antibiotics. Microbiol. Rev. **51**:439–457.
29. **Tai, P. C., B. J. Wallace, and B. D. Davis.** 1974. Selective action of erythromycin on initiating ribosomes. Biochemistry **13**:4653–4659.
30. **Walter, A., and J. Gutknecht.** 1984. Monocarboxylic acid permeation through lipid bilayer membranes. J. Membr. Biol. **77**:255–264.
31. **Weisblum, B.** 1983. Inducible resistance to macrolides, lincosamides, and streptogramins type-B: the resistance phenotype, its biological diversity, and structural elements that regulate expression, p. 91–121. *In* J. Beckwith, J. Davies, and J. A. Gallant (ed.), Gene function in prokaryotes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Wertz, D. H.** 1980. Relationship between the gas-phase entropies of molecules and their entropies of solvation in water and 1-octanol. J. Am. Chem. Soc. **102**:5316–5322.
33. **Wiley, P. F., L. Baczynskyj, L. A. Dolak, J. I. Ciandella, and V. P. Marshall.** 1987. Enzymatic phosphorylation of macrolide antibiotics. J. Antibiot. **40**:195–201.
34. **Zalacain, M., and E. Cundliffe.** 1989. Methylation of 23S rRNA caused by *tlrA* (*ermSF*), a tylosin resistance determinant from *Streptomyces fradiae*. J. Bacteriol. **171**:4254–4260.