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Novel Mechanism for Plasmid-Mediated Erythromycin Resistance by pNE24 from *Staphylococcus epidermidis*

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We describe an unusual type of erythromycin resistance (Em^r) mediated by a plasmid designated pNE24 from *Staphylococcus epidermidis*. This 26.5-kilobase plasmid encodes resistance strictly to 14-membered macrolide antibiotics, erythromycin, and oleandomycin. Resistance to other macrolide-lincosamide-streptogramin B (MLS) antibiotics was not observed even after a prior induction stimulus with various MLS antibiotics. Plasmid pNE24 was found to express resistance constitutively and manifested a low to intermediate MIC (62.5 μg/ml) for erythromycin. The resistance gene, designated *erpA*, appears to mediate resistance by altering the permeability of the host cell for erythromycin, because the measured uptake of ^{14}C-labeled erythromycin by strain 958-2 (containing pNE24) was lower than for the erythromycin-susceptible, isogenic strain 958-1. No inactivation of erythromycin in overnight broth culture supernatants could be detected. In addition, no significant loss in binding affinity between [^{14}C]erythromycin and ribosome could be detected for ribosomes isolated from strain 958-2 relative to 958-1, indicating that pNE24 probably does not produce a modification of the bacterial ribosome. No other selectable marker was found associated with pNE24; however, a 60,000-dalton protein was present only in the membrane fractions of cells (958-2) containing pNE24 and may play a role in mediating resistance to erythromycin.

Macrolides, lincosamides, and type B streptogramins (MLSs) are chemically distinct antibiotics with a similar mode of action and spectrum of activity. A biochemical alteration of the 50S subunit of the bacterial ribosome, namely N^6-dimethylation of a single adenine residue at position 2058 in 23S rRNA, confers resistance to all members belonging to the MLS group of antibiotics (14, 33, 34). The result is a reduction of the affinity between the antibiotic and the ribosome and the inability of the drug to inhibit the synthesis of proteins in resistant microorganisms (14). In bacteria resistant to MLS antibiotics, the gene responsible encodes a rRNA methylase and usually resides on a plasmid (6, 32).

The presence of inactivating enzymes for the macrolide erythromycin, which are encoded by a plasmid originally found in *Escherichia coli* (2, 28), as well as for lincomycin-clindamycin (17) and streptogramin B (18), has also been reported to be encoded by plasmids in staphylococci. In this report, we describe in *Staphylococcus epidermidis* an unusual type of plasmid-mediated resistance only to the 14-membered macrolides erythromycin and oleandomycin by a mechanism which appears to affect the permeability of the cell for the antibiotic.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *S. epidermidis* recipient strain 958 was obtained from England during a phage typing study. Determination of the resistance phenotype was done by the in situ induction test on solid medium with Kirby-Bauer antibiotic susceptibility disks, as described by Weisblum et al. (40).

Transduction. Transduction of *S. epidermidis* recipient strain 958 was done with the *S. epidermidis* transducing phages 108 and 112. Both phages were from the phage typing collection of J. Parisi (30). The transduction procedure was the same as reported by Olson et al. (27).

MIC for MLS antibiotics. Determination of the MIC for erythromycin, oleandomycin phosphate, and tylosin tartrate (Sigma Chemical Co., St. Louis, Mo.) and clindamycin hydrochloride (The Upjohn Co., Kalamazoo, Mich.) was by the macro broth dilution method (39); and susceptibility to the streptogramin B antibiotic, ostreogrycin B (gift from B. Weisblum), and other clinically important antibiotics was by the disk diffusion method (23). Resistance to heavy metals was assayed by the method of Novick and Roth (26) with paper disks soaked with different concentrations of inorganic salt solutions. Resistance to different dyes was also determined with presoaked paper disks.

Microbiological assay for inactivation of erythromycin. A simple plate test, with *Micrococcus luteus* as a biological indicator, was done based on the test described by Gots (9) to detect inactivation of erythromycin. A quantitative method was used to detect any inactivation of erythromycin in culture supernatants after overnight growth and was similar to published methods (25, 42).

Erythromycin accumulation by cells. The experimental procedure used to measure the uptake and accumulation of ^{14}C-labeled erythromycin was based on previously published methods (21, 41) and is outlined as follows. Cells of three isogenic strains of *S. epidermidis* from overnight slant cultures were used to inoculate a flask containing yeast extract-Trypticase soy (YETS; BBL Microbiology Systems, Inc., Cockeysville, Md.) (27) broth to an optical density of 0.1 (A_{600}). The flask was incubated by shaking at 37°C until the optical density reached 0.35. Various concentrations of [^{14}C]erythromycin (0.13 μCi/μg; a gift from J. Martin) were added, and the flasks were incubated for 30 min. Cells were

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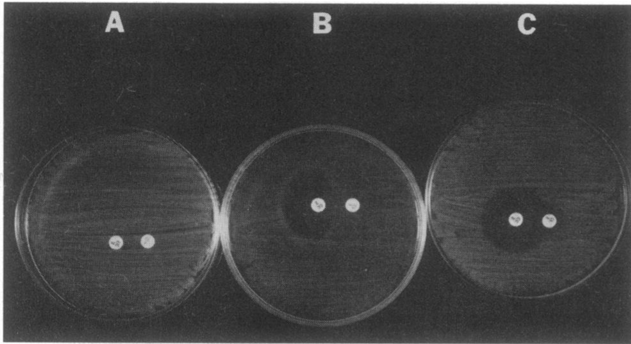

FIG. 1. Em^r phenotypes displayed by various clinical isolates of *S. epidermidis*. Three phenotypes, constitutive (A), inducible (B), and low-level (C), were distinguished by placing standard Kirby-Bauer antibiotic susceptibility disks on YETS agar inoculated with *S. epidermidis* and incubated overnight. The disk on the left of each pair contains 2 μg of clindamycin, and the disk on the right of each pair contains 15 μg of erythromycin.

collected by filtering 3-ml fractions through 0.45-μm-pore size filters (25 millimeter; type HA; Millipore Corp., Bedford, Mass.). The filters had been prewashed with standard buffer (0.01 M Tris [pH 7.6], 0.01 M $MgCl_2$, 0.06 M KCl, 0.007 M 2-mercaptoethanol [41]) containing 1 μg of cold erythromycin per ml. Cells were washed three times by passing 2-ml volumes of standard buffer containing cold erythromycin through the filters. The filters were dried, and radioactivity was counted by dissolving the filters in 2-methoxyethanol (Eastman Co.) and counting in a liquid scintillation counter.

Erythromycin binding to ribosomes. Preparation of purified ribosomes from *S. epidermidis* was based on methods described for *Staphylococcus aureus* (20) and *Bacillus megaterium* (36). Cultures were grown overnight in YETS broth; for resistant strains, 2 μg of erythromycin per ml was added to the broth. Cells were collected and washed with cold standard buffer. Cells were broken by grinding the wet cell pellet in the presence of 2 volumes of alumina (Sigma) with a mortar and pestle on ice. The broken cells and alumina were suspended in 4 ml of RS buffer (0.01 M Tris [pH 7.6], 0.016 M magnesium acetate, 0.05 M NH_4Cl) and centrifuged twice at 10,000 × *g* for 15 min to remove alumina and cell debris. The supernatants were centrifuged at 105,000 × *g* for 90 min to pellet the ribosomes. The ribosomes were washed in RS buffer and centrifuged again. The final ribosome pellet was suspended in RS buffer and stored at −20°C.

The procedure for in vitro binding of erythromycin to ribosomes was similar to methods reported previously (22, 41) and is briefly described as follows. Isolated ribosomes were mixed with labeled erythromycin in a 0.5-ml reaction volume containing standard buffer, 1 μg of [^{14}C]erythromycin, and various concentrations of ribosomes. The ribosomes were added last to start the reaction, followed by incubation at 37°C for 10 min. The reaction was stopped by adding 3 ml of cold standard buffer. The reaction mixture was then passed through prewashed filters, and the filters were washed twice with standard buffer and then dried. The filters were counted as described above.

Isolation of membrane proteins. *S. epidermidis* membrane proteins were isolated by a procedure similar to those reported for the isolation of *S. aureus* membranes (10, 38) and is outlined as follows. *S. epidermidis* isogenic strains were incubated by shaking at 37°C to late-logarithmic phase in YETS broth containing 0.5% glycine. Cells were centrifuged, washed in cold 50 mM sodium phosphate buffer (pH 7.5), and suspended in phosphate buffer to which lysostaphin was added at a concentration of 50 μg/ml. After incubation at 37°C for 1 h, the protoplast suspension was lysed by sonication for 5 min. A low-speed spin (5,000 × *g*) of the lysate to remove cell debris was followed by a high-speed centrifugation (100,000 × *g*) to pellet the cell membrane fraction. The membrane fraction was washed several times in 50 μM sodium phosphate buffer, and fractions stored at −70°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein concentration of membrane fractions was determined by the procedure described by Lowry et al. (19). The procedure for sodium dodecyl sulfate-polyacrylamide gel electrophoresis was similar to that of Laemmli (13), with some modifications (31).

RESULTS

Em^r phenotypes. Standard Kirby-Bauer antibiotic disks were placed on a lawn of bacterial growth to distinguish different forms of expression of Em^r. In Fig. 1 is shown an example of the results of a survey of Em^r clinical isolates of *S. epidermidis* and *S. aureus* by this simple in situ assay. A total of 59 *S. epidermidis* and 21 *S. aureus* cultures obtained over a 6-month period from the Health Sciences Center of the University of Missouri-Columbia were tested. The results indicate that *S. epidermidis* isolates fall into one of three different phenotypic groups: Constitutive MLS resistance (group A), inducible MLS resistance (group B), and low-level resistance to erythromycin (group C). The constitutive phenotype is manifested by the unconditional or constitutive expression of resistance to all MLS antibiotics, in this case to the macrolide erythromycin (disk on the right, Fig. 1A) and the lincosamide clindamycin (the disk on the left, Fig. 1A). The distorted zone of inhibited growth around the clindamycin disk (Fig. 1B) is indicative of the inducible phenotype. Here, full expression of resistance to MLS antibiotics occurs on induction by a subinhibitory concentration of erythromycin diffusing away from the erythromycin disk. A third kind of resistance phenotype (Fig. 1C), which we call low-level resistance (because of its lower MIC) displayed resistance to erythromycin but susceptibility to clindamycin and to other MLS antibiotics similarly tested. In this strain resistance appears to be constitutively expressed. Of the 80 clinical isolates of staphylococci tested, 48 *S. epidermidis* and 14 *S. aureus* isolates, or 77.5%, expressed constitutive MLS resistance (Fig. 1A); 9 *S. epidermidis* and 7 *S. aureus* isolates, or 20%, expressed inducible MLS resistance (Fig. 1B); and only 2 *S. epidermidis* isolates, or 2.5%, expressed low-level resistance to erythromycin (Fig. 1C). Others have similarly classified macrolide-resistant *S. aureus* strains into three groups; however, our group C phenotype differs from that reported by Yamagishi et al. (42).

Curing experiments were carried out as reported previously (27) and revealed the presence of a plasmid associated with each of the three phenotypes (data not shown). The *S. epidermidis* strains which displayed the low-level resistance phenotype (Fig. 1C) were found to contain a large, 26.5-kilobase (kb) plasmid. One of these plasmids, designated pNE24, was chosen for further study.

Transduction. To define better the resistance phenotype coded for by pNE24 and to purify plasmid DNA for restriction mapping, pNE24 was transduced into the antibiotic-susceptible, plasmid-free recipient strain 958-1. The consti-

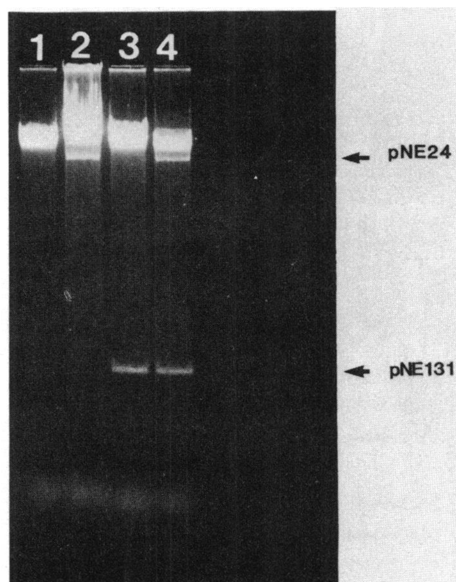

FIG. 2. Agarose gel electrophoresis of plasmid DNA extracted from the recipient strain 958-1 and the transduced derivatives. DNA extracted from the recipient strain 958-1 (lane 1) appears plasmid free, with only the thick chromosomal band present. The large 26.5-kb Em^r plasmid pNE24 appears in the DNA extract from the transduced derivative 958-2 (lane 2). The derivative strain 958-3 was transduced to MLS resistance and contains the small, previously described plasmid pNE131 (lane 3). Phage 108 was propagated in strain 958-3 and used to transduce strain 958-2 to clindamycin resistance to give the derivative 958-4 (lane 4) which contains both compatible plasmids pNE24 and pNE131.

tutive MLS resistance plasmid pNE131 (15) was also transduced into the same recipient strain for comparison. Agarose gel electrophoresis of plasmid DNA extracted from recipient and transduced derivatives is shown in Fig. 2. Not surprisingly, pNE24 was compatible with pNE131 when pNE131 was transduced into strain 958-2 (Fig. 2, lane 4). Both plasmids were stably maintained in the same host strain, indicating their dissimilarity regarding replication and maintenance functions.

Southern blot hybridization experiments (35) showed that pNE24 lacks DNA sequence homology with two staphylococcal MLS plasmids. Labeled probes made from pNE131, which has recently been sequenced in our laboratory (16), and the inducible MLS resistance plasmid pE194 from *S. aureus* (40) failed to cross-hybridize with pNE24 DNA (data not shown).

Resistance phenotype. To define better the resistance phenotype mediated by pNE24, MICs of MLS antibiotics were determined (Table 1). Plasmid pNE131 (strain 958-3) mediated high-level resistance to all MLS antibiotics tested with an MIC greater than 1,000 μg/ml. In contrast, pNE24 (strain 958-2) mediated a lower level of resistance to erythromycin (62.5 μg/ml) and to the closely related macrolide oleandomycin (125 μg/ml). The plasmid did not provide detectable resistance to the other macrolide tylosin, the lincosamide antibiotic clindamycin, or to the streptogramin B antibiotic ostreogrycin B (data not shown).

Results from the in situ disk sensitivity assay (Fig. 1C) suggested that expression of Em^r by pNE24 was constitutive. A second experiment to confirm constitutive expression employed a growth curve assay (11) with broth cultures which had been induced (or left uninduced) by a low concentration of erythromycin prior to the start of the growth curve. At the mid-logarithmic growth phase, erythromycin was added to a final concentration of 75 μg/ml, and the effect of this challenge on the growth relative to a known constitutively resistant culture was measured. Results showed that regardless of whether the culture was induced previously for 1 h, the growth curves appeared the same after challenge, indicating that there had been no enhancement in the level of resistance. Thus, Em^r conferred by pNE24 was expressed constitutively.

From analysis of restriction fragments, the size of pNE24 was estimated to be about 26.5 kb. Because of the large size and potential coding capacity of pNE24, a battery of tests was done on recipient strain 958-1 and transductant strain 958-2 to screen for the presence of other selectable markers which may be associated with this plasmid. Tests for susceptibilities to 24 antibiotics, 3 dyes, and 6 heavy metals and tests for the production of a bacteriocin were done. However, no marker other than Em^r was found to be associated with pNE24.

Mechanism of resistance. Given the unusual Em^r phenotype displayed by pNE24, alternative mechanisms (other than methylation of rRNA) by which this plasmid may mediate resistance were explored. A microbiological assay similar to the test described by Gots (9) did not detect inactivation of erythromycin by strain 958-2 containing pNE24 (data not shown). A second, more quantitative experiment was also done in which broth cultures containing a known concentration of erythromycin were grown overnight. Supernatants were obtained, various dilutions were made, and paper disks were soaked with the dilutions to quantitate the amount of erythromycin remaining in the supernatants. This experiment (data not shown) also did not detect any loss in the antimicrobial potency of erythromycin in the supernatants after overnight culture with 958-2.

An experiment was conducted to compare the extent of uptake and accumulation of increasing concentrations of erythromycin by the three isogenic strains of *S. epidermidis*, 958-1, 958-2, and 958-3. The results (Fig. 3) show a marked decrease in uptake of [^{14}C]erythromycin by strain 958-2 containing pNE24 in comparison with the susceptible, plasmid-free strain 958-1. However, strain 958-3 containing the MLS resistance plasmid pNE131 showed the lowest level of accumulation of erythromycin by the three isogenic strains.

Ribosomes from isogenic strains of *S. epidermidis* were tested for their ability to bind erythromycin (Fig. 4).

TABLE 1. MICs for recipient and transduced derivatives of *S. epidermidis*

Strain	Plasmid	MIC (μg/ml)			
		Erythromycin	Oleandomycin	Tylosin	Clindamycin
958-1	Plasmid-free recipient	0.065	0.50	0.125	<0.015
958-2	pNE24	62.5	125	0.125	<0.015
958-3	pNE131	>1,000	>2,000	>1,000	>2,000

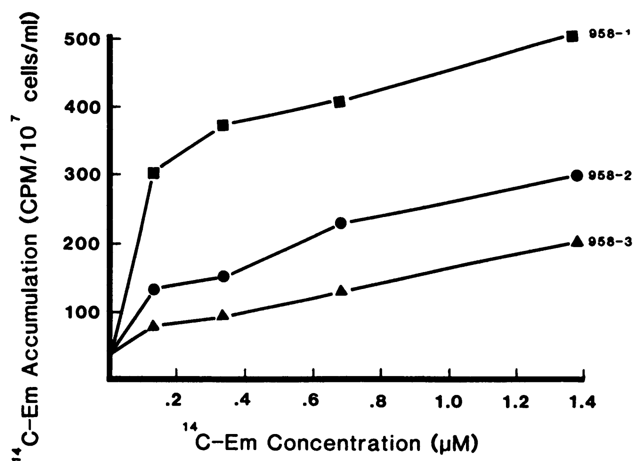

FIG. 3. Uptake of labeled erythromycin by *S. epidermidis* cells. To broth cultures containing about 10^7 cells per ml were added various amounts of [^{14}C]erythromycin to give the indicated concentrations. Fractions (3 ml) were removed, the bacteria were collected on filters and washed, and the uptake of erythromycin was measured as the amount of radioactivity retained on the filters. Strain 958-1 (■) is the plasmid-free, Em^s recipient. Strain 958-2 (●) is a transduced derivative containing pNE24, and strain 958-3 (▲) is a transduced derivative containing pNE131.

Ribosomes isolated from cells (958-3) that contained the MLS resistance plasmid pNE131 bound very little labeled erythromycin in vitro, indicating that the ribosomes were modified (methylation of 23S rRNA). In contrast, ribosomes isolated from cells (958-2) that contained pNE24 bound as much as a fourfold greater level of erythromycin, indicating that the ribosomes are probably significantly more susceptible to erythromycin. However, ribosomes from cells containing pNE24 did not bind as high a level of erythromycin as ribosomes from the susceptible, plasmid-free strain (958-1).

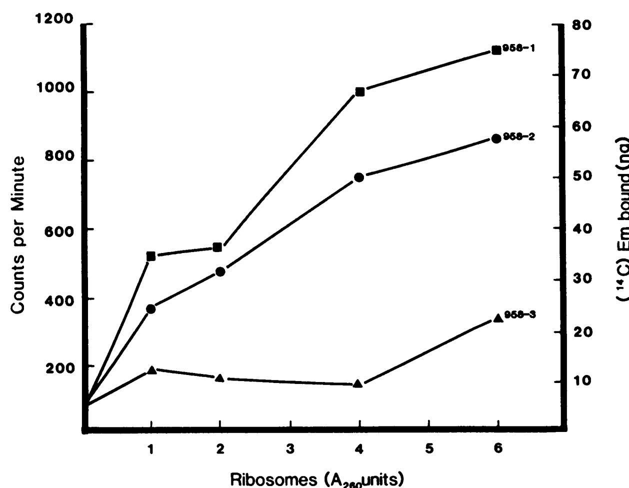

FIG. 4. Binding of labeled erythromycin to ribosomes in vitro. Different concentrations of ribosomes, isolated and purified from each strain, were mixed with 1 μg of [^{14}C]erythromycin and incubated at 37°C. The ribosomes were collected on filters, and the amount of erythromycin bound was determined by counting radioactivity. Ribosomes were isolated from the plasmid-free recipient strain 958-1 (■), strain 958-2(pNE24) (●), and strain 958-3(pNE131 (▲).

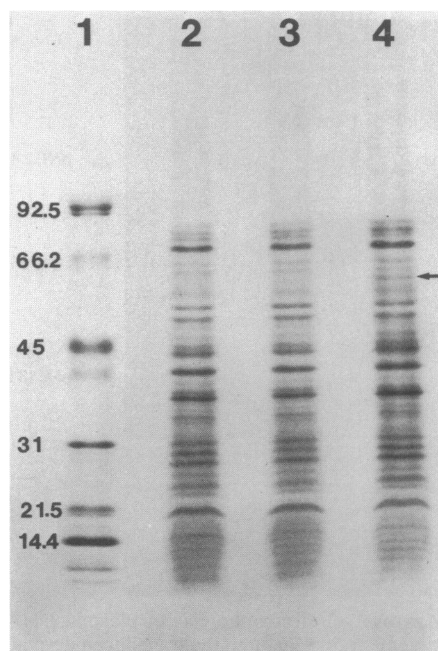

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane proteins obtained from *S. epidermidis* 958-1 and transduced derivatives. The protein bands are discernible after the gel was stained with Coomassie brilliant blue. Lane 1, molecular weight standards; the numbers to the left indicate apparent molecular weight in thousands; lane 2, membrane proteins from recipient strain 958-1 (plasmid-free); lane 3, proteins from transductant 958-3(pNE131); lane 4, membrane proteins from transductant 958-2(pNE24). The arrow shows the apparent 60,000-dalton protein present only in the membrane fraction from strain 958-2(pNE24).

Membrane proteins. In Fig. 5 the cell membrane fractions isolated from the isogenic strains 958-1, 958-2, and 958-3 are compared. The membrane protein fractions were separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. An extra protein band (or perhaps the increased production of a protein) of about 60,000 molecular weight appeared in the membrane fraction obtained from strain 958-2 containing pNE24 (Fig. 5, lane 4) but was not present (or was weakly present) in the membrane fractions obtained from plasmid-free 958-1 (Fig. 5, lane 2) and 958-3(pNE131) (Fig. 5, lane 3). Cytoplasmic protein fractions from strain 958-2 that were analyzed similarly appeared identical to those obtained from the other strains (data not shown).

DISCUSSION

Several characteristics of pNE24 set this plasmid apart from plasmids which mediate MLS resistance. (i) The size (26.5 kb) of the plasmid was larger than MLS resistance plasmids reported in staphylococci (16, 29), although MLS resistance plasmids from *Streptococcus* species have been reported in this size range (4). (ii) pNE24 mediated resistance strictly to the 14-membered macrolides erythromycin and oleandomycin. (iii) pNE24 provided only low- or at best, intermediate-level resistance (Table 1). (iv) Resistance was expressed constitutively, and no antibiotic tested was capable of inducing resistance to other MLS antibiotics.

pNE24 did not display characteristics of known plasmids which encode an MLS resistance gene and, therefore, probably does not mediate Em^r via a methylase which modifies

the 50S ribosome target site of the drug. Results from experiments presented here support this hypothesis and suggest that pNE24 may provide resistance to erythromycin (and oleandomycin) by reducing the permeability of the cell to the antibiotic (we designated the responsible gene *erpA*). For example, no detectable loss in the antimicrobial potency of erythromycin could be measured in culture supernatant of strain 958-2(pN24), indicating that pNE24 probably does not encode an erythromycin-inactivating enzyme. However, the possibility that an erythromycin-inactivating enzyme exists that remains intracellularly cannot be excluded. In addition, in vitro binding of [^{14}C]erythromycin to ribosomes isolated from strain 958-2 suggests that pNE24 does not mediate resistance by a modification of ribosomes because there appeared to be no significant loss in affinity between ribosomes and erythromycin, in comparison with ribosomes isolated from the susceptible recipient strain 958-1 (Fig. 4). In contrast, when the degree of accumulation of labeled erythromycin was compared among the three isogenic strains (Fig. 3), the strain containing pNE24 appeared to accumulate considerably less erythromycin than the susceptible recipient strain.

Other findings support a permeability barrier to erythromycin as a possible mechanism by which pNE24 mediates resistance. For instance, permeability mutants (12, 37) which affect the transport of antibiotic into the bacterial cell share some characteristics in common with strain 958-2(pNE24). These include a limited degree of resistance to the antibiotic and a target site still sensitive to the action of the antibiotic. This latter characteristic is likely to be true for ribosomes obtained from cells containing pNE24 since they had a significantly high affinity for erythromycin (Fig. 4). Moreover, plasmids have been found in gram-negative bacteria which confer resistance to chloramphenicol but do not encode a chloramphenicol acetyltransferase-inactivating enzyme (7, 24). These plasmids generally mediate a lower level of resistance to chloramphenicol (50 versus 200 μg/ml for a chloramphenicol acetyltransferase gene) and are thought to specify a cytoplasmic membrane permeability barrier (8). In addition to chloramphenicol, plasmid-mediated resistance mechanisms which result in a decrease in antibiotic accumulation have been reported for fusidic acid (3) and tetracycline (5).

Many questions remain, however, concerning pNE24 and resistance to erythromycin. The two- to threefold reduction in uptake of erythromycin observed for cells containing pNE24 relative to the susceptible recipient strain (Fig. 3) does not reflect the 1,000-fold difference in MICs between these two strains (Table 1). It is also interesting that cells (958-3) containing the MLS resistance plasmid pNE131 (Fig. 3) show an even greater reduction in the level of [^{14}C]erythromycin uptake than cells (958-2) containing pNE24. This observation supports results of earlier experiments of others (21, 42) which suggest that accumulation of erythromycin may be intimately connected with the degree of affinity of the ribosome for erythromycin. A similar effect has been shown to occur in the uptake of aminoglycosides in *E. coli* in which ribosomal mutants cause a reduction in the rate of accumulation of gantamicin (1). Experiments to detect the presence of methylated adenine in 23S rRNA from cells containing pNE24 were inconclusive (unpublished data). At this time we cannot rule out the possibility that pNE24 produces a novel modification of the ribosome that renders it resistant to the inhibitory action of erythromycin but still able to bind the antibiotic.

It seems likely, however, that pNE24 reduces the uptake of erythromycin into the host cell, not by a modification of the ribosome but by some other mechanism. The 60,000-dalton protein present in the cell membrane fraction of cells containing pNE24 (Fig.5) may be involved in resistance. A more meaningful interpretation of the results presented here is precluded by a poor understanding of the mechanisms involved in the transport of antibiotics by gram-positive cells. Further study of the means by which pNE24 confers resistance may provide insights into this important process.

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