ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1984, p. 563–569
0066-4804/84/100563-07$02.00/0

Vol. 26, No. 4

Gentamicin Uptake in *Staphylococcus aureus* Possessing Plasmid-Encoded, Aminoglycoside-Modifying Enzymes

LAURA J. MANDEL,[1,2] ELLEN MURPHY,[3] NEAL H. STEIGBIGEL,[1] AND MICHAEL H. MILLER[1]*

Division of Infectious Diseases, Department of Medicine, Montefiore Medical Center, Albert Einstein College of Medicine, Bronx, New York 10467[1]; Public Health Research Institute of the City of New York, Inc., New York, New York 10016[3]; and Division of Infectious Diseases, Downstate Medical Center, Brooklyn, New York 11203[2]

Received 6 March 1984/Accepted 13 July 1984

[^{3}H]gentamicin uptake and killing were studied in three strains of gentamicin-resistant *Staphylococcus aureus* possessing plasmid-encoded, gentamicin-modifying enzymes and in three isogenic, enzyme-free, gentamicin-susceptible derivatives. At low (≤2.0 μg/ml) concentrations of gentamicin, uptake by resistant organisms was impaired compared with that of susceptible strains, and no killing was noted. In contrast, at higher (2.5 to 10.0 μg/ml) concentrations (which were below the MIC for the resistant strains), rapid gentamicin uptake similar to that seen in susceptible isolates was observed. Although growth inhibition at these concentrations was apparent, there was no loss of viability in resistant strains. Consistently, the membrane H^+-ATPase inhibitor *N,N'*-dicyclohexyl carbodiimide caused resistant strains to take up low concentrations (1.0 μg/ml) of gentamicin at rates comparable to those seen in susceptible organisms without causing an associated loss of viability. These studies show differences between gentamicin uptake in *S. aureus* and streptomycin uptake in *Escherichia coli* (Dickie et al., Antimicrob. Agents Chemother. 14:569–580, 1978) regarding the kinetics of uptake in resistant strains with plasmid-encoded aminoglycoside-modifying enzymes. Specifically, they suggest that for 2-deoxystreptamine compounds such as gentamicin, ribosomal binding followed by accelerated uptake and subsequent interference with cell growth may occur without invariably being associated with lethal effect.

Clinical important resistance to the aminoglycoside-aminocyclitol antibiotics in bacteria is generally associated with plasmid-encoded aminoglycoside-modifying enzymes (4, 6, 15, 16). The mechanisms by which these enzymes mediate resistance is unclear. Enzymatic modification per se does not provide an adequate explanation since only a small proportion of antibiotic is modified and bacterial cells are able to grow in media containing active antibiotic (7, 8). Moreover, the presence of aminoglycoside-modifying enzymes does not necessarily correlate with clinical resistance (7).

After initial ionic binding of aminoglycosides to the outer surface of aminoglycoside-susceptible *Escherichia coli* and *Staphylococcus aureus* cells, these bacteria actively take up aminoglycosides (3, 24). Subsequent uptake occurs in a linear fashion for a period of several minutes. The rate and duration of this phase of aminoglycoside uptake are related to the external drug concentration and the MIC, and it is abolished by the addition of metabolic poisons. This first energy-dependent phase (EDP I) is followed by a second, more rapid, aminoglycoside-induced phase of uptake termed EDP II (8), which is also dependent on metabolic energy (3, 24). Streptomycin-resistant, R^+ *E. coli*, however, fails to demonstrate the second energy-dependent phase (EDP II) at antibiotic concentrations below the MIC (8). In strains capable of 3'-adenylylation, intracellular streptomycin appears to be present in modified form (8) and adenylated streptomycin demonstrates altered ribosomal binding in cell-free preparations (31). These observations have suggested a model in which the presence or absence of an inhibitory effect (i.e., drug resistance) is dependent upon a "competition" between the rate of drug modification at the level of the cytoplasmic membrane and the rate of drug transport across this membrane barrier (7, 8). This model proposes that as long as the rate of drug transport (EDP I) does not exceed the maximal rate of drug modification, i.e., at concentrations below the MIC, only modified drug is accumulated and EDP II uptake does not occur. Since streptomycin lethality appeared to be associated with EDP II kinetics (3, 8), this model was consistent with earlier studies in streptomycin-resistant *rpsl* (*strA*) *E. coli* mutants in which the absence of EDP II was shown to correlate with impaired ribosomal binding (5).

Preliminary studies performed in our laboratory with *S. aureus* suggested that the kinetics of gentamicin uptake in two resistant clinical isolates with 2''-*o*-phosphotransferase [APH (2'')] and 6'-*N*-acetyltransferase [AAC (6')] enzymes differed from that described in streptomycin-resistant, gram-negative bacilli (M. H. Miller, S. C. Edberg, M. A. Wexler, and N. H. Steigbigel, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 18th, Atlanta, Ga., abstr. no. 286, 1978). Moreover, there are important differences recognized between streptomycin (a streptidine) and gentamicin (a 2-deoxystreptamine) regarding ribosomal binding and effects on protein synthesis (12, 17, 28, 29).

To shed further light on the mechanisms of the lethal effect of this class of antibiotics, we investigated gentamicin and streptomycin uptake as it relates to resistance in clinical isolates with aminoglycoside-modifying enzymes and in isogenic derivatives selected for aminoglycoside susceptibility.

(The results of this study were presented in part at the 20th Interscience Conference on Antimicrobial Agents and Chemotherapy, 22 to 24 September, 1980, New Orleans, La.).

* Corresponding author.

MATERIALS AND METHODS

Organisms and susceptibilities. The clinical isolates of *S. aureus* (LM 48, LM 102, LM 36) used in these studies were resistant to gentamicin as determined by the standard Bauer-Kirby technique (10). Each produced coagulase and fermented mannitol (24). MICs of gentamicin for these strains were determined in nutrient broth supplemented with yeast extract (NBYE) as previously described (24) and were equal to 12.5 μg/ml (Table 1) (corresponding MICs in Mueller-Hinton broth were ≥50 μg/ml). All three strains produced penicillinase and were resistant to cadmium. In addition to the resistances shown in Table 1, LM 48 was also resistant to tetracycline and erythromycin and LM 102 was resistant to chloramphenicol. SA 121 is a clinical isolate previously described (23), and SA 121 *strA* is a one-step, high-level, streptomycin-resistant mutant derived from it (selected by plating SA 121 on agar containing 1,000 μg of streptomycin per ml). RN 450 is a plasmid-free, gentamicin- and streptomycin-susceptible *S. aureus* strain obtained from Richard Novick, Public Health Research Institute of the City of New York, Inc., New York.

Selection of isogenic gentamicin-susceptible isolates. Gentamicin-susceptible isolates were selected from each of the three strains described above by incubating cells with various subinhibitory concentrations of the intercalating dye ethidium bromide (Sigma Chemical Co., St. Louis, Mo.) (LM 36), or by overnight growth at 47°C (LM 48, LM 102) (1, 21). Subcultures were made on blood agar plates (BBL Microbiology Systems, Cockeysville, Md.). After overnight incubation, single colonies were then streaked, in duplicate, onto the surface of nutrient agar plates with and without gentamicin (5 μg/ml) (Schering Corp., Bloomfield, N.J.). Identification and susceptibility testing of susceptible isolates were performed as described for the parent strains. Gentamicin MICs for these strains ranged from 0.1 to 0.4 μg/ml in NBYE (Table 1). Other than loss of aminoglycoside resistances (Table 1), no resistance markers present in the parent strains were lost, except for chloramphenicol resistance by LM 1021.

Plasmid transfer procedures. Plasmid transfer in mixed cultures was performed as described by Jaffe et al. (15). The recipient strain LM 200 is a mutant of *S. aureus* RN 450 that was selected sequentially for chromosomal high-level resistance to rifampin and streptomycin. Each of the donor strains (LM 48, LM 102, or LM 36) and the recipient strain LM 200 were incubated overnight in NBYE (pH 6.8), centrifuged, combined, and suspended in transfer medium at a final cell density of approximately 10^{10} CFU/ml. Gentamicin-resistant progeny were selected in NBYE agar with gentamicin (10 μg/ml), rifampin (25 μg/ml), and streptomycin (1,000 μg/ml). Isolates that grew on the selective media were characterized as described above. Only transcipients of LM 48 were isolated. The approximate transfer frequency was 10^{-9} for this strain (LM 250).

Plasmid DNA. Plasmid DNA was prepared from 500-ml early stationary phase cultures by standard methods, as previously described (11, 26, 27). Restriction endonucleases were obtained from various manufacturers and used according to their specifications. Agarose gel electrophoresis was carried out in Tris-borate buffer at 2 V/cm for 16 h. Estimates of plasmid size were made by comparing restriction fragments with the migration of known molecular weight standards, and summing the sizes of the fragments.

Characterization of aminoglycoside-modifying enzymes. The analysis of modifying enzymes was performed by the method of Haas and Dowding (13). Crude lysates of *S. aureus* were prepared by osmotic lysis of lysostaphin-treated cells and incubated with radiolabeled acetylcoenzyme A or ATP in the presence of appropriate aminoglycoside substrates. The reaction mixture (containing modified radiolabeled antibiotics) was spotted onto phosphocellulose paper, washed, dried, and counted in a liquid scintillation counter; the presence of counts significantly above control indicates that acetylation, phosphorylation, or adenylylation of the aminoglycoside substrate has occurred.

Media. The broth used for gentamicin uptake studies and MICs was nutrient broth (BBL) supplemented with 0.1% yeast extract (Difco Laboratories, Detroit, Mich.) NBYE. Solid media used in these studies were NBYE plus 1.5% agar (Difco), heart infusion agar (Difco), sheep blood agar (BBL), and Mueller-Hinton agar (BBL).

Aminoglycoside uptake and killing. For uptake studies, strains were grown overnight in NBYE. Cells were diluted 10^{-3} in NBYE, and growth was monitored until cells were in log-phase growth at a density of ca. 10^8 CFU/ml, as determined by measuring absorbance at 600 nm (Coleman spectrophotometer model 6-20/A). The relationship between absorbance and CFU/ml was established as previously described (24). [^{14}C]gentamicin was supplied by Schering (bioactivity, 652 μg/mg; specific activity, 637 mCi/mmol).

TABLE 1. Characteristics of *S. aureus* strains used

S. aureus strain	Aminoglycoside phenotype	MIC (μg/ml)		Source/comment
		Gentamicin	Streptomycin	
LM36	Gm^r, Str^s	12.5	1.6	Clinical blood culture isolate
LM 361	Gm^s, Str^s	0.4	1.6	Derivative of LM36
LM 48	Gm^r, Str^r	12.5	62	Clinical blood culture isolate
LM481	Gm^s, Str^r	0.1	62	Derivative of LM48
LM 102	Gm^r, Str^s	12.5	ND[a]	Clinical blood culture isolate
LM 1021	Gm^s, Str^s	0.1	ND	Derivative of LM102
RN 450	Gm^s, Str^s	0.2	1.6	Previously characterized plasmid-free strain
LM 200	Gm^s, Str^r	0.2	≥1,000	Derivative of RN 450 selected for resistance to streptomycin and rifampin
LM 250	Gm^r, Str^r	12.5	≥1,000	Gm^r transcipient of LM 200 obtained from mixed culture of LM 200 and LM 48
SA 121	Gm^s, Str^s	0.4	1.6	Clinical blood culture isolate (23)
SA 121 *strA*	Gm^s, Str^r	0.4	≥1,000	Derivative of SA 121 selected for high-level resistance to streptomycin

[a] ND, Not done.

[^{3}H]gentamicin (Amersham Corp., Arlington Heights, Ill.) had a specific activity of 86 mCi/mmol (0.12 mCi/mg) and was mixed with standard gentamicin powder (588 μg/mg; Schering) to a final specific activity of 1 to 5 μCi/mg just before use. [^{3}H]dihydrostreptomycin sesquisulfate (Amersham) (1.2 mCi/mg) was mixed with streptomycin powder (Sigma) to a final specific activity of 5 to 10 μCi/mg. Radiolabeled aminoglycoside was then added to strains in log-phase growth (at 37°C) in a rotary shaker bath to the desired final concentration. Uptake was measured by periodic sampling of 5-ml samples. Samples were immediately filtered through membrane filters (Millipore Corp., Bedford, Mass.), 0.45 μm pore size, and washed with 3% sodium chloride as previously described (20, 24). The filters were placed into counting vials and dried overnight. Samples were counted in a Packard Tri-Carb liquid scintillation counter after the addition of 10 ml of toluene containing (per liter) 5.0 g of 2,5-diphenyloxazole and 0.4 g of 1,4-bis-2-(5-phenyloxazole) benzene. Amingolycoside uptake is expressed as total uptake (nanograms of aminoglycoside) per 10^8 CFU (as determined by absorbance at 600 nm).

For the determination of cell viability, 1-ml samples were taken at indicated times and cell viability (CFU/ml) was determined by the standard pour plate technique (10, 25), using heart infusion agar adjusted to pH 5.0 to inhibit aminoglycoside carry-over.

Chemicals. *N,N*-Dicyclohexylcarbodiimide (DCCD) (Sigma) was prepared as a fresh solution (4 mM in 100% ethanol) and was added to cultures simultaneously with gentamicin to a final concentration of 20 μM DCCD.

RESULTS

Isolation of isogenic pairs of gentamicin-susceptible and -resistant organisms and characterization of plasmids and aminoglycoside-modifying enzymes. Three gentamicin-resistant *S. aureus* strains (LM 36, LM 48, and LM 102) and susceptible derivatives (LM 361, LM 481, LM 1021) selected as described above were studied.

Overnight incubation of mixed cultures of the gentamicin-resistant isolate LM 48 (Gm^r) and LM 200 (Rif^r, Str^r) and subsequent selection with gentamicin, rifampin, and streptomycin resulted in isolates resistant to all of these antibiotics (LM 250). None of the other known markers of LM 48, including penicillin resistance or resistance to cadmium or arsenate ions, were cotransferred. No similar transcipients were isolated for LM 36 or LM 102.

LM 36 and LM 48 each contained a single large plasmid, pLJM 36 (52 kilobases [kb]) and pLJM 48 (46 kb), respectively, which were not present in the gentamicin-susceptible derivatives LM 361 or LM 481. pLJM 36 and pLJM 48 are closely related to one another, having in common at least five *Hin*dIII fragments (Fig. 1); similarly related cleavage patterns obtained with endonucleases *Hae*III, *Hha*I, *Msp*I, and *E*coRI were also observed (data not shown). On the basis of their *Hin*dIII and *Hae*III digests, pLJM 48 and pLJM 36 appear to be very closely related to the gentamicin resistance plasmids previously identified by Jaffe et al. (16) and Cohen et al. (4). The plasmid present in the gentamicin-resistant transcipient LM 250 is identical to the plasmid (pLJM 48) present in the donor strain LM 48 (Fig. 1). LM 102 contained three plasmids with molecular sizes of approximately 48, 32, and 4.5 kb (data not shown). Restriction digests of the plasmid DNA from this strain contained too many bands to allow a rigorous comparison with plasmid digests from LM 36 and LM 48. The *Hin*dIII and other restriction digests of the plasmid DNA from LM 102, however, contained several bands in common with pLJM 48 and pLJM 36. Plasmids were absent in the gentamicin-susceptible derivatives LM 361, LM 1021, and LM 481.

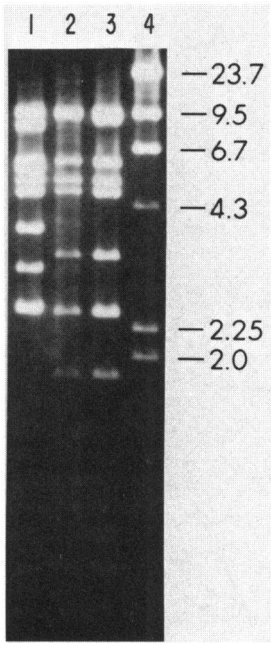

FIG. 1. Agarose (0.7%) gel electrophoresis (2 V/cm, 16 h, Tris-borate buffer) of restriction endonuclease *Hin*dIII digests of plasmid DNA in gentamicin-resistant strains; lane 1, LM 36; lane 2, LM 48; lane 3, LM 250; lane 4, *Hin*dIII-digested DNA standard (sizes in kb indicated on the right). The 9.5- and 2.5-kb fragments of LM 48 and LM 250, and the 2.5-kb fragment of LM 36, were determined to be doublet bands by direct fluorimetry of the ethidium bromide-stained gel with the Shimadzu CS 910 scanning densitometer.

Crude lysates of all strains were screened for aminoglycoside-modifying activity as described above, using kanamycin B, gentamicin C_{1A} and C_1, and amikacin as substrates. Gentamicin-resistant strains (LM 36, LM 48, LM 102, and LM 250) phosphorylated both kanamycin B and gentamicin C_{1A} and acetylated gentamicin C_{1A}, but not gentamicin C_1, as determined by nitrocellulose binding of labeled substrate. In contrast, the susceptible strains had no demonstrable aminoglycoside-modifying activity. Broader substrate profiles with kanamycin, gentamicin, tobramycin, amikacin, butirosin, paromomycin, netilmicin, and lividomycin as substrates confirmed these results (David Bobey, Bristol Laboratories, Syracuse, N.Y.). This spectrum of activity is consistent with the interpretations that all three resistant strains contained AAC (6′) and APH (2″) enzyme activity (7). The cured derivatives LM 361, LM 481, and LM 1021 demonstrated no aminoglycoside-modifying activity.

Gentamicin uptake and killing in isogenic *S. aureus* strains. Figure 2A shows a representative plot of uptake dose-response curves for gentamicin in strains LM 36 (MIC, 12.5 μg/ml) and LM 361 (MIC, 0.4 μg/ml). At the lower gentamicin concentrations (1.0 to 2.5 μg/ml), there was diminished gentamicin uptake in LM 36 as compared with the susceptible strain LM 361. As the external concentration of gentamicin was increased, accelerated rates of uptake were observed. Notably, at 5 and 10 μg/ml, similar uptake was observed in both susceptible and resistant strains. Figure 2B demonstrates the effect of this concentration-dependent up-

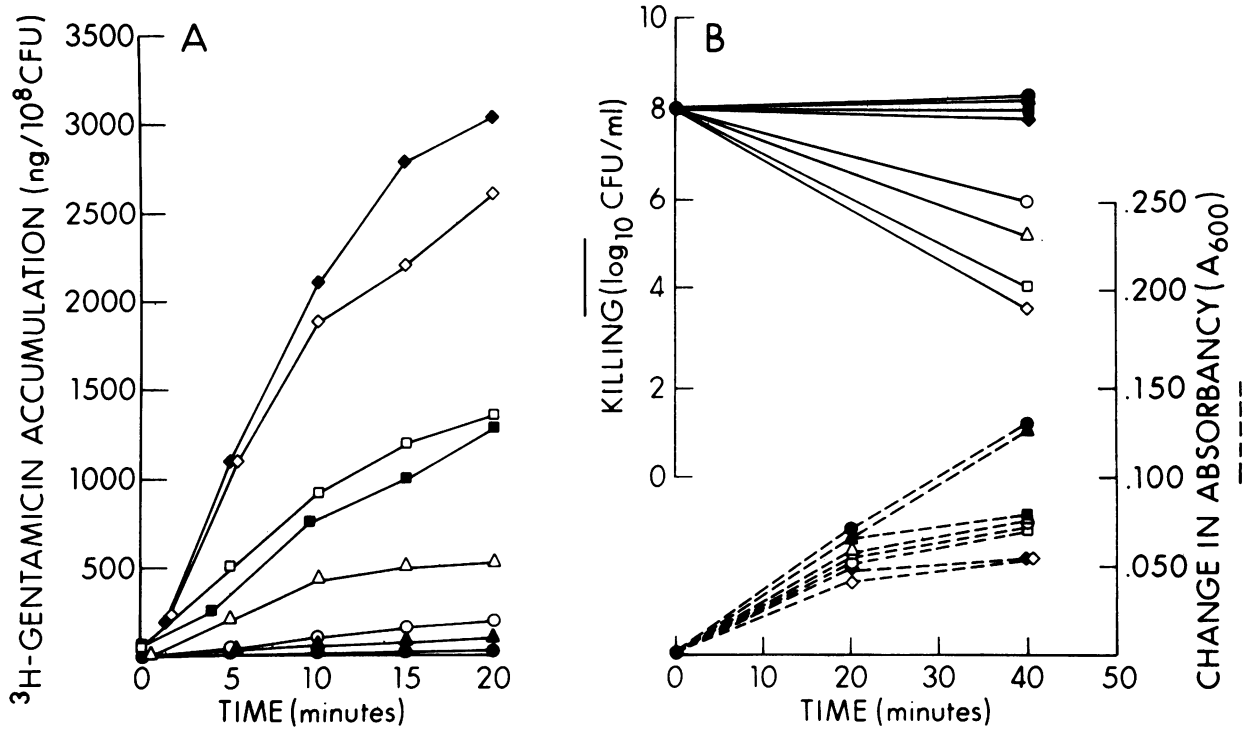

FIG. 2. (A) Dose-response data showing the relationship between external gentamicin concentration and [^{3}H]gentamicin uptake in gentamicin-resistant strain LM 36 (MIC, 12.5 μg/ml) at 1.0 (●), 2.5 (▲), 5.0 (■) and 10.0 (◆) μg/ml. Open symbols show data for gentamicin-susceptible LM 361 (MIC, 0.4 μg/ml) at the same concentrations. (B) Rate of growth as a function of change in absorbancy (lower panel, dashed lines) versus time and viability (upper panel, solid lines) of these strains. Symbols correspond to those used in A.

take on bacterial viability (upper panel) and on growth as monitored spectrophotometrically (lower panel) for both susceptible and resistant strains. The upper panel shows that in the resistant strain (LM 36), as the gentamicin concentration was increased up to 10 μg/ml (MIC, 12.5 μg/ml), the drug uptake observed in Fig. 2A was not associated with any bactericidal effect. In comparison, the susceptible strain demonstrated a decrease (2 to 5 $\log_{10}$) in CFU/ml that was proportional to the external gentamicin concentration. Standard time-kill studies over 24 h confirmed that there was no bactericidal effect at all gentamicin concentrations that were below the MIC for the resistant, plasmid-containing strain. Importantly, however, growth measured spectrophotometrically (Fig. 2B, lower panel) was shown to be diminished in the susceptible strain at all concentrations of gentamicin, but in the resistant strain only at concentrations above the inductive concentration (that concentration associated with accelerated uptake but below the MIC) of 2.5 μg/ml (one-fifth the MIC for gentamicin). There was no effect on growth monitored spectrophotometrically in LM 36 at subinductive gentamicin concentrations (1.0 and 2.5 μg/ml) when compared with a control not treated with gentamicin (data not shown). Similar studies that determined dose-response aminoglycoside uptake, bactericidal effect, and growth measured spectrophotometrically in LM 48, LM 481, LM 102, and LM 1021 gave identical results (data not shown).

Figure 3 shows drug uptake plotted as area under the gentamicin uptake-time curves for the three pairs of gentamicin-resistant and -susceptible strains. In the susceptible strains, uptake was proportional to external drug concentration at all drug concentrations tested. In contrast, for the resistant strains, total uptake was impaired at the lower concentrations. However, at higher but still subinhibitory (below the MIC) concentrations, the amount of drug accumulated for resistant strains increased markedly and thereafter appeared to be proportional to the external drug concentration.

Effect of DCCD on aminoglycoside uptake and killing in *S. aureus*. Figure 4A shows comparative gentamicin (1 μg/ml) uptake and killing in gentamicin-resistant strain LM 36 with and without the addition of the proton-translocating ATPase (H^+-ATPase) inhibitor DCCD. DCCD increases aminoglycoside uptake in *S. aureus* by increasing the membrane potential for drug transport due to its effect on the magnitude of the electrical potential across the cytoplasmic membrane (9).

The resistant strain (LM 36) showed decreased uptake as compared with the susceptible strain (LM 361), and there was no bactericidal effect (Fig. 4A). The addition of 20 μM DCCD caused the resistant strain (LM 36) to take up gentamicin at a rate comparable to that seen in the susceptible strain in the absence of DCCD, but there was no loss of viability as determined by measuring CFU/ml. DCCD alone at these concentrations was not lethal to *S. aureus*, and it stimulated both gentamicin uptake and killing in LM 361 (data not shown), as has been previously described with other gentamicin-susceptible *S. aureus* isolates (18, 24).

The effect of 20 μM DCCD on streptomycin uptake was determined in the susceptible strain SA 121 and its single-step, high-level streptomycin-resistant mutant SA 121 *strA* (Fig. 4B). In SA 121 at 20 μg of streptomycin per ml, 20 μM DCCD stimulated the uptake of streptomycin, as has been previously shown with gentamicin in this strain (24). In SA 121 *strA* (MIC > 1,000 μg/ml), there was little stimulation of streptomycin uptake and no bactericidal effect relative to that seen in streptomycin-susceptible SA 121. Moreover, 20

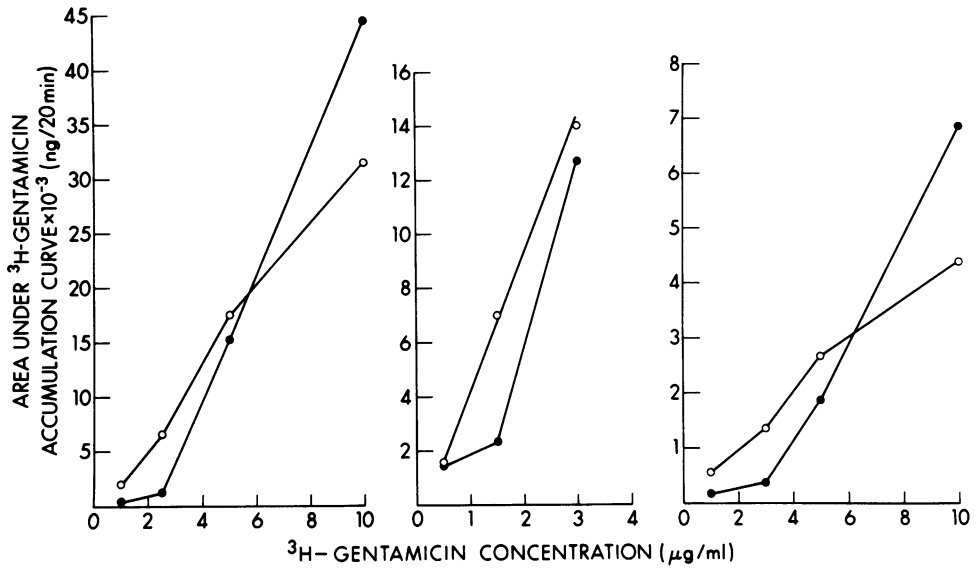

FIG. 3. Total gentamicin uptake as a function of external drug concentration in resistant (●) and susceptible (○) isolates. LM 36 and LM 361 are shown in the left panel, LM 48 and LM 481 in the middle panel, and LM 102 and LM 1021 in the right panel.

μM DCCD stimulated gentamicin uptake in both SA 121 *strA* and SA 121 (data not shown). These studies further support the specificity of the effect of DCCD on membrane energization (9). Moreover, they suggest both that a ribosomal binding sink is required to demonstrate EDP II kinetics (2, 3) and that there are multiple ribosomal binding sites for gentamicin (2, 12, 28, 29).

DISCUSSION

The aminoglycoside-resistant strains studied (LM 36, LM 48, and LM 102) had aminoglycoside-modifying enzymes commonly found in gentamicin-resistant isolates of *S. aureus* and *Staphylococcus epidermidis* (22, 30, 31). LM 250, the gentamicin-resistant transcipient of LM 200 (derived from plasmid-free RN 450) was found to possess the same aminoglycoside-modifying activity as the donor strain LM 48. The gentamicin-susceptible isolates (LM 361, LM 481, and LM 1021) selected from gentamicin-resistant clinical isolates had lost the APH (2'')- and AAC (6')-modifying enzymes present in resistant strains.

Studies of the plasmid content of gentamicin-resistant clinical isolates confirmed that both LM 48 and LM 250 contained a plasmid (pLJM 48) of 48 kb that yielded identical cleavage patterns with *Hin*dIII and other restriction endonucleases. LM 36 was found to contain a similar, although not identical plasmid (pLJM 36) that was absent in the gentamicin-susceptible derivative LM 361. The simplest interpretation of the cleavage pattern of pLJM 36 and pLJM 48 would be that pLJM 36 carries two insertions relative to pLJM 48. By comparison with published data, these plasmids are closely related to plasmids previously identified in gentamicin-resistant strains (4, 16). These data suggest that the AAC (6')- and APH (2'')- modifying enzymes associated with gentamicin resistance in LM 36 and LM 48 are encoded by genes carried by pLJM 36 and pLJM 48.

Kinetic studies examining the uptake and lethal effect of the aminoglycoside gentamicin in isogenic susceptible and resistant strains of *S. aureus* demonstrated that at higher external drug concentrations (inductive concentrations), uptake in resistant and suceptible strains was similar. In contrast, when resistant cells are exposed to subinductive concentrations of gentamicin, decreased uptake is observed as compared with uptake in susceptible stains. Notably, however, when the inductive external concentration of gentamicin is reached, there is diminution of the rate of cell replication as determined spectrophotometrically in resistant as well as susceptible strains. Despite this growth inhibition associated with gentamicin uptake, there is no loss of viability of resistant strains, as measured by ability to form colonies in agar.

Studies with DCCD confirm our data indicating that gentamicin uptake is not necessarily associated with a bacterial lethal effect. We have recently shown that DCCD (a proton translocating ATPase inhibitor) increases the magnitude of the electrical potential across the cytoplasmic membrane (9). Previous studies from our laboratory have demonstrated that DCCD increases the uptake of gentamicin by susceptible strains at both inhibitory (24) and subinhibitory gentamicin concentrations (18) in a dose-dependent manner. Although 20 μM DCCD stimulates gentamicin uptake in the plasmid-containing, gentamicin-resistant isolates, there is no associated loss of viability noted.

Since the accelerated gentamicin uptake seen in resistant strains is not associated with a bactericidal effect, uptake could represent increased extracytoplasmic binding rather than transport. This is highly unlikely because (i) concentration-associated uptake is shown to diminish the rate of cell replication spectrophotometrically, suggesting that cellular uptake, ribosomal binding, and partial drug effect have occurred; (ii) as noted above, in gentamicin-susceptible strains, DCCD stimulates both uptake and killing in a dose-dependent fashion, suggesting that DCCD-associated aminoglycoside uptake correlates with drug transport; (iii) in both susceptible and resistant (plasmid containing) *S. aureus* (24) and *E. coli* (3) cells, surface aminoglycoside binding is virtually instantaneous, and continued incubation is associated with uptake only in the presence of oxidative energy.

Examination of the kinetics of gentamicin uptake (Fig. 2), as well as that of total uptake (Fig. 3), suggests that at the highest external drug concentrations uptake is greater in the

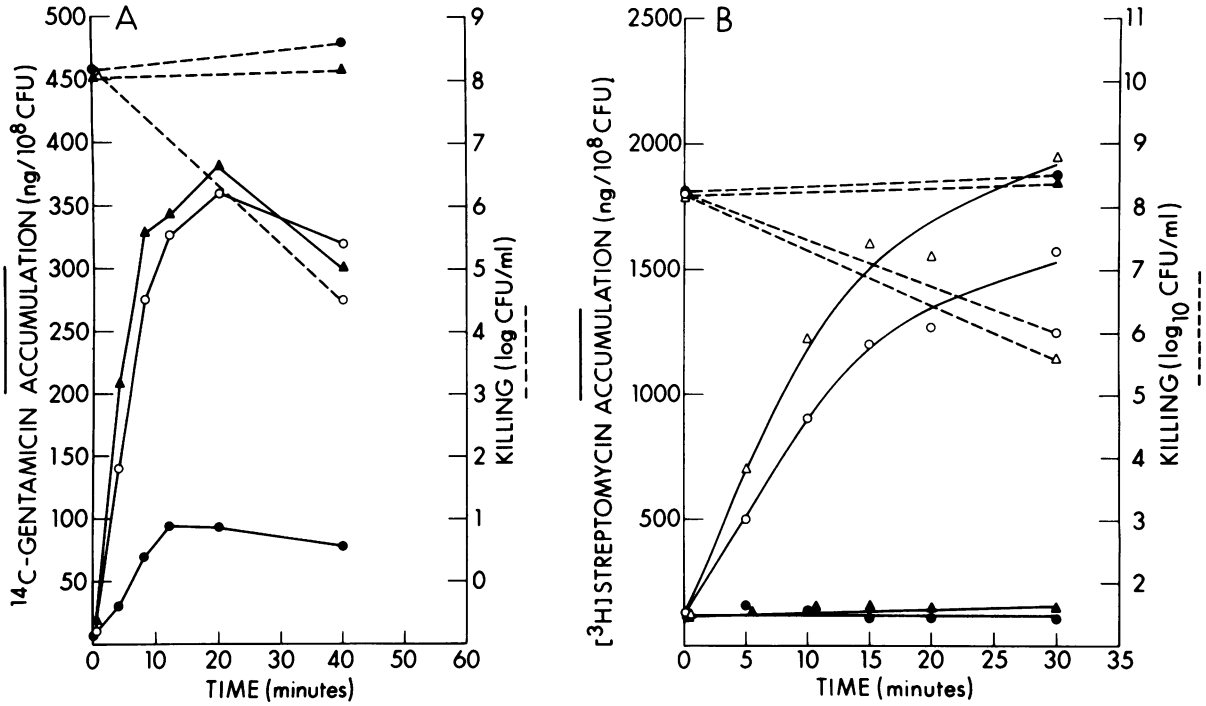

FIG. 4. (A) [^{14}C]gentamicin accumulation at 1 μg/ml in gentamicin-resistant *S. aureus* LM 36 (MIC of gentamicin, 12.5 μg/ml) with (▲) and without (●) 20 μM DCCD. Uptake in the gentamicin-susceptible strain LM 361 without DCCD (○) is also shown. Dashed lines show corresponding bacterial killing. (B) [^{3}H]streptomycin uptake at 20 μg/ml in streptomycin-susceptible SA 121 with (△) and without (○) 20 μM DCCD. Dark symbols show corresponding data for a single-step, high-level, streptomycin-resistant mutant of SA 121 (MIC, ≥1,000 μg/ml). Corresponding killing is shown (dashed lines).

resistant than in the susceptible strains. This observation, however, probably represents inhibition of the cellular processes or killing of susceptible strains and a secondary decrease in the antibiotic uptake measured for these strains. Furthermore, it has recently been suggested that, in susceptible *E. coli*, aminoglycosides themselves diminish the magnitude of the electrical potential across the cell membrane (2).

Previous studies measuring streptomycin uptake in resistant *E. coli* (ribosomal binding *strA* mutants and aminoglycoside-modifying R^+ clinical isolates) have demonstrated EDP I but not EDP II kinetics (3, 8). Ribosomes from *strA* mutants do not bind native streptomycin, and this failure of binding mediates resistance (5). In a similar fashion, plasmid-mediated modification of streptomycin also alters binding affinity to ribosomes of susceptible strains (8, 31). These data suggested that in gram-negative bacilli, the occurrence of EDP II is associated with susceptibility, and that ribosomal binding is required for this phase of uptake to occur. Moreover, these studies suggested that strategically located, membrane-associated modifying enzymes mediate resistance when the rate of modification exceeds the rate of transport as described by EDP I (2, 8).

Recently, Hurwitz et al. (14) have demonstrated that the absence of streptomycin accumulation seen in *strA* mutants of *E. coli* can be overcome by the addition of puromycin which, like the aminoglycosides themselves, induces premature termination of the nascent protein chain and a subsequent increase in the number of runoff ribosomes. By this manipulation, these authors were able to induce EDP II-type accumulation kinetics in the resistant strain without causing loss of cell viability. Whether these observations are due to an increase in the ribosomal binding sites, as suggested, or are secondary to some other alteration of cell function, is not clear from the available data. However, these observations provide additional evidence that the ability, under certain circumstances, to dissociate the phenomenon of deficient aminoglycoside uptake from that of resistance is not limited to *S. aureus*, nor to a specific aminoglycoside.

Our studies suggest that there are important differences between the kinetics of gentamicin uptake in *S. aureus* with gentamicin-modifying enzymes, and streptomycin uptake in the streptomycin-resistant R^+ *E. coli* (8) regarding the complex relationships between rate of uptake, drug modification, ribosomal binding, and resistance. These differences were anticipated because of the recognized differences between the effects of streptomycin and gentamicin on protein synthetic events (initiation, chain elongation, miscoding, termination, etc.) and ribosomal binding (2, 12, 28, 29). However, there are additional variables that may play a role such as differences between aminoglycoside-modifying enzymes (6, 7) and between 30S ribosomes of gram-negative and gram-positive bacteria (23). We are not aware of any studies examining the effects of modified deoxystreptamine aminoglycosides (i.e., gentamicin, tobramycin, amikacin) on ribosomal binding and protein synthesis. Clearly, such studies are important.

In any event, regardless of these mechanistic considerations, it is important to point out, given the observations presented here, that an understanding of enzyme-mediated aminoglycoside resistance requires studies with 2-deoxystreptamine aminoglycosides, as well as with streptomycin. This is particularly true since most clinically useful aminoglycosides belong to the former class of compounds (19).

ACKNOWLEDGMENTS

L.J.M. was supported in part by a stipend from a Public Health Service Institutional Research Training Award of the National Institute of Allergy and Infectious Diseases (5T 32 AI 07183 03).

We are grateful to Julian Davies, Thomas Dougherty, and Ruy Soeiro for their helpful advice and review of this manuscript, to David Bobey (Bristol Laboratories) for his assistance in the characterization of the enzymes in LM 36, LM 48, LM 102, and LM 250, and to Joyce Williams for her technical assistance.

LITERATURE CITED

1. **Bouanchaud, D. H., M. R. Scavizzi, and Y. A. Chabbert.** 1969. Elimination by ethidium bromide of antibiotic resistance in enterobacteria and staphylococci. J. Gen. Microbiol. **54**:417–425.
2. **Bryan, L. E., and S. Kwan.** 1983. Roles of ribosomal binding, membrane potential, and electron transport in bacterial uptake of the streptomycin and gentamicin. Antimicrob. Agents Chemother. **23**:835–845.
3. **Bryan, L. E., and H. M. Van den Elzen.** 1976. Streptomycin accumulation in susceptible and resistant strains of *Escherichia coli* and *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **9**:928–938.
4. **Cohen, M. L., E. S. Wong, and S. Falkow.** 1982. Common R-plasmids in *Staphylococcus aureus* and *Staphylococcus epidermidis* during a nosocomial *Staphylococcus aureus* outbreak. Antimicrob. Agents Chemother. **21**:210–215.
5. **Davies, J.** 1964. Studies on the ribosomes of streptomycin-sensitive and resistant strains of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **51**:659–664.
6. **Davies, J.** 1971. Bacterial resistance to aminoglycoside antibiotics. J. Infect. Dis. **124**(Suppl.):7–10.
7. **Davies, J., and S. A. Kagan.** 1977. What is the mechanism of plasmid-determined resistance to aminoglycoside antibiotics? p. 207–219. *In* J. Drews and G. Hogenau (ed.), R-factors: their properties and possible control. Topics in infectious diseases, vol. 2. Springer-Verlag, New York.
8. **Dickie, P., L. E. Bryan, and M. A. Pickard.** 1978. Effect of enzymatic adenylylation on dihydrostreptomycin accumulation in *Escherichia coli* carrying an R-factor: model explaining aminoglycoside resistance by inactivating mechanisms. Antimicrob. Agents Chemother. **14**:569–580.
9. **Eisenberg, E. S., L. J. Mandel, H. R. Kaback, and M. H. Miller.** 1984. Quantitative association between electrical potential across the cytoplasmic membrane and early gentamicin uptake and killing in *Staphylococcus aureus*. J. Bacteriol. **157**:863–867.
10. **Ericsson, H. M., and J. C. Sherris.** 1971. Antibiotic sensitivity testing-report of international collaborative study. Acta Pathol. Microbiol. Scand. Sect. B **217**:(Suppl):1–99.
11. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064–1066.
12. **Hancock, R.** 1981. Aminoglycoside uptake and mode of action-with special reference to streptomycin and gentamicin. J. Antimicrob. Chemother. **8**:249–276.
13. **Hass, M. J., and J. E. Dowding.** 1975. Aminoglycoside-modifying enzymes. Methods Enzymol. **43**:611–625.
14. **Hurwitz, C., C. B. Braun, and C. L. Rosano.** 1981. Role of ribosome recycling in uptake of dihydrostreptomycin in sensitive and resistant *E. coli*. Biochem. Biophys. Acta **652**:168–176.
15. **Jaffe, H. W., M. H. Sweeney, C. Nathan, R. A. Weinstein, S. A. Kabins, and S. Cohen.** 1980. Identity and interspecies transfer of gentamicin-resistance plasmids in *Staphylococcus aureus* and *Staphylococcus epidermidis*. J. Infect. Dis. **141**:738–747.
16. **Jaffe, H. W., H. M. Sweeney, R. A. Weinstein, S. A. Kabins, C. Nathan, and S. Cohen.** 1982. Structural and phenotypic varieties of gentamicin resistance plasmids in hospital strains of *Staphylococcus aureus* and coagulase-negative staphylococci. Antimicrob. Agents Chemother. **21**:773–779.
17. **Kuhberger, R. W., A. Piepersberg, P. Petzet, P. Buckel, and A. Bock.** 1979. Alteration of ribosomal protein L6 in gentamicin-resistant strains of *Escherichia coli*. Effects on fidelity of protein synthesis. Biochemistry **18**:187–193.
18. **Mandel, L. J., E. S. Eisenberg, N. J. Simkin, and M. H. Miller.** 1983. Effect of *N,N'*-dicyclohexylcarbodiimide and nigericin on *Staphylococcus aureus* susceptibility to gentamicin. Antimicrob. Agents Chemother. **24**:440–442.
19. **Mandel, L. J., and M. H. Miller.** 1983. Aminoglycosides (including aminocyclitols), p. 37–50. *In* S. C. Edberg and S. Berger (ed.), Antibiotics and infections. Churchill-Livingston, Inc., New York.
20. **Mates, S. M., E. S. Eisenberg, L. J. Mandel, L. Patel, H. R. Kaback, and M. H. Miller.** 1982. Membrane potential and gentamicin uptake in *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U.S.A. **79**:6693–6697.
21. **May, J. W., R. H. Houghton, and C. J. Perry.** 1964. The effect of growth at elevated temperatures on some heritable properties of *Staphylococcus aureus*. J. Gen. Microbiol. **37**:157–169.
22. **McGovern, J. E., Jr., P. M. Terry, T. S. R. Huang, C. L. Houk, and J. Davies.** 1979. Nosocomial infection with gentamicin resistant *Staphylococcus aureus*: plasmid analysis as an epidemiologic tool. J. Infect. Dis. **140**:864–872.
23. **McLaughlin, J. R., C. L. Murray, and J. C. Rabinowitz.** 1981. Unique features in the ribosome binding site sequence of the gram positive *Staphylococcus aureus* β-lactamase Gene. J. Biol. Chem. **2456**:11283–11291.
24. **Miller, M. H., S. C. Edberg, L. J. Mandel, C. F. Behar, and N. H. Steigbigel.** 1980. Gentamicin uptake in wild-type and aminoglycoside-resistant small-colony mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **18**:722–729.
25. **Miller, M. H., M. A. Wexler, and N. H. Steigbigel.** 1978. Single and combination antibiotic therapy of *Staphylococcus aureus* experimental endocarditis: emergence of gentamicin-resistant mutants. Antimicrob. Agents Chemother. **14**:336–343.
26. **Novick, R. P., and D. Bouanchaud.** 1971. Extra chromosomal nature of drug resistance in *Staphylococcus aureus*. Ann. N.Y. Acad. Sci. **182**:279–294.
27. **Novick, R. P., E. Murphy, T. J. Gryczan, E. Baron, and I. Edelman.** 1979. Penicillinase plasmids of *Staphylococcus aureus*: restriction-deletion maps. Plasmid **2**:109–129.
28. **Pestka, S.** 1977. Inhibitors of protein synthesis, p. 467–535. *In* H. Weissbach and S. Pestka (ed.), Mechanisms of protein biosynthesis. Academic Press, London.
29. **Tai-Phang, C., and B. D. Davis.** 1979. Triphasic concentration effects of gentamicin on activity and misreading in protein synthesis. Biochemistry **18**:193–198.
30. **Vogel, L., C. Nathan, H. M. Sweeney, S. A. Kabins, and S. Cohen.** 1978. Infections due to gentamicin-resistant *Staphylococcus aureus* strain in a nursey for neonatal infants. Antimicrob. Agents Chemother. **13**:466–472.
31. **Yamada, T., D. Tipper, and J. Davies.** 1968. Enzymatic inactivation of streptomycin by R-factor-resistant *Escherichia coli*. Nature (London) **219**:288–291.