Gentamicin Uptake in Wild-Type and Aminoglycoside-Resistant Small-Colony Mutants of *Staphylococcus aureus*

MICHAEL H. MILLER,¹* STEPHEN C. EDBERG,² LAURA J. MANDEL,¹ CAREN F. BEHAR,¹ AND NEAL H. STEIGBIGEL¹

Division of Infectious Diseases, Department of Medicine,¹ and Division of Microbiology, Department of Pathology,² Montefiore Hospital and Medical Center, and Departments of Medicine,¹ Pathology,² and Microbiology and Immunology,² and Albert Einstein College of Medicine, Bronx, New York 10467

Gentamicin uptake and killing were studied in aminoglycoside-susceptible wild-type *Staphylococcus aureus* strains and aminoglycoside-resistant small-colony mutants selected by gentamicin from these strains. In wild-type *S. aureus* three phases of gentamicin accumulation were noted, and killing occurred during the last and most rapid phase of uptake. Uptake and killing were abolished by anaerobic growth and sodium azide, suggesting that energy-dependent active drug transport required respiration. Treatment of wild-type strains with the uncouplers *N,N'*-dicyclohexyl carbodiimide (DCCD) and carbonyl cyanide-*m*-chlorophenyl hydrazone showed disparate effects on gentamicin uptake, producing enhanced and diminished accumulations, respectively. Small-colony mutants demonstrated markedly deficient uptake compared with the wild-type strains and were not killed by gentamicin in concentrations up to 10 µg/ml. Several classes of aminoglycoside-resistant mutant strains are described. One mutant strain was a menadione auxotroph which, when grown in the presence of menadione, exhibited normal gentamicin uptake and killing. Gentamicin uptake and killing in this strain were abolished by KCN when the strain was grown in a medium supplemented with menadione. The membrane adenosine triphosphatase inhibitor DCCD was lethal for this mutant but not for other mutants or wild-type strains. Preincubation with menadione prevented the lethal effect of DCCD, and this strain demonstrated normal gentamicin accumulation when exposed to both DCCD and menadione. A second mutant strain demonstrated both gentamicin uptake and killing in the presence but not the absence of DCCD. Studies with small-colony mutants of *S. aureus* indicated that the defect in aminoglycoside uptake is very likely related to an inability to generate or maintain energized membranes from respiration. These studies suggest that the membrane energization associated with active aminoglycoside accumulation requires electron transport for the generation of a protonmotive force.

In vitro time kill studies have demonstrated that gentamicin is rapidly bactericidal for *Staphylococcus aureus*; however, low concentrations of gentamicin (0.5 µg/ml) select for mutants which demonstrate increased resistance to gentamicin and other aminoglycosides and which form nonhemolytic small colonies on sheep blood agar (21). Treatment of animals with *S. aureus* endocarditis with gentamicin dosages giving serum concentrations comparable to those in treated patients only minimally diminishes the bacterial densities in valvular vegetations and selects for these resistant mutants (21). The clinical significance of dwarf-colony mutants of *S. aureus* is uncertain, although such mutants have been isolated occasionally from clinical material (1). These mutants have been shown to be pathogenic when evaluated in sev-

eral models of infection in animals (21, 24). The decreased susceptibilities to a wide variety of aminoglycosides observed in these mutant strains suggest a more general mechanism of resistance than specific enzymatic modification or altered ribosomal binding of particular aminoglycosides. In view of data indicating that similar small-colony variants of *S. aureus* have impairments in oxidative processes (19, 20) and the demonstration that oxidatively generated energy is required for aminoglycoside uptake in gram-negative bacilli (4-6), we were interested in studying gentamicin accumulation and its relationship to oxidative metabolism in wild-type and aminoglycoside-resistant small-colony mutants of *S. aureus*.

(The results of this study were presented in part at the 18th Interscience Conference on An-

timicrobial Agents and Chemotherapy, October 1978.)

MATERIALS AND METHODS

Organisms and susceptibilities. The wild-type *S. aureus* strains used in these studies were blood culture isolates from this hospital, which were resistant to penicillin G but susceptible to nafcillin and gentamicin by the Kirby-Bauer technique (Table 1). These isolates were identified by the determination of coagulase production and mannitol fermentation (21). Several mutants resistant to gentamicin were selected in vitro with 0.5 μ g of gentamicin (Schering Corp., Bloomfield, N.J.) per ml, as previously described (21). An additional mutant strain, *S. aureus* 15956 Mar, was selected in vivo from a gentamicin-treated animal with experimental endocarditis (21). These mutant strains were resistant to gentamicin, streptomycin, kanamycin, amikacin, and tobramycin by the Kirby-Bauer technique (10). The minimum inhibitory concentrations for *S. aureus* strains 15956 and 15956 Mar were determined in nutrient broth (BBL Microbiology Systems, Cockeysville, Md.), as previously described (21). Overnight incubations of the other mutant strains in liquid media showed reversion with mixed populations of wild-type and mutant strains after subculture to blood agar plates. The minimum inhibitory concentrations of gentamicin, *N,N'*-dicyclohexyl carbodiimide (DCCD), carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), and menadione for these unstable mutant strains and the wild-type parent strains were determined by a modification of an agar dilution technique (10), using nutrient broth containing 0.1% yeast extract and 1.5% agar. Strains were stored on agar slants at -70°C before use.

Media. The broth used for aerobic studies was nutrient broth (BBL Microbiology Systems) containing 0.1% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.) (NBYE). Broth for anaerobic studies contained the following (in grams per liter): yeast extract (Difco), 5; tryptone (Difco), 15; dextrose (Difco), 5; L-cysteine (Sigma Chemical Co., St. Louis, Mo.), 0.25; NaCl, 2.5; and sodium thioglycolate, 0.3 (5). The broth for the aerobic control consisted of the same reagents without cysteine or thioglycolate. The agars used in these studies were brain heart infusion agar (Difco), sheep blood agar (BBL Microbiology Systems), and Mueller-Hinton agar (BBL Microbiology Systems).

Growth rates and absorbance. Bacterial densities (in colony-forming units [CFU] per milliliter) were determined in duplicate by using a standard pour plate technique in heart infusion agar adjusted to a pH of 5.5 to inhibit gentamicin carry-over (21). Growth was determined for 6 h at 37°C in NBYE, using as an initial inoculum a 10^{-8} dilution of an overnight culture. Absorbance readings were measured with a Coleman spectrophotometer (model 6-20/A) at 600 nm. The conversion of absorbance to CFU per milliliter was done by using these data. Linear absorbance in all experiments indicated that the strains were in log-phase growth at a bacterial density of at least 10^7 CFU/ml before the addition of gentamicin.

Gentamicin uptake. The [^{14}C]gentamicin used in

TABLE 1. Wild-type and mutant *S. aureus* strains used

Strains ^a	Characteristics of mutant	Gentamicin minimal inhibitory concn ($\mu\text{g/ml}$)
15956		0.5
15956 Mar	Selected in vivo; stable in liquid media; growth rate similar to wild type	16
121		0.4
121 Mar	Selected in vitro; unstable in liquid media; growth rate similar to wild type	12.8
86		0.8
86 Mar	Selected in vitro; intermediate stability in liquid media; slow growing compared with wild type	6.4

^a Mutant strains (Mar) showed multiple aminoglycoside resistance and formed small, nonhemolytic colonies on sheep blood agar plates.

these studies was supplied by Schering Corp. The bioactivity (652 $\mu\text{g/mg}$) and specific activity (637 mCi/mmol) were confirmed by using a standard gentamicin powder (568 $\mu\text{g/mg}$; Schering) and a ^{14}C standard (10^6 dpm; Packard). Several strains (Table 1) were unstable in broth. From these strains nonhemolytic colonies were selected from the surfaces of sheep blood agar plates after 48 to 72 h of incubation at 37°C and inoculated into NBYE. In these experiments the wild-type strains were also taken from blood agar plates. In unstable strains reversion was monitored in pour plates by noting colonial morphology and on blood agar plates by the presence or absence of hemolysis after overnight incubation (21). Experiments in which revertants represented less than 1 in 1,000 CFU were included in the results. [^{14}C]gentamicin at different concentrations was added to cells in log-phase growth, and 5-ml portions were periodically sampled in duplicate by using bacterial membrane filters (0.45 μm ; Millipore Corp., Bedford, Mass.) pretreated with 1 ml of unlabeled gentamicin (250 $\mu\text{g/ml}$). Filtration time was less than 10 s; after filtration, filters were washed with 3% (wt/vol) NaCl, placed into counting vials, and dried overnight at 37°C . Vials were counted for 5 min in a Packard Tri-Carb liquid scintillation counter with 10 ml of a solution containing 5.0 g of 2,5-diphenyloxazole per liter, 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)benzene per liter, and toluene. Gentamicin uptake is expressed as total accumulation (nanograms of gentamicin) per 10^8 CFU. Absorbance and cell viability (in CFU per milliliter) were measured during uptake. DCCD and CCCP were used as fresh solutions prepared in 100% ethanol and were added to cultures simultaneously with [^{14}C]gentamicin. Anaerobic cultures were incubated overnight in a 100% nitrogen environment, and uptake experiments were done in a hood with nitrogen running at 5 liters/min. All samples were collected through a sterile catheter without

breaking the anaerobic system. Statistical analyses of gentamicin uptake in the presence and absence of DCCD were performed by using Student's *t* test; for the analysis of the association between total gentamicin accumulation and drug concentration we used the Pearson product moment correlation.

Identification of growth-stimulating factors and effect on gentamicin uptake. Mutant strains were grown and harvested from the surfaces of blood agar plates, suspended in saline, and seeded immediately onto the surfaces of Mueller-Hinton agar and blood agar plates at densities which gave confluent growth. A sterile filter paper strip saturated with gentamicin (200 $\mu\text{g}/\text{ml}$) was placed onto the agar of each plate. A second strip, which was saturated with one of several potential growth factors for small-colony mutants, was placed at right angles to the gentamicin strip, and plates were incubated overnight at 37°C (1). The growth factors tested included menadione (100 $\mu\text{g}/\text{ml}$), fructose (100 $\mu\text{g}/\text{ml}$), pyruvate (100 $\mu\text{g}/\text{ml}$), and thiamine (100 and 400 $\mu\text{g}/\text{ml}$). DCCD at a concentration of 4 mM was tested in a similar fashion.

Effect of growth factors on gentamicin uptake. Strains were preincubated for 1 to 2 h at 37°C in NBYE with and without menadione (10 $\mu\text{g}/\text{ml}$), and gentamicin uptake was assayed as described above.

The effect of menadione on gentamicin uptake in *S. aureus* 86 Mar was studied by the addition of KCN (final concentration, 0.2 mM) to culture flasks after preincubation with menadione and immediately before the addition of gentamicin.

Chemicals. The KCN used was reagent grade (J. T. Baker Chemical Co., Phillipsburg, N.J.). Menadione (sodium bisulfate salt), phthiocol, 1,4-naphthoquinone, DCCD, and CCCP were all obtained from Sigma Chemical Co. Phyloquinone was from Nutritional Biochemicals Corp., Cleveland, Ohio, and menaquinone (MK 7) was a gift from Meir Lev.

RESULTS

Gentamicin accumulation and killing studies in wild-type and mutant strains. Figure 1 shows gentamicin accumulation in two wild-type *S. aureus* strains and mutants derived from these strains. Gentamicin uptake in *S. aureus* 15956 and *S. aureus* 121 was rapid during the first 20 min. Thereafter, accumulation occurred at a decreasing rate, which was associated with a decrease in the rate of growth, as measured by linear absorbance. There was no significant gentamicin uptake in either *S. aureus* 15956 Mar or *S. aureus* 121 Mar. *S. aureus* 15956 and *S. aureus* 121 showed decreases of more than 2 logs (in CFU per milliliter), compared with no killing in the mutant cultures. Growth as measured by absorbance was similar for these strains during this period. The intracellular gentamicin concentration calculated for *S. aureus* 15956 by using a value of 1.5 ml of cell water per g (dry weight) (23) at 20 min was 1,010 $\mu\text{g}/\text{ml}$ (100-fold that in broth).

Additional experiments were performed to de-

FIG. 1. Accumulation kinetics for 10 μg of gentamicin per ml in *S. aureus* 15956 (●) and *S. aureus* 15956 Mar (○) (A) and corresponding bacterial killing and growth (B). (C and D) Similar data for *S. aureus* 121 (●) and *S. aureus* 121 Mar (○) with 1 μg of gentamicin per ml.

fine whether *S. aureus*, like gram-negative bacilli, demonstrates biphasic aminoglycoside uptake after initial aminoglycoside binding to cells (18). Gentamicin uptake and killing in *S. aureus* 15956 and *S. aureus* 121 were biphasic (Fig. 2). The duration of the first phase was shorter and the rate of uptake was more rapid with a higher gentamicin concentration (1.0 $\mu\text{g}/\text{ml}$) (Fig. 2A). Figure 2B shows that killing primarily occurred during the second, more rapid phase of gentamicin accumulation.

Dose-response accumulation studies in *S. aureus* 121 using four concentrations of gentamicin

FIG. 2. (A) Accumulation kinetics for $0.5 \mu\text{g}$ of gentamicin per ml ($\blacktriangle$) and $1.0 \mu\text{g}$ of gentamicin per ml ($\blacksquare$) in *S. aureus* 15956. (B) Data for a second strain, *S. aureus* 121, showing the association between drug accumulation ($\bullet$) and killing ($\circ$) at a gentamicin concentration of $0.5 \mu\text{g}/\text{ml}$. Ordinate values are the same as those in (A).

(1.0, 2.5, 5.0, and $10 \mu\text{g}/\text{ml}$) suggested concentration-dependent aminoglycoside uptake kinetics; growth was similar before the addition of gentamicin and for 30 min thereafter. The areas under the gentamicin uptake curves were determined by using the trapezoid rule (25) and were shown to be directly proportional to the concentration of gentamicin ($r > +0.999$) despite at least a 2-log decrease in CFU per milliliter at all concentrations.

Relationship between respiration and gentamicin accumulation and killing. To determine the requirement for oxidatively generated energy for gentamicin uptake and killing, gentamicin accumulation was measured under both aerobic and anaerobic conditions. For the anaerobic studies, the gentamicin concentration used was $15 \mu\text{g}/\text{ml}$, and uptake was measured for 45 min; anaerobic broth was associated with less accumulation than was NBYE. Gentamicin uptake and killing in *S. aureus* 121 was noted in the aerobic flask but not in the anaerobic flask. Growth measurements as determined by linear absorbance were identical. Similarly, cells treated with 0.1% sodium azide showed marked reductions in both gentamicin uptake ($1 \mu\text{g}/\text{ml}$) and killing.

Effect of uncouplers on gentamicin accumulation and killing. Membrane energization for aminoglycoside transport was investigated further by using the uncouplers CCCP and DCCD. Gentamicin accumulation was completely inhibited by both 10 and $20 \mu\text{M}$ concentrations of the proton-translocating ionophore CCCP, and no diminution in cell viability was noted (Fig. 3). Gentamicin uptake and killing were observed in the control flask which contained no CCCP. The function of membrane adenosine triphosphatase (ATPase) in the generation of energized membranes for gentamicin

transport was investigated by using DCCD, which inhibits the ATPase complex in bacterial cell membranes (27). Gentamicin uptake and killing at a DCCD concentration of $10 \mu\text{M}$ were identical to the uptake and killing in the control, whereas a higher DCCD concentration produced a stimulation of gentamicin uptake in each of three wild-type strains tested (Fig. 4). This enhancement of aminoglycoside uptake was significant ($P < 0.01$) at 10 min. There was an associated increased rate of killing in the presence of DCCD; however, this difference was not significant. DCCD at these concentrations was not inhibitory to growth in the absence of gentamicin.

Characterization of defects and their relationship to resistance in mutant strains. An initial characterization of mutant strains was made by noting the effects of potential growth factors placed on the surfaces of agar plates or in NBYE with the mutant strains. *S. aureus* 15956 Mar and *S. aureus* 121 Mar were not stimulated with hemin, fructose, pyruvate, thiamine, or menadione. On the other hand, *S. aureus* 86 Mar was stimulated by menadione, and the zone of inhibition around the gentamicin strip was widened, suggesting a decrease in resistance to gentamicin. Growth stimulation was detected by noting colony size after overnight incubation. However, this strain was inhibited by concentrations of menadione of more than $12.5 \mu\text{g}/\text{ml}$. A subculture of menadione-stimulated *S. aureus* 86 Mar on sheep blood agar

FIG. 3. Accumulation kinetics for gentamicin ($5 \mu\text{g}/\text{ml}$) in *S. aureus* 121 ($\bullet$) and *S. aureus* 121 with CCCP at concentrations of $10 \mu\text{M}$ ($\blacktriangle$) and $20 \mu\text{M}$ ($\blacksquare$). Corresponding bacterial killing is shown.

without menadione produced reversion to small nonhemolytic colonies. Gentamicin uptake in *S. aureus* 86 Mar was markedly increased in the presence of menadione, and uptake was associated with killing (Fig. 5A). *S. aureus* 86 and *S. aureus* 15956 Mar were not affected by menadione. *S. aureus* 15956 Mar incubated with DCCD, however, showed uptake kinetics similar

FIG. 4. Accumulation kinetics for gentamicin (5 $\mu\text{g}/\text{ml}$) in three wild-type strains of *S. aureus* (86, 15956, and 121) in the presence (■) and absence (●) of 20 μM DCCD. The data represent average accumulations for each of three strains. Corresponding bacterial killing is shown.

FIG. 5. (A) Gentamicin (6.5 $\mu\text{g}/\text{ml}$) accumulation kinetics in *S. aureus* 86 Mar in the presence (■) and absence (●) of menadione (10 $\mu\text{g}/\text{ml}$) and corresponding bacterial killing. (B) Gentamicin (5.0 $\mu\text{g}/\text{ml}$) accumulation kinetics in *S. aureus* 15956 Mar in the presence (■) and absence (●) of 20 μM DCCD and corresponding killing.

to those of *S. aureus* 86 Mar incubated with menadione (Fig. 5B). In the presence of DCCD, *S. aureus* 15956 Mar demonstrated both gentamicin uptake and killing.

To attempt to characterize the role of menadione (2-methyl-1,4-naphthoquinone) in *S. aureus* 86 Mar as it relates to oxidatively generated energy for aminoglycoside transport, we examined growth stimulation with related naphthoquinones. Neither menaquinone (vitamin K₂), phyloquinone (vitamin K₁), 1,4-naphthoquinone, nor phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone) showed any stimulation of growth. To define further the possible role of menadione in respiration, gentamicin accumulation was examined in *S. aureus* 86 Mar by using subinhibitory concentrations (for *S. aureus* 86) of KCN and DCCD. KCN inhibited gentamicin uptake and killing in *S. aureus* 86 Mar cells which had been preincubated with menadione (Fig. 6). DCCD alone had a killing effect on *S. aureus* 86 Mar which was comparable to that with gentamicin in the presence of menadione (Fig. 7B). However, growth of *S. aureus* 86 Mar with menadione (data not shown) was not inhibited by DCCD. Moreover, gentamicin uptake was normal in *S. aureus* 86 Mar incubated with both menadione and DCCD (Fig. 7A).

DISCUSSION

Previous studies with small-colony mutants of *S. aureus* resistant to multiple aminoglycoside-aminocyclitol antibiotics suggested that this

FIG. 6. Gentamicin (5 $\mu\text{g}/\text{ml}$) accumulation kinetics in *S. aureus* 86 Mar (●) and *S. aureus* 86 Mar preincubated with menadione (10 $\mu\text{g}/\text{ml}$) with (▲) and without (■) KCN (0.2 mM) and corresponding killing.

FIG. 7. (A) Accumulation kinetics for gentamicin (5 $\mu\text{g}/\text{ml}$) in *S. aureus* strain 86 Mar with gentamicin alone (▲) and with gentamicin in the presence of DCCD (20 μM) (★), menadione (10 $\mu\text{g}/\text{ml}$) (●), and both menadione and DCCD (■). (B) Corresponding bacterial killing. Also shown is killing on exposure to DCCD alone (no gentamicin or menadione) (◆).

general resistance was due to a failure of drug uptake associated with defects in oxidative membrane energization processes (21; 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). Recent aminoglycoside accumulation kinetic studies by Bryan and Van den Elzen in gram-negative bacilli have demonstrated two energy-dependent phases of uptake, the second of which is associated with the lethal action of these antibiotics (4-6). A model for aminoglycoside uptake based upon these observations has been proposed recently, in which uptake of aminoglycosides in susceptible strains involves binding to a membrane aerobic energization complex (6). Our studies with *S. aureus* support and expand upon the previous work by these investigators and demonstrate the mechanism of aminoglycoside resistance in small-colony mutants of *S. aureus*.

These studies have also demonstrated that gentamicin uptake in wild-type *S. aureus* occurs in three phases. The initial phase is virtually complete at the time that the first sample is taken and probably represents ionic adherence to the cell surface (18). The duration and rate of the second phase are concentration dependent; the third and most rapid phase of accumulation is associated with the lethal effect of gentamicin, as determined by the inability of cells to form

colonies in agar. Rapid gentamicin uptake with continued cell growth as measured by absorbance occurs during a period when most cells are acquiring a lethal amount of antibiotic. Uptake studies at multiple gentamicin concentrations have demonstrated no antibiotic inhibitory effect on those cell functions associated with aminoglycoside uptake during the early initial phases of accumulation. Anaerobic growth and sodium azide inhibit gentamicin uptake and killing, indicating that energy-dependent active drug transport requires respiration. The requirement of aerobic growth for [^{14}C]streptomycin uptake and killing has been reported previously in *S. aureus* (15).

Membrane energization in facultative bacteria may utilize energy from respiration or adenosine 5'-triphosphate hydrolysis (12, 31). The latter requires a functional membrane ATPase complex (2, 13, 16, 31, 34); both generate a proton motive force used in energy transduction for the active transport of substrates according to the chemiosmotic hypothesis of Mitchell (12, 22, 31). The disparate effects of the uncouplers DCCD and CCCP on aminoglycoside accumulation are best explained in terms of this well-accepted model of energy transduction. The proton-translocating ionophore CCCP dissipates the membrane proton gradient and thereby uncouples respiration from adenosine 5'-triphosphate synthesis (3, 13, 17). Inhibition of aminoglycoside uptake by CCCP in wild-type *S. aureus* strains suggests that a proton motive force is required for aminoglycoside uptake. A similar inhibition of aminoglycoside uptake occurs in CCCP-treated *Escherichia coli* cells (9), and a naturally proton-permeable *unc* mutant of *E. coli* demonstrates increased resistance associated with reduced aminoglycoside uptake (6). DCCD, an uncoupling agent which inactivates the ATPase complex used in the anaerobic generation of a proton motive force, did not inhibit gentamicin uptake. In fact, enhancement of uptake in wild-type *S. aureus* was noted when a 20 μM concentration of DCCD was used. Similarly, enhanced aminoglycoside uptake has been observed in a proton-impermeable *unc* mutant of *E. coli* (6); *unc* mutants and DCCD-treated wild-type strains are similar in that both have inactive membrane ATPase but normal redox activity.

Enhanced gentamicin uptake in the presence of DCCD (as well as in *unc*, proton-impermeable mutants) is consistent with the specific requirement for respiration for the generation of a proton motive force used for aminoglycoside uptake. According to the chemiosmotic view of energy transduction, the energy derived from proton extrusion which is related to respiration is normally coupled to and thus partially dissi-

pated by adenosine 5'-triphosphate synthesis via the membrane ATPase complex. Therefore, strains with an inactive membrane ATPase complex would be expected to have enhanced aerobic membrane energization for aminoglycoside uptake. The inability of facultative bacteria to utilize an anaerobically generated proton motive force suggests that either anaerobic growth is associated with insufficient membrane energization for aminoglycoside uptake or a carrier system coupled to respiration is required for the transport of hydrophilic aminoglycosides across bacterial cell membranes (6).

Resistance in small-colony mutants of *S. aureus* appears to be due to a failure to accumulate gentamicin intracellularly. This resistance is not due to a diminished rate of growth. Several classes of Mar mutants have been identified. One strain, *S. aureus* 86 Mar, showed both growth stimulation and gentamicin accumulation in the presence of menadione. The lethal effect of DCCD on *S. aureus* 86 Mar but not on *S. aureus* 86 or *S. aureus* 15956 Mar suggested that the former was a respiratory auxotroph which required a functional ATPase to provide energization for normal growth. The DCCD-induced lethal effect on *S. aureus* 86 Mar was prevented by supplementation with menadione. With menadione supplementation normal gentamicin accumulation occurred with or without exposure to DCCD. These observations, in conjunction with the observation that KCN inhibited gentamicin uptake in *S. aureus* 86 Mar incubated with menadione, suggest that menadione or a related compound functions in the respiration associated with membrane energization for gentamicin uptake. Menaquinone, the primary naturally occurring naphthoquinone in *S. aureus*, and benzoquinones, such as ubiquinone, function in electron transport in bacteria (7, 8, 11, 29, 32). Menadione in staphylococci acts as a precursor to menaquinone or related naphthoquinones (14, 30) and is found in *S. aureus* cell membranes (17).

Another small-colony mutant, *S. aureus* 15956 Mar, failed to show growth stimulation or alteration of gentamicin susceptibility when incubated with hemin, fructose, pyruvate, thiamine, or menadione. Growth of this mutant in the presence of DCCD was associated with normal gentamicin uptake and killing, indicating that this strain has a different defect and that it may be relatively proton permeable and thus unable to energize membranes for aminoglycoside transport (2, 12, 26-28). A third class of *S. aureus* small-colony mutants has been reported, which is selected with aminoglycosides and is stimulated by heme (19). Slow-growing small-colony mutants of *Bacillus subtilis* which are cyto-

chrome and respiration deficient demonstrate both increased resistance to kanamycin and decreased drug uptake (33); a similar mechanism of resistance seems likely in heme mutants of *S. aureus*.

ACKNOWLEDGMENTS

We are grateful to Meir Lev for helpful advice and review of this manuscript and to Wanda Gaynor for secretarial assistance.

During the course of this study L.J.M. was supported by Public Health Service Training Grant T01-A1-00405 from National Institute of Allergy and Infectious Diseases, and C.F.B. was supported by a stipend from the Schering Corp.

LITERATURE CITED

1. Acar, J. F., F. W. Goldstein, and P. Lagrange. 1978. Human infections caused by thiamine- or menadione-requiring *Staphylococcus aureus*. *J. Clin. Microbiol.* 8: 142-147.
2. Altendorf, K., F. M. Harold, and R. D. Simoni. 1974. Impairment and restoration of the energized state in membrane vesicles of a mutant of *Escherichia coli* lacking adenosine triphosphatase. *J. Biol. Chem.* 249: 4587-4593.
3. Asghar, S. S., E. Levin, and F. M. Harold. 1973. Accumulation of neutral amino acids by *Streptococcus faecalis*. Energy coupling by a proton-motive force. *J. Biol. Chem.* 248:5225-5233.
4. Bryan, L. E., and H. M. Van den Elzen. 1975. Gentamicin accumulation by sensitive strains of *Escherichia coli* and *Pseudomonas aeruginosa*. *J. Antibiot.* 28:696-703.
5. 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.
6. Bryan, L. E., and H. M. Van den Elzen. 1977. Effects of membrane-energy mutations and cations on streptomycin and gentamicin accumulation by bacteria: a model for entry of streptomycin and gentamicin in susceptible and resistant bacteria. *Antimicrob. Agents Chemother.* 12:163-177.
7. Burke, K. A., and J. Lascelles. 1975. Nitrate reductase system in *Staphylococcus aureus* wild type and mutants. *J. Bacteriol.* 123:308-316.
8. Cox, G. B., and F. Gibson. 1974. Studies on electron transport and energy linked reactions using mutants of *Escherichia coli*. *Biochim. Biophys. Acta* 346:1-25.
9. 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.
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. Goldenbaum, P. E., and D. C. White. 1974. Role of lipid in the formation and function of the respiratory system of *Staphylococcus aureus*. *Ann. N.Y. Acad. Sci.* 236: 115-123.
12. Haddock, B. A., and C. W. Jones. 1977. Bacterial respiration. *Bacteriol. Rev.* 41:47-99.
13. Hamilton, W. A. 1975. Energy coupling in microbial transport. *Adv. Microb. Physiol.* 12:1-53.
14. Hammon, R. K., and D. C. White. 1969. Separation of vitamin K₂ isoprenologues by reversed phase thin layer chromatography. *J. Chromatogr.* 45:446-452.
15. Hancock, R. 1962. Uptake of ¹⁴C-streptomycin by some

- micro-organisms and its relation to their streptomycin sensitivity. *J. Gen. Microbiol.* **28**:493-501.
16. Harold, F. M. 1977. Membrane and energy transduction in bacteria. *Curr. Top. Bioenerg.* **6**:83-149.
 17. Harold, F. M., and J. R. Baarda. 1968. Inhibition of membrane transport in *Streptococcus faecalis* by uncouplers of oxidative phosphorylation and its relationship to proton conduction. *J. Bacteriol.* **96**:2025-2034.
 18. Hurwitz, C., and C. L. Rosano. 1962. Accumulation of label from C¹⁴-streptomycin by *Escherichia coli*. *J. Bacteriol.* **83**:1193-1201.
 19. Kaplan, M. L., and W. Dye. 1976. Growth requirements of some small-colony-forming variants of *Staphylococcus aureus*. *J. Clin. Microbiol.* **4**:343-348.
 20. Lacey, R. W. 1969. Dwarf-colony variants of *Staphylococcus aureus* resistant to aminoglycoside antibiotics and to a fatty acid. *J. Med. Microbiol.* **2**:187-197.
 21. 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.
 22. Mitchell, P. 1979. Keilin's respiratory chain concept and its chemiosmotic consequences. *Science* **206**:1148-1159.
 23. Mitchell, P., and J. Moyle. 1956. Osmotic function and structure in bacteria. *Symp. Soc. Gen. Microbiol.* **6**:150-180.
 24. Musher, D. M., R. E. Baughn, G. B. Templeton, and J. N. Minuth. 1977. Emergence of various forms of *Staphylococcus aureus* after exposure to gentamicin and infectivity of the variants in experimental animals. *J. Infect. Dis.* **136**:360-369.
 25. Notari, R. E., J. L. DeYoung, and R. C. Anderson. 1975. Biopharmaceutics and pharmacokinetics. Marcel Dekker, New York.
 26. Rosen, B. P. 1973. β -Galactoside transport and proton movements in an adenosine triphosphatase-deficient mutant of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **53**:1289-1296.
 27. Rosen, B. P. 1973. Restoration of active transport in a Mg²⁺ adenosine triphosphatase-deficient mutant of *Escherichia coli*. *J. Bacteriol.* **116**:1124-1129.
 28. Rosen, B. P., and L. W. Adler. 1975. The maintenance of the energized membrane state and its relation to active transport in *Escherichia coli*. *Biochem. Biophys. Acta* **387**:23-36.
 29. Sasarman, A., P. Purvis, and V. Portelance. 1974. Role of menaquinone in nitrate respiration in *Staphylococcus aureus*. *J. Bacteriol.* **117**:911-913.
 30. Sasarman, A., M. Surdeanu, V. Portelance, R. Dobardzk, and S. Sonea. 1971. Classification of vitamin K-deficient mutants of *Staphylococcus aureus*. *J. Gen. Microbiol.* **65**:125-130.
 31. Simoni, R. D., and P. W. Postma. 1975. The energetics of bacterial active transport. *Annu. Rev. Biochem.* **44**:523-554.
 32. Taber, H. 1979. Functions of vitamin K₂ in micro-organisms, p. 177-187. In J. W. Suttie (ed.), *Vitamin K metabolism and vitamin K dependent proteins*. University Park Press, Baltimore.
 33. Taber, H., and G. M. Halfenger. 1976. Multiple-aminoglycoside-resistant mutants of *Bacillus subtilis* deficient in accumulation of kanamycin. *Antimicrob. Agents Chemother.* **9**:251-259.
 34. Van Thienen, G. T., and P. W. Postma. 1973. Coupling between energy conservation and active transport of serine in *Escherichia coli*. *Biochim. Biophys. Acta* **323**:429-440.