

## Membrane Potential in Anaerobically Growing *Staphylococcus aureus* and Its Relationship to Gentamicin Uptake

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The electrical potential ( $\Delta\psi$ ) across the cytoplasmic membranes of *Staphylococcus aureus* cells growing under aerobic and anaerobic conditions was determined by measuring the equilibrium distribution of [<sup>3</sup>H]tetraphenyl phosphonium. In conjunction, gentamicin uptake and killing were studied in the same cells under identical conditions. Under aerobic conditions,  $\Delta\psi$  was -169 mV, gentamicin uptake was readily demonstrable, and the number of viable cells decreased by almost four orders of magnitude in the presence of antibiotic. In contrast,  $\Delta\psi$  was -142 mV anaerobically, gentamicin uptake was essentially nonexistent, and the aminoglycoside had no effect on viability. Remarkably, when the ionophore nigericin was added under anaerobic conditions,  $\Delta\psi$  increased to the level observed aerobically, gentamicin uptake tripled to about 18% of the aerobic level, and viability decreased by one order of magnitude. The results are consistent with other observations (Mates et al., Proc. Natl. Acad. Sci. U.S.A. 79:6693-6697, 1982), indicating that the relationship between  $\Delta\psi$  and gentamicin uptake is gated, and suggest that diminution of  $\Delta\psi$  may be an important factor in aminoglycoside resistance under anaerobic conditions.

Aminoglycoside antibiotics are not accumulated significantly by either obligate (1) or facultative (2, 8) anaerobes grown under anaerobic conditions. It has recently been demonstrated that effective aminoglycoside uptake is related to the magnitude of the electrical potential ( $\Delta\psi$ ) in *Staphylococcus aureus* (13), *Escherichia coli* (2, 6), and *Bacillus subtilis* (2). This proposal suggests that chemiosmotic forces (15, 16) play an important role in the accumulation of aminoglycosides. Proton extrusion during respiration or ATP hydrolysis creates an electrochemical gradient of protons ( $\Delta\mu_{H^+}$ ) that is the immediate driving force for a wide variety of biological processes (20). This thermodynamic entity is composed of electrical and chemical parameters according to the following relationship:  $\Delta\mu_{H^+} = \Delta\psi - Z \Delta pH$ , where  $\Delta\psi$  represents the electrical potential across the membrane and  $\Delta pH$  is the chemical difference in proton concentrations across the membrane ( $Z$  is equal to 61.7 mV at 38°C).

It has previously been demonstrated that manipulations which change the magnitude of  $\Delta\psi$  in *S. aureus* (ionophores, external pH changes) affect gentamicin uptake and killing (13). The association between  $\Delta\psi$  and gentamicin effect

suggested that deficient uptake under anaerobic conditions in these facultative organisms may be due to a low magnitude of  $\Delta\psi$  when the electrochemical proton gradient is generated via ATP hydrolysis (14).

It has also been suggested, however, that electron transport per se is necessary for aminoglycoside uptake (5) and that aminoglycoside resistance under anaerobic conditions is due to a lack of electron transport (1). Thus, it is important to investigate the relationship between the magnitude of  $\Delta\psi$  and anaerobic gentamicin uptake and killing in the absence of an added terminal electron acceptor. The results presented here indicate that a low magnitude of  $\Delta\psi$  is an important factor in anaerobic resistance to gentamicin.

### MATERIALS AND METHODS

**Organisms.** *S. aureus* 86W is a clinical isolate (Montefiore Hospital and Medical Center) which was identified by the determination of coagulase production and mannitol fermentation (14). The minimal inhibitory concentration of gentamicin for this organism is 0.8 µg/ml.

**Media.** The broth used for both aerobic and anaerobic studies was nutrient broth (BBL Microbiology Systems) containing 0.1% (wt/vol) yeast extract (Difco Laboratories). Control studies were done with and without 0.5% (wt/vol) glucose supplementation, and

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no difference in gentamicin uptake was found under either aerobic or anaerobic conditions. All experiments were carried out at the nonadjusted pH (6.5) of the anaerobic experiment after incubation with hydrogen and carbon dioxide generator envelopes. Hydrochloric acid was added to the aerobic experiments to reach pH 6.5. Heart infusion agar (Difco) was used.

**Bacterial growth and protein determination.** Experiments were carried out at 38°C either aerobically in a rotary shaker bath or anaerobically in a GasPak (BBL) jar modified in such a way that the contents of a flask inside the jar could be sampled through an airtight, rubber-stoppered glass conduit. The anaerobic atmosphere was created with hydrogen and carbon dioxide generator envelopes (GasPak). Bacterial cultures were grown and split, and one sample was put into the anaerobic jar and then grown further until it reached the logarithmic phase of growth. Anaerobic and aerobic experiments were carried out as nearly simultaneously as possible.

Absorbance readings were measured with a Coleman spectrophotometer (model 6-20/A) at 600 nm. Cells were determined to be in the logarithmic phase of growth by linear absorbance with time before the addition of radiolabeled solute.

A linear relationship ( $y = 0.00625x - 0.017$ ; where  $x$  is equal to protein [in micrograms per milliliter] and  $y$  is equal to absorbance at 600 nm in nutrient broth containing 0.1% [wt/vol] yeast extract) was found between absorbance readings (from 0.05 to 0.20 nm) and bacterial protein (in milligrams per milliliter), as determined by the method of Lowry et al. (12), with crystalline bovine serum albumin as the standard. During experiments, absorbance readings were taken at regular intervals, and bacterial protein was subsequently calculated. Bacterial densities (in CFU per milliliter) were determined by standard pour-plate techniques in heart infusion agar. Control experiments with and without pH adjustment of the agar to pH 5.5 (to inhibit gentamicin carry-over) demonstrated no difference in observed bacterial density; subsequently, the heart infusion agar was not pH adjusted.

**Gentamicin uptake.** [ $^3\text{H}$ ]gentamicin (5  $\mu\text{g}/\text{ml}$  [10  $\mu\text{Ci}/\text{mg}$ ]) was added to cells in the logarithmic phase of growth at a density of 15 to 40  $\mu\text{g}/\text{ml}$  of protein, and 5-ml portions were sampled with glass microfiber filters (GF/C; Whatman, Inc.). Filters were washed with 5 ml of 0.9% NaCl. Control experiments demonstrated no difference between 0.9 and 3% NaCl washes. Filters were then dried and counted by liquid scintillation counting in a toluene-based scintillation fluid.

**Determination of  $\Delta\psi$ .** Determination of  $\Delta\psi$  (interior negative) was made by measuring the equilibrium distribution of [ $^3\text{H}$ ]tetraphenyl phosphonium (TPP $^+$ ) (26  $\mu\text{M}$  [5.8 mCi/mM]) by filtration under conditions identical to those described for gentamicin uptake. The use of permeable ions such as TPP $^+$  to measure  $\Delta\psi$  has been well described in several bacterial species, including *S. aureus* (7, 11, 13).  $\Delta\psi$  was calculated from the Nernst equation ( $\Delta\psi = 61.7 \log_{10} \frac{[\text{TPP}^+]_{\text{out}}}{[\text{TPP}^+]_{\text{in}}}$ ). TPP $^+$  concentration gradients were calculated with a value of 4.2  $\mu\text{l}$  of intracellular fluid per mg of cell protein, which was determined with [ $^{14}\text{C}$ ]inulin and  $^3\text{H}_2\text{O}$  as described previously (18).

**Chemicals.** [ $^3\text{H}$ ]gentamicin (637 mCi/mmol) was purchased from Amersham Corp. [ $^3\text{H}$ ]tetraphenyl phosphonium was prepared by the Isotope Synthesis

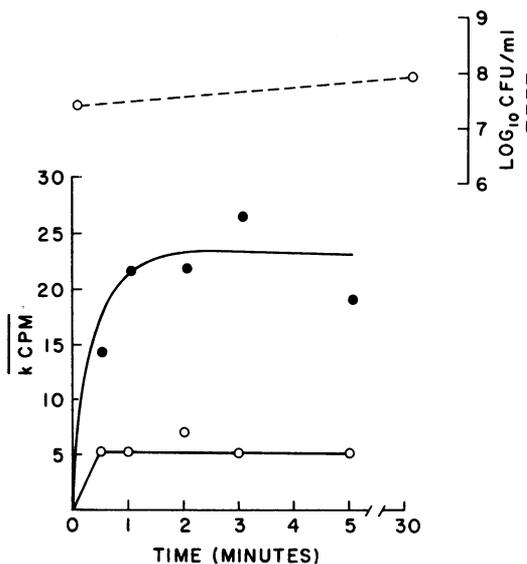


FIG. 1. [ $^3\text{H}$ ]TPP $^+$  accumulation in *S. aureus* 86 at pH 6.5 as determined by filtration (5-ml samples). [ $^3\text{H}$ ]TPP $^+$  (26  $\mu\text{M}$  [5.8 mCi/mM]) accumulation is shown under aerobic (●) and anaerobic (○) conditions. Aerobic  $\Delta\psi$  was  $-167$  mV and anaerobic  $\Delta\psi$  was  $-133$  mV when calculated as described in the text. During the experiment, cells were in the logarithmic phase of growth under both aerobic and anaerobic conditions.

Group at Hoffmann-La Roche Inc. under the direction of Arnold Liebman. Gentamicin sulfate was purchased from Shering Corp. (potency, 588  $\mu\text{g}/\text{mg}$ ). Nigericin was the generous gift of J. Wesley (Hoffmann-La Roche Inc.).

## RESULTS

**Effect of anaerobiosis on the magnitude of  $\Delta\psi$  with and without nigericin.** To investigate the relationship between anaerobic growth and the magnitude of  $\Delta\psi$ , we measured  $\Delta\psi$  under anaerobic and aerobic conditions. In addition, the effect of nigericin on  $\Delta\psi$  was measured. Nigericin is an ionophore which causes an electroneutral exchange of  $\text{K}^+$  (and  $\text{Na}^+$ ) for  $\text{H}^+$ , thus collapsing  $\Delta\text{pH}$  with an increase in  $\Delta\psi$  and relatively little effect on the magnitude of  $\Delta\bar{\mu}_{\text{H}^+}$  (17).

The data in Fig. 1 represent a typical accumulation of TPP $^+$  (26  $\mu\text{M}$ ) in the logarithmic phase of growth of *S. aureus* 86 under aerobic and anaerobic conditions. When  $\Delta\psi$  was calculated as described in Materials and Methods, average aerobic  $\Delta\psi$  was  $-169$  mV ( $n = 3$ ; standard deviation, 14), and average anaerobic  $\Delta\psi$  was  $-142$  mV ( $n = 3$ ; standard deviation, 12). The average difference between  $\Delta\psi$  measured aerobically and anaerobically was 27 mV.

The data in Fig. 2 represent both anaerobic

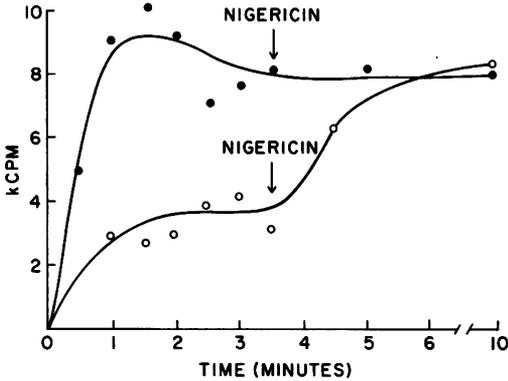


FIG. 2.  $[^3\text{H}]\text{TPP}^+$  accumulation in *S. aureus* 86 at pH 6.5 as determined by filtration (5-ml samples).  $[^3\text{H}]\text{TPP}^+$  (26  $\mu\text{M}$  [5.8 mCi/mM]) accumulation is shown under aerobic ( $\bullet$ ) and anaerobic ( $\circ$ ) conditions. Nigericin (0.05  $\mu\text{M}$ ) was added as indicated by arrows. Aerobic  $\Delta\psi$  was  $-155$  mV both before and after the addition of nigericin. Anaerobic  $\Delta\psi$  was  $-136$  mV before and  $-151$  mV after the addition of nigericin.

and aerobic effects of added nigericin on the magnitude of  $\Delta\psi$  at pH 6.5. When nigericin was added, aerobic  $\Delta\psi$  was not measurably changed, but anaerobic  $\Delta\psi$  was increased to the aerobic level. Nigericin at this concentration had no effect on cell growth (see Fig. 4).

**Effect of anaerobiosis on the magnitude of gentamicin uptake and killing with and without nigericin.** Because the magnitude of  $\Delta\psi$  under anaerobic conditions was demonstrated to be lower than that under aerobic conditions, aerobic and anaerobic gentamicin uptake with and without nigericin was investigated.

Figure 3 shows gentamicin (5  $\mu\text{g}/\text{ml}$ ) uptake aerobically and anaerobically. Under anaerobic conditions, there was minimal uptake and no killing. Under aerobic conditions, there was significant uptake and an average of 3.6 ( $n = 3$ ; standard deviation, 1.6)  $\log_{10}$  unit killing.

When nigericin was added, however, gentamicin uptake under anaerobic conditions could be stimulated (Fig. 4) and was accompanied by killing of the organisms. This uptake averaged 3.5  $\mu\text{g}/\text{mg}$  of protein ( $n = 6$ ; standard deviation, 0.7) and was accompanied by an average 1.1  $\log_{10}$  unit killing ( $n = 8$ ; standard deviation, 1.1). The maximal anaerobic uptake after addition of nigericin averaged 18% of the maximal aerobic uptake at the same pH (6.5).

## DISCUSSION

It has been known for over 30 years that anaerobiosis decreases the action of the aminoglycoside antibiotics (9). This decreased killing

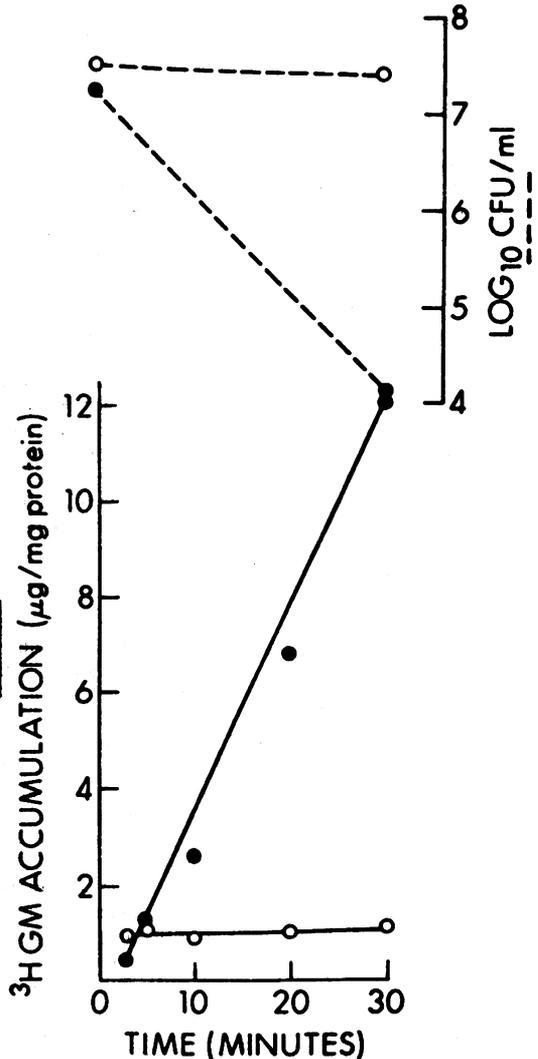


FIG. 3.  $[^3\text{H}]\text{gentamicin}$  uptake in *S. aureus* 86 at pH 6.5 as determined by filtration.  $[^3\text{H}]\text{gentamicin}$  (5  $\mu\text{g}/\text{ml}$  [10  $\mu\text{Ci}/\text{mg}$ ]) uptake is shown under aerobic ( $\bullet$ ) and anaerobic ( $\circ$ ) conditions. Anaerobically there was no killing of the organisms; aerobically there was 3  $\log_{10}$  CFU killing per ml.

is associated with decreased uptake of the antibiotics under anaerobic conditions (1, 5, 8).

Bryan et al. have suggested that the effect of anaerobiosis on aminoglycoside activity is due to inhibition of electron transport which results in inadequate energization of a membrane complex needed to transport aminoglycosides (1, 3, 4). These authors and others (5) have induced anaerobic electron transport and shown increased uptake and killing, although not to the aerobic level.

The results of our study show that the cytoplasmic membrane potential of growing *S. aureus*

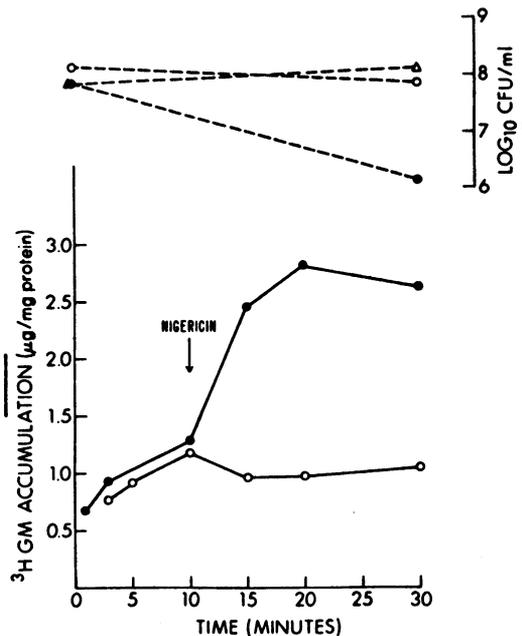


FIG. 4. [ $^3\text{H}$ ]gentamicin uptake in *S. aureus* 86 at pH 6.5 as determined by filtration. [ $^3\text{H}$ ]gentamicin (5  $\mu\text{g}/\text{ml}$  [10  $\mu\text{Ci}/\text{mg}$ ]) uptake is shown under anaerobic conditions with (●) and without (○) the addition of nigericin at 10 minutes. Neither nigericin ( $\Delta$ ) nor gentamicin (○) alone killed the organism, but when nigericin was added to gentamicin (●), there was approximately 2  $\log_{10}$  CFU killing per ml in this experiment.

*us* cells under anaerobic conditions is lower than that under aerobic conditions, an observation in accordance with previous data (11), and that raising the magnitude of this membrane potential with nigericin induces gentamicin uptake and killing. This evidence for the role of  $\Delta\psi$  in gentamicin uptake and lethal effect is further supported by data obtained under aerobic conditions. We have shown that at acidic external pH, at which  $\Delta\psi$  is reduced, gentamicin is not taken up. Either raising external pH (which increases  $\Delta\psi$ ) or increasing  $\Delta\psi$  by the addition of nigericin at acid external pH is associated with gentamicin uptake and killing. Furthermore, addition of valinomycin, an ionophore which collapses  $\Delta\psi$ , at alkaline pH abolishes gentamicin uptake and killing (13). In addition, the ATPase inhibitor *N,N'*-dicyclohexyl carbodiimide, added to sub-inhibitory concentrations of gentamicin, induces uptake (14). *N,N'*-Dicyclohexyl carbodiimide is a carboxyl reagent which binds to the  $F_0$  portion of the  $\text{H}^+$ -ATPase, blocking proton conductance (19). We have shown that the addition of *N,N'*-dicyclohexyl carbodiimide increases  $\Delta\psi$  (M. H. Miller, L. J. Mandel, S. M. Mates, N. J. Simkin, and H. R. Kaback, Program Abstr. In-

tersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no. 50, 1981), probably owing to the blocking of proton conductance. These results are in accordance with recent observations correlating minimal inhibitory concentrations of streptomycin in *E. coli* with the magnitude of  $\Delta\psi$  (6).

Although we have shown a definite relationship between decreased magnitude of  $\Delta\psi$  under anaerobic conditions and decreased gentamicin effect, it is clear that this relationship is complex. The addition of nigericin raises  $\Delta\psi$  to the aerobic level, but gentamicin uptake is raised to only 18% of the aerobic level. This is similar to the effect of nitrate addition to stimulate electron transport, which also induces an anaerobic aminoglycoside effect, although not to the aerobic level (5). Stimulation of electron transport may lead to an increase in  $\Delta\psi$  through more efficient proton extrusion. The inverse causal relationship, i.e., that increases in  $\Delta\psi$  reflect an increase in respiration in the presence of nigericin, is unlikely, since it has been shown that manipulations of the proton electrochemical gradient do not affect oxygen consumption (10).

Preliminary results suggest that intracellular binding may play a role in differences between aerobic and anaerobic aminoglycoside effect. When the protonophore carbonyl cyanide-*m*-chlorophenyl hydrazone, which collapses the electrochemical proton gradient, was added to anaerobically growing *S. aureus* after gentamicin uptake had been stimulated by nigericin, gentamicin was shown to efflux from the cells (S. Mates and M. Miller, unpublished data). Under aerobic conditions, no such effect occurred, suggesting that a relatively large proportion of intracellular gentamicin may remain unbound under anaerobic conditions. Effective aminoglycoside binding possibly requires not only membrane transport and sensitive ribosomes but also critical intracellular conditions (e.g., pH, redox potential, etc.) not present in anaerobic growth.

#### ACKNOWLEDGMENTS

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