

## Streptomycin Accumulation by *Bacillus subtilis* Requires both a Membrane Potential and Cytochrome *aa*<sub>3</sub>

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**Cytochrome *aa*<sub>3</sub> concentrations in the cytoplasmic membrane of *Bacillus subtilis* were altered by growth conditions, and the effects on the membrane potential ( $\Delta\psi$ ) in whole cells were measured. When cytochrome *aa*<sub>3</sub> was absent, the magnitude of  $\Delta\psi$  was not diminished by comparison with the  $\Delta\psi$  measured in cells containing normal cytochrome *aa*<sub>3</sub> concentrations. In addition, the energy-dependent uptake of proline and glutamate was comparable at both cytochrome *aa*<sub>3</sub> concentrations. However, in the cytochrome *aa*<sub>3</sub>-deficient cell preparation, accumulation of the aminoglycoside antibiotic streptomycin was much lower than that of the cytochrome *aa*<sub>3</sub>-sufficient cells. When cells were cultured under conditions that stimulated higher than normal concentrations of cytochrome *aa*<sub>3</sub>,  $\Delta\psi$  was also increased, and enhanced streptomycin accumulation was observed. Phenazine methosulfate-ascorbate was used both in  $\Delta\psi$  measurements and in uptake studies to provide high rates of electron transport and maximal  $\Delta\psi$  values. These results, taken together with those previously published (A. S. McEnroe and H. W. Taber, *Antimicrob. Agents Chemother.* 26:507-512, 1984) suggest that the uptake of streptomycin by *B. subtilis* requires adequate levels both of  $\Delta\psi$  and cytochrome *aa*<sub>3</sub>.**

The involvement of cytochromes in aminoglycoside accumulation by bacteria has been suggested by several studies with cytochrome-deficient mutants. Bryan and Van den Elzen (7) found that a heme-deficient mutant of *Escherichia coli* showed enhanced aminoglycoside resistance and decreased aminoglycoside uptake in the presence of limiting heme precursor ( $\delta$ -aminolevulinic acid). Similar observations were made by Campbell and Kadner (8) and by Damper and Epstein (9). Bryan et al. (6) studied a cytochrome- and nitrate reductase-deficient mutant of *Pseudomonas aeruginosa* which appeared to be defective in aminoglycoside uptake due to reduced cytochrome oxidase activity. Decreased aerobic uptake of aminoglycosides was also correlated with cytochrome and nitrate reductase deficiencies in several additional aminoglycoside-resistant mutants of *P. aeruginosa* (4). Taber and Halfenger (26) isolated *Bacillus subtilis* mutants with cytochrome deficiencies that showed multiple aminoglycoside-resistant phenotypes.

Since aminoglycoside accumulation by bacterial cells requires at least the  $\Delta\psi$  component of the proton electrochemical gradient ( $\Delta\mu_{H^+}$ ) (5, 6, 9, 10, 16), cytochromes presumably are needed to help maintain this gradient. However, a decreased concentration of ubiquinone was shown by Bryan and Kwan (5) to decrease gentamicin uptake in *E. coli* while leaving glutamine and proline transport unaffected. This suggests that specific electron transport components may be required for aminoglycoside entry, in addition to maintenance of  $\Delta\mu_{H^+}$ . McEnroe and Taber (17) have described growth conditions for *B. subtilis* that change the concentration of cytochrome *aa*<sub>3</sub> in a systematic way. Utilizing these growth conditions, a relationship between cytochrome *aa*<sub>3</sub> content and streptomycin accumulation was demonstrated (17), suggesting a requirement of cytochrome *aa*<sub>3</sub> for streptomycin uptake in *B. subtilis*. It was possible that this apparent dependence of streptomycin uptake on cytochrome *aa*<sub>3</sub> actually was due to enhanced functioning of the electron

transport chain under physiological conditions in which cytochrome *aa*<sub>3</sub> was the principal terminal oxidase. The present work was undertaken to investigate this possibility and to further explore the role of cytochrome *aa*<sub>3</sub> in streptomycin accumulation.

### MATERIALS AND METHODS

**Bacterial strains.** Strain RB1 (*trpC2*) is a laboratory stock of *B. subtilis* 168. Strain RB95 (*strC2*) is a spontaneous streptomycin-resistant mutant obtained from J. Hoch (23) and is conditionally cytochrome *aa*<sub>3</sub> deficient (17).

**Media.** The minimal salts medium (MG) was that of Anagnostopoulos and Spizizen (1) to which was added 0.5% glucose. When further supplemented with 50  $\mu$ g of tryptophan per ml, it is designated MGT. Casamino Acids, vitamin-free (CV; Difco Laboratories, Detroit, Mich.) were added in the concentrations specified in each experiment. MG agar was prepared by adding 15 g of Bacto-Agar (Difco) per liter of liquid MG. Tryptose-blood agar base medium (TBAB) was prepared as previously described (11). The liquid equivalent of TBAB (T medium) contained (in grams per liter): tryptose (Difco), 10; beef extract (Difco), 3.0; and NaCl, 5.0. TG medium was prepared by adding sterile glucose (final concentration, 0.5%) to previously autoclaved TBAB or T medium.

**Growth of cells.** For small cultures (10 to 25 ml), cells were grown in 250-ml nephelometer flasks. For batch cultures (300 to 1,000 ml), cells were grown in 2,800-ml Fernbach flasks. All cultures were incubated at 37°C with vigorous shaking (200 rpm) in a New Brunswick G-25 incubator. The increase in bacterial cell mass in liquid culture was then monitored by the change in turbidity on a Klett-Summerson photoelectric colorimeter.

**Preparation of membrane vesicles and whole cells for uptake experiments.** Membrane vesicles were prepared from late-logarithmic-phase cells grown in the appropriate medium, as described by Bisschop and Konings (2). For whole-cell preparations, strains were grown in MGT medium with concentrations of CV to obtain cellular cytochrome *aa*<sub>3</sub> concentrations specified for each experiment. These levels

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were established by spectroscopic measurements at liquid nitrogen temperature as previously described (17, 24), and are represented on a scale of 0 to 5. Level *5aa<sub>3</sub>* represents the maximal concentration of cytochrome *aa<sub>3</sub>* normally formed by early-stationary-phase cultures of strain RB1 growing in MG medium plus 1.0% CV; *0aa<sub>3</sub>* represents a concentration less than 5% of the maximum and occurs when strains are cultured in MGT in the absence of CV. Under special growth conditions (extended incubation time in the stationary phase), the normal maximum concentrations can be exceeded, and cytochrome *aa<sub>3</sub>* formation occurs at a level 15 to 20% higher. This latter level is referred to as *5<sup>+</sup>aa<sub>3</sub>*.

Cells were washed in 50 mM potassium phosphate buffer having a pH as designated in each experiment and were resuspended in the same buffer to a concentration of 8 mg (dry weight) per ml. Samples containing 40  $\mu$ l of cells were immediately frozen in dry ice-ethanol and stored at  $-70^{\circ}\text{C}$ . Frozen cells were thawed just before use and were not refrozen.

**Uptake measurements.** The transport studies were conducted, with minor modifications, according to the procedures of Konings and Freese (15) in 100- $\mu$ l incubation mixtures at  $37^{\circ}\text{C}$ . The mixtures contained 50 mM potassium phosphate with a pH as designated for each experiment, 10 mM  $\text{MgSO}_4$ , 10 mM potassium ascorbate, 100 mM phenazine methosulfate (PMS), and 0.032 mg (dry weight) of cells per ml. Reactions were terminated by the addition of 2 ml of 0.1 M LiCl and rapidly filtered on 0.45- $\mu\text{m}$ -pore-size membrane filters (Millipore Corp., Bedford, Mass.) that had been prewashed with 0.1 M LiCl. The filters were then washed with an additional 2 ml of 0.1 M LiCl, removed immediately, air dried, and counted in 4 ml of PPO (2,5-diphenyloxazole)-POPOP [1,4-bis-(5-phenyloxazolyl)benzene] scintillation cocktail.

For uptake of [ $^{14}\text{C}$ ]glutamic acid (final concentration,  $9.7 \times 10^{-6}$  M; final specific activity, 0.66  $\mu\text{Ci/ml}$ ) and [ $^{14}\text{C}$ ]proline (final concentration,  $4.6 \times 10^{-6}$  M; final specific activity, 0.62  $\mu\text{Ci/ml}$ ), the reaction mix was preincubated for 5 min in the presence of the respective labeled amino acid, and the reaction was started by the addition of PMS-ascorbate. Reactions were terminated at 30-s intervals for a total of 4.5 min.

For uptake of [ $^3\text{H}$ ]dihydrostreptomycin sulfate (final concentration,  $8 \times 10^{-5}$  M; final specific activity, 0.3  $\mu\text{Ci/ml}$ ; Amersham Corp., Arlington Heights, Ill.), the reaction mix was preincubated in the presence of both unlabeled streptomycin sulfate and PMS-ascorbate for the time designated in each experiment. Measurement of uptake was initiated by the addition of the labeled dihydrostreptomycin. Reactions were terminated at 1-min intervals for a total of 9 min or at the time points specified in a particular experiment.

**Electrical potential measurement.** The magnitude of the membrane potential  $\Delta\psi$  was determined from the distribution ratio of lipid-soluble [ $^3\text{H}$ ]tetraphenylphosphonium ion, using the flow dialysis procedure (21). Several modifications in the procedure were made to adapt the system for use with *B. subtilis* whole cells, rather than *E. coli* membrane vesicles. Whole cells were prepared as described above for uptake measurements, except that samples to be used for  $\Delta\psi$  measurements were concentrated to give 0.4-ml samples containing the equivalent of 4 mg of membrane protein per ml. This resulted in a final concentration in the upper chamber (total volume, 0.8 ml) of 2 mg of membrane protein per ml, the same amount of membrane protein utilized for studies with membrane vesicles. Potassium phosphate buffer

(50 mM) having the same pH as the cell suspension was pumped through the lower chamber at a rate of 6 ml/min. Ascorbate, PMS, and whole cells were added in that order, at fraction 12, once equilibrium of [ $^3\text{H}$ ]tetraphenylphosphonium across the membrane had occurred; 2  $\mu$ l of 1 mM valinomycin (Sigma Chemical Co., St. Louis, Mo.) was added at fraction 25, and sampling continued until fraction 40. Nonspecific binding of [ $^3\text{H}$ ]tetraphenylphosphonium was determined by measuring residual cation uptake in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (21). The magnitude of  $\Delta\psi$  was calculated from the Nernst equation, using an intracellular volume of 2  $\mu\text{l/mg}$  of membrane protein as determined for *B. subtilis* cells by Miller and Koshland (18).

## RESULTS

**Measurement of electron transport chain function in relation to cytochrome *aa<sub>3</sub>* content.** Strain RB95 (*strC*) is cytochrome *aa<sub>3</sub>* deficient (23, 25), streptomycin resistant (23), and does not transport this aminoglycoside under common growth conditions (un-supplemented broth medium); this phenotype can be overcome by the addition of CV to the medium (17). As previously described, the cytochrome *aa<sub>3</sub>* content of both the *strC* mutant and a standard strain (RB1) can be altered in a systematic fashion by the addition of CV to a minimal salts growth medium (17). However, creating a cytochrome *aa<sub>3</sub>* deficiency in the standard strain in this way could cause other physiological changes which might affect the experimental results. To try to ensure that this did not occur, we utilized both strains in this study.

To determine whether limitation of electron transfer to oxygen was the cause of decreased streptomycin accumulation under conditions of cytochromes *aa<sub>3</sub>* deficiency, we conducted oxygen uptake studies on both strains RB1 and RB95 under a wide variety of growth conditions and a wide range of cytochrome *aa<sub>3</sub>* levels. No significant difference in oxygen consumption occurred regardless of the concentration or composition (i.e., cytochromes *aa<sub>3</sub>*, *o*, or 617) of the terminal oxidases present (data not presented).

In addition to a potential effect on oxygen consumption, decreases in cytochrome *aa<sub>3</sub>* and increases in cytochrome 617 (e.g., by growth in un-supplemented minimal medium) could result in decreased  $\Delta\psi$  values and a subsequent diminution in streptomycin uptake. Strains RB1 and RB95 were grown under conditions so as to obtain several different concentrations of cytochrome *aa<sub>3</sub>* (17), and the magnitude of  $\Delta\psi$  in the presence of PMS-ascorbate was measured by flow dialysis as described above.

For comparison of  $\Delta\psi$  in the two extreme cases, strains RB1 and RB95 were obtained with *0aa<sub>3</sub>* and with *5aa<sub>3</sub>* (see Materials and Methods). In addition, cells with greater than normal levels of cytochrome *aa<sub>3</sub>* (*5<sup>+</sup>aa<sub>3</sub>*) were also obtained for comparative purposes.

Because  $\Delta\psi$  is known to vary with external pH (21), these measurements were made at several external pH values (pH 5.0, 6.85, 7.5). The result of a series of representative flow dialysis experiments with *5<sup>+</sup>aa<sub>3</sub>* cells is presented in Fig. 1; the corresponding  $\Delta\psi$  values calculated from experiments conducted on cells with all three levels of cytochrome *aa<sub>3</sub>* (*0aa<sub>3</sub>*, *5aa<sub>3</sub>*, *5<sup>+</sup>aa<sub>3</sub>*) are summarized in Table 1. From comparisons of the  $\Delta\psi$  values in this table, several points have become apparent: (i) the magnitude of  $\Delta\psi$  in cells lacking cytochrome *aa<sub>3</sub>* is sufficient to support uptake of aminoglycosides; (ii) there is a slight increase in  $\Delta\psi$  as external pH increases for *0aa<sub>3</sub>* and *5aa<sub>3</sub>* cells, while the *5<sup>+</sup>aa<sub>3</sub>* cells show

a substantial change in  $\Delta\psi$  as the pH increases from 5 to 7.5; (iii) there is an increase in the magnitude of  $\Delta\psi$  when greater than normal concentrations of cytochrome  $aa_3$  are present ( $5^+aa_3$ ) at all three external pH values.

**Uptake of amino acids dependent on PMS-ascorbate.** It appeared from the flow dialysis experiments presented above that the inability of cells lacking cytochrome  $aa_3$  to accumulate streptomycin (17) was not due to insufficient  $\Delta\psi$ . The  $\Delta\psi$  experiments were conducted on whole cells with the addition of PMS-ascorbate as an artificial electron donor system to obtain values of  $\Delta\psi$  under conditions of maximal electron flow. Consequently, it was desirable to measure streptomycin uptake under similar conditions. For these experiments it was necessary to adapt the uptake system designed for measurement of amino acid uptake in membrane vesicles with PMS-ascorbate as electron donors (15) to measure aminoglycoside accumulation in whole cells. The latter preparation, rather than membrane vesicles, was utilized because stable membrane vesicles having cytochrome compositions similar to intact cells could not be prepared from cells lacking cytochrome  $aa_3$ . It was also clear from the work of Bryan and Kwan (5) that membrane vesicles of *E. coli* lacking protein synthesis components were inactive in aminoglycoside uptake. In addition, preliminary amino acid uptake experiments in whole cells of strain RB1 with PMS-ascorbate indicated that whole cells were much more efficient in the uptake process than membrane vesicles with the same concentration of membrane protein. When the PMS-ascorbate was omitted, glutamate uptake fell to <5%. It was apparent that on a dry weight basis, whole cells will accumulate glutamate with at least 40-fold-higher efficiency than membrane vesicles (data not shown).

Uptake experiments accordingly were carried out with

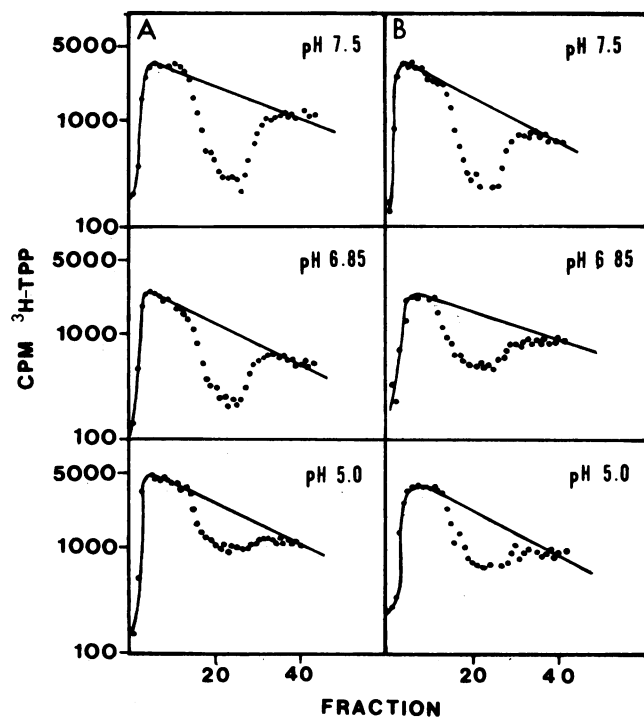


FIG. 1. Measurement of  $\Delta\psi$  by the flow dialysis technique. Experiments were carried out at the pH values shown, using whole cells of strain RB1 (A) and strain RB95 (B) having  $5^+aa_3$  concentrations of cytochrome  $aa_3$ .  $^3\text{H-TTP}$ , [ $^3\text{H}$ ]tetraphenylphosphonium.

TABLE 1. Summary of  $\Delta\psi$  values derived from flow dialysis experiments

Strain	External pH	$\Delta\psi$ value (mV) at the following concn of cytochrome $aa_3$ :		
		$0aa_3$	$5aa_3$	$5^+aa_3$
RB1	5.00	-128	-132	-142
	6.85	-128	-147	-164
	7.50	-139	-137	-178
RB95	5.00	-136	-133	-153
	6.85	-135	-137	-154
	7.50	-137	-134	-179

whole-cell preparations of strains RB95 and RB1, cultured so as to have each of the three cytochrome  $aa_3$  concentrations used for the  $\Delta\psi$  measurements (i.e.,  $0aa_3$ ,  $5aa_3$ ,  $5^+aa_3$ ). The capacity of each of these cell preparations to accumulate glutamic acid and proline is shown in Fig. 2 and 3, respectively. PMS-ascorbate effectively served to energize uptake of both of these amino acids, regardless of the cytochrome  $aa_3$  content of the cells. For example, panels E and F in Fig. 2 and 3 show that cells of either strain RB1 or RB95

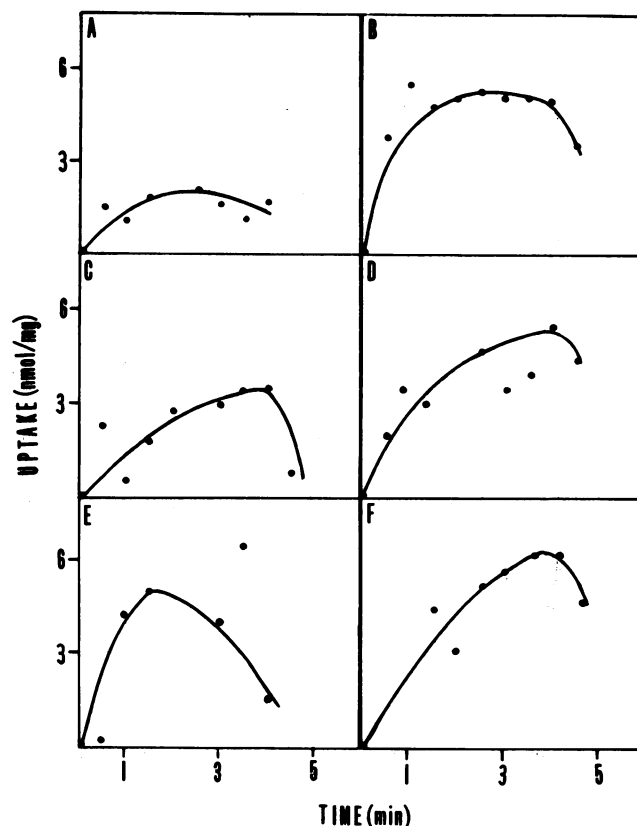


FIG. 2. Uptake of glutamic acid ( $9.7 \times 10^{-6}$  M) in the presence of PMS-ascorbate by whole cells of strain RB95 (A, C, and E) and strain RB1 (B, D, and F). Cells were cultured to yield cytochrome  $aa_3$  concentrations of  $5^+aa_3$  (A and B),  $5aa_3$  (C and D), and  $0aa_3$  (E and F). Culture conditions, cytochrome concentrations, and uptake measurements are described in the Materials and Methods. Uptake is expressed as nanomoles of glutamate accumulated per milligram (dry weight) of cells. The suspending buffer had a pH of 7.5.

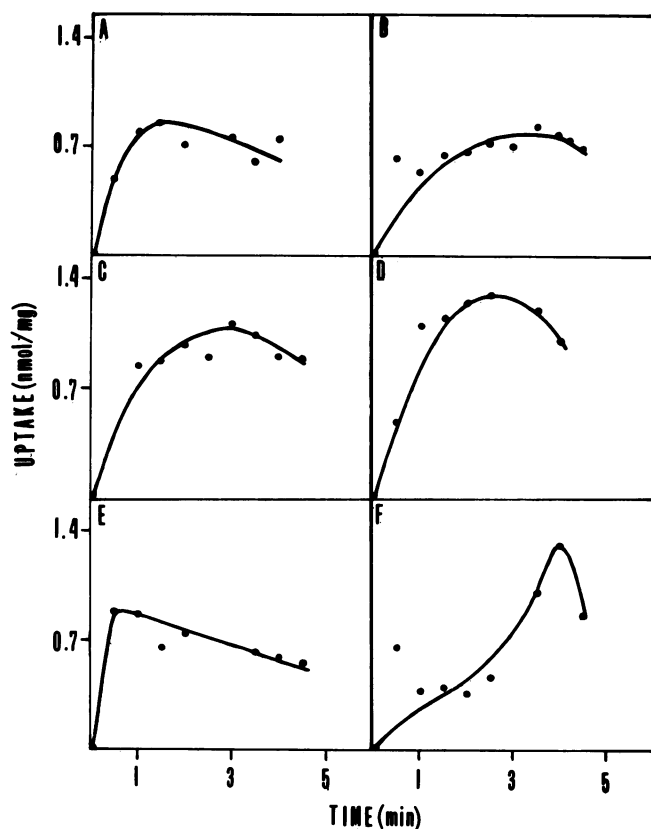


FIG. 3. Uptake of proline ( $4.6 \times 10^{-6}$  M) in the presence of PMS-ascorbate by whole cells of strain RB95 (A, C, and E) and strain RB1 (B, D, and F). Cells were cultured to yield cytochrome  $aa_3$  concentrations of  $5^+aa_3$  (A and B),  $5aa_3$  (C and D), and  $0aa_3$  (E and F). Culture conditions, cytochrome concentrations, and uptake measurements are described in the Materials and Methods. Uptake is expressed as nanomoles of proline accumulated per milligram (dry weight) of cells. The suspending buffer had a pH of 7.5.

containing no spectroscopically demonstrable cytochrome  $aa_3$  ( $0aa_3$ ) have uptake rates and final intracellular levels of glutamate and proline comparable to those of cells containing either normal ( $5aa_3$ ; panels C and D) or greater than normal ( $5^+aa_3$ ; panels A and B) amounts of cytochrome  $aa_3$ . Also, the presence of the *strC* mutation in strain RB95 seemed to have little or no effect on the uptake of glutamate and proline (compare panels A, C, and E with panels B, D, and F in Fig. 2 and 3).

**Streptomycin accumulation dependent on PMS-ascorbate.** Having established the ability of strains RB1 and RB95 to take up amino acids when supplied with PMS-ascorbate, we next measured PMS-ascorbate-dependent uptake of streptomycin in whole-cell preparations of these strains containing differing levels of cytochrome  $aa_3$  ( $0aa_3$ ,  $5aa_3$ ,  $5^+aa_3$ ). These results are shown in Fig. 4. [ $^3$ H]dihydrostreptomycin added to cells preincubated with unlabeled streptomycin was taken up rapidly by both strains RB1 (Fig. 4A) and RB95 (Fig. 4B), provided that the cells contained normal (circles) or greater than normal (crosses) concentrations of cytochrome  $aa_3$ . Cells deficient in cytochrome  $aa_3$  (triangles) accumulated [ $^3$ H]dihydrostreptomycin poorly. The residual uptake observed presumably was due to the low but detectable levels of cytochrome  $aa_3$  remaining in the cells when cultured on MGT medium. The data shown in Fig. 4 are from a representative experiment; however, the relative peak uptake

levels at 9 to 10 min were the same in each of the three repetitions of the experiment. The result shows that not only is cytochrome  $aa_3$  necessary for efficient streptomycin entry into *B. subtilis* but that enhanced concentrations of cytochrome  $aa_3$  in the  $5^+aa_3$  cell preparations allowed higher levels of streptomycin accumulation under the experimental conditions used.

## DISCUSSION

Aminoglycoside resistance has been noted in mutants blocked in the pathway for heme biosynthesis (8), in menaquinone mutants (11, 19, 26), and in anaerobes grown under conditions in which no cytochrome formation was detected (3). While the resistances were not specific for streptomycin (but usually included streptomycin resistance), all of these mutations would result in deficiencies of several cytochromes. This would include cytochrome  $aa_3$  in the organisms that synthesize this cytochrome (in organisms such as *E. coli*, which do not synthesize cytochrome  $aa_3$ , apparently another cytochrome serves a function in aminoglycoside transport analogous to that of cytochrome  $aa_3$  in *B. subtilis*). Supplementation of the appropriate precursor to these heme and menaquinone mutants, as well as the anaerobes, resulted in both cytochrome formation and increased aminoglycoside susceptibility.

A requirement for the  $\Delta\psi$  component of the proton electrochemical gradient ( $\Delta\bar{\mu}_{H^+}$ ) to promote aminoglycoside accumulation by bacterial cells has been well documented (5, 6, 9, 10, 16). However, there are additional data which suggest the involvement of specific electron transport components in aminoglycoside uptake, in conjunction with the establishment of  $\Delta\bar{\mu}_{H^+}$ . Bryan and Kwan (5) have shown in an *E. coli ubi* mutant depleted of 75 to 80% of its ubiquinone that  $O_2$  consumption and gentamicin uptake are proportionately decreased, whereas glutamine and proline uptake are unaffected. Similarly, Taber et al. (27) found that a *B. subtilis* mutant when severely depleted (<10%) of menaquinone accumulated gentamicin poorly. Previous work from this laboratory has demonstrated a relationship between cytochrome  $aa_3$  levels and streptomycin accumulation in *B. subtilis* (17). However, these experiments did not exclude the possibility that the observed correlation between cytochrome  $aa_3$  levels and streptomycin uptake was a result of altered proton extrusion, causing changed  $\Delta\psi$  values. However, if this was the case, then accumulation of streptomycin should be related to the magnitude of  $\Delta\psi$ , rather than to cytochrome  $aa_3$  levels.

In the work reported here, we found that the magnitude of  $\Delta\psi$  was sufficient to support uptake of streptomycin even under conditions in which no cytochrome  $aa_3$  was present (Table 1). The  $\Delta\psi$  values obtained were in the same range as those reported for whole cells of *E. coli* (20) or *Staphylococcus aureus* (16), utilizing a similar assay system. Slightly higher  $\Delta\psi$  values have been reported by other investigators utilizing whole cells (9, 13, 14) including *B. subtilis* (22). However, the latter measurements were obtained by a method shown to be subject to the degree of aerobiosis (13) and consequently could have yielded results at variance with the flow dialysis assay system, in which the cells are well aerated.

The question of a threshold  $\Delta\psi$  value that is necessary for aminoglycoside uptake, as indicated from the studies of Mates et al. (16) and Bryan and Kwan (5), cannot be addressed from the present studies, since the  $\Delta\psi$  values in our experiments (see Table 1) are well above the apparent thresholds published. However, we do have evidence that

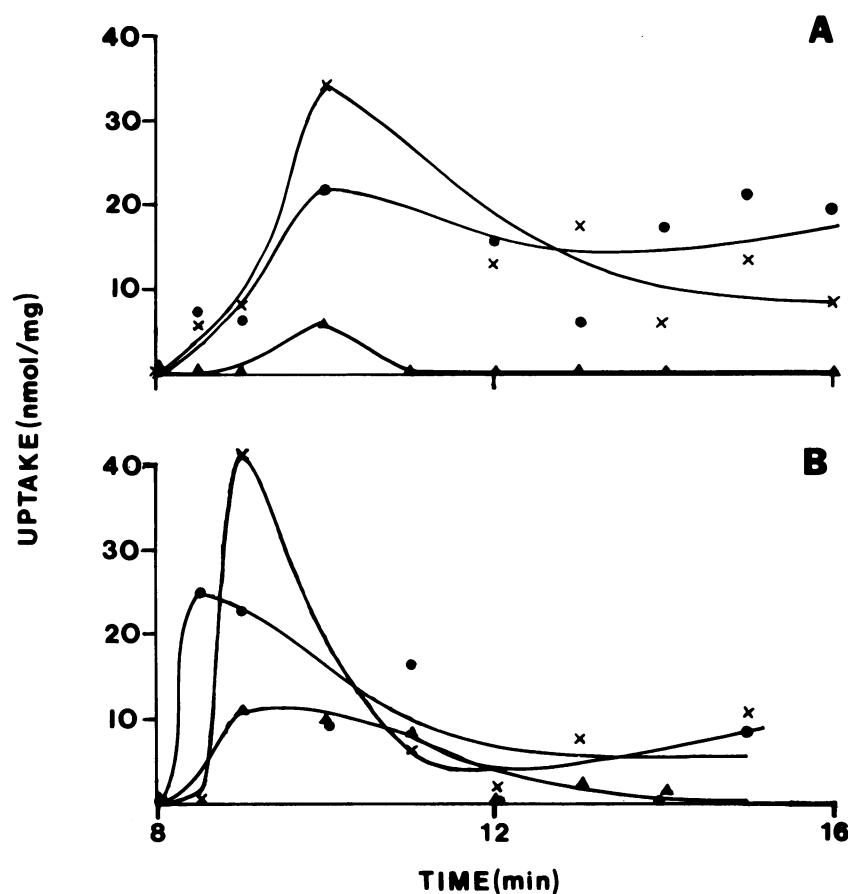


FIG. 4. Uptake of streptomycin by strains RB1 (A) and RB95 (B). The final streptomycin concentration was 87.3  $\mu\text{g/ml}$  ( $8 \times 10^{-5}$  M), with an 8-min preincubation period in the presence of PMS-ascorbate and unlabeled streptomycin. Measurements were made on cells with different cytochrome *aa*<sub>3</sub> concentrations: 0*aa*<sub>3</sub> (▲); 5*aa*<sub>3</sub> (●); and 5<sup>+</sup>*aa*<sub>3</sub> (×). The suspending buffer had a pH of 7.5.

enhanced synthesis of cytochrome *aa*<sub>3</sub> (to the 5<sup>+</sup>*aa*<sub>3</sub> level as defined in Materials and Methods) is associated with a marked increase in  $\Delta\psi$ , from the  $-130$  to  $-140$  mV range for cells containing 0*aa*<sub>3</sub> and 5*aa*<sub>3</sub> to  $> -175$  mV for 5<sup>+</sup>*aa*<sub>3</sub> cells at pH 7.5. This causes an increased accumulation of streptomycin in a fashion reminiscent of the dicyclohexylcarbodiimide-enhanced gentamicin uptake observed by Eisenberg et al. (see Fig. 4 in reference 10) which occurred (at pH values of 5.0 and 6.0) only when  $\Delta\psi$  exceeded  $-155$  mV. Since in our measurements we did not find substantial decreases in  $\Delta\psi$  at lower pH values (Table 1), we carried out our studies primarily at pH 7.5, at which  $\Delta\psi$  is the predominant component of  $\Delta\bar{\mu}_{\text{H}^+}$ .

To make the  $\Delta\psi$  and uptake measurements comparable, we carried out both types of experiments in the presence of an artificial electron donor system, PMS-ascorbate. This allowed us to make meaningful comparisons of amino acid uptake, streptomycin accumulation, and membrane potential in relation to cellular cytochrome *aa*<sub>3</sub> concentrations. Proline uptake, which is dependent solely on  $\Delta\psi$  (21), was roughly equivalent at all cytochrome *aa*<sub>3</sub> concentrations, including 0*aa*<sub>3</sub>. The uptake of glutamate, shown by Bisschop and Konings (2) to be dependent on the electron carrier menaquinone in *B. subtilis*, was likewise refractory to the presence or absence of cytochrome *aa*<sub>3</sub>. However, streptomycin uptake was markedly influenced by the cellular cytochrome *aa*<sub>3</sub> concentration (data not shown) (Fig. 4; 17). Although the data obtained with cells containing 0*aa*<sub>3</sub> and

5*aa*<sub>3</sub> concentrations of cytochrome *aa*<sub>3</sub> are relatively straightforward, the enhanced uptake of streptomycin seen in cells containing 5<sup>+</sup>*aa*<sub>3</sub> concentrations of cytochrome *aa*<sub>3</sub> cannot be simply attributed to the higher amount of cytochrome *aa*<sub>3</sub> present, since  $\Delta\psi$  also is increased in these cell preparations.

It should be added that a limited number of experiments parallel to those reported here have been carried out on the relationship between gentamicin accumulation and cellular cytochrome *aa*<sub>3</sub> concentration in *B. subtilis* (A. Arrow and H. Taber, submitted for publication). By contrast with the results shown here and obtained previously (17) with streptomycin, susceptibility to growth inhibition by and uptake of gentamicin show an inverse relationship to cytochrome *aa*<sub>3</sub> concentrations and a direct relationship to cytochrome 617 concentrations in this organism. In consequence of all of our findings thus far, we would like to agree with and extend the conclusion of Bryan and Kwan (5) that there is a role in aminoglycoside uptake for electron transport in addition to its requirement for development of  $\Delta\psi$ . Not only do we feel that certain molecular components of the electron transport chain play some sort of direct role in aminoglycoside uptake (e.g., cytochrome *aa*<sub>3</sub> for streptomycin in *B. subtilis* and ubiquinone for gentamicin and streptomycin in *E. coli* [5, 7]) but that in some instances this role may be specific to a particular aminoglycoside or structural class of aminoglycosides.

In summary, the studies reported here and previously (17)

show (i) that functional cytochrome *aa<sub>3</sub>* must be present in the *B. subtilis* cytoplasmic membrane for rapid streptomycin entry to occur; (ii) that in the absence of cytochrome *aa<sub>3</sub>*, a normal  $\Delta\psi$  can be established by electron flow to other terminal oxidases, but that this is insufficient to promote streptomycin entry; and (iii) that hyperpolarization of the membrane associated with enhanced cytochrome *aa<sub>3</sub>* formation can result in increased streptomycin uptake. These studies also underscore the importance of defining carefully the physiological state of bacterial cell preparations that are used in aminoglycoside uptake experiments. The flexibility of bacterial electron transport systems, with their multiple terminal oxidases, branching pathways, and rapidly adjustable composition, make such definition imperative for meaningful conclusions to be drawn about the mechanisms by which these systems participate in aminoglycoside entry into bacterial cells.

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