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Correlation Between Cytochrome aa_3 Concentrations and Streptomycin Accumulation in *Bacillus subtilis*

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Accumulation of aminoglycosides by *Bacillus subtilis* appears to require specific components of the electron transport chain. These components include cytochromes and the lipophilic quinone vitamin K_2. The present study concerns the importance of cytochrome aa_3, a terminal oxidase, in the uptake of streptomycin. Growth conditions have been established such that the concentration of cytochrome aa_3 can be modified over a wide range; on defined minimal salts agar, the wild-type strain (RB1) and an *strC* mutant (RB95) synthesized cytochrome aa_3 only when adequate amounts of Casamino Acids (Difco Laboratories, Detroit, Mich.) were present. A positive correlation between cytochrome aa_3 levels and streptomycin accumulation was observed. The same correlation was seen when cytochrome aa_3 was measured in relation to growth susceptibility. These correlations suggest that cytochrome aa_3 is necessary for accumulation of streptomycin by *B. subtilis*.

The requirement of an electrochemical proton gradient ($\Delta\bar{\mu}_{H+}$) for aminoglycoside accumulation by bacterial cells has been well documented through the use of uncoupling agents and of mutants defective in electron transport or oxidative phosphorylation (2, 6, 7, 11, 14). Also, there is data suggesting the involvement of specific electron transport components in aminoglycoside uptake, in conjunction with the establishment of $\Delta\bar{\mu}_{H+}$. Components of the electron transport chain may participate directly in aminoglycoside uptake, serving either as binding sites or as carrier molecules (7, 8) or by channeling electron flow through particular segments of the chain.

Based on the decreased accumulation of aminoglycosides by ubiquinone-deficient *Escherichia coli* strains (7), it has been suggested that lipophilic quinones may serve this function, as well as participating in generation of $\Delta\bar{\mu}_{H+}$. Studies with menaquinone-deficient mutants of *Bacillus subtilis* (18, 19) and *Staphylococcus aureus* (14) are consistent with this proposal. However, in *B. subtilis* the menaquinone concentration regulates the amounts of cytochromes aa_3, *b*, and *c* (10), and quinone deficiency could limit aminoglycoside accumulation by decreasing the amount of one or more cytochromes crucial to the uptake process. The present work is concerned with the involvement of cytochrome aa_3 in uptake of streptomycin. Growth conditions were devised that limited cytochrome aa_3 formation in the standard strain. These conditions enhanced resistance to growth inhibition by streptomycin, while decreasing rates of uptake of the antibiotic. A mutation (*strC*) associated with cytochrome aa_3 deficiency under most growth conditions caused resistance to growth inhibition by streptomycin and very low rates of uptake.

MATERIALS AND METHODS

Bacterial strains. Strain RB1 (*trpC2*) is a laboratory stock of *B. subtilis* strain 168. Strain RB95 (*strC2*) is a spontaneous streptomycin-resistant mutant obtained from J. Hoch (15).

Media. The minimal salts medium (MG) was that of Anagnostopoulos and Spizizen (1) supplemented with 0.5% glucose. MG supplemented with 50 μg of tryptophan per ml was designated MGT. Casamino Acids (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 (10). TG medium was prepared by adding sterile glucose (final concentration 0.5%) to previously autoclaved TBAB. The liquid equivalent of TBAB contained (in grams per liter) tryptose (Difco), 10; beef extract (Difco), 3.0; and NaCl, 5.0.

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.

Cytochrome measurements. The cytochrome components of intact cells grown for 48 h on solid media were determined at liquid nitrogen temperature with a Hartree low-dispersion microspectroscope, as previously described (16). An example of the absorption band spectrum obtained with the microspectroscope is shown in Fig. 1B (inset), and Fig. 1A gives the concentration values assigned to the band intensities by visual estimation. The concentration scale is 0 to 5, with 5 as the maximal intensity observed. The designation ± indicates that a cytochrome absorption band was detectable but had an intensity of less than 1. The recorded absorption spectrum (Fig. 1B) was obtained with a Cary Model 14 spectrophotometer equipped with a high-intensity light source and a scattered-transmission accessory (see reference 16).

Pyridine hemochromogens of membrane preparations. After 48 h of growth on MGT supplemented with 10 mg of CV per ml, 20-ml samples of whole-cell suspensions with the appropriate cytochrome content were chilled to 10 to 15°C and treated with 0.75 μg of lysozyme per ml at 10 to 15°C for 15 min. The treated cell culture was placed in a French pressure cell cylinder and kept overnight at −70°C in a Revco freezer. The frozen suspension was disrupted by passage through the pressure cell at 16,000 lb/in^2. A second lysozyme treatment, followed by freezing and passage

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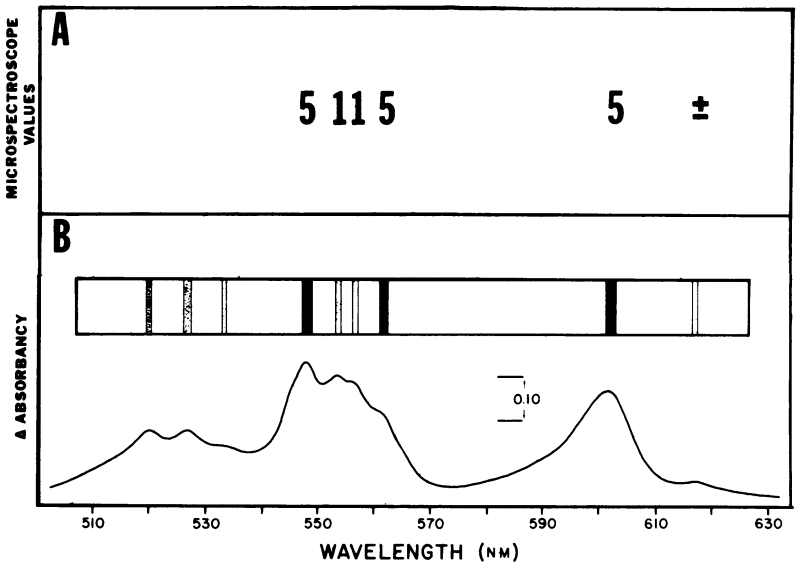

FIG. 1. Absorption spectrum of intact cells of strain RB1 at the temperature of liquid nitrogen. (A) Relative concentrations assigned to absorption intensities of the band spectrum (B, insert). At the bottom is a recorded spectrum of the same preparation. Cultures were grown as described in the text, and spectra were measured as described previously (16, 17) and in the text.

through the French pressure cell, was necessary for complete disruption of the cell suspension. Cellular debris and unbroken cells were removed by centrifugation in a Sorvall SS-34 rotor at 15,000 rpm for 40 min. The supernatant was removed, lyophilized, and suspended in 2.1 ml of filtered (Millipore Corp., Bedford, Mass.) water–0.5 ml of pyridine–0.25 ml of 1 N NaOH. Sodium dithionite (2 mg) was added and spectra recorded immediately, utilizing a Beckman model 320 spectrophotometer.

Antibiotic accumulation. Strains RB1 and RB95 were grown overnight in the same medium subsequently utilized for the uptake experiment. The next morning, cells were diluted and allowed to grow to a density of 60 Klett units. At this time, unlabeled streptomycin sulfate (58.2 μg/ml) and ^{3}H-labeled dihydrostreptomycin (DHS) sulfate (final specific activity, 0.2 μCi/ml) were added to 14.7 ml of cells to give a final streptomycin concentration of 58.5 μg/ml. Samples (1.0 ml) were taken at 3- or 10-min intervals and filtered rapidly onto polycarbonate filters (Unipore; pore size, 0.4 μm) that had been prewashed with 10× MG containing 100 μg of streptomycin sulfate per ml. Filters were washed with 5 ml of 1.5 M NaCl, air dried, and counted in 5 ml of PPO (2,5-diphenyloxazole)-POPOP [1,4-bis-(5-phenyloxazolyl)benzene] scintillation cocktail.

Antibiotic susceptibility. A sample of an overnight culture (0.1 ml), grown in the medium subsequently used for plating, was suspended in 2.5 ml of 0.8% soft agar (of the same medium) and poured onto 25 ml of the desired medium. After the agar had solidified, antibiotic-impregnated disks were placed on the plate. The antibiotic disks were prepared by adding 3 μl of a sterile 100-mg/ml solution of streptomycin sulfate to a sterile blank disk (diameter, 7 mm). Results were recorded after 24 h, and zones of inhibition were measured with a vernier caliper. Averages of duplicates were computed, with a deviation of no more than ± 10% ordinarily noted.

RESULTS

Modification of cytochrome concentrations by growth conditions. Conditions of growth can modify susceptibility of *B. subtilis* to aminoglycosides (18, 19) and also have effects on cytochrome synthesis in this organism (9, 20). We sought to relate cytochrome content to antibiotic susceptibility by systematic alteration of growth conditions. The visible absorption spectrum of a wild-type strain (RB1) after growth on TBAB without added glucose is shown in Fig. 1. Absorption maxima characteristic of cytochromes aa_3 (601 nm), *b* (562 nm), *o* (557 nm), c_1 (554 nm), and *c* (549 nm) are present (17, 20). In addition, a maximum at 617 nm has been noted (16). As will be reported elsewhere (McEnroe and Taber, manuscript in preparation), this absorption maximum appears to be associated with a *d* type cytochrome.

An *strC* mutant (15), previously shown to be cytochrome aa_3-deficient when grown on TBAB (17), also had a considerably elevated content of cytochrome-617 on this medium. This is shown in Table 1, which summarizes relative cytochrome concentrations of the *strC* strain (RB95), together with those of strain RB1, after growth on TBAB, TG, and TG + 10 mg of CV per ml. The effect of the *strC* mutation, which was to lower cytochrome aa_3 concentration and to increase that of cytochrome-617, could be partially reversed by addition of high concentrations of CV to TBAB.

Glucose had several regulatory effects on both strains; concentrations of cytochromes *b* and *c* were markedly decreased, whereas cytochromes c_1 and *o* were increased. Glucose also caused a partial decrease of cytochrome aa_3 and a slight increase of cytochrome-617 in strain RB1. The effects of CV on the contents of cytochromes aa_3 and -617 in strain RB95 are exerted even in the presence of glucose (Table 1). Previously, we had found that CV must be added to MGT to obtain appreciable rates of aminoglycoside accumulation (19). To relate the effects of CV on cytochrome concentrations and antibiotic sensitivity to aminoglycoside uptake, increasing amounts of CV were added to MGT. For convenience, solid media were used for the cytochrome and antibiotic-sensitivity measurements, whereas liquid media were used for studies of aminoglycoside uptake. Relationships between results from the two types of media are discussed below.

The changes in cytochrome concentrations of strains RB1 and RB95 in response to increasing amounts of CV in MGT

TABLE 1. Relative cytochrome concentrations of wild-type (RB1) and mutant (RB95) strains of *B. subtilis*[a]

Growth medium and strain	Relative concn of cytochrome:					
	c	c_1	*o*	*b*	aa_3	−617
TBAB						
Alone						
RB1	5	1	1	5	5	±
RB95	5	0	0	5	±	4
+CV[b]						
RB1	5	±	±	4	5	0
RB95	5	0	0	5	2	±
TG						
Alone						
RB1	1	5	5	1	3	1
RB95	±	5	5	±	±	5
+CV[b]						
RB1	5	2	2	4	5	±
RB95	2	5	5	2	2	0

[a] Strains were grown on solid media for 2 days, and measurements were made as previously described (16). Units are as defined in the text.
[b] 10 mg/ml.

are summarized in Table 2. The most striking result was an increased formation of cytochromes aa_3 and *c* as the CV concentration was increased. A smaller increase in cytochrome *b* also occurred, whereas cytochromes c_1 and *o* were virtually unchanged. In addition, the formation of cytochrome-617 was reduced when the CV concentration was 5 mg/ml or more. Although the presence of the *strC* mutation in strain RB95 diminished the effects of a given level of CV on concentrations of the cytochromes, cytochrome aa_3 formation could be detected in strain RB95. To confirm that the cytochrome formed was an aa_3 type cytochrome, strains RB1 and RB95 were grown on media to enhance synthesis of cytochrome aa_3 (MGT plus 10 mg of CV per ml), and the pyridine hemochromogen spectra of membrane preparations were measured. The pyridine hemochromogen of heme *a* could be detected in strain RB1 with an absorption maximum at ca. 587 nm and no absorption in the longer-wavelength region (Fig. 2). Similarly, strain RB95 showed an absorption maximum of slightly smaller magnitude at ca. 587 nm, also

TABLE 2. Effect of CV on cytochrome concentrations of strains RB1 and RB95[a]

Strain and concn of CV (mg/ml) added to MGT	Relative concn of cytochrome:					
	c	c_1	*o*	*b*	aa_3	−617
RB1						
0.01	±	4	4	±	±	5
0.1	1	4	4	1	1	5
1.0	3	4	4	2	3	5
5.0	4	3	3	1	4	±
10	5	4	4	2	4–5	0
RB95						
0.01	±	3–4	3–4	±	0	5
0.1	1	4	4	1	±	5
1.0	1–2	4	4	1–2	±	4
5.0	3	5	5	2–3	2	2
10	5	5	5	3	3–4	±

[a] Measurements were made as described in footnote *a* of Table 1.

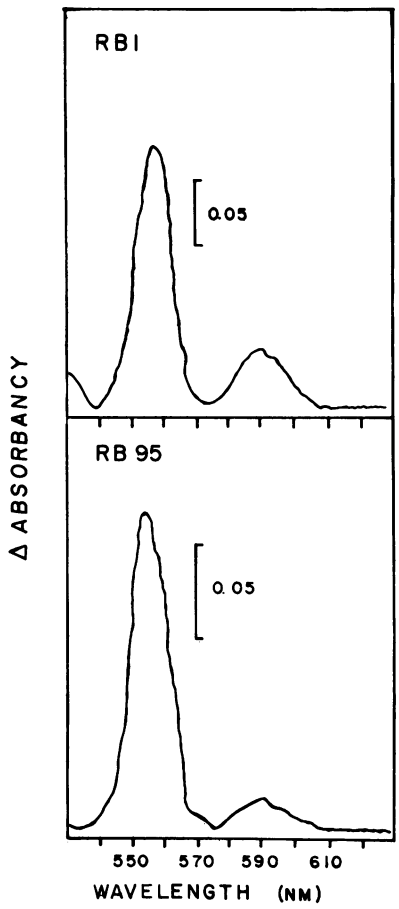

FIG. 2. Spectra of the pyridine hemochromogen derivatives of strains RB1 and RB95. Strains were grown in MGT + 10 mg of CV per ml as described in the text, and spectral measurements of membrane preparations were made as described in the text.

with no other absorption in the longer-wavelength region (Fig. 2). These findings indicate the formation of cytochrome aa_3 in strain RB95 when this strain is grown under the appropriate conditions.

Relationship of streptomycin susceptibility to cytochrome aa_3 concentration. A correlation between the concentration of CV and cytochrome aa_3 levels was demonstrated for both strains on glucose (Table 2), as well as with lactate as the sole carbon source (data not shown). A similar relationship was observed between the concentration of CV and streptomycin susceptibility (Table 3). Strain RB1 showed enhanced susceptibility to streptomycin as the concentration of CV in the growth medium increased; this effect was independent of the carbon source, as it could be demonstrated on both lactate and glucose (Table 3). The finding that as the CV concentration in the growth medium increased, both cytochrome aa_3 levels and streptomycin susceptibility increased suggested a functional relationship between these two properties. A similar correlation was observed between cytochrome aa_3 and streptomycin sensitivity in strain RB95, although the increase in sensitivity was not as markedly dependent on CV concentration when this strain was grown on glucose (Table 3). This could have been due to the failure to observe appreciable stimulation of cytochrome aa_3 formation unless the CV concentration was 5 mg/ml or higher (Table 2). Decreases in zone diameters were noted at higher

TABLE 3. Effect of CV on streptomycin susceptibility of strains RB1 and RB95

Strain and concn of CV (mg/ml) added to MGT	Relative diameter of zones of inhibition by carbon sources[a]:	
	Lactate	Glucose
RB1[b]		
0.01	100	109
0.03	190	100
0.1	210	140
0.3	320	158
1.0	350	152
3.0	350	171
5.0	280	159
10.0	300	148
RB95[c]		
0.01	100	100
0.03	111	106
0.1	152	107
0.3	164	121
1.0	150	104
5.0	182	119
10.0	182	109

[a] All values normalized to 100.
[b] Disks contained 300 μg of streptomycin sulfate.
[c] Disks contained 600 μg of streptomycin sulfate.

(>3 mg/ml) CV concentrations, probably due to the increased growth rates of both strains under these conditions.

Effect of growth conditions on streptomycin accumulation. We have previously demonstrated that respiration-deficient *B. subtilis* mutants having multiple-aminoglycoside resistance (Mar) phenotypes exhibit reduced rates of aminoglycoside accumulation (18, 19). Therefore, we measured streptomycin uptake by strains RB1 and RB95 after subjecting the cultures to growth conditions that would stimulate or limit cytochrome aa_3 formation. The uptake of [^{3}H]DHS by the two strains after growth in TG liquid medium is compared in Fig. 3. We measured the cytochrome concentrations of the two strains in this liquid medium and found values (data not shown) comparable to those in Table 1; strain RB1 formed sufficient cytochrome aa_3 to permit substained uptake over a 50-min period. Strain RB95 formed only trace amounts of cytochrome aa_3 in this medium and did not show time-dependent accumulation of [^{3}H]DHS (Fig. 3).

Strains RB1 and RB95 were then grown in MGT containing varying concentrations of CV, and rates of accumulation were measured. The dependence of uptake rates by strain RB1 on the concentration of CV in MGT is shown in Fig. 4A. At the lowest concentration tested (0.01 mg/ml), the rate of [^{3}H]DHS accumulation was very low. Cultures grown in higher CV concentrations showed progressively higher rates of uptake (Fig. 4A). The effect of CV in stimulating [^{3}H]DHS uptake by strain RB95 was similar to that observed for strain RB1 (Fig. 4B). From the data on cytochrome aa_3 concentrations (Table 2), we would have supposed [^{3}H]DHS uptake by strain RB95 to be less responsive to the CV than that by strain RB1. However, when the relative cytochrome concentrations of cells grown in liquid MGT with various levels of added CV were measured, ca. 20% more cytochrome aa_3 was formed at a given CV concentration than when cells were grown on the corresponding solid medium. For example, strain RB95 grown in liquid MGT plus 0.01 μg of CV per ml had a level of 1, whereas on solid media (cf. Table 2), strain RB95 lacked cytochrome aa_3 completely. Strain RB1

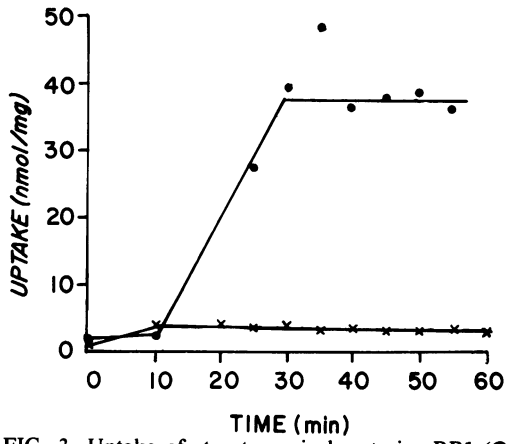

FIG. 3. Uptake of streptomycin by strains RB1 (●) and RB95 (x). Cells were grown in liquid TG medium. The external streptomycin concentration was 58.2 μg/ml, with 10-min sampling times for the first 20 min and 5-min sampling times for the remainder of the experiment.

did not show this differential in cytochrome formation when liquid and solid media were compared. A limited number of uptake measurements were made with cells grown on solid (rather than liquid) media. Although there was some variability in uptake rates between the two types of media for a given CV concentration, the uptake rates were well correlated with the cellular cytochrome aa_3 content.

DISCUSSION

The involvement of cytochromes in aminoglycoside accumulation by bacteria is suggested by several studies with cytochrome-deficient mutants. Using an *E. coli* mutant blocked in the heme biosynthetic pathway, Campbell and Kadner were able to show that the rate of dihydrostreptomycin uptake is proportional to the amount of heme present (8). Bryan et al. (5) studied a mutant of *Pseudomonas aeruginosa* deficient in cytochrome c_{552} and nitrate reductase. This mutant appeared to be defective in streptomycin and gentamicin uptake because of reduced cytochrome oxidase activity. Two additional aminoglycoside-resistant mutants of *P. aeruginosa* have been described (4) in which decreased aerobic uptake of streptomycin and gentamicin were correlated with cytochrome *d* and nitrate reductase deficiencies and decreased terminal oxidase activity. Taber and Halfenger (18) isolated multiple aminoglycoside-resistant (*mar*) mutants of *B. subtilis* that showed cytochrome aa_3 deficiencies and decreased kanamycin uptake. Inhibitors of electron transport have also been used to show that electron flow to oxygen is necessary for aminoglycoside accumulation (cf. reference 12). Uptake of these compounds is, for example, quite sensitive to cyanide, an agent that reacts with terminal oxidases. In bacteria, these oxidases correspond to cytochromes aa_3, *d*, and *o* (13).

We have been able to alter the cytochrome aa_3 concentration of *B. subtilis* strains by growing cultures in minimal salts media containing various concentrations of CV. This has been accomplished both for a standard strain (RB1) and for a mutant (RB95) that is cytochrome aa_3-deficient when grown in complex media lacking additional CV. This accumulation is associated with the presence of cytochrome aa_3 in the standard strain but is lacking in the cytochrome aa_3-deficient strain, as shown in Fig. 3, which compares accumulation of dihydrostreptomycin by the standard and mutant strains in unsupplemented complex media. Systematic increases in

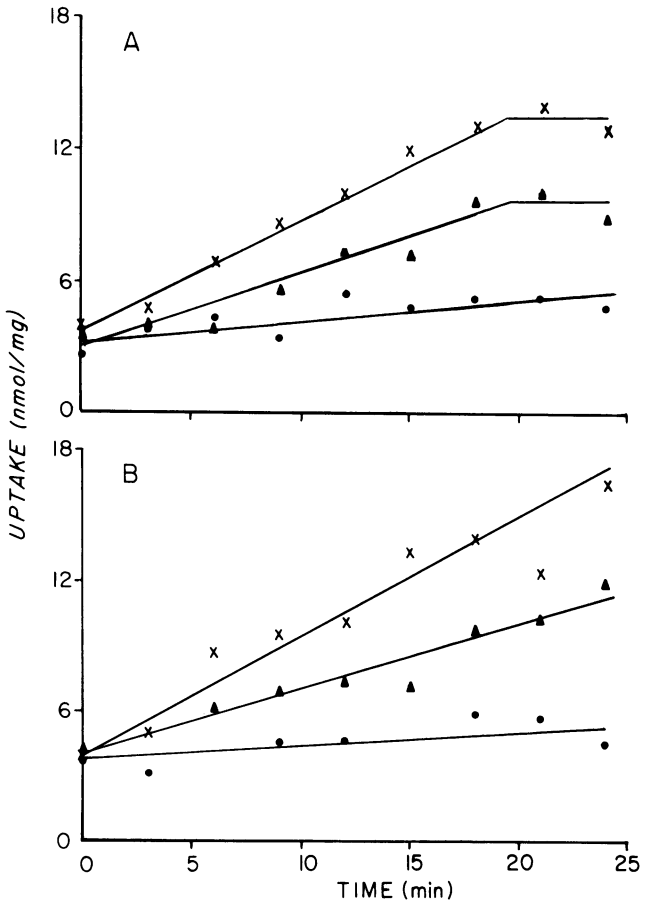

FIG. 4. Uptake of streptomycin by strains RB1 (A) and RB95 (B). The external streptomycin concentration was 58.2 μg/ml, with 1.5-min sampling times. Cells were grown in MGT + 0.01 mg of CV (●) per ml; 0.1 mg of CV (▲) per ml; or 1.0 mg of CV (x) per ml.

cytochrome aa_3 levels brought about by addition of CV to minimal salts media result in corresponding increases in rates of dihydrostreptomycin accumulation (Fig. 4). We suggest, then, that cytochrome aa_3 is necessary for dihydrostreptomycin uptake by *B. subtilis*. The antibiotic-susceptibility data (Table 3) show a general correlation with this suggestion; as the cytochrome aa_3 concentration increases, the cells become progressively more susceptible to growth inhibition by streptomycin. It should be noted that the relationship between antibiotic susceptibility and CV concentration is not one of strict proportionality, since a number of factors contribute to zone size. In this circumstance, these would include the increased rate of streptomycin accumulation at higher CV concentrations leading to larger zone sites, which would be partially offset by the more rapid cell growth caused by these conditions. Also, both from the susceptibility measurements (Table 3) and from the observed rates of streptomycin uptake (Fig. 4), it would appear that a full complement of cytochrome aa_3 is not necessary for antibiotic accumulation to occur. Of the normally regulated level, 50% or less seems to be sufficient to allow uptake rates consistent with growth inhibition. This threshold level also helps to account for the relatively minor differences between strains RB1 and RB95 in rates of streptomycin uptake in minimal media (Fig. 4). The liquid-grown RB95 culture synthesized sufficient cytochrome aa_3 under CV stimulus to accumulate substantial streptomycin, although this accumulation seemed to stop after 25 min.

Other explanations are of course possible. For example, CV could alter the level of an intracellular effector (such as a polyphosphorylated nucleotide), which in turn could exert independent regulatory effects on aminoglycoside accumulation and on cytochrome aa_3 formation. However, this seems to introduce an unnecessary complication, especially in light of the association between cytochrome concentrations and aminoglycoside accumulation in *E. coli* (8) and *P. aeruginosa* (4, 5). It is also possible that the apparent correlation between cytochrome aa_3 levels and streptomycin uptake is a result of enhanced functioning of the electron transport chain under conditions in which cytochrome aa_3 has replaced cytochrome-617 as terminal oxidase. (Cytochromes *d* have been demonstrated to function as terminal oxidases [13].) This enhanced functioning could result in either increased oxygen consumption or increased proton extrusion, hence in greater $\Delta\psi$ values, as a function of the concentration of cytochrome aa_3. If this is the case, then accumulation of streptomycin should be related to the magnitude of $\Delta\psi$ rather than to cytochrome levels. Measurements of these parameters have been made, and accumulation has been shown to be related to cytochrome aa_3 concentrations, not to $\Delta\psi$ values (A. S. McEnroe, Ph.D. thesis, Albany Medical College, 1983; McEnroe and Taber, in preparation).

In addition, we are intrigued by the modulation of cytochrome aa_3 concentrations by CV and are proceeding to characterize this in a more detailed fashion. The reciprocal-concentration relationship between cytochrome aa_3 and cytochrome-617 is also under study, since it appears that strain RB95 bears a defect in the regulation of these two membrane components.

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