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Multiple-Aminoglycoside-Resistant Mutants of *Bacillus subtilis* Deficient in Accumulation of Kanamycin

H. TABER* AND G. M. HALFENGER

Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

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Three classes of spontaneous multiple-aminoglycoside-resistant *(mar)* mutants of *Bacillus subtilis* were isolated by plating on a low (1.2 μg/ml) concentration of kanamycin sulfate and were found to be resistant also to low concentrations of paromomycin, neomycin and gentamicin. The three classes could be distinguished one from another by their degree of cytochrome deficiency, respiration deficiency, and susceptibility to kanamycin lethality. A fluctuation test showed that the mutations were spontaneous and not induced by the conditions of selection. Representative strains from two classes of mutants *(mar-2* and *mar-3)* accumulated aminoglycoside very poorly in comparison with the parent strain, whereas a strain of the third class *(mar-1)* inactivated aminoglycoside present in the growth medium. The *mar-3* strain studied *(aroD163)* had previously been shown to be a menaquinone auxotroph (Farrand and Taber, 1973) and to be deficient in amino acid uptake (Bisschop et al., 1975). Such mutants, which are resistant to low concentrations of aminoglycosides, may be of use in elucidating the biochemical and genetic bases of certain bacterial transport systems.

Two general mechanisms of resistance to inhibition by aminoglycoside antibiotics have been recognized in bacteria (reviewed by Price et al. [21], Benveniste and Davies [3], and Gale et al. [13]). The ribosomal target may be altered by mutation and prevent binding of the drug: a single amino acid change in the S12 protein of the 30S ribosomal subunit can give rise to streptomycin resistance in *Escherichia coli* (12). A second resistance mechanism, mediated by R factor-coded gene products, involves enzymatic inactivation of aminoglycoside antibiotics by covalent addition of acetyl, adenyl, or phosphoryl groups (3, 20, 31). Although natural resistance to aminoglycosides appears to arise most frequently by spread of these plasmids to new bacterial populations (cf. e.g. reference 6), physiological conditions such as anaerobiosis (17) can suppress entry of aminoglycosides into the cell and confer temporary resistance. Indeed, Hancock (14) showed a correlation between uptake of [^{14}C]streptomycin and susceptibility to the antibiotic among several genera of bacteria.

While isolating cytochrome-deficient mutants of *Bacillus subtilis* by using the kanamycin resistance selection devised by Tien and White (30) for *Staphylococcus aureus*, we encountered a large number of small-colony mutants that were resistant not only to kanamycin but to several other aminoglycosides as well. Preliminary reports of this work (28; H. Taber, P. Bitoun, and G. M. Halfenger, Bacteriol. Proc., p. 139, 1970) have stressed the alterations in membrane proteins, particularly cytochrome deficiencies. One of the mutants (strain RB163) has been extensively characterized and shown to be a "tight" *aroD* mutant, completely blocked in menaquinone synthesis unless shikimic acid is supplied to metabolizing cells (8). Recently Bisschop et al. have shown that strain RB163, when menaquinone deficient, is blocked in the active transport of L-glutamate (4). The present communication is concerned with physiological properties of the several classes of *mar* mutants.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* strain RB1 *(trpC2)* was originally derived from strain 168. RB1 has a normal complement of cytochromes and sporulates well (27, 29). This strain is resistant to 10 μg of streptomycin per ml but susceptible to the other aminoglycosides used in this study.

Media. Tryptose blood agar base (TBAB; Difco) was used for routine culturing on solid media. Tryptose-beef extract medium and TG medium (tryptose-beef extract medium + 0.2% glucose) have been described (8). Brain heart infusion (BHI) was obtained from Baltimore Biological Laboratories, and antibiotic medium no. 3 (Penassay) was from Difco. Tryptone-yeast broth contained (per liter): 10 g of tryptone (Difco); 5.0 g of yeast extract (Difco); 5.0 g

of NaCl; and 0.5 ml of 0.01 M $MnSO_4$. Minimal salts medium was that of Anagnostopoulos and Spizizen (1); minimal salts-glucose medium had 0.5% glucose added to minimal salts medium.

Growth. To estimate growth, cells were cultured and optical density at 540 nm was measured as described previously (8).

Isolation of *mar* mutants. Spontaneous, independent *mar* mutants were isolated by resistance to low concentrations of kanamycin according to the following procedure. A single clone of strain RB1 was inoculated into 15 ml of BHI medium and shaken for 18 h at 37 C. The culture was diluted to 2 $\times 10^3$ to 3 $\times 10^3$ cells/ml in fresh BHI and distributed into 80 growth tubes, 0.5 ml per tube. The 80 independent cultures were shaken 18 h at 37 C and diluted 1:10 with fresh BHI, and 0.1 ml was removed from each tube for spreading on 80 petri dishes containing TBAB + 1.2 μg of kanamycin sulfate per ml (Mann Laboratories, 805 μg/mg). These were incubated for 2 days at 37 C, and single resistant clones of each of several small-colony morphology types were purified from each petri dish.

Antibiotic uptake. A zone-of-inhibition assay (15) was devised, based on loading of cells with kanamycin. The strain to be tested was grown in 15 ml of Penassay to t_1 (1 h after the measured end of exponential growth, which is t_0). The optical density at 540 nm was 0.7–0.8 at this time in the culture cycle. Sufficient kanamycin was added to give a final concentration of 10 μg/ml, and the culture was shaken gently at 37 C. At successive times, 1.0-ml samples were removed, cooled quickly in an ice bath, and centrifuged at 10,000 rpm (4 C) to pellet the cells. The supernatant was discarded, and the cells were resuspended in 2.0 ml of ice-cold water and recentrifuged. After resuspending the cell pellet in 200 μl of lysis solution [300 μg of lysozyme per ml, 0.1 μg of deoxyribonuclease per ml, 0.03 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5], the mixture was incubated at 37 C until the suspension had cleared (usually 15 min). Lysozyme was inactivated by heating the lysate at 60 C for 15 min, and 10-μl volumes were spotted on disks (0.88-cm diameter) of membrane filter material (Millipore Corp.). After air-drying, these disks were distributed on petri dishes containing 3.0 ml of Tris agar (20 g of agar per liter of 0.1 M Tris-hydrochloride, pH 7.0). An overlay of kanamycin-susceptible cells was prepared by growing strain RB1 to mid-exponential phase and adding 10^5 cells to 3.0 ml of tryptone-yeast soft (0.7%) agar at 50 C. This was poured over the filter disks, and plates were incubated for 18 h at 37 C. The zones of inhibition surrounding the disks were measured with a micrometer. The measurements were always made in duplicate.

A calibration curve was constructed by lysing cells not exposed to kanamycin and then adding known amounts of antibiotic to the lysed cells and measuring the zones of inhibition as described above. A plot of diameter of inhibition zone versus log kanamycin concentration yielded a straight line (Fig. 1). Uptake of antibiotic was expressed as micrograms of kanamycin per 10^8 cells (the latter measured by Petroff-Hauser count).

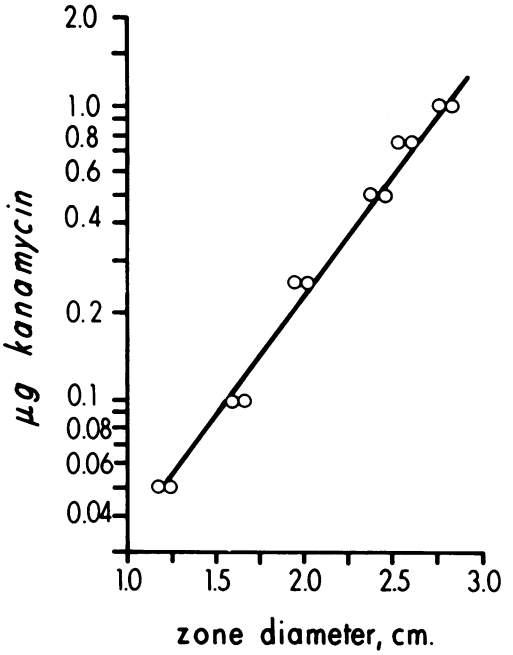

FIG. 1. *Calibration plot for zone-of-inhibition assay uptake of kanamycin, as described in the text.*

Cytochrome concentrations. For rapid estimation of relative cytochrome concentrations, 24- or 48-h cultures grown on TBAB were observed at lipid N_2 temperature in a Hartree microspectroscope, as described previously (8, 27).

Cell respiration. Rates of respiration were measured at 37 C with a Clark-type oxygen electrode (9). Cultures were grown in BHI to t_1 and diluted 1:10 into minimal medium plus either 1% sodium succinate or 1% glucose. A 10-ml sample of cells was simultaneously centrifuged and washed with water for measurement of dry weight. Respiration rates were expressed as micromoles of O_2 per minute per milligram (dry weight).

RESULTS

Multiple-aminoglycoside resistance phenotype. Although more than 100 independent mutants were initially isolated by selection on TBAB + kanamycin, many were unstable and reverted readily in the absence of kanamycin. By plate testing on TBAB into which antibiotic had been incorporated, 33 strains were found to grow in the presence of each of several aminoglycosides (Table 1); growth was measured as colony size after 2 days of incubation at 37 C compared with TBAB containing no antibiotic. The different aminoglycosides, in high enough concentrations, inhibited the resistant mutants but were differentially effective in doing so; kanamycin and paromomycin were less inhibitory than neomycin and gentamicin.

TABLE 1. *Resistance of mar mutants to growth inhibition by several structurally related aminoglycoside antibiotics incorporated into solid media (TBAB)*

Aminoglycoside[a]	Potency, (μg/mg)[b]	Concn (μg/ml) at which *mar* mutants will grow[c]
Kanamycin	805	1.2
Paromomycin	709	1.2
Neomycin	713	0.10
Gentamicin	—[d]	0.10

[a] Streptomycin was not tested, since the parent strain was resistant to this compound.

[b] For example, 1 mg of the kanamycin preparation is equivalent to 805 μg of highly purified kanamycin in inhibiting the growth of test organisms. All of the compounds were used as their sulfate salts; kanamycin, paromomycin, and neomycin were from Mann Laboratories; gentamicin was Garamycin (Schering Corp.).

[c] The parent strain, RB1, was completely growth inhibited by the concentrations indicated.

[d] Received as an aqueous solution containing (per milliliter) 40 mg of gentamicin sulfate, 1.8 mg of methyl paraben, 0.2 mg of propyl paraben, 3.2 mg of sodium bisulfite, and 0.1 mg of sodium edetate.

Selection of *mar* mutants in the presence of kanamycin. There have been repeated suggestions (19, 30) that aminoglycosides can act to induce mutations to antibiotic resistance and small-colony formation, as well as to select for these traits. If, in our procedure, induction were occurring, the mutation rate would be high; in addition, plating of parallel cultures would result in recovery of similar numbers of mutants from each culture (because of induction occurring on the plates). We were able to test the induction-versus-selection hypothesis by counting the numbers of *mar* mutants formed during our selection procedure (see Materials and Methods) and subjecting the data to a Delbrück-Luria fluctuation analysis (7).

Repeat samples from the same culture showed good agreement between sample mean and variance, despite the limited number of mutant clones per plate (Table 2). However, among parallel cultures grown from small inocula, the variance was 100-fold greater than the sample mean (Table 2). In the latter case, the mutants could not have been induced by the presence of kanamycin in the selection plates, otherwise similar numbers of mutants would have been found on all plates and there would not have been plates containing zero mutants.

Following the Delbrück-Luria analysis (7) and the suggestions of Ryan (22), we have estimated the spontaneous mutation rate a from the fraction of tubes containing zero mutants (assuming a Poisson distribution) according to the equations $P_0 = e^{-m}$ and $a = (m \cdot \ln 2)/(n_t - n_0)$ where P_0 is the fraction of tubes containing no mutants, m is the average number of mutations occurring during time t, and $n_t - n_0$ is the increase in the number of bacteria during time t. Substituting the experimental values, we find: $P_0 = 25/80 = e^{-m}$, $m = 1.33$; and $a = (1.33 \cdot 0.693)/(5.3 \cdot 10^6) = 1.7 \cdot 10^{-7}$. The mutation rate to the Mar phenotype of $1.7 \cdot 10^{-7}$ per cell generation is very similar to spontaneous rates exhibited by many other genes in bacteria (22). The calculation overestimates the rate of mutation per *mar* gene, since several *mar* genes probably exist in the *B. subtilis* genome, and their individual rates of mutation are combined by the above analysis.

Cytochrome phenotypes of *mar* mutants. Each *mar* strain was tested for cytochrome deficiencies; on this basis, three classes of *mar* mutants could be identified, based on the relative concentrations of cytochromes observed spectroscopically (28). These are summarized in Table 3, together with estimates of colony sizes of the mutant strains. Altogether, 14 strains were cytochrome deficient; the remaining 19 strains, although they were of the small-colony type, showed essentially the same cytochrome complement as the parent strain.

TABLE 2. *Fluctuation analysis of mar mutant selection in the presence of kanamycin*

No. of *mar* mutants in 17 different samples from the same culture[a]		Distribution of *mar* mutant cells in a series of 80 parallel cultures[b]	
Sample no.	No. of *mar* mutants present per sample	No. of *mar* mutants present in a culture sample	No. of cultures
1	6	0	25
2	5	1	12
3	11	2	7
4	12	3	6
5	7	4	4
6	9	5	3
7	5	6	1
8	3	7	3
9	9	8	2
10	2	9	2
11	8	10	1
12	5	11–20	3
13	10	21–30	4
14	9	31–100	4
15	6	>100	3
16	11		
17	5		

[a] Sample mean, 7.2; variance, 8.0.

[b] Total mutants, 3,156; sample mean, 39.5; variance, 3,818.

Effect of altered cytochrome phenotype on cell respiration. Cytochrome deficiencies previously have been shown to be associated with depressed rates of oxygen consumption in *B. subtilis* (27). In the present study, the three classes of *mar* mutants having altered cytochrome concentrations also showed lower whole-cell respiration rates when compared with the parent strain (Table 4). The strains listed are representative of the *mar* class to which each belongs. The *mar-2* and *mar-3* classes (which are severely cytochrome *a* deficient) were capable of oxidation rates only 30 to 40% of the parent strain when supplied with either glucose or succinate as substrate. *mar-1* strains were similarly depressed in succinate oxidation but oxidized glucose at about 60% the wild-type rate. Detailed information on oxygen consumption by strain RB163 *(mar-3)* has been published (9). The cytochrome deficiencies and depressed respiration rates were manifested in lowered growth rates of *mar-2* and *mar-3* mutants in either BHI broth or minimal glucose medium, whereas *mar-1* strains were virtually wild type in their growth characteristics (data not shown).

TABLE 3. *Cytochrome phenotypes of B. subtilis mar mutants*

Class	Colony size (mm)	No. of strains	Relative cytochrome concn				
			c	c_1	*o*	*b*	$a + a_3$
Parent	5–6		5	0	0	5	5
mar-1	3.4–4.5	6	1	5	5	1	2
mar-2	2–3	4	5	0	0	5	1
mar-3	0.5–1.5	4	±	5	5	±	±

TABLE 4. *Oxygen consumption by cultures of mar strains*

Class	Strain no.	Oxygen consumption[a]	
		Glucose	Succinate
Parent	RB1	250	260
mar-1	RB106	150	90
mar-2	RB120	70	99
mar-3	RB163	69	72

[a] Nanomoles of O_2 per minute per milligram (dry weight).

Depression of growth rates by aminoglycosides. We noted that the growth of *mar* mutants on solid media could be inhibited by increasing the concentration of aminoglycoside. We also found that both mutants and parent strain were susceptible to growth inhibition by neomycin at a much lower concentration than for kanamycin or paromomycin. To study this quantitatively, we determined the doubling times of parent and mutant strains in the presence of increasing concentrations of several aminoglycosides (Fig. 2). Each of the strains had a characteristic susceptibility to all three aminoglycosides; strain RB120 *(mar-2)* was least susceptible and strain RB163 *(mar-3)* was more susceptible, whereas strain RB106 *(mar-1)* had approximately the same susceptibility as strain RB1 (parent). The similarity in the responses of a given strain to several aminoglycosides suggested that the multiple-resistance

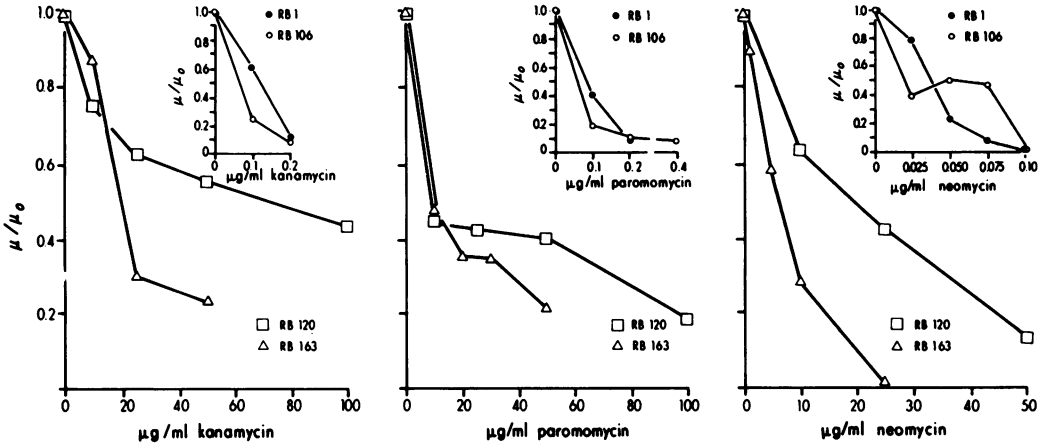

FIG. 2. *Decrease in growth rate of mar mutants on TG medium in the presence of three aminoglycosides. Shown are data for the parent strain (RB1), mar-1 (RB106), mar-2 (RB120), and mar-3 (RB163) mutants. μ/μ₀ is defined as the ratio [growth rate constant (+ antibiotic)]/[growth rate constant (− antibiotic)], which is equivalent to the experimentally derived ratio [1/doubling time (+ antibiotic)]/[1/doubling time (− antibiotic)].*

phenotype within a given *mar* class might have a unitary basis.

Inactivation of neomycin by a *mar*-1 mutant. As noted above, strains RB1 (parent) and RB106 *(mar-1)* were growth inhibited by very low concentrations of neomycin. Growth curves in TG medium are shown in Fig. 3. Strain RB1 remained inhibited by 0.075 μg of neomycin per ml up to 6.5 h of exposure, but strain RB106 began to grow after about 4 h of exposure to neomycin. This was not due to secondary mutations that might have arisen during the 4-h time period, since sampling of the culture of strain RB106 after the upturn in growth did not yield any isolates having resistance to a higher concentration of neomycin. We performed the following experiment to determine whether cells of strain RB106 were inactivating the neomycin present in the medium. TG medium containing 0.075 μg of neomycin per ml was conditioned by exposure to strain RB106 for 5 h (i.e., until growth began) and then freed of cells by centrifugation and membrane filtration. Strain RB1 was suspended in this conditioned medium, and growth was compared with that in fresh TG with and without neomycin. The result (Fig. 4) shows clearly that neomycin-containing medium, when exposed to strain RB106, lost its capacity subsequently to inhibit growth of strain RB1. A similar experiment, substituting strain RB1 for RB106 during the conditioning regimen, resulted in no loss of growth-inhibiting capacity.

Uptake of kanamycin by *mar* mutants. The cytochrome- and respiration-deficient properties of *mar-2* and *mar-3* mutants, together with the similar growth susceptibilities to several aminoglycosides, suggested to us that these strains might not be transporting the antibiotics efficiently to the cell interior. Uptake of kanamycin was measured as described in Materials and Methods (Fig. 5). The parent strain

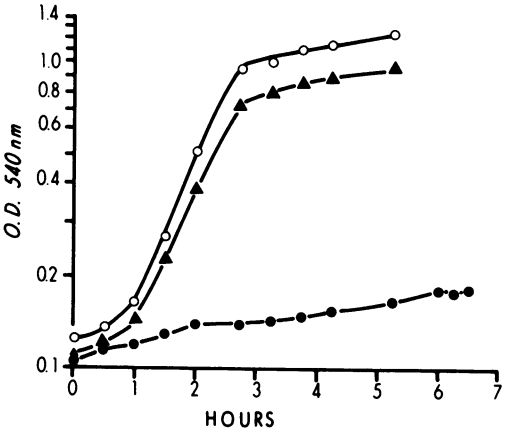

FIG. 4. *Growth of strain RB1 (parent) in TG medium + 0.075 μg of neomycin per ml, previously conditioned by growth of strain RB106 (see text). No neomycin (○); +neomycin, conditioned (▲); a control culture of RB1 in fresh (i.e., unconditioned) TG medium + 0.075 μg of neomycin per ml (●).*

(RB1) showed a time course of kanamycin uptake remarkably similar to streptomycin uptake by *E. coli* (2. 26). There was an initial rapid binding, complete within 30 s (the time required for sampling after addition of the drug). There appeared to be an ionic interaction with the cell surface, since immediate washing of the cells with 1.0 M NaCl reduced the value from ~0.25 to <0.05 μg/10^8 cells. The binding phase was followed by a phase of intracellular accumulation, which was unaffected by a salt wash; this phase was complete within 20 min, after which the concentration remained constant for about 30 min. A third uptake phase began at about 50 min and continued until the experiment was terminated at 90 min. The *mar-2* (strain RB120) and *mar-3* (strain RB163) mutants tested (Fig. 5) showed only the initial binding reaction, and the values could be uni-

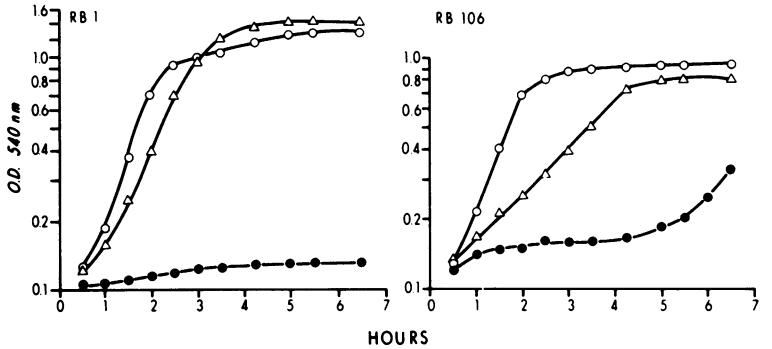

FIG. 3. *Effect of neomycin on growth of strains RB1 (parent) and RB106 (mar-1) in TG medium. No neomycin (○); 0.025 μg/ml (△); 0.075 μg/ml (●).*

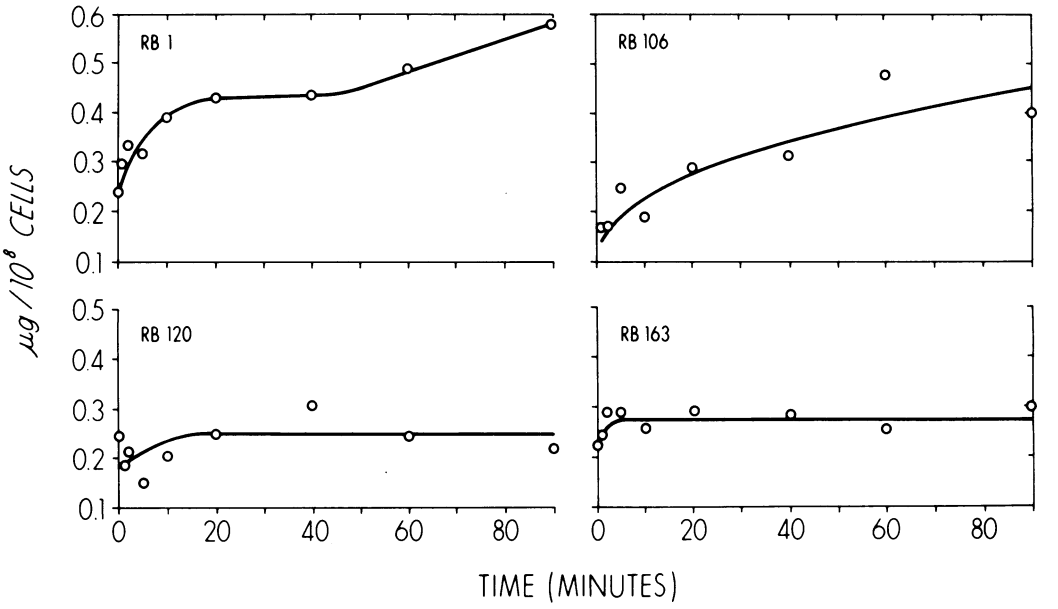

FIG. 5. *Uptake of kanamycin by the parent strain (RB1) and mar-1 (RB106), mar-2 (RB120), and mar-3 (RB163) mutants. The external kanamycin concentration was 10 μg/ml. The procedures for sampling and measuring kanamycin accumulation are described in detail in the text.*

formly decreased to <0.05 μg/10⁸ cells by washing with 1.0 M NaCl. The *mar-1* mutant (strain RB106) took up kanamycin with complex kinetics not referable to the parent strain (Fig. 5). In contrast to the other *mar* mutants, strain RB106 accumulated kanamycin in an intracellular compartment (i.e., not removable by a 1.0 M NaCl wash), although less rapidly than the parent strain. We would conclude from these experiments that the *mar-2* and *mar-3* mutants tested are deficient in intracellular accumulation of kanamycin in comparison with the parent strain.

Loss of viability of *mar* mutants in the presence of kanamycin. The uptake experiments were carried out in the presence of 10 μg of kanamycin per ml (see Materials and Methods), which had only a slight growth-inhibitory effect on *mar-2* and *mar-3* mutants but permited no growth at all by *mar-1* mutants and the parent strain (Fig. 2). It was therefore of interest to determine the effect of 10 μg of kanamycin per ml on the viability of *mar* mutants and the parent strain during the experimental period of kanamycin uptake. In Fig. 6 are shown survival curves for colony-forming ability of the parent strain and *mar-1*, *-2*, and *-3* mutants. The parent strain was killed most rapidly, but killing could be delayed during the first 20 min by washing with salt before dilution and plating. Strain RB106 (*mar-1*) was

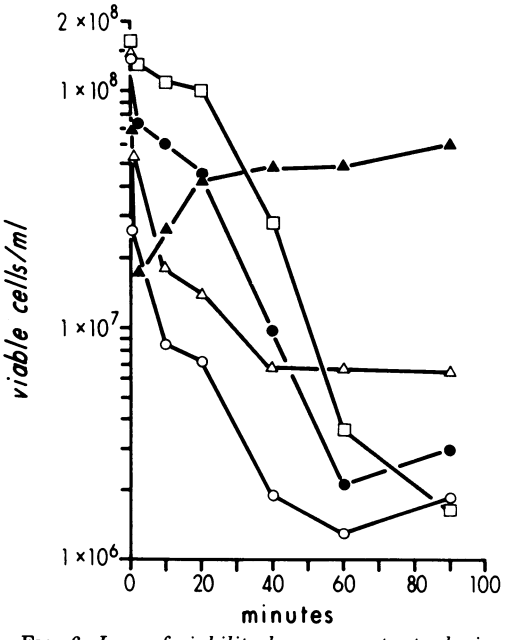

FIG. 6. *Loss of viability by mar mutants during exposure to 10 μg of kanamycin per ml in Penassay medium. Conditions of growth and exposure to antibiotic were as described in the text. Cultures were sampled at successive times, diluted rapidly in Penassay medium, and plated on BHI agar for measurement of colony-forming units. Colony-forming units were taken as a measure of viability. Strain RB1*

killed rapidly up to about 30 min, after which no further killing occurred; the resistant fraction was six- to sevenfold higher than strain RB1. Strain RB120 (*mar-2*) exhibited an initial decline and then recovered and survived to the extent of about 50%. Strain RB163 (*mar-3*) showed virtually no killing up to about 20 min, after which it declined with kinetics similar to those of salt-washed strain RB1 samples.

Several conclusions can be drawn by comparing Fig. 5 with Fig. 6. (i) Sufficient kanamycin is bound to the cell surface within 1 to 2 min to reduce viability of the parent strain by 90%; if this "exterior" kanamycin is removed before plating, killing is significantly reduced. (ii) Killing by kanamycin is reduced in the *mar-1* mutant, which takes up kanamycin more slowly than the parent. (iii) Although *mar-2* and *mar-3* are similar in that they do not accumulate appreciably intracellular kanamycin, they differ markedly from each other in their responses to its killing action: the *mar-2* strain shows relatively little killing on continued exposure, whereas 99% of the *mar-3* cells eventually are killed by surface-bound kanamycin.

DISCUSSION

The *mar* mutants described in this communication appear to be related to the small-colony, slowly growing *B. subtilis* mutants described by Marjai et al. (19). The latter mutants were isolated by plating strain 168 (*trpC2*) on nutrient agar containing 100 to 200 μg of streptomycin per ml and were found to be *aro* (aromatic amino acid requiring), *hem* (heme-requiring), or *nda* (nondefined auxotrophy) types. The isolates showed substantially higher resistance to both the bacteriostatic and bactericidal effects of streptomycin, although the effects of other aminoglycosides were not measured. We would conclude that *mar* mutations are not induced by the presence of kanamycin during our isolation procedure because wide fluctuations in numbers of mutants from parallel cultures were observed and because a mutation rate compatible with spontaneous mutation events could be calculated.

Staal and Hoch (25) reported two classes of conditional streptomycin-resistant mutants of *B. subtilis*. Both classes (*strB* and *strC*) were resistant to streptomycin during vegetative growth but developed susceptibility during sporulation. *strC* mutants were, in addition, cytochrome *a* deficient. However, neither class was more resistant than the parent strain to neomycin or kanamycin, and thus they appear to be different from the *mar* mutants described here.

Because of our interest in the use of mutants to study oxidative energy metabolism in *B. subtilis*, we have concentrated in the present report upon *mar* mutants that are cytochrome and respiration deficient. Representative strains from two classes (*mar-2* and *mar-3*) that are most deficient in oxidative metabolism also appear (within the considerable technical limitations of our uptake experiments) to be deficient in the ability to accumulate kanamycin from the external medium. One of these (the *mar-3* strain RB163) has been shown to be an *aroD* mutant, deficient in menaquinone when grown in media lacking shikimic acid but with the aromatic amino acids and *p*-aminobenzoic acid present (8, 9). When menaquinone-deficient, strain RB163 is kanamycin resistant, but it becomes kanamycin susceptible after a short period of growth in the presence of the menaquinone precursor shikimic acid (28; Taber and Halfenger, unpublished experiments). Bisschop et al. (4) have studied the active transport of L-glutamate by strain RB163 and have found that menaquinone deficiency prevents transport, but this is restored by growth of the cells on shikimic acid. Comparable studies have not been carried out on the *mar-2* strain RB120.

Although we have not established, with the aid of appropriate inhibitors, that the uptake of aminoglycosides by *B. subtilis* is an active transport process, a rough calculation of the amount taken up by the parent strain (Fig. 5) indicates that *B. subtilis* can concentrate kanamycin at least 100-fold over the surrounding medium.

The Mar-1 phenotype is apparently not due to a defect in accumulation of aminoglycosides since, for strain RB106, the uptake of kanamycin and susceptibility to growth inhibition by kanamycin, neomycin, and paromomycin is similar to that of the parent strain. Rather, this strain can negate the inhibitory effect of aminoglycoside present in the medium, possibly by chemical inactivation or by secretion of a competitive inhibitor for uptake and/or antiribosomal activity. The growth response of RB106 to medium containing neomycin is reminiscent of the inducible resistance to tetracycline displayed by *B. cereus* (5); Connamacher has suggested that the latter phenomenon is due to an increased rate of efflux of antibiotic (5) although, as discussed by Franklin (11), active efflux is difficult to establish unequivocably.

Decreased rates of tetracycline entry have

(parent) (○); strain RB1, washed with 1.0 M NaCl immediately after exposure to kan (●); strain RB106 (mar-1) (△); strain RB120 (mar-2) (▲); strain RB163 (mar-3) (□).

been shown to accompany R factor-mediated inducible tetracycline resistance in *E. coli* (10, 11, 17). Levy and McMurry (18) have detected at 50,000-dalton membrane protein in *E. coli* minicells carrying a tetracycline resistance R factor. The synthesis of this protein is inducible by tetracycline and may well be involved in the decreased tetracyline uptake in such strains. Induced tetracycline resistance has also been correlated with decreased uptake in *S. aureus* (24).

The present experiments do not shed much light on the mode of aminoglycoside lethality for bacterial cells, but the general correlation between decreased uptake of kanamycin and decreased lethality agrees with the recent results of Sakai and Cohen, in which a *rel*$^-$ strain of *E. coli* was found to be simultaneously more susceptible to streptomycin killing and more permeable to the drug in comparison with an isogenic *rel*$^+$ strain (23). This suggests that aminoglycosides must enter the cell, at least in small concentrations, in order to kill.

Genetic studies of *mar* strains and measurements of uptake using radioactively labeled aminoglycosides are now under way. Such studies should aid in establishing some principles governing the transport of this structurally related group of antibiotics and in discovering the normal cellular functions of this transport system.

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