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Streptomycin Resistance in a Streptomycin-Producing Microorganism

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Cell-free extracts of *Streptomyces bikiniensis* contain an adenosine 5'-triphosphate-dependent kinase which inactivates streptomycin (Sm) and dihydrostreptomycin by phosphorylation. The products have been identified as streptomycin 6-phosphate and dihydrostreptomycin 6-phosphate. Activity was not present in logarithmic-phase cells, which were susceptible to 25 μg of Sm per ml. In stationary-phase cells, activity appeared 12 h before detectable Sm in the medium. These cells were resistant to more than 200 μg of Sm per ml. Certain *S. bikiniensis* isolates selected from cultures treated with acriflavine or ethidium bromide lost the ability to produce Sm and became susceptible to 10 μg of Sm per ml throughout their growth. Cell-free extracts of the dye-treated isolates did not inactivate Sm and lacked streptomycin kinase activity at all stages in development. Ribosomes from resistant cells bound the same amount of [^{3}H]dihydrostreptomycin as ribosomes from susceptible cells, and there was no correlation between the uptake of [^{3}H]dihydrostreptomycin and resistance. The Sm-inactivating enzyme was identified as streptomycin-6-kinase. These results suggest that phosphorylation by streptomycin-6-kinase is a major factor in resistance in *S. bikiniensis*.

Streptomycin (Sm) is a potent inhibitor of procaryotic protein synthesis (17). Some microorganisms, however, are resistant to Sm because of either a decreased permeability to the drug (28), altered binding sites for the drug (1), or enzymes which modify the drug (1). Sm-producing procaryotes must also have mechanisms for escaping the effects of Sm, at least during the antibiotic production phase of their growth. It has been suggested (1, 10, 16) that the mechanisms of resistance developed by antibiotic-producing microorganisms to their own antibiotics are probably the same as those evolved by resistant microorganisms that do not produce antibiotics. Sm-modifying enzymes have been reported in Sm producers (16, 21, 22). These enzymes may be involved in the detoxification of Sm and the resistance mechanism in Sm-producing strains (1, 4, 16; L. C. Vining, Annu. Meet. Am. Chem. Soc., Abstr. no. 42, 1978); however, there is only circumstantial evidence to support this suggestion. In addition, studies on an Sm-producing *Streptomyces griseus* strain suggest that decreased permeability may be involved in resistance in that microorganism (4).

Streptomyces bikiniensis ATCC 11062 produces and is resistant to Sm. Treatment of spores of *S. bikiniensis* with acriflavine or ethidium bromide results in loss of both the ability to produce Sm and resistance to Sm in 2 to 16% of the colonies obtained from surviving cells (19). We examined the mechanism of Sm resistance in *S. bikiniensis* by using as controls dye-treated, Sm-susceptible isolates derived from the parent strain. Dye-treated isolates and parent strain cells were examined for the presence of Sm-inactivating enzymes, differences in Sm permeation, and differences in the susceptibility of the protein synthesis system to Sm.

MATERIALS AND METHODS

Bacterial strains and media. Stock cultures of *S. bikiniensis* ATCC 11062 and all isolates derived from it (dye-treated isolates) were maintained in sterile soil. Cultures were transferred to yeast malt extract (YMX) agar (Difco Laboratories) for use in the experiments. Unless otherwise stated, cells were grown in 2% peptone -0.2% yeast extract (PYX) medium. Spores from YMX agar slants were suspended in 5 ml of 0.05% Triton X-100. A 1-ml amount of the suspension was transferred to 100 ml of PYX medium. Cells were incubated at 26°C in a reciprocal shaker. *Escherichia coli* C600 was maintained on nutrient agar (Difco) and was grown in nutrient broth (Difco).

Growth curve determination. Spores from an agar slant were suspended in 10 ml of 0.05% Triton X-100. A 1-ml amount of the suspension was transferred to 30 ml of PYX medium in a 125-ml flask. Cells were harvested by centrifugation at various times after inoculation, and cell weight was determined after drying to constant weight in a vacuum desiccator over phosphorous pentoxide.

Sm resistance and production assays. *S. bikiniensis* was grown in Lumb medium, and Sm production was determined by paper disk assay as previously described (19). Portions (approximately 0.2 ml) of the same culture were removed at 0, 12, 24, 36, and 48 h after inoculation and applied to YMX agar containing 0, 10, 25, 50, 75, 100, 150, 200, 300, 400, or 500 μg of streptomycin sulfate per ml for determination of Sm susceptibility. Resistance was defined as the maximum Sm concentration allowing visible colony formation.

[^{3}H]DHSm and ^{14}C-amino acid uptake. Cells used for uptake studies were prepared as described by Cella and Vining (4) and suspended in PYX medium. A 15-ml amount of the PYX cell suspension was stirred slowly at room temperature. After 5 min, 4.0 μmol of [^{3}H]dihydrostreptomycin (DHSm; specific activity, 10 Ci/mol) or 4.0 μmol of ^{14}C-amino acid mixture (Amersham/Searle; specific activity, 1.25 Ci/mol) was added to the suspension. Samples (1 ml) were removed at 0, 5, 10, 15, and 30 min after the addition of DHSm or amino acid mixture and filtered on 0.45-μm membrane filters (Millipore Corp.). The cells were rinsed with 10 ml of 0.1 M Na_2SO_4 and 10 ml of distilled water and then air dried. Radioactivity was determined by liquid scintillation counting in a toluene-based scintillation fluid (4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-phenyloxazole per liter of toluene). The rate of DHSm or amino acid uptake was calculated from the linear portion of the uptake curve and is expressed as micromoles per milligram (dry weight) of cells per minute.

The extent of [^{3}H]DHSm uptake was measured by incubating 0, 5, 10, 25, 50, 75, 100, 150, 200, or 300 μg of [^{3}H]DHSm (specific activity, 20 Ci/mol) per ml with 5 ml of PYX cell suspension. After 2 h, 1-ml samples were removed, and radioactivity was measured on Millipore filters as described above.

In vitro binding of [^{3}H]DHSm to ribosomes. Cells from 30 ml of PYX were harvested by centrifugation at 300 × *g* for 5 min and washed twice in 10 ml of TMK buffer [10 mM tris(hydroxymethyl)-aminomethane, 10 mM magnesium acetate, 60 mM potassium chloride, pH 7.3]. Rinsed cells were homogenized in a Potter-Elvehjem homogenizer with 1.5 volumes of TMK buffer and passed through a French pressure cell at 8,000 lb/in^2. Cell extracts were centrifuged at 20,000 rpm in a Sorvall SS34 rotor for 15 min. The supernatant fraction was centrifuged for 4 h at 147,000 rpm in a Beckman 65 rotor. The ribosomal pellet was suspended in 2 ml of TMK buffer and used for binding studies without further purification.

The binding mixture (110 μl) contained 0.016 μg of [^{3}H]DHSm (specific activity, 3.0 Ci/mmol) and 0.275 mg of crude ribosome preparation (16 absorbance units [260 nm] per mg of ribosomes) (27). After a 10-min incubation at 4°C, the binding mixture was layered onto a linear sucrose gradient (15 to 30%, wt/vol, in glass-distilled water). The gradients were centrifuged for 4 h at 30,000 rpm in a Beckman SW41 rotor. The number of DHSm molecules bound per 30S ribosomal subunit was calculated by assuming that 1 absorbance unit (260 nm) equaled 70 pmol of ribonucleic acid (18).

Sm-inactivating enzymes. Cell-free extracts were prepared by the technique of Walker and Walker (22) in TMS buffer [10 mM tris(hydroxymethyl)aminomethane hydrochloride, 10 mM magnesium chloride, 25 mM ammonium chloride, 0.6 mM β-mercaptoethanol, pH 7.8]. Protein determinations were made by using the colorimetric assay of Lowry et al. (15). Assay mixtures containing equal volumes of cell-free extracts of dye-treated isolates or parent strain cells, 1 mM streptomycin sulfate, 40 mM adenosine 5′-triphosphate, disodium salt (ATP) (in water, pH 7.6), and 100 mM magnesium acetate were incubated for 1 h at 30°C. Portions of the reaction mixtures were assayed for biological activity as previously described (19). Streptomycin kinase (Sm kinase) activity was assayed by the phosphocellulose-paper binding assay of Davies et al. (8). Reaction volumes of 155 μl contained: 0.32 mM streptomycin sulfate, 0.32 mM dihydrostreptomycin sulfate, or 1 mM streptidine sulfate; 0.65 mM [γ-^{32}P]ATP (specific activity, 20 Ci/mol); 1, 10, or 100 μl of cell-free extract (10.6 mg of protein per ml); and glycylglycine buffer (GGB; 0.5 M glycylglycine, 40 mM magnesium chloride, pH 8.0) to volume. Sm kinase was partially purified from 48-h PYX cultures of parent strain cells by the procedure of Miller and Walker (16).

Identification of streptomycin phosphate (SmP). Phosphorylated Sm derivatives labeled with [^{32}P]phosphate were identified by ascending paper chromatography (22), high-voltage electrophoresis (pH 10.4) (22), and ion-exchange chromatography (16). Radioactivity was detected on the paper chromatograms and electrophoretograms by autoradiography, using Kodak No Screen X-ray film (Eastman Kodak Co.), and in the ion-exchange column effluents by liquid scintillation counting in Bray scintillation fluid (2).

RESULTS

Sm resistance. Sm-producing organisms have been reported to be susceptible to their own antibiotic during the logarthmic phase of their growth cycle. They develop resistance to Sm during the stationary phase of growth when antibiotic production begins (24). Figure 1 shows the relationship between growth, Sm resistance, and antibiotic production in the parent strain of *S. bikiniensis*. Cells became resistant to Sm at the beginning of the stationary phase of growth, 12 h before commencement of antibiotic excretion. Dye-treated isolates of *S. bikiniensis*, which did not make detectable antibiotic at any stage in their growth cycle (19), grew on control plates without Sm, but not on plates with 10 to 500 μg of Sm per ml. In contrast to the phenomenon observed in the parent strain, no resistance to Sm was seen in the stationary phase of the growth cycle.

Sm and amino acid uptake. The rate of DHSm uptake by the parental strain of *S. bikiniensis* and four dye-treated isolates was measured to determine the possible role of Sm permeability in Sm resistance. The rate of [^{3}H]DHSm uptake in the parental strain cells and dye-treated isolate 3A138 is shown in Table

1. During the late stationary phase of growth (36-h cultures), the rate of [^{3}H]DHSm uptake decreased to about the same level in the parent strain and dye-treated isolate. At this stage in development dye-treated isolates were susceptible to 5 μg of Sm per ml, whereas parent strain cells were resistant to more than 400 μg of Sm per ml. The rate of ^{14}C-amino acid uptake, used as a measure of cell permeability, decreased to approximately the same levels in both the parent strain and the dye-treated isolate 3A138 during development. The effect of DHSm concentration on DHSm uptake in the parent strain *S. bikiniensis* and one of the four isolates (3E68) examined in the rate studies was examined at all stages in the growth cycle. In the parent strain, total uptake at each DHSm concentration decreased from logarithmic to late stationary phase (Fig. 2A). Figure 2B shows [^{3}H]DHSm uptake in dye-treated isolate 3E68. The total DHSm uptake by 3E68 cells at any DHSm concentration was always equal to or less than the total DHSm uptake by the parent strain at the same stage in the growth cycle. In the presence of 15 mM sodium azide, [^{3}H]DHSm uptake in the parent strain was inhibited 95%.

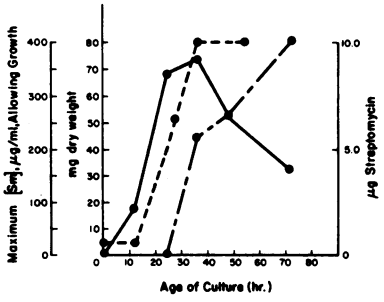

FIG. 1. *Growth, antibiotic production, and resistance in parent strain S. bikiniensis. Symbols: —, dry weight (in milligrams); – – – –, Sm production (in micrograms of Sm per 100 μl of culture medium; - - - -, Sm resistance, expressed as maximum Sm concentration ([Sm]; in micrograms per milliliter) allowing visible colony formation.*

In vitro binding of Sm to ribosomes. Ribosome preparations were separated into three major components on linear sucrose gradients. [^{3}H]DHSm comigrated with the 30S ribosomal subunits and 70S ribosomes, but no [^{3}H]DHSm was associated with the 50S ribosomal subunit. Ribosomes from *E. coli* bound 0.4 [^{3}H]DHSm molecules per 30S subunit, as did dye-treated isolate 3A138 (Table 2). Both of these strains are susceptible to low levels of Sm. Ribosomes from stationary-phase *S. bikiniensis* cells bound 0.3 [^{3}H]DHSm molecules per 30S subunit, the same number of [^{3}H]DHSm molecules bound by ribosomes from logarithmic-phase cells, which are 20 times more susceptible to Sm (Table 2).

Sm-inactivating enzyme. Cell-free extracts of stationary-phase parental strain cells biologically inactivated more than 16 mg of Sm per mg of protein per h in the presence of 40 mM ATP and 100 mM Mg^{2+}. Less than 0.05 mg of Sm per mg of protein per h was inactivated in the absence of ATP and Mg^{2+} or with a boiled, cell-free extract in the presence of Mg^{2+} and ATP. Antibiotic activity was restored by treatment with *E. coli* alkaline phosphatase for 1 h at 37°C and pH 8.0. Cell-free extracts also formed ^{32}P-labeled SmP when incubated with Sm, [γ-^{32}P]-ATP, and Mg^{2+}. Alkaline phosphatase removed all of the radioactivity from the SmP. Figure 3 shows the relationship between antibiotic activity of Sm and extent of Sm phosphorylation. After 1 h, 63 nmol of Sm was inactivated and 53 nmol of [^{32}P]SmP was formed. Approximately one equivalent of [^{32}P]SmP was formed for each equivalent of Sm inactivated during this period. When the mixture was incubated with *E. coli* alkaline phosphatase, one equivalent of antibiotic activity was restored for each equivalent of phosphate lost from SmP.

Increases in cell-free extract concentration of 100-fold resulted in no increase in rates of phosphorylation. After 5 min the amount of radioactive SmP decreased, approaching background levels after 15 min. Cell-free extracts of *S. bikiniensis* have been reported to contain phosphatases which act on SmP (23). Addition of inorganic phosphate to these cell-free extracts was

TABLE 1. *Rate of DHSm and amino acid uptake by S. bikiniensis isolates*

Isolate	Culture age (h)	Rate of DHSm uptake (pmol of [^{3}H]DHSm/mg [dry wt]/min)	Rate of amino acid uptake (pmol of ^{14}C-amino acid/mg [dry wt]/min)
Parent strain	12	349	184
	24	1,065	81
	36	200	0
Dye-treated isolate 3A138	12	886	215
	24	254	91
	36	117	42

reported to inhibit phosphatase activity (23). Inorganic phosphate (40 mM KH_2PO_4) both increased the initial rate of phosphorylation of Sm and prevented loss of radioactive SmP in our assays. Addition of ATP after 5 min also prevented loss of radioactive SmP upon subsequent incubation. As a result of these observations, all subsequent Sm kinase assays were carried out in the presence of 40 mM inorganic phosphate to minimize dephosphorylation. In assays measuring biological inactivation by Sm kinase, the addition of inorganic phosphate was unnecessary because of the high levels of ATP used in the mixture (data not shown).

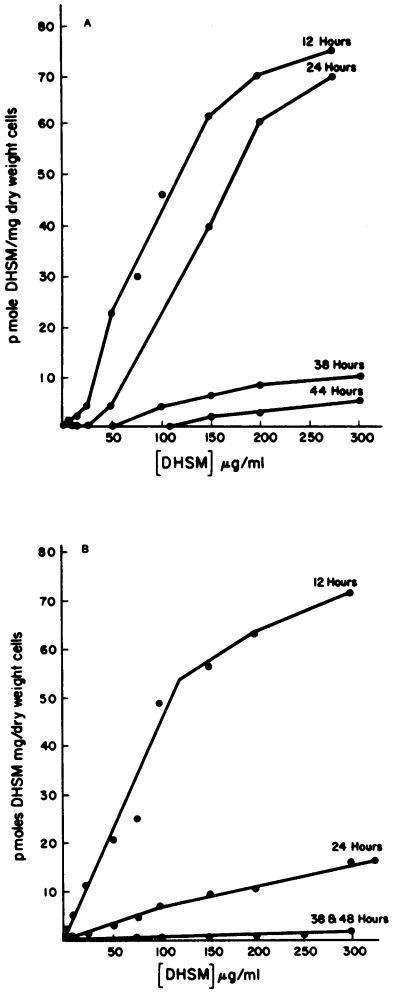

FIG. 2. *(A) Extent of [^{3}H]DHSm uptake by parent strain S. bikiniensis at various culture ages and external DHSm concentrations ([DHSm]). (B) Extent of [^{3}H]DHSm uptake by dye-treated isolate 3E68 at various culture ages and external DHSm concentrations.*

Sm kinase activity was separated from competing reactions by elution of a 0 to 70% ammonium sulfate fraction of the crude cell extract through a Sephadex G-200 column (16). With these preparations, phosphorylation of Sm was directly proportional to enzyme concentration (Fig. 4). The partially purified enzyme inactivated and phosphorylated Sm. The phosphorylated product formed by the purified enzyme had the same R_f on paper chromatograms and the same elution pattern on Bio-Rex 70 (NH_4^+) columns as SmP formed by the crude cell extracts.

Sm kinase activity could not be detected in cell-free extracts of logarithmic-phase, parent strain cells. Cells were susceptible to 50 µg of Sm per ml at this stage in their growth cycle (Fig. 1). Activity appeared at the beginning of the stationary phase of growth (24 h), coinciding with the appearance of increased Sm resistance in these cells. Sm kinase activity increased from 0.2 to 0.5 pmol of SmP formed per µg of protein per min during the stationary phase of the growth cycle (Fig. 5). Sm resistance increased during this period from 200 to more than 500 µg of Sm per ml.

A total of 30 dye-treated isolates were assayed for Sm kinase activity. Cell-free extracts from stationary-phase cells of all 30 isolates failed to inactivate Sm when incubated with ATP and Mg^{2+}. Ten of these isolates were tested for Sm-phosphorylating enzymes. Cell-free extracts of these 10 isolates did not phosphorylate Sm in the kinase assay. Dye-treated isolate 3A47 was selected arbitrarily from the original 30 isolates for further study. Cell-free extracts from logarithmic-phase and early- and late-stationary-phase cells of 3A47 had no detectable Sm kinase activity in the presence or absence of inorganic phosphate. It appears that all *S. bikiniensis* cells which are susceptible to low levels of Sm,

TABLE 2. *Binding of DHSm to purified ribosomes*

Source of ribosomes	pmol of [^{3}H]DHSm/ pmol of 30S subunits
E. coli (Sm susceptible)	0.4
S. bikiniensis (parent strain)	
Logarithmic phase	0.3
Stationary phase	0.3
Dye-treated isolate 3A138 stationary phase	0.4

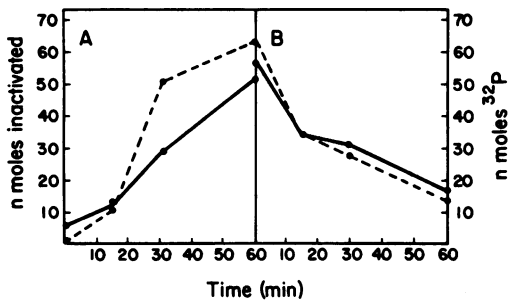

FIG. 3. *(A) Biological inactivation and phosphorylation of Sm by cell-free extracts of S. bikiniensis. (B) Regeneration of Sm activity and dephosphorylation of SmP by alkaline phosphatase. ----, nanomoles of Sm inactivated; —, nanomoles of [^{32}P]SmP. The reaction mixture (295 μl) contained 0.26 nM streptomycin sulfate in GGB, 0.35 nM [^{32}P]ATP in GGB (specific activity, 20 Ci/mol), 15 μl of cell-free extract, and GGB to volume. After 60 min, 0.6 U of E. coli alkaline phosphatase (type III; Sigma Chemical Co.) was added. Samples were removed at various times and assayed for biological activity and radioactivity as described in the text.*

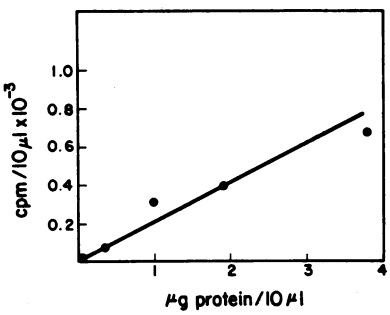

FIG. 4. *Effect of purified enzyme concentration on phosphorylation of Sm. Enzyme activity was assayed by the Sm kinase assay. Samples were removed after 30 min, and the net phosphorylation is expressed in counts per minute per 10 μl of reaction mixture.*

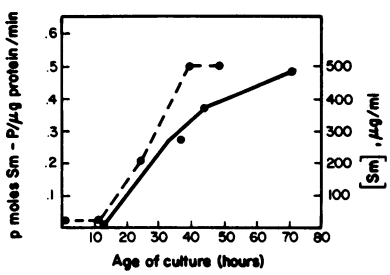

FIG. 5. *Sm kinase activity in crude extracts at various culture ages. —, Sm kinase activity (picomoles of [^{32}P]SmP formed per microgram of protein per minute); ----, resistance of culture (maximum Sm concentration [Sm], in micrograms per milliliter, allowing visible colony formation).*

whether they are logarithmic-phase parental strain cells or cells from dye-treated isolates, lack detectable Sm kinase. Sm kinase, however, was always present in cells resistant to Sm levels greater than 50 μg/ml.

SmP identification. The location of the phosphate(s) group on SmP or DHSmP formed by the incubation of Sm or DHSm with cell-free extracts of *S. bikiniensis*, ATP, and Mg^{2+} has been reported previously (16, 21, 22). Paper chromatography showed the cell-free extracts of *S. bikiniensis* formed one phosphorylated compound when incubated with Sm or DHSm plus [^{32}P]ATP and Mg^{2+}, which was not formed when cell-free extracts were incubated with [^{32}P]ATP and Mg^{2+} alone. SmP had an R_f of 0.36, and DHSmP had an R_f of 0.65. SmP and DHSmP were eluted from a Bio-Rex 70 column by 0.8 M ammonium formate. DHSmP from the 0.8 M ammonium formate wash was analyzed by paper chromatography. Only one phosphorylated compound was present, and its R_f corresponded to DHSmP formed in the reaction mixture. When analyzed by high-voltage electrophoresis, SmP and DHSmP had mobilities of −0.08 and −0.24 cm from the origin, respectively. These results are consistant with the phosphate being located on position 6 of the streptidine ring of Sm (20). In addition, partially purified Sm kinase phosphorylated streptidine sulfate, although the extent of streptidine phosphorylation per milligram of protein was 20% that of Sm.

DISCUSSION

Development of antibiotic resistance at or shortly before antibiotic production begins has been reported in many antibiotic-producing organisms (11, 26), including Sm producers (3, 24). The mechanisms by which resistance in antibiotic-producing microorganisms increases during their production phase have been reviewed recently (10). *S. bikiniensis*, an Sm producer, appears to follow this same pattern of resistance development. Logarithmic-phase cells are susceptible to Sm, whereas stationary-phase cells are resistant to high levels of Sm (Fig. 1). Treatment of *S. bikiniensis* with dyes results in loss of Sm resistance, as well as Sm production, with high frequency (19), and the dye-treated isolates appeared to differ from parent strain cells only in their Sm susceptibility and lack of Sm production. Comparisons of parent strain cells and dye-treated isolates at each stage of development therefore offered a means of distinguishing between factors that were involved only in the development of the culture (13) and those specifically involved in Sm resistance.

The rate of DHSm uptake by the parent strain

and dye-treated isolates decreased to approximately the same level during the stationary phase of growth (Table 1). The rate of amino acid uptake also decreased by the same amount in both the parent strain cells and cells of the dye-treated isolate. The extent of DHSm uptake at various external DHSm concentrations in the dye-treated isolate was always equal to or less than the extent of DHSm uptake by the parent strain cells at the same external concentration, regardless of the stage of culture development (Fig. 2). If permeability barriers to Sm were responsible for resistance in *S. bikiniensis*, one would expect the rate and extent of uptake to be inversely proportional to the level of resistance. This is not the case. The rate and extent of DHSm uptake by dye-treated isolates decrease to approximately the same levels as observed in the parent strain during the stationary phase of growth, with no corresponding increase in resistance. The decreased amino acid uptake that also occurred during this time suggests that the observed decrease in DHSm uptake was due to a general decrease in cell permeability during this stage of development. Cella and Vining (4) observed a decrease in DHSm uptake during the stationary growth phase of an Sm-producing *S. griseus* strain. They attributed this decrease to a blockade in the uptake mechanism which resulted in increased resistance. This does not appear to occur in all Sm producers, and, at least in *S. bikiniensis*, decreased uptake does not appear to play a significant role in protecting cells from the lethal effects of Sm.

Alternatively, stationary-phase *S. bikiniensis* may escape the lethal effects of Sm by developing an Sm-insensitive protein synthesis system. In non-antibiotic-producing procaryotes this occurs through alteration of the 30S ribosomal subunit (7, 9). Ribosomes from these organisms no longer bind Sm or DHSm (5, 12, 25). Ribosomes from cells of *S. bikiniensis* which were resistant to Sm bound approximately the same amount of antibiotic as did ribosomes from cells which were susceptible to Sm (Table 2). It seems unlikely, then, that Sm resistance in stationary-phase *S. bikiniensis* was due to alteration in the binding of Sm to ribosomes. Similarly, the Sm tolerence of an Sm-producing *S. griseus* strain cannot be attributed to the presence of Sm-resistant ribosomes (3).

An Sm-inactivating enzyme was detected in cell-free extracts from parent strain *S. bikiniensis* cells which were resistant to high levels of Sm (Fig. 1). Cell-free extracts from these cells also catalyzed the transfer of a phosphate group from ATP to the Sm molecule; 1 pmol of Sm was inactivated for each picomole of phosphate lost from the molecule (Fig. 3A), suggesting that the inactivating enzyme was a kinase which phosphorylated Sm. In addition, biological activity could be restored by treating SmP with bacterial alkaline phosphatase (Fig. 3B), and partially purified Sm kinase from these cells phosphorylated and biologically inactivated Sm.

Several Sm-phosphorylating enzymes have been reported (16, 22), but their roles in Sm metabolism have not been clearly defined. Miller and Walker (16) have suggested that ATP:streptomycin-6-phosphotransferase (Sm-6-kinase), a stationary-phase enzyme found in *S. bikiniensis*, may serve to keep Sm in an inactive phosphorylated form before its secretion from the cell. Two additional phosphorylating enzymes have been reported in streptomycete species. *S. griseus* ATCC 10971 contains a kinase that phosphorylates the 3 position of the *N*-methyl-L-glucosamine ring. This enzyme phosphorylates the same position of the Sm molecule as Sm-inactivating enzymes from plasmid-containing enterobacteria (8). A second phosphorylating enzyme has been reported in *S. bikiniensis* (ATP:dihydrostreptomycin-6-P-3α'-phosphotransferase) which phosphorylates the 3α' position of the dihydrostreptose ring of DHSm. The products formed by all three of these enzymes can be identified by several means (20). Results of paper chromatography, ion-exchange chromatography, and high-voltage electrophoresis of SmP and DHSmP formed by cell-free extracts and partially purified kinase are all consistent with the phosphate being located on the 6 position of the streptidine ring. The product of the kinase reaction and the ability of the enzyme to phosphorylate streptidine indicated that the enzyme involved in the inactivation of Sm is the Sm-6-kinase reported by Miller and Walker (16).

Clinical isolates of Sm-resistant bacteria containing Sm-phosphorylating enzymes modify Sm by addition of a phosphate group at the 3 position of the *N*-methyl-L-glucosamine ring of Sm. The only known exception to this is a strain of *Pseudomonas aeuriginosa* which also phosphorylates the 6 position of the streptidine ring of Sm (14). The possibility that antibiotic-producing organisms may be the source of genes for resistance-associated enzymes in these organisms has been suggested (1). There is evidence to support this suggestion in kanamycin, gentamicin (1), and neomycin resistance (6). Sm resistance seems an exception to this observation because the Sm-inactivating enzyme in *S. bikiniensis* differs from inactivating enzymes in plasmid-containing clinical isolates.

Sm-6-kinase activity paralleled the level of resistance in the parent strain (Fig. 5). No enzyme activity was detected in dye-treated isolates at any stage in development and the iso-

lates were susceptible to Sm throughout their growth. This indicates that Sm-6-kinase activity was closely related to the level of Sm resistance in *S. bikiniensis*. Our studies on the mechanism of Sm resistance in *S. bikiniensis* suggest that Sm-6-kinase plays a major role in the resistance mechanism. To our knowledge this is the first evidence that an Sm-phosphorylating enzyme confers resistance in a producing organism. In addition, the absence of Sm-6-kinase in the dye-treated isolates (19) suggests that resistance in *S. bikiniensis* may result from phosphorylation of Sm at the 6 position of the streptidine ring by a plasmid-encoded kinase.

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