JOURNAL OF BACTERIOLOGY, May 1982, p. 598–603
0021-9193/82/050598-06$02.00/0

Vol. 150, No. 2

Control of the Actinomycin Biosynthetic Pathway in and Actinomycin Resistance of *Streptomyces* spp.

KOZO OCHI†

Department of Microbiology, Georgetown University Schools of Medicine and Dentistry, Washington, D.C. 20007

Received 24 August 1981/Accepted 7 January 1982

Using actinomycin-producing and nonproducing strains of *Streptomyces antibioticus*, I studied several steps in the biosynthetic pathway of this antibiotic. Actinomycin-nonproducing strains derived after acriflavine or novobiocin treatment showed activity of kynurenine formamidase and phenoxazinone synthase as high as that of the parental strain, but these nonproducing strains failed to convert 4-methyl-3-hydroxy-anthranilic acid to actinomycin. In addition, accumulation of 4-methyl-3-hydroxyanthranilic acid (in the presence of D-valine) was not detected in the nonproducing isolates. Actinomycin-nonproducing strains derived after acriflavine treatment of *Streptomyces parvulus* showed a drastic decrease of resistance to the antibiotic. However, these strains regained resistance after preincubation with a small amount of actinomycin D.

A plasmid seems to be involved in the synthesis of several antibiotics, as has been suggested by genetic analyses (1, 23). In particular, the synthesis of actinomycin may require a plasmid in both *Streptomyces parvulus* (15, 18) and *Streptomyces antibioticus* (15). No actinomycin was detected in the culture filtrate or the mycelium of presumptively plasmid-cured strains (17). In this communication, the biosynthesis of actinomycin in *S. antibioticus* and the resistance to the antibiotic are examined in actinomycin nonproducing (presumptively plasmid-cured) strains.

MATERIALS AND METHODS

Organisms and growth conditions. The actinomycin-nonproducing variants AF-A4 and AF-A7 were derived after acriflavine treatment, and the strains NB-A7 and NB-A8 were derived after novobiocin treatment of *S. antibioticus* strain 3720 (Rutgers collection) as previously described (17). The actinomycin-nonproducing variant AF3-1 of *S. parvulus* ATCC 12434 was also obtained after acriflavine treatment (17). These strains were maintained on slants containing soluble starch-yeast extract-malt extract agar (6) at 4°C. None of the cured strains produced actinomycin on soluble starch-yeast extract-malt extract agar, in glutamic acid-galactose-glucose medium (9), or in glutamic acid-histidine-fructose medium (22), as determined by both a photometric assay (11) and a bioassay (16).

The strains were grown in NZ-amine medium (8) for 2 days, and harvested mycelia were washed with cold water. The washed cells were inoculated (8) into a chemically defined medium, and the synthesis of actinomycin was measured as previously described (8).

Determination of resistance to actinomycin. For the study of resistance, NZ-amine medium was used because it does not allow actinomycin production. After growth for 2 days, 1-ml samples were transferred into 25 ml of the same medium (in a 250-ml flask) containing various amounts of actinomycin D. After 48 h of incubation at 30°C, the optical density at 600 nm (OD_{600}) of the broth were measured. The actinomycin concentration causing 50% growth inhibition was determined by interpolation. It was confirmed in some cases that the OD_{600} values and measurements of dry cell weight per volume increased in parallel. One OD_{600} unit corresponded to 300 μg of dry cell weight.

Uptake of actinomycin D into protoplasts. The protoplast preparation and the composition of protoplast buffer were previously reported (5, 16). The protoplasts were washed twice with 10 ml of protoplast buffer and suspended in protoplast buffer at an OD_{600} of 2.0. ^{3}H-labeled actinomycin D (final concentration, 8 μM; 0.006 μCi/ml) was added to 5 ml of the protoplast suspension in a 25-ml flask, which was then incubated at 30°C using a Dubnoff metabolic shaker (120 cycles per min). At the indicated times the suspension was chilled in an ice bath and immediately centrifuged for 1 min at 10,000 × *g* at 4°C; the centrifuge was rapidly decelerated. The pelleted protoplasts were washed twice with 5 ml of cold protoplast buffer. Three milliliters of deionized water was then suddenly added to rupture the protoplasts. After standing for 30 min at room temperature the suspension was centrifuged (10 min at 12,000 × *g*). One milliliter of the supernatant was added to 10 ml of Bray scintillation fluid for measurement of radioactivity in a refrigerated Nuclear Chicago Mark I spectrometer.

Preparation of extracts for enzyme assay. Mycelia (5 g, wet weight) were suspended in 4 ml of deionized water and broken in a French pressure cell at 1,200 lb/

† Present address: Laboratory of Molecular Biology, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, MD 20205.

in^2 (two times) followed by centrifugation at 27,000 × *g* for 20 min. The supernatant was used for enzyme assays.

Enzyme assays. Phenoxazinone synthase was assayed in 80 mM potassium acetate buffer (pH 5.3) with 3-hydroxyanthranilic acid as substrate by following the increase of OD_{452} (10). Kynurenine formamidase was assayed in 50 mM potassium phosphate buffer (pH 7.4) with *N*-formylkynurenine as substrate by following the increase of OD_{365} in an automatic Gilford recording spectrophotometer (model 2000; Gilford Instrument Laboratories, Inc., Oberlin, Ohio) (3). Enzyme activity was expressed as nanomoles product per milligram of protein per minute. Protein concentration was assayed by the procedure of Lowry et al. (14) with serum albumin as standard.

Short-term labeling experiments. Five milliliters of a 48-h-old culture of *S. antibioticus* in glutamic acid-galactose-glucose medium was transferred into a Dubnoff metabolic flask (50 ml) containing the ^{14}C-labeled amino acids indicated below and shaken at 30°C. At the indicated times the culture was filtered through tightly packed glass wool. To prepare a neutral extract, 0.2 ml of 2 M Tris-hydrochloride (pH 7.5) was added to 2 ml of filtrate, and the mixture was extracted with 4 ml of ethyl acetate. Subsequently, the aqueous layer was removed, adjusted to pH 2.0 by 6 N HCl, and again extracted with 4 ml of ethyl acetate (acidic extract).

Chromatographic procedures. The neutral and acidic ethyl acetate extracts were evaporated to dryness at 30°C. Samples (along with standards) were then dissolved in 50 μl of methanol; 50 μl was applied to 20- by 20-cm thin-layer plates containing silica gel 60 F_{254} (0.25-mm thickness; Merck & Co., Inc., Rahway, N.J.). The plates were eluted with chloroform-acetic acid (70:30, by volume). The metabolites on the chromatograms were visualized under a UV lamp (Mineralite UVS-11; Ultraviolet Products, Inc., San Gabriel, Calif.). Radioactive metabolites were detected by autoradiography with either a Du Pont Cronex daylight pack (Du Pont Co., Wilmington, Del.) or Kodak SB-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.; 3 to 5 days exposure).

Reagents and radioisotopes. 3-Hydroxyanthranilic acid was purchased from Mann Research Laboratories, Orangeburg, N.Y.; L-3-hydroxykynurenine (free base) was from Calbiochem-Behringer Corp., La Jolla, Calif.; *N*-formyl-L-kynurenine (B grade, ring monoformyl, catalog no. 34423) was from Calbiochem-Behringer; crystalline bovine serum albumin (fraction V) was from Sigma Chemical Co., St. Louis, Mo.; L-[*methyl*-^{14}C]methionine (48.9 mCi/mmol) was from New England Nuclear Corp., Boston, Mass.; and DL-[benzene ring-*U*-^{14}C]tryptophan (60 mCi/mmol) was from Amersham/Searle, Des Plaines, Ill. [benzene ring-^{14}C]4-Methyl-3-hydroxyanthranilic acid (33,000 cpm/μmol) was generously provided by T. Troost (20). ^{3}H-labeled actinomycin was prepared in this laboratory by using L-[*methyl*-^{3}H]methionine as a substrate.

RESULTS

Phenoxazinone synthase and kynurenine formamidase activity in actinomycin-producing and nonproducing strains. The biosynthetic pathway of actinomycin in *S. antibioticus* has been suggested by Katz and Weissbach (7, 12) and is summarized in Fig. 1. The specific activity of two enzymes in this pathway was measured in both actinomycin-nonproducing strains of *S. antibioticus* and their parent (Table 1). The activity of kynurenine formamidase was similar in all strains. The activity of phenoxazinone synthase (10; H. Weissbach and E. Katz, J. Biol. Chem. **236**:PC17-18, 1961) differed in the different strains by up to a factor of 3.6. These differences may result from the fact that this enzyme is under the control of catabolite repression (4). The results suggest that neither the genes coding for phenoxazinone synthase nor kynurenine formamidase is carried on or controlled by the presumptive plasmid that may mediate actinomycin formation.

Inhibition of actinomycin formation by D-valine. 4-Methyl-3-hydroxyanthranilic acid is one of the intermediates in actinomycin biosynthesis (20, 21) (Fig. 1). D-Valine causes its accumulation and inhibits actinomycin synthesis (2) (Table 2). Incorporation was inhibited 90 to 100% by D-valine. In contrast, the radioactivity in the acidic extract slightly increased. In two actinomycin nonproducing strains (AF-A4 and NB-A7) no incorporation of radioactivity into material extractable at neutral pH was detected, even without D-valine addition. However, a large amount of [benzene ring-^{14}C]tryptophan was incorporated into material extractable at acidic pH.

Determination of metabolites. To determine which metabolites in the actinomycin pathway

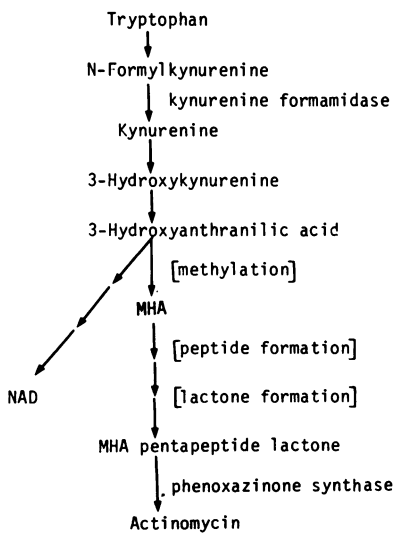

FIG. 1. Biosynthetic pathway of actinomycin. MHA, 4-Methyl-3-hydroxyanthranilic acid.

TABLE 1. Kynurenine formamidase and phenoxazinone synthase in actinomycin-producing and nonproducing strains of *S. antibioticus*[a]

Strain[b]	Sp act (nmol of product/mg of protein/min)	
	Kynurenine formamidase	Phenoxazinone synthase
Parent (3720)	43.5	122
AF-A4	44.2	71
AF-A7	22.7	190
NB-A7	48.1	139
NB-A8	44.2	254

[a] The cells for the crude enzyme preparation were obtained after 48 h of culture of each strain in glutamic acid-galactose-glucose medium (9).

[b] Actinomycin-nonproducing strains AF-A4 and AF-A7 were derived from producing strain 3720 by acriflavine treatment; other nonproducing strains NB-A7 and NB-A8 were made by novobiocin treatment. All strains are prototrophic.

accumulated during D-valine treatment or in the nonproducing mutants, the acidic extract was chromatographed on thin layers (Fig. 2). 4-Methyl-3-hydroxyanthranilic acid accumulated when the parent (3720) of *S. antibioticus* was incubated with [*methyl*-^{14}C]methionine in the presence of D-valine (about a sixfold increase above the counts without D-valine). No accumulation was observed in the actinomycin nonproducing strain AF-A4, without and with D-valine (less than 1/30 of the parent). In six other nonproducing isolates obtained after acriflavine or novobiocin treatment, again no accumulation of 4-methyl-3-hydroxyanthranilic acid was observed (data not shown).

To confirm the lack of accumulation of 4-methyl-3-hydroxyanthranilic acid in the actinomycin-nonproducing strain, [benzene ring-^{14}C]tryptophan was also used to label this compound. The acid extracts, analyzed by thin-layer chromatography (Fig. 3), again did not show accumulation of 4-methyl-3-hydroxyanthranilic acid in the nonproducing isolate, whereas it did in the parental strain (accumulation was more pronounced in the presence of D-valine). Note that 3-hydroxyanthranilic acid (precursor of 4-methyl-3-hydroxyanthranilic acid) also did not accumulate in the nonproducing strain (Fig. 3). Other differences between parent and mutants were observed in the chromatographs (Figs. 2 and 3), but the metabolites corresponding to these spots were not identified.

Incorporation of labeled 4-methyl-3-hydroxyanthranilic acid into actinomycin. To study whether the pathway from 4-methyl-3-hydroxyanthranilic acid to actinomycin was still functional in actinomycin-nonproducing strains, conversion of labeled 4-methyl-3-hydroxyanthranilic acid to actinomycin was examined. In the parental strain, 4-methyl-3-hydroxyanthranilic acid was incorporated into actinomycin (data not shown). In contrast, there was no incorporation of radioactivity into actinomycin in the nonproducing strains (less than 1/30 of the parental strain, data not shown). These results suggest that at least one step in the biosynthetic pathway from 4-methyl-3-hydroxyanthranilic acid to actinomycin has been lost concurrently with the ability of the strains to accumulate 4-methyl-3-hydroxyanthranilic acid in the presence of D-valine.

Resistance of nonproducing strains to actinomycin D. Using actinomycin-nonproducing variants of *S. parvulus*, derived after acriflavine treatment, I studied the relationship between actinomycin-producing ability and resistance to the antibiotic (Fig. 4). The concentration of

TABLE 2. Incorporation of ^{14}C-labeled amino acids into ethyl acetate-extractable metabolites in the presence or absence of D-valine[a]

Labeled amino acid	Strain	Radioactivity (cpm/0.1 ml) of extract			
		Without D-valine		With D-valine	
		Neutral	Acidic	Neutral	Acidic
L-[*methyl*-^{14}C]methionine	Parent (3720)	6,810	1,114	670	1,774
	AF-A4	11	1,020	14	715
	NB-A7	6	1,568	0	1,605
DL-[benzene ring-*U*-^{14}C]tryptophan	Parent (3720)	1,312	633	0	1,648
	AF-A4	9	3,390	0	4,282
	NB-A7	0	5,990	0	4,995

[a] The strains were grown in glutamic acid-galactose-glucose medium. After 47 h of incubation each culture was divided into two parts, to one of which D-valine was added (final concentration, 1 mM). After another 1 h incubation, 5 ml of each culture was added to 0.1 ml of L-[*methyl*-^{14}C]methionine (1 μCi, 50 nmol) or DL-[benzene ring-*U*-^{14}C]tryptophan (2 μCi, 100 nmol) in a 50-ml flask. The flasks were shaken for 1 h at 30°C. Neutral (with actinomycin) and acidic ethyl acetate extracts were prepared as described in the text, and the radioactivity in the extracts was determined.

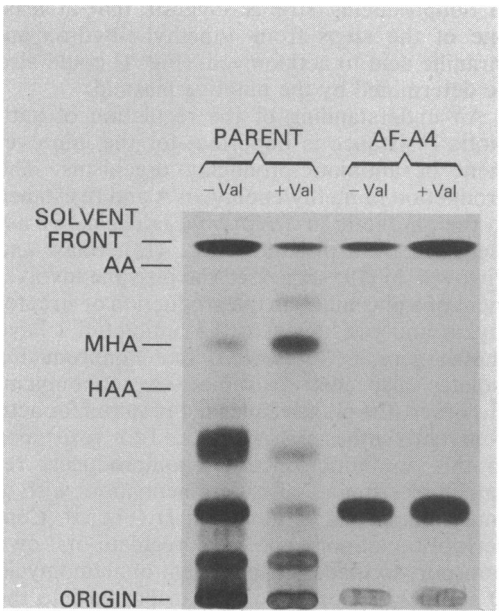

FIG. 2. Thin-layer chromatography and autoradiography of ethyl acetate extracts of acidic material produced after incubation with L-[*methyl*-^{14}C]methionine. The parent (3720) and the nonproducing variant (AF-A4) were grown and extracted by ethyl acetate and the extracts were chromatographed as described in the text. AA, Anthranilic acid; MHA, 4-methyl-3-hydroxyanthranilic acid; HAA, 3-hydroxyanthranilic acid.

actinomycin D needed for 50% growth inhibition was 110 to 240 μg/ml in isolates from the parental strain, whereas the actinomycin-nonproducing strains (isolated after acriflavine treatment) were more sensitive (less than 20 μg of actinomycin D per ml was needed to cause 50% growth inhibition in all 15 isolates tested). Actinomycin-producing cultures isolated after acriflavine treatment showed intermediate resistance between nonproducing and parental isolates (18 to 140 μg/ml for 50% inhibition). Thus, the resistance level of each isolate to actinomycin D was roughly correlated with the ability to produce the antibiotic. Similar observations have been made in *Streptomyces bikiniensis*, a streptomycin-producing organism (19).

Although actinomycin-nonproducing isolates had lost resistance to actinomycin D, they regained it when they were precultured with 10 μg of actinomycin D per ml (Fig. 4). Uptake of actinomycin D into protoplasts was studied. In the actinomycin-nonproducing strain AF3-1, the rate of actinomycin D uptake into protoplasts prepared from cells preincubated with 10 μg of actinomycin D per ml was only 1/4 that observed for protoplasts prepared from cells grown in the absence of actinomycin D (data not shown). The same was observed for protoplasts of the parent. These results suggest that the development of resistance may be due, at least partially, to the development of a permeability barrier to actinomycin.

DISCUSSION

Since neither the kynurenine formamidase nor phenoxazinone synthase activities disappeared from nonproducing strains believed to have arisen by plasmid loss (Table 1), the synthesis of these enzymes apparently is not controlled by the putative plasmid. A stable chromosomal location of the gene for kynurenine formamidase is reasonable because this enzyme is also needed for the biosynthesis of NAD, which is essential for the growth of the cell.

4-Methyl-3-hydroxyanthranilic acid did not accumulate in actinomycin-nonproducing strains, even in the presence of D-valine (Fig. 2 and 3). Therefore, at least one of the metabolic steps from tryptophan to 4-methyl-3-hydroxyanthranilic acid could be under control of the putative plasmid. Considering that the pathway from tryptophan to 3-hydroxyanthranilic acid is used for the de novo synthesis of NAD, it seems likely that the step from 3-hydroxyanthranilic

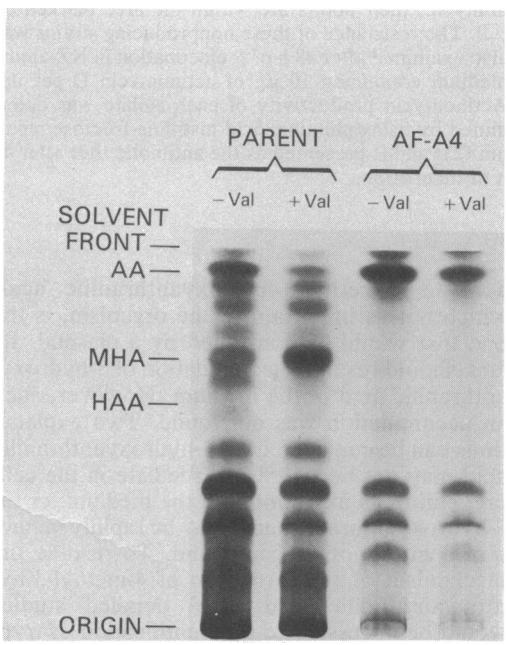

FIG. 3. Thin-layer chromatography and autoradiography of ethyl acetate extracts of acidic material produced after incubation with DL-[benzene ring-*U*-^{14}C]tryptophan. Conditions and definitions are the same as in Fig. 2.

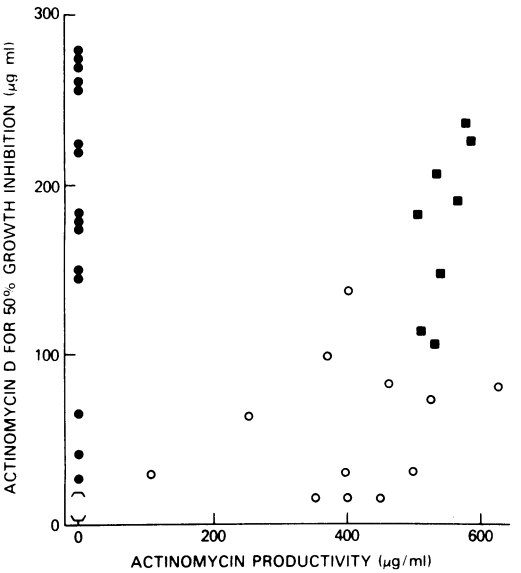

FIG. 4. Actinomycin resistance of parent and actinomycin-producing and nonproducing strains of *S. parvulus* derived after acriflavine treatment. Eight parental isolates (■), actinomycin-producing strains (○), and nonproducing (●) strains derived after acriflavine treatment were grown in NZ-amine medium containing various concentrations of actinomycin D. The antibiotic concentration needed to inhibit growth by 50% was determined. All 15 actinomycin-nonproducing strains tested were extremely sensitive to actinomycin; their points are within the area marked by (□). The resistance of these nonproducing strains was also examined after 48 h of preincubation in NZ-amine medium containing 10 μg of actinomycin D per ml. Actinomycin productivity of each isolate was determined by using glutamic acid-histidine-fructose medium (23) and is presented as the antibiotic titer after 48 h of incubation.

acid to 4-methyl-3-hydroxyanthranilic acid, which is less important to the organism, is the one that would be controlled by a plasmid. Its lack should result in accumulation of 3-hydroxyanthranilic acid in the medium. However, such an accumulation was not found. Two explanations can be considered: (i) 3-hydroxyanthranilic acid may not be a free intermediate in the cell, preventing its excretion into the medium; or (ii) 3-hydroxyanthranilic acid may be rapidly metabolized into another compound. To resolve the mechanism of the formation of 4-methyl-3-hydroxyanthranilic acid more detailed studies would be necessary (e.g., identification of tryptophan metabolites which show marked quantitative differences between actinomycin-producing and nonproducing strains in Fig. 3).

The fact that labeled 4-methyl-3-hydroxyanthranilic acid was not converted to actinomycin in nonproducing strains suggests that at least one of the steps from 4-methyl-3-hydroxyanthranilic acid to actinomycin (Fig. 1) could also be determined by the putative plasmid.

An understanding of the regulation of antibiotic resistance is important for the improvement of antibiotic-producing organisms. The production of methylenomycin A and resistance to the antibiotic in *Streptomyces coelicolor* are mediated by a plasmid, SCP1 (13). Shaw and Piwowarski (19) suggested the possible involvement of a plasmid(s) in the production of streptomycin and resistance to the antibiotic. I have shown here in *S. parvulus* that nonproducing isolates also lost resistance to actinomycin. However, the putative plasmid required for actinomycin synthesis is not needed for resistance to this antibiotic since the nonproducers regained resistance after preincubation with a small amount of actinomycin D (Fig. 4). Conceivably, actinomycin may regulate its own transport so that a small amount of actinomycin D synthesized de novo or transported into the cell reduces the transport of exogenous actinomycin D present at high concentrations.

ACKNOWLEDGMENTS

The investigation was supported by Public Health Service research grant CA-06926 and contract N01-CM-43732 from the National Cancer Institute.

This work was carried out in Edward Katz's laboratory at Georgetown University, and I thank him for his encouragement to proceed with this research. I also thank N. Vasantha and C. L. Marks for revision of this manuscript and E. Freese and S. Silver for their kind help in editing it.

LITERATURE CITED

1. **Akagawa, H., M. Okanishi, and H. Umezawa.** 1975. Plasmid involved in chloramphenicol production in *Streptomyces venezuelae*: evidence from genetic mapping. J. Gen. Microbiol. **90**:336–346.
2. **Beaven, V., J. Barchas, E. Katz, and H. Weissbach.** 1967. Studies on the inhibition of actinomycin biosynthesis by D-valine. J. Biol. Chem. **242**:657–660.
3. **Brown, D. D., M. J. M. Hitchcock, and E. Katz.** 1980. Evidence for a constitutive and inducible form of kynurenine formamidase in an actinomycin-producing strain of *Streptomyces parvulus*. Arch. Biochem. Biophys. **202**:18–22.
4. **Gallo, M., and E. Katz.** 1972. Regulation of secondary metabolite biosynthesis: catabolite repression of phenoxazinone synthase and actinomycin formation by glucose. J. Bacteriol. **109**:659–667.
5. **Hitchcock, M. J. M., and E. Katz.** 1978. Actinomycin biosynthesis by protoplasts derived from *Streptomyces parvulus*. Antimicrob. Agents Chemother. **13**:104–114.
6. **Hook, D. J., and L. C. Vining.** 1973. Biosynthetic precursors of etamycin, a peptidelactone antibiotic from *Streptomyces griseoviridus*. Can. J. Biochem. **51**:1630–1637.
7. **Katz, E.** 1967. Actinomycin, p. 276–341. *In* D. Gottlieb and P. D. Shaw (ed.), Antibiotics II. Springer-Verlag, New York.
8. **Katz, E., and W. A. Goss.** 1959. Controlled biosynthesis of actinomycin with sarcosine. Biochem. J. **73**:458–465.
9. **Katz, E., P. Pienta, and A. Sivak.** 1958. The role of nutrition in the biosynthesis of actinomycin. Appl. Microbiol. **6**:236–241.

10. **Katz, E., and H. Weissbach.** 1962. Biosynthesis of the actinomycin chromophore: enzymatic conversion of 4-methyl-3-hydroxyanthranilic acid to actinocin. J. Biol. Chem. **237**:882–886.
11. **Katz, E., and H. Weissbach.** 1963. Incorporation of ^{14}C-labeled amino acids into actinomycin and protein by *Streptomyces antibioticus*. J. Biol. Chem. **238**:666–675.
12. **Katz, E., and H. Weissbach.** 1967. Studies on the cell-free synthesis of actinomycins. Dev. Ind. Microbiol. **8**:67–87.
13. **Kirby, R., L. F. Wright, and D. A. Hopwood.** 1975. Plasmid determined antibiotic synthesis and resistance in *Streptomyces coelicolor*. Nature (London) **254**:265–267.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Ochi, K.** 1982. Protoplast fusion permits high-frequency transfer of a *Streptomyces* determinant which mediates actinomycin synthesis. J. Bacteriol. **150**:592–597.
16. **Ochi, K., M. J. M. Hitchcock, and E. Katz.** 1979. High-frequency fusion of *Streptomyces parvulus* or *Streptomyces antibioticus* protoplasts induced by polyethylene glycol. J. Bacteriol. **139**:984–992.
17. **Ochi, K., and E. Katz.** 1978. The possible involvement of a plasmid(s) in actinomycin synthesis by *Streptomyces parvulus* and *Streptomyces antibioticus*. J. Antibiot. **31**:1143–1148.
18. **Ochi, K., and E. Katz.** 1980. Genetic analysis of the actinomycin-producing determinants (plasmid) in *Streptomyces parvulus* using the protoplast fusion technique. Can. J. Microbiol. **26**:1460–1464.
19. **Shaw, P. D., and J. Piwowarski.** 1977. Effects of ethidium bromide and acriflavine on streptomycin production by *Streptomyces bikiniensis*. J. Antibiot. **30**:404–408.
20. **Troost, T., and E. Katz.** 1979. Phenoxazinone biosynthesis: accumulation of precursor, 4-methyl-3-hydroxyanthranilic acid, by mutants of *Streptomyces parvulus*. J. Gen. Microbiol. **111**:121–132.
21. **Weissbach, H., B. Redfield, V. Beaven, and E. Katz.** 1965. 4-Methyl-3-hydroxyanthranilic acid, an intermediate in actinomycin biosynthesis. Biochem. Biophys. Res. Commun. **19**:524–530.
22. **Williams, W. K., and E. Katz.** 1977. Development of a chemically defined medium for the synthesis of actinomycin D by *Streptomyces parvulus*. Antimicrob. Agents Chemother. **11**:281–290.
23. **Wright, L. F., and D. A. Hopwood.** 1976. Identification of the antibiotic determined by the SCP1 plasmid of *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. **95**:96–106.