# THE POSSIBLE INVOLVEMENT OF A PLASMID(S) IN ACTINOMYCIN SYNTHESIS BY *STREPTOMYCES PARVULUS* AND *STREPTOMYCES ANTIBIOTICUS*

Kōzō Ochi and Edward Katz

Department of Microbiology, Georgetown University
Schools of Medicine and Dentistry,
Washington, D. C. 20057, U.S.A.

(Received for publication July 6, 1978)

The treatment of *Streptomyces parvulus* and *Streptomyces antibioticus* with acriflavine or novobiocin resulted in the loss of ability to produce actinomycin. The concomitant loss of ability to form aerial mycelium and the incidence of auxotrophic progeny (*S. parvulus*) were both low relative to the loss of the antibiotic-producing property. Protoplast fusion induced by polyethylene glycol 4000, using suitable auxotrophic strains of *S. parvulus*, resulted in high recombination frequencies to prototrophy (9.6~15%). When fusion was carried out between auxotrophic *act*$^+$ and *act*$^-$ strains, respectively, there was a high frequency (84~95%) of the actinomycin synthesizing character among the prototrophic recombinants examined. No actinomycin-producing recombinant was detected in similar experiments between auxotrophic non-producing strains.

Numerous investigations with *Streptomyces* species have suggested the role of plasmids as genetic determinants of a number of phenotypic properties (*e.g.*, fertility[1,2], aerial mycelium formation[3,4], antibiotic production[3,5~11], antibiotic resistance[6,9] and melanin synthesis[12,13]). Of particular interest is the finding that antibiotic production, *e.g.*, methylenomycin A, may depend on the presence of a plasmid[6,8]. Most of the evidence for the involvement of an extrachromosomal element has been based on the loss of one or more of these properties following treatment with acriflavine, ethidium bromide or elevated temperature.

In this communication we report the results of experiments with *Streptomyces parvulus* and *Streptomyces antibioticus* using dyes and novobiocin to effect a loss of actinomycin-producing ability. In addition, the isolation of recombinant prototrophic strains derived by protoplast fusion with polyethylene glycol suggest the possibility that a plasmid(s) is involved in actinomycin synthesis.

## Materials and Methods

### Cultures

*S. parvulus* (ATCC 12434) and *S. antibioticus* strain 3720 and all strains isolated from these organisms were generally maintained on slants of GYM agar medium[14]. Auxotrophic strains were cultivated on the same medium supplemented with a suitable growth requirement at a concentration of 50 $\mu$g per ml (10 $\mu$g per ml of riboflavine).

### Treatment to eliminate plasmids

Spores of *S. parvulus* or *S. antibioticus* from a GYM agar slant were suspended in a small volume of NZ amine medium[15] and transferred to 100 ml of the same medium in a 250-ml Erlenmeyer flask. After two days cultivation at 30°C, 1-ml aliquots were inoculated into 100 ml of NZ amine medium containing acriflavine, ethidium bromide or novobiocin. Control cultures were incubated in the same medium without addition of dye or antibiotic. Novobiocin was employed since it was shown to cure R plasmids in several bacterial species with high frequency[16]; in addition, the effect of elevated tempera-

ture was examined. The organism was cultivated on a reciprocating shaking incubator for 2~5 days at the appropriate temperature. The mycelium was harvested by filtration on Whatman #2 filter paper and washed with 10% sucrose solution. Protoplasts were prepared from the mycelium with lysozyme as described previously[17] in order to obtain 'single cell' preparations. The protoplasts were diluted in a protoplast buffer and suitable dilutions were plated on a medium favorable for regeneration at high efficiency. The reversion medium employed was a modification of the one described by OKANISHI *et al.*[18] and consisted of sucrose (150 g), fructose (40 g), $CaCl_2 \cdot 2H_2O$ (3.7 g), $MgCl_2 \cdot 6H_2O$ (5.1 g), $KH_2PO_4$ (0.05 g), L-glutamic acid·HCl (2.5 g), L-histidine (0.776 g), casamino acids (1 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), $ZnSO_4 \cdot 7H_2O$ (0.025 g), $FeSO_4 \cdot 7H_2O$ (0.025 g) and TES buffer (250 ml of 0.1 M pH 7.2) and agar (20 g) in a total volume of 1 liter of deionized water. Following reversion (5 days for *S. parvulus*; 9 days for *S. antibioticus*), the colonies were randomly selected and transferred to GYM agar plugs (diam. 9.0 mm × thickness 5.0 mm). After 5-day incubation, the agar plugs were tested for the presence or absence of antibiotic by a bioassay procedure using antibiotic medium seeded with spores of *Bacillus subtilis*.

Determination of actinomycin and aerial mycelium formation

The plates were incubated overnight at 37°C and actinomycin-negative strains (*act*$^-$) were detected by the absence of a zone of inhibition around the agar plug. The lack of ability to produce the antibiotic was subsequently confirmed by cultivation of the strains in liquid medium. Antibiotic synthesis was measured in the culture filtrate and mycelium by spectrophotometric[19] and bioassay methods. Further sensitivity with respect to antibiotic synthesis was achieved by incubation of the organism with radioactive precursors followed by thin-layer chromatography and autoradiography[17]. The parental strains subjected to the same procedures (without AF, EB or novobiocin treatment) generally synthesize 500~600 μg/ml (*S. parvulus*, GHF medium[20]) or 70~110 μg/ml (*S. antibioticus*, GGG medium[21]).

Aerial mycelium formation was checked microscopically after cultivation of treated cells on both GYM and SYM (in which soluble starch replaced glucose) agar slants. The incidence of auxotrophic strains after treatment was determined by observing growth on GHF agar medium (minimal medium) and GHF agar medium supplemented with 0.1% casamino acids (complete medium).

Protoplast fusion

The protoplast fusion experiments that were carried out will be described in detail elsewhere. For fusion of protoplasts ($2 \times 10^9$ for each auxotrophic strain), polyethylene glycol 4000 (final concentration, 42% w/v) was used. Fused protoplast mixtures were plated on minimal medium (GHF-reversion medium) for 5~10 days at 30°C. Prototrophic recombinants, which appeared, were then streaked for two successive passages to purify progeny.

Auxotrophic strains, used for fusion experiments, were obtained by NTG treatment according to the procedure of DELIĆ *et al.*[22]

Reagents

Novobiocin was a gift from Dr. O. SEBEK, Upjohn & Co., Kalamazoo, Mich.; $^{14}C$-Labeled 4-methyl-3-hydroxyanthranilic acid was kindly provided by Dr. T. TROOST of this laboratory. Polyethylene glycol (PEG) 4000 was purchased from Sigma Chem. Co., St. Louis, Mo. Other reagents employed were from commercial sources and were analytical grade.

## Results

A preliminary experiment was carried out with acriflavine (AF) and ethidium bromide (EB) to determine whether the loss of antibiotic producing ability would be observed. *S. parvulus* was cultivated for 5 days in NZ amine medium containing the highest concentration of AF (5 μg/ml) or EB (5 μg/ml) permitting growth. After incubation, regeneration of protoplasts was carried out on GHF-reversion medium supplemented with 0.1% casamino acids (see Materials and Methods) and actinomycin synthesis was assayed by the agar plug method. With *S. parvulus* we observed that 1.1% (EB, 5/438) and 5.1% (AF, 26/513) of the progeny tested had lost the ability to synthesize actinomycin. By contrast, in

the case of the untreated control, only 0.3% (1/349) colonies tested was a non-producing strain. These findings suggested the possibility that a plasmid(s) may be involved in actinomycin biosynthesis.

A second experiment was conducted in which the organism was successively transferred in NZ amine medium containing increasing concentrations of EB or AF as shown in Table 1. Since AF and EB are known to restrict plasmid replication during cell growth, it was considered likely that repeated cultivation would provide a more effective means for loss of a plasmid(s). To establish that this treatment did not result in a significant deletion of chromosomal DNA, the incidence of auxotrophic progeny requiring components of casamino acids was also determined. The effect of elevated temperature and novobiocin for obtaining antibiotic-negative strains are also presented in Table 1. The incidence of *S. parvulus* progeny lacking the ability to synthesize actinomycin was found to be relatively high with AF (15%) and novobiocin (13.5%), whereas EB (2.8%) and high temperature (0.7%) were not nearly as effective treatments. In contrast, with *S. antibioticus*, acriflavine proved to be far more effective than novobiocin for obtaining *act*$^-$ strains. In the investigation with *S. parvulus* we observed that loss of ability to form aerial mycelium and the incidence of auxotrophic progeny were both low relative to the loss of antibiotic producing ability. The data shown in Table 1 represent the number of stable, non-producing or non-aerial mycelium-forming strains as determined after seven successive transfers on GYM and SYM agar media. Further evidence that the strains were *act*$^-$ was obtained by growth of *S. parvulus* and *S. antibioticus* in chemically defined liquid media suitable for antibiotic production. No antibiotic was detected in the culture filtrate and 1-butanol extracts of the mycelium assayed by microbiological and spectrophotometric procedures. Using $^{14}CH_3$-L-methionine, DL-[$^{14}$C-benzene-ring labeled] tryptophan and $^{14}$C-labeled 4-methyl-3-hydroxyanthranilic acid as radiolabeled precursors, we were unable to observe synthesis of actinomycin. This procedure would permit the detection of nanomole quantities of antibiotic[17]. Cosynthesis studies between *act*$^-$ strains using

Table 1. Effect of various treatments on actinomycin production, aerial mycelium formation and auxotrophy.

| Organism | Treatment[1] | Number of colonies tested | Number of colonies lacking ability to form | | Number of auxotrophs observed |
|---|---|---|---|---|---|
| | | | Actinomycin | Aerial mycelium | |
| | | | (%) | (%) | (%) |
| *S. parvulus*[2] | None | 324 | 0 (0) | 0 (0) | 0 (0) |
| | Ethidium bromide | 394 | 11 (2.8) | 1 (0.25) | 1 (0.25) |
| | Acriflavine | 326 | 49 (15.0) | 12 (3.7) | 12 (3.7) |
| *S. parvulus*[3] | None | 673 | 1 (0.1) | 0 (0) | 0 (0) |
| | Novobiocin, 15 μg/ml | 195 | 12 (6.2) | 3 (1.5) | 3 (1.5) |
| | Novobiocin, 30 μg/ml | 185 | 25 (13.5) | 5 (2.7) | 1 (0.5) |
| | High temperature, 40°C | 701 | 5 (0.7) | 9 (1.3) | 4 (0.6) |
| *S. antibioticus*[4] | None | 319 | 1 (0.3) | — | — |
| | Novobiocin | 266 | 13 (4.9) | — | — |
| | Acriflavine | 318 | 63 (20.8) | — | — |

[1] All experiments were carried out in NZ amine medium at 30°C except for high temperature (40°C).

[2] Cultures were incubated at 3-day intervals without treatment or with increasing amounts of ethidium bromide (2, 2, 3, 10, 10, 10, 10 μg/ml) or AF (3, 3, 5, 10, 30, 30, 30 μg/ml).

[3] Cultures were incubated without or with treatment for 5 days.

[4] Cultures were incubated at 3-day intervals without treatment or with increasing amounts of novobiocin (5 and 10 μg/ml) or AF (2 and 5 μg/ml).

many combinations failed to show synthesis of antibiotic in liquid media.

Table 2 shows the frequency of recombination between protoplasts prepared from various auxotrophic strains of *S. parvulus* induced by polyethylene glycol treatment. Strain #44-2 (*cys*$^-$, *act*$^-$) was derived from strain #44 (*cys*$^-$) by acriflavine treatment. NTG treatment was used to obtain all the nutritionally-dependent strains isolated. In order to determine the back mutation frequency, two hundred progeny from strain #44-2 (*cys*$^-$, *act*$^-$) were selected at random and tested by the agar plug method; none of the progeny were found to produce actinomycin. Examination of Table 2 reveals that high recombination frequencies to prototrophy (9.6~15%) were obtained by the protoplast fusion method. As will be described elsewhere, the colonies seen on minimal medium were true recombinants and not heterokaryons or diploids. It was shown that when PEG was omitted, recombinants were not observed even when high densities of protoplast mixtures were plated on reversion medium.

Table 2. Frequency of prototrophic recombination after protoplast fusion between various auxotrophic strains of *S. parvulus*.

| Fusion between strain #44-2 (*cys*$^-$ *act*$^-$) and | Colony formation/ml ($\times 10^{-5}$) on | | Recombination frequency (A/B) (%) |
|---|---|---|---|
| | MM (A) | MM+ requirements[1,2] (B) | |
| Strain # | | | |
| 14 (*try*$^-$ *act*$^+$) | 8 | 83 | 9.6 |
| 16 (*threo*$^-$ *act*$^+$) | 4 | 41 | 9.8 |
| 30 (*ura*$^-$ *act*$^+$) | 6 | 40 | 15 |
| 32 (*rib*$^-$ *act*$^+$) | 6 | 45 | 13 |
| 34 (*try*$^-$ *act*$^+$) | 5 | 51 | 9.8 |

[1] The nutritional requirement of auxotrophic strains was added at a concentration of 50 μg/ml except for riboflavine (10 μg/ml).

[2] Nutritional requirements are designated cysteine (*cys*$^-$), tryptophan (*try*$^-$), threonine (*threo*$^-$), uracil (*ura*$^-$), riboflavine (*rib*$^-$) and tyrosine (*tyr*$^-$); *act*$^-$ indicates inability to produce actinomycin.

The recombinants, after purification by streaking on GHF-minimal medium to isolate single colonies, were examined for their ability to synthesize actinomycin by the agar plug method using GYM agar medium and representative colonies were also checked in GHF liquid medium. The results, shown in Table 3, reveal that there is a high frequency (84~95%) of the actinomycin synthesizing character among the prototrophic recombinants examined. Protoplast fusion experiments were also carried out between auxotrophic, non-producing strains #44-2 (*cys*$^-$, *act*$^-$) and #303-1 (*ura*$^-$, *lys*$^-$, *act*$^-$) or #330-2 (*rib*$^-$, *meth*$^-$, *act*$^-$). While prototrophic recombinants were obtained efficiently, no actinomycin-producing recombinants were ever detected. Although a genetic map for *S. parvulus* has not been determined as yet, it is reasonable to assume from previously published studies[23] with *S. coelicolor* and other *Streptomyces* that the auxotrophic mutations induced are randomly distributed on the chromosome and that, at least, some of the genetic determinants involved with actinomycin synthesis

Table 3. Analysis of prototrophic recombinants[1] that synthesize actinomycin after fusion.

| Fusion between strain #44-2 and | Number of recombinant colonies tested | Number of colonies | | Percent *act*$^+$ |
|---|---|---|---|---|
| | | *act*$^+$ | *act*$^-$ | |
| Strain # | | | | |
| 14 | 227 | 200 | 27 | 88 |
| 16 | 234 | 216 | 18 | 92 |
| 30 | 219 | 183 | 36 | 84 |
| 32 | 233 | 212 | 21 | 91 |
| 34 | 238 | 226 | 12 | 95 |

[1] See Table 2.

are not linked with the prototrophic markers examined (try, threo, ura, rib and tyr). The results of a more detailed genetic analysis for the determinants concerned with actinomycin production will be published elsewhere.

## Discussion

The results described herein strongly support the view that a plasmid(s) plays a role in actinomycin formation. Loss of antibiotic-producing ability was seen when several 'curing' agents such as AF, EB and novobiocin were used. The *act*$^-$ phenotypic character was established by bioassays, a spectrophotometric procedure and radiolabeling experiments. Moreover, the loss appeared to be a stable property of the progeny isolated. By comparison, the loss of ability to form aerial mycelium as well as the incidence of nutritional dependency occurred at much lower frequencies in these experiments. Additional evidence for the role of a plasmid(s) in actinomycin synthesis was provided by the protoplast fusion experiments and cosynthesis studies. Prototrophic recombinants obtained from fusion between actinomycin-negative strains were never found to be *act*$^+$, whereas similar experiments involving different auxotrophic *act*$^+$ genotypes with an *act*$^-$ auxotrophic strain always showed a high frequency of the *act*$^+$ genotype in the prototrophic recombinants. Although transfer of certain plasmids has not been shown to occur by conjugation[7,24], it may be possible to mediate the transfer by application of protoplast fusion techniques. This procedure provides a new, efficient method for genetic exchange and may have wide application for examining interstrain and interspecific combinations[25].

Acknowledgements

The investigation was supported by Public Health Service research grant CA-06926 and contract NO1-CM-43732 from the National Cancer Institute.

We thank Dr. M. J. M. HITCHCOCK for his advice and many useful discussions.

## References

1) VIVIAN, A. & D. A. HOPWOOD: Genetic control of fertility in *Streptomyces coelicolor* A 3 (2): The IF fertility type. J. Gen. Microbiol. 64: 101~117, 1970
2) VIVIAN, A.: Genetic control of fertility in *Streptomyces coelicolor* A 3 (2): Plasmid involvement in the interconversion of UF and IF strains. J. Gen. Microbiol. 69: 353~364, 1971
3) OKANISHI, M.; T. OHTA & H. UMEZAWA: Possible control of aerial mycelium and antibiotic in *Streptomyces* by episomic factors. J. Antibiotics 23: 45~47, 1970
4) REDSHAW, P. A.; P. A. MCCANN, L. SANKARAN & B. M. POGELL: Control of differentiation in streptomycetes: Involvement of extrachromosomal deoxyribonucleic acid and glucose repression in aerial mycelia development. J. Bacteriol. 125: 698~705, 1976
5) KAHLER, R. & D. NOACK: Action of acridine orange and ethidium bromide on growth and antibiotic activity of *Streptomyces hygroscopicus* JA 6599. Z. Allg. Mikrobiol. 14: 529~533, 1974
6) KIRBY, R.; L. F. WRIGHT & D. A. HOPWOOD: Plasmid determined antibiotic synthesis and resistance in *Streptomyces coelicolor*. Nature 254: 265~267, 1975
7) AKAGAWA, H.; M. OKANISHI & H. UMEZAWA: A plasmid involved in chloramphenicol production in *Streptomyces venezuelae*: Evidence from genetic mapping. J. Gen. Microbiol. 90: 336~346, 1975
8) WRIGHT, L. F. & D. A. HOPWOOD: Identification of the antibiotic determined by the SCPI plasmid of *Streptomyces coelicolor* A 3 (2). J. Gen. Microbiol. 95: 96~106, 1976
9) SHAW, P. D. & J. PIWOWARSKI: Effect of ethidium bromide and acriflavine on streptomycin production by *Streptomyces bikiniensis*. J. Antibiotics 30: 404~408, 1977
10) HOTTA, K.; Y. OKAMI & H. UMEZAWA: Elimination of the ability of kanamycin producing strain to biosynthesize deoxystreptamine moiety by acriflavine. J. Antibiotics 30: 1146~1149, 1977
11) UMEZAWA, H.; Y. OKAMI & K. HOTTA: Transfer of the leupeptin producing ability of the strain, *Streptomyces roseus* MA839-A1, by conjugation. J. Antibiotics 31: 99~102, 1978
12) GREGORY, K. F. & J. C. C. HUANG: Tyrosinase inheritance in *Streptomyces scabies*. I. Genetic recombination. J. Bacteriol. 87: 1281~1286, 1964

13) GREGORY, K. F. & J. C. C. HUANG: Induction of tyrosinase deficiency in *Streptomyces scabies*. II. Induction of tyrosinase deficiency by acridine dyes. J. Bacteriol. 87: 1287~1294, 1964
14) HOOK, D. J. & L. C. VINING: Biosynthetic precursors of etamycin, a peptide lactone antibiotic from *Streptomyces griseoviridus*. Canad. J. Biochem. 51: 1630~1637, 1973
15) KATZ, E. & W. A. GOSS: Controlled biosynthesis of actinomycin with sarcosine. Biochem. J. 73: 458~465, 1959
16) MCHUGH, G. L. & M. N. SWARTY: Elimination of plasmids from several bacterial species. Antimicr. Agents & Chemoth. 12: 423~426, 1977
17) HITCHCOCK, M. J. M. & E. KATZ: Actinomycin biosynthesis by protoplasts derived from *Streptomyces parvulus*. Antimicr. Agents & Chemoth. 13: 104~114, 1978
18) OKANISHI, M.; K. SUZUKI & H. UMEZAWA: Formation and reversion of streptomycete protoplasts: Cultural conditions and morphological study. J. Gen. Microbiol. 80: 389~400, 1974
19) KATZ, E. & H. WEISSBACH: Incorporation of $^{14}$C-labeled amino acids into actinomycin and protein by *Streptomyces antibioticus*. J. Biol. Chem. 238: 666~675, 1963
20) WILLIAMS, W. K. & E. KATZ: Development of a chemically defined medium for the synthesis of actinomycin D by *Streptomyces parvulus*. Antimicr. Agents & Chemoth. 11: 281~290, 1977
21) KATZ, E.; P. PIENTA & A. SIVAK: The role of nutrition in the biosynthesis of actinomycin. Appl. Microbiol. 6: 236~241, 1958
22) DELIĆ, V.; D. A. HOPWOOD & E. J. FRIEND: Mutagenesis by N-methyl-N′-nitro-N-nitrosoguanidine (NTG) in *Streptomyces coelicolor*. Mutation Research 9: 167~182, 1970
23) HOPWOOD, D. A.; K. F. CHATER, J. E. DOWDING & A. VIVIAN: Advances in *Streptomyces coelicolor* genetics. Bacteriol. Rev. 37: 371~405, 1973
24) NOVICK, R. P.: Extrachromosomal inheritance in bacteria. Bacteriol. Rev. 33: 210~235, 1969
25) HOPWOOD, D. A.; H. M. WRIGHT & M. J. BIBB: Genetic recombination through protoplast fusion in *Streptomyces*. Nature 268: 171~174, 1977