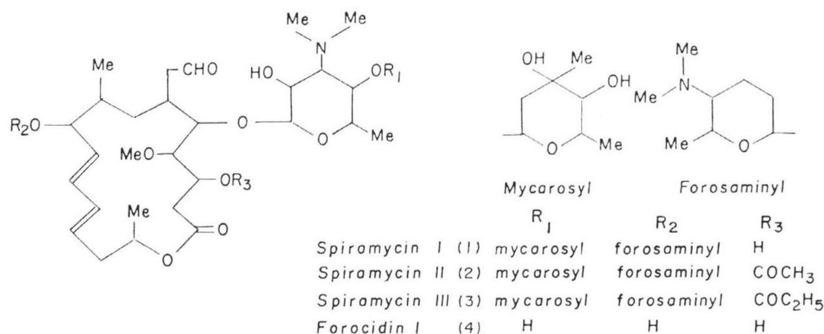
THE DETECTION OF A PLASMID IN *STREPTOMYCES AMBOFACIENS* KA-1028 AND ITS POSSIBLE INVOLVEMENT IN SPIRAMYCIN PRODUCTION

Sir:

The role of plasmids as a genetic determinant of antibiotic production has been suggested by numerous investigations[1~3]. We have studied the isolation, structural determination and biosynthesis of 16-membered macrolides[4,5]. Of particular interest regarding the production of 16-membered macrolides is the observation that many related components are coproduced by the same strain; this phenomenon is an important problem in development of the production of single components to be evaluated as practical antibiotics. A study of the involvement of plasmids in 16-membered macrolide synthesis may provide us with fundamental information concerning genetic control of macrolide production.

In this communication we report the detection of plasmid DNA in a spiramycin-producing strain, *Streptomyces ambofaciens* and provide evidence for the possible involvement of the plasmid in the biosynthesis of spiramycin which is a 16-membered macrolide with a well characterized biosynthetic scheme[5].

S. ambofaciens KA-1028 (ISP-5053) produced 100 μg/ml of spiramycin when fermentation was performed in 500 ml SAKAGUCHI flasks with 100 ml of medium consisting of 1.0% glucose, 1.0% dried yeast, 0.5% NaCl, 1.0% $CaCO_3$, and 0.1% $NaNO_3$ (pH 7.5), with aeration at 27°C for 72 hours. Spiramycin non-producing strains were obtained by treatment of KA-1028 with 10 μg/ml of acriflavine[6] at a frequency of 10%. Plasmid DNA from the parent strain KA-1028 and the spiramycin non-producing strain AF-30 (obtained by acriflavine treatment) were analyzed by CsCl-ethidium bromide equilibrium centrifugation and agarose gel electrophoresis. Seed culture of strains for plasmid analysis were incubated in 5 ml of medium containing 1.0% glucose, 0.4% glycine, 0.4% casamino acid, 0.02% yeast extract, 0.1% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 1.0% 1 M phosphate buffer (pH 7.2) and 0.4% of a solution of trace elements containing 40 mg $ZnCl_2$, 200 mg $FeCl_2 \cdot 6H_2O$, 10 mg $CuCl_2 \cdot 2H_2O$, 10 mg $MnCl_2 \cdot 4H_2O$, 10 mg $(NH_4)_6Mo_7O_{24} \cdot H_2O$ and 10 mg $Na_2B_4O_7 \cdot 10H_2O$ in one liter, at 27°C for 72 hours. The seed culture was transferred to 10 ml of the same medium with glycerol replaced glucose, without yeast extract and containing 5 μCi of [methyl-^{3}H] thymidine per ml and 200 μg of uridine per ml. Incubation was continued for 24 hours at 27°C. Mycelia were harvested on Millipore filters and washed with 20 ml of TES buffer (50 mM NaCl, 5 mM EDTA, 30 mM Tris-HCl, pH 8.0) and homogenized with a Teflon tissue homogenizer. The homogenized cells were lysed by incubation with 1 mg of lysozyme per ml for 10 minutes at 37°C followed by the addition of 1.5 ml of 0.75% sodium lauryl sarcosinate solution. A cleared lysate was obtained by the treatment of the lysate with 200 μg of proteinase K per ml at 37°C for 1 hour and centrifugation at 30,000 × *g* for 30 minutes. Two ml of the cleared lysate was mixed with 5.00 g of CsCl and 2.76 ml of TES buffer, and 0.5 ml of ethidium bromide solution (4.67 mg/ml in water) in a polyallomer tube. The contents of the tube were covered with liquid paraffin, and centrifuged with Hitachi RP-65 angle rotor for 36 hours at 36,000 rpm. Forty fractions were collected for the estimation of distribution of radioactivity. The cleared



	R1	R2	R3
Spiramycin I (1)	mycarosyl	forosaminyl	H
Spiramycin II (2)	mycarosyl	forosaminyl	$COCH_3$
Spiramycin III (3)	mycarosyl	forosaminyl	COC_2H_5
Forocidin I (4)	H	H	H

Fig. 1. CsCl-ethidium bromide equilibrium centrifugation of a cleared lysate of strain KA-1028.

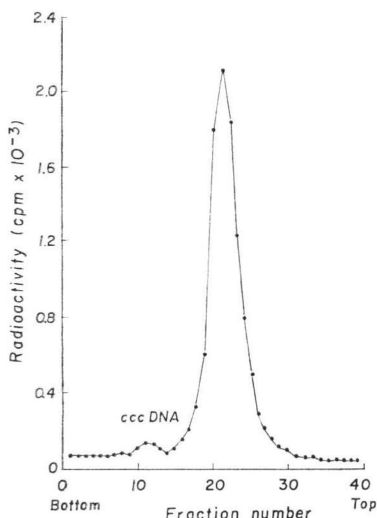


Fig. 2. Agarose gel electrophoresis.
(A) a cleared lysate of strain S-1.
(B) a cleared lysate of strain AF-30.
(C) a cleared lysate of strain KA-1028 (parent strain).
(D) a portion (40 μl) of fractions of denser DNA satellite band (fractions 10~13 in Fig. 1).

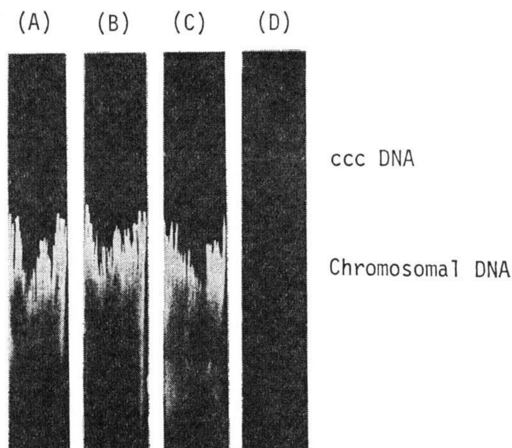
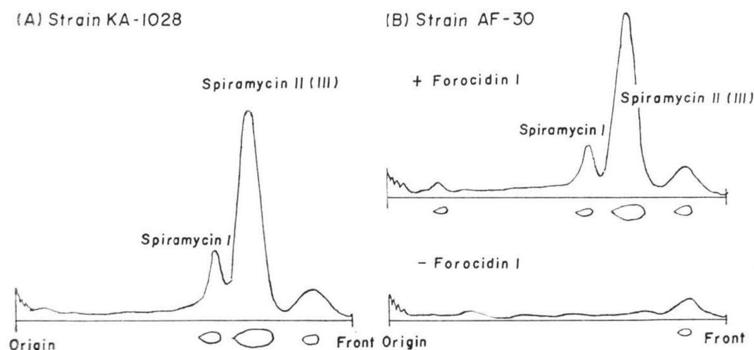


Fig. 3. Bioconversion of forocidin I to spiramycins by the spiramycin-non-producing strain, AF-30.
The chromatograms developed with $CHCl_3$-MeOH-1.5 N aqueous ammonia (2:1:1, bottom layer) were scanned at 232 nm. Spiramycin II and III show identical Rf values under these conditions. Forocidin I was added after 48 hour of cultivation of the strain AF-30.



lysates were also analyzed by agarose gel electrophoresis according to the procedure of MEYERS *et al.*[7]

The presence of plasmid DNA (ccc DNA) could be detected by both analytical methods in the spiramycin-producing strain KA-1028 as shown in Figs. 1 and 2. However, the plasmid DNA was not detected in the cleared lysate from the spiramycin-non-producing strains AF-30 and S-1.

In order to ascertain if a plasmid-controlled function was involved in the biosynthetic pathway to spiramycin, we examined the plasmid cured strain for its ability to convert forocidin I (**4**) which is an important intermediate in the biosynthesis of spiramycin[5], into spiramycin II (**2**). Fig. 3 indicates silica-gel thin-layer chromatograms of extracts obtained from 72-hour fermentation broths of KA-1028 and AF-30 scanned with dual wave-length chromatogram scanner. The chromatogram of the extract from KA-1028 (Fig. 3A) indicates two peaks corresponding to spiramycin II (**2**) and spiramycin I (**1**), respectively. Strain AF-30 did not produce

spiramycins as shown in Fig. 3B. However, the production of spiramycins in strain AF-30 could be detected when forocidin I (**4**) was added to the fermentation broth, at 48-hour cultivation (Fig. 3B). Conversion of forocidin I (**4**) to spiramycin II (**2**) was also observed in the majority of spiramycin-non-producing strains derived from strain KA-1028 by curing the plasmid by acriflavine treatment. Table 1 summarizes information on strains KA-1028, AF-30 and S-1; the latter was randomly chosen from spiramycin-non-producing strains spontaneously derived from KA-1028. Formation of soluble pigment and aerial mycelium, both of which presumably require some plasmid-coded function were absent in strains AF-30 and S-1 (Table 1). The production of two enzymes, spiramycin I-3 hydroxyl acylase[8] and TDP-mycarose synthetase[9] in strains AF-30 and S-1 were comparable to those of strain KA-1028. From this evidence we conclude that at some stage before the formation of forocidin I (**4**), in the biosynthesis of spiramycin, one or more plasmid-encoded functions are required.

Table 1. Characterizations of *Streptomyces ambofaciens* strains KA-1028, S-1 and AF-30

	KA-1028 (parent strain)	S-1	AF-30
Production of spiramycin (μg/ml)	100	0	0
Transformation of forocidin I into spiramycin II	+	+	+
Resistance to spiramycin (μg/ml)	>800	>800	>800
Formation of soluble pigment*	+	−	−
Formation of aerial mycelium**	+	−	−
Color of colony***	yellow	white	white

* Oatmeal agar medium (SHIRLING and GOTTLIEB[10])

** Inorganic salts-starch agar medium (SHIRLING and GOTTLIEB[10])

*** Glucose-CZAPEK agar medium +1% casamino acid

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(Received June 1, 1979)

References

1) HOPWOOD, D. A. & M. J. MERRICK: Genetics of antibiotics production. Bact. Rev. 41: 595~635, 1977
2) YAGISAWA, M.; T-S. R. HUANG & J. E. DAVIES: Possible involvement of plasmids in biosynthesis of neomycin. J. Antibiotics 31: 809~813, 1978
3) OCHI, K. & E. KATZ: The possible involvement of a plasmid(s) in actinomycin synthesis by *Streptomyces parvulus* and *Streptomyces antibiotics*. J. Antibiotics 31: 1143~1148, 1978
4) ŌMURA, S. & A. NAKAGAWA: Chemical and biological studies on 16-membered macrolide antibiotics (Review article). J. Antibiotics 28: 401~433, 1975
5) ŌMURA, S.; C. KITAO, H. HAMADA & H. IKEDA: Final steps in the biosynthesis of spiramycin, using enzyme inhibitor: cerulenin. Chem. Pharm. Bull. 27: 176~182, 1979
6) SHAW, P. D. & J. PIWOWARSKI: Effects of ethidium bromide and acriflavine on streptomycin production by *Streptomyces bikiniensis*. J. Antibiotics 30: 404~408, 1977
7) MEYERS, J. A.; D. SANCHEZ, L. P. ELWELL & S. FALKOW: Simple agarose gel electrophoresis method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bact. 127: 1529~1537, 1976
8) ŌMURA, S.; H. IKEDA & C. KITAO: J. Biochem. submitted.
9) PAPE, H. & G. U. BRILLINGER: Biosyntheses von Thymidin-diphosphymycarose durch ein zellfreies System aus *Streptomyces rimosus*. Arch. Mikrobiol. 88: 25~35, 1973
10) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bact. 16: 313~340, 1966