POSSIBLE INVOLVEMENT OF PLASMIDS IN BIOSYNTHESIS OF NEOMYCIN

Sir:

The involvement of plasmids in the production of antibiotics in *Streptomyces* has been suggested in several cases. OKANISHI *et al.*[1] showed that the ability to produce kasugamycin and aureothricin was lost following treatment of the producing organism, *Streptomyces kasugaensis*, with acridine dyes or high temperature. Such treatment is known to eliminate plasmids from bacterial cells. Three different plasmids have been identified in *S. kasugaensis*[2]; an 1.5 ± 0.5 μ plasmid that is thought to be involved in the biosynthesis of kasugamycin, since kasugamycin non-producers lack this plasmid; a 3.35 ± 0.05 μ plasmid associated with biosynthesis of aureothricin or thiolutin, and an uncharacterized 0.59 μ plasmid. Production of, and resistance to, methylenomycin in *S. coelicolor*[3] and streptomycin in *S. bikiniensis*[4] have been reported to be controlled by plasmids. HOTTA *et al.*[5] have described the possible involvement of a plasmid in the biosynthesis of the 2-deoxystreptamine (DOS) moiety of kanamycin by *S. kanamyceticus*. Genetic studies of a chloramphenicol-producing organism, *S. venezuelae*, indicated that the genes required for the production of chloramphenicol were independent of chromosomally determined auxotrophic markers and thus thought to be encoded on a plasmid[6]. Apart from the plasmids isolated from *S. kasugaensis*, the only other plasmids isolated from *Streptomyces* come from *S. coelicolor* A3(2); Two sex factors have been identified in this strain. SCP1 determines synthesis of the antibiotic methylenomycin in addition to its sex factor activity, however the plasmid DNA has not been isolated.[7] A second sex factor SCP2 has been isolated[7,8] as a plasmid of molecular weight $18 \sim 20 \times 10^6$.

In this paper, we report the isolation of plasmids from *S. fradiae* and their possible involvement in the biosynthesis of, and resistance to, the antibiotic neomycin.

S. fradiae ATCC10745 was selected for this study by the following reasons: (1) The biosynthetic pathway of neomycin has been studied extensively[9]. (2) Production of neomycin is rapid, stable and in high quantity. (3) The strain is highly resistant to neomycin. (4) Production of a neomycin 3′-phosphotransferase [APH (3′) that is different from R-plasmid coded aminoglycoside 3′-phosphotransferases I, II or III][10]. (5) The strain can be readily lysed with lysozyme even when cultivated in neomycin-production medium.

Non-antibiotic producing variants of *S. fradiae* ATCC10745 were obtained by acridine dye treatment. Logarithmically growing cells of *S. fradiae* in tripticase soy broth (TSB, Difco) medium were dispersed into small fibrous mycelium in a teflon tissue homogenizer and inoculated into 10 ml TSB medium containing 5 μg/ml of acridine orange. The culture was incubated for 4 days at 30°C in the dark, the cells were washed and resuspended in TSB medium, homogenized and spread on TSB-agar plates. Each colony was transferred onto a 0.25 ml TSB-agar plug in a microtiter plate (Falcon) and incubated 4 days. The agar plugs were removed and placed on assay plates for neomycin production, seeded with *Bacillus subtilis* as the test organism. Three of 191 clones tested (AO80, AO83 and AO144) had lost the capacity to produce neomycin, this was confirmed by the usual shake-culture and paper disc-plate assay. After over 20 successive subcultures, the three variants did not regain neomycin production.

As shown in Table 1, the three variants were classified into two groups by their sensitivity to neomycin; AO80 and AO83 were inhibited by 1.56 μg/ml of neomycin, AO144 by 100 μg/ml, and the parent ATCC10745 and CMP 487 (a neomycin non-producing mutant kindly supplied by the Upjohn Co.,) were resistant to greater than 100 μg/ml. By radioenzymatic assay of APH(3′)[11], AO80 and AO83 were found not to produce phosphotransferase but AO144 and

Table 1. Sensitivity to neomycin and production of phosphotransferase [APH(3′)] of plasmid-cured variants

	MIC (μg/ml) of neomycin	APH(3′) activity (cpm)
S. fradiae		
ATCC10745	>100	5,116
AO80	1.56	0
AO83	1.56	0
AO144	100	5,341
CMP487	>100	6,760

Table 2. Effect of DOS feeding on neomycin production by acridine orange-treated variants

	Addition of DOS*	Neomycin production after the addition of DOS (mm)**		
		0-day	3-day	5-day
S. fradiae ATCC10745	−	18.0	20.0	20.0
AO80	+	0	0	0
AO83	+	0	0	0
AO144	+	0	20.0	20.0

* DOS was added at 100 μg/ml to the 2 day-old culture.

** Neomycin production was assayed by the paper disc-plate assay method using *Bacillus subtilis* as the test organism.

CMP487 showed approximately the same phosphotransferase activity as the parent strain. This suggests that resistance of *S. fradiae* ATCC10745 to neomycin requires the production of APH(3′) and that the gene for this enzyme may be coded on a plasmid, which is not present in AO80 and AO83.

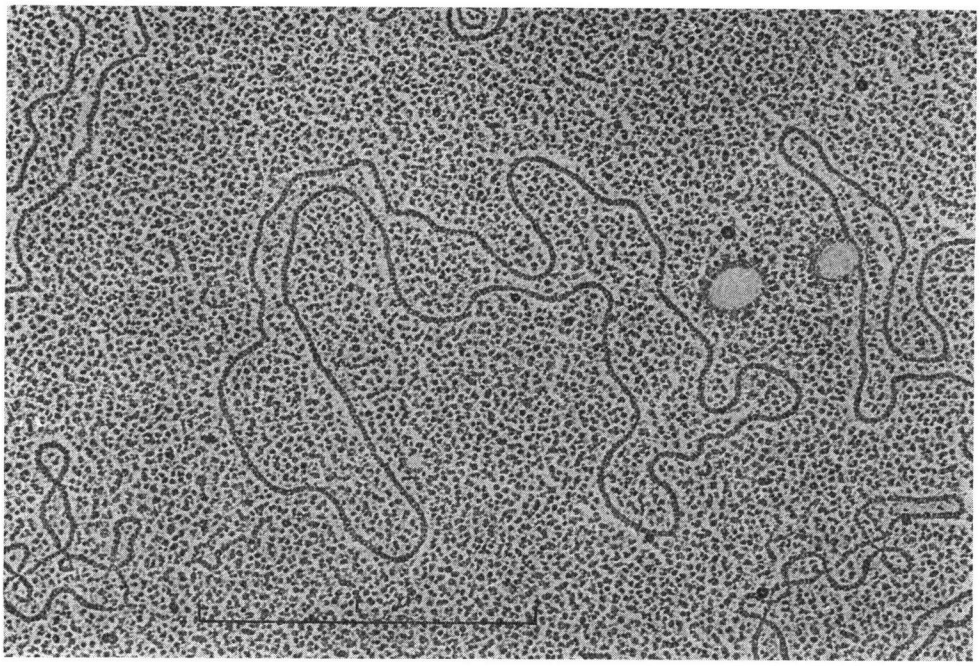
Idiotrophs requiring DOS for the production of neomycin[12], paromomycin[13], ribostamycin[14], kanamycin[14] and sisomicin[15] have been isolated and the possible involvement of a plasmid in the biosynthesis of DOS has been suggested[5]. Accordingly, we attempted feeding experiments in which DOS at a concentration of 100 μg/ml was added to 2 day-old cultures of the neomycin non-producing variants. Only AO144 and CMP-487 (to a much lesser extent), possessing phosphotransferase activity, produced neomycin under these conditions (Table 2). These results suggest the possibility of the existence of two or more plasmids in the parent strain, one encoding the gene(s) for the biosynthesis of DOS and the other for the production of the phosphotransferase; AO144 has presumably lost the former and AO80 and AO83 the latter or both [APH(3′) may play a role in the biosynthesis of neomycin similar to streptomycin phosphotransferase which has been indicated to be important for streptomycin biosynthesis[16]]. When AO144 was subjected to successive subculture, it no longer produced phosphotransferase and became sensitive to neomycin; such clones were no longer capable of producing neomycin when grown in the presence of DOS.

Plasmid DNA was isolated from logarithmically growing cells of *S. fradiae* ATCC10745 by the procedure of PALCHAUDHURI & CHAKRABARTY[17] with modifications as follows: 11 g of fresh cells from 500 ml of a 3 day culture in TSB medium were washed and resuspended in 40 ml of 25% sucrose-50 mM tris-hydrochloride buffer (pH 8.0), homogenized into small fibrous mycelium. Two ml of 100 mg/ml lysozyme solution and 2 ml of 0.25 M disodium-EDTA (pH 8.0) were added to the cell suspension and the mixture was incubated at 30°C for 2 hours with rotary shaking (150 rpm). Almost all cells were shown to form protoplasts when examined under phase contrast. Addition of 25 ml of 2% sodium lauryl sarcosinate - 1.5 M sodium chloride solution and shearing by passing 5 times through a 60 ml disposable syringe (without needle) caused immediate lysis of the protoplasts. Sodium hydroxide (4 N) was added dropwise to the lysate with stirring to pH 12.0 and the lysate was stirred for 1 minute to denature chromosomal DNA. The pH was adjusted to 8.0 by addition of saturated tris-hydrochloride (pH 7.0), and 25 g (wet weight) of prewashed nitrocellulose powder was added to the solution. After gentle mixing for 15 minutes, the nitrocellulose powder, which binds single-stranded DNA, was removed by centrifugation and filtration through fiber glass. The DNA remaining in the filtrate was precipitated by the addition of polyethyleneglycol 6000 to 10% (w/v) at 4°C. The resulting precipitate was dissolved in 10 ml of TES buffer (10 mM tris-hydrochloride - 1 mM EDTA - 10 mM sodium chloride, pH 8.0), and 16 g of cesium chloride was added to the solution. Insoluble materials were removed by low speed centrifugation. The solution was placed in two 5/8″ × 3″ centrifuge tubes and 2 ml of 3 mg/ml ethidium bromide was added. The mixture was centrifuged at 40,000 rpm for 48 hours in a Ti50 rotor and the plasmid DNA, well separated from contaminating chro-

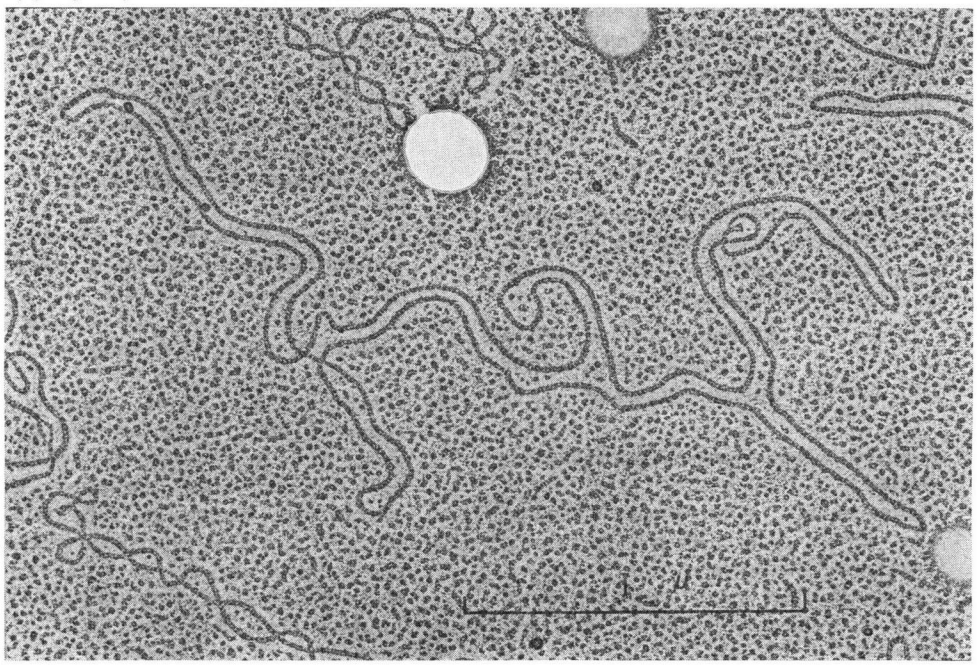
mosomal DNA, was detected under UV light. The plasmid DNA was purified by cesium chloride-ethidium bromide buoyant density gradient centrifugation. After extraction of ethidium bromide by iso-propanol and dialysis against TES, plasmid DNA was obtained without any visible contamination of chromosomal DNA or RNA on agarose gel electrophoresis. Examina-

Fig. 1. Electron micrographs of plasmids isolated from *S. fradiae* ATCC10745

(A): pSF1, 14.9×10^6 dalton DNA.



(B): pSF2, 21.9×10^6 dalton DNA.



tion of the preparation by electron microscopy revealed the presence of two plasmids, pSF1 and pSF2 (Fig. 1), molecular weight (from contour length measurements) 14.9 and 21.9×10^6, respectively, using RSF2124 (7.4×10^6) as the reference plasmid. The plasmids, pSF1 and pSF2, were not digested by EcoRI nor HindIII restriction endonuclease but were cut into more than 5 fragments by SalI, BamI, SmaI, KpnI and PstI. By the same procedure used for the isolation of pSF1 and pSF2, it has not been possible to isolate plasmid DNA from the two variants AO80 and AO83.

We have demonstrated the presence of at least two plasmids in a neomycin-producing organism, *S. fradiae* ATCC10745, and based on curing experiments we can suggest that they play roles in the biogenesis of DOS and APH(3′). Further experiments using plasmid-cured clones as recipients for transformation with isolated plasmid DNA will be important in elucidating the involvement of these plasmids in the biosynthesis of neomycin. Transformation in *Streptomyces*[18] and *Thermoactinomyces*[19] have been studied using DNA preparation obtained by MARMUR's method which contained both chromosomal and plasmid DNA. Intraspecific transformation of *S. griseus* NCIB 8891 (candicidin producer, streptomycin non-producer) with DNA of *S. griseus* MA8 (streptomycin producer) gave streptomycin producers at the frequency as high as 2.5%[18]. However, general methods of high frequency transformation for *Streptomyces* are not yet available.

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