ELIMINATION OF THE ABILITY OF A KANAMYCIN-PRODUCING STRAIN TO BIOSYNTHESIZE DEOXYSTREPTAMINE MOIETY BY ACRIFLAVINE

Sir:

Involvement of plasmids have been reported in production of several antibiotics including kasugamycin[1], aureothricin[1], methylenomycin A[2,3], chloramphenicol[4], turimycin[5], streptomycin[6,7,8] and chlortetracycline[6,7]. Evidences of plasmid involvement have been realized by elimination of antibiotic productivities by treatment with acriflavine or ethidium bromide or incubation at high temperature. Since the kanamycin (KM) productivity was eliminated with a high frequency when a KM-producing strain (*Streptomyces kanamyceticus*) was subjected to acriflavine (AF) treatment, properties of the isolates were examined with reference to KM biosynthesis.

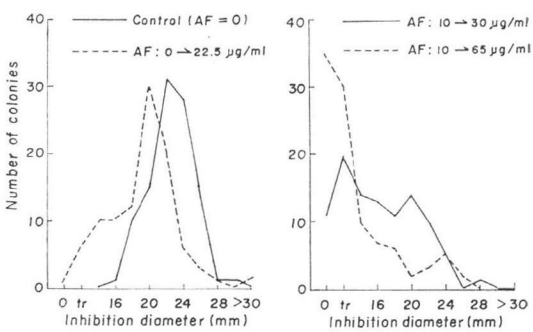
Streptomyces kanamyceticus K-2j grown on maltose (1%)-yeast extract (0.4%) agar slant (indicated as MY hereafter) was transferred into 10 ml of GPY medium consisting of glucose 1.0%, peptone 0.4%, yeast extract 0.4%, KH_2PO_4 0.2% and K_2HPO_4 0.4% in a test tube (24 mm in diameter) and incubated at 27°C for 24 hours under reciprocal shaking. Thereafter, 0.1 ml of the cultured broth was inoculated to fresh GPY medium added with increasing concentrations of AF (0~30 μg/ml with interval of 2.5 μg/ml increase). After 4 days' incubation the cultured broth containing the highest concentration of AF (22.5 μg/ml) permitting the growth was centrifuged, washed twice with saline and treated by a blender (Micronizer, Nihon Seiki Co. product) to cut mycelia. The mycelial suspension thus obtained was diluted, spread on GPY agar plate and incubated at 27°C for 3~4 days. Each of randomly selected 100 colonies grown on the plates was transferred to agar pieces (3 mm in height, 8 mm in diameter) containing glucose 0.1%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.25% and agar 1.5%, pH 7.0 (KM production medium). These agar pieces after 7 days incubation at 27°C were placed on agar plates seeded with *Bacillus subtilis* PCI 219 in order to detect the antibiotic production. One among 100 isolates showed no detectable KM production. On the other hand, when 0.1 ml portion of the cultured broth containing 10 μg/ml of AF was transferred to the medium containing 20~80 μg/ml of AF and further cultured, the frequency of isolating KM-nonproducing strains increased with the increased concentrations of AF. Among randomly selected 100 isolates obtained from media containing 30 or 65 μg/ml of AF, 11 and 35 isolates lost the ability to produce the antibacterial activity, respectively (Fig. 1).

Similarly, the frequency of isolating strains of low KM-productivity increased when *S. kanamyceticus* K-2j was incubated at 35°C which was the highest temperature permitting the growth (for 5 days without AF). One among 100 isolates did not produce any antibiotic activity.

Isolates No. 33, 211, 311 and 811 thus obtained by AF treatment did not regain antibiotic productivity after three repeated transfers on GPY and MY agar media, whereas many of apparent KM-nonproducing isolates regained the productivity gradually by repeated transfer. This reversion seemed to be due to that segregation of the isolates of multicellular mycelium. The above four isolates which showed no reversion were studied on their KM biosynthesis, resistance to KM and morphology in comparison with the original strain (*S. kanamyceticus* K-2j).

In order to know which steps in KM biosynthesis were defficient in the KM-nonproducers, activities of KM acetyltransferase and N-acetylkanamycin amidohydrolase, which had been suggested to be involved in the later steps[9,10,11], were examined. KM-acetyltransferase was assayed as follows: mycelia in log phase grown in a KM-production medium were harvested by centrifugation (10,000 *g*, 15 minutes, 5°C), washed twice with 10 mM Tris-HCl buffer (pH 8.0)

Fig. 1. Elimination of kanamycin productivity of *Streptomyces kanamyceticus* K-2j by acriflavine (AF) treatment.



containing 5 mM 2-mercaptoethanol, resuspended in 3~4 fold volume of the above buffer, disrupted twice by French press (1,200 kg/cm^2) following a blender treatment, centrifuged at 30,000 *g* for 30 minutes at 5°C, and subjected to the enzyme activity assay. The supernatant was used as the enzyme solution. The reaction mixture (1 ml) was consisted of the enzyme solution, ATP 16 mM, Co-enzyme A 0.24 mM, Mg-acetate 10 mM, KCl 60 mM and KM 0.4 mM in 100 mM Tris-HCl buffer (pH 8.0) containing 5 mM 2-mercaptoethanol. The reaction was carried out at 27°C for 2 hours and stopped by heating for 3 minutes in a boiling water bath. The antibiotic activity remaining in the supernatant was determined by cylinder assay method. As shown in Table 1, KM was inactivated remarkably indicating the presence of KM-acetyltransferase in the KM-nonproducing isolates described above, although the specific activities of these isolates were about 66~80% of that of the parent.

N-Acetylkanamycin amidohydrolase activity was also detected by incubating the washed mycelia of stationary phase of growth with 6′-N-acetylkanamycin according to the method previously reported[12].

In the next experiment, we examined KM-production by the KM-nonproducers in media supplemented with compounds known as precursors such as D-glucosamine, *myo*-inositol, 2-deoxystreptamine (DOS) and 3-amino-3-deoxyglucose (3AG)[13,14], singly and in combination. As shown in Table 2, KM-nonproducers produced a significant amount of the antibiotic only in media supplemented with DOS. *S. kanamyceticus* K-2j produced the antibiotic in all media tested. The effect of DOS on KM production by the KM-nonproducing isolates was also confirmed in a liquid medium as shown in Table 3. Addition of DOS at the concentration of 750 μg/ml resulted in 9 μg/ml of KM production by the isolate No. 311 and in 23 μg/ml by the isolate No. 811. The antibiotic produced in the presence of DOS was identified to be KM (kanamycin A) by high voltage paper electrophoresis. The results indicated that all the isolates had lost the ability to biosynthesize DOS moiety and suggested that a plasmid should control the formation of enzymes involved in biosynthesis of this characteristics structural part of kanamycins.

Table 1. Activity of kanamycin acetyltransferase

Organism	KM inactivated nmoles	Protein in the reaction mixture, mg	Enzyme activity	
			nmoles/ml·mg protein	Specific activity %
S. kanamyceticus				
K-2j	400	24	16.7	100
33	400	35	11.5	69.0
211	200	15	13.3	79.8
311	400	30	13.3	79.8
811	320	29	11.0	66.0

Table 2. Effect of precursors on strains** of *Streptomyces kanamyceticus*

Additives	Concn. (μg/ml)	Strains of *S. kanamyceticus*			
		K-2j	33	311	811
None	1,000	28.0***	tr	0	0
DOS*	〃	30.0	19.0	16.5	18.0
GLS*	〃	29.0	tr	0	0
3AG*	〃	26.5	tr	0	0
myo-Inositol	〃	29.5	tr	0	0
DOS+GLS+3AG	each 700	29.5	21.0	tr	14.5
DOS+GLS	〃	30.0	18.0	15.5	15.5
DOS+3AG	〃	30.0	23.0	13.5	tr
GLS+*myo*-Ino.	〃	29.5	tr	0	0

* DOS: 2-deoxystreptamine, 3AG: 3-amino-3 deoxyglucose, GLS: D-glucosamine.

** Strains were incubated on agar piece (3 mm in height, 8 mm in diameter) containing glucose 0.1%, yeast extract 0.2%, peptone 0.4%, $MgSO_4 \cdot 7H_2O$ 0.25%, agar 1.5% and additive, pH 7.0.

*** Inhibition diameter (mm) measured by cylinder assay using *Bacillus subtilis* PCI 219 as a test organism.

Table 3. Effect of 2-deoxystreptamine on the production of kanamycin by isolates in a liquid medium

Additive	Concn. (μg/ml)	Kanamycin production (μg/ml)					
		K-2j		311		811	
		2*	4*	2*	4*	2*	4*
None	—	17	15	0	0	0	0
DOS	750	15	19	9	8	23	20
D-Glucosamine	〃	20	18	0	0	0	0
myo-Inositol	〃	15	18	0	0	0	0

* Organisms were incubated on a reciprocal shaker for 2 and 4 days.

Since it was reported that the loss of antibiotic productivity by AF treatment was accompanied by the loss of resistance to the own antibiotic[3] or formation of aerial mycelium[1], the isolates were also examined.

When their mycelia were streaked on MY medium containing KM (100~1,000 μg/ml with interval of 100 μg/ml), the resistance to KM was about 200 μg/ml in the isolates Nos. 211, 311 and 811 and about 500 μg/ml in the isolate No. 33 and the parent (*S. kanamyceticus* K-2j). Similar results were obtained when they were cultured in a liquid medium containing KM according to the method previously reported[15]. Increasing KM concentrations resulted in the delay of the growth at the start. In the presence of 100 μg/ml of KM, the time-lag before the start of the growth was 24 hours in the strain K-2j and the isolate No. 33 and 48 hours in the isolates Nos. 211, 311 and 811, respectively. All the isolates retained a high resistance to KM concentrations.

Formation of aerial mycelium of the isolates was influenced by carbon sources in a medium. All the KM-nonproducing isolates did not form aerial mycelium after 14 days incubation at 27°C on MY and GPY agar media. They formed, however, aerial mycelium on ISP No. 9 medium[16] supplemented with 1% of L-arabinose or D-fructose, but not on the medium supplemented with 1% of glucose or mannitol, while *S. kanamyceticus* K-2j formed aerial mycelium on all above media. It suggested that the aerial mycelium formation of these KM-nonproducing isolates became sensitive to catabolite repression as the result of AF treatment.

As described above, the ability of a KM-producing strain to biosynthesize 2-deoxystreptamine moiety was eliminated by acriflavine treatment, and the involvement of plasmid(s) in biosynthesis of this characteristic structure moiety of kanamycins was suggested.

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