Communication to the editor

TRANSFER OF THE LEUPEPTIN-PRODUCING ABILITY OF THE STRAIN, *STREPTOMYCES ROSEUS* MA839-A1, BY CONJUGATION

Sir:

Plasmids have been reported to be involved in the phenotypic expression of several characters such as fertility[1,2] and the formation of melanin[3], antibiotics[4~10] and aerial mycelium[4,11] in *Streptomyces* species. The elimination of these characters by treatment with acridinium or phenanthrium dyes has been used to recognize the involvement of a plasmid. The ability to produce an antibiotic or a small molecular weight enzyme inhibitor is restricted to certain strains of a given species. However, the same antibiotic or enzyme inhibitor may be produced by other strains belonging to other species. These observations suggest that the presence of plasmids may be required for the synthesis of microbial secondary metabolites[12]. Leupeptin (acetyl or propionyl-L-leucyl-L-leucyl-L-arginal)[13] which inhibits the enzymes; trypsin, cathepsin B and papain has been found in culture filtrates of many strains belonging to more than 17 species of *Streptomyces*[14]. We, therefore, investigated the possible involvement of a plasmid in leupeptin biosynthesis and its possible transfer by conjugation.

Streptomyces roseus MA839-A1, a leupeptin producer, was subjected to acriflavine (AF) treatment. Since the growth of the strain was completely inhibited by a low concentration of AF (5 μg/ml) in a medium consisting of glucose, 1.0%; peptone, 0.4%; yeast extract, 0.4%; KH_2PO_4, 0.2% and K_2HPO_4, 0.4% (GPY medium), a strain resistant to AF was selected by cultivation in the above medium containing 2.5 μg/ml of AF. The strain thus selected exhibited the same ability to produce leupeptin as the original culture and was resistant to considerably higher concentrations of AF than that used for the selection process. The resistant strain was incubated at 27°C or 35°C in the presence of increasing concentrations of AF. After 3 days' incubation, the medium containing the highest concentration of AF permitting growth of the organism was centrifuged. The mycelium was washed twice with saline and treated in a blendor (Polytron, Kinematica Gmbh, Switzerland) to fragment the mycelium. The suspension of the fragmented mycelium was diluted with saline and spread on GPY agar medium.

About 100 colonies thus obtained were inoculated into a leupeptin production medium consisting of soluble starch, 2.0%; glucose, 1.0%; peptone, 2.0% and NaCl, 0.5% and cultivated for 3 days at 27°C on a reciprocal shaker. The leupeptin titer in culture filtrates was measured as inhibition of trypsin activity by the method of AOYAGI *et al.*[15]

As shown in Table 1, the maximal concentration of AF which allowed growth was 50 μg/ml at 27°C and 30 μg/ml at 35°C, and strains which had lost the ability to produce leupeptin appeared with a frequency of about 2.0% to 3.0%. Of the 5 leupeptin-nonproducing strains obtained, 4 lacked aerial mycelium, however, the fifth one produced spores comparable to the parental strain.

Table 1. Isolation of leupeptin-nonproducing strains by acriflavine treatment

Organism	Culture condition			No. of colonies tested	No. of leupeptin nonproducing colonies
	Temp. (°C)	AF* (μg/ml)	Time (days)		
Streptomyces roseus MA839-A1	27	2.5	3	98	0
	〃	50	〃	100	2
	35	2.5	3	100	0
	〃	30	〃	100	3

* Acriflavine

Table 2. Frequency of leupeptin producers in arginine-requiring mutants after conjugation between leupeptin-producing and nonproducing auxotrophs

	Number of colonies of 0.05 ml suspension			Leupeptin production in arginine⁻ colonies		
	MM*1	MM+ arginine	MM+ methionine	Colonies tested	Leupeptin producing colonies	Frequency of leupeptin producers
Experiment 1	0(1)*2	12*3(10^{-1})*2	2.7*3(10^{-5})*2	75*4	4	5.3%
〃 2	0(1)	10 (10^{-1})	4.0 (10^{-5})	45	5	11.1%

*1 Minimal medium

*2 Numbers in parenthesis indicate dilution of the suspension; (1) means undiluted and (10^{-1}) and (10^{-5}) mean 10^{-1} and 10^{-5} dilutions, respectively.

*3 Mean colony numbers on a plate. 0.05 ml of each diluted suspension was spread on 10 plates.

*4 75 and 45 colonies were randomly selected from 120 and 100 colonies on 10 plates in two experiments, respectively.

To obtain additional evidence for the involvement of a plasmid in leupeptin formation we attempted to transfer the ability to produce leupeptin by conjugation. Three methionine-requiring mutants were obtained by UV-irradiation (killing rate, 99.9%) of a spore suspension (10^8/ml) of *S. roseus* MA839-A1. All three mutants produced leupeptin and formed spores. More than 10 arginine-requiring mutants were obtained by similar means from the spore-forming leupeptin-nonproducing strain described above. All of the latter mutants lacked the ability to produce leupeptin; three of them produced spores similar to the original strain. Each of the auxotrophs (met⁻, arg⁺, leupeptin⁺, spore⁺ and met⁺, arg⁻, leupeptin⁻, spore⁺) was cultured on a sheet of cellophane spread over the surface of a maltose (1.0%)-yeast extract (0.4%) agar medium (MY) at 27°C for 10 days to sporulate. Spores were then harvested with 5 ml of a salt solution consisting of NaCl, 0.5%; $MgSO_4 \cdot 7H_2O$, 0.05% and Aerosol OT, 0.005%. The suspension was vigorously shaken in a mixer to break up spore chains and then passed through cotton wool to remove large pieces of mycelium or spore aggregates. One ml of the spore suspension (8.8×10^8/ml) of the leupeptin-producing auxotroph was mixed with 1 ml (8.6×10^6/ml) of the leupeptin-nonproducing auxotroph and 0.025 ml of the mixture was then spread on plates of MY agar medium and incubated at 27°C for 2 weeks. The spores thus formed were harvested with 5 ml of saline, shaken, filtered through cotton wool, centrifuged at 3,000 *g* for 10 minutes and washed twice with the above salt solution. The spores were resuspended in 5 ml of the salt solution and shaken vigorously in a mixer and diluted. Then 0.05 ml of a $1 \sim 10^{-5}$ dilution of the suspension was spread on each of 10 plates of a minimal medium (MM) consisting of glucose, 1.0%; L-asparagine, 0.1%; NaCl, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.1%; trace element solution[16] ($ZnCl_2$, 40 mg; $FeCl_3 \cdot 6H_2O$, 200 mg; $CuCl_2 \cdot 2H_2O$, 10 mg; $MnCl_2 \cdot 4H_2O$, 10 mg; $NH_4Mo_7O_{24}$, 10 mg and $Na_2B_4O_7$ 10 mg/liter), 0.4% (v/v); KH_2PO_4, 0.2%; K_2HPO_4, 0.4% and special Noble agar (Difco) 2.0% and MM supplemented with L-methionine (0.1%) or L-arginine (0.1%).

As shown in Table 2, no colonies appeared on MM. In two experiments 12 and 10 (as the mean) colonies, respectively, appeared on a plate of MM supplemented with arginine after 4 days' incubation at 27°C when a 10^{-1} dilution of the spore suspension was used. On the other hand, 2.7 and 4.0 (as the mean) colonies were observed on the plate of MM supplemented with methionine after 4 days' incubation even when a 10^{-5} dilution of the spore suspension was employed. To purify the clones, colonies grown on MM supplemented with arginine were transferred with sterile toothpicks to the same fresh medium. After it was confirmed that these colonies did not grow on MM supplemented with methionine, they were randomly selected and inoculated into the leupeptin production medium described above. Thus, 4/75 and 5/45 colonies tested were confirmed to produce leupeptin, respectively. As a control the strains derived from 100 spores of the arginine-requiring strain used for this experiment were tested and found to lack the ability to produce leupeptin. These results indicate that the ability to produce leupeptin was transferred from the leupeptin-producing

strain to the leupeptin-nonproducing strain previously treated with AF and the rate of the transfer was significantly higher than that of transfer of arginine or methionine markers which are considered to be located on the chromosome[17]. Accordingly, it is suggested that a plasmid is involved in leupeptin production and that this plasmid is transferred by conjugation.

HORI *et al.*[18,19] have succeeded in isolating a multienzyme system from strain MA839-A1 which was used in the present experiments. The multienzyme complex catalyzes the biosynthesis of leupeptin acid (acetylleucylleucyl-arginine) in reaction mixtures containing the following components: (1) acetate, leucine, arginine and ATP; (2) acetylleucine, leucine, arginine and ATP; or (3) acetylleucylleucine, arginine and ATP. It has also been shown that a homogenate of the leupeptin-producing strain contains a labile enzyme that catalyzes the reduction of leupeptin acid to leupeptin. An analogous multienzyme complex was not detected in homogenates of the leupeptin-nonproducing strains obtained by AF treatment. These findings indicate that a plasmid specifies the biosynthesis of leupeptin through the formation of a multienzyme system which catalyzes leupeptin acid synthesis.

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