STUDIES ON NEW AMINOGLYCOSIDE ANTIBIOTICS, ISTAMYCINS, FROM AN ACTINOMYCETE ISOLATED FROM A MARINE ENVIRONMENT

II. POSSIBLE INVOLVEMENT OF PLASMID IN ISTAMYCIN PRODUCTION

KUNIMOTO HOTTA, YOSHIRO OKAMI and HAMAO UMEZAWA

Institute of Microbial Chemistry
14–23 Kamiosaki 3-Chome, Shinagawa-ku, Tokyo, Japan

(Received for publication August 28, 1980)

Acriflavine treatment of an istamycin-producing *Streptomyces tenjimariensis* strain designated SS-939 resulted in a high frequency of isolates with reduced istamycin production. Some of these were shown to have lost a particular plasmid present in the parent strain. Istamycin production by these isolates was largely restored by the addition of 2-deoxystreptamine (DOS) to the medium whereas the effect of DOS was small in the strain SS-939. Sodium palmitate also stimulated production, especially when added together with DOS. These stimulative effects by DOS and palmitate, however, were not exhibited in the presence of glucose (1.0%).

In previous papers[1,2], we reported that *Streptomyces tenjimariensis* SS-939 harbors plasmids and produces new aminoglycoside antibiotics, istamycins A and B. In this paper, the effect of acriflavine treatment on istamycin production was studied to examine the possible involvement of plasmid(s) in istamycin production.

Materials and Methods

Acriflavine treatment

Streptomyces tenjimariensis SS-939 was treated with acriflavine according to the method described in a previous paper[3].

Production of istamycin

The parent strain (SS-939) and acriflavine-treated isolates were incubated at 27°C on agar (1.5%) pieces and in a liquid medium. Both media contained the following ingredients; potato starch 1.0%, soy bean meal (Prorich S; Ajinomoto Co.) 1.5%, glucose 0.2%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1% and K_2HPO_4 0.1%, pH 7.0. Antibiotic activity was measured as istamycin A production using *Bacillus subtilis* PCI 219 as the test organism.

Detection of plasmids

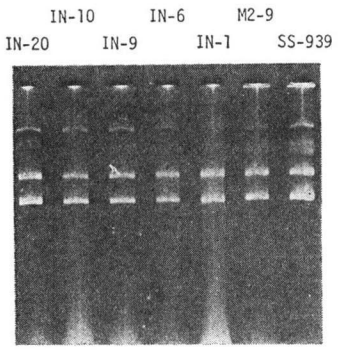
Plasmid DNA was separated from chromosomal DNA by ethidium bromide-cesium chloride ultracentrifugation[1], dialyzed against TES[1], loaded on a sucrose neutral density gradient solution (5~20%) and centrifuged at 36,000 r.p.m. for 105 minutes at 20°C in a Hitachi RPS 40T swing rotor. Agarose gel electrophoresis was performed by the method described in a previous paper[1].

Results

Effect of Acriflavine Treatment on the Plasmid Profile of *S. tenjimariensis* SS-939

When *S. tenjimariensis* SS-939 was treated with 15 μg/ml of acriflavine for 4 days, isolates without

Fig. 1. Agarose gel electrophoresis of plasmids isolated from SS-939 and its acriflavine-treated isolates.



antibiotic activity on the agar piece medium appeared at a frequency of more than 20%. All the isolates still harbored plasmids detected by EtBr-CsCl ultracentrifugation. However, a few of them seemed to have lost a certain species of plasmid, which became evident when agarose gel electrophoretic patterns were examined (Fig. 1) and compared with that of the parent strain. Sucrose neutral density gradient centrifugation (Figs. 2 and 3) supported this result. As shown in Fig. 2, five peaks were detected in the case of the parent strain and three of them (indicated as I, II and IV) contained plasmids when they were examined by agarose gel electrophoresis. In contrast, only two plasmid-containing peaks were observed in the acriflavine treated isolate IN-9 as shown in Fig. 3: *i.e.* the peak III observed in the parent indicated by an arrow could not be detected in the isolate. Peaks II and V were identified as chromosomal DNA and sheared DNA pieces.

Fig. 2. Sucrose neutral density gradient centrifugation of satellite DNA fractions in ethidium bromide-cesium chloride ultracentrifugation of SS-939.

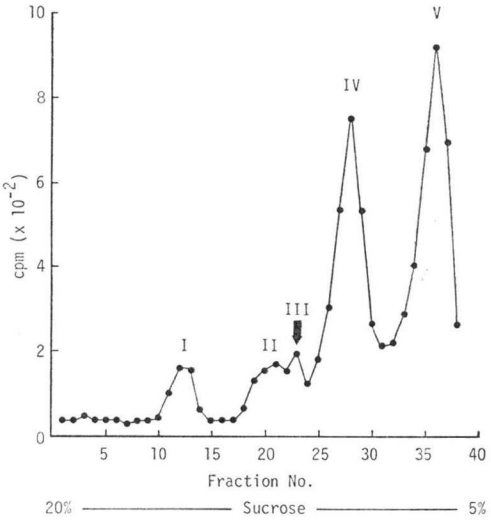
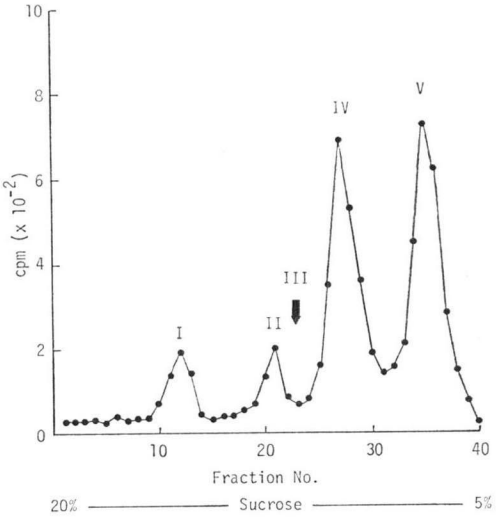


Fig. 3. Sucrose neutral density gradient centrifugation of plasmid DNA fractions from the acriflavine treated isolate IN-9.



Istamycin Production by Acriflavine-Treated Isolates

Acriflavine treatment of *S. tenjimariensis* SS-939 yielded isolates which lost both the capacity to produce istamycin on the agar medium and a particular plasmid. Istamycin production by these isolates was further examined in a liquid medium on a rotary shaker (180 r.p.m.) and compared to that of the parent strain.

Istamycin has an aminocyclitol moiety (2-deoxyfortamine). This moiety is similar to 2-deoxystreptamine (DOS) which is a moiety common to several aminoglycoside antibiotics, such as kanamycin and neomycin. Idiotrophs requiring DOS were reported to be obtained by acridine dye treatment

Fig. 4. Effect of deoxystreptamine on istamycin production by acriflavine-treated isolates.

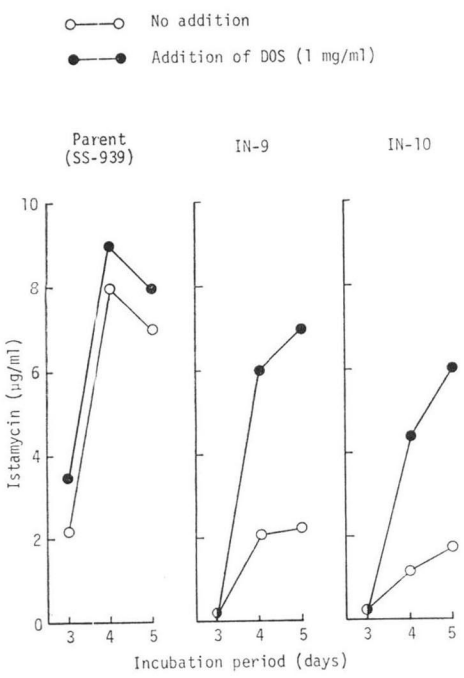
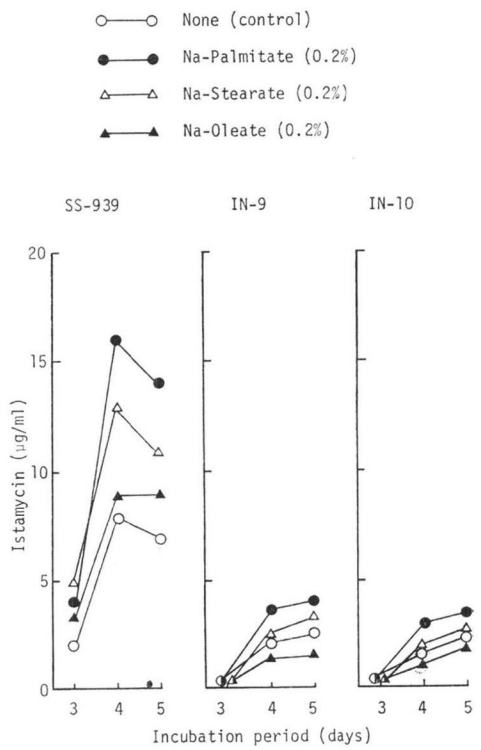


Fig. 5. Effect of fatty acid on istamycin production.



of kanamycin-[3,4], neomycin-[5] or paromomycin-[6] producing *Streptomyces* species. Streptamine also induced antibiotic production in a DOS-requiring idiotroph of a kanamycin producer[4]. In view of these results, the effect of aminocyclitol on istamycin production by acriflavine-treated isolates was examined using DOS instead of 2-deoxyfortamine. As shown in Fig. 4, the isolates which were selected as low istamycin producers produced istamycins prior to addition of DOS at a level about 1/3 of the amount produced by the parent. Addition of DOS (0.1%) increased antibiotic production by isolates IN-9 and IN-10 up to a level comparable to that of the parent. DOS stimulated production in the parent too, but the effect of DOS was considerably lower than in the isolates.

It is also known that treatment of antibiotic-producing streptomycetes with curing agents often causes an apparent pleiotropic change in their phenotypes. It has been suggested that these effects can be explained as a result of altered membrane permeability[7]. The effect of aliphatic fatty acids was examined since it has been reported that administration of fatty acids such as oleic and palmitic acids to an *S. fradiae* crypto-producer of neomycin resulted in restoration of neomycin production to the level of its parent strain, in addition to altering the fatty acid composition of its cellular membrane[8,9]. As shown in Fig. 5, all fatty acids (palmitic, stearic and oleic acids) tested stimulated istamycin production by the parent strain. The strongest effect was shown by palmitic acid which doubled istamycin production. Palmitic acid also stimulated production by the acriflavine-treated isolates.

Table 1 shows the effect of glucose on istamycin production. Increasing the concentration of glucose in the medium from 0.2% to 1.0% resulted in a marked decrease of istamycin production by the acriflavine treated isolates, but not by the parent strain.

Since it was thus found that at least three chemical factors, DOS, palmitate and glucose, influenced

Table 1. Effect of glucose on istamycin production by acriflavine-treated isolates.

Addition of glucose	Istamycin produced*					
	SS-939		IN-9		IN-10	
	pH	μg/ml	pH	μg/ml	pH	μg/ml
0.2 %	8.4	4.5	8.4	0.8	8.4	1.5
0.5	8.2	4.5	8.2	0.9	8.2	1.3
1.0	8.2	4.5	7.4	0.5	8.0	0.3
1.5	7.6	4.5	7.2	0.3	7.6	0.2
2.0	6.6	4.5	6.2	0.2	7.0	0.2

* Basal medium consists of starch (potato) 1.0 %, soy bean meal 1.5 %, NaCl 0.3 %, $MgSO_4 \cdot 7H_2O$ 0.1 %, and K_2HPO_4 0.1 %. Organisms were incubated at 27°C for 4 days.

Table 2. Combined effect of glucose, deoxystreptamine and palmitate on istamycin production.

Additive* (%)			Istamycin (μg/ml)		
Glu	DOS	Pal	SS-939	IN-9	IN-10
0.2	—	—	4.2	1.0	1.5
	0.1	—	4.2	3.0	3.0
	—	0.2	8.0	1.8	1.8
	0.1	0.2	11.0	5.0	4.2
0.5	—	—	4.0	0.6	0.6
	0.1	—	4.6	3.2	3.0
	—	0.2	5.4	1.3	0.6
	0.1	0.2	5.0	2.7	0.8
1.0	—	—	4.0	0.6	0.4
	0.1	—	4.2	0.6	0.8
	—	0.2	5.8	0.6	0.4
	0.1	0.2	5.0	1.5	0.8

* Basal medium: starch (potato) 1.0 %, soy bean meal 1.5 %, NaCl 0.3 %, $MgSO_4 \cdot 7H_2O$ 0.1 % and K_2HPO_4 0.1 %.

istamycin production, the combined effect of these factors was examined (Table 2). When both DOS and palmitate were added to a medium containing a low concentration of glucose (0.2%), istamycin production by both the parent and the isolates was synergistically enhanced. This effect, however, was depressed by increasing glucose concentration up to 0.5% or 1.0%. The effect of DOS on the isolates and of palmitate on the parent were also markedly depressed by the presence of 1.0% glucose. A synergistic effect of DOS and palmitate was not exhibited in the presence of 1.0% glucose.

Discussion

Acriflavine treatment of istamycin producing *S. tenjimariensis* strain SS-939 resulted in the occurrence of isolates which exhibited markedly lowered istamycin productivity accompanied by loss of a particular plasmid. Istamycin production by these isolates was restored by the addition of DOS to a level comparable to that of the parent strain, although the parent strain was not strongly influenced by DOS. Sodium palmitate also showed a stimulative effect on istamycin production, especially in the parent strain, and combined addition of DOS and palmitate further increased istamycin production by both the parent and the isolates. This marked stimulative effect on istamycin production, however, was not exhibited when the glucose concentration in the medium was increased from 0.2% to 1.0%. These observations suggest that a certain species of plasmid is involved in istamycin production by *S. tenjimariensis* SS-939 and the composition of membrane is an important factor in istamycin production as was shown by ARIMA *et al.*[8,9] to be the case in neomycin production by an *S. fradiae* crypto-producer of neomycin, although detailed studies are needed.

Plasmids participate in antibiotic production as carriers for genes involved in biosynthesis, as demonstrated in methylenomycin A production by *S. coelicolor* A3(2)[10,11], in self-resistance[10,12~14] and possibly in membrane permeability[7]. In istamycin production, at least one plasmid which was cured by acriflavine treatment may be involved through membrane permeability. However, the other plasmids observed in the strain could not be cured by acriflavine treatment, and their roles remain completely unknown. Further investigation is now in progress.

It is also of interest that DOS stimulated antibiotic production by acriflavine-treated isolates. A dose response to the stimulation was also observed (data not shown). We are now studying whether DOS causes new antibiotic formation as result of intact incorporation during synthesis of istamycin(s).

Acknowledgements

This investigation was supported by a grant No. 410710 of the Ministry of Education, Science and Culture in Japan. The authors are grateful to Miss N. SAITO for her technical assistance and Miss M. HAYGOOD, Misaki Marine Biological Station, Department of Science, University of Tokyo, for revision of this manuscript.

References

1) HOTTA, K.; N. SAITO & Y. OKAMI: Studies on new aminoglycoside antibiotics, istamycins, from an actinomycete isolated from a marine environment. I. The use of plasmid profiles in screening antibiotic-producing streptomycetes. J. Antibiotics 33: 1502~1509, 1980
2) OKAMI, Y.; K. HOTTA, M. YOSHIDA, D. IKEDA, S. KONDO & H. UMEZAWA: New aminoglycoside antibiotics, istamycins A and B. J. Antibiotics 32: 964~966, 1979
3) HOTTA, K.; Y. OKAMI & H. UMEZAWA: Elimination of the ability to biosynthesize deoxystreptamine moiety by acriflavine. J. Antibiotics 30: 1146~1149, 1977
4) CHANG, L.; D. A. BEHR & R. P. ELANDER: Effects of plasmid-curing agents on cultural characteristics and kanamycin formation in a production strain of *Streptomyces kanamyceticus*. Abstr. 3rd Int. Symp. Genet. Ind. Microorganisms, Madison, Wis., Abstr. No. 73, p. 36, 1978
5) YAGISAWA, M.; T-S. R. HUANG & J. E. DAVIES: Possible involvement of plasmids in biosynthesis of neomycin. J. Antibiotics 31: 809~813, 1978
6) WHITE, T. J. & J. DAVIES: Possible involvement of plasmids in the biosynthesis of paromomycin. Abstr. 3rd Int. Symp. Genet. Ind. Microorganisms, Madison, Wis., Abstr. No. 79, p. 36, 1978
7) OKANISHI, M.: Plasmids and antibiotic synthesis in streptomycetes. *In* Genetics of Industrial Microorganisms (*eds.* SEBEK, O. K. & A. I. LASKIN), pp. 134~140, American Society for Microbiology, Washington, D. C., 1979
8) ARIMA, K.; H. OKAZAKI, H. ONO, K. YAMADA & T. BEPPU: Effect of exogenous fatty acids on the cellular fatty acid composition and neomycin formation in a mutant strain of *Streptomyces fradiae*. Agr. Biol. Chem. 37: 2313~2318, 1973
9) OKAZAKI, H.; H. ONO, K. YAMADA, T. BEPPU & K. ARIMA: Relationship among cellular fatty acid composition, amino acid uptake and neomycin formation in a mutant strain of *Streptomyces fradiae*. Agr. Biol. Chem. 37: 2319~2325, 1973
10) KIRBY, R. & D. A. HOPWOOD: Genetic determination of methylenomycin synthesis by the SCP 1 plasmid of *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. 98: 239~252, 1977
11) HOPWOOD, D. A.: Extrachromosomally determined antibiotic production. Ann. Rev. Microbiol. 32: 373~392, 1978
12) BORONIN, A. M. & L. G. SADOVNIKOVA: Elimination by acridine dyes of oxytetracycline resistance in *Actinomyces rimosus*. Genetika 8: 174~176, 1972
13) KIRBY, R.; L. F. WRIGHT & D. A. HOPWOOD: Plasmid-determined antibiotic synthesis and resistance in *Streptomyces coelicolor*. Nature 254: 265~267, 1975
14) SHAW, P. D. & J. PIWOWARSKI: Effect of ethidium bromide and acriflavine on streptomycin production by *Streptomyces bikiniensis*. J. Antibiotics 30: 404~408, 1977