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

KUNIMOTO HOTTA, NORIKO SAITO and YOSHIRO OKAMI

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

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Plasmid profiles were used to screen streptomycetes for production of new antibiotics. Among about 100 strains isolated from sea muds, an isolate designated SS-939 was revealed to harbor several plasmids of different sizes, and to produce istamycins, new aminoglycoside antibiotics. Based on the characteristics of the strain, a new *Streptomyces* species is proposed: *S. tenjimariensis*.

Reports[1~17] of plasmid involvement in antibiotic production by streptomycetes have increased since OKANISHI *et al.*[10] suggested that plasmids are involved in kasugamycin and aureothricin production by *Streptomyces kasugaensis*. It has also been shown that various antibiotic-producing streptomycetes harbor plasmids which vary in size and/or form depending on the antibiotic produced[6~9,16~27]. These observations led us to attempt a search for new antibiotics based on plasmid profiles.

Using other techniques, we had been screening actinomycetes isolated from sea muds and discovered some new antibiotics such as SS-228Y[28] and aplasmomycin[29], indicating that marine environments provide a good source of new antibiotic-producing strains.

Therefore, about 100 strains of antibiotic-producing streptomycetes newly isolated from coastal sea muds were screened for the presence of plasmids, and isolates having both antibiotic productivity and plasmids were studied. This paper deals mainly with a strain designated SS-939 which harbors plasmids and produces istamycins, new aminoglycoside antibiotics. We propose that it is named *Streptomyces tenjimariensis* nov. sp. HOTTA *et* OKAMI.

Materials and Methods

Isolation of organisms

Sea muds were collected on the shore of Sagami Bay at the Tenjin-Island Marine Biological Garden of the Yokosuka City Museum. Approximately 1 ml of each sea mud sample was suspended in 0.85% NaCl solution (4 ml), stirred vigorously and a 0.05 ml portion was spread on Maltose (1.0%)-Yeast Extract (0.4%) agar and/or Inorganic Salts-Starch (ISP No. 4) agar supplemented with 20 μg/ml of antibiotics such as nystatin and kanamycin. Colonies were picked after incubating 4~10 days at 27°C.

Detection and isolation of plasmids

The method employed for the detection and isolation of plasmids was almost the same as that described by OKANISHI *et al.*[26] After rotary shaking at 27°C in Tryptic Soy Broth (Difco) for 2 days or in a synthetic medium containing 6-[^{3}H]-thymidine for 24 hours, mycelia were harvested by centri-

fugation, washed twice by centrifugation in TES buffer (50 mM Tris, 50 mM 3Na-EDTA and 50 mM NaCl, pH 7.4) and, if necessary, treated with 0.1 N NH_4OH - 10 mM 3Na-EDTA solution at room temperature for 20 minutes followed by washing with TES buffer. Several methods were used to lyse the mycelia and precipitate the DNA. One g of the washed mycelium was suspended in 5 ml of the buffer, then lysed by incubation at 37°C with lysozyme (2~4 mg/ml) for 10~30 minutes followed by addition of either sarkosyl (1.0%) or SDS (1.5%). When sarkosyl was used, a solution cleared by centrifugation of the mycelial lysate at 20,000×*g* for 30 minutes at 20°C was either dialyzed against TES buffer at 4°C overnight or the solution was brought to a concentration of 1 M NaCl and 10% polyethylene glycol (PEG) 6000 to precipitate DNA. In the case of SDS lysis, NaCl was added to the mycelial lysate cleared by centrifugation which was then placed in an ice bath and treated with pronase and PEG 6000 according to OKANISHI *et al.*[26] omitting RNase treatment. The precipitated DNA was collected by centrifugation (1,000×*g*, for 5 minutes) and dissolved in TES buffer.

In order to detect plasmid DNA, the DNA-containing solution was subjected to agarose gel electrophoresis. Gels containing 0.7% agarose (Seakem ME) were made in a horizontal type apparatus. Agarose was melted by autoclaving and then concentrated (10×) TBE buffer (90 mM Tris, 90 mM boric acid and 4 mM 3Na-EDTA, pH 8.3) was added in a ratio of 1/10 the volume of the agarose solution. Ethidium bromide solution (1 mg/ml) was also added up to a concentration of 0.5 μg/ml. Samples, 30~40 μl, containing 5% glycerol, 0.1% bromphenol blue and TBE buffer were loaded onto the gel. Gels were electrophoresed at 40 mA and 120 V for about 2 hours at room temperature. The gel was photographed under UV light through UV and orange filters using Kodak Tri-X film.

When a plasmid band was observed under UV light (short wave) illumination, the original DNA-containing solution was centrifuged in an ethidium bromide-cesium chloride density gradient according to OKANISHI *et al.*[26] Plasmid DNA separated from chromosomal DNA was collected in fractions. Radioactivity was measured by usual methods when DNA was labeled with 6-[^{3}H]-thymidine. Plasmid DNA was further purified by EtBr-CsCl recentrifugation.

Plasmids were also examined by electron microscopy after treatment according to the formide technique[31].

Classification of organisms

Organisms were taxonomically classified with reference to the 8th ed. of BERGEY's Manual of Determinative Bacteriology[32], ISP descriptions[33] and recent reports on classification of actinomycetes. Methods described by SHIRLING and GOTTLIEB[30] were employed for classification. The chemical composition of the cell wall was analyzed by the method of BECKER and LECHEVALIER[34]. Cultural characteristics were observed on media described by SHIRLING and GOTTLIEB[30] and WAKSMAN[35]. Incubation was carried out at 27°C.

Results

Plasmids Observed in Streptomycetes Isolated from Sea Muds

Agarose gel electrophoresis of cleared lysates of 110 strains yielded 8 strains with satellite bands indicative of plasmids. Among these, plasmids from 5 strains could be separated from chromosomal DNA by EtBr-CsCl density gradient centrifugation, while the other three could not be separated, suggesting they are open circular or linear plasmids. At least 6 satellite bands in SS-939, 5 in SS-980, 3 in SS-996, 1 in SS-1000 and 7 in SS-1125 were observed when the separated plasmids were electrophoresed in an agarose gel again (Fig. 1). Plasmid profiles of the 5 strains differed and all the strains produced antibiotics active against bacteria when cultured in a medium containing starch and soy bean meal. Strains SS-939, SS-980 and SS-996 produced water soluble basic antibiotics and the others lipophilic ones. Water soluble basic antibiotics produced by SS-939 were found to be active against both Gram-positive and Gram-negative bacteria including clinically isolated strains resistant to known aminoglycoside antibiotics. A detailed chemical analysis of the antibiotics revealed the struc-

Fig. 1. Agarose gel electrophoresis of plasmids observed in *Streptomyces* isolates from sea muds.

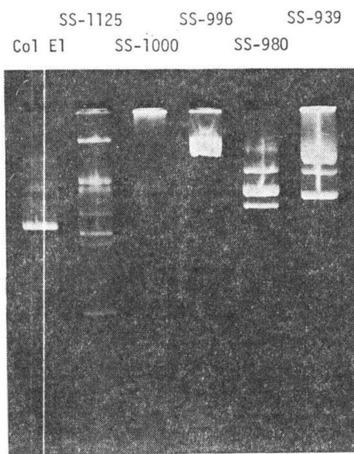
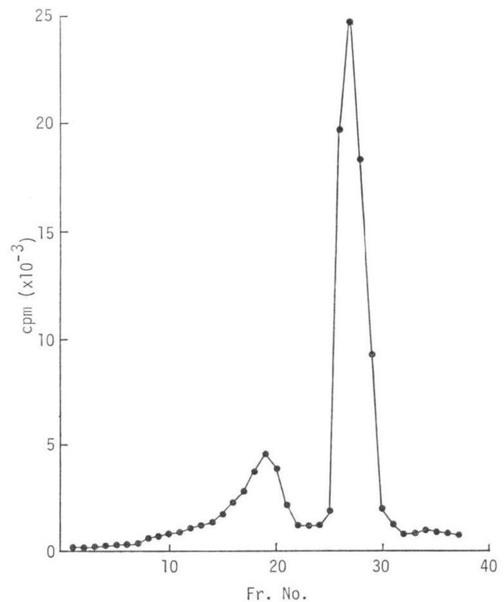


Fig. 2. Ethidium bromide-cesium chloride density gradient centrifugation of a cleared lysate from *Streptomyces tenjimariensis* SS-939.



tures of new aminoglycoside antibiotics which were named istamycins A and B[36]. Another strain, SS-980, also produced istamycin-like antibiotics judging from results obtained by thin-layer chromatography and high voltage paper electrophoresis, although the plasmid profile of SS-980 differed from that of SS-939. Identification of the structures of the antibiotics produced by the other plasmid-containing strains is now in progress.

Fig. 2 shows the separation of closed circular plasmid DNA from chromosomal DNA by EtBr-CsCl ultracentrifugation of a cleared lysate from SS-939. A satellite peak (fractions No. 16 ~ 23) indicated the presence of closed circular plasmid DNA. Different kinds of plasmids were observed in these peak fractions by electron microscopy as shown in Fig. 3. There were plasmids about 2.7 and 5.5 μm in contour length together with much bigger ones.

Fig. 3. Plasmids observed in *Streptomyces tenjimariensis* SS-939.

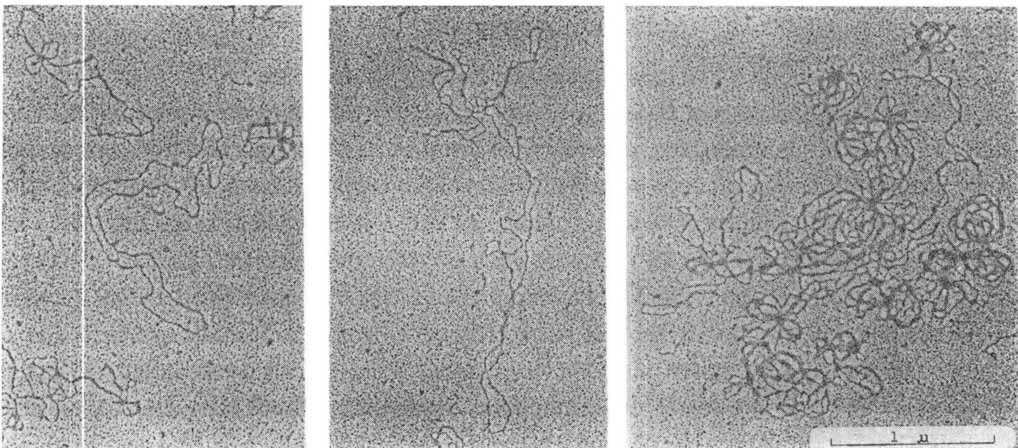


Fig. 4. Morphology of *Streptomyces tenjimariensis* SS-939.

(A) Spore chains (×5,000) (B) Aerial mycelium (×100) (C) Substrate mycelium (×100)

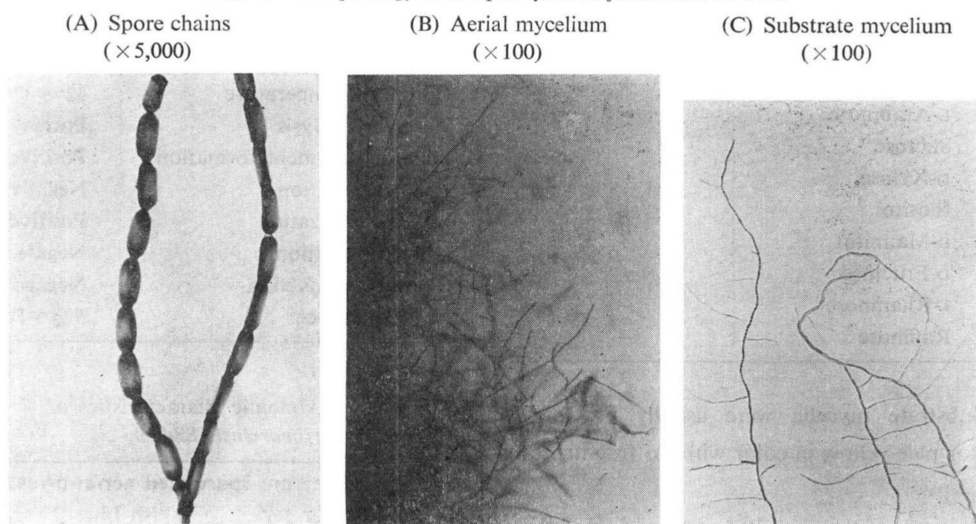


Table 1. Cultural characteristics of strain SS-939.

Agar medium	Growth	Substrate mycelium	Aerial mycelium	Soluble pigment
Sucrose-nitrate	Poor	Colorless	White (a)*	None
Glucose-asparagine	Poor	Colorless	—	None
Glycerol-asparagine (ISP No. 5)	Moderate	Brownish white (2 ec)	White (a)	None
Inorganic salts-starch (ISP No. 4)	Abundant	Yellowish white (3 ca)	Grayish blue (17 ec)	Not distinctive
Tyrosine (ISP No. 7)	Good	Pale yellowish brown (2 gc)	White (a)	None
Nutrient	Good	Brownish white (2 ca)	White (a)	Not distinctive
Yeast extract-malt extract (ISP No. 2)	Good	Pale yellowish brown (2 gc)	Bluish gray (19 dc)	Not distinctive
Oat meal (ISP No. 3)	Abundant	Colorless	Grayish blue (17 ec)	None
Peptone-yeast extract-iron (ISP No. 6)	Good	Brown (3 lg)	—	None

* Designation Number of Color according to the Color Harmony Manual.[35]

Classification of Strain SS-939

As summarized in Table 1, strain SS-939 showed abundant raised growth on Inorganic Salts-Starch (ISP No. 4), Yeast Extract-Malt Extract (ISP No. 2), Oat Meal (ISP No. 3) and Inositol (ISP No. 9) agars. Incubation for several days on these media yielded an aerial mycelium of a grayish blue (17 ec) or bluish gray (19 dc) color. Relatively good growth with a white aerial mycelium was observed on Tyrosine (ISP No. 7) and Nutrient (Difco) agars. White aerial mycelia were poorly formed at the periphery of the growth region on Glycerol-Asparagine (ISP No. 5) and Sucrose-Nitrate (Waksman No. 1) agars. No aerial mycelium was observed after growth on Glucose-Asparagine (Waksman No. 2) and Peptone-Yeast Extract (ISP No. 6) agars even after 2 to 3 weeks incubation at 27°C.

The aerial mycelia which developed on the above media were straight or slightly bent (rectus flexibilis) and bore 10~50 smooth surfaced cylindrical spores (0.4~0.6 μm × 1~2 μm) as shown in Fig. 4. No special structures such as verticils, screlotia or sporangia were observed.

Table 2. Carbon utilization by strain SS-939.

Carbon source	Utilization
D-Glucose	+
L-Arabinose	−
Sucrose	−
D-Xylose	−
Inositol	+
D-Mannitol	−
D-Fructose	±
L-Rhamnose	−
Raffinose	−

Table 3. Physiological properties of strain SS-939.

Temperature for growth	18°∼41°C
Optimum temperature	32°∼37°C
Starch hydrolysis	Positive
Melanoid pigment formation	Positive
Milk coagulation	Negative
Milk peptonization	Positive
Nitrate reduction	Negative
Gelatin liquefaction	Negative
NaCl tolerance	5%∼7%

Table 4. Taxonomic characteristics of *Streptomyces tenjimariensis* SS-939.

1. Color of mature sporulated aerial mycelium
   Blue color series: Grayish blue (17 ec∼19 dc) on ISP No. 2, 3 & 4 media.
2. Spore chain morphology
   Rectus flexibilis (RF)
   Spore surface: Smooth
3. Melanoid pigment formation: Positive
4. Carbon utilizing ability on ISP No. 9 medium: Glucose & inositol
5. Cell wall type: I
6. Antibiotic productivity: Istamycins (aminoglycoside)

Substrate mycelia were usually brownish white or pale yellow in color with no fragmentation.

Analysis of the chemical composition of the cell wall indicated that strain SS-939 produces a type I cell wall according to the scheme of LECHEVALIER and LECHEVALIER[38], since LL-diaminopimelic acid and glycine were detected as major components of its cell wall.

Physiological properties of strain SS-939 are summarized in Table 2. Growth was observed at 18∼41°C (optimum temperature 32∼37°C) on Inorganic Salts-Starch agar. Melanoid pigment was formed in Tyrosine, Tryptone Yeast Extract Broth (ISP No. 1). No other soluble pigment was formed in any of the media used. Strain SS-939 showed a unique carbon utilization profile; *i.e.* it showed good growth only on glucose (without aerial mycelium formation) and inositol (with abundant bluish gray colored mycelium) as indicated in Table 3.

In addition to these properties, strain SS-939 was resistant to istamycins A and B (its own antibiotics), fortimicin A, neamine, kanamycin A, butirosin A and ribostamycin at a concentration of 400 μg/ml, and to tobramycin at 100 μg/ml, but was sensitive to gentamicin, lividomycin, neomycin B, paromomycin and streptomycin at 50 μg/ml when about 3 μl of spore suspension was dropped on ISP No. 4 medium containing each of the above antibiotics and incubated at 27°C for 4 days.

The above results, summarized in Table 4, indicate that the characteristics of strain SS-939 are typical of the genus *Streptomyces*. Among *Streptomyces* species, it most resembles *S. polychromogenes* with respect to the color of the aerial mycelium, spore chain morphology and melanoid pigment formation. However, *S. polychromogenes*'s carbon utilization profile is quite different from that of strain SS-939; *i.e.* it utilizes glucose, xylose, arabinose and fructose for growth on ISP No. 9 medium, but not inositol. On the other hand, there are some *Streptoverticillium* species showing the same carbon utilization profile as that of strain SS-939; however, no verticil was observed in SS-939. Therefore, we concluded that SS-939 represents a new *Streptomyces* species, and named it *S. tenjimariensis* HOTTA

and OKAMI after Tenjin island where it was collected.

Strain SS-939 is nominated as type for the species. Strain SS-980 was also classified as *S. tenjimariensis*.

Discussion

A search for new antibiotics on the basis of plasmid profiles in a variety of streptomycetes resulted in the discovery of new aminoglycoside antibiotics, istamycins A and B. The number of antibiotics reported so far has already exceeded 4,000 and it increases year by year. However, the probability of discovering new antibiotics by conventional screening methods has been rapidly declining. In order to find new antibiotics more efficiently, therefore, it has become important to select organisms prior to screening for new antibiotics. In the light of this situation, our approach might provide a useful way of searching for new antibiotics.

Detection and isolation of plasmids from streptomycetes are not always satisfactory although increasing numbers of reports have accumulated. For instance, the SCP1 plasmid which carries structural genes for methylenomycin A biosynthesis by *S. coelicolor* A3(2)[4] and certain plasmid(s) involved in chloramphenicol formation by *S. venezuelae*[1,2] and in actinomycin formation by *S. paruvulus*[5] have resisted isolation efforts, although genetic analyses have clearly indicated the involvement of plasmids in the production of these antibiotics. Such results support the existence of plasmids which are difficult to detect and isolate. Another problem is that different isolation methods can yield radically different results. For example, YAGISAWA *et al.*[16] isolated a 14.9 and a 21.9×10^{6} dalton plasmid from a neomycin-producing strain of *S. fradiae* while CHUNG and MORRIS[17] found a 33.7 and a 53.2×10^{6} dalton plasmid. We also encountered difficulty in confirming the presence of plasmids in some strains of streptomycetes in which plasmids were detected by agarose gel electrophoresis of cleared lysates but disappeared during subsequent procedures. Occasionally smeared DNA bands appeared in the agarose gel, suggesting the existence of a highly active DNase in the corresponding strains. In addition, adequate cell lysis may prove to be a problem. All streptomycetes tested were routinely lysed almost completely with lysozyme followed by detergent, but it may be possible to increase plasmid yield by improved lysis techniques.

The role of plasmid(s) in istamycin production by *S. tenjimariensis* SS-939 will be discussed in the next paper and characterization of the plasmids will be provided elsewhere.

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