STUDIES ON *ACTINOMYCETALES* PRODUCING ANTIBIOTICS ONLY ON AGAR CULTURE

I. SCREENING, TAXONOMY AND MORPHOLOGY-PRODUCTIVITY RELATIONSHIP OF *STREPTOMYCES HALSTEDII*, STRAIN SF-1993

TAKASHI SHOMURA, JUNKO YOSHIDA, SHOICHI AMANO, MICHIO KOJIMA, SHIGEHARU INOUYE and TARO NIIDA

Central Research Laboratories, Meiji Seika Kaisha, Ltd., Morooka-cho, Kohoku-ku, Yokohama 222, Japan

(Received for publication January 11, 1979)

Soil *Actinomycetales* that produce antibiotics during growth on agar but not in submerged culture were searched for and about 25 strains were obtained. One of these, *Streptomyces halstedii*, strain SF-1993, which produces an antibiotic designated SF-1993 was studied taxonomically and morphologically. The antibiotic-productivity of strain SF-1993 was correlated with mycelial morphology. The vegetative mycelium was filamentous in antibiotic-producing agar cultures, but fragmented in non-producing submerged cultures. By maintaining submerged cells filamentous, production of antibiotic in the submerged fermentations was accomplished. Filamentation was maintained by the use of diluted media or non-fragmented mutant substrains.

It has been reported that some microorganisms produce antibiotics when grown on agar but not in submerged culture[1~4]. However, no systematic investigation has been reported which explains antibiotic production under such circumstances.

Our attention was focussed on this problem, because of the possibility of discovering a new antibiotic not detected by the submerged culture studies, and of throwing light on the mechanism of production of antibiotics in such circumstances.

Such microorganisms can easily be selected by examining the bioactivity of agar plugs in the initial screening step, followed by confirming inactivity in shaken-broth fermentations. Among 25 strains isolated, a *Streptomyces* strain (SF-1993) was selected as a candidate for detailed study, because it produced on agar dishes an antibiotic showing activity against some Gram-negative bacteria.

In this paper, screening, taxonomy and morphology-productivity relationships of strain SF-1993 are described. Physico-chemical and biological characterization, as well as structural elucidation of the antibiotic will be described in a subsequent paper[5].

Materials and Methods

1. Screening methods:

Primary screening was carried out by using an agar plug method. *Actinomycetales* isolated from soil samples were streaked on a glycerol bouillon (GB) agar medium containing 2.0% glycerol, 1.0% Polypeptone, 0.5% meat extract, 0.3% $CaCO_3$ and 2.0% agar. Agar cultures (25 ml of medium/Petri-dish, 80 mm, i.d.) were grown at 28°C. At maximal growth, which required 3~7 days after inoculation, agar plugs were cut out with a cork borer (8 mm, i.d.), and assayed against several test organisms *i.e.*, *Escherichia coli* IFO 12734, *Escherichia coli* IFO 13168, *Pseudomonas aeruginosa* IAM

1007, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538P, *Mycobacterium smegmatis* ATCC 607 and *Candida albicans* IAM 4888. Active strains selected from the primary screen were subjected to submerged culture for secondary screening. Submerged cultures were grown in 20 ml of medium in 100-ml Erlenmeyer flasks at 28°C for 2~7 days with shaking on a rotary shaker (220 rpm). The liquid media employed were as follows (pH was adjusted before sterilization):

1.	GB medium		4.	Sucrose	40 g
2.	Soluble starch	20 g		Soybean meal	30 g
	Soybean meal	25 g		Wheat embryo	20 g
	Wheat embryo	10 g		NaCl	6 g
	NaCl	2.5 g		Tap water	1,000 ml
	Tap water	1,000 ml		(pH 7.0)	
	(pH 7.0)		5.	Glucose	20 g
3.	Glucose	15 g		Polypeptone	5 g
	Glycerol	10 g		Soybean meal	2 g
	Pharmamedia	25 g		Yeast extract	3 g
	Tap water	1,000 ml		Tap water	1,000 ml
	(pH 7.0)			(pH 7.0)	

Resulting culture filtrates were tested for antibiotic activity against the same test organisms as mentioned above by an agar hole method. Strains active only after agar culture were selected. Of the 25 strains selected, strain SF-1993 was studied in detail.

2. Taxonomy of strain SF-1993:

Strain SF-1993 was isolated from a soil sample collected at Yokohama City, Japan. Stock cultures were maintained on a starch-yeast extract (SY) agar medium containing 1.0% soluble starch, 0.2% yeast extract and 2.0% agar, adjusted to pH 7.0 before sterilization. Cultural characteristics were determined by methods outlined in WAKSMAN[6] and SHIRLING and GOTTLIEB[7]. Diaminopimelic acid isomer in whole cell hydrolysates was determined by the method of BECKER *et al.*[8]

3. Fermentation and bioassay:

GB medium with or without agar was used for the fermentation studies of strain SF-1993. Microbiological assays were carried out by the agar diffusion method using *Escherichia coli* IFO 13168 as a test organism. Potency was assayed using a standard sample of antibiotic SF-1993 described elsewhere[5].

Growth was measured by weighing freeze-dried cells harvested by centrifugation. With agar cultures, growth was measured as follows: each agar culture was placed in a boiling water bath for 10 minutes, and the melted agar was removed by filtration through gauze. Harvested mycelium was washed with hot water several times. The mycelium then was suspended in hot water, centrifuged and the residue freeze-dried.

4. Microscopy:

Smears of submerged cells after centrifugation and washing were heat-fixed on microscope slides and stained with 2% aqueous solution of Crystal Violet. Slides were examined with a light microscope at 600× magnification. The morphology of vegetative mycelium in the agar medium was studied with a slide culture method.

For scanning electron microscopy, cell suspensions, after centrifugation and washing, were fixed in 2.5% glutaraldehyde, and postfixed with osmium tetraoxide. The cells then were gradually dehydrated by washing with increasing amounts of acetone and finally dried by the critical-point method[10]. Specimens were coated with evaporated gold, and examined with a scanning electron microscope model JEM100C-ASID (JEOL Ltd.) at 1,000~20,000× magnification.

5. Mutation and selection of mutants by the filtration method:

The original strain SF-1993 was treated with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) as follows: Spores of strain SF-1993 obtained from SY agar slant were suspended in 0.1 M Tris-malate buffer (pH 6.0). NTG was added to a final concentration of 500 μg/ml, and the suspensions were gently shaken on a Monod shaker at 37°C for 15 minutes. Treated spores were washed twice with

saline, and used to inoculate GB liquid medium (20 ml/100-ml Erlenmeyer flask). After shaking on a rotary shaker (220 rpm) at 28°C for 48 hours, 0.5 ml of each culture was filtered through sterilized cotton. Residual organisms trapped on the cotton were re-inoculated into fresh GB medium, and shaken at 28°C for 24 hours. Filtration and shaking was repeated at least 10 times. The final cultures were diluted with sterile saline (1: 1,000) and plated on SY agar dishes. The plates were incubated at 28°C for 3 days, and about 100 colonies were transferred to SY agar slants. From these cultures, 14 mutant substrains were obtained which showed filamentous growth and antibiotic production in submerged cultures.

Results

1. Screening for *Actinomycetales* Producing Antibiotic Activity only in Agar Medium

In the primary screen, about 1,300 isolates of 6,500 tested showed antimicrobial activity against one or more of the test organisms by the agar plug method. In the secondary screen, about 1.9% (25 strains) of the 1,300 were non-producers in submerged culture. When all 25 strains were compared, they showed no common feature in morphology, but it was noted that mycelial fragmentation had occurred in more than one-half of the strains during submerged culture. Strain SF-1993 was selected for further study because it produced an antibiotic active against Gram-negative bacteria in agar dishes and showed fragmented mycelium in non-producing liquid cultures.

Table 1. Cultural characteristics of strain SF-1993 and *Streptomyces halstedii*, strain ISP 5068 (IMRU 3328).

		Strain SF-1993	*Stm. halstedii*
Glycerol asparagine agar (ISP 5)	G:	moderate	moderate
	A:	abundant, gray[e]	gray[2fe to e]
	R:	light yellowish brown	light yellowish brown
	S:	none	none
Inorganic salts-starch agar (ISP 4)	G:	good	good
	A:	abundant, gray[g]	gray[g to 3fe]
	R:	light olive	light olive gray
	S:	none	none
Oatmeal agar (ISP 3)	G:	good	good
	A:	abundant, gray[3ig]	gray[e]
	R:	colorless	light grayish yellow
	S:	none	none
Yeast extract-malt extract agar (ISP 2)	G:	moderate	moderate
	A:	gray[e to 3fe]	gray[3fe]
	R:	yellowish brown	yellowish brown
	S:	none	none
Glucose asparagine agar	G:	good	moderate
	A:	abundant, gray[e]	scant, light gray[2dc]
	R:	cream to light yellow	light gray
	S:	none	none

G: growth, A: aerial mycelium, R: reverse, S: soluble pigment
[ ]: Color Harmony Manual[9] designations.

Table 2. Physiological properties of strain SF-1993 and *Streptomyces halstedii*, strain ISP 5068 (IMRU 3328).

	Strain SF-1993	*Stm. halstedii*
Hydrolysis of starch	positive	positive
Liquefaction of gelatin	positive	positive
Peptonization of skim milk	positive	positive
Coagulation of skim milk	positive	positive
Formation of melanoid pigment		
in tryptone-yeast extract broth	negative	negative
in peptone yeast extract iron agar	negative	negative
in tyrosine agar	negative	negative

Table 3. Utilization of carbon sources of strain SF-1993 and *Streptomyces halstedii*, strain ISP 5068 (IMRU 3328).

	Strain SF-1993	*Stm. halstedii*
D-Glucose	+	+
D-Xylose	+	+
L-Arabinose	+	+
D-Fructose	+	+
i-Inositol	−	−
D-Mannitol	−	−
Raffinose	−	−
Rhamnose	−	−
Sucrose	±	−

+: good growth, ±: poor growth, −: no growth.

2. Taxonomic Characterization of Strain SF-1993

Aerial mycelium of strain SF-1993 was monopodially branched with spore chains of *Rectus-Flexibilis* type on most of the media used. Spores were in chains of more than ten, cylindrical in shape, $0.7 \sim 0.9 \times 0.9 \sim 1.5\ \mu$ in size and had smooth surfaces. On most media, the aerial mass color was gray and the reverse color was pale yellowish brown. Cultural characteristics of strain SF-1993 are summarized in Table 1.

Physiological properties of strain SF-1993 are summarized in Tables 2 and 3. LL-Diaminopimelic acid was detected in whole cell hydrolysates.

Based on its characteristics, strain SF-1993 belongs to the genus *Streptomyces*, among which, *Streptomyces halstedii*[11] seems most closely related. Therefore, *Streptomyces halstedii*, strain ISP 5068 (IMRU 3328) was compared with strain SF-1993 by simultaneous cultivation, and the results are shown in Tables 1, 2 and 3. Morphological and physiological properties were in good agreement. Differences in sporulation and reverse colors on glucose-asparagine agar and oatmeal agar were not sufficient to designate strain SF-1993 as a new species, so it was identified as a strain of *Streptomyces halstedii*. Strain SF-1993 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Chiba, Japan, with an accession number of FERM-P 4332.

3. Comparison of Agar and Submerged Cultures of Strain SF-1993

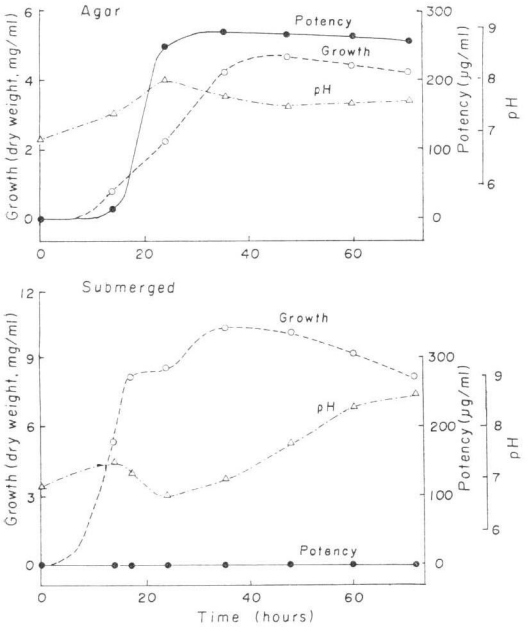
The time course of antibiotic production of agar and submerged cultures of strain SF-1993 is shown in Fig. 1. With agar cultures the production of antibiotic SF-1993 activity was maximum at 36 hours after inoculation, reaching 270 μg/ml. In contrast, no potency was detected in submerged cultures.

When antibiotic SF-1993, isolated from agar cultures as described in a separate paper[5], was added to submerged cultures, antibiotic activity was not diminished. It appeared, therefore, that lack of potency in submerged cultures was, in fact, due to lack of antibiotic-productivity and not to inactivation of the antibiotic, as with fumaramidmycin[12].

It was expected from the microscopic observations described below that, if the mycelium could maintain a filamentous form under submerged conditions, the antibiotic also may be produced in shaken culture. To ascertain this possibility, an attempt was made to select cultural conditions so as to

Fig. 1. Comparison of pH, growth and antibiotic activity of agar and submerged cultures of strain SF-1993.

Potencies of the culture filtrates were measured by an agar hole method using *E. coli* IFO 13168 as test organism. Culture filtrate from agar cultures were prepared as follows: A whole agar culture in a Petri-dish was frozen once at −20°C and then melted in water bath at 80°C. The melted material was quickly filtered through gauze. The resulting culture filtrates were used for assay.



maintain the filamentous form in shaken cultures. This was accomplished by dilution of the medium.

The influence of GB medium concentration upon the cellular morphology and the antibiotic production is summarized in Table 4. Mycelial fragmentation was inhibited by diluting medium concentration, with concomitant production of the antibiotic.

Table 4. Antibiotic-productivity of strain SF-1993 with various concentrations of GB medium.

Condition	Concentration of medium	Mycelial fragmentation	Potency (μg/ml)*
Submerged	2×	++	0
	1×	+	0
	1/2×	−	380
	1/4×	−	150
Agar	1×	−	270

++: marked, +: positive, −: negative

* Antibiotic SF-1993 isolated in separate paper[5] used as a standard (1,000 μg/mg).

Fig. 2. Time course of pH, growth and antibiotic activity of strain SF-1993 in 1/2×GB and 2×GB medium.

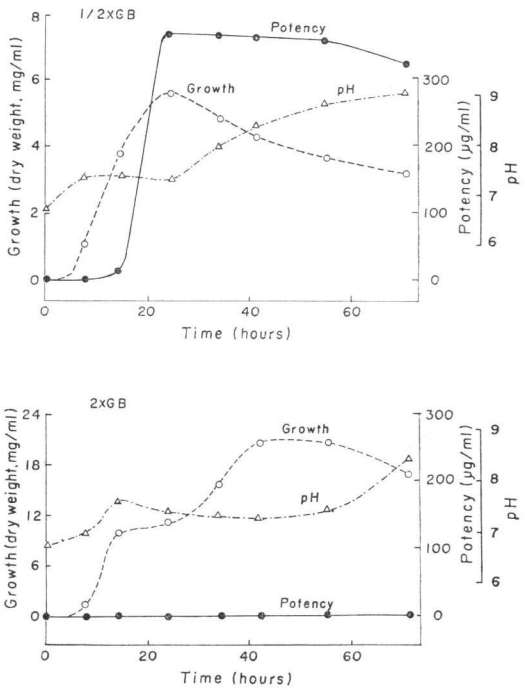


Fig. 2 indicates the time course of submerged cultures in half-diluted (1/2×GB) and two times concentrated (2×GB) media. The time course with 1/2×GB medium was similar to that in GB agar medium. At the same time the filamentous form was maintained during growth. It was noted in the liquid cultivation that the antibiotic not only was produced, but at higher potency than on GB agar medium. In sharp contrast, with 2×GB medium the mycelium changed from filamentous to fragmented forms during growth, and there was no antibiotic production.

4. Microscopy

By examination with the light and scanning electron microscopes, we found that the morphologic cell forms in non-producing submerged cultures were different from those in antibiotic-producing agar cultures. As shown in Plate 1, the vegetative mycelium of strain SF-1993 was filamentous in agar cultures (photo a), but rod-like in shaken cultures (photo b), apparently caused by fragmentation of mycelia.

Thus, productivity of the antibiotic seemed to be positively correlated with the filamentous form in strain SF-1993. This relationship between morphology and antibiotic productivity was further re-

Plate 1. Photomicrograph of strain SF-1993.

(a) Filamentous mycelia at 24 hours in GB agar medium, ×600.

(b) Fragmented cells after 20 hours shaking in GB liquid medium, ×600.

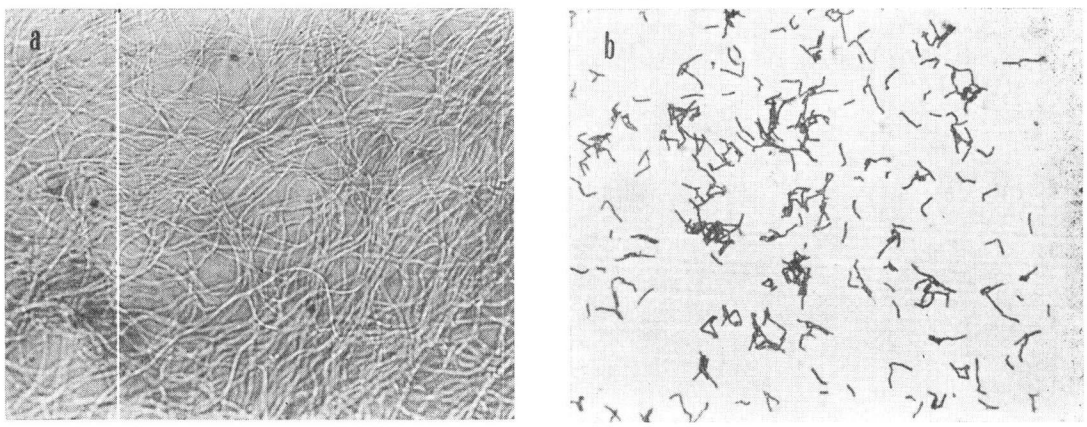


Plate 2. Morphological changes in submerged cultures of strain SF-1993 on 2×GB medium.
(A, B) Filamentous mycelia at 11 hours cultivation, A: ×1,000, B: ×5,000

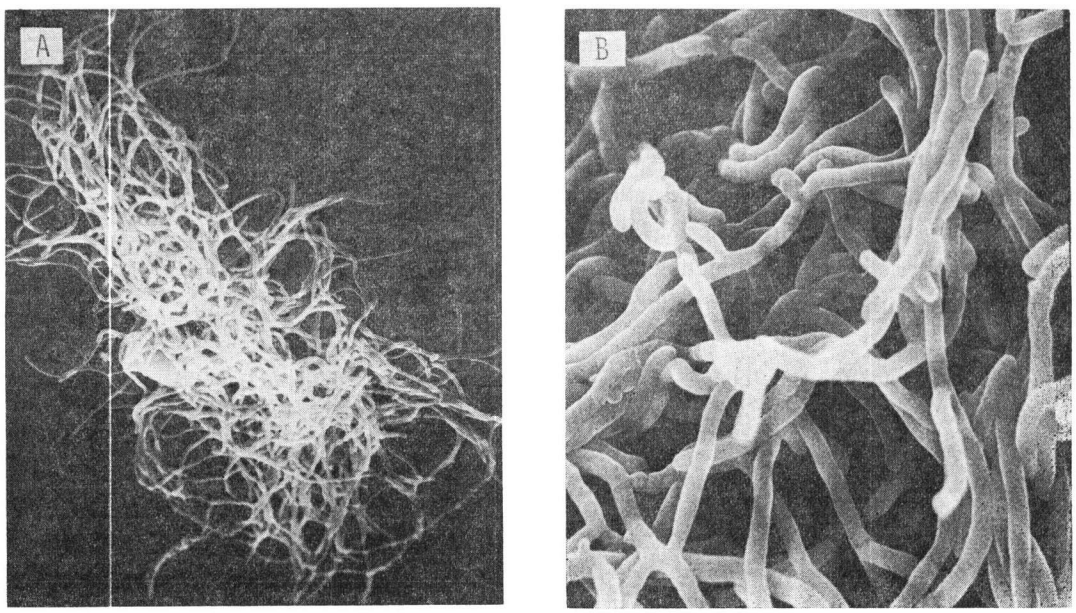
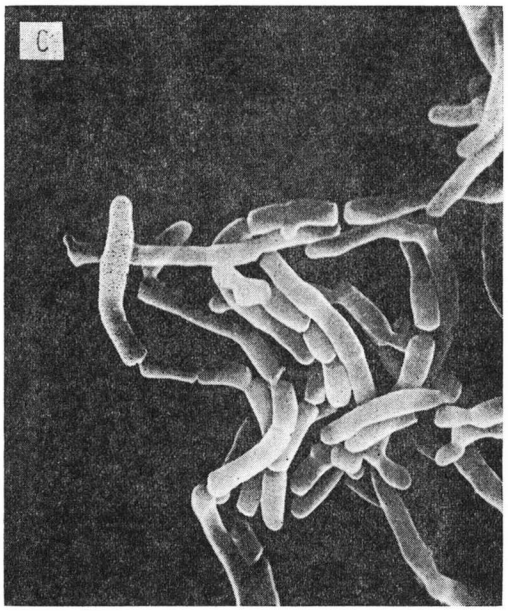
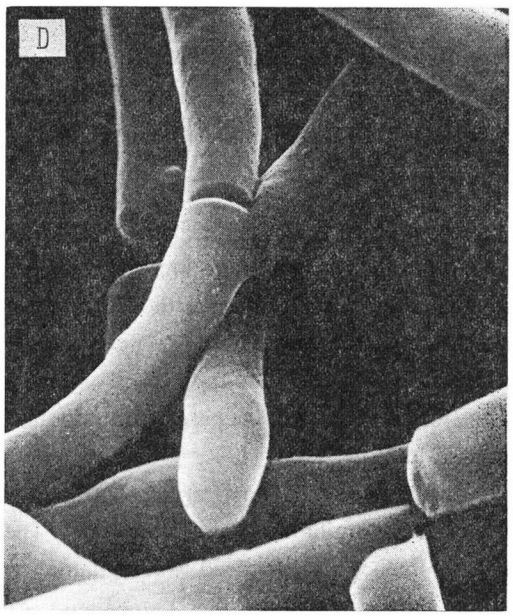


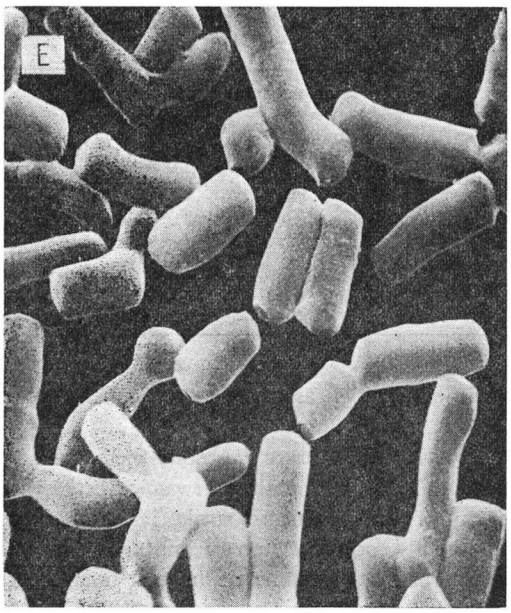
Plate 2.

(C, D) Mycelia at initiating of fragmentation at 17 hours, C: ×5,000, D: ×20,000

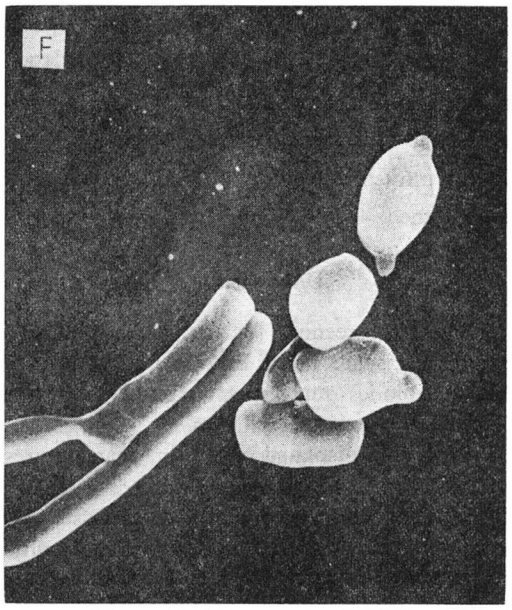




(E) Fully fragmented cells at 20 hours, ×10,000



(F) Fragmented cells budding at 24 hours, ×10,000



cognized in the other strains selected by our screening.

Morphological changes on 2×GB medium are illustrated in the scanning electron micrographs of Plate 2. During the first 14 hours of incubation, filamentous forms were dominant (photo A, B). Fragmentation was initiated at 17~24 hours and breaks occurred in many portions of the mycelium (photo C, D, E). After 24 hours, each fragmented cell budded and developed again into a filamentous

form (photo F, G). Filamentous growth and re-fragmentation were repeated until about 45 hours of cultivation. During the first period of fragmentation, the mycelial dry weight remained almost constant, so that the growth curve was characterized as double sigmoid (Fig. 2).

5. Mutant Substrains and Their Fermentative Productivity

Non-fragmented mutants were successfully isolated using the filtration method mentioned in "Materials and Methods". Fermentative comparison of two non-fragmented mutants (Nos. 101 and 105) with the parent strain is summarized in Table 5.

As expected, the non-fragmented mutants produced the antibiotic in shaken culture. The highest potency was obtained with mutant No. 105 in submerged culture in GB medium. As will be shown in a subsequent paper[5], the antibiotic produced by the parent strain in 1/2×GB medium and by non-fragmented mutants in GB medium is identical with antibiotic SF-1993 produced in agar culture. This suggests no significant biochemical differences in agar and liquid cultures, so far as antibiotic production is concerned.

Plate 2.

(G) New Filamentous forms growing at 35 hours, ×5,000.

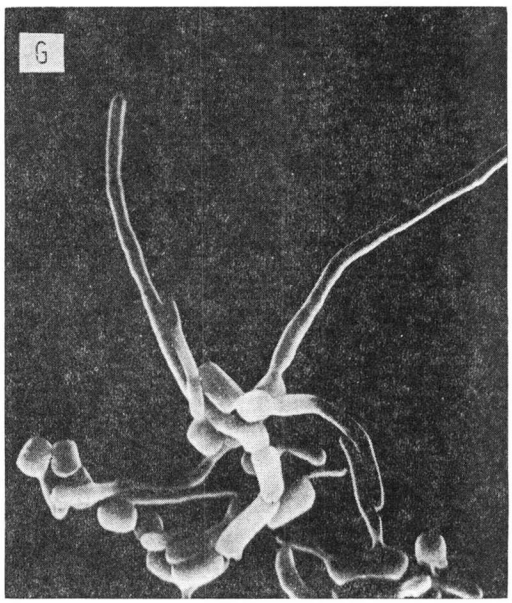


Table 5. Comparison of antibiotic-productivity of non-fragmented mutant substrains with parent strain SF-1993.

	GB medium		1/2×GB medium	
	Potency (μg/ml)	Mycelial fragmentation	Potency (μg/ml)	Mycelial fragmentation
Mutant No. 101	620	−	400	−
No. 105	750	−	440	−
Parent	0	+	380	−

Discussion

During screening for new antibiotics, microorganisms are often encountered that produce antibiotics only in agar culture. However, very little is known as to why such activity is not detected in submerged cultures.

Antibiotics isolated only from agar cultures of *Actinomycetales* are rare. Among them, fumaramidmycin[12] is one example. According to MARUYAMA *et al.*[12], fumaramidmycin was detected with difficulty in submerged cultures, because the mycelium of the producing strain inactivated the antibiotic more readily than that in agar culture. Apparently, non-detection of antibiotic SF-1993 in submerged culture cannot be ascribed to the same reason as that for fumaramidmycin, because the antibiotic added to submerged cultures was not inactivated.

We have noted a close relationship between morphology and productivity. As shown, the vegetative mycelium of strain SF-1993 is filamentous when antibiotic SF-1993 is produced in agar cultures. It is fragmented in submerged cultures and no antibiotic activity can be detected. Furthermore, filamentous cells in diluted medium (1/2×GB) and non-fragmented mutants can produce the same antibiotic as that in the agar cultures. It is suggested, therefore, that antibiotic synthesis with strain SF-1993 has some connection with cellular morphology.

The reason why filamentous cells can produce antibiotic but fragmented cells cannot remains

for future study. Strain SF-1993 and its non-fragmented mutants provide useful materials for study on control mechanisms of cellular differentiation and production of secondary metabolites such as antibiotics.

References

1) KUROYA, M.; N. OUCHI & M. KATSUNO: Studies on the antibiotic substances from *Actinomyces*. II. On the classification of antibiotic-producing *Actinomyces* by the "Streak plate method". J. Antibiotics 2 (Suppl. A): 74~78, 1949
2) UMEZAWA, H. & T. TABATA: On some differences of the antibacterial spectra of the antibiotic *Streptomyces* growing in the broth and the agar. J. Antibiotics 2 (Suppl. B): 55~59, 1949
3) OGATA, K.; I. TADOKORO & K. NAKAZAWA: Studies on *Actinomyces* and its antibiotics. I. Isolation of active strains. J. Antibiotics 3: 297~301, 1950
4) UESAKA, I.: Studies on the antibiotic action of *Nocardia*. I. Isolation and classification of antibiotic *Actinomycetales*. J. Antibiotics 3: 730~735, 1950
5) OMOTO, S.; T. SHOMURA, H. SUZUKI & S. INOUYE: Studies on *Actinomycetales* producing antibiotics only on agar culture. II. Isolation, structure and biological properties of N-carbamoyl-D-glucosamine (substance SF-1993). J. Antibiotics 32: 436~441, 1979
6) WAKSMAN, S. A.: The actinomycetes. Vol. II. Classification, identification and description genera and species. pp. 328~334. The Williams & Wilkins Co., Baltimore, 1961
7) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Intern. J. Syst. Bact. 16: 313~340, 1966
8) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
9) Color Harmony Manual, 4th edition: Container Corporation of America, Chicago, U.S.A., 1958
10) ANDERSON, T. F.: Techniques for the preservation of three-dimentional structure in preparing specimens for the electron microscope. Trans. N. Y. Acad. Sci. Ser. II. 13: 130, 1951
11) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species description from first study. Intern. J. Syst. Bact. 18: 128~131, 1968
12) MARUYAMA, H. B.; Y. SUHARA, J. SUZUKI-WATANABE, Y. MAESHIMA, N. SHIMIZU, M. OGURA-HAMADA, H. FUJIMOTO & K. TAKANO: A new antibiotic, fumaramidmycin. I. Production, biological properties and characterization of producer strain. J. Antibiotics 28: 636~647, 1975