# STUDIES ON A NEW AMINOGLYCOSIDE ANTIBIOTIC, DACTIMICIN

## I. PRODUCING ORGANISM AND FERMENTATION

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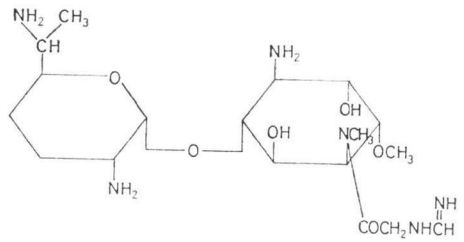
The producing organism of a new antibiotic, dactimicin, is described. The presence of finger-shaped sporangia bearing motile spores, the absence of aerial mycelium and its cell wall of type II, ascribe this organism to the genus *Dactylosporangium*. The cultural and physiological features distinguish this organism from all the described *Dactylosporangium* species. Therefore, it is considered to be a new species for which the name *Dactylosporangium matsuzakiense* sp. nov. is proposed. Fermentation of dactimicin is also described.

In the course of our screening program for antibiotic producers from rare genera of the order *Actinomycetales*, we have isolated a strain of *Dactylosporangium* that produces a new aminoglycoside antibiotic, dactimicin (Fig. 1), which is previously designated as substance SF-2052[1].

Fig. 1. Structure of dactimicin.



In this paper, taxonomy of the producing organism and fermentation of dactimicin are described. Physico-chemical and biological characterization, as well as structural elucidation of the antibiotic will be described in a subsequent paper[2].

## Materials and Methods

### Microorganism

Strain SF-2052 was isolated from a soil sample collected at Matsuzaki-cho, Izu Peninsula, Japan. The organism was selected as a slow grower appeared on agar plate after 4-week incubation at 28°C. A fifth-diluted inorganic salts-starch agar medium (ISP No. 4) was used for isolation.

Strain SF-2052 was kept in lyophilized form and cultured on slants of inorganic salts-starch agar.

### Medium and cultural condition for general taxonomic studies

The media and procedures used for the cultural and physiological characterization of strain SF-2052 were those recommended by SHIRLING and GOTTLIEB[3]. Additional culture media described by WAKSMAN[4] and by THIEMANN[5] were also used.

Carbon utilization test was performed in LUEDEMANN's basal medium[6], because the strain shows no growth on the medium according to THIEMANN[5] and PRIDHAM and GOTTLIEB[7]. Sodium chloride tolerance was tested on LUEDEMANN's agar media[8] containing NaCl, constituted 0%, 1.5%, 3%, 5% and 7%, respectively.

The medium used for morphological observation was mainly inorganic salts-starch agar.

Microscopy

The morphology of sporangia on the agar medium was studied with a slide culture method, and the slides were examined with a light microscope at 600× magnification.

For scanning electron microscopy, an agar block with numerous sporangia was fixed in 2.5% glutaraldehyde. The block then was gradually dehydrated by washing with increasing amounts of acetone and finally dried by the critical-point method[9]. Specimen was coated with evaporated gold, and examined with a scanning electron microscope model JEM100C-ASID (JEOL Ltd.) at 1,000~20,000 × magnification.

Cell wall and whole-cell analysis

Cell wall preparations were obtained by the method of YAMAGUCHI[10]. Hydrolyzed cell walls were analyzed by the procedure of BECKER[11]. Whole cells were analyzed by the procedure of LECHEVALIER[12].

Fermentation procedure

Most of a slant culture of strain SF-2052 was inoculated into 20 ml of a seed culture medium consisting of 2.0% soluble starch, 0.5% Polypepton, 0.3% yeast extract, 0.2% meat extract, 0.2% soybean meal and 0.1% $CaCO_3$ (pH 7.0) in a 100-ml Erlenmeyer flask. The inoculated flask was shaken on a rotary shaker (220 rpm) at 28°C for 6 days. Six milliliters of the first seed were inoculated into 100 ml of the same medium in a 500-ml Erlenmeyer flask. After shaking at 28°C for 3 days, 800 ml of the second seed were transferred to 20 liters of the same medium in a 30-liter fermentor. It was incubated at 28°C for 48 hours with an air-flow rate of 20 liters per minute and an agitation rate of 300 rpm. The whole culture of the third seed was added to a 300-liter fermentor containing 200 liters of the following production medium: 3.0% soluble starch, 0.9% wheat germ, 0.75% Polypepton, 0.45% yeast extract, 0.15% $CaCO_3$ and 0.0005% $CoCl_2 \cdot 6H_2O$ in tap water. The medium was adjusted to pH 7 before sterilization. Silicon KM68-2F (Shinetsu-Chemical Co., Ltd.) was used as an antifoam agent.

Cultivation was carried out at 28°C with an air-flow rate of 200 liters per minute and an agitation at 100 rpm during first 24 hours and 150 rpm during the remainder of the fermentation. After 50 hours of cultivation, the pH was continuously kept below pH 7.0 with 25% solution of phosphoric acid. The growth was measured as packed mycel volume (PMV, %).

Assay of dactimicin

Antibiotic activity was determined by a microbial paper-disc agar diffusion assay, using *Bacillus subtilis* ATCC 6633 as the test organism. The diameter of the inhibition zones was proportional to the log of the antibiotic concentration in the range of 10 to 50 $\mu$g/ml. Potency of the fermentation broths was determined against a standard curve of dactimicin.

## Results and Discussion

### Taxonomic Studies

Morphology

Colonies of strain SF-2052 are compact, tough and somewhat leathery. Vegetative mycelia are long, irregularly branched, twisted and penetrating into the agar. Fragmentation of hyphae does usually not occur both on agar and in submerged condition.

Aerial mycelium is not formed on any of the media used.

Strain SF-2052 is characterized by the formation of finger-shaped sporangia. Sporangia are formed abundantly on inorganic salts-starch agar and sometimes on glycerol-asparagine agar, tyrosine agar and oatmeal agar. They appear after 10~15 days of incubation at 28°C singly or in clusters on surface of agar media (Plate 1). Sporangia are 0.9~1.4 by 4.0~6.0 $\mu$ in size, and are formed on short sporangiophores (0.5~1.5 $\mu$) which emerge from the vegetative hyphae.

Plate 1. Finger-shaped sporangia of strain SF-2052. (×600)

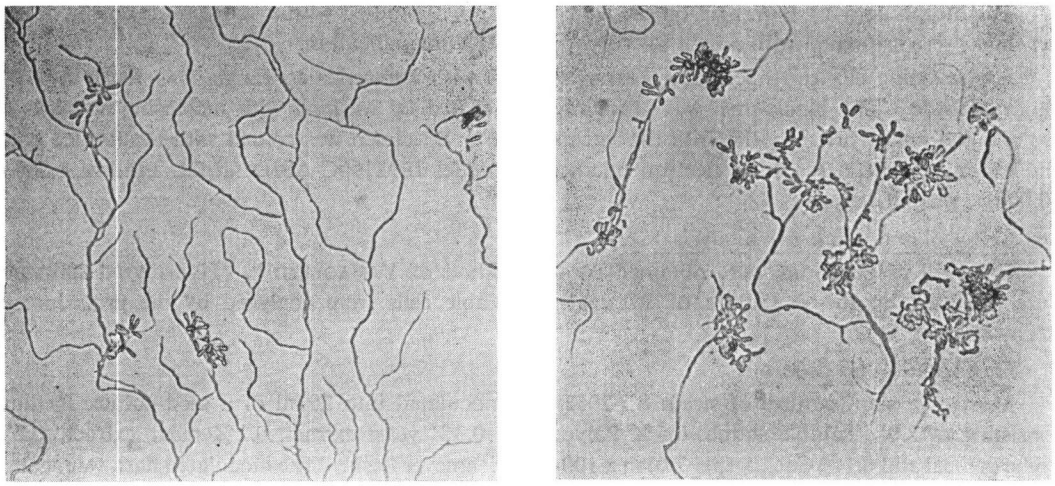
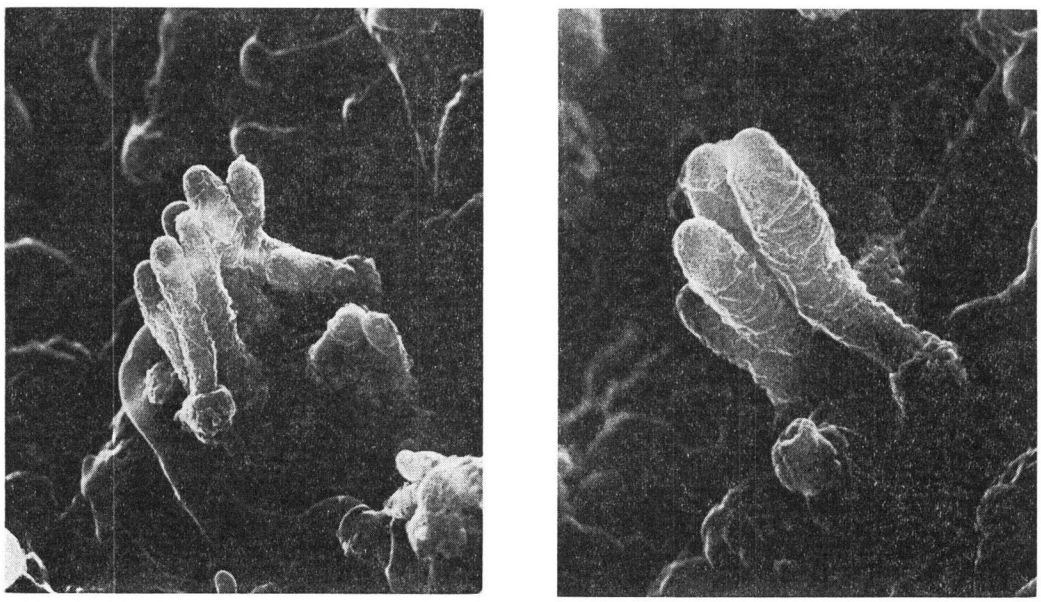


Plate 2. Scanning electron micrograph of a cluster of sporangia of strain SF-2052.



(×10,000) (×15,000)

Under the scanning electron microscope a cluster of sporangia can be vividly observed on the background of vegetative mycelia as shown in Plate 2. Each sporangium contains usually three spores, rarely four, arranged always in a single row inside a sporangium. The naked sporangiospores are shown in Plate 3, which was fortunately taken when the sporangial membrane was broken. Spores are cylindrical, others oblong, and are 0.8~1.3 by 1.1~1.6 $\mu$ in size.

The spore release was observed by flooding the surface of sporangia-formed cultures with sterile water or soil extract solution. Spores are motile, but there is a time lag (up to 30 minutes) before they became highly active. While the motile spore could be observed as vigorous swimmers, flagella

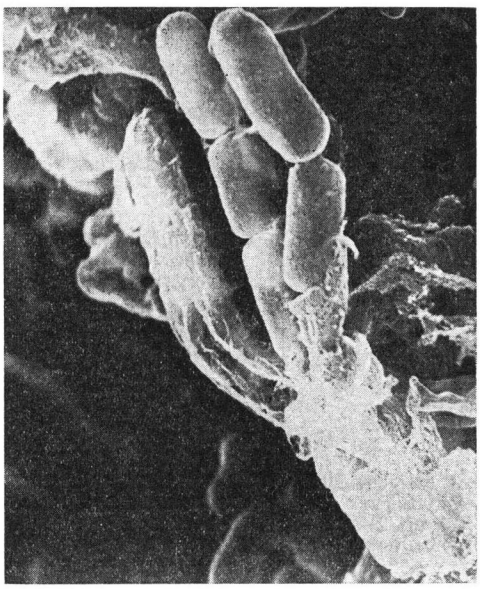
staining was not successful as yet. Formation of globose bodies[5,13] was not clearly observed in strain SF-2052.

Cultural and Physiological Characteristics

Cultural characteristics on various media were observed during the cultivation of strain SF-2052 at 28°C for 2~3 weeks, and the results are shown in Table 1. Aerial mycelium and soluble pigments (except for tyrosine agar) were not formed in any of the media tested.

The optimum growth temperature ranges from 25 to 35°C. No growth is observed at 45°C, minimal growth at 15°C and 42°C. Sodium chloride tolerance of strain SF-2052 is very low, and it does not grow even in 1.5% NaCl. Other physiological properties including utilization of carbon sources are listed in Table 2.

Plate 3. Scanning electron micrograph of sporangiospores of strain SF-2052 (×20,000).



Chemical Analysis of Cell Constituents

Analysis of cell wall hydrolysates by paper chromatography demonstrated the presence of 3-hydroxy-diaminopimelic acid, which gave the same greenish color with ninhydrin reagent as diaminopimelic acid (DAP) but moved more slowly than meso-DAP. In addition to 3-hydroxy-DAP, the hydrolysates also contained large amounts of muramic acid, glutamic acid, glycine, alanine and a trace of meso-DAP as the amino acid composition.

Whole cell hydrolysates contained xylose, glucose, mannose, galactose and glucosamine as the major carbohydrates, and a trace of arabinose. Qualitative differences of carbohydrate composition were not found between cell wall and whole cell as shown in Table 3.

Consequently, strain SF-2052 is assigned to cell wall type II and to whole cell sugar pattern D according to the classification of LECHEVALIER *et al.*[14]

Comparisons with Known Species

Strain SF-2052, because of its formation of finger-shaped sporangia and motile spores as well as cell wall composition, can doubtlessly be assigned to the genus *Dactylosporangium* THIEMANN[5].

THIEMANN (1967) have described two species, *Dactylosporangium aurantiacum*[5] and *D. thailandense*[5,15]. Thereafter, *D. variesporum*[16] and *D. salmoneum*[17], which have been reported to produce capreomycins and a new polyether antibiotic CP-44,161, respectively.

Comparative studies of strain SF-2052 with these four species of the genus *Dactylosporangium* showed that strain SF-2052 is a new species by the reasons described below: *D. salmoneum* can be clearly differentiated from strain SF-2052 in that the color of growth is cream to salmon, while that of strain SF-2052 is orange. *D. variesporum* produces reddish orange soluble pigment on yeast extract-malt extract agar and sucrose-nitrate agar, whereas no soluble pigments on the same media are observed in strain SF-2052. In addition, the best medium for formation of sporangia is yeast extract-malt extract agar in *D. variesporum*, while that of strain SF-2052 is inorganic salts-starch agar.

Table 1. Cultural characteristics of strain SF-2052, *D. aurantiacum* (strain ATCC 23491) and *D. thailandense* (strain ATCC 23490).

| Medium | Strain SF-2052 | *D. aurantiacum* | *D. thailandense* |
|---|---|---|---|
| Sucrose-nitrate agar | G: moderate, amber (31c) | moderate, light apricot (4ea) | moderate to good, light brown (4ng) |
| | SP: none | none | light tan |
| | S: poor | none | none |
| Glucose-asparagine agar | G: moderate, russet orange (4pc) | poor to moderate, light ivory (2ca) | moderate, light orange (4ia) |
| | S: none | none | none |
| Glycerol-asparagine agar (ISP no. 5) | G: poor, light melon yellow (3ea) | poor, hyaline | poor, colorless to light orange (4ia) |
| | S: very poor | poor | moderate |
| Inorganic salts-starch agar (ISP no. 4) | G: moderate to good, russet orange (4pc) | good, whitish to light apricot (4ea) | good, dusty orange (4lc) |
| | S: abundant | poor to moderate | very abundant |
| Oatmeal agar (ISP no. 3) | G: moderate, dusty orange to burnt orange (4lc to 5nc) | good, raised, light apricot (4ea) | good, russet orange (4pc to 4nc) |
| | S: poor | poor | poor |
| Yeast extract-malt extract agar (ISP no. 2) | G: moderate, wrinkled, amber to light brown (3lc to 4ng) | moderate, amber (3lc) | good, pastel orange (4ic) |
| | SP: none | none | light yellow |
| | S: none | none | none |
| Tyrosine agar (ISP no. 7) | G: moderate, dusty orange to light brown (4lc to 4ng) | poor to moderate, bisque (4ec) | poor to moderate, light brown (3lg) |
| | SP: light brownish pink | faint grayed pink | none |
| | S: poor | poor | moderate |
| Nutrient agar | G: very poor, light orange (4ia) | moderate, light amber (3ic) | moderate, tan (3ie) |
| | S: none | none | none |
| Ca-malate agar | G: poor, melon yellow to apricot (3ga to 4ga) | poor, hyaline | very poor, light orange (4ia to 3gc) |
| | S: none, or very poor | very abundant | very abundant |
| Potato plug | G: no growth | poor, hyaline | moderate, light brown (4ng) |

G: Growth, SP: Soluble pigment, S: Sporangia

( ): Color number designations taken from Color Harmony Manual, 4th edition, Container Corporation of America, Chicago, Illinois, 1958.

With respect to the growth color (orange) and the lack of distinctive soluble pigments, strain SF-2052 is similar to *D. aurantiacum* and *D. thailandense*. Therefore, standard strains of these two species were compared with strain SF-2052 by simultaneous cultivation, and the results are shown in Tables 1 and 2. The growth colors of these three strains are all orange by rough observation, but are different each other in details as shown in Table 1. *D. aurantiacum* and *D. thailandense* form abundant sporangia on Ca-malate agar, whereas strain SF-2052 forms no or only trace of sporangia on the same medium. Furthermore, *D. aurantiacum* and strain SF-2052 differ in physiological properties such as gelatin liquefaction and nitrate reduction. *D. thailandense* grows moderately on potato plug, but strain SF-2052 does not.

In view of the above-described characteristics and its ability of producing dactimicin, strain SF-2052 is considered a new species of the genus *Dactylosporangium* and named *Dactylosporangium matsuzakiense* sp. nov. SHOMURA *et* NIIDA from the place where the soil was collected. The type strain, SF-2052, has been deposited in the American Type Culture Collection, and in Fermentation Research

Table 2. Physiological properties of strain SF-2052, *D. aurantiacum* (strain ATCC 23491) and D. *thailandense* (strain ATCC 23490).

| | Strain SF-2052 | *D. aurantiacum* | *D. thailandense* |
|---|---|---|---|
| Hydrolysis of starch | positive | positive | positive |
| Liquefaction of gelatin | negative | positive | negative |
| Reduction of nitrate | negative | positive | negative |
| Peptonization of skim milk | negative | negative | negative |
| Coagulation of skim milk | negative | negative | negative |
| Formation of melanoid pigment | | | |
| in tryptone-yeast extract broth | negative | negative | negative |
| in peptone yeast extract iron agar | negative | negative | negative |
| in tryosine agar | negative | negative | negative |
| Growth at 42°C | very poor | poor | none |
| Utilization of D-glucose | ++ | ++ | ++ |
| D-xylose | ++ | ++ | ++ |
| L-arabinose | ++ | ++ | ++ |
| D-fructose | + | + | ++ |
| D-mannitol | ++ | ++ | ++ |
| *i*-inositol | − | − | − |
| D-melibiose | − | + | − |
| L-rhamnose | ++ | ++ | ++ |
| raffinose | − | − | − |
| sucrose | ++ | + | ++ |
| glycerol | − | − | − |

++: Strongly positive utilization, +: Positive utilization, −: Utilization negative.

Table 3. Carbohydrate composition of cell wall and whole cell hydrolysates of strain SF-2052.

| | Arabinose | Xylose | Galactose | Glucose | Mannose | Rhamnose | Glucosamine |
|---|---|---|---|---|---|---|---|
| Cell wall | Tr* | ++ | Tr | Tr | ++ | − | + |
| Whole cell | Tr | ++ | + | ++ | ++ | − | + |

* Tr: Trace

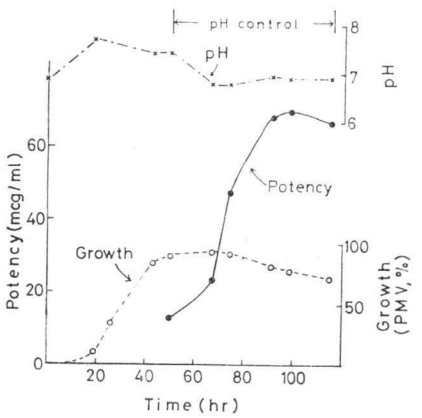
Institute, Japan, and accessioned as ATCC 31570 and as FERM-P 4670, respectively.

## Fermentation of Dactimicin

A typical time-course of dactimicin production in a 300-liter fermentor is shown in Fig. 2. As is the case with the production of many secondary metabolites including antibiotics, the synthesis of dactimicin also begins toward the end of the trophophase. The production of dactimicin was maximum at 98 hours after inoculation, reaching 70 $\mu$g/ml.

Because of the unstability of dactimicin in alkaline condition, the pH control during fermentation was indispensable. The pH was con-

Fig. 2. Time course of fermentation of *Dactylosporangium matsuzakiense* illustrating pH, growth and dactimicin production.



tinuously kept below pH 7.0 from 50 hours corresponding with the start of antibiotic production to the end of fermentation.

The characteristics of the fermentation by *Dactylosporangium* strain is that there is a time lag in the course of seed preparations, and that a large inoculum size is necessary. In the course of production, however, no difference is recognized as compared with that by *Streptomyces* strain.

Acknowledgement

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