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# Studies of Cirratiomycin. Part I. Taxonomy of Producing Organism, and Production, Isolation, Physico-chemical Activities of Cirratiomycin A and B†

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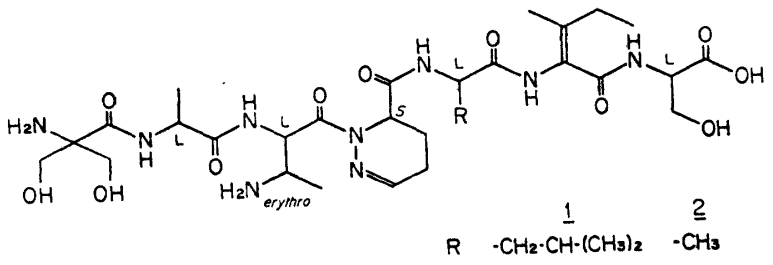
In our screening program for antifolic metabolites using *Lactobacillus casei* as a test organism grown on a synthetic medium with a limiting amount of folic acid, two peptide antibiotics, cirratiomycin A (**1**) and B (**2**), have been isolated from the culture filtrate of *Streptomyces cirratus* 248-Sq2.

*Taxonomic studies of strain* 248-*Sq*2

The cirratiomycin producing strain 248-Sq2 was isolated from a soil sample collected at Norikura Highland, Nagano Prefecture, Japan. The characterization of the strain was performed by the methods of the International Streptomyces Project (ISP) and Waksman. Determination of the cell wall type was carried out by Becker *et al.* for an analysis of diaminopimelic acids ($A_2pm$) and by Lechevalier and Lechevalier for sugars.

The strain produced long and branched vegetative mycelium on agar media but fragmented to coryneform cells in the broth media with shaking. The aerial mycelium formed clusters with monopodial branching on the short main stem. The spore chain was straight, belonging to the section *Rectiflexibiles* (*RF*), and rarely formed open loops and terminal hooks which are suggestive of *Retinaculiaperti* (*RA*). The mature spore chains were generally long and had 10 to 50 or more spores per chain. Electron microscopy revealed that the mature spores were oval to cylindrical (1.3~2.0 by 0.6~0.8 $\mu$m) in shape with a smooth surface. The following special morphologies were also observed; long aerial hyphae which were entangled and the formation of knots and masses of spores on some agar media. The strain contained LL-$A_2pn$ but not sugars in its whole cell hydrolyzate, indicating the cell wall type as I.

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The cultural and physiological characteristics of the strain are examined together with the utilization of carbon sources. These characteristics can be summarized as follows; aerial mass color was of both Red and Gray color-series, the reverse side of the colony showed no distinctive pigments (colorless or pale yellow to dull yellow or yellowish brown) and soluble pigments (pale brown or pale yellowish brown) were formed in some media. The strain was mesophilic and possessed weak diastatic and proteolytic properties but did not form melanoid pigments.

From the above description the strain 248-Sq2 was considered to belong to the genus *Streptomyces*. Among the known *Streptomyces* species described in the 8th edition of Bergey's Manual, ISP reports and any other publications listed in "Approved Lists of Bacterial Names," the strain resembles *Streptomyces cirratus* and *Streptomyces polychromogenes* as shown in Table I. The spore chain morphology, *RF* (a few *RA*), of the strain is different from *S. cirratus* but *S. polychromogenes*. From our experience, however, it was observed that some strains which showed both *RF* and *RA* were affected in their expression rate of spore chain morphology by single colony isolation or by changing the culture conditions. Therefore, distinction of the strain by its spore chain morphology alone may be difficult in this case. On the other hand, the aerial mass color, Red to Gray color-series, of the strain is more similar to that of *S. cirratus* than *S. polychromogenes*. From these observations, it can be concluded that the strain is most closely related to *S. cirratus*. Although the strain had some different properties from *S. cirratus*, such as melanoid and soluble pigment formation which was also affected by single colony isolation, these were not sufficient to classify the strain as a new species. Thus, strain

Table I. Comparison of Strain 248-Sq2 with *S. cirratus* and *S. polychromogenes*

| | 248-Sq2 | *S. cirratus* | *S. polychromogenes* |
|---|---|---|---|
| Spore chain | *RF* (a few *RA*) | *RA* | *RF* (a few *RA*) |
| Spore surface | Smooth | Smooth | Smooth |
| Special morphology | Knots, nest-like tangles, masses of spores | Knots and moist, nest-like tangles | Knots, nest-like tangles (some fragment into spore-like bodies) |
| Color of colony | Red and/or Gray | Red or Gray | Red or Blue* |
| Color of reverse | Not distinct | Not distinct | Not distinct |
| Melanoid pigments + | | PYIA, TYB, (TA) | PYIA, TYB, TA |
| − | PYIA. TYB, TA | (TA) | (TA) |
| Soluble pigments | NP or trace of yellowish brown or brown | Yellow | NP or trace yellow |
| Carbon utilization + | G, A, X, F, S | G, A, X, F, S | G, A, X, F, S |
| ±or − | I, M, Rh, Ra | I, M, Rh, Ra | I, M, Rh, Ra |

*RF*, rectiflexibiles; *RA*, retinaculiaperti; PYIA, pepton-yeast extract iron agar; TYB, trypton-yeast extract broth; TA, tyrosine agar; NP, no pigment; G, D-glucose; A, arabinose; X, D-xylose; F, D-fructose; S, sucrose; I, *i*-inositol; M, D-mannitol; Rh, rhamnose; Ra, raffinose.

* Blue color-series in Bergey's Manual.

248-Sq2 was designated as *S. cirratus* Koshiyama, Okanishi, Ohmori, Miyaki, Tsukiura, Matsuzaki and Kawaguchi 1963 (Approved List No. 1, 1980).

*Test-organism and medium*

The test organism used was *Lactobacillus casei* subsp. *rhamnosus* IAM 1118 which required folic acid as a growth factor. The composition of the assay medium is listed in Table II. Activity against *L. casei* was assayed by agar diffusion tests using a 6 mm paper disk. Because no supplement of folic acid was added to the assay medium, the test organism should have use the limited amount of folic acid which existed in the agar powder as an impurity (final concentration, $10^{-2}$ γ/liter). Consequently, this assay system seems to be hypersensitive to folic acid metabolites.

Table II. Composition of the Assay Medium (1 lit er)

| | | | |
|---|---|---|---|
| Casamino acid | 4.5mg | Pyridoxal | 1 mg |
| Glucose | 20 g | Biotin | 0.01 mg |
| Sodium acetate | 20 g | *p*-Aminobenzoic acid | 0.2 mg |
| L-Cystine | 100 mg | $K_2HPO_4$ | 500 mg |
| L-Tryptophan | 100 mg | $KH_2PO_4$ | 500 mg |
| L-Glutamic acid | 100 mg | $MgSO_4 \cdot 7H_2O$ | 200 mg |
| L-Arginine | 100 mg | NaCl | 10 mg |
| Thiamine | 1 mg | $FeSO_4 \cdot 7H_2O$ | 10 mg |
| Riboflavin | 1 mg | $MnSO_4 \cdot 4H_2O$ | 10 mg |
| Nicotinic acid | 1 mg | Agar | 10 g |
| Pantothenic acid | 1 mg | | |

*Production and isolation*

Seed flasks were inoculated with stock cultures of *S. cirratus* 248-Sq2 maintained at −20°C and incubated for 48 hours at 27°C. The seed medium consisted of 2.0% soybean meal, 2.5% dextrin, 0.5% NaCl and 0.4% $CaCO_3$ (pH 6.4). Inoculum (2%) was transferred to the production medium of the same composition and cultivated at 27°C dor 72 hours in jar fermenters. The whole broth was filtered with the aid of 5% celite. The filtrate (50 liters) was treated with activated carbon (1%) and the carbon cake eluted with 2×10 liters of 70% aqueous acetone (pH 3.0). After concentration, the eluate was applied to a column of Dowex 50W-X2 (7.5×50 cm, $H^+$ form). After being washed with water (3 liters), the column was developed with 5 liters of 5% pyridine. The active eluate was concentrated and chromatographed over a clumn of Dowex 50W-X2 (4.2×65 cm), which was eluted with a 0.5 M pyridine-AcOH buffer, pH 6.4. The active principles were separated into two components **2** and **1** in the order of elution. These were further purified separately by repeated column chromatographies using a Sephadex G-25 partition (3.4×30 cm, *n*-BuOH-AcOH-water, 4 : 1 : 2) and Toyopearl HW 40 F (2.1×100 cm) to afford 50 mf of **1** and 30 mg of **2**.

Table III. Physico-chemical Properties of Cirratiomycins

| | | Cirratiomycin A | | Cirratio mycin B | |
|---|---|---|---|---|---|
| Appearence | | Amorphous white powder | | | |
| M.P. | | 200°C (dec.) | | 215°C (dec.) | |
| p*Ka'* | | 3.4, 6.7, 8.8 | | 3.4, 6.4, 8.5 | |
| FD mass | | 728 $(M+H)^+$, 750 $(M+Na)^+$ | | 686 $(M+H)^+$, 708 $(M+Na)^+$ | |
| Mol. form. | | $C_{31}H_{53}N_9O_{11}\cdot HCl$ | | $C_{28}H_{47}N_9O_{11}\cdot HCl$ | |
| Elemental | | Calcd. % | Found % | Calcd. % | Found % |
| Analysis | C | 48.72 | 48.39 | 46.57 | 46.11 |
| | H | 7.07 | 7.20 | 6.65 | 6.69 |
| | N | 16.50 | 16.98 | 17.46 | 17.82 |
| | O | 23.05 | 24.11 | 24.39 | 24.71 |
| | Cl | 4.65 | 4.16 | 4.92 | 4.67 |
| Ninhydrin | | Pale purple | | | |
| TLC* | No. 1 | 0.26 | | 0.16 | |
| | No. 2 | 0.75 | | 0.63 | |

* No. 1, *n*-BuOH-AcOH-water (4 : 1 : 2); No. 2, $CHCl_3$-MeOH-conc. $NH_3$ (2 : 3 : 1)

*Physico-chemical properties*

Some physico-chemical properties are listed in Table III. The molecular formular of **1** and **2** were determined by the data from elemental analysis together with $^{13}C$-NMR and FD mass spectra. The IR spectrum of **1** suggested the presence of peptide bonds in the molecules.

*Biological activities*

Cirratiomycin A and B are active against a narrow range of *Lactobacillus* including *L. casei* and some strains of *Streptococcus* and *Mycobacterium* but substantially inactive against filamentous fungi and yeasts.