Actinosporangium vitaminophilum sp. nov.

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A new species of *Actinosporangium* is described, for which we propose the name *Actinosporangium vitaminophilum*. This organism produces new antibiotics called pyrrolomycins and is characterized by pseudosporangia lacking sporangial walls, nonmotile spores, and a type I cell wall. It differs significantly from previously described species of the genus *Actinosporangium* in the cultural conditions necessary for pseudosporangium formation. The type strain of *A. vitaminophilum* is SF-2080 (= ATCC 31673).

In the course of our screening program for new antibiotics, we isolated an actinomycete designated strain SF-2080^T (type strain). This organism produces new chlorinated nitropyrrole antibiotics called pyrrolomycins (8, 13), which show both antibacterial and antifungal activities.

In an early stage of our taxonomic study, strain SF-2080^T was considered to belong to the genus *Streptomyces* Waksman and Henrici 1943 because of its chemotaxonomic features, but it did not show any of the morphological characteristics necessary for genus determination. Further extensive study revealed that this strain forms pseudosporangia and nonmotile spores on nutritionally restricted media, instead of the spore chains typically found among the species of the genus *Streptomyces*.

The taxonomic characteristics of this actinomycete are very similar to those of the genus *Actinosporangium*, which was described by Krasil'nikov and Tsi-shen (16). Based on a taxonomic comparison with known species, strain SF-2080^T has been identified as a new species of the genus *Actinosporangium*.

In this paper we describe the morphological, cultural, and physiological characteristics of strain SF-2080^T and our whole-cell analysis of this organism. We also discuss the taxonomic position of strain SF-2080^T.

MATERIALS AND METHODS

Actinomycete strains. Strain SF-2080^T was isolated in our laboratory from a soil sample collected at the Chikuma River (Nagano City, Japan) by using inorganic salts-starch agar (ISP medium 4) containing 10 µg of novobiocin per ml. For comparative purposes, *Actinosporangium violaceum* strain KCC A-0052 (= ATCC 15813) was also studied; this strain was provided by the KCC Culture Collection (Kaken Seiyaku Co. Ltd., Tokyo, Japan).

Morphological characterization. As described below, the formation of aerial mycelia and pseudosporangia

occurred on calcium malate agar diluted 1:4 (0.25 × calcium malate agar) and supplemented with 1 µM vitamin B₁₂, and this medium was used for light microscopy and electron microscopy studies.

For scanning electron microscopy, an agar block bearing numerous pseudosporangia was fixed in 2.5% glutaraldehyde for 16 h and in osmium tetroxide fumes for 4 h. The block was then gradually dehydrated by washing with increasing proportions of acetone and finally dried by the critical-point method (1). An alternative preparation was made by touching a collodion film mounted on a grid to the surface of a heavily sporulating agar culture and fixing the resulting preparation in osmium tetroxide fumes for 4 to 20 h. The block or collodion film prepared as described above was coated with a gold-palladium sputter and then examined with a scanning electron microscope (model JEM100C-ASID; Japan Electron Optics Laboratory Co. Ltd.).

Cultural characterization. To examine the cultural characteristics of strain SF-2080^T washed cells were inoculated onto the following media: ISP media 2 to 7 (24); the sucrose-nitrate agar, glucose-asparagine agar, nutrient agar, Bennett agar, and calcium malate agar of Waksman (27); and the water agar and thin potato-carrot agar of Higgins et al. (10). The effect of 1 µM vitamin B₁₂ on cultures was investigated for all media used. Inoculated plates were incubated at 28°C and were observed for 5 to 30 days.

Physiological characterization. The temperature range for growth was determined on ISP medium 2. Starch hydrolysis was examined by testing for the presence of starch by the iodine reaction on ISP medium 4 plates. Melanoid pigment production was observed in ISP media 1, 6, and 7. Antibiotic susceptibilities were determined by using susceptibility disks placed onto the surfaces of ISP medium 2 plates seeded with 2% vegetative cells. NaCl tolerance was measured on Luedemann agar medium (21) containing various concentrations of NaCl (0, 1.5, 3, 4, 5, and 7%).

Carbon utilization tests were performed by using ISP medium 4 without starch as the basal medium, because strain SF-2080^T showed no growth on ISP medium 9, even if vitamin B₁₂ was added.

Chemical analyses of whole cells. The presence and form of diaminopimelic acid were determined by the method of Becker et al. (2), and a carbohydrate analysis of whole-cell hydrolysates was performed by the method of Lechevalier (19).

The glycolate test was carried out by the method of Uchida and Aida (26). Phospholipids were analyzed by the procedure of Lechevalier et al. (20). A menaquinone analysis was performed by using reverse-phase thin-layer chromatography, as described by Collins et al. (5), and mass spectrometry also was used to determine unequivocally the quinone system (4, 29).

RESULTS AND DISCUSSION

Morphological characteristics. The vegetative mycelium of strain SF-2080^T was long, narrow, branched, and 0.5 to 0.6 μm in diameter. A microscopic examination of our agar and liquid cultures did not yield any morphological characteristics useful for identification (e.g., aerial mycelia, sporangia, spores, nocardioform growth, synnemata, sclerotia, etc.). Therefore, we added supplements to the media and altered the cultural conditions; i.e., we varied the carbon sources, nitrogen sources, inorganic salts, trace metals, vitamins, pH, temperature, and atmosphere. The carbon source supplements were tested at concentrations of 0.05 and 0.5% and included D-glucose, glycerol, D-mannitol, maltose, L-rhamnose, starch, sucrose, D-xylose, sodium acetate, and calcium citrate. The nitrogen source supplements were tested at concentrations of 0.01 and 0.5% and included NH_4NO_3 , NaNO_3 , $(\text{NH}_4)_2\text{SO}_4$, $(\text{NH}_4)_2\text{HPO}_4$, urea, L-asparagine, L-glutamine, Casamino Acids (vitamin-free), peptone, and yeast extract. The inorganic salt supplements, which were tested at concentrations of 0.01 and 0.1 M, were CaCO_3 , KCl, KH_2PO_4 , MgSO_4 , NaCl, and Na_2HPO_4 , and the trace metal supplements, which were tested at concentrations of 0.01 and 0.1 mM, were Co^{2+} , Cu^{2+} , Fe^{3+} , Mn^{2+} , and Ni^{2+} . The vitamins ascorbic acid, biotin, calcium pantothenate, folic acid, inositol, niacin, *p*-aminobenzoic acid, pyridoxin, riboflavin, thiamine, and vitamin B₁₂ were tested at concentrations of 0.1, 1, and 10 μM . The cultural conditions which we varied were pH (pH 5.0, 6.0, 7.0, 8.0, and 9.0), temperature (15, 20, 24, 28, 32, 37, and 42°C), and atmosphere (air, air containing 5% CO_2 , and 90% N_2 –5% H_2 –5% CO_2). In all of these tests the basal media used were calcium malate agar and ISP media 2, 3, and 4. Most of our alterations were ineffective; however, vitamin B₁₂ was effective not only in stimulating the growth of strain SF-2080^T on the various media tested (see below), but also in stimulating the formation of aerial mycelia and sporangium-like vesicles (pseudosporangia) (only on calcium malate agar). Aerial mycelia and pseudosporangia were formed more abundantly on 0.25 $\times$ calcium

malate agar than on full-strength calcium malate medium when both were supplemented with 1 μM vitamin B₁₂. The former medium was used for our morphological study of strain SF-2080^T.

Aerial mycelia appeared after 5 to 7 days of incubation at 28°C, and these mycelia formed pseudosporangia several days later. Aerial hyphae were long, tufted, branched, and 0.7 to 0.8 μm in diameter. The pseudosporangia were spherical to oval and sometimes irregular in shape, and their diameters ranged from 3 to 30 μm (Fig. 1 and 2). Although the pseudosporangia had no definite envelope (sporangial wall), they appeared to keep their compact shape. However, the pseudosporangia broke immediately when water was added (Fig. 3). The spores in each pseudosporangium had smooth surfaces and were elliptical, 0.5 to 0.6 μm in diameter, and nonmotile. About 20 to more than 100 spores were contained in each pseudosporangium. Strain SF-2080^T did not form straight, flexuous, or spiral spore chains, as observed commonly in the genus *Streptomyces*.

Electron microscopic observations were made to investigate the mode of pseudosporangium formation in strain SF-2080^T. As shown in Fig. 4a and b, the formation of pseudosporangia started with coiling and intertwining of aerial hyphae, which eventually resulted in the forma-

FIG. 1. Pseudosporangia of strain SF-2080^T on 0.25 $\times$ calcium malate agar supplemented with 1 μM vitamin B₁₂, as seen with a light microscope at $\times 600$. The organism was incubated at 28°C for 15 days.

FIG. 2. Pseudosporangium of strain SF-2080^T on 0.25 × calcium malate agar supplemented with vitamin B₁₂, as seen with a scanning electron microscope. The organism was incubated at 28°C for 15 days. (a) ×8,000. (b) ×3,000.

tion of a network of coiled hyphae. Spores were formed by septation of coiled hyphae (Fig. 4c and d). Throughout the development of the pseudosporangium there was no distinct sporangial wall. This mode of pseudosporangium formation in strain SF-2080^T differs from the mode of sporangium formation in the family *Actinoplanaceae* Couch 1955, (e.g., in *Actinoplanes* Couch 1950 and *Streptosporangium* Couch 1955). The members of the *Actinoplanaceae* form their sporangia by growing a hypha inside a bag formed by the extension of the outer sheath of the sporangiophore (17, 18).

Cultural characteristics. The cultural characteristics of strain SF-2080^T on various media are shown in Table 1. Generally, growth was poor on the chemically defined media but moderate on most of the organic media. The reverse sides of the colonies were colorless to pale tan or pale grayish yellow on all of the media used.

Vitamin B₁₂ stimulated the growth of vegetative mycelia on several media, such as glucose-asparagine agar, nutrient agar, and ISP media 2, 3, 4, and 7 (Table 1). This vitamin also stimulated the formation of aerial mycelia on calcium malate agar. The addition of cobalt ions or any

FIG. 3. Spores scattered from mature pseudosporangia when water was added, as seen with a light microscope at ×600.

FIG. 4. Process of pseudosporangium formation in strain SF-2080^T, as seen with a scanning electron microscope. (a and b) Network formation by coiling and intertwining of aerial mycelia at an early stage (10 days of incubation). (c and d) Spore formation by septation of coiled hyphae in an immature pseudosporangium. (a) $\times 5,000$. (b) $\times 10,000$. (c) $\times 10,000$. (d) $\times 20,000$.

TABLE 1. Cultural characteristics of strain SF-2080^T on various media with or without vitamin B₁₂

Medium	Vitamin B ₁₂ ^a	Growth ^b	Reverse color	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	A	±	Colorless	None	None
	P	±	Colorless	None	None
Glucose-asparagine agar	A	±	Colorless	None	None
	P	++	Pale yellow	None	None
Glycerol-asparagine agar (ISP medium 5)	A	+	Colorless	None	None
	P	+	Colorless	None	None
Inorganic salts-starch agar (ISP medium 4)	A	+	Colorless	None	None
	P	++ or +++	Pale grayish yellow	None	None
Calcium malate agar	A	±	Colorless	None	None
	P	±	Colorless	Very scant, white	None
0.25 × calcium malate agar	A	±	Colorless	None	None
	P	±	Colorless	Scant, white	None
Oatmeal agar (ISP medium 3)	A	+ or ++	Colorless	None	None
	P	++	Pale yellow	None	Faint yellow
Yeast extract-malt extract agar (ISP medium 2)	A	++ or +++	Colorless to pale tan	None	None
	P	+++	Colorless to pale tan	None	None
Tyrosine agar (ISP medium 7)	A	+ or ++	Colorless to pale tan	None	None
	P	++	Colorless to pale tan	None	Faint yellow
Nutrient agar	A	+	Pale tan	None	None
	P	++ or +++	Pale tan	None	None
Bennett agar	A	++	Pale grayish yellow	None	None
	P	++	Pale grayish yellow	None	None
Thin potato-carrot agar	A	+	Colorless	None	None
	P	+	Colorless	None	None
Water agar	A	±	Colorless	None	None
	P	±	Colorless	None	None

^a A, Absent; P, present, (1 µM).^b ±, Very poor; +, poor; ++, moderate; +++, good.

other vitamin had no effect. We observed no difference between the effects of 0.5 and 50 µM vitamin B₁₂. When media were supplemented with vitamin B₁₂, calcium citrate was used in place of calcium malate, and NH₄NO₃ and (NH₄)H₂PO₄ were used in place of NH₄Cl, but NaNO₃ was not.

The color of the aerial mycelium was white. A distinct diffusible pigment was not produced, except for a faint yellow color which was observed with ISP media 3 and 7 supplemented with vitamin B₁₂.

Physiological characteristics. The physiological characteristics of strain SF-2080^T were as follows. Hydrolysis of starch, liquefaction of gelatin, and reduction of nitrate were positive. Peptonization and coagulation of skim milk and formation of melanoid pigment were negative. Strain SF-2080^T grew between 15 and 45°C; optimum growth occurred between 25 and 34°C. Poor growth was observed on agar medium

containing 1.5% NaCl, and no growth occurred on media containing more than 3% NaCl. Strain SF-2080^T utilized D-glucose, D-xylose, L-rhamnose, and glycerol, but not L-arabinose, D-fructose, D-mannitol, *D*-inositol, sucrose, or raffinose.

Whole-cell analysis. An analysis of whole-cell hydrolysates by paper chromatography demonstrated the presence of LL-diaminopimelic acid, but not *meso*-diaminopimelic acid. No arabinose, madurose, or xylose was detected in whole-cell hydrolysates. A glycolate test of whole cells was negative. Thus, strain SF-2080^T may have an acetyl group in the muramic acid of its peptidoglycans. Strain SF-2080^T had a type PII phospholipid according to the classification scheme of Lechevalier et al. (20). This strain contained MK-9(H₆) (*m/z*, 790), MK-9(H₈) (*m/z*, 792), and MK-9(H₁₀) (*m/z*, 794) as its major menaquinones; these menaquinones are common in *Streptomyces* strains.

TABLE 2. Comparison of some morphological characteristics of strain SF-2080^T and related genera

Taxon	Aerial mycelium	Spore chains	Sporangia or pseudosporangia		Zoospores
			Shape	Location ^a	
Strain SF-2080 ^T	+	—	Spherical, irregular	AM	—
<i>Actinopycnidium</i>	+	+	Spherical (pycnidium)	SC	—
<i>Actinosporangium</i>	+	+	Spherical, irregular	AM, VM	—
<i>Elytrosporangium</i>	+	+	Club shaped	VM	—
<i>Intrasporangium</i>	—	—	Spherical, lemon shaped	VM	—
<i>Kineosporia</i>	—	—	Spherical, pyriform	VM	+
<i>Kitasatoa</i>	+	+	Club shaped	AM, VM	+
<i>Microellobosporia</i>	+	—	Club shaped	AM, VM	—

^a AM, Aerial mycelium; SC, surface of colony; VM, vegetative mycelium.

Identity of strain SF-2080^T. Among the genera of the order *Actinomycetales* Buchanan 1918 on the Approved Lists of Bacterial Names (25), seven genera are related to strain SF-2080^T as aerobic actinomycetes that form sporangia or sporangium-like vesicles and possess type I cell walls; these genera are *Actinopycnidium* Krasil'nikov 1962 (14), *Actinosporangium* Krasil'nikov and Tsi-shen 1961 (16), *Elytrosporangium* Falcao de Moraes et al. 1966 (9), *Intrasporangium* Kalakoutskii et al. 1967 (12), *Kineosporia* Pagani and Parenti 1978 (23), *Kitasatoa* Matsumae et al. 1968 (22), and *Microellobosporia* Cross et al. 1963 (7). Table 2 shows a comparison of strain SF-2080^T and the related genera mentioned above. Strain SF-2080^T can be clearly distinguished from *Elytrosporangium*, *Intrasporangium*, *Kineosporia*, *Kitasatoa*, and *Microellobosporia* on the basis of the shape and location of sporangia.

The distinguishing characteristic of the genus *Actinopycnidium* is the presence of pycnidia containing numerous spores. As described by Krasil'nikov (14), the pycnidia of this genus are spherical or oval and 70 to 100 µm (sometimes 200 µm) in diameter and are found on the surfaces of the colonies. They have distinct walls and are colorless at first, later becoming brownish or black. The pseudosporangia of strain SF-2080^T clearly differ from the pycnidia of *Actinopycnidium* in shape and structure.

In 1961 Krasil'nikov and Tsi-shen (16) proposed the new genus *Actinosporangium* and the new species *Actinosporangium violaceum*. According to the description of these authors, the genus *Actinosporangium* is characterized by the formation of pseudosporangia that lack a solid envelope and contain nonmotile spores. The cell wall analysis of Yamaguchi (30) demonstrated that *A. violaceum* contains LL-diaminopimelic acid. Thus, strain SF-2080^T must be morphologically related to *A. violaceum* strain KCC A-0052. *A. violaceum* formed abundant spore chains and pseudosporangia when it was cultivated at 28°C for 10 to 15 days on thin potato-

carrot agar and moderate numbers when it was cultivated on ISP media 2 and 4. As Fig. 5 shows, the pseudosporangia of *A. violaceum* resemble closely the pseudosporangia of strain SF-2080^T in shape, in location, and in the lack of a definite envelope. Hussein and Krasil'nikov (11) reported that there are three modes of formation of pseudosporangia in the genus *Actinosporangium*; i.e., pseudosporangia form from spiral sporophores or from hyphae of aerial and vegetative mycelia. The pseudosporangia of strain SF-2080^T correspond to the type formed

FIG. 5. Pseudosporangium of *A. violaceum* strain KCC A-0052 on thin potato-carrot agar, as seen with a scanning electron microscope at ×6,000. The organism was incubated at 28°C for 15 days.

from the hyphae of aerial mycelia. Accordingly, we believe that strain SF-2080^T belongs to the genus *Actinosporangium*.

There is some question about the legitimacy of the genus *Actinosporangium*, and, according to Cross and Goodfellow (6) and Williams et al. (28), the name *Actinosporangium* is a synonym of *Streptomyces*. According to these authors, the pseudosporangia are just tightly coiled chains of *Streptomyces* spores. However, the coiling or intertwining of aerial hyphae before sporulation seems to be irregular and heavy in *Actinosporangium*, whereas it is more regular and slight in *Streptomyces*. Although the legitimacy of the genus *Actinosporangium* cannot be considered further here because information about this genus is too scanty, we believe that the pseudosporangia of strain SF-2080^T resemble those of *Actinosporangium* rather than the masses of *Streptomyces* spores.

A. violaceum is the only species of the genus *Actinosporangium* on the Approved Lists of Bacterial Names (25). As Table 3 shows, strain SF-2080^T differs clearly from *A. violaceum* in aerial mass color and various physiological properties. Krasil'nikov (15) proposed a systematic identification of the species of the genus *Actinosporangium*, in which 16 species, including *A. violaceum*, were described. Recently, Bush et al. (3) described a new species, "*Actinosporangium bohemicum*" (not on the Approved Lists). However, strain SF-2080^T can be distinguished from the known species of the genus *Actinosporangium* as described below. All of the previously described species have been reported to form both spore chains and pseudosporangia on usual agar media, whereas strain SF-2080^T forms only pseudosporangia on a limited medium (that is, calcium malate agar supplemented with vitamin B₁₂).

In view of the characteristics described above, strain SF-2080^T can be considered a new species of the genus *Actinosporangium*, for which we propose the name *Actinosporangium vitaminophilum* sp. nov. (vi.ta.mi.no'phi.lum. L. n. *vita* life; M.L. n. *aminum* amine, hence vitamin; Gr. adj. *philon* loving; M.L. adj. *vitaminophilum* vitamin-loving.). Strain SF-2080^T is the type strain of *A. vitaminophilum*; a culture of this strain has been deposited in the American Type Culture Collection as strain ATCC 31673.

Summary description of *Actinosporangium vitaminophilum* sp. nov. Pseudosporangia are 3 to 30 µm in diameter, spherical to oval, and sometimes irregular and are formed on aerial mycelium; there is no definite sporangial wall. Spores are elliptical, 0.5 to 0.6 µm in diameter, and nonmotile, with smooth surfaces. Aerial mycelium white and 0.7 to 0.8 µm in diameter. The morphology described above is observed only

TABLE 3. Characteristics that differentiate strain SF-2080^T and *A. violaceum*

Strain	Aerial mass color	Nitrate reduction	Milk peptonization	Melanin formation	NaCl tolerance (%)	Antibiotic susceptibilities ^a		Carbon utilization ^b			
						Novobiocin (5-µg disk)	Polymyxin B (300-U disk)	D-Fructose	D-Mannitol	L-Arabinose	D-Sucrose
SF-2080 ^T	White	Positive	Negative	Negative	<1.5	R	S	+	+	+	+
<i>A. violaceum</i>	Pinkish	Negative	Positive	Positive	<5	S	R	-	-	-	-

^a R, Resistant (no zone of inhibition); S, susceptible (zone of inhibition).
^b +, Compound utilized; -, compound not utilized.

on calcium malate agar supplemented with vitamin B₁₂.

Poor growth on chemically defined media and moderate growth on organic media. Growth on all media is pale tan or pale grayish yellow. No distinct diffusible pigment is produced.

Utilizes D-glucose, D-xylose, L-rhamnose, and glycerol for growth, but not L-arabinose, D-fructose, D-mannitol, D-inositol, sucrose, or raffinose.

Cell wall type I; aerobic; mesophilic.

Produces antibiotics of the pyrrolomycin complex.

Habitat: soil.

Type strain: strain SF-2080 (= ATCC 31673).

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