ACTINOSYNNEMA MIRUM, A NEW PRODUCER OF NOCARDICIN ANTIBIOTICS

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β-Lactam antibiotics from actinomycetes so far reported have been limited to those produced by Genera *Streptomyces* and *Nocardia*. Nocardicins, monocyclic β-lactam antibiotics, have been first reported as products from *Nocardia uniformis* subsp. *tsuyamanensis*[1].

During the course of our screening for new antibiotics selectively active against an anaerobe, *Eubacterium moniliforme* ATCC 25546, we have isolated from a fallen leaf a strain designated NR 0364 which produced nocardicins. Strain NR 0364 was characterized by the formation of synnemata and zoospores. It was identified as *Actinosynnema mirum*.

This is the first report to show that the strain belonging to *Actinosynnema* produces β-lactam antibiotics. The type strain of *Actinosynnema mirum* KCC A-0225 was also found to produce nocardicin A. This paper describes the taxonomical characterization of strain NR 0364 and the fermentation and isolation of nocardicins in comparison with *Actinosynnema mirum* KCC A-0225.

Strain NR 0364 was isolated from a fallen leaf collected in Fukuyama, Hiroshima Prefecture, Japan (March, 1979), by following the isolation method of TUBAKI[2]. The strain was deposited in American Type Culture Collection as ATCC 31896.

The taxonomic studies were carried out mostly according to the procedures adopted by the International Streptomyces Project (ISP) using the media recommended by SHIRLING and GOTTLIEB[3] and by WAKSMAN[4]. The color description including color names and hue numbers was in accordance with the Color Harmony Manual (4th edition)[5].

White aerial mycelia generally arise at the tip of yellow orange synnemata (40~100×50~450 μm) formed on the tested agar media (Fig. 1). Observations were made using the method of HASEGAWA[6]. Light microscopic examination of aerial mycelia showed tufts or often curls (Fig. 2). Electron microscopic studies revealed that usual spore chain morphology was not observed, but the septate spore chains showed a bamboo-like appearance with smooth surface (Fig. 3). The spores were easily separated from each other in an aqueous environment as rod-shaped cells with smooth surface or with flagella in some cases. Thus, the aerial mycelia formed on agar media such as inorganic salts-starch agar

Fig. 1. Photomicrograph of synnemata.
(ISP medium No. 4, 6 days, bar represents 10 μm).

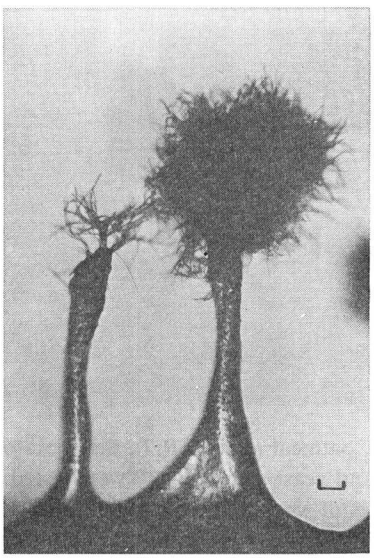


Fig. 2. Photomicrograph of aerial mycelia.
(ISP medium No. 4, 10 days, bar represents 10 μm).

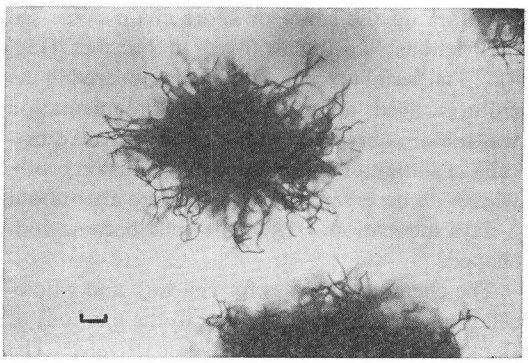


Fig. 3. Electronmicrograph of aerial mycelia. (ISP medium No. 2, 6 days, bar represents 1 μm).

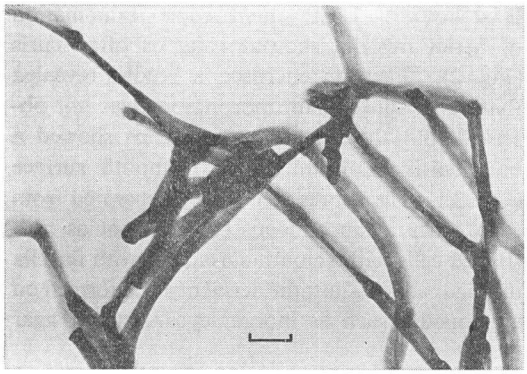
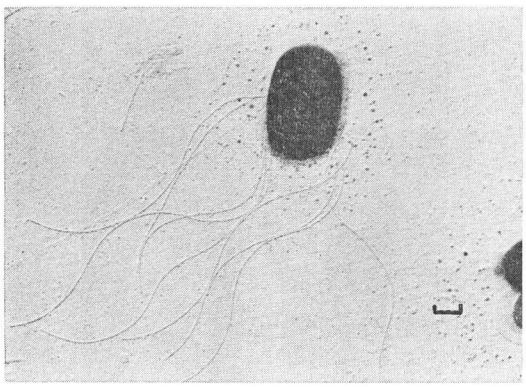


Fig. 4. Electronmicrograph of a zoospore. (ISP medium No. 4, 6 days, bar represents 0.25 μm).



(ISP 4), oatmeal agar (ISP 3), thin potato carrot agar[7] and yeast starch agar[8] were tested for the presence of spores by the method of HIGGINS *et al.*[7] using water or brain heart infusion broth (Difco): Motile spores were recognized after about 1 hour under the light microscope.

With the electron microscope, these motile spores showed a rod-shape ($0.4\sim0.8\times1.2\sim2.5$ μm) and the spore or spore chain possessed peritrichous flagella arranged in bunches (Fig. 4). The aerial mycelia usually disappeared in the cultures aged about 3 weeks. Fragmentation was seldom observed in long-term cultivated mycelia of static culture in brain heart infusion broth, whereas it occurred in shaking culture after about 3 days incubation. Then the cells became rod-shaped.

The chemical analyses for cell-wall and whole-cell were carried out according to the methods of BECKER[9] and LECHEVALIER[10], respectively. The cell-wall analysis confirmed the presence of *meso*-diaminopimelic acid in addition to alanine, glucosamine, glutamic acid, muramic acid, and a small amount of aspartic acid and glycine. Galactose, glucose and mannose were detected in the whole-cell hydrolysate, while neither arabinose nor madurose was detected.

Cultural characteristics, physiological properties and carbon utilization are summarized in Tables 1, 2 and 3, respectively. Unless otherwise stated, all cultures were incubated at 27°C for 3 weeks. Strain NR 0364 grew well on synthetic and organic media and was aerobic and mesophilic.

According to the literature on the genus belonging to the order Actinomycetales, such an actinomycete as its aerial mycelia bear chains of conidia capable of forming flagella in an aqueous environment and are formed at the tip of synnemata originating from the substrate mycelia, is differentiated from all other genera of Actinomycetales, and is placed in the genus *Actinosynnema*

Fig. 5. Isolation of nocardicins from strain NR 0364.

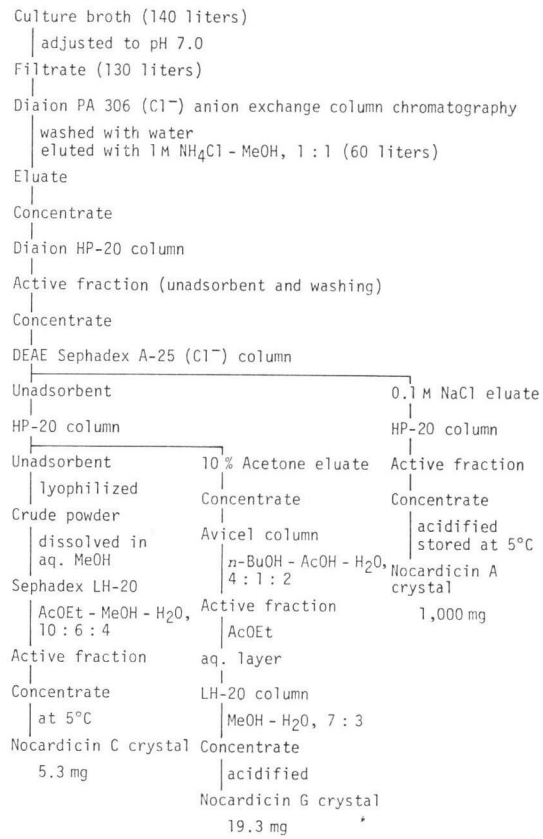


Table 1. Cultural characteristics of strain NR 0364.

	Growth	Reverse	Aerial mycelium	Diffusible pigment
Sucrose nitrate agar (Waksman medium No. 1)	Yellow orange (2lc, gold) synnemata	Dull yellow orange (2ic, light gold)	Scant, white (a, white)	None
Glucose asparagine agar (Waksman medium No. 2)	Dull yellow orange (3nc, amber~2gc, bamboo) synnemata	Pale yellow orange (2ie, light mustard tan)	Scant, white (a, white)	None
Glycerol asparagine agar (ISP medium No. 5)	Pale yellow orange (2nc, brite gold), synnemata	Dull yellow orange (2ic, light gold)	Thin, white (a, white)	None
Inorganic salts-starch agar (ISP medium No. 4)	Yellow orange (2lc, gold)~dull yellow orange (2ic, light gold)synnemata	Dull yellow orange (2ic, light gold~2gc, bamboo)	Moderate, white (a, white)~grayish white (2db, ivory)	Faint brownish
Tyrosine agar (ISP medium No. 7)	Yellowish brown (2pe, mustard gold~2pg, mustard gold) synnemata	Yellowish brown (2pe, mustard gold~2pg, mustard gold)	Moderate, white (a, white)	None
Nutrient agar (Waksman medium No. 14)	Yellow orange (2lc, gold)~dull yellow orange (3nc, amber)	Yellow orange (2lc, gold)~pale yellow orange (2nc, brite gold)	None	None
Yeast-malt extract agar (ISP medium No. 2)	Yellowish brown (2pe, mustard gold) ~dull yellow orange (3nc, amber) synnemata	Yellowish brown (2pe, mustard gold) ~dull yellow orange (3nc, amber)	Moderate, white (a, white)	None
Oatmeal agar (ISP medium No. 3)	Dull yellow orange (2ic, light gold~2gc, bamboo) synnemata	Dull yellow orange (2ic, light gold~2gc, bamboo)	Thin, white (a, white)	None

Table 2. Physiological characteristics of strain NR 0364.

Gelatin liquefaction (Waksman medium No. 19)	Positive, faint
Starch hydrolysis (ISP medium No. 4)	Positive
Milk coagulation (Difco 10% skimmed milk)	Positive, faint
Milk peptonization (Difco 10% skimmed milk)	Positive
Nitrate reduction (Waksman medium No. 37)	Positive
Casein hydrolysis (Nutrient agar containing 10% skimmed milk)	Positive
Melanoid pigment formation	
Tyrosine agar (ISP medium No. 7)	Negative
Peptone-yeast-iron agar (ISP medium No. 6)	Negative
Tryptone-yeast broth (ISP meidum No. 1)	Negative
Growth under anaerobic condition (ISP medium No. 2)	No growth
Temperature range for growth (ISP medium No. 4)	15~37°C
Optimum temperature for aerial mycelium formation (ISP medium No. 4)	27°C

as proposed by HASEGAWA *et al.*[4]. In comparison with the type strain of *Actinosynnema mirum* described by HASEGAWA *et al.*, strain NR 0364 differs in the production of diffusible pigment on tyrosine agar and inorganic salts-starch agar. However, in other properties including morphological, physiological properties and the utilization of carbon sources, strain NR 0364 is quite similar to *Actinosynnema mirum*. Thus, the present taxon should be identified as *Actinosynnema mirum* Hasegawa *et al.*

The original nocardicin producer was reported in 1976 by AOKI *et al.* to be *Nocardia uniformis* subsp. *tsuyamanensis*, although no mention on its capability to form synnemata and zoospores was made. However, when we examined the claimed strain (ATCC 21806) purchased from ATCC under the same conditions as above, we confirm-

Table 3. Utilization of carbon sources (ISP medium No. 9).

Substrate	Reaction	Substrate	Reaction
L-Arabinose	±	Inositol	−
D-Xylose	±	L-Rhamnose	+
D-Glucose	+	Raffinose	±
D-Fructose	±	D-Mannitol	+
Sucrose	+	Control	−

+: Utilization, ±: probable utilization, −: no utilization.

ed the formation of synnemata and zoospores in 11 days culture of ISP medium No. 7.

The strain NR 0364 was grown on an inorganic salts - starch agar slant (ISP No. 4) at 27°C for at least 10 days. A loopful of the aerial mycelia was transferred to a 500-ml Erlenmeyer flask containing 200 ml of sterile medium consisting of 4% glycerol, 2.5% soybean meal, 0.2% NaCl, 0.1% glycine, 0.1% L-asparagine, 0.1% L-tyrosine and 0.001% $CoCl_2 \cdot 6H_2O$. This was incubated at 27°C on a rotary shaker set at 180 rpm for 3 days. One liter of the thus obtained culture was inoculated as seed in five 50-liter jar fermentors containing 30 liters each of the same sterile medium mentioned above. Fermentation was done under the following conditions: agitation with an impeller speed of 300 rpm; aeration at a rate of 0.5 vvm and incubation at 27°C. The pH of the culture was controlled below 8.5 with 2 N HCl. Productivity of nocardicins was monitored by a paper disc-plate assay using *Eubacterium moniliforme* ATCC 25546 as the test organism. When the solution contained 125 μg/ml of nocardicin A, it gave 25 mm of inhibitory zone. After 72 hours fermentation, the potency of nocardicins reached 94 μg/ml in the broth. The isolation and purification of nocardicin A, C and G was carried out by the procedure shown in Fig. 5. Thus, 1.0 g of nocardicin A, 5.3 mg of C and 19.3 mg of G were obtained as colorless crystals from 140 liters of fermentation broth. Their physicochemical properties including IR, UV and NMR spectra agreed well with those reported[1,11].

On the other hand, the cultivation of *Actinosynnema mirum* KCC A-0225 was carried out under the identical conditions as the above in 500-ml Erlenmeyer flask. 100 μg/ml of nocardicins was thus produced in the broth. From 2 liters of broth, 15 mg of purified nocardicin A was obtained (not shown).

Acknowledgments

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