NANAOMYCINS, NEW ANTIBIOTICS PRODUCED BY A STRAIN OF *STREPTOMYCES*

I. TAXONOMY, ISOLATION, CHARACTERIZATION AND BIOLOGICAL PROPERTIES

HARUO TANAKA, YASUAKI KOYAMA, JUICHI AWAYA, HIROFUTO MARUMO*, RUIKO ŌIWA, MICHIKO KATAGIRI, TOSHIAKI NAGAI and SATOSHI ŌMURA**

The Kitasato Institute and Kitasato University, Minato-ku, Tokyo, Japan

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Nanaomycins are new antibiotics produced by the strain OS-3966 which was designated *Streptomyces rosa* var. *notoensis*. Nanaomycins A and B were isolated from the culture filtrate by extraction with organic solvent and silica gel chromatography. The physical and chemical properties suggest that nanaomycins A and B are quinone-related compounds having the molecular formulae, $C_{16}H_{14}O_6$ and $C_{16}H_{16}O_7$, respectively. Nanaomycins A and B inhibit mainly mycoplasmas, fungi and Gram-positive bacteria. The acute toxicities (LD_{50}, ip) of nanaomycins A and B in mice are 28.2 and 169 mg/kg, respectively.

Screening of *Streptomyces* culture filtrates for antimycoplasmal activities has been conducted in order to discover new antibiotics by ŌMURA *et al.*[1], IKEUCHI *et al.*[2] and KITAME *et al.*[3] In the course of our screening program of antibiotics more active against *Mycoplasma gallisepticum* KP-13 (a pathogen of chronic respiratory disease of chickens) than against bacteria, new antibiotics, nanaomycins A and B, effective especially against mycoplasmas and fungi were obtained from the culture broth of strain OS-3966 which had been isolated from a soil sample collected at Nanao-shi in the Noto Peninsula, Japan. The successful isolation and properties of the antibiotics have been reported briefly in the preliminary report[4].

The present paper deals with taxonomy of the producing strain, isolation, characterization and biological activities of the antibiotics.

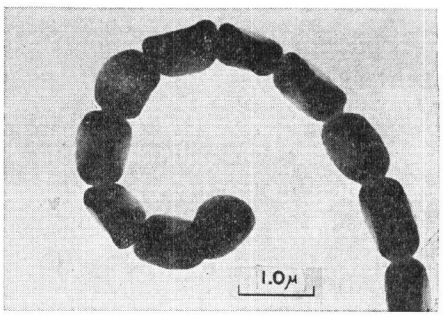
Characteristics of the Nanaomycin-producing Strain

The nanaomycin-producing culture, strain OS-3966, is a *Streptomyces* isolated from a soil sample obtained at Nanao-shi in the Noto Peninsula, Japan.

1. Morphological characteristics.

The morphology of the strain cultured on yeast extract-malt extract agar and inorganic salt-starch agar for 14 days at 27°C was

Fig. 1. Electronmicrograph of the spores of strain OS-3966 (JSM-2, JEDL Co., Ltd.)



* Present address: Pharmaceutical Res. Labs., Kyōwa Hakkō Kōgyō Co. Ltd., Nagaizumi-chō, Suntō-gun, Shizuoka-ken, Japan.

** To whom all correspondence should be addressed.

microscopically observed (Fig. 1). The aerial mycelium of the strain is abundant on either synthetic or organic agar medium. It forms no whorls, but extends aerial hyphae forming open or compact spirals. The spores are oval or cylindrical and their surfaces are smooth.

2. Cultural and physiological characteristics.

The strain OS-3966 was cultivated on various media described by WAKSMAN[5] and ISP (International Streptomyces Project)[6] at 27°C or 37°C, and the changes of growth, aerial

Table 1. Cultural characteristics of strain OS-3966

Medium	Growth	Reverse	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	good, light ivory to light melon yellow	light melon yellow to apricot	light apricot	pearl pink to light melon yellow
Glucose-nitrate agar	good, dusty yellow to golden brown	golden brown to chocolate brown	white to pearl pink	light wheat to sepia brown
Glycerol-asparagine agar	good, light melon yellow to orange rust	apricot	light apricot	melon yellow to apricot
Inorganic salts-starch agar	moderate, light melon yellow	pearl pink to golden brown	white to flesh pink	dark luggage tan to sepia brown
Tyrosine agar	good, light wheat to amber topaz	light melon yellow to nude tan	light melon yellow to pearl pink	light wheat to melon yellow
Nutrient agar	moderate, colorless to pearl pink	squash yellow to bright yellow	white, scant	none
Glucose-peptone agar	moderate, colorless to golden brown	golden brown to sepia brown	white	ivy to dark laurel
Yeast extract-malt extract agar	good, colorless to golden brown	golden brown to orange rust	light melon yellow to light apricot	ivy
Oatmeal agar	moderate, colorless to light melon yellow	light melon yellow to nude tan	light melon yellow to light apricot	light tan
Peptone-yeast extract iron agar	moderate, cream to light wheat	colonial yellow	scant, white to colonial yellow	none
Tryptone-yeast extract broth	surface growth, moderate, light ivory	light ivory	white	none
Milk	surface growth. moderate	pearl pink	none	light apricot to pearl pink
Gelatin	surface growth, good	pearl pink to chartreuse tint	white to celadon gray	laurel
Nitrate broth	surface growth, moderate	light ivory	white	none
Cellulose	none	none	none	none

Table 2. Physiological properties of strain OS-3966

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Nitrate reduction	+
Hydrolysis of starch	+
Liquefaction of gelatin	+
Peptonization of milk	+
Coagulation of milk	+
Cellulolytic activity	−
Temp. range for growth	15~45°C

Table 3. Utilization of carbon sources by strain OS-3966

Response	Carbon source
Positive	D-glucose, D-fructose, D-xylose, L-arabinose, glycerol, mannose, rhamnose, maltose, D-mannitol
Negative	sucrose, raffinose, *i*-inositol

mycelium and soluble pigment were observed after a period of 7, 14 and 21 days. Utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[6] containing 1 % of various carbon sources. Color names and hue numbers indicated were those of the Color Harmony Manual (4th edition) published by Container Cooperation of America.

Table 4. Comparison between the strain OS-3966 and *Streptomyces rosa* ISP 5533

	OS-3966	ISP 5533
Morphology		
Spore chain	Spirals	Spirals
Spore surface	Smooth	Smooth
Inorganic salt-starch agar		
AM*	Flesh pink (4 ca)	Lt. melon yellow (3 ea)
R*	Beige camel (3 ea)	Bamboo chamois (3 gc)
SP*	Slate (15 ih)	Chocolate (4 nl)
Tyrosine agar		
AM	Flesh pink (4 ca)	Flesh pink (4 ca)
R	Lt. melon yellow (4 ea)	Lt. melon yellow (4 ea)
SP	Dusty yellow ($1\frac{1}{2}$ gc)	Flesh pink (4 ca)
Carbon utilization		
Sucrose	−	±
Inositol	−	±
Nanaomycin production	+	−

* AM: Aerial mycelium, R: Reverse, SP: Soluble pigment.

The cultural and physiological characteristics of the strain OS-3966 are listed in Tables 1 and 2, respectively. The utilization of carbon sources by the strain is shown in Table 3. The cultural and physiological characteristics can be summarized as follows: growth is yellow to brown on either synthetic or organic media; aerial mass color is yellowish orange to pink on various media; soluble pigment is yellowish brown on various media; it produces no melanoid pigment.

From the above results, the strain is non-chromogenic and belongs to the red color series. Among known *Streptomyces* species described in "BERGEY's Manual of Determinative Bacteriology", 8th ed.[7], "The Actinomycetes" Vol. II by WAKSMAN[5] and the ISP reports by SHIRLING and GOTTLIEB[8~11], *Streptomyces rosa*[11] was closely related to the strain OS-3966. In comparison of strain OS-3966 with the type culture, *Streptomyces rosa* ISP 5533, all of the morphological characteristics and most of the cultural and physiological characteristics of the former were in agreement with those of the latter. However, as shown in Table 4, such characteristics of the former as the formation of soluble pigment on some media and the utilization of sucrose and *i*-inositol were slightly different from those of the latter. Also, the former produced the new antibiotics, nanaomycins, but the latter did not.

Therefore, the strain OS-3966 should be assigned to a new variety of *Streptomyces rosa*, and was designated as *Streptomyces rosa* var. *notoensis* AWAYA var. nov. The strain OS-3966 has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology with accession number FERM-P No. 2209.

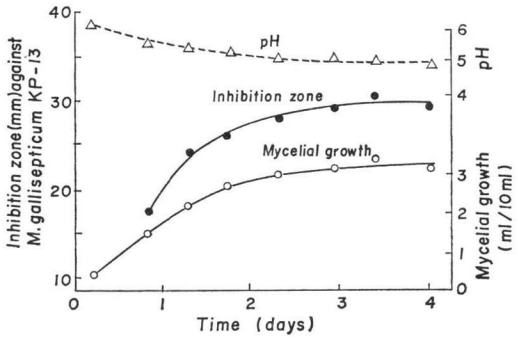
Production of Nanaomycins A and B

The stock culture of strain OS-3966 (*Streptomyces rosa* var. *notoensis*) was maintained as an agar slant (KRAINSKY's agar medium). A 7-day culture of the agar slant was inoculated into

a medium (100 ml) in a SAKAGUCHI's flask, incubated for two days at 27°C, and then used as a seed culture for production of nanaomycin. Fermentation was carried out using a 30-liter jar or a 400-liter tank fermentor containing 20 or 200 liters, respectively, of medium for 4 days at 27°C. The composition of the seed or fermentation medium was 2% glycerol, 2% soybean meal and 0.3% sodium chloride (the pH value was adjusted to 7.0 with 6.0 N sodium hydroxide before sterilization). The time course of a typical fermentation is shown in Fig. 2. The nanaomycin produced was assayed by the methed reported by ITOH *et al.*[16] using *Mycoplasma gallisepticum* KP-13. The nanaomycin production started 1 day after the inoculation and the maximum concentration was reached at 3~4 days. The total amount of nanaomycins A and B accumulated at the 4th day was about 100 μg/ml.

Fig. 2. Time course of nanaomycin production by *Streptomyces rosa* var. *notoensis*.

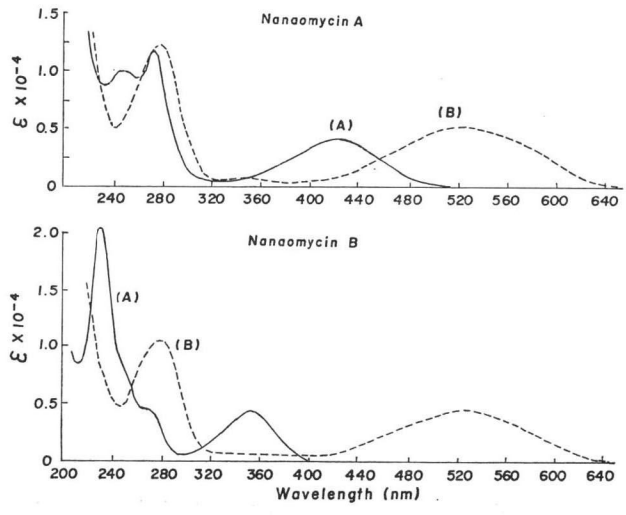
Cultivation was performed using a 30-liter jar fermentor containing 20 liters of the medium described in the text. Culture conditions were as follows: agitation, 300 rpm; temp., 27°C; aeration, 10 liters/minute. Antimycoplasma activity was assayed by the method reported by ITOH *et al.*[16]



Isolation and Characterization of Nanaomycins

Culture broth (200 liters) of *Streptomyces rosa* var. *notoensis*, obtained by incubation in a 400-liter tank, was used as a starting material for the isolation of nanaomycins. Nanaomycins were detected by antimicrobial activity[16]. After the culture supernatant was adjusted to pH 2.0 with 6 N hydrochloric acid, nanaomycins were extracted with butyl acetate and then transferred into 1% sodium bicarbonate solution. From the aqueous solution, nanaomycins were extracted with ethyl acetate after adjusting to pH 2.0 with 6 N hydrochloric acid. A crude powder (10.9 g) of nanaomycins was obtained by evaporating the solvent layer dried with sodium sulfate (anhydrous). The crude powder was chromatographed on a column of silica gel No. 923 (Davison Chemical Co.) with benzene - ethyl acetate. The first active component (designated as nanaomycin A) was eluted with benzene - ethyl acetate (4 : 1) from the column, and then the second active component (designated as nanaomycin B) with benzene - ethyl acetate (3 : 1).

Fig. 3. UV-Spectra of nanaomycins A and B
(A) 90% MeOH or in 0.1 N HCl-90% MeOH.
(B) in 0.1 N NaOH-90% MeOH.



The fractions containing the first active component were

combined and concentrated under reduced pressure to dryness. Orange needles of nanaomycin A were obtained from an ethanol solution of the powder. The crystals were recrystallized from an ethanol solution: yield 317 mg; mp 178~180°C.

Anal. Found: C, 63.35; H, 4.47; N, 0.

Calcd. for $C_{16}H_{14}O_6$: C, 63.57; H, 4.66; N, 0%.

UV λ_{max}^{MeOH} nm(ε): 250(9,850), 274(12,200), 423(4,040). $[\alpha]_D^{26}$: −27.5° (*c* 1.0, MeOH). Mass M^+ (*m/e*): 302.079. The UV and IR spectra are shown in Figs. 3 and 4, respectively. The molecular formula, $C_{16}H_{14}O_6$, was assigned to the compound from elementary analysis and its mass spectrum.

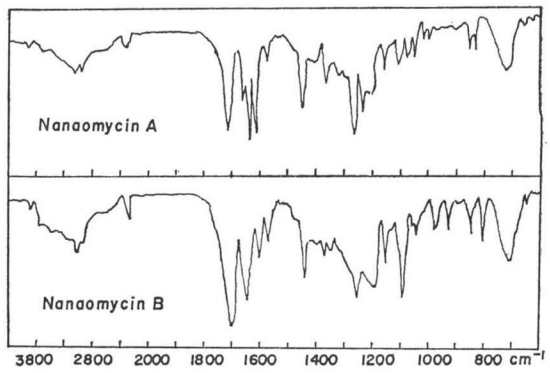
The fractions containing the second active component were combined and concentrated under reduced pressure to give a powder of nanaomycin B (4.5 g). A pure powder of nanaomycin B was obtained by rechromatography: yield 2.7 g; mp 84~86°C.

Anal. Found: C, 59.70; H, 4.90; N, 0.

Calcd. for $C_{16}H_{16}O_7$: C, 59.99; H, 5.03; N, 0%.

UV λ_{max}^{MeOH} nm(ε): 231(20,800), 248(8,700, sh), 269(5,030, sh), 352(4,970). $[\alpha]_D^{26}$: −74.5° (*c* 1.0, MeOH). Mass M^+ (*m/e*): 320.090. The UV and IR spectra are shown in Figs. 3 and 4, respectively. The molecular formula, $C_{16}H_{16}O_7$, was assigned to nanaomycin B from elementary analysis and its mass spectrum.

Fig. 4. IR-Spectra of nanaomycins A and B in $CHCl_3$



Nanaomycins A and B are soluble in methanol, ethanol, ethyl acetate, chloroform, acetone and ethyl ether, but insoluble in water, *n*-hexane and petroleum ether. They gave positive reactions to ferric chloride, 2,4-dinitrophenyl hydrazine and formaldehyde-*O*-dinitrobenzene reagents, but negative reactions to ninhydrin, SAKAGUCHI, EHRLICH, FEHLING, MOLISCH and DRAGENDORFF reagents. Rf values of nanaomycins A and B on silica gel thin-layer chromatography using chloroform - methanol (5 : 1, v/v) were 0.25 and 0.15, respectively.

From the above data, it was concluded that nanaomycins A and B had quinone groups and structures closely related to each other.

Biological Activities of Nanaomycins

The antimicrobial spectra of nanaomycins A and B are shown in Table 5. This test was conducted by agar dilution method using nutrient agar for bacteria, potato agar for fungi, and Hokken and Eiken PPLO agars for mycoplasmas. Nanaomycins A and B inhibit mainly Gram-positive bacteria, and mycoplasmas. Especially against *Trichophyton* species among fungi and *Mycoplasma gallisepticum* and *M. pneumoniae* among mycoplasmas, the antibiotics were active at low concentrations. However, MIC of nanaomycin B shown in Table 5 may be misleading because of its instability discussed in the following section.

Because KITAME *et al.*[3] reported that the activity of laidlomycin against *Acholeplasma*

Table 5. Antimicrobial spectra of nanaomycins A and B

Test organisms	Medium*	Minimal inhibitory concentration (μg/ml)	
		A	B
Bacillus subtilis PCI 219	N	7.8	7.8
Staphylococcus aureus FDA 209P	N	3.9	3.9
Staphylococcus aureus FDA 209P (JC-1)	N	2.0	2.0
Sarcina lutea PCI 1001	N	2.0	2.0
Mycobacterium smegmatis	N	62.5	125
Escherichia coli NIHJ	N	31.3	15.6
Escherichia coli NIHJ (JC-2)	N	250	500
Klebsiella pneumoniae PCI 602	N	31.3	31.3
Salmonella typhimurium	N	62.5	62.5
Shigella flexneri	N	31.3	62.5
Xanthomonas oryzae N-5824	N	62.5	125
Pseudomonas aeruginosa	N	500	>500
Candida albicans	P	31.2	31.2
Saccharomyces sake	P	31.2	62.5
Aspergillus niger ATCC 6275	P	62.5	62.5
Aspergillus fumigatus IAM 2612	P	12.5	>100
Piricularia oryzae	P	7.8	15.6
Microsporum gypseum 704	P	0.8	12.5
Trichophyton asteroides	P	1.6	12.5
Trichophyton ferrugineum	P	1.6	12.5
Trichophyton interdigitale	P	1.6	12.5
Trichophyton mentagrophytes	P	0.8	25
Trichophyton pedis 804	P	0.2	3.1
Trichophyton purpureum	P	3.1	25
Trichophyton roseum	P	0.4	12.5
Trichophyton rubrum	P	<0.1	3.1
Trichophyton schoenleini	P	0.2	3.1
Trichophyton violaceum	P	0.4	3.1
Mycoplasma gallisepticum KP-13	H	<0.013	<0.013
	E	0.05	0.10
Mycoplasma gallisepticum S-6	H	<0.013	<0.013
	E	0.10	0.10
Mycoplasma gallisepticum 333P (Spiramycin resistant)	H	<0.013	0.013
	E	<0.013	0.05
Mycoplasma gallinarum	H	1.56	3.12
Mycoplasma iners	H	3.12	3.12
Mycoplasma pneumoniae	E	0.013	3.05
Acholeplasma laidlawii (A) PG 8	H	>25	>25
	E	>25	>25
Acholeplasma laidlawii (B) Bm 1	H	25	25
	E	>25	>25

* Abbreviations used: N, nutrient agar (pH 7.0, 2 days, 37°C), P, potato agar (pH 6.4, 4 days, 27°C), H, Hokken PPLO agar (pH 7.8, 8 days, 37°C); E, Eiken PPLO agar (pH 7.8, 8 days, 37°C).

laidlawii A was inhibited by addition of serum to a culture medium, the effect of serum concentration in a culture medium on the activity of nanaomycins A and B against *M. gallisepticum* was investigated. As shown in Table 6, nanaomycins A and B were a little more active at high concentrations than at low concentrations of serum; the results is contrary to the case of laidlomycin.

Toxicity of nanaomycin in mice was determined using 5 mice from one group, and the LD_{50} was calculated by BEHRENS-KARBER's method. The toxicities (LD_{50}) of nanaomycins A and B are 28.2 and 169 mg/kg intraperitoneally, respectively, and 290 mg/kg orally.

The Conversion of Nanaomycin B to Nanaomycin A and their Biological Activities

The irreversible conversion of nanaomycin B to nanaomycin A in alkaline media was observed. At pH values over 10 and at room temperature, nanaomycin B was immediately converted to the substance having a UV-spectrum identical with

Table 6. Influence of horse serum on antimycoplasmal activities of nanaomycins A and B

Horse serum (%)	Minimal inhibitory concentration* (μg/ml)			
	M. gallisepticum KP-13		*M. gallisepticum* 333P**	
	A	B	A	B
1	0.2	0.2	0.8	0.2
2	0.1	0.1	0.05	0.05
5	0.2	0.2	0.1	0.2
10	0.05	0.1	0.05	0.1
20	0.05	0.05	0.025	0.025

* Agar dilution method (Eiken PPLO agar, 37°C, 8 days).
** Spiramycin-resistant strain.

Table 7. Influence of pH values of the assay medium on antibacterial activities of nanaomycins A and B

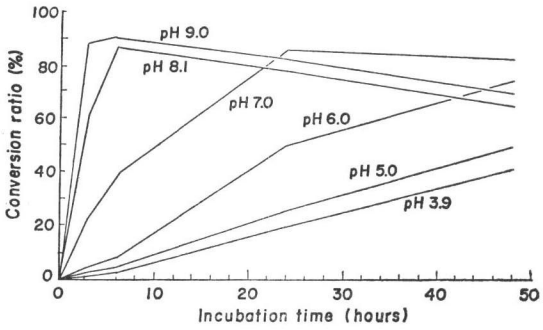
pH	Minimal inhibitory concentrations* (μg/ml)			
	St. aureus FDA 209P		*St. aureus* FDA 209P JC-1	
	A	B	A	B
6.1	3.1	50	0.4	25
7.4	6.3	12.5	1.6	12.5
8.4	12.5	12.5	3.1	6.3

* Agar dilution method (nutrient agar, 2 days, 37°C).

that of nanaomycin A. The converted substance was isolated and identified as nanaomycin A by thin-layer chromatography, melting point, UV- and IR-spectra. Effect of pH values on the conversion of nanaomycin B to nanaomycin A was investigated. As shown in Fig. 5, nanaomycin B was found to convert to nanaomycin A even at pH 7.0 by incubation for 24 hours at 37°C; the conversion ratio was 85 %.

Fig. 5. Conversion of nanaomycin B to nanaomycin A at various pH values

Solutions (5.0×10^{-5} M) of nanaomycin B in the buffers having various pH values were incubated at 37°C. Concentration of nanaomycin A formed was determined from the optical density at 420 nm of the reaction mixture.



On the other hand, influence of pH values of the media on antimicrobial activities of nanaomycins A and B against *Staphylococcus aureus* was investigated. Consequently, as shown in Table 7, in an acidic medium (pH 6.1) the antimicrobial activities of nanaomycin B were weaker than those of nanaomycin A, but in an alkaline medium (pH 8.4) the former was nearly equal to the latter. In Table 5, correlation between the A and B activities is better for the assays in PPLO agar (pH 7.8, 37°C) and nutrient agar (pH 7.0, 37°C) than those in potato agar (pH 6.4, 27°C).

The results of the experiments described above can be explained as follows: Nanaomycin B itself has no or very weak activity and it is activated by the non-enzymatic conversion to nanaomycin A in assay medium.

Discussion

From the above data, it was found that nanaomycins A and B isolated from the culture broth of the strain OS-3966, *Streptomyces rosa* var. *notoensis*, are quinone-related antibiotics having inhibitory activities against mycoplasmas, fungi and Gram-positive bacteria (especially strong activities against *Trichophyton* species, *M. gallisepticum* and *M. pneumoniae*).

Among known quinone antibiotics, kalafungin,[12] frenolicin[13~15] and deoxyfrenolicin[14,15] resemble nanaomycins A and B. However, nanaomycins A and B differ from these compounds in molecular formula, UV-spectra, and other physical and chemical properties. Thus, it can be concluded that nanaomycins A and B are new antibiotics.

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