DELTAMYCINS, NEW MACROLIDE ANTIBIOTICS. I

PRODUCING ORGANISM AND FERMENTATION

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A streptomycete strain, P3409, which produced new basic macrolide antibiotics, deltamycins A_1, A_2 and A_3 and carbomycin A (deltamycin A_4) was considered to be a new subspecies for which the name *Streptomyces halstedii* subsp. *deltae* was proposed. Deltamycins A_1, A_2, A_3 and A_4 which were active against Gram-positive bacteria were produced in organic complex media.

In the course of a screening program for antibiotics, a streptomycete strain P3409 was found to produce a complex of antibiotics which was collectively named deltamycin. The complex consisted of four components designated deltamycins A_1, A_2, A_3 and A_4. From their physicochemical properties, it was found that deltamycins A_1, A_2 and A_3 were new basic macrolide antibiotics while deltamycin A_4 was identical with carbomycin A[1,2].

In this paper, the taxonomy of the producing strain and fermentation studies of deltamycin production are reported together with the antimicrobial activity of the different components.

1. Taxonomy

Strain P3409 was isolated from a soil sample collected in the Botanical Garden of Hokkaido University, Sapporo, Japan, and was deposited under number FERM-P 2504 in the collection of the Fermentation Research Institute, Agency of Industrial Science and Technology, Chiba, Japan.

Taxonomical studies were performed principally using the methods recommended by SHIRLING and GOTTLIEB[3~7] as well as the methods reported by PRIDHAM and TRESNER[8] and WAKSMAN[9]. Reference strains employed in the studies were received from the Institute for Fermentation, Osaka.

Morphological Characteristics

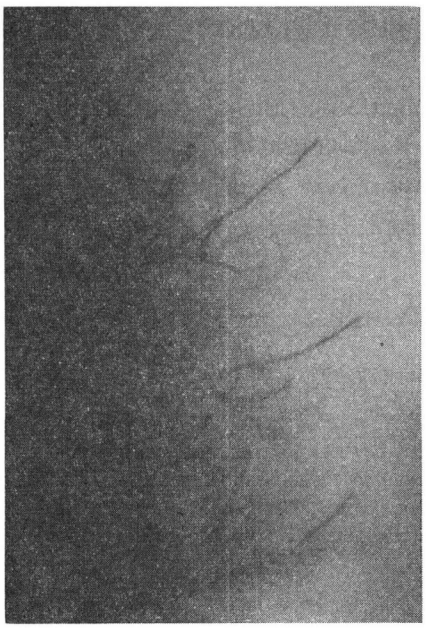
Morphological features were observed after 1~3 weeks incubation at 28°C on the various ISP media[3].

The aerial mycelia branch without verticils. The spore chains are predominantly straight, but occasionally flexuous (Plate 1). Very rarely, hook-like or loop-like shapes are seen on yeast extract-malt extract agar or inorganic salts-starch agar. No or little sporulation is observed on oat meal agar and glycerol-asparagine agar and, where present, only straight spore chains are observed.

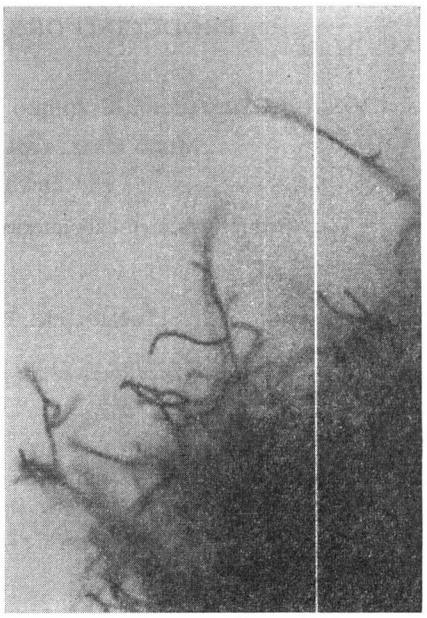
Mature spore chains are often moderately long with 10 to 50 spores per chain on the top of aerial mycelia. Rarely longer chains are observed.

Plate 1. Photomicrograph of strain P3409 on yeast extract-malt extract agar (×680)

Predominantly straight spore chains



Hook-like spore chains are occasionally observed.



It is therefore believed that this strain belongs to the Section *Rectiflexibiles* (*RF*) of the genus *Streptomyces*.

Electron microscopy reveals that the mature spores are cylindrical and $0.3 \sim 0.5\ \mu \times 0.9 \sim 1.5\ \mu$ with a smooth surface (Plate 2).

Cultural and Physiological Characteristics

The cultural characteristics of strain P3409 on the designated media are shown in Table 1. The results were based on observation after 2 weeks incubation unless otherwise noted. The color of sporulated aerial mycelia and substrate mycelia was designated mainly on the basis of seven color series of the color wheel made by TRESNER and BACKUS[11] and also on the color table of "Guide to Color Standard", a manual published by Nippon Shikisai Kenkyusho, Tokyo, Japan.

Plate 2. Electron micrograph of spores of strain P3409 (×12,000)

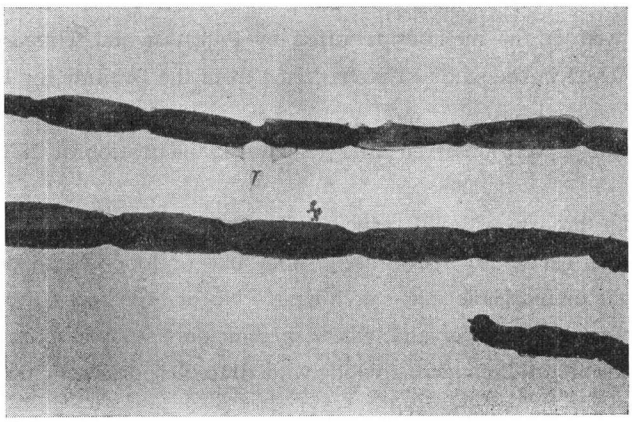


Table 1. Cultural characteristics of strain P3409

Medium	Cultural characteristic
Sucrose nitrate agar	G: Abundant or moderate. Am: None; occasionally thinly formed, white. Rs: Pale yellow (2ca-2db). Sp: None.
Glucose asparagine agar	G: Abundant. Am: Grayish yellowish pink (5cb-5dc). Rs: Light orange yellow (3ea) and later yellowish brown. Sp: None or slightly yellow.
Glycerol asparagine agar	G: Abundant. Am: None or poor; when incubated for 3 weeks, if any, dotted, white or grayish yellowish pink (5cf). Rs: Light orange yellow (3ea) and later yellowish brown. Sp: None or slightly yellow.
Inorganic salts - starch agar	G: Abundant; surface smooth and powdery. Am: Light gray (d) at 2 weeks; grayish yellowish pink (5dc) at 3 weeks. Rs: Light yellow (2fb) and later grayish yellow (3ec) or light brown (4ie). Sp: None.
Tyrosine agar	G: Abundant; surface wrinkled or folded. Am: None or poor; occasionally, if any, white dotted, after 3 weeks. Rs: Grayish yellow (3ec) and later light brown (4ie). Sp: Brown.
Nutrient agar	G: Abundant or moderate. Am: Absent. Rs: Pale yellow (2db). Sp: None.
Yeast extract - malt extract agar	G: Abundant; surface folded. Am: Grayish yellowish pink (5dc) or light gray (d) at 2 weeks; light grayish reddish brown (5fe) at 3 weeks. Rs: Light yellow (2fb) and later light grayish brown (4ig). Sp: None or slightly brown.
Oatmeal agar	G: Abundant; surface wrinkled or folded. Am: Almost absent, but when developed, white or grayish yellowish pink (5cb). Rs: Moderate yellowish pink (4gc) and later brown (4ie). Sp: None or slightly brown.
Peptone yeast extract - iron agar	G: Abundant. Am: None. Rs: Yellow or pale yellow (2db). Sp: None.
Glucose-peptone - gelatine (20°C)	G: Moderate. Am: None.
Skimmed milk	G: Ring formed. Am: None. Sp: Yellow or yellowish brown.

G: Growth, Am: Aerial mycelium, Rs: Reverse side of substrate mycelium, Sp: Soluble pigment, ( ): Color code of the Color Harmony Manual[10].

The physiological characteristics of strain P3409 are shown in Table 2.

Comparison with the Known Strains

It is clear from the above characteristics that strain P3409 belongs to a group of the genus *Streptomyces* having the following characteristics: color of mature sporulated aerial mycelium is in the gray color series; spore chains are predominantly straight belonging to the Section *RF*; spore

surface is smooth; and melanoid pigment production is positive only in tyrosine agar.

As species which morphologically resemble P3409, the following 8 species in the genus *Streptomyces* were selected: *Streptomyces halstedii* ISP 5068[4], *S. nitrosporeus* ISP 5023[4], *S. misakiensis* ISP 5222[5], *S. omiyaensis* ISP 5552[7], *S. xanthocidicus* ISP 5575[7], *S. flavovirens* ISP 5062[4], *S. griseinus* ISP 5047[5], and *S. osterogriseus* ISP 5511[7].

The spore chains of these 8 strains are straight or flexuous (Section *RF*) with a smooth surface. *S. halstedii* ISP 5068 has been assigned into the Section *RF* because the spore chains are predominantly flexuous, although some hooks and irregular coils are observed on yeast extract-malt extract agar or glycerol-asparagine agar medium.

Table 2. Physiological characteristics and carbon source utilization of strain P3409

Hydrolysis of starch	Positive (weak)
Liquefaction of gelatine	Positive
Hydrolysis of casein	Positive (slow)
Coagulation of milk	Negative
Formation of melanoid pigment	
in peptone yeast extract iron agar	Negative
in tryptone yeast extract broth	Negative
in tyrosine agar	Positive
Reduction of nitrate	Positive
Temperature range for growth	20~32°C
Optimum	28~30°C
No growth	37°C
pH range for growth	3.0~10.0
Optimum	6.0~8.0
Utilization of carbon sources	
D-Glucose, D-xylose, L-arabinose, rhamnose, D-fructose, sucrose, raffinose, D-mannitol and *i*-inositol are utilized for growth.	

Further, there are other strains resembling strain P3409 with regard to characteristics other than the morphology of the spore chain. *S. aureofaciens* ISP 5127[5] and *S. recifensis* ISP 5115[4] have been reported to produce spore chains indistinguishable from that of the Section *RF* strains even though they are assigned into the Section *Retinaculiaperti* (*RA*).

These 10 strains were compared with the strain P3409 after cultivation under the same conditions. Color of the mature sporulated aerial mycelium of all strains was in the gray color series. Though a few of the strains were distinguishable from each other by the difference in color shade of the mature sporulated aerial mycelium, most of the strains could be distinguished from each other after precise comparison of other cultural characteristics. After such an examination, these 10 strains could be satisfactorily distinguished from each other. In a similar way, the strain P3409 could also be distinguished from the 10 strains. Thus all the 10 strains did not produce melanoid pigment in tyrosine agar while strain P3409 did. Also the 10 strains showed utilization patterns of carbon sources different from that of the strain P3409. Cultural characteristics and utilization of carbon sources of the most similar 3 strains among the 10 strains tested are shown in Tables 3 and 4.

S. tendae ISP 5101[4,12] and *S. halstedii* NRRL B-2331[13], a different strain from the type strain *S. halstedii* ISP 5068 are producers of carbomycin. They were compared with strain P3409 after cultivation under the same conditions.

S. tendae ISP 5101, showing morphological characteristics of the Section *RA* or *S*, is clearly different from strain P3409 not only in morphology but also in cultural characteristics and utilization of carbon sources.

The characteristics of *S. halstedii* NRRL B-2331 are shown in Tables 3 and 4. It seems reasonable to assign strain NRRL B-2331 to Section *RF* or *RA* but not to the Section *S*. Despite several different characteristics from those of the type strain, strain NRRL B-2331 has been designated *S. halstedii*. Many indistinguishable points are found between strain P3409 and *S. halstedii* NRRL B-2331 not

Table 3. Comparison with the most resembling strains (1)

		S. halstedii ISP 5068	*S. xanthocidicus* ISP 5575	*S. omiyaensis* ISP 5552	*S. halstedii* NRRL B-2331	Strain P3409
Morphology:*1						
Section		*RF.*	*RF.*	*RF.*	*RF.*	*RF.*
Variation		(*RA*) on YMA & GAA.	None.	None.	(*RA*) on YMA & TA.	(*RA*) rarely on YMA.
Sporulation*2		None or poor on OA, good on others.	All good.	None or poor on GAA, good on others.	All good.	None or poor on OA & GAA, good on others.
Spore chain length (No. per chain)		Short. (3～10)	Long. (More than 50).	Medium. (10～50).	Short. (3～10 or 20).	Medium. (10～50).
Culture:						
Oat meal agar (OA)	G*3	A.*4	A.	A.	A.	A.
	Am*6	Light grayish reddish brown (5*fe*).	Light grayish reddish brown (5*fe*).	Yellowish gray (2*dc*).	Light olive gray (2*ih*).	None or dotted, if any, white to grayish yellowish pink (5*cb*).
	Rs*7	Grayish yellow (3*ec*).	Light brownish gray (3*fe*).		Light yellow.	Moderate yellowish pink (4*gc*).
	Sp*8	None or slightly brown.	None.	None.	None.	None or slightly brown.
Yeast extract-malt extract agar (YMA)	G	A.	A.	A.	A.	A.
	Am	Medium gray (*e*).	Light grayish reddish brown (5*fe*).	Yellowish gray (2*dc*).	Light olive gray (2*ih*).	Light gray (*d*) or light grayish reddish brown (5*fe*).
	Rs	Grayish yellowish brown (3*ig*).	Light grayish yellowish brown (3*ge*).	Light yellow (2*fb*).	Light orange yellow (3*ea*).	Light yellow (2*fb*).
	Sp	None.	None.	None.	None.	None or slightly brown.
Glycerol asparagine agar (GAA)	G	A.	A.	A.	A.	A.
	Am	Light grayish reddish brown (5*fe*).	Light brownish gray (3*fe*).	None or poor; if any, white partly formed.	Light olive gray (2*ih*).	None or poor; if any, dotted, white.
	Rs	Light brownish gray (2*fe*).	Light grayish yellowish brown (3*ge*).	Pale yellow (2*db*).	Dark greenish gray (24*ml*).	Light orange yellow (3*ea*).
	Sp	None.	None.	None.	None.	None.

(to be continued)

Table 3. (Continued)

		S. halstedii ISP 5068	*S. xanthocidicus* ISP 5575	*S. omiyaensis* ISP 5552	*S. halstedii* NRRL B-2331	Strain P3409
Inorganic salts-starch agar (ISSA)	G	A.	A.	A.	A.	A.
	Am	Medium gray (*g*), partly formed.	Light grayish reddish brown (5*fe*).	Yellowish gray (2*dc*).	Medium gray (2*fe*).	Light gray (*d*) to grayish yellowish pink (5*dc*).
	Rs	Light olive gray (2*ih*).	Light brownish gray (3*fe*).	Light yellow ($1\frac{1}{2}$ *fb*).	Dark greenish gray (24*ml*).	Light yellow (2*fb*).
	Sp	None.	None.	None.	None.	None.
	Hydrolysis of starch	Positive.	Positive.	Positive.	Positive.	Positive (slow).
Tyrosine agar (TA)	G	A.	A.	A.	A.	A.
	Am	Medium gray (*e*).	White with yellowish tinge.	Yellowish gray (2*dc*).	White with grayish tinge.	None or poor; if any, dotted, white.
	Rs	Brownish gray (4*li*).	Light brownish reddish brown (4*ge*).	Light yellow (2*fb*).	Pale yellow (2*db*).	Grayish yellow (3*ec*).
	Sp	None.	None.	None.	None.	Brown.
Sucrose nitrate agar	G	A.	M.*5	M.	A.	M.
	Am	White	Light brownish gray (3*fe*).	None or poor.	White or light gray (*d*).	None or poor.
	Rs	Grayish yellow (3*ec*).	Grayish yellow (3*ec*).	Pale yellow (2*db*).	Light yellow (2*fb*).	Pale yellow (2*db*).
	Sp	None.	None.	None.	None.	None.
Glucose asparagine agar	G	A.	A.	A.	A.	A.
	Am	Light gray (*d*).	Grayish yellowish pink (5*cb*).	White or yellowish gray (2*dc*).	Medium gray (2*fe*) or light brownish gray (3*fe*).	Grayish yellowish pink (5*dc*).
	Rs	Light brownish gray (3*fe*).	Pale yellow (2*db*).	Light yellow ($1\frac{1}{2}$ *fb*).	Light olive gray (2*ih*) or dark greenish gray (24*ml*).	Light orange yellow (3*ea*).
	Sp	None.	None.	None.	None.	None.

*1: Based on the observation on OA, YMA, GAA & ISSA media after 10 to 20 days incubation,
*2: Based on the observation on OA, YMA, GAA & ISSA media after 2 or 3 weeks incubation,
*3: Growth, *4: Abundant growth, *5: Moderate growth, *6: Aerial mycelium,
*7: Reverse side of substrate mycelium, *8: Soluble pigment,
( ): Color code of the Color Harmony Manual.

Table 4. Comparison with the most similar strains (2)

	S. halstedii ISP 5068	*S. xanthocidicus* ISP 5575	*S. omiyaensis* ISP 5552	*S. halstedii* NRRL B-2331	Strain P3409
D-Glucose	+	+	+	+	+
D-Xylose	+	+	+	+	+
L-Arabinose	+	+	±	+	+
Rhamnose	−	−	+	+	+
D-Fructose	+	+	±	+	+
Sucrose	−	+	−	+	+
Raffinose	−	+	−	+	+
D-Mannitol	−	−	−	+	+
i-Inositol	−	−	±	−	+

+: Good growth, ±: Poor growth, −: No growth.

only in morphological and cultural characteristics and but also in the utilization of carbon sources. *S. halstedii* NRRL B-2331 among the strains tested is the most similar to strain P3409.

Nevertheless, strain P3409 is distinguishable from *S. halstedii* NRRL B-2331 in spore chain morphology, carbon utilization pattern and melanoid pigment production. These differences are considered insignificant to designate a new species for strain P3409. It is adequate to assign strain P3409 to a new subspecies of *S. halstedii*.

The authors propose a new subspecies, *Streptomyces halstedii* subsp. *deltae* subsp. nov. KOUNO *et* ISHIKURA. The strain P3409 is nominated as the type strain of *S. halstedii* subsp. *deltae*.

2. Fermentation

Fermentation conditions suitable for the production of deltamycins were studied and media shown in Table 5 were found useful. For the production of deltamycins, a 20-liter jar fermentor containing 10 liters of the fermentation medium was inoculated with 0.2 liters of a seed culture grown in 500-ml Erlenmeyer flasks containing 100 ml of seed medium on a rotary shaker for 48 hours. The fermentor was incubated aerobically under stirring at 26°C. Deltamycin production was followed by microbiological assay using the paper disk-agar diffusion method with *Bacillus subtilis* ATCC 6633 as the assay organism. The growth was measured using the packed volume of sediment from 3 ml of broth after centrifugation at 1,500 *g* for 10 minutes. One ml of the packed volume contained approximately 75 mg of dry cells.

A representative time course of deltamycin fermentation is shown in Fig. 1. In this case,

Fig. 1. Time course of deltamycin fermentation
Medium: MML-5, 20-liter jar fermentor.
Temp.: 26°C
PV: packed volume

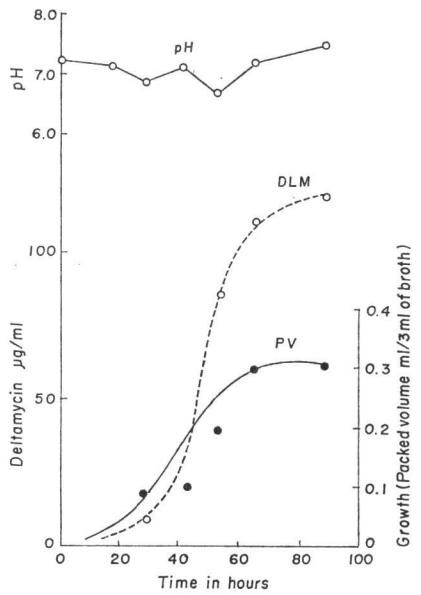


Table 5. Seed medium and fermentation medium

Seed medium		Fermentation medium (MML-5)	
Soybean meal	2.0%	Glycerol	6.0%
Glycerol	2.0	Glucose	2.0
Yeast extract	0.1	Corn steep liquor	2.0
K_2HPO_4	0.05	Dried yeast	0.3
$MgSO_4 \cdot 7H_2O$	0.05	$CaCO_3$	0.35
NaCl	0.05	NaCl	0.5
pH 7.0 (prior to sterilization)		pH 7.0 (prior to sterilization)	

production of deltamycin complex after about 90 hours fermentation was determined to be 120 μg/ml as deltamycin A_1.

3. Antimicrobial Activity

The deltamycin complex was separated into four components by isolation processes using extraction and column chromatography. As reported in detail in a subsequent paper, it was found that deltamycin A_1, A_2 and A_3 were new while deltamycin A_4 was identified as carbomycin A[2]. They were active against Gram-positive bacteria. The minimal inhibitory concentrations against a variety of microorganisms were determined by the broth dilution method using an inoculum size of 1×10^5 cells per ml. After 20 hours incubation at 35°C, growth of the assay organisms in a series of tubes

Table 6. Minimal inhibitory concentrations of deltamycins (Broth dilution method)

Microorganism	Medium	Deltamycin (μg/ml)			
		A_1	A_2	A_3	A_4
Staphylococcus aureus FDA 209P	1	0.8	0.4	0.4	0.2
〃 EM^r	1	6.25	0.8	0.8	0.8
〃 OM^r	1	50	12.5	12.5	12.5
〃 SPM^r	1	50	12.5	12.5	12.5
〃 Smith	1	1.6	0.8	0.8	0.8
〃 BX-1633 Pc-G^r	1	3.13	0.8	0.8	0.8
〃 Russell Pc-G^r	1	3.13	0.8	0.8	0.8
Streptococcus pyogenes NY5	2	0.4	0.2	0.2	≦0.2
Streptococcus pneumoniae type 1	2	0.2	<0.2	<0.2	≦0.2
〃 type 3	2	0.2	<0.2	<0.2	≦0.2
Neisseria meningitidis	2	6.25	0.8	1.6	0.8
〃 *catarrhalis*	2	25	12.5	25	12.5
Shigella sonnei	1	12.5	25	25	6.25
Sarcina lutea	1	0.4	<0.2	<0.2	<0.2
Klebsiella pneumoniae ATCC 10031	1	6.25	3.1	3.1	3.1
Bacillus subtilis ATCC 6633	1	0.8	0.4	0.4	0.2
Mycobacterium smegmatis 607	3	3.1	3.1	3.1	3.1
Escherichia coli K 12	1	>100	100	100	100

Medium 1: Nutrient broth.
〃 2: Nutrient broth containing 10% horse blood.
〃 3: Nutrient broth containing 10% glycerine.

containing various amounts of deltamycin in nutrient broth was observed. The results are shown in Table 6.

Deltamycins A_1, A_2 and A_3 showed similar antibacterial spectra against Gram-positive bacteria and *Klebsiella* as well as *Mycobacterium*. Deltamycins A_1, A_2 and A_3 are somewhat less active than carbomycin A (deltamycin A_4).

Acknowledgement

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