ANTIBIOTIC 5879 PRODUCED BY *STREPTOMYCES AIZUNENSIS*, IDENTICAL WITH BICYCLOMYCIN

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Antibiotic 5879 has been found to be identical with bicyclomycin which was reported simultaneously with antibiotic 5879. *Streptomyces* sp. strain No. 5879 was classified as a new species and was given the name *Streptomyces aizunensis*. *Streptomyces aizunensis* is different from *Streptomyces sapporonensis* which produces bicyclomycin.

In the previous paper[1], we presented a preliminary report on a new water-soluble antibiotic 5879 produced by *Streptomyces* No. 5879. Differences in the taxonomic properties of the strain from those of recognized species were considered to be significant enough to propose the new species *Streptomyces aizunensis* nov. sp. for this strain, and antibiotic 5879 was named aizumycin. This report deals with the taxonomy of the producing organism, the isolation of crystalline antibiotic 5879, and the comparison of the physical, chemical and biological properties of the antibiotic with those of bicyclomycin[2].

Taxonomy

The strain *Streptomyces* No. 5879 was isolated from a soil sample collected in Aizu area, Fukushima Prefecture, Japan.

I. Morphological Characteristics

The morphology of the culture grown on glucose asparagine agar and starch ammonium agar at 28°C for 4~10 days was microscopically observed. The aerial mycelia of strain No. 5879 are well branched, 1.0~1.25 μ in diameter. Conidia are elliptical to spherical in shape and 0.6~0.8 by 1.3 μ in size. The surface appearance of conidia is smooth (Plates 1 and 2).

2. Cultural and Physiological Characteristics

The cultural characteristics and summarized physiological properties of strain No. 5879 are

Plate 1. Aerial mycelia of strain No. 5879. (×150×2.5)

Plate 2. Electronmicrograph of the spores of strain No. 5879. (×5,000×2.0)

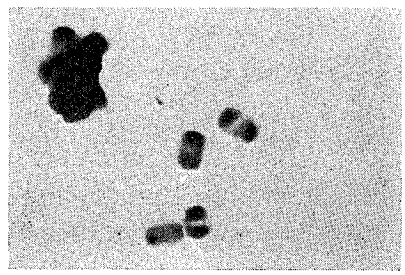

Table 1. Cultural characteristics of strain No. 5879

Medium	Growth	Aerial mycelium	Soluble pigment	Remarks
CZAPEK's agar	white, faint growth	white, powdery	none	
Starch inorganic agar	colorless	yellow grayish white, powdery	pale yellow	
Starch ammonium agar	colorless	white, powdery	none	
Glucose asparagine agar	white, spreading growth	pale brownish gray and white, powdery	pale yellow	
Glycerol asparagine agar	white	pale yellow	pale yellow	
Tyrosine agar	yellow	pinkish gray, powdery	pale brownish yellow	
Nutrient agar	pale yellow, small colonies	none	none	
Yeast malt extract agar	pale yellow	pale pinkish gray, powdery	brown	
Peptone yeast iron agar	colorless, faint growth	none	dark brown	
Oat meal agar	pale yellow, small colonies	gray, powdery	none	
Egg albumin agar	white	grayish white	none	
Egg	blackish brown	none	brown	
Glucose CZAPEK's solution	white, small colonies at bottom	none	none	
Glucose bouillon	pale brown, small colonies at bottom	none	none	
Milk	pale yellowish white	none	none	peptonization and coagulation: positive
Gelatin stab (20°C, 20 days)	no growth			liquefaction: negative
Potato plug	yellow, wrinkled colonies	none	none	
Carrot plug	no growth			
Cellulose	no growth			

shown in Tables 1 and 2, respectively. Each of the media used in this study was prepared according to WAKSMAN[3] or International *Streptomyces* Project[4]. All culture were incubated at 28°C for 2 weeks before observation.

Table 2. Physiological properties of strain No. 5879

Optimum temperature for growth*	25~30°C
Optimum pH range for growth	6.0~8.0
Tyrosinate reaction	positive
Melanoid reaction	positive
Reduction of nitrate	positive
Liquefaction of gelatin	negative
Coagulation of milk	positive
Peptonization of milk	positive
Hydrolysis of starch	positive
Cellulose decomposition	negative
Product	antibiotic 5879

* On glucose asparagine agar

Table 3. Carbon utilization pattern for strain No. 5879

Source of carbon	Growth
D-Xylose	+
L-Arabinose	+
D-Fructose	+
D-Glucose	+
D-Mannose	+
L-Rhamnose	−
D-Mannitol	−
Saccharose	−
Lactose	+
Raffinose	±
Inositol	−
Salicin	−
Starch	+
Negative control	−

(+) utilization, (−) no utilization
(±) doubtful

In general, the organism makes good growth with white to yellow color. The aerial mycelium on most of the tested media is white to yellow or pale pinkish gray in color and the reverse side of the colonies was white to yellow or brown. A pale yellow soluble pigment is produced when it is grown on most of the media tested, but a pale brown color is formed when grown on peptone-yeast extract, iron agar and tyrosine agar. Starch and milk is hydrolyzed, but cellulolytic activity, gelatin liquefaction are negative. Nitrate reduction is positive.

The utilization of carbon sources by strain No. 5879 was investigated with the basal medium described by PRIDHAM and GOTTLIEB[5]. The results are shown in Table 3. D-Glucose, D-fructose, D-xylose, L-arabinose, D-mannose and starch are utilized, but saccharose, L-rhamnose, D-mannitol and inositol are not.

From the observations described above, strain No. 5879 may be characterized as follows: It forms well-branched aerial hyphae and straight or wavy sporophores. A pale yellow soluble pigment are produced on most of the media. A brown soluble pigment is produced on tyrosine agar and peptone-yeast iron agar. The color of vegetative growth is white to yellow or brown. The aerial mycelia is white to yellow or pale pinkish gray. The hydrolytic activities on starch and milk are positive, but gelatin liquefaction is negative.

3. Comparison of Strain No. 5879 with Related *Streptomyces*

After comparison of the characteristics of those *Streptomyces* species described in "The *Actinomycetes*, vol. 2" by WAKSMAN[3], "BERGEY's Manual of Determinative Bacteriology" (1957)[6] and other recent literature, some related strains were selected by further detailed comparison. They were *Streptomyces griseolus* and *Streptomyces flavogriseus*. However, these organisms were differentiated from strain No. 5879 by the following characteristics.

(1) *Streptomyces griseolus* produces generally soluble brown pigment on various media, grows spreadingly with brown color on bouillon agar, liquefies gelatin and gives an alkaline reaction on litmus-milk media.

(2) *Streptomyces flavogriseus* bears spherical conidia and grows excellently with abundant aerial hyphae on potato plug, but grows poorly on starch agar and forms white aerial hyphae on bouillon agar.

4. Comparison of Strain No. 5879 with *Streptomyces sapporonensis*

Strain No. 5879 also should be compared with *S. sapporonensis* which produces bicyclomycin[2], similar to antibiotic 5879. The organism was different from strain No. 5879 as follows:

S. sapporonensis exhibits typical whorl formation and the conidia are oval and cylindrical in shape. Strain No. 5879 forms no whorl and the shape of conidia is elliptical or spherical. The aerial mass color of *S. sapporonensis* is white with grayish or yellowish tinge, whereas that of strain No. 5879 is white to yellow or pale pinkish gray. *S. sapporonensis* produces no soluble pigment on tyrosine agar, nutrient agar, yeast malt agar or other proteinous media, whereas strain No. 5879 produces a plale yellow pigment on the same media. In the biological examination, nitrate reduction, coagulation and peptonization of milk, tyrosinate reaction, and the formation of melanoid pigment are all negative in *S. sapporonensis*, whereas they are positive in strain No. 5879. In addition, the former easily utilizes inositol, but does not utilize lactose in contrast to the latter. These characters apparently differentiate *S. sapporonensis* from strain No. 5879.

Differences in taxonomic properties given above and those of recognized species in the genus *Streptomyces* are considered to be significant enough to propose the new species *Streptomyces aizunensis* nov. sp. OTSUKA, OGASAWARA *et* MIYAMURA for the strain No. 5879. A culture of the new species has been deposited in the American Type Culture Collection, with accession number ATCC 21775.

Fermentation Conditions

The antibacterial activity in the production, isolation and purification processes was assayed by paper-disc plate method with *Escherichia coli* K-12 as the test organism. In a 20-liter jar fermenter, 15 liters of a medium consisting of 2% glucose, 2% soybean meal, 0.2% peptone, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, and 0.0001% $FeSO_4 \cdot 7H_2O$(pH 7) was inoculated with 400 ml of the seed culture obtained by 48 hours shaking culture at 30°C. Antibiotic production was carried out at 30°C under aeration of 10 liters per minute and stirring at 300 rpm. The potency of the broth reached maximum of about 40 mcg/ml after 60~65 hours of inoculation.

Isolation

The fermented broth was acidified to pH 2, filtered to remove a precipitate, and the acidic filtrate was adjusted to pH 8 with anion-exchange resin (Daia-ion A-6) under stirring. After removing the resin by filtration, the filtrate was passed through a column of IRC-50(H^+). The column was washed with water and 0.01 N hydrochloric acid, and the antibiotic was eluted with 0.5 N hydrochloric acid. Fractions containing strong activity were pooled, neutralized to pH 7 by mixing with A-6 and then filtered. The filtrate was concentrated under reduced pressure, and the concentrated solution was freeze-dried. This powder was extracted with approximately 5 volumes of 50% aqueous ethanol, and the aqueous ethanol was filtered to separate the residue. After concentration of the filtrate, the resulting solution was adjusted to pH 8 with dilute aqueous sodium hydroxide and passed through a column of activated charcoal (Wako Pure Chem. Ind. Co.). The column was washed with water and the antibiotic was eluted with 80% aqueous ethanol. The crude antibiotic was isolated as an off-white powder by lyophilization of combined fractions. This

substance was further purified by means of column chromatography on cellulose (Whatman CF 11) with a solvent system consisting of water-saturated *n*-butanol. Active fractions were combined, concentrated *in vacuo* to dryness. The resulting purified powder was dissolved in a small volume of hot methanol and insoluble material was removed by filtration. This solution was mixed with approximately 10 volume of acetone, concentrated under reduced pressure until crystallization occurred, and the mixture was then refrigerated overnight. The crystalline antibiotic was recrystallized from a mixture of methanol and acetone to yield colorless prisms.

Characterization

Antibiotic 5879 forms colorless prism-shaped crystals, and the physicochemical properties of the antibiotic are presented in Table 4. The infrared spectrum comparing antibiotic 5879 with bicyclomycin is shown in Fig. 1.

Biological Properties

The antimicrobial activity of antibiotic 5879 was determined by the agar dilution method, using mostly heart infusion agar for bacteria and SABOURAUD's agar for fungi. As shown in Table 5, the antibiotic is especially active against Gram-negative bacteria, including *Klebsiella*, *Salmonella*, *Escherichia*, *Shigella*, *Brucella* and *Vibrio* sp., but not active against *Proteus*, *Morganella*, *Ret-*

Table 4. Physicochemical properties of antibiotic 5879

Melting point	170°~171°C (decomp.)
Specific rotation $[\alpha]_D^{31}$ (c 0.63, H_2O)	+82.6°
Ultraviolet absorption	end absorption
Infrared absorption	Fig. 1 (A: antibiotic 5879 B: bicyclomycin)
Molecular weight	302 (isothermal distillation)
Elementary analysis calcd. for $C_{12}H_{18}N_2O_7$	C 47.76, H 6.01, N 9.27,
found	C 47.86, H 6.05, N 9.17
Solubility	soluble: water, methanol. scarecely soluble: ethanol. insoluble: acetone, ether, hexane, *etc.*
Color reaction: positive	ferric hydroximate, FEHLING, vic-diol splitting test with periodic acid, ninhydrin after degradation with 1N aqueous potassium hydroxide

Fig. 1. Infrared absorption spectra of antibiotic 5879 and bicyclomycin (KBr tablet).

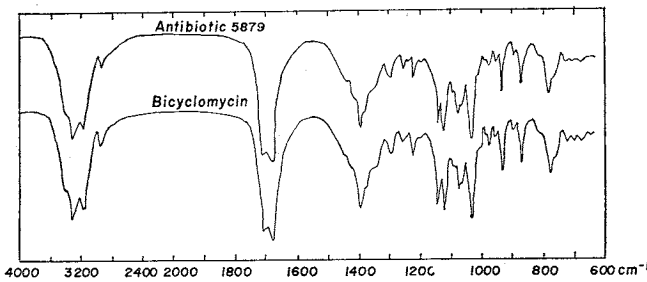

Table 5. Antibacterial activity of antibiotic 5879

Test organism	Minimal inhibitory concentration (mcg/ml)
Staphylococcus aureus FDA 209 P	500
Sarcina lutea PCI 1001	62.5
Streptococcus faecalis 5	>500
Bacillus anthracis 1	>500
Bacillus subtilis ATCC 6633	>500
Pseudomonas aeruginosa 35	>500
Klebsiella pneumoniae S	15.6
Salmonella typhosa 376	15.6
Salmonella derby 3299	31.2
Salmonella enteritidis NG 567	7.8
Escherichia coli B	31.2
Escherichia coli K-12	31.2
Escherichia coli H-3*	31.2
Shigella flexneri 3a 3196	15.6
Shigella flexneri R-4*	15.6
Shigella sonnei R-1*	15.6
Brucella melitensis K-3	0.9
Vibrio comma 384	3.9
Proteus vulgaris X-19	>500
Serratia marcescens 2	>500
Mycobacterium phlei 607	>500
Morganella 3	>500
Rettgerella 15	>500
Candida albicans YU-1200	>500
Aspergillus niger N-1	>500

* Resistant strain to streptomycin, chloramphenicol, tetracycline and kanamycin

tgerella, *Pseudomonas*, Gram-positive bacteria and fungi. The antibacterial spectrum shows the similarity of antibiotic 5879 with bicyclomycin.

The acute toxicity test was carried out with mice weighing 15~17 g for up to 2 weeks. Antibiotic 5879 showed low toxicity. The mice tolerated intravenous and intraperitoneal injection of 1 g/kg of the antibiotic.

Discussion

From the data of physical and chemical measurements, and of the antibacterial spectrum and toxicity, and also from the studies of the producing organism, we previously reported[1] that antibiotic 5879 appears to be a new water-soluble antibiotic, effective against Gram-negative bacteria. However, from a comparison of physicochemical and biological properties, antibiotic 5879 has been found to be identical to bicyclomycin[2], which was reported simultaneously with our preliminary report in this journal.

Streptomyces aizunensis, antibiotic 5879-producing strain, has been classified as a new species and is different from *Streptomyces sapporonensis*[2].

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