CERVINOMYCIN A_1 AND A_2, NEW ANTIBIOTICS ACTIVE AGAINST ANAEROBES, PRODUCED BY *STREPTOMYCES CERVINUS* SP. NOV.

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(Received for publication March 25, 1982)

Two new antibiotics, cervinomycin A_1 and A_2, were isolated from the culture filtrate of strain AM-5344, a soil isolate. The strain was found to belong to a new species of the genus *Streptomyces* for which the name *Streptomyces cervinus* is proposed. The antibiotics possess a strong inhibitory activity against anaerobic bacteria, such as *Clostridium perfringens*, *Peptococcus prevotii* and *Bacteroides fragilis*.

In the course of screening for antimycoplasmal antibiotics of actinomycetes origin, we found that strain AM-5344 isolated from a soil sample collected at Saiwai-cho, Chiba City, Japan, produces new antibiotics which have been designated cervinomycin A_1 and A_2. These antibiotics were active against anaerobic bacteria at low concentrations. The producing strain was classified as a new species of the genus *Streptomyces* and designated *Streptomyces cervinus* sp. nov.

The present paper deals with the taxonomy of strain AM-5344 and the production, isolation, and biological and physicochemical properties of cervinomycin A_1 and A_2.

Taxonomy of the Producing Organism

Morphology

The vegetative mycelium grows abundantly on both synthetic and complex agar media, and does not show fragmentation into coccoid or bacillary elements. Though moderate growth of aerial mycelium was observed on glycerol - asparagine agar and tyrosine agar, the aerial mycelium on other agar media was poor or absent.

The spore chains are of the *Rectus* or *Flexibilis* type (Plate 1). Mature spore chains on glycerol-asparagine agar have more than ten spores per chain. The spores are cylindrical in shape, 0.6×1.2 μm in size, and have a smooth surface (Plate 2). The electronmicrographs of strain AM-5344 were taken with a scanning electron microscope (Model S-430, Hitachi). Sclerotic granules, sporangia and flagellated spores were not observed.

Chemical Compositions

The chemical analyses of sugars in whole cells and amino acids in cell walls were carried out by the methods of BECKER *et al.*[1] and LECHEVALIER & LECHEVALIER[2], respectively. Strain AM-5344 shows no characteristic sugar pattern and LL-diaminopimelic acid (DAP) is present.

Plate 1. Scanning electronmicrograph of aerial hyphae of strain AM-5344 (×437).

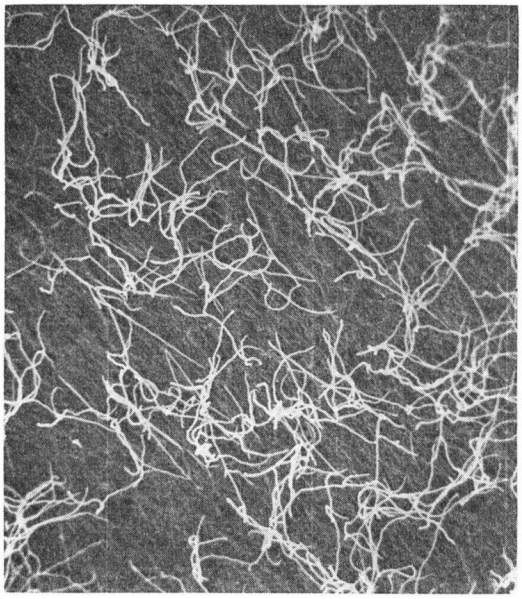
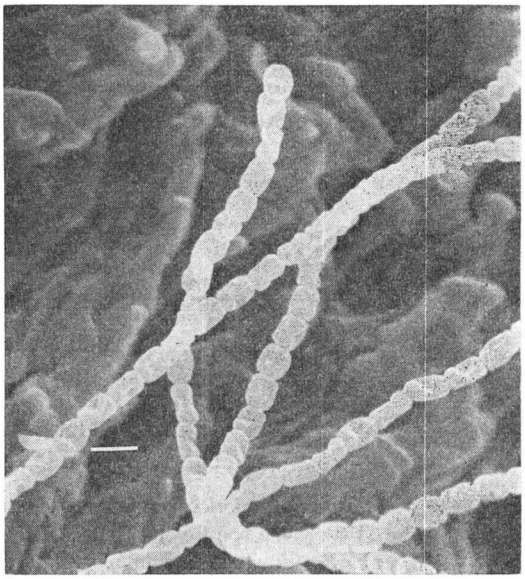


Plate 2. Scanning electronmicrograph of spore chains of strain AM-5344. Bar represents 1 μm.



Cultural and Physiological Characteristics

The International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[3] and those recommended by WAKSMAN[4] were used for these experiments. Cultures were observed after incubation at 27°C for two weeks. Color names and hue numbers indicated are those of the Color Harmony Manual (4th edition) published by the Container Cooperation of America. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% carbon source each. The cultural and physiological characteristics, and the utilization of carbon sources of strain AM-5344 are shown Tables 1, 2 and 3, respectively.

Strain AM-5344 exhibits the following properties. Sporophore, *Rectus* or *Flexibilis*; spore, cylindrical and smooth surface; color of aerial mycelium, yellow or gray; melanoid pigment, none; soluble pigment, yellow tint to bright gold; DAP in cell wall, LL-type. Based on the taxonomic properties described above, strain AM-5344 is considered to belong to the genus *Streptomyces* being a strain of the yellow or gray series of the PRIDHAM and TRESNER grouping[5].

According to the taxonomic criteria[4~10] of the genus *Streptomyces*, strain AM-5344 resembles *Streptomyces flavochromogenes*, *S. alboniger*, *S. willmorei* and *S. gedanensis*. Direct comparison of strain AM-5344 with the above four *Streptomyces* strains for cultural characteristics was carried out. It was found that the strain is different from these strains in the following properties.

S. flavochromogenes (ISP 5541): The color of the substrate mycelium is ivory or yellow on oatmeal agar and inorganic salts - starch agar. Raffinose, D-mannitol and *i*-inositol are not utilized.

S. alboniger (ISP 5043): The color of the substrate mycelium is chocolate brown on glycerol-asparagine agar. Aerial mycelium is abundantly formed on most media. L-Rhamnose and raffinose are not utilized.

S. willmorei (ISP 5459): The color of the aerial mycelium is pearl on some media. Aerial mycelium is abundantly formed on most media. Raffinose and *i*-inositol are not utilized.

Table 1. Cultural characteristics of strain AM-5344.

Medium	Cultural characteristics**
Yeast extract - malt extract agar (ISP)*	G : Good, raised & penetrant, honey gold (2ic) R : Honey gold (2ic) AM: Very poor, white (a) SP : None
Oatmeal agar (ISP)*	G : Good, penetrant, mustard brown (2pi) R : Mustard brown (2pi) AM: Very poor, white (a) SP : None
Inorganic salts - starch agar (ISP)*	G : Good, penetrant, inner; mustard gold (2ne), outer; mustard brown (2pl) R : Dull gold (2ng) AM: Poor, powder, light ivory (2ca) SP : None
Glucose - asparagine agar	G : Good, light antique gold (1½ic) R : Light antique gold (1½ic) AM: None SP : Light yellow (1½ea)
Glycerol - asparagine agar (ISP)*	G : Good, penetrant, antique gold (1½ne) R : Antique gold (1½ne) AM: Moderate, velvety, white (a) SP : None
Peptone - yeast extract iron agar (ISP)*	G : Good, raised, cream (1½ca) R : Light wheat (2ea) AM: None SP : None
Tyrosine agar (ISP)*	G : Good, raised, biscuit ecru (2ec) R : Citron (1gc) AM: Moderate, velvety, dark covert gray (2ih) SP : None
Sucrose - nitrate agar	G : Good, penetrant, inner; mustard tan (2lg), outer; bamboo (2gc) R : Inner; mustard brown (2ni), outer; bamboo (2gc) AM: Thin, velvety, covert tan (2ge) SP : Cream (1½ca)
Glucose - nitrate agar	G : Good, raised, bright gold (2pc) R : Bright gold (2pc) AM: None SP : Bright gold (2pc)
Glycerol - calcium malate agar	G : Good, penetrant, inner; bamboo (2gc), outer; pearl (3ba) R : Inner; bamboo (2gc), outer; light ivory (2ca) AM: Poor, powder, natural (2dc) SP : None
Glucose - peptone agar	G : Good, raised & penetrant, sunlight yellow (1½ia) R : Sunlight yellow (1½ia) AM: None SP : Butter yellow (1½ga)
Nutrient agar	G : Moderate, raised & penetrant, light wheat (2ea) R : Light wheat (2ea) AM: None SP : None

* Medium employed by International Streptomyces Project.

** Abbreviation; G: growth of vegetative mycelium, R: reverse, AM: aerial mycelium, SP: soluble pigment.

S. gedanensis (ISP 5518): The color of the substrate mycelium is light yellow on inorganic salts - starch agar. D-Xylose and raffinose are not utilized.

Consequently, the strain is reasonably concluded to be a new species of the genus *Streptomyces* and designated as *Streptomyces cervinus* Takahashi and Ōmura sp. nov. The name *cervinus* is derived from its Latin meaning of "yellowish brown color" in English and related to the vegetative mass color of strain AM-5344. The type strain has been deposited in the Fermentation Research Institute, Agents of Industrial Science and Technology, Japan, as FERM-BP 67.

Table 2. Physiological properties of strain AM-5344.

Melanin formation	—
Tyrosinase reaction	—
H_2S production	—
Nitrate reduction	—
Liquefaction of gelatin	−(20°C)
Peptonization of milk	+(36°C)
Cellulolytic activity	—
Coagulation of milk	−(36°C)
Hydrolysis of starch	+
Temperature range for growth	6~36°C

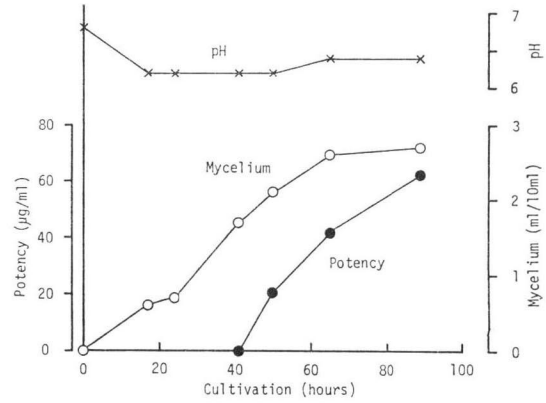
Table 3. Utilization of carbon sources by strain AM-5344.

Carbon source	Response
D-Glucose	+
D-Xylose	±
D-Mannitol	+
D-Fructose	+
L-Arabinose	+
Sucrose	—
i-Inositol	+
L-Rhamnose	+
Raffinose	+
Maltose	+

Production and Isolation

The stock culture of strain AM-5344 was inoculated into 100 ml of a seed medium consisting of 1.0% glucose, 2.0% starch, 0.5% yeast extract, 0.5% peptone, 0.4% $CaCO_3$ in a 500-ml Sakaguchi flask and incubated at 27°C for 48 hours. Three hundred ml of a thus obtained seed culture was transferred to 30 liters of the production medium in a 50-liter jar fermentor and the fermentation was carried out at 27°C for 89 hours with 10 liters of air per minute and agitation of 250 rpm. The composition of the production medium was 2.0% glycerol, 2.0% soybean meal, 0.3% NaCl (pH 7.0 before sterilization). The antibiotic production started at 40 hours after inoculation, then gradually increased and reached a maximum at 89 hours as shown in Fig. 1.

Fig. 1. Time course of cervinomycin production in a 50-liter jar fermentor.



A 89-hour culture (30 liters) was clarified with a Sharples centrifuge to obtain about 25 liters of supernatant. The antibiotic in the supernatant was extracted with 10 liters of ethyl acetate. The solvent layer was concentrated *in vacuo* to dryness. The resultant oily material was treated with 300 ml of *n*-hexane to give 3 g of brown powder. The crude powder was dissolved in a small amount of chloroform, and then chromatographed over silica gel (Merck, Kieselgel 60, 120 g) eluting with a mixed solvent of chloroform and methanol (50: 1, v/v). The active fractions were concentrated *in vacuo* to give 500 mg of reddish brown powder. The crude powder was purified by preparative thin-layer chromatography eluting with a mixed solvent of chloroform and methanol (40: 1, v/v) to isolate A_1 (25 mg, yellow powder) and A_2 (150 mg, reddish orange powder).

The antibiotic activity was assayed by paper disc method against *Acholeplasma laidlawii* PG-8 on agar plates. Cervinomycin A_1 and A_2 were also detected by thin-layer chromatography on silica gel (Merck, GF_{254}), develop ing with chloroform - methanol (40: 1, v/v): the Rf values of A_1 and A_2 show 0.39 and 0.32, respectively.

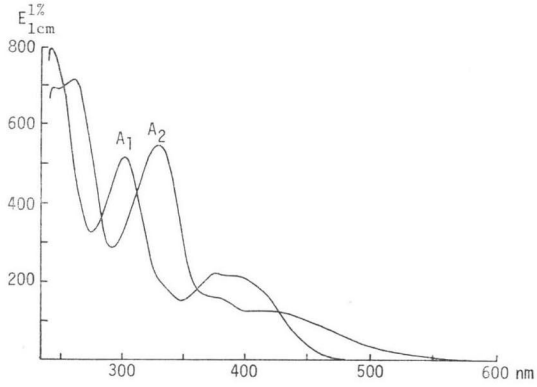
Table 4. Physico-chemical properties of cervinomycin A_1 and A_2.

	A_1	A_2
Appearance	Yellow powder	Reddish orange powder
Melting point	>240°C (decomp.)	>290°C (decomp.)
Optical rotation	$[\alpha]_D^{23}$ −92° (*c* 0.05, $CHCl_3$)	$[\alpha]_D^{20}$ −214° (*c* 0.25, $CHCl_3$)
Elemental analysis (%)	C 64.51, H 4.19, N 2.46	C 63.87, H 3.96, N 2.29
Molecular formula	$C_{29}H_{23}NO_9$ (EI-Mass: M^+, *m/z* 529.135)	$C_{29}H_{21}NO_9$ (EI-Mass: M^+, *m/z* 527.124)
UV, $\lambda_{max}^{CHCl_3}$ nm ($E_{1cm}^{1\%}$)	303 (516), 376 (219), 385 (214)	260 (719), 329 (546), 375 sh (159), 420 sh (125)
IR, ν_{max}^{KBr} cm^{-1}	3550, 3370, 2980, 1660, 1639, 1618, 1558, 1500, 1460, 1445, 1425	3450, 2980, 1685, 1619, 1499, 1460, 1425, 1275
Solubility: Slightly soluble	chloroform, benzene, ethyl acetate, acetone, methanol, ethanol	Same as A_1
Insoluble	water, *n*-hexane, ethyl ether	Same as A_1
Rf values (silica gel TLC) $CHCl_3$ - MeOH (40: 1)	0.39	0.32
C_6H_6 - Me_2CO (1: 1)	0.69	0.68
C_6H_6 - MeOH (4: 1)	0.63	0.58
EtOAc	0.27	0.20
n-BuOH - CH_3COOH - H_2O (4: 1: 1)	0.67	0.61

Physico-chemical Properties

Some physico-chemical properties of cervinomycin A_1 and A_2 are summarized in Table 4. The UV spectra of both antibiotics are shown in Fig. 2. The molecular formulas of cervinomycin A_1 and A_2 were proposed to be $C_{29}H_{23}NO_9$ and $C_{29}H_{21}NO_9$, respectively from the FD and EI-mass spectra and elemental analyses. In the IR spectra (Fig. 3) of components A_1 and A_2, the presence of a characteristic ketone carbonyl group was observed at 1685 cm^{-1} in A_2 but this signal was absent in A_1. Instead of it, a hydroxyl absorption was observed at 3370 cm^{-1}. These spectral evidences, in addition to both UV spectral data, clearly demonstrate that the structure of component A_2 is the oxidized form of component A_1.

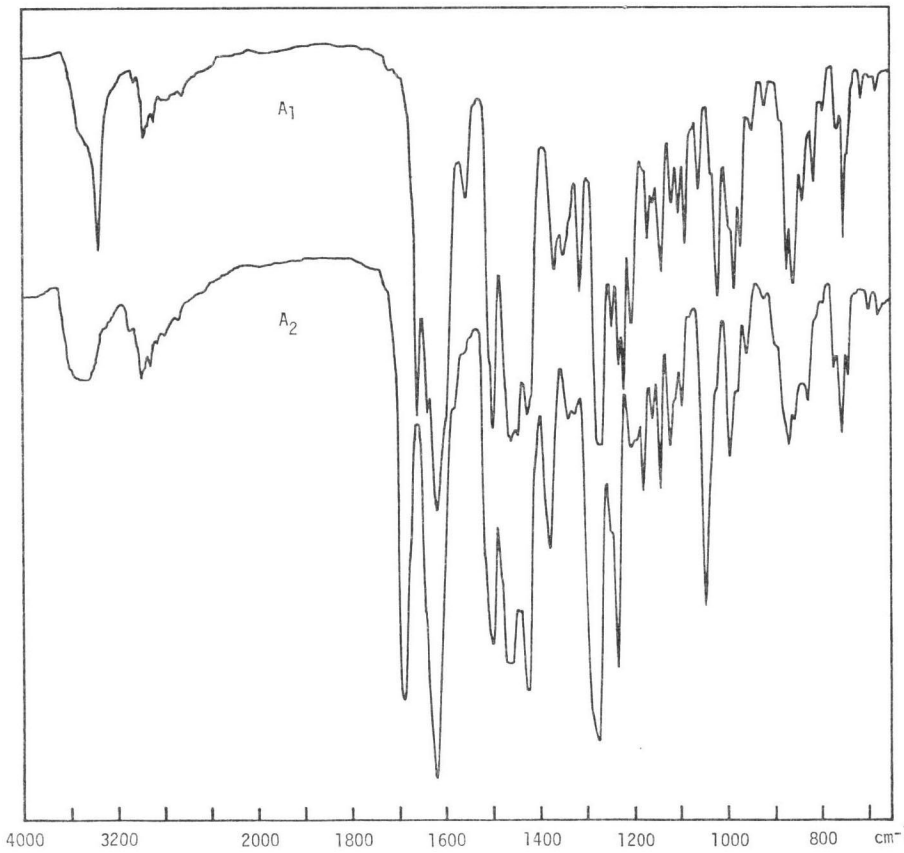
Fig. 2. UV spectra of cervinomycin A_1 and A_2 ($CHCl_3$).



Biological Properties

The antimicrobial spectra of cervinomycin A_1 and A_2 were determined by conventional agar dilution method using heart infusion agar for aerobic bacteria, GAM agar for anaerobic bacteria, Eiken PPLO agar for mycoplasmas and glucose - potato agar for fungi. The minimum inhibitory concentration (MIC) of cervinomycin A_1 and A_2 is given in Table 5. Both components are highly active against anaerobic bacteria and to a lesser extent against mycoplasma and some Gram-positive bacteria, but inactive against Gram-negative bacteria and fungi. Antitrichomonas activity was examined by liquid dilution method using *Trichomonas foetus*. The MICs of cervinomycin A_1 and A_2 were 0.4 and 0.05 μg/

Fig. 3. IR spectra of cervinomycin A_1 and A_2 (KBr).



ml, respectively. The acute toxicities (LD_{50}) of cervinomycin A_1 and A_2 in mice show the same value, 50 mg/kg, by intraperitoneal injection.

Discussion

Strain AM-5344, the cervinomycin-producing strain, was found to be a new species of the genus *Streptomyces* and named *Streptomyces cervinus* sp. nov. Since the parent strain produced cervinomycin A_1 as a minor component and the total productivity of components A_1 and A_2 was low (Fig. 1), improvement of the production of the antibiotics was carried out by the monospore-culture method. As result, strain AM-5344 M-81 (FERM-P 6006) was selected. The strain was found to produce more than 600 μg/ml of cervinomycin A_1 as main component. This high production made the isolation of the A_1 component easy.

The UV spectra of cervinomycin A_1 and A_2 show absorption maxima at 303, 376, 385, and 260, 329, 375 (sh.), 420 (sh.) nm, respectively. Both UV spectra were found to exhibit similar absorption maxima to those of known antibiotics such as albofungin (240, 255, 305 and 375 nm)[11], chloroalbofungin (233, 254, 305, 371 and 384 nm)[11], chartreusin (236, 266, 334, 380, 401 and 424 nm)[12], cerulomycin (265~270, 335, 380, 405 and 425 nm)[13], mekemycin (235~236, 264 and 398 nm)[14] and thermorubin A (300, 328, 414 and 420 nm)[15]. However, none of their physico-chemical properties were identical with those of cervinomycin A_1 and A_2. It is reasonable to conclude that cervinomycin A_1 and A_2 are new antibiotics.

Cervinomycin A_1 and A_2 were at first screened as antimycoplasmal antibiotics. Later it was found

Table 5. Antimicrobial activity of cervinomycin A_1 and A_2.

Test organism	MIC (μg/ml)		Test organism	MIC (μg/ml)	
	A_1	A_2		A_1	A_2
Staphylococcus aureus TPR 23	0.20	0.05	*Peptococcus prevotii* ATCC 9321	0.098	0.098
Staphylococcus aureus NIHJ JC-1	>100	>100	*Peptococcus variabilis* ATCC 14955	0.098	0.049
Streptococcus faecalis ATCC 8043	0.78	0.20	*Lactobacillus acidofilus* IFO 3205	0.049	0.049
Micrococcus flavus IFO 3242	0.39	0.20	*Bacteroides fragilis* 5550	0.049	0.049
Micrococcus luteus ATCC 9341	25	3.13	*Bacteroides fragilis* NIAH 2	0.098	0.049
Bacillus subtilis ATCC 6633	0.20	0.10	*Bacteroides fragilis* ATCC 23745	0.049	0.049
Bacillus cereus IFO 3466	>100	>100	*Fusobacterium necrophorum* NIAH 1	50	50
Escherichia coli NIHJ JC-2	>100	>100	*Fusobacterium varium* ATCC 8501	100	100
Salmonella typhimurium	>100	>100	*Veillonella alcalescens* ATCC 17745	100	100
Klebsiella pneumoniae IFO 3512	>100	>100	*Mycoplasma gallisepticum* KP-13	12.5	25
Enterobacter aerogenes IFO 5467	>100	>100	*Mycoplasma gallisepticum* S-6	25	25
Proteus vulgaris A 33	>100	>100	*Mycoplasma gallisepticum* 333P	12.5	25
Pseudomonas aeruginosa IAM 1054	>100	>100	*Mycoplasma pneumoniae*	12.5	25
Clostridium perfringens ATCC 13124	0.012	0.006	*Acholeplasma laidlawii* (A) PG8	12.5	25
Clostridium perfringens ATCC 19574	0.024	0.024	*Acholeplasma laidlawii* (B) Bml	6.25	25
Eubacterium lentum ATCC 25559	0.012	0.006	*Candida albicans* KF-1	>100	>100
Eubacterium limosum ATCC 8486	0.024	0.024	*Saccharomyces sake* KF-26	>100	>100
Bifidobacterium bifidum ATCC 11146	0.098	0.098	*Aspergillus niger* KF-102	>100	>100
Bifidobacterium bifidum ATCC 11147	0.195	0.049	*Mucor racemosus* IFO 4851	>100	>100

that the antibiotics are more active against anaerobic bacteria than against mycoplasmas (Table 5). In the screening program for new antimycoplasmal antibiotics, we previously found nanaomycins[16,17], frenolicin B[18] and 2′-amino-2′-deoxyadenosine[19]. These antibiotics, too, are more active against some microorganisms other than mycoplasmas: Nanaomycins and frenolicin B show activity against fungi and 2′-amino-2′-deoxyadenosine is active against a virus[20]. It is noteworthy that in the course of screening for antibiotics of antimycoplasmal activity, substances are found which inhibit various other microorganisms.

Acknowledgement

The authors wish to thank Messrs. K. OTOGURO and K. YAMASHITA for their assistance.

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