IRUMAMYCIN, AN ANTIFUNGAL 20-MEMBERED MACROLIDE PRODUCED BY A *STREPTOMYCES*

TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

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(Received for publication August 18, 1984)

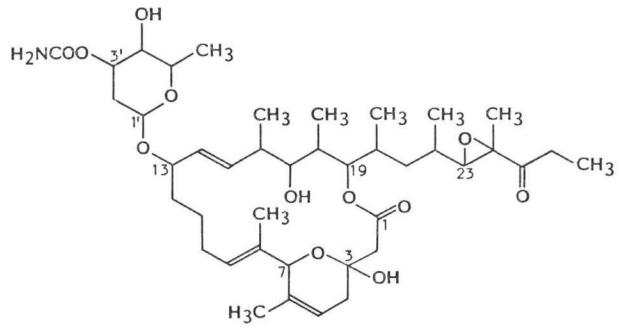
Irumamycin is a new 20-membered macrolide antibiotic isolated from a culture broth of a soil isolate which was named *Streptomyces subflavus* subsp. *irumaensis* AM-3603. It is active *in vitro* against some phytopathogenic fungi, but inactive against most aerobic and anaerobic bacteria and mycoplasmas.

The potent *in vitro* activity and the results of preliminary pot tests indicated that the antibiotic is practicable as an agricultural antifungal agent.

Several antifungal antibiotics have been successfully used to control plant diseases. These include blasticidin S, kasugamycin, polyoxin and validamycin. Recently, an excellent activity of mildiomycin was reported (for review, see ref 1). However, the occurrence of drug-resistant fungal strains and, in some cases, water and soil pollution are increasing in the agricultural fields treated with these natural and other synthetic chemicals. Therefore, new antifungal antibiotics which are active against resistant strains, are less toxic, and are degraded more promptly in soil are still needed. Agricultural antibiotics which currently have practical applications are in the nucleoside and aminoglycoside classes. From the above points of view, compounds of other classes such as macrolides are interesting.

In the course of our search for new antifungal antibiotics, a new macrolide antibiotic named irumamycin was obtained from the culture broth of a streptomycete, strain AM-3603.[2] The structure proposed for irumamycin is shown in Fig. 1.[3] It is a 20-membered macrolide antibiotic with a neutral sugar attached to the epoxidic aglycone. The gross structure resembles those of venturicidins,[4] although the latter compounds possess no epoxide group. The antibiotic is active against the phytopathogenic *Piricularia oryzae*, *Sclerotinia cinerea* and *Botrytis cinerea*.

Fig. 1. Structure of irumamycin.



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The present paper describes the taxonomy of the producing organism, fermentation and biological properties. The biosynthesis of the aglycone moiety,[3] and the production of irumanolides I and II which correspond to the aglycone moiety were described previously.[5]

Taxonomy of Producing Organism

Strain AM-3603 was isolated from a soil sample collected in Iruma-shi, Saitama Prefecture, Japan. Taxonomy of the strain was studied according to the method described by SHIRLING and GOTTLIEB[6] and WAKSMAN.[7]

Morphological Characteristics

Abundant vegetative mycelia and no fragmention of the hyphae were observed. Aerial mycelia are straight or flexious or open loops in shape (Fig. 2, left), and produce more than ten spores per chain (Fig. 2, right). The spores, which have a smooth surface, are cylindrical in shape and $0.4\sim0.5\times1.0\sim1.1$ μm in dimension. Sporangia, flagellated spores, sclerotia and verticils were not observed.

Cultural and Physiological Characteristics

The cultural characteristics of strain AM-3603 shown in Table 1 were observed after two weeks of incubation at 27°C on various media. Color names and hue numbers indicated are those of the Color Harmony Manual (4th edition) published by Container Cooperation of America. The strain shows good growth on all the media used. Yellow substrate mycelia on oatmeal agar are characteristic of strain AM-3603. Aerial mycelia colored in white or pale yellow were formed abundantly. The physiological properties of strain AM-3603 are shown in Table 2. Because no soluble pigment is produced, the strain is classified as non-chromogenic. The utilization of carbon sources as studied by the method of PRIDHAM & GOTTLIEB[8] is shown in Table 3. Using the procedures described by LECHEVALIER *et al.*,[9] the hydrolysate of whole cells was found to contain LL-diaminopimeric acid. Strain AM-3603 showed no characteristic sugar pattern. These results and the morphological characteristics described above indicate that strain AM-3603 belongs to the genus *Streptomyces*.

Fig. 2. Scanning electron micrographs of aerial mycelia of *S. subflavus* subsp. *irumaensis* subsp. nov. AM-3603.

Left, *Rectus-Flexibilis* or *Retinaculum-Apertum* morphology of cells grown on glycerol - asparagine agar for 14 days, and right, spore chains of cells grown on inorganic salts - starch agar for 14 days are shown.

Bars denote 1 μm.

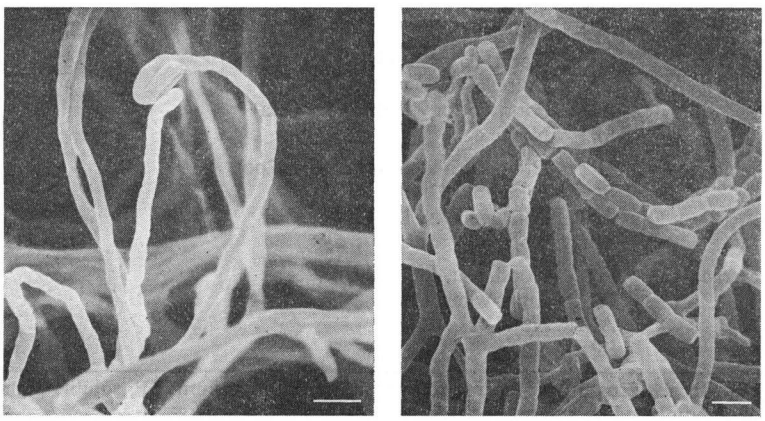


Table 1. Cultural characteristics of strain AM-3603 in comparison with those of *S. subflavus* ATCC 19846.

Medium	Cultural characteristics	
	Strain AM-3603	*S. subflavus* ATCC 19846
Yeast extract - malt extract agar (ISP)	G: Good, wrinkled, honey gold (2ic) R: Honey gold (2ic) AM: Moderate, velvety, white (a) SP: None	Moderate, raised, bamboo (2gc) Honey gold (2ic) Moderate, velvety, white (a) None
Oatmeal agar (ISP)	G: Good, raised, squash yellow (2ia) R: Cream (1ca) AM: Moderate, cottony, white (a) SP: None	Good, flat, light ivory (2ca) Light ivory (2ca) Abundant, velvety, white (a) ±, light ivory (2ca)
Inorganic salts - starch agar (ISP)	G: Good, wrinkled, honey gold (2ic) R: Colonial yellow (2ga) AM: Abundant, cottony, white (a) SP: None	Good, honey gold (2ic) Honey gold (2ic) Abundant, velvety, pearl pink (3ca) or white (a) ±
Glycerol - asparagine agar (ISP)	G: Good, raised, colonial yellow (2ga) R: Honey gold (2ic) AM: Abundant, velvety and cottony, light ivory (2ca) SP: None	Good, raised, honey gold (2ic) Colonial yellow (2ga) Abundant, velvety, white (a) ±, yellow
Glucose - asparagine agar	G: Good, raised, honey gold (2ic) R: Honey gold (2ic) AM: Abundant, cottony, white (a) SP: None	Good, raised, brite gold (2nc) Honey gold (2ic) Abundant, velvety, white (a) None
Peptone - yeast extract - iron agar (ISP)	G: Good, wrinkled, light wheat (2ea) R: Bamboo (2gc) AM: None SP: None	
Tyrosine agar (ISP)	G: Good, raised, wrinkled, honey gold (2ic) R: Honey gold (2ic) AM: Abundant, velvety, light ivory (2ca) SP: None	Good, raised, light amber (3ic) Honey gold (2ic) Abundant, velvety, flesh pink (4ca) None
Sucrose - nitrate agar	G: Good, flat, light wheat (2ea) R: Light wheat (2ea) AM: Moderate, velvety, white (a) SP: None	Good, penetrant, light ivory (2ca) Light ivory (2ca) Moderate, velvety, white (a) None
Glucose - nitrate agar	G: Moderate, flat, light ivory (2ca) R: Light ivory (2ca) AM: None SP: None	
Glycerol - calcium malate agar	G: Good, flat, colonial yellow (2ga) R: Colonial yellow (2ga) AM: Poor, cottony, light ivory (2ca) SP: None	
Glucose - peptone agar	G: Good, wrinkled, brite gold (2pc) R: Brite gold (2pc) AM: Poor, velvety, light ivory (2ia) SP: None	
Peptone - beef extract agar	G: Good, flat, light wheat (2ea) R: Light wheat (2ea) AM: None SP: None	

Abbreviations: G, growth; R, reverse color; AM, aerial mycelium; SP, soluble pigment production; and ISP, medium employed by International Streptomyces Project.

Table 2. Physiological properties of strain AM-3603.

Melanin formation	−*
Tyrosinase reaction	−
H_2S production	−
Nitrate reduction	+
Liquefaction of gelatin (22°C)	+
Hydrolysis of starch	+
Coagulation of milk (37°C)	−
Peptonization of milk (37°C)	+
Cellulolytic activity	−
Temp range for growth	22~42°C

* + Positive, − negative.

Table 3. Utilization of carbon sources by strain AM-3603 and *S. subflavus* ATCC 19846.

Carbon source	Strain AM-3603	*S. subflavus* ATCC 19846
None	−*	−
D-Glucose	+	+
L-Arabinose	−	+
Sucrose	−	−
D-Fructose	+	+
D-Xylose	+	±
L-Lhamnose	−	−
i-Inositol	+	±
Raffinose	+	−
D-Mannitol	+	±

* + Utilized, ± poorly utilized, − not utilized.

The properties of strain AM-3603 are summarized as follows: genus, *Streptomyces*; spore-chain morphology, section *Rectus-Flexibilis* or *Retinaculum-Apertum*; production of melanoid pigment, negative; mass color of aerial mycelia, white or yellow; spore surface, smooth.

Comparison of Strain AM-3603 with Related Organisms

As a result of the comparison with cultures described in BERGEY's Manual of Determinative Bacteriology (8th edition)[10] and International Streptomyces Project,[11~14] this strain was found to be similar to *Streptomyces chryseus* and *Streptomyces subflavus*. The growth characteristics and aerial mass color of strain AM-3603 (Table 1) resemble those of *S. chryseus*. However, the morphological characteristics of aerial mycelia clearly differentiate the two strains. Strain AM-3603 has spores in open loops, whereas *S. chryseus* has spores in spiral chains. The comparison of strain AM-3603 with *S. subflavus* (morphology, *Retinaculum-Apertum*) in the utilization of carbon sources and in the cultural characteristics indicates that they are slightly different in the following properties: utilization of L-arabinose and raffinose (Table 3), and particularly, the yellow vegetative mass color of strain AM-3603 on oatmeal agar in contrast to the brown substrate mycelia of *S. subflavus* on the same medium and other minor differences (Table 1).

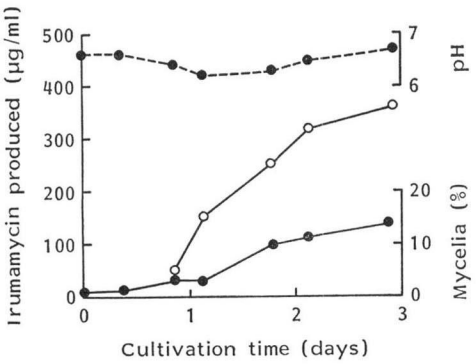
On the basis of these results, it is concluded that strain AM-3603 is a new subspecies of *S. subflavus*, and is named *Streptomyces subflavus* subsp. *irumaensis* subsp. nov. This strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, and assigned as *Streptomyces* sp. AM-3603 with an accession number of FERM-P 5619.

Fermentation

A loopful of spores and mycelia of strain AM-3603 grown on an agar slant were inoculated into a 500-ml Sakaguchi flask containing 100 ml of a seed medium (glycerol 1%, glucose 0.2%, soybean meal 1% and NaCl 0.3%, pH 7.0 before sterilization), and cultivated for 2 days at 27°C. The seed culture (700 ml) thus obtained was transferred into a 30-liter jar fermentor containing 20 liters of a production medium: glycerol 2%, glucose 0.4%, soybean meal 1% and NaCl 0.3%, pH 7.0 prior to autoclaving. The fermentor was run at 27°C for 3 days with agitation of 250 rpm and with aeration of 10 liters/minute. The antibiotic activity in the culture broth was monitored by a paper disc method using *Aspergillus niger* KF-102 (a stock culture in our laboratories) as a test organism. The test organism was seeded in potato - glucose agar (pH 6.0), and incubated for two days at 27°C. Fig. 3

Fig. 3. Irumamycin production by strain AM-3603 in a 30-liter jar fermentor.

Antibiotic potency (○—○), growth (●—●) and pH (●---●) are shown.



shows a typical time course of irumamycin production in a 30-liter jar fermentor. The antibiotic production started one day after inoculation and reached a maximum (*ca.* 350 μg/ml) at day 3. The antibiotic activity did not decrease untill day 4. A larger scale fermentation was carried out using a 2,000-liter fermentor containing 1,000 liters of the production medium described above.

Table 4. Antimicrobial spectrum of irumamycin.

Microorganism	MIC* (μg/ml)
Staphylococcus aureus FDA 209P	100
Micrococcus luteus PCI 1001	>100
Mycobacterium smegmatis ATCC 607	100
Corynebacterium paurometabolum KB-21	0.2
Streptococcus pyogenes C 203	25
Escherichia coli NIHJ	>100
Pseudomonas aeruginosa P-3	>100
Xanthomonas oryzae KB-88	>100
Candida albicans KF-1	>100
Saccharomyces sake KF-26	>100
Aspergillus niger KF-102	25
A. brevipus KF-201	1.6
Penicillium chrysogenum KF-97	0.2
Piricularia oryzae KF-180	<0.1
Botrytis cinerea KF-184	6.3
Sclerotinia cinerea KF-181	<0.1
Alternaria kikuchiana KF-185	3.1
Mucor racemosus KF-223	>100
Fusarium oxysporum KF-166	>100
Trichophyton interdigitale KF-62	3.1
Microsporum gypseum KF-65	50
Pellicularia sasakii KF-219	25

* Bacteria were grown on heart infusion agar overnight at 37°C, and fungi were grown on potato-glucose agar for 2 days at 27°C.

Biological Properties

The antimicrobial spectrum of irumamycin was determined by a conventional agar dilution method. As shown in Table 4, irumamycin inhibits the growth of filamentous fungi including plant pathogens such as *P. oryzae*, *S. cinerea* and *B. cinerea* in a range of 0.1 to 12.5 μg/ml. It is inactive against most aerobic bacteria, except for members of restricted genera. Also no or very little growth inhibition was observed at 1,000 μg/ml (paper disc method) with the following microorganisms: *Bacteroides fragilis*, *Fusobacterium varium*, *Clostridium perfringens*, *Mycoplasma gallicepticum* and *Acholeplasma laidlawii*.

The drug-sensitivity of a fungus alters occasionally during storage under laboratory conditions. To exclude this possibility, pathogenic fungal strains freshly isolated from infected plants were used as test organisms. Table 5 shows that irumamycin is nearly equally active against laboratory strains and plant-derived strains. It is more active than known antibiotics of agricultural use. Kasugamycin did not exhibit *in vitro* activity under the conditions employed. Irumamycin is of low acute toxicity: the LD_{50} value was 300 mg/kg on mice when administered intraperitoneally.

Table 6 illustrates the protective effect of irumamycin in a pot test. The antibiotic showed excellent activity at 200 ppm for protection of cucumber and rice plant from infection by *B. cinerea*, *Colletotrichum lagenarium* and *Cochliobolus miyabeanus*. No phytotoxicity was observed under these conditions. These results promise the feasibility of this antibiotic as a new type of agricultural antifungal material. Larger scale experiments in greenhouses are now in progress. A good activity of irumamycin for the

Table 5. Comparison of irumamycin with other antibiotics in anti-phytopathogenic activity.

Organism		MIC (μg/ml)*1					
		Iruma-mycin	Venturi-cidin A	Botry-cidin	Aabo-mycin A	Blasti-cidin S	Prumycin
Piricularia oryzae	A*2	0.05	<0.03	0.05	<0.2	>100	>100
	B	<0.03	<0.03	0.03	<0.2	25	100
Sclerotinia cinerea	A	0.05	0.78	0.4	0.2	50	1.56
	B	0.1	0.78	0.78	0.2	25	6.25
Pellicularia sasakii	A	0.4	1.56	12.5	>100	>100	>100
	B	25	>25	>25	>100	>100	>100
Botrytis cinerea	A	—*3	—	—	>100	50	100
	B	6.25	>25	12.5	>100	100	50
Alternaria kikuchiana	A	50	—	—	>100	50	>100
	B	12.5	>25	12.5	100	12.5	50

*1 Glucose - potato agar, 2~3 days.
*2 A, isolate from infected plant; B, laboratory stock culture.
*3 — Not tested.

Table 6. Protection of plants from fungal infection by irumamycin in pot tests.

Pathogenic organism	Plant infected	Irumamycin (ppm)	Protection value (%)*
Botrytis cinerea	Cucumber	200	98
		100	97
		50	97
Colletotrichum lagenarium	Cucumber	200	100
		100	99
		50	92
Cochliobolus miyabeanus	Rice plant	200	92

* Calculated from an equation:

$$\left(1-\frac{\text{No. of infectious lesions on treated plants}}{\text{No. of infectious lesions on control plants}}\right)\times 100$$

control of polyoxin- or thiophanate (a synthetic pesticide)-resistant pathogenic fungi has been observed. Detailed results of these experiments will be reported elsewhere.

Acknowledgments

The authors are grateful to Dr. T. MISATO and Dr. K. KŌ, Inst. Phys. Chem. Res., for conducting pot tests, and for their kind supply of aabomycin A. They are indebted to Prof. H. ZÄHNER, Inst. für Biologie II, Universitat Tübingen, for his generous gift of venturicidin A and botricidin. Thanks are also due to Dr. Y. ARAI, Fuji Plant, Kyowa Hakko Kogyo Co., Shizuoka, for large-scale fermentations and purification for the preparation of irumamycin samples.

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