PRODUCTION, ISOLATION AND PROPERTIES OF PYRACRIMYCIN A, A NEW ANTIBIOTIC FROM *STREPTOMYCES ERIDANI* N. SP.

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Two new compounds, pyracrimycins A and B, have been isolated from a *Streptomyces* species named *Streptomyces eridani* CORONELLI *et al.* n. sp. The morphological and physiological characteristics of this strain are described in the present paper together with the production and properties of the two metabolites. Pyracrimycin B is biologically inactive whereas pyracrimycin A is active *in vitro* against both Gram-positive and Gram-negative bacteria.

During the course of our screening program for new antimicrobial agents two chemically-related substances, one of which active *in vitro* against Gram-positive and Gram-negative bacteria, were isolated from a *Streptomyces* strain. The producing organism, isolated from a soil sample collected in Pavia (Italy) was named *Streptomyces eridani* CORONELLI *et al.* n. sp. (ATCC 21619) and the products isolated were named pyracrimycins A and B.

The present paper deals with the description of the producing strain, the isolation of the two metabolites and the biological properties of pyracrimycin A.

Description of the Producing Strain

For the investigation of the growth characteristics *S. eridani* was grown on a variety of standard media according to GOTTLIEB and SHIRLING[1]; in addition some media recommended by WAKSMAN[2] were used. The optimum temperature range for development of the colonies was from 28° to 37°C, no growth was observed at 50°C and very little at 20°C. Cultural and physiological characteristics of the *Streptomyces* are shown in Table 1.

Aerial mycelium is produced by *S. eridani* only on oat-meal agar and potato agar. On the latter the aerial mycelium produced is scanty and no sporulation was observed; in oat-meal agar the aerial mycelium is white, velvety with long, flexous and branched hyphae having a diameter of about 1.3 μ. Aerial mycelium produces sporophores in fairly closed spirals different in their lengths. The spores are oval with smooth surface and have diameters of about 1~1.3 $\mu \times$ 1.3~1.5 μ. Based on the form of the sporophores and on the color of the aerial mycelium, *S. eridani* was assigned to the Section Spira, White Series according to the classification of PRIDHAM, HESSELTINE and BENEDICT[5]. The test for utilization of carbon sources performed according to PRIDHAM and GOTTLIEB[6] are shown in Table 2. All the compounds tested are utilized carbon sources tested except cellulose.

Table 1. Cultural and physiological characteristics of *Streptomyces eridani* (ATCC 21619)

Culture medium	Vegetative mycelium	Aerial mycelium	Soluble pigment	Physiological characteristics
Oat-meal agar	Good growth, smooth surface cream	Whitish in traces	Cream to light amber 12/G/7*	
Medium 2 (GOTTLIEB and SHIRLING)	Good growth, slightly wrinkled amber 13/E/7*	Absent	Amber brown 13/F/9*	
Oat-meal agar (Medium 3 GOTTLIEB and SHIRLING)	Moderate growth, smooth surface hyaline	White, velvety, not much abundant	Absent	
Glycerol asparagine agar (Medium 5)	Good growth, smooth surface cream	Absent	Absent	
HICKEY and TRESNER's agar[3]	Good growth, slightly wrinkled brown 16/A/10*	Absent	Deep amber brown 15/L/12*	
BENNETT's agar	Good growth, wrinkled surface amber brown 14/4/7*	Absent	Amber brown 14/1/9*	
CZAPEK glucose agar	Moderate growth, smooth surface straw 9/B/1*	Absent	Absent	
Glucose asparagine agar	Good growth, smooth surface with wax aspect, cream 9/D/2*	Absent	Traces, cream	
Nutrient agar	Growth scarce, thin smooth surface amber 13/H/8*	Absent	Amber 13/H/8*	
Potato agar	Good growth, slightly wrinkled surface, light brown 15/E/7*	Whitish in traces	Light brown 15/E/7*	
Starch agar (Medium 4)	Moderate growth, smooth surface, straw 11/C/2*	Absent	Straw 11/C/2*	Good hydrolysis
Peptone-yeast extract iron agar (Medium 6)	Moderate growth, smooth surface brown 16/A/8*	Absent		Production of H_2S
Tyrosine agar (Medium 7)	Good growth, wrinkled surface black brown	Absent	Black-brown at the edges of the growth, brown in the medium 15/A/12*	Tyrosinase reaction: positive-production of melanoid pigment (strong)
Calcium malate agar	Good growth, smooth surface straw 10/B/1*	Absent	Absent	Strong digestion of Ca-malate
Gelatin			Absent	Liquefaction
Nitrate broth			Dark brown	Nitrate reduction positive
Litmus milk	Brown ring		Absent	No peptonization no coagulation
Skim milk agar	Good growth, smooth surface 8/A/12*	Absent	Brown not much soluble on the medium 8/A/12*	Hydrolysis of casein: negative

* Color determinations were performed according to MAERZ and PAUL[4].

A comparison of *S. eridani* with some related strains is reported in Table 3. Among the known species of *Streptomyces* belonging to Section Spira, White Series, only *Streptomyces albidus* CBS 100.34, and *Streptomyces armillatus* NANCY-COURTILLET and PINNET-SINDICO, 1954, show some similarity to *Streptomyces eridani*. However they differ from our strain mainly for the color of vegetative mycelium on several

Table 2. Utilization of carbon compounds by *S. eridani* (ATCC 21619)

Carbon source	Growth	Carbon source	Growth
Sucrose	++	Mannitol	++
Xylose	++	Fructose	++
Arabinose	++	Rhamnose	++
Inositol	++	Cellulose	−
Raffinose	++	Glucose (positive control)	++

++ : Strongly positive utilization − : No utilization

Table 3. Comparison of *S. eridani* with related strains

	S. eridani n. sp.	*S. albidus* CBS 100.34	*S. armillatus*	*S. diastaticus* ATCC 3315	*S. odorifer* ATCC 6246	*S. felleus* CBS 49168
Vegetative mycelium	Amber to brown	Colorless to cream	Colorless, yellow, gray	Yellowish	Cream to brown	Yellowish, brown
Aerial mycelium	Scarce, white, spirals	Whitish, spirals	Scarce, white, spirals	White to gray, spirals	White and cream, spirals	White-gray, straight sporophores
Soluble pigment	Amber to brown	Yellowish to brownish	Absent	Colorless to brown	Light brown	Yellowish brown
Nitrate broth	Reduction	Slow reduction	No reduction	Weak reduction	Reduction	ND
Litmus milk	No coagulation No peptonization	Weak coagulation, peptonization	Coagulation, peptonization	Coagulation, weak peptonization	No coagulation, weak peptonization	No coagulation, peptonization
Gelatin	Liquefaction	Liquefaction	Liquefaction	Liquefaction	Weak liquefaction	No liquefaction
Nutrient agar	V.*: amber A.*: absent P.*: amber brown	ND**	ND	V.: cream A.: white to gray P.: brown	V.: brown A.: white P.: light brown	V.: yellowish brown A.: absent P.: light brownish yellow
Glucose asparagine agar	V.: cream A.: absent P.: cream	ND	V.: yellow-gray A.: white P.: absent	V.: yellowish A.: absent P.: absent	V.: cream to brown A.: cream P.: light brown	V.: yellowish brown A.: white-gray P.: brownish
Glycerol asparagine agar	V.: light brown A.: absent P.: absent	ND	V.: yellow-gray A.: white P.: absent	ND	ND	V.: yellowish A.: white-gray P.: yellowish brown
Czapek glucose agar	V.: straw A.: absent P.: absent	V.: colorless A.: white P.: yellowish	V.: colorless A.: absent P.: absent	V.: gray A.: white to gray P.: ND	V.: cream to light brown A.: cream P.: absent	ND
Starch agar	V.: straw A.: absent P.: straw hydrolysis	ND hydrolysis	ND No hydrolysis	V.: colorless A.: gray P.: ND hydrolysis	V.: cream to brown A.: straw P.: absent hydrolysis	V.: colorless A.: white P.: absent hydrolysis

* V. = vegetative mycelium ; A. = aerial mycelium ; P. = soluble pigment.
** No puplished data.

media and for their ability to peptonize milk and to sporulate on media on which *S. eridani* does not. *Streptomyces diastaticus* ATCC 3315, *Streptomyces odorifer* ATCC 6246 and *Streptomyces felleus* CBS 49168, although not belonging to Section Spira, White Series, are similar to our strain in the production of brown pigment and in some other characteristics, but differ in the color of aerial mycelium on several media.

Production of the Antibiotic

The culture of *S. eridani* was maintained on oat-meal agar. Fermentation conditions suitable for the production were studied and the following media were found to be useful:

1) Vegetative medium for shake flask culture (g/liter): beef extract, 5; yeast extract, 5; peptone, 5; enzymatic casein hydrolysate, 3; dextrose, 20; sodium chloride, 1.5.

2) Vegetative medium for fermentors (g/liter): beef extract, 4; peptone, 4; NaCl, 2.5; yeast extract, 1; soybean meal, 10; dextrose, 20; $CaCO_3$, 5.

3) Fermentative medium: the same as medium 2 with 50 g/liter dextrose.

For the production of the antibiotic jar fermentors containing 10 liters of medium 3 were inoculated with one liter of vegetative culture and incubated aerobically (1 liter air/liter/min.) under stirring (800 r.p.m.) at 28°C.

Maximum antibiotic activity was obtained after 48 hours of fermentation. A paper disc agar plate assay with *Escherichia coli* as the test organism was used to determine the antibiotic levels.

Isolation of Pyracrimycins A and B

The isolation and purification of the two metabolites was followed spectroscopically as both pyracrimycins A and B show intense ultraviolet absorptions.

The fermented broth was filtered with the aid of 2% Hyflo-Super Cel and the mycelial cake discarded. The filtrate, after the addition of 20% of NaCl was extracted twice with one-half volume of butanol, the combined extracts were washed with a small volume of slightly alkaline water and concentrated to one fifth of the original volume. A precipitation of inorganic salts, containing less than 1% of pyracrimycins, was obtained by cooling the concentrated solution for a few hours at 4°C; the salts were filtered off and the solution concentrated again to a small volume. A crude mixture of pyracrimycins A and B was obtained by adding a large excess of petroleum ether to the concentrated extracts. The crude material has about 30~35% purity, pyracrimycins A and B being present in roughly one to one proportion. The product was suspended in hot methanol (2 g/100 ml) and filtered from insoluble particles; the solution was treated with charcoal to remove impurities and concentrated until crystallization occurred; both metabolites co-crystallize on cooling giving a product with about 95% total purity. A separation of the two was achieved by treatment of the product on water bath for one hour with chloroform (1 g/liter) in which only pyracrimycin B is soluble. Pyracrimycin A, containing only 1~2% of B, was filtered and pyracrimycin B crystallized after concentration of the chloroform solution to a small volume. A final crystallization from methanol for pyracrimycin A and from chloroform for B gave pure products.

Chemico-physical Characteristics

Pyracrimycin A is a white crystalline substance, m.p. 215~216°C; the product appeared to be unitary by paper and thin-layer chromatographic analyses; the Rf

Table 4. Chromatographic behavior of pyracrimycin A

Solvent system	Rf*
Water-saturated *n*-butanol	0.60
Water-saturated *n*-butanol + 2 % *p*-toluensulfonic acid	0.29
Water-saturated *n*-butanol + 2 % concentrated ammonia	0.53
n-Butanol-saturated water	0.14
Ammonium chloride (20 % solution in water)	0.71
n-Butanol - methanol - water (40 : 10 : 20) containing 0.75 g methyl orange	0.65
n-Butanol - methanol - water (40 : 10 : 30)	0.71
Water-acetone (1 : 1)	0.52
Water-saturated ethyl acetate	0.0
Chloroform-methanol (9 : 1) (TLC)**	0.22

* Paper chromatography on Whatman No. 1, antibiotic visualized on agar plates seeded with *S. aureus*.

** TLC performed on Silica-gel HF/UV_{254} plates to a distance of 10.0 cm; spot detected under UV light.

Table 5. Antimicrobial activity of pyracrimycin A

Microorganism tested	Minimal inhibitory concentration (mcg/ml)
Staphylococcus aureus FDA 209P	20
Staphylococcus aureus Tour	20
Streptococcus hemolyticus C 203	20
Diplococcus pneumoniae UC 41	10
Candida albicans SKF 2270	>100
Trychophyton mentagrophytes SKF 17410	>100
Mycobacterium tuberculosis H37Rv ATCC 9360	10
Proteus vulgaris X19 ATCC 881	10
Pseudomonas aeruginosa ATCC 10145	50
Escherichia coli SKF 12140	10
Escherichia coli M. Leod ATCC 10536	20
Escherichia coli M. Leod ATCC 10536-Caf/R	20
Escherichia coli M. Leod ATCC 10536-Tetra/R	20
Escherichia coli M. Leod ATCC 10536-Strepto/R	10
Escherichia coli M. Leod ATCC 10536-Neo/R	10
Escherichia coli M. Leod ATCC 10536-Kana/R	10

values obtained with different solvent systems are reported in Table 4. The following microanalytical data C 60.99, H 7.50, N 20.20 % are consistent for a molecular formula $C_7H_{10}N_2O$ (theoretical values: C 60.85, H 7.30, N 20.27 with a molecular weight 138.17). The product has slightly basic character. It is almost insoluble in the common organic solvents, with the exception of dimethylsulfoxide and dimethylformamide and slightly soluble in acidic water and methanol. The ultraviolet spectrum in methanol solution shows a maximum at 235 mμ (ε 24,000).

Pyracrimycin B is a light yellow crystalline substance, m.p. 222~224°C. Thin-layer chromatographic analysis gave Rf 0.30 in the same conditions as for pyracrimycin A. The following microanalytical data C 54.38, H 6.59, N 18.29, are consistent for a molecular formula $C_7H_{10}N_2O_2$ (theoretical values C 54.54, H 6.54, N 18.17 with a molecular weight 154.7). The product is soluble in dimethylsulfoxide, dimethylformamide and methanol, slightly soluble in chloroform and alkaline water. The ultraviolet spectrum in methanol solution shows a maximum at 332 mμ (ε 16,000).

The structure of *trans* 3-(1-pyrrolin-2-yl)acrylamide for pyracrimycin A was determined as described in the following paper.[7]

Biological Properties of Pyracrimycin A

The antibiotic is active *in vitro* against a variety of Gram-positive and Gram-negative bacteria and it presents no cross-resistance with a series of *E. coli* strains resistant to different antibiotics. The antibacterial spectrum of pyracrimycin A determined according to the serial dilution method in Difco Pennassay broth is reported in Table 5. The acute toxicity in mice is about 150 mg/kg by intraperitoneal route.

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