BIOXALOMYCINS, NEW ANTIBIOTICS PRODUCED BY THE MARINE *Streptomyces* sp. LL-31F508: TAXONOMY AND FERMENTATION

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(Received for publication July 14, 1994)

An actinomycete strain designated LL-31F508 was isolated from an intertidal sediment sample collected in Key West, Florida. Culture LL-31F508 was assigned to the *Streptomyces* genus based on the presence of LL-diaminopimelic acid (DAP) in the cell wall and observations of spiny spores using scanning electron microscopy (SEM). Excellent antimicrobial activity against *Staphyloccoccus* and *Enterococcus* spp. were detected in both the supernatant and cell extract samples from fermentations of culture LL-31F508. Production of antibiotic activity peaked at 48~50 hours and closely paralleled cell growth, during which time glucose was more rapidly assimilated than dextrin. A series of new antibiotics called the bioxalomycins was identified as the antibacterial products from fermentations of this culture. Fermentation conditions for production of bioxalomycin α differed substantially from those required for production of a related compound, naphthyridinomycin, by the reference culture *Streptomyces lusitanus* NRRL 8034.

Since the discovery of penicillin, much of the effort in natural products screening programs has focused on microbial metabolites from terrestrial sources[1]. To increase the likelihood of discovering novel compounds, we expanded our program to include isolation of microorganisms from the marine environment[2]. During the course of our screening program for antibacterial agents, a fermentation sample of culture LL-31F508 exhibited activity against methicillin-sensitive and -resistant *Staphylococcus aureus* (MSSA, and MRSA, respectively), and vancomycin-resistant *Enterococcus faecium* (VREF). Based on these data and the fact that the organism was isolated from a marine environment, we further investigated the taxonomy of the culture and the conditions for antibiotic production.

Materials and Methods

Microorganisms

Strain LL-31F508 was compared to *Streptomyces olivoviridis* ATCC 23944, *S. viridiviolaceus* ATCC 27478, *S. macrosporeus* ATCC 19783, *S. cuspidosporus* ATCC 33340, *S. griseorubens* ATCC 19909, *S. werraensis* ATCC 14424, *S. albaduncus* LL-BL0620, *S. gancidicus* NRRL B-1872, *S. lusitanus* NRRL 8034, and *S. viridodiastaticus* ATCC 5518. Clinical isolates were collected from various medical centers in the United States and quality control strains were obtained from ATCC (American Type Culture Collection, Rockville, MD). Identification of each culture was done by conventional methods: Gram-negative rods by API 20E (Analytab Products, Plainville, N. Y.) and NF systems (Remel, Lenexa, Kans.), and staphylococci by Staph Trac (Analytab Products). All isolates were stored frozen with 20% DMSO at $-70°C$.

Media

Nutrient agar (pH 6.8) and Luria agar (Difco Laboratories, Detroit, MI) were prepared in distilled

deionized water. Medium A-1 contained glucose 1%, NZ-Amine A 0.5%, $CaCO_3$ 0.1%, dextrin 2%, and yeast extract 0.5% in tap water.

Isolation

One gram of an intertidal sediment sample from Key West, Florida, was placed in a sterile 25 × 150 mm glass tube and mixed with 5 ml of a solution containing yeast extract 6%, sodium dodecyl sulphate 0.05%, $CaCl_2$ 0.01 M, and Bacto agar 0.1% in deionized water. After incubating the suspension for 20 minutes at 40°C in a water bath, 20 ml of 0.1% agar solution were added. The suspension was inoculated onto agar containing NZ-amine A 0.3%, Bromocresol purple sodium salt 0.7%, agar 1.8%, cycloheximide (50 μg/ml), nystatin (25 μg/ml), and novobiocin (25 μg/ml). After 10 days of incubation at 28°C, strain LL-31F508 was isolated and subsequently deposited with the Northern Regional Research Center's Culture Collection Laboratory, Peoria, Illinois, under the accession number NRRL 21082.

Physiological Characteristics

Physiological studies were carried out as described by the International Streptomyces Project (ISP) and GORDON *et al.*[3,4]. For the evaluation of cultural characteristics, the strains were incubated from 14 to 28 days at 28°C.

Morphological Observations

Cultural characteristics were recorded after incubation at 28°C for 7, 14, and 28 days according to ISP standards. The color and hue numbers indicated are those of the National Bureau of Standard Centroid Color Charts, Publication 440, Washington, D.C. Scanning electron micrographs were prepared using a modified method of Locci[5] and photographed using a JEOL 6300V scanning electron microscope.

Chemotaxonomic Analysis

Cultures were grown in 50 ml of medium containing yeast extract 1% and glucose 1% in a 250 ml Erlenmeyer flask at 28°C, 200 RPM. After 72 hours, the mycelium was harvested by centrifugation, thoroughly washed with sterile distilled water, and lyophilized to dryness. Whole-cell sugars were determined using standard gas chromatography and mass spectroscopy (GC/MS) methods[6]. To determine the isomers of diaminopimelic acid (DAP) present in the cell wall, *N*-heptafluorobutyryl *n*-butyl ester derivatives were prepared before analysis by GC/MS using a Chirasil-Val column[7]. Monosaccharide standards and 2,6-diaminopimelic acid (racemic) were obtained from Sigma Chemicals St. Louis, MO, USA. Heptafluorobutyric anhydride (HFBA) was obtained from Pierce Chemical Co., Rockford, IL, USA. Phospholipids were extracted and analyzed using two-dimensional thin-layer chromatography[8]. The preparation and analysis of whole cell fatty acid methyl esters was carried out using the Microbial Identification System[9].

In vitro Assays for Bioxalomycins

Antibacterial activity was determined by measuring the diameter of the inhibition zone in agar diffusion assays.

HPLC Assay for Bioxalomycins

Fermentation samples were centrifuged and 10 ml of supernatant were applied to a Sep-Pack C-18 cartridge. The column was washed with 10 ml of water and excess water was removed by injecting air from an empty syringe. Addition of 2 ml MeOH produced a turbid eluate which was clarified by centrifugation. The MeOH supernatant was taken to dryness on a rotovap and reconstituted in 1 ml of 0.1% trifluoroacetic acid (TFA). After injecting 5 μl of the latter solution onto a Vydac C-18 column (0.4 × 25 cm; Cat. #218TP54), the column was equilbrated with 0.1% TFA in water containing 5% acetonitrile. The column was eluted with a gradient of 5 to 50% acetonitrile at a flow rate of 1.5 ml/minute. Dual wavelengths of 264 nm and 294 nm were utilized to detect the beta and alpha components, respectively. The alpha component eluted at 2.9 minutes and the beta component eluted at 6.5 minutes.

Results and Discussion

Taxonomy of the Producing Culture

Microscopic examination of strain LL-31F508 revealed non-fragmented, extensively branched substrate mycelium. Abundant aerial mycelium which transformed into *Rectiflexibiles* chains of 15~20 spiny, oval, non motile arthrospores was also formed (Fig. 1). Analyses of whole-cell hydrolysates revealed the presence of only the LL isomer of DAP and the sugars ribose, mannose, inositol, and galactose. A

Fig. 1. Scanning electron micrograph of a spore chain of strain LL-31F508 .grown on yeast extract-malt-extract agar.

Bar represent 1 μm.

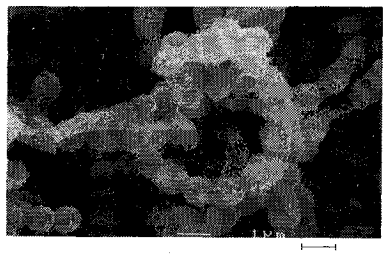


Table 1. Characterization of *Streptomyces* spp.[1]

Characteristic	LL-31F508	*S. viridodiastaticus*	*S. werraensis*
Spore chain morphology	*Rectiflexibilis*	*Rectiflexibilis*	*Rectiflexibilis*
Fragmentation of substrate mycelium	None	None	None
Zoospores and sporangia	None	None	None
Number of spore per chain	15~20	15~20	15~20
Spore shape	Oval	Oval	Oval
Spore surface	Spiny	Spiny	Spiny
Growth temperature	22~37°C	22~50°C	22~50°C
Salt tolerance	9%	6%	4%
DAP isomer	LL-DAP	LL-DAP	LL-DAP
Whole cell sugars	Ribose, mannose, inositol, galactose	Not determined	Not determined

[1] Seven *Streptomyces* spp. were compared; the data for the two most similar to LL-31F508 are presented.

Table 2. Cultural characteristics of *Streptomyces* spp.[1]

Medium		LL-31F508	*S. viridodiastaticus*	*S. werraensis*
Yeast-malt (ISP 2)	G:	Abundant	Abundant	Abundant
	AM:	Medium gray	Medium gray	Medium gray
	SM:	Yellow-brown	Medium olive-brown	Dark gray-yellow
	SP:	None	None	None
Oatmeal (ISP 3)	G:	Abundant	Abundant	Abundant
	AM:	Mecdium gray	Medium gray	Medium gray to dark gray
	SM:	Light olive-gray	Dark gray to dark yellow	Dark gray
	SP:	None	None	None
Inorganic salts starch (ISP 4)	G:	Abundant	Abundant	Abundant
	AM:	Light to medium gray	Medium gray	Medium gray
	SM:	Medium gray to gray-yellow	Medium gray	Medium gray
	SP:	None	None	None
Glycerol-asparagine (ISP 5)	G:	Moderate	Moderate	Moderate
	AM:	Medium gray	Medium gray	Light gray
	SM:	Colorless	Colorless	Colorless
	SP:	None	None	None

[1] G, growth; AM, aerial mycelium; SM, subtrate mycelium; SP, soluble pigment. ISCC, National Bureau of Standard Centroid Color Charts, Publication 440, Washington, D.C., 1976.

type 2c fatty acid pattern consisting mainly of saturated iso and anteiso fatty acids was noted. Since the culture contained only phosphatidylethanolamine, the phospholipid pattern was classified as type PII. These analytical data indicated that strain LL-31F508 represents a member of the genus *Streptomyces*, and is most closely related to *S. viridodiastaticus* and *S. werraensis*.

A comparison of the macromorphology of the three strains revealed only subtle differences among the cultures (Tables 1 and 2). Strains LL-31F508, *S. viridodiatstaticus*, and *S. werraensis* also exhibited few differences in their biochemical reactions (Table 3). Scanning electron microscopy revealed that all three strains exhibited spiny, oval-shaped spores. However, the spines on the spores of strains LL-31F508

Table 3. Biochemical reactions of *Streptomyces* spp.[1]

Test	Compound	LL-31F508	*S. viridodiastaticus*	*S. werraensis*
Carbon utilization:	D-Glucose	+	+	+
	L-Arabinose	+	+	+
	Sucrose	+	+	+
	Cellulose	−	−	−
	D-Xylose	+	+	+
	I-Inositol	+	+	+
	D-Mannitol	+	+	+
	β-D-Fructose	+	+	+
	α-L-Rhamnose	+	+	+
	Raffinose	−	−	−
Decarboxylation of:	Acetate	+	+	+
	Benzoate	−	−	−
	Citrate	+	+	+
	Lactate	±	±	±
	Malate	+	+	+
	Mucate	−	−	−
	Oxalate	+	−	+
	Propionate	+	+	+
	Pyruvate	+	+	+
	Succinate	+	+	+
	Tartrate	−	−	−
Hydrolysis of:	Casein	+	+	+
	Xanthine	+	+	+
	Hypoxanthine	+	+	+
	Tyrosine	+	+	+
	Adenine	−	−	−
	Esculin	−	−	+
Production of:	Urease	+	−	+
	Melanin like pigments	−	−	−
Acid production from:	Arabinose	−	+	−
	Dulcitol	−	−	−
	Erythritol	−	−	−
	Glucose	+	+	+
	Inositol	+	+	+
	Lactose	+	+	+
	Mannitol	+	+	+
	Mannose	+	+	+
	Methyl-α-D-glucoside	+	+	+
	Melibiose	+	+	+
	Raffinose	−	−	−
	α-L-Rhamnose	+	+	+
	Sorbitol	−	−	−
	Trehalose	+	+	+

[1] Reaction: positive, +; negative, −; weak, ±.

Fig. 2. Cluster analysis of culture LL-31F508, *S. werraensis* ATCC 14424, and *S. viridodiastaticus* ATCC 25518 by fatty acid profiles.

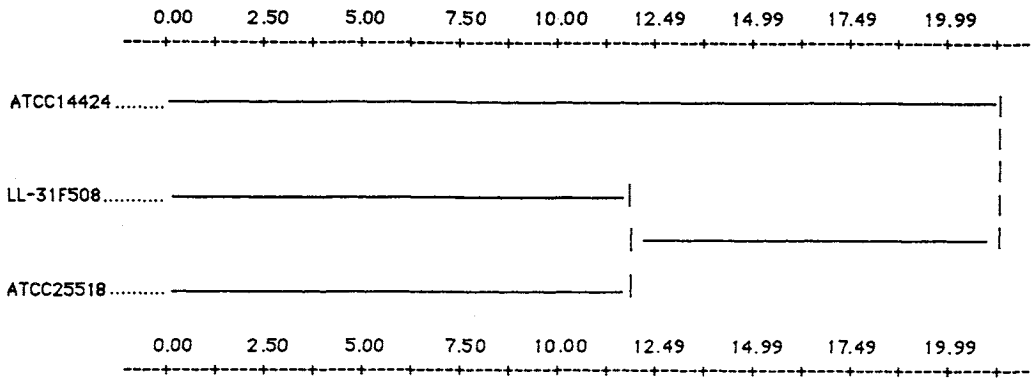


Table 4. Antimicrobial activity in fermentation samples.[1]

Culture	Sample type	Zone diameter (mm)		
		S. aureus MSSA	*S. aureus* MRSA	*E. faecium* VREF
LL-31F508	Whole Broth	19	21	14
	C-18 effluent	0	0	0
	C-18 eluate	21	25	13
	Pellet extract	16	18	12
S. lusitanus	Whole broth	13	11	11
NRRL 8034	C-18 effluent	0	0	0
	C-18 eluate	18	19	18
	Pellet extract	0	0	0

[1] Organisms were grown for 5 days at 28°C, 200 RPM, in A-1 medium.

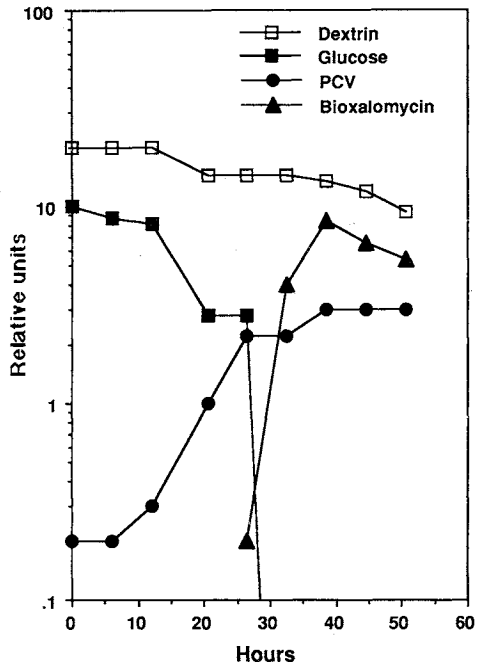
and *S. viridodiatstaticus* were more rounded at their tips than those found on *S. werraensis*. Finally, a dendrogram analysis of fatty acid profiles revealed that strain LL-31F508 was more closely related to *S. viridodiatstaticus* than to *S. werraensis* (Fig. 2).

Based on similarities in spores morphologies, fatty acid profiles, and salt tolerances, strain LL-31F508 was classified as a *S. viridodiatstaticus*. However, strain LL-31F508 demonstrated a higher salt tolerance (9 vs 6%), a maximum temperature for growth at 37°C vs 50°C, and differed in several biochemical reactions from the type strain. Hence, we concluded that strain LL-31F508 represents a new subspecies. Based on the guidelines recommended in the International Code of Nomenclature of Bacteria, we propose the subspecies designation "litoralis", the latin term for "shoreline", for strain LL-31F508.

Production of Bioxalomycins

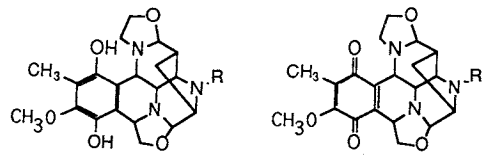
Culture LL-31F508 was compared to *S. lusitanus* NRRL 8034, an organism that produces a closely related antibiotic, naphthyridinomycin[10]. The two organisms were morphologically distinct and neither culture exhibited much antimicrobial activity when fermented in the Tomato Paste medium recommended for production of naphthyridinomycin (data not shown). In contrast, culture LL-31F508 exhibited excellent activity when fermented in A-1 medium (Table 4). Although activity was detected in the pellet extracts, the majority of the bioactive material was found in the supernatant (C-18 eluates). *S. lusitanus* produced much less activity in A-1 medium and none was detected in pellet extracts.

Fig. 3. Production of bioxalomycins.



Two seed stages in medium A-1 were used to scale up inoculum for a 300 liters fermentation. The fermentation in medium A-1 supplemented with antifoam agent (HODAG FD82 0.3%) was carried out at 200 liters per minute, 200 rpm, 28°C. PCV: packed cell volume for a 10 ml sample. Dextrin and glucose are reported in mg/ml. Bioxalomycin α (mg/liter) was determined by HPLC.

Fig 4. The structure of bioxalomycins α1, α2, β1, and β2.



α1 R=H
α2 R=CH_3

β1 R=H
β2 R=CH_3

Production of antibacterial activity was enhanced by further modification of the fermentation medium. Addition of NaCl 2% to medium A-1 increased biomass by 33% and antibacterial activity four-fold (data not shown). This observation is not surprising since this culture was isolated from a marine environment, and salt may be required for optimum growth and production of bioxalomycins. When *S. lusitanus* was fermented in a medium containing NZ-amine A, nutrisoy, ammonium chloride, and glucose, antimicrobial activity increased (data not shown). Interestingly, only the bioxalomycins were detected in fermentations of either culture; naphthyridinomycin was never detected[11]. In addition, neither antibiotic was detected in fermentations of the type strain *S. viridodiastaticus* ATCC 5518.

Fermentations were carried out in a 300 liters volume at 28°C, 200 liters per minute, and 200 rpm to provide material for isolation and structure elucidation studies. The fermentor was sampled every 6 hours and C-18 eluates were assayed by HPLC. Production of bioxalomycins peaked approximately 36 hours after inoculation during the idiophase (Fig 3). As determined by packed cell volume measurements, exponential growth ceased at the same time the glucose became depleted. Since the level of dextrin did not change significantly, it appears that the culture cannot utilize dextrin as a carbon source.

Isolation and Structure Elucidation

The purification of the bioxalomycins was accomplished utilizing preparative reverse phase HPLC and the structures were elucidated using a variety of NMR and Mass Spectral techniques[11]. The purification of the bioxalomycins resulted in the isolation of four novel antibiotics (31F508α1, 31F508α2, 31F508β1, 31F508β2). The UV maximum of the α1 and α2 components in methanol solution was 290 nm (ε 2,729). High resolution fast atom bombardment mass spectroscopy (FAB-MS) for α1 gave a molecular formula of $C_{20}H_{25}N_3O_5$ 387 MW, and $C_{21}H_{27}N_3O_5$ 401.2 MW for α2. The β1 form (MW 385) and β2 form (MW 399) have UV maxima at 270 (ε 10,508) and 370 nm in methanol. The β species were found to be the quinone form of the corresponding α components. The β components are distinguished from the antibiotic naphthyridinomycin by the presence of a second oxazolidine ring in a region of the molecule

Table 5. Antibacterial activity of bioxalomycin α2 against gram-positive clinical isolates.[1]

Organism [no. of strains]	MIC (μg/ml)			
	Bioxalomycin α2	Piperacillin	Vancomycin	Erythromycin
MSSA [4]	≤0.002～0.015	1～4	1	0.25～>128
MRSA [33]	0.004～0.015	>128	1～2	4～>128
SCN [6]	≤0.002～0.004	1～>128	1～2	20.06～>128
Staphylococcus hemolyticus [1]	≤0.002	>128	1	>128
Streptococcus pyogenes [1]	≤0.002	≤0.06	0.25	≤0.06
Streptococcus agalactiae [1]	≤0.002	≤0.06	0.50	≤0.06
Streptococcus pneumoniae [1]	0.015	≤0.06	0.25	≤0.06
Enterococcus faecalis VS [4]	≤0.002～0.25	1～>128	0.50～1	2～>128
Enterococcus faecium VR [2]	0.03～0.06	128～>128	>128	>128
Bacillus cereus [1]	0.12	2	1	≤0.06

[1] Agar dilution method. MSSA and MRSA, methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*, respectively; SCN, coagulase-negative staphylococci; VS, vancomycin-sensitive; VR, vancomycin-resistant.

analogous to quinocarcin (Fig. 4).

Biological Activity

Bioxalomycin α_2, the major component of the complex exhibited excellent activity against Gram-positive organisms with MICs between ≤0.002～0.25 μg/ml (Table 5). Mechanistic studies determined that bioxalomycin α2 inhibited bacterial DNA synthesis (94%) while RNA and protein synthesis were inhibited by only 33% and 30%, respectively.[12] Further studies on the cytotoxicity and mode of action of bioxalomycin α2 in eucaryotic cells is currently under investigation.

Acknowledgments

The authors are grateful to Mr. BRIAN STRASSLE for assistance in the scanning electron microscope, to Dr. D. LABEDA for kindly providing the actinomycete fatty acid database, and to Ms. TAMI EVANS for her technical assisstance in the taxonomic studies.

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