LL-E19085α, A NOVEL ANTIBIOTIC FROM *MICROMONOSPORA CITREA*: TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITY

W. M. MAIESE, M. P. LECHEVALIER†, H. A. LECHEVALIER†, J. KORSHALLA, J. GOODMAN, M. J. WILDEY, N. KUCK and M. GREENSTEIN

Medical Research Division, American Cyanamid Company, Lederle Laboratories, Pearl River, New York 10965, U.S.A.
†Rutgers University, Piscataway, New Jersey 08855, U.S.A.

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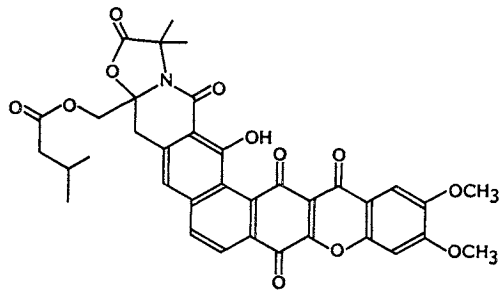
A new antibacterial antibiotic, designated LL-E19085α, was isolated from the fermentation broth of an actinomycete strain. Based on cultural, physiological, morphological and chemical characteristics, culture LL-E19085 was identified as a new subspecies of *Micromonospora citrea*. Antibiotic LL-E19085α demonstrated potent activity against a spectrum of Gram-positive aerobic and anaerobic bacteria.

In the course of our search for novel antibacterial antibiotics produced by microorganisms, a culture designated LL-E19085 was found to produce a novel antibiotic, LL-E19085α[1] (Fig. 1). This antibiotic exhibited potent antibacterial activity against a spectrum of Gram-positive aerobic and anaerobic organisms. This paper describes the fermentation and biological activity of LL-E19085α and the taxonomy of the producing culture.

Fig. 1. The structure of LL-E19085α.



Materials and Methods

Microorganism

Culture LL-E19085 was isolated from a soil sample collected at Lake Manyara, Tanzania, Africa. This culture was deposited at the Northern Regional Research Center's Culture Collection Laboratory under the accession No. NRRL 18351.

Taxonomic Studies

The taxonomic studies were carried out as described by the International Streptomyces Project (ISP)[2] and GORDON *et al.*[3]. For the evaluation of cultural characteristics, the strains were incubated for 14~31 days at 28°C. Cell wall and whole cell composition were analyzed by the methods of LECHEVALIER and LECHEVALIER[4].

Media and Fermentation

Culture LL-E19085 was stored as a frozen seed suspension at −70°C in growth medium. To prepare seed inoculum for the production of the antibiotic, 1.0 ml of a thawed suspension was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium consisting of yeast extract 0.5%, NZ-Amine type A (Sheffield Chemical Company) 0.5%, dextrin 2.0%, glucose 1.0%, and $CaCO_3$ 0.1%. After 48 hours incubation at 32°C on a rotary shaker at 210 rpm, 100 ml of this suspension was added into a 12-liter fermenter containing 10 liters of seed medium. Following 48 hours

incubation at 32°C (aeration: 1 v/v/m, 450 rpm), the contents from this fermenter were inoculated into a 410-liter fermenter containing 300 liters of seed medium (aeration: 0.75 v/v/m, 250 rpm). After 48 hours growth, these 300 liters were used to inoculate a production fermenter containing 3,000 liters of medium consisting of dextrin 3.0%, glucose 0.5%, Nutrisoy 1.5%, corn steep liquor 0.5%, $CaCO_3$ 0.5% and antifoam 0.3%. This fermentation was carried out at 28°C for up to 120 hours (aeration: 0.66 v/v/m, 450 rpm). The pH of the seed and production media used in these studies was adjusted to 6.8~7.0 prior to sterilization. Antibiotic production was monitored by a paper-disk agar diffusion assay using *Bacillus subtilis* and by analytical HPLC.

Isolation

The antibiotic was isolated by extraction into ethyl acetate from the acidified (pH 2.5) whole fermentation mash. The crude extract was concentrated, subjected to chromatography on silica gel (60~200 mesh) and eluted with 2% acetone in methylene chloride. Fractions containing LL-E19085α were pooled for rechromatography by reversed phase HPLC. The reversed phase HPLC system consisted of a C_{18} column (2.14×25 cm) with 12 μm packing and eluted with 65% acetonitrile - 35% 0.05 M NH_4OAc buffer (pH 4.5). Fractions were pooled on the basis of analytical HPLC to yield pure LL-E19085α. Analytical HPLC was conducted with the same system, but a 2.1×100 mm, 5 μm packing C_{18} column was used. Detection of antibiotic was by absorbance at 325 nm.

In Vitro Antimicrobial Activity

The *in vitro* antibacterial activity of LL-E19085α against a spectrum of Gram-positive and Gram-negative bacteria was determined by the agar dilution method employing Mueller-Hinton medium. The lowest concentration that inhibited growth of a bacterial strain after 18 hours incubation at 35°C was recorded as the MIC.

In Vivo Activity

The *in vivo* efficacy of LL-E19085α was tested in mice against an experimental infection of *Streptococcus pyogenes* C 203. Mice were challenged with a lethal dose of this organism, and the drug was administered subcutaneously at 0.5 and 3 hours after infection. The antibiotic was solubilized in DMSO and mixed in a 0.2% aqueous agar suspension immediately before administration to the animals.

Table 1. Comparison of macromorphology of culture LL-E19085 and *Micromonospora citrea* NRRL B16101 on ISP agar media.

ISP agar medium	Spores	Vegetative mycelium[a]	Soluble pigments
LL-E19085			
Yeast - malt (ISP 2)	Slight black at edge	Strong orange (50) to medium orange-yellow (71)	Slight brown-black
Oatmeal (ISP 3)	None	Light orange-yellow (70) to vivid orange-yellow (66)	Slight brownish
Inorganic salts - starch (ISP 4)	None	Light orange-yellow (70) to vivid orange-yellow (66)	Slight brownish
Glycerol - asparagine (ISP 5)	Slight brownish at edge	Light tone of brownish-orange (54)	Slight brownish
NRRL B16101			
Yeast - malt	Excellent spores black (267)	Medium orange-yellow (71)	Slight brownish
Oatmeal	Sparse spores light gray-black	Colorless to dark orange-yellow (72)	Slight brownish
Inorganic salts - starch	Excellent spores black (267)	Light yellow-brown (76)	—
Glycerol - asparagine	—	Colorless to pale orange-yellow (73)	—

[a] ISCC, National Bureau of Standard Centroid Color Charts, Publication 440, Washington, D.C., 1976.

Results

Taxonomic Studies of the Producing Culture

Culture LL-E19085 was isolated from the Lake Manyara region of Tanzania, Africa. Examination of the culture grown at 28°C for 14~31 days on various media revealed that monospores were present either singly or in masses on vegetative hyphae, but no aerial hyphae were observed. The results are summarized in Table 1. Electron microscopy examination showed the spores were warty (Fig. 2). A summary of the culture's growth characteristics on various media is presented in Table 2. Whole cell analysis showed that the strain contained the *meso*-isomer as well as traces of the L-isomer of diaminopimelic acid. Additionally, the strain contained xylose plus traces of arabinose in its whole cell sugar hydrolysate, indicating a whole cell sugar pattern of type D. The carbohydrate utilization

Fig. 2. Electron micrograph of spores of culture LL-E19085.

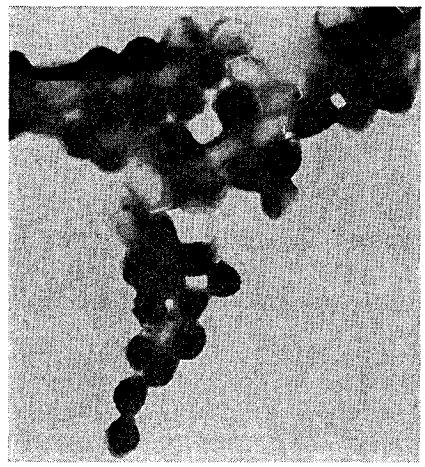


Table 2. Comparison of macromorphology of culture LL-E19085 with *Micromonospora citrea* [NRRL B16101 on various agar media[a].

Agar medium	LL-E19085	NRRL B16101
Pablum	V: Brown (58)	Brown (58)
	S: Sparse	Brown-black (62)
	P: Soluble dark brown (78)	Slight soluble dark brown (78)
Yeast - CZAPEK	V: Brownish tan (77)	Brownish tan (77)
	S: Sparse	Black (267)
	P: Slight soluble dark brownish (59)	Slight soluble brown-black (62)
CZAPEK	V: Vegetative color	Vegetative color
	S: Black (267)	Black (267)
	P: Slight dark	Slight dark
Yeast extract - glucose	V: Vegetative color	Vegetative color
	S: Black, dry (267)	Black, moist (267)
	P: Soluble brownish (78)	Soluble brownish (78)
Nutrient	V: Orange-brown (57)	Orange (50)
	S: Sparse black (267)	Sparse black (267)
	P: Moderate brown (78)	Slight soluble brown (47)
Nutrient - glycerol	V: Blackish-tan (80)	Orange (50)
	S: Sparse	Sparse black (267)
	P: Intense brown-black (65)	Slight soluble brown (47)
BENNETT's dextrin	V: Tan (76)	Orange (50)
	S: Moderate black (267)	Moderate black (267)
	P: Soluble reddish brown (44)	Soluble brownish-rose (20)
Glucose - asparagine	V: Orange-tan (57)	Orange-tan (57)
	S: None	Sparse black (267)
	P: Slight soluble dark (65)	Slight soluble dark (65)

[a] ISCC, National Bureau of Standard Centroid Color Charts Publication 440, Washington, D.C., 1976.
V: Growth of vegetative mycelium, S: spores, P: soluble pigment.

patterns and physiological reactions of culture LL-E19085 are summarized in Tables 3 and 4, respectively. From the macromorphological and physiological studies, it was concluded that culture LL-E19085 and *Micromonospora citrea* NRRL B16101 are closely related but differ in production of nitrate reductase, decarboxylation of lactate, and production of acid from glycerol and α-methyl D-glucoside (Tables 1~4). For these reasons, culture LL-E19085 is considered a new subspecies of *M. citrea*.

Table 3. Carbohydrate utilization of culture LL-E19085 and *Micromonospora citrea* NRRL B16101.

	LL-E19085	NRRL B16101
Arabinose	+	±
Cellulose	−	−
Fructose	±	±
Glucose	+	+
Inositol	−	−
Mannitol	−	−
Raffinose	−	−
Rhamnose	−	−
Sucrose	±	+
Xylose	+	+

+: Utilized, ±: weakly utilized, −: not utilized.

Fermentation and Isolation

Culture LL-E19085 was grown in a 3,000-liter fermenter at 28°C for 120 hours. A typical time course for the production of LL-E19085α is presented in Fig. 3. Antibiotic production started at approximately 20~24 hours postinoculation and reached a maximum at 110~120

Table 4. Physiological reactions of culture LL-E19085 and *Micromonospora citrea* NRRL B16101.

	LL-E19085	NRRL B16101		LL-E19085	NRRL B16101
Hydrolysis of			Acid from		
Casein	+	W	Adonitol	−	−
Xanthine	−	−	Arabinose	+	+
Hypoxanthine	−	−	Cellobiose	+	+
Tyrosine	+	+	Dextrin	+	+
Adenine	+	W	Dulcitol	−	−
Gelatin	+	+	Erythritol	−	−
Potato starch	+	+	Fructose	+	+
Esculin	+	+	Galactose	+	+
Production of			Glucose	+	+
Nitrate reductase	−	+	Glycerol	+	−
Phosphatase	+	+	Inositol	−	−
Urease	−	−	Lactose	+	+
Growth on			Maltose	+	+
Salicylate	−	−	Mannitol	−	−
5% NaCl	−	−	Mannose	+	+
Lysozyme broth	−	−	α-Methyl D-glucoside	+	−
Decarboxylation of			Melibiose	+	+
Acetate	+	+	Raffinose	+	+
Benzoate	−	−	Rhamnose	−	−
Citrate	−	−	Salicin	+	+
Lactate	+	−	Sorbitol	−	−
Malate	−	−	Sucrose	+	+
Mucate	−	−	Trehalose	+	+
Oxalate	−	−	Xylose	+	+
Propionate	+	+	β-Methyl D-xyloside	−	−
Pyruvate	+	+	Growth at		
Succinate	−	−	10°C	−	−
Tartrate	−	−	42°C	+	+
			45°C	+	+

W: Weak.

Fig. 3. Fermentation profile of culture LL-E19085.
■ LL-E19085α, ○ packed cell volume, □ pH.

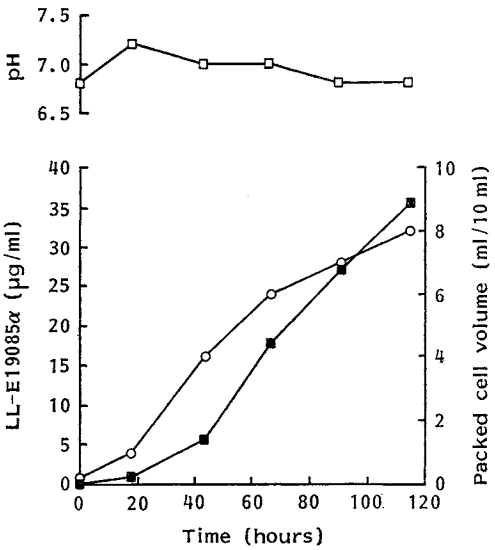


Table 5. *In vitro* antimicrobial spectrum of LL-E19085α.

Organisms (No. of strains tested)	MIC range (μg/ml)[a]
Escherichia coli (2)	>128
Klebsiella pneumoniae (1)	>128
Serratia sp. (1)	>128
Citrobacter sp. (1)	>128
Pseudomonas aeruginosa (2)	>128
Staphylococcus aureus (8)	≤0.06~0.12
S. epidermidis (2)	≤0.06
Enterococcus sp. (5)	≤0.06~0.12
Streptococcus sp. (β-hemolytic) (4)	≤0.06
S. pneumoniae (2)	≤0.06
Bacteroides fragilis (1)	16
B. thetaiotaomicron (2)	4
Clostridium perfringens (1)	≤0.06
C. difficile (1)	≤0.06

[a] MIC values were determined by the standard agar dilution method in Mueller-Hinton medium.

hours into the fermentation cycle. The antibiotic was distributed between the mycelium and the growth medium.

Biological Properties

The antimicrobial spectrum of LL-E19085α was determined by the agar dilution method employing Mueller-Hinton medium and is shown in Table 5. The antibiotic exhibited potent antibacterial activity against Gram-positive aerobic and anaerobic organisms with MIC values less than 0.12 μg/ml. However, no activity was observed against Gram-negative organisms. The *in vivo* activity of LL-E19085α is presented in Table 6. Although the antibiotic protects mice against a lethal *S. pyogenes* infection, no dose response was observed. This is probably due to the extremely poor solubility of LL-E19085α, which could account for poor distribution in the animal.

Table 6. *In vivo* activity of LL-E19085α against *Streptococcus pyogenes* C203.

Dose (mg/kg)[a]	Survival ratios[b]
128	3/5
64	2/5
32	2/5
16	3/5
8	2/5
Nontreated infected controls	0/10

[a] Two individual subcutaneous doses administered at 0.5 and 3 hours after infection.
[b] Number mice alive 7 days after infection/number mice infected.

Discussion

Culture LL-E19085, identified as a new subspecies of *M. citrea*, was found to produce a new antibiotic, LL-E19085α. This compound possesses potent activity against Gram-positive aerobic and anaerobic bacteria *in vitro* and was shown to be active against *S. pyogenes* infections in mice. This compound is extremely insoluble, and this property will probably impact its efficacy in animal studies. Poor solubility is also a characteristic of the structurally related antibiotics, the lysolipins[5], the actinoplanones[6] and the cervinomycins[7,8].

Acknowledgments

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