LL-E19020α AND β, ANIMAL GROWTH PROMOTING ANTIBIOTICS: TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITY

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Antibacterial antibiotics LL-E19020α and β were isolated from the fermentation broth of an actinomycete strain. Based on cultural and physiological characteristics, culture LL-E19020 was identified as a new subspecies of *Streptomyces lydicus*. The LL-E19020α and β antibiotics were found to possess a very narrow antibacterial spectrum against human pathogens. In studies in chickens, LL-E19020α demonstrated excellent growth promoting activity.

In the course of our search for novel antibacterial antibiotics produced by microorganisms, a culture designated LL-E19020 was found to produce antibiotics, LL-E19020α and β. These antibiotics possessed a very narrow antibacterial spectrum against human pathogens. This paper describes the taxonomy of the producing culture, fermentation and biological activity of LL-E19020α and β.

Materials and Methods

Microorganism

Culture LL-E19020 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 number NRRL 18036.

Taxonomic Studies

The taxonomic studies were carried out as described by the International Streptomyces Project (ISP)[1] and GORDON *et al.*[2]. 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[3].

Media and Fermentation

Culture LL-E19020 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 vol/vol/minute, 450 rpm), the contents from this fermenter were inoculated into a 410-liter fermenter these 300 liters were used to inoculate a production fermenter containing 3,000 liters of medium consisting

of dextrin 3.0%, cane molasses 2.0%, yeast extract 0.25%, soy peptone 0.75%, $CaCO_3$ 0.5% and antifoam 0.3%. This fermentation was carried out at 28°C for 96 hours (aeration: 0.66 vol/vol/minute, 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 analytical HPLC.

Isolation

Antibiotics LL-E19020α and β were isolated from the fermentation broth by solvent extraction. Purification was achieved by chromatography on silica gel and reverse-phase HPLC[4].

HPLC Analysis

Antibiotic titers of LL-E19020α and β were determined by reverse-phase HPLC employing a C_{18} column developed with 65% acetonitrile in 0.05 M ammonium acetate buffer, pH 4.5[4].

In Vitro Antimicrobial Activity

The *in vitro* antibacterial activities of LL-E19020α and β were tested against a spectrum of Gram-positive and Gram-negative bacteria. Assays were conducted 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* efficacies of LL-E19020α and β were determined 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.

Growth Promotion Studies

One day old Vantress X Hubbard chickens were administrated LL-E19020α or bacitracin methylene disalicylate (BMD) in the feed for 7 weeks. Each treatment had 6 replicates of 31 females and 31 males per pen. Measurements of weight gain were taken during the trial and were subjected to analysis.

Results

Taxonomic Studies of the Producing Culture

Culture LL-E19020 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 short spiral spore chains, 10~50 spores long, with occasional longer chains. Electron microscopy examination showed the spores to have

Fig. 1. Electron micrograph of spores of culture LL-E19020.

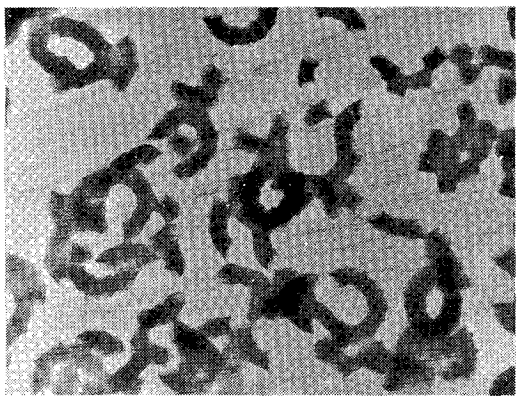


Table 1. Carbohydrate utilization of culture LL-E19020 and *Streptomyces lydicus* ISP 5461.

	LL-E19020	ISP 5461
Arabinose	+	+
Cellulose	−	−
Fructose	+	+
Glucose	+	+
Inositol	+	+
Mannitol	+	+
Raffinose	+	+
Rhamnose	+	+
Sucrose	+	+
Xylose	+	+

+: Utilized, −: not utilized.

Table 2. Physiological reactions of culture LL-E19020 and *Streptomyces lydicus* ISP 5461.

	LL-E19020	ISP 5461		LL-E19020	ISP 5461
Hydrolysis of			Acid from		
Casein	+	+	Adonitol	+	+
Xanthine	−	+	Arabinose	+	+
Hypoxanthine	+	+	Cellobiose	+	+
Tyrosine	+	+	Dextrin	+	+
Adenine	+	+	Dulcitol	−	−
Production of			Erythritol	+	−
Amylase	+	+	Fructose	+	+
Esculinase	+	+	Galactose	+	+
Gelatinase	+	+	Glucose	+	+
Nitrate reductase	−	−	Glycerol	+	+
Phosphatase	+	+	Inositol	+	+
Urease	+	+	Lactose	+	+
Growth on			Maltose	+	+
Salicylate	−	−	Mannitol	+	+
5% NaCl	+	+	Mannose	+	+
Lysozyme broth	Trace	Trace	α-Methyl D-glucoside	+	+
Utilization 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	−	−
			50°C	−	−

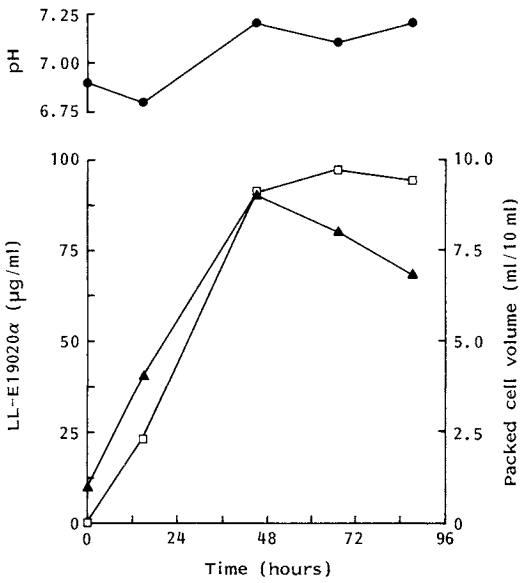
smooth surfaces (Fig. 1). Whole cell analysis showed that the strain contained the L-isomer of diaminopimelic acid and may thus be assigned to the genus *Streptomyces*. The carbohydrate utilization patterns and physiological reactions of culture LL-E19020 are summarized in Tables 1 and 2, respectively. From these studies, it was concluded that culture LL-E19020 and *Streptomyces lydicus* ISP 5461 are closely related but differ in xanthine hydrolysis, decarboxylation of oxalate and production of acid from erythritol, rhamnose and β-methyl-D-xyloside. For these reasons, culture LL-E19020 is considered a new subspecies, *S. lydicus* subspecies *tanzanius*.

Fermentation and Isolation

Culture LL-E19020 was grown in a 3,000-liter fermenter at 28°C for 96 hours. A typical time course for the production of LL-E19020α is presented in Fig. 2. Antibiotic production reached a maximum at roughly 48~72 hours into the fermentation cycle and remained relatively constant. Antibiotic production for the β component paralleled the α component; however, the yield was approximately 10% of α. The antibiotic was distributed between the mycelium and the growth medium and was recovered by ethyl acetate extraction of the acidified whole broth. Purification was achieved by chromatography on silica gel developed with methylene chloride.

Fig. 2. Production profile of culture LL-E19020α.

□ LL-E19020α, ▲ packed cell volume, ● pH.



Biological Properties

The antimicrobial spectrum of LL-E19020α and β was determined by the agar dilution method employing Mueller-Hinton medium and is shown in Table 3. These antibiotics exhibited an exceptionally narrow antimicrobial spectrum against human pathogens, showing meaningful activity against *Streptococcus* species and certain anaerobes. No activity was observed against Gram-negative organisms. The *in vivo* activities of LL-E19020α and β are presented in Table 4. The antibiotics protected mice against a lethal *S. pyogenes* infection. Antibiotic LL-E19020α was also evaluated in growth promotion assays in chickens. In comparison trials, this component demonstrated excellent growth promoting activity which was superior to that observed with BMD (Table 5).

Discussion

Culture LL-E19020, identified as a new subspecies of *S. lydicus*, was found to produce antibiotics LL-E19020α and β. These compounds possess a very narrow antibacterial spectrum against

Table 3. *In vitro* antimicrobial spectrum of LL-E19020α and β.

Organism (strains tested)	MIC range (μg/ml)	
	LL-E19020α	LL-E19020β
Gram-positive		
Staphylococcus aureus (6)	128～>256	>128
S. epidermidis (1)	>256	>128
S. saprophyticus (1)	>256	>128
Enterococcus sp. (5)	>256	>128
Streptococcus sp. (β-hemolytic) (6)	0.12～1	0.12～0.5
S. pneumoniae (6)	0.25～2	0.25～1
Clostridium difficile (1)	4	1
C. perfringens (1)	16	4
Peptococcus magnus (2)	0.12	0.5
Gram-negative		
Escherichia coli (1)	>256	>128
Klebsiella pneumoniae (1)	>256	>128
Enterobacter cloacae (1)	>256	>128
Morganella morganii (1)	>256	>128
Serratia marcescens (1)	>256	>128
Pseudomonas aeruginosa (1)	>256	>128
Bacteroides fragilis (1)	>128	>128

Table 4. *In vivo* activity of LL-E19020α and β against *Streptococcus pyogenes* C 203.

Dose (mg/kg)[a]	Survival ratios[b]	
	LL-E19020α	LL-E19020β
64	5/5	5/5
32	5/5	5/5
16	5/5	5/5
8	4/5	3/5
4	2/5	2/5
Nontreated infected controls	0/10	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.

Table 5. Performance of LL-E19020α in broiler floor pen trials.

Treatment	Dosage[a]	Body weight (g)	Improvement over controls (%)
Control	—	1,983	—
BMD	55	2,015	1.6
LL-E19020α	20	2,073	4.5
	10	2,086	5.2
	5	2,068	4.3
	2.5	2,064	4.1

[a] Grams per metric ton million of drug administered in feed.

human pathogens *in vitro* and are active against *S. pyogenes* infections in mice. LL-E19020α and β are members of the elfamycin class of antibiotics (*cf.* aurodox[5], efrotomycin[6], and L-681,217[7]) and are apparently identical with the recently described antibiotics phenelfamycins E and F, respectively, produced by *Streptomyces violaceoniger*[8]. In comparison trials in poultry, antibiotic LL-E19020 demonstrated excellent growth promoting activity.

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