KEDARCIDIN, A NEW CHROMOPROTEIN ANTITUMOR ANTIBIOTIC

I. TAXONOMY OF PRODUCING ORGANISM, FERMENTATION AND BIOLOGICAL ACTIVITY

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Strain L585-6 (ATCC 53650) is an actinomycete isolated from a soil sample collected in Maharastra State, India. It produces a new chromoprotein antitumor antibiotic, designated kedarcidin. Taxonomic studies demonstrated that strain L585-6 is an unidentified and unknown actinomycete. Kedarcidin shows potent antitumor activity against implanted P388 leukemia (3.3 μg/ml/kg) and B16 melanoma (2 μg/kg) in mice. Kedarcidin also shows potent antimicrobial activity against Gram-positive bacteria but no activity against Gram-negative bacteria.

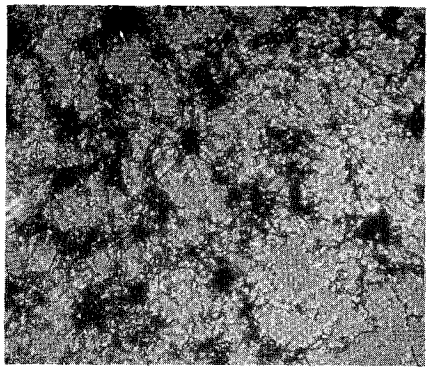
A novel antitumor antibiotic, kedarcidin, was isolated from the culture supernatant of an actinomycete strain L585-6 (ATCC 53650). Strain L585-6 was isolated from a soil sample collected in Maharastra State, India. Kedarcidin is a chromoprotein with a MW of 12,400. Its physico-chemical characteristics[1] are different from those of the known peptide antibiotics having antitumor activity such as neocarzinostatin[2], macromomycin[3] and largomycin[4]. Kedarcidin exhibits potent *in vivo* antitumor activity against P388 leukemia and B16 melanoma in murine models. It is active against ip implanted P388 leukemia and B16 melanoma in mice at a dosage of 3.3 and 2 μg/kg, respectively. The potency of kedarcidin against murine tumor is similar to that of esperamicin[5,6] and calicheamicin[7] class of antibiotics with the unique 1,5-diyn-3-ene structure. Kedarcidin also shows potent activity against Gram-positive bacteria but no activity against Gram-negative bacteria. Taxonomic studies showed that strain L585-6 is an unidentified and unknown actinomycete. Production titers of kedarcidin in shake flask and fermenter cultures are 1,300 μg/ml and 1,050 μg/ml, respectively. The present paper describes the taxonomy of the producing organisms, fermentation and biological properties. The isolation and physico-chemical characteristics of kedarcidin will be described in the following paper[1].

Taxonomy of the Producing Strain

Morphology

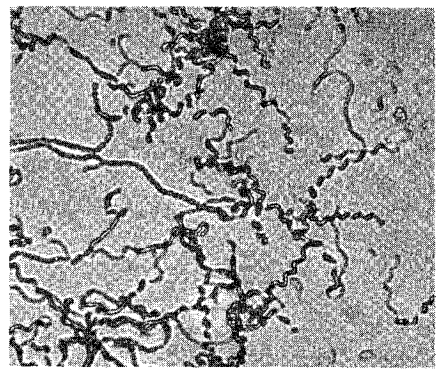
Strain L585-6 is a Gram-positive, filamentous organism that forms substrate and aerial mycelia. The substrate mycelium penetrates the agar and is not fragmented. Globose dense aggregates of hyphae, 5 to 25 μm i.d., along with coalesced vegetative hyphae, are observed. The aerial mycelium is well branched and develops straight slewing or spiral long hyphae, in which spores are formed in continuous or discontinuous chains. Dense tufts of branched short spore chains are formed predominantly in International

Fig. 1. Slimy tufts of branched short spore chains on the aerial mycelium.



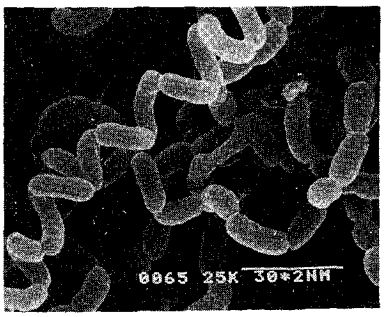
Photomicrograph of strain L585-6 (Bar = 40 μm). medium: ISP 5. cultivation: 28°C for 3 weeks.

Fig. 2. Slewing spore chains on the aerial mycelium.



Photomicrograph of strain L585-6 (Bar = 10 μm). medium: ISP 7. cultivation: 28°C for 3 weeks.

Fig. 3. Slewing spore chains.



Scanning electron micrograph of strain L585-6. medium: ISP 7. cultivation: 28°C for 3 weeks.

Table 1. Carbon utilization of strain L585-6.

Utilization:	D-Glucose, D-ribose
No utilization:	L-Rhamnose, D-galactose, D-fructose, D-mannose, L-sorbose, sucrose, lactose, cellobiose, melibiose, soluble starch, cellulose, dulcitol, inositol, D-mannitol, D-sorbitol, salicin, glycerol, D-arabinose, L-arabinose, D-xylose

Streptomyces Project (ISP) medium No. 5. Both types of spores are oval to short-cylindrical (0.4 to 0.6 by 1.0 to 2.0 μm), non-motile, and have a smooth surface. Colorless balloon-like bodies (5 to 20 μm i.d.) are observed singularly or in mass on the aerial mycelium after incubation for 5 to 10 days. After incubation for 3 weeks or more, these balloon-like bodies develop into yellowish-brown sclerotic granules (40 to 100 μm i.d.) which are covered with further elongated aerial hyphae.

Carbon Utilization

The carbon utilization pattern of strain L585-6 was determined by the method of SHIRLING and GOTTLIEB[8] excepting the inclusion of a 3-hour starvation period between the harvesting and inoculation steps. Washed vegetative cells were shaken at 250 rpm and 28°C on a rotary shaker in a liquid version of ISP medium No. 9 with no carbon source. Among 25 sugars tested, only D-ribose and D-glucose are utilized for growth by strain L585-6 (Table 1).

Cell Wall Chemistry

The cell wall content of strain L585-6 was examined according to the methods described by BECKER *et al.*[9], YAMAGUCHI[10], and LECHEVALIER and LECHEVALIER[11]. Purified cell wall contains *meso*-diaminopimelic acid, galactose, mannose, ribose and rhamnose. Whole cell sugars do not contain additional sugar. Hence, the cell wall type belongs to type III_c. Phospholipids are type P-II containing two

Table 2. Cultural characteristics of strain L585-6.

Medium	Growth of vegetative mycelium	Aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose-nitrate agar (CZAPEK-Dox agar)	None or scant	None	Colorless	None
Tryptone-yeast extract broth (ISP 1)	Moderate; not turbid, floccose	None	Colorless	Deep yellowish-brown (75)
Yeast extract-malt extract agar (ISP 2)	Moderate	None	Dark yellowish-brown (78)	Deep yellowish-brown (75)
Oatmeal agar (ISP 3)	Scant	None or scant; white when present	Colorless	None
Inorganic salts-starch agar (ISP 4)	Scant	None or scant; white when present	Colorless	None
Glycerol-asparagine agar (ISP 5)	Moderate	Moderate; yellowish-white (92)	Colorless	None
Peptone-yeast extract-iron agar (ISP 6)	Moderate	None	Light grayish-yellowish brown (79)	Brownish black (65)
Tyrosine agar (ISP 7)	Moderate	Abundant, yellowish-white (92)	Black	Black
Glucose-asparagine agar	Poor	None	Dark orange-yellow (72)	None
BENNETT's agar	Moderate	None or scant; white when present	Dark grayish-yellowish brown (81)	Moderate yellowish-brown (77)

Observation after incubation at 28°C for 3 weeks.
Color and number in parenthesis follows ISCC-NBS designation.

phosphatidylethanolamines, phosphatidylglycerol and phosphatidylinositol. The major menaquinone are MK-9 (H_4) and MK-9 (H_6) (30% and 48%, respectively). Glycolate test is negative.

Cultural Characteristics

The cultural characteristics of strain L585-6 are shown in Table 2. Strain L585-6 grows moderately in most descriptive media except on CZAPEK's sucrose-nitrate agar, oatmeal agar and starch-mineral salts agar. The aerial mycelium is formed on the tyrosine agar and glycerol-asparagine agar, but not on ISP media Nos. 2, 3, 4 and 6, and BENNETT's agar. The color of the aerial mycelium is yellowish-white. Blackish melanoid pigments are formed in ISP media Nos. 6 and 7.

Table 3. Physiological characteristics of strain L585-6.

Hydrolysis of:	
Gelatin	+
Soluble starch	−
Potato starch	−
Milk coagulation	+
Milk peptonization	+
Production of:	
Nitrate reductase	− or +[a]
Tyrosinase	+
Tolerance to:	
Lysozyme, 0.01% (w/v)	+
NaCl, 1%~4% (w/v)	+
5% (w/v)	−
pH 5.0~11.0	+
pH 4.5 and 12	−
Temperature:	
Growth range	18~39°C
No growth	15 and 41°C
Optimal growth	30~35°C

[a] Negative in CZAPEK's sucrose-nitrate broth and positive in peptone-nitrate broth.

Physiological Characteristics

The physiological characteristics of strain L585-6 are shown in Table 3. The optimal growth range

Table 4. Differential characteristics of strain L585-6 from *Streptoalloteichus* and *Saccharothrix*.

	Strain L585-6	*Streptoalloteichus*	*Saccharothrix*
Morphology:			
Fragments in aerial and substrate mycelia	−	−	+
Tufts of branched short spore chains	+	+	−
Sporangium-like enveloping flagellate spores	−	+	−
Physiology:			
Growth at 50°	−	+	−
Cell chemistry:			
Cell wall	*meso*-DAP, gal, man, rib, rham	*meso*-DAP, gal, man, rham	*meso*-DAP, gal, rham
Phospholipids	PE (2 types), PG, PI	PE (2 types), PG, PI	PE (2 types), PG, PI
Major menaquinone	MK-9 (H_6) and MK-9 (H_4)	MK-9 (H_6) and MK-10 (H_6)	MK-9 (H_4) and MK-10 (H_4), or MK-9 (H_4)

Abbreviations: *meso*-DAP, *meso*-diaminopimelic acid; gal, galactose; man, mannose; rib, ribose; rham, rhamnose. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol. MK-9 (H_6), menaquinone with nine isoprene subunits, three of which are saturated.

of strain L585-6 was 30~35°C. The temperature range for growth was 18~39°C but no growth at 15°C and 41°C. There was no growth on yeast extract-malt extract supplemented with more than 5% NaCl. Nitrate was reduced in peptone-nitrate broth but not in CZAPEK's sucrose-nitrate broth. Gelatin liquefaction gave positive reaction but starch was not hydrolyzed.

Taxonomic Position

The above-mentioned morphological, cultural and physiological characteristics as well as chemotaxonomic compositions of strain L585-6 indicate that the strain is related to two genera, *Streptoalloteichus*[12] and *Saccharothrix*[13~15]. As shown in Table 4, strain L585-6 differs from *Streptoalloteichus* in the absence of flagellate spore, the lack of growth at 50°C and the menaquinone composition, and from *Saccharothrix* in the absence of fragmentation in aerial and substrate mycelia, the lack of branched short spore chains and the menaquinone composition. Therefore, further morphological observation and additional chemotaxonomic data are required to determine the genus. Thus, at the present stage, strain L585-6 is considered an unidentified and unknown actinomycete.

Fermentation

Strain L585-6 was grown in test tubes on agar slants of yeast extract-malt extract agar supplemented with $CaCO_3$. This medium consisted of glucose 0.4%, yeast extract 0.4%, malt extract 1%, calcium carbonate 0.15% and agar 1.5%. The culture was incubated for 10 days at 28°C. To prepare an inoculum for the production phase, the surface growth from the slant culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of sterile medium consisting of glucose 3%, Pharmamedia 1%, Nutrisoy 1% and $CaCO_3$ 0.3%. The vegetative culture was incubated at 28°C for 72 hours on a Gyrotary shaker (Model 53, New Brunswick Scientific Co.) set at 250 rpm. Five ml of vegetative culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of production medium consisting of glycerol 3%, Pharmamedia 1%, distillers solubles 1.5%, fish meal 1% and $CaCO_3$ 0.6%. The produc-

tion culture was incubated at 28°C and 250 rpm on the same shaker for 5~6 days. For production in fermenters 1.5 liters of vegetative culture was transferred to 30-liter production medium. The incubation temperature was 28°C, the agitation rate was 250 rpm and the air flow was 0.7 volume/minute. The back pressure of the fermenter was set at 0.35 kg/cm^2.

The production of kedarcidin in the fermentation was monitored by HPLC using a TSK-G2000SW column (7.5 × 300 mm, LKB Produkter AB, Sweden). The solvent system was 0.05 M Tris-HCl buffer (pH 7.4) and the flow rate was 1 ml/minute. The detector wavelength was set at 220 nm. The fermentation broth was centrifuged at 1,500 × *g* for 15 minutes. One ml of the supernatant was passed through an Accell QMA sep-pak cartridge (Waters Associates, PN 10835) previously equilibrated with 0.05 M Tris-HCl buffer (pH 7.4). The cartridge was washed with 20 ml of Tris-HCl buffer. Kedarcidin was eluted from the cartridge by washing the cartridge with 1 ml of 0.05 M Tris-HCl (pH 7.4) containing 1 M NaCl. Fifty μl of the eluate was used for HPLC analysis. Kedarcidin was eluted at around 11.6 minutes. The production of kedarcidin in shake flask and fermenter culture after 144 hours of fermentation averaged 1,300 μg/ml and 1,050 μg/ml, respectively.

Biological Activity

The *in vivo* antitumor activity of kedarcidin was evaluated against lymphocytic leukemia P388 in CDF_1 mice and B16 melanoma in BDF_1 mice according to previously described protocols[16,17]. The results are summarized in Tables 5 and 6. Against ip-implanted P388 leukemia (Table 5), the mice were treated

Table 5. Effect of kedarcidin on P388 leukemia.

Treatment schedule	Dose, ip (μg/kg/injection)	Effect (% T/C)
QD 1 to 5 days	90	95
	30	155
	10	145
	3.3	140

Tumor inoculum: 10^6 ascites, ip.
Host: CDF_1 mice.
Evaluation: MST = medium survival time.
Effect: % T/C = (MST treated/MST control) × 100.
Criteria: % T/C ≥ 125 considered significant antitumor activity.

Table 6. Effect of kedarcidin on B16 melanoma.

Treatment schedule	Dose, ip (μg/kg/injection)	Effect (% T/C)
QD 1 to 9 days	256	97
	128	148
	64	161
	32	191
	16	197
	8	206
	4	164
	2	130

Tumor inoculum: 0.5 ml 10% cell homogenate, ip.
Host: BDF_1 mice.
Evaluation: MST = medium survival time.
Effect: % T/C = (MST treated/MST control) × 100.
Criteria: % T/C ≥ 125 considered significant antitumor activity.

Table 7. Antimicrobial spectrum of kedarcidin.

Test organism	MIC (μg/ml)	Test organism	MIC (μg/ml)
Enterococcus faecalis A20688	0.03	*E. coli* A20697	>500
E. faecalis A25707 (ATCC 29212)	0.004	*E. coli* A9751 (ATCC 33176)	>500
E. faecalis A25708 (ATCC 33186)	0.016	*Klebsiella pneumoniae* A9664	>500
Staphylococcus aureus A9537	0.008	*K. pneumoniae* A20468	>500
S. aureus A20698	0.008	*Proteus vulgaris* A21559	>500
S. aureus A24407 (ATCC 29213)	0.008	*Pseudomonas aeruginosa* A9843	>500
Bacillus subtilis A9506-A (ATCC 6633)	0.002	*P. aeruginosa* A20235 (ATCC 23389)	>500
Escherichia coli A15119	>500	*P. aeruginosa* A21508 (ATCC 27853)	>500

ip with either saline (control mice) or doses of kedarcidin once daily for five consecutive days beginning one day post-tumor inoculation. The effective dose levels ranged from 3.3 to 30 μg/kg, with the maximum effect (55% increase of life span) achieved at a dose of 30 μg/kg on a day 1 to 5 treatment schedule. Prolongation of survival of mice inoculated (ip) with B16 melanoma (Table 6) was observed at dose levels ranging from 2 to 128 μg/kg, with the maximum effect (106% increase of life span) was observed at a dose of 8 μg/kg on a day 1 to 9 treatment schedule. The results given in Tables 5 and 6 demonstrate that kedarcidin is a potent antibiotic displaying *in vivo* antitumor activity against murine leukemia P388 and B16 melanoma.

The antimicrobial spectrum of kedarcidin was determined by serial broth dilution method using nutrient broth (Difco). The results are summarized in Table 7. Kedarcidin was effective against Gram-positive bacteria but inactive against Gram-negative bacteria tested.

Conclusion

Strain L585-6 (ATCC 53650), an unidentified and unknown actinomycete, was found to produce a new antitumor antibiotic. This compound, designated kedarcidin, possesses potent *in vivo* activity against P388 leukemia in CDF_1 mice and B16 melanoma in BDF_1 mice. Kedarcidin also possesses potent *in vitro* activity against Gram-positive bacteria. The production of kedarcidin by strain L585-6 in shake flask and fermenter cultures was over 1 mg/ml. This titer is comparable to the production of macromomycin by *Streptomyces macromomyceticus* (800 μg/ml)[18] and neocarzinostatin by *Streptomyces carzinostaticus* (500 μg/ml)[19]. The isolation and physico-chemical characteristics of kedarcidin will be described in the following paper.

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