CL307-24, A NEW ANTIBIOTIC COMPLEX FROM *Saccharopolyspora aurantiaca* sp. nov.

I. TAXONOMY, FERMENTATION AND PURIFICATION

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CL307-24, a complex of new antibiotics has been isolated from the fermentation broth of *Saccharopolyspora aurantiaca* sp. nov. The complex was purified by cation-exchange and hydrophobic interaction chromatographies. It was then resolved as one major and three minor components by silica gel chromatography and HPLC.

In the course of a screening program for pesticidal substances from actinomycetes, we have isolated 30 strains which produced insecticidal activities[1]. All of these strains, except one, produced known metabolites such as avermectins (1 strain), piericidin (6 strains) or valinomycin (22 strains). A complex of new compounds was isolated from the culture broth of the remaining strain, CL307-24. The metabolites produced by this strain were orally active against larval and adult *Musca domestica*, and showed antimicrobial activity against some species of coryneform bacteria and *Saccharomyces cerevisiae* by inhibiting oxidative metabolism.

This paper reports the taxonomy of the producing organism, as well as the production and purification of the CL307-24 complex.

Physico-chemical properties and biological activities of the CL307-24 complex are described in the accompanying paper[2].

Materials and Methods

Taxonomic Studies

The cultural characteristics of the microorganism which produces the CL307-24 complex were determined following the method of SHIRLING and GOTTLIEB[3]. The cultures were incubated at 27°C for 14 days and were observed at regular intervals. Microscopic and macroscopic examinations were carried out using GAPY (glucose 1%, soluble starch 2%, yeast extract 0.5%, soya peptone 0.5%, $CaCO_3$ 1% and agar 1.5%)[4], ISP2 (yeast extract-malt extract agar) and CZAPEK's media.

Whole-cell hydrolysates were prepared and analyzed according to the method of KUTZNER[5]. The presence of mycolic acid was determined as described by DAFFE *et al.*[6].

Fermentation

The fermentation was carried out in a 14-liter fermentor containing 10 liters of a production medium composed of soluble starch 4%, yeast extract (Difco) 1%, soybean meal (Sio) 1%, KH_2PO_4 0.05%, $MgSO_4$

0.03% and $CaCO_3$ 0.2% in 1 liter tap-water and adjusted to pH 7 using NH_4OH before sterilization by autoclaving for 30 minutes at 121°C.

A spore suspension of a 7 day old culture grown on GAPY medium was made in sterile distilled water. One liter Erlenmeyer flasks containing 200 ml of a seed medium were inoculated using 5 ml of the spore suspension. The seed medium consisted of soluble starch 3%, glucose 0.5%, soybean meal (Sio) 1.5%, corn steep liquor (Roquette) 0.5%, yeast extract (Difco) 0.2%, NaCl 0.3%, $MgSO_4$ 0.05% and $CaCO_3$ 0.3% in 1 liter tap-water, adjusted to pH 7 using NH_4OH before sterilization by autoclaving for 20 minutes at 121°C. The flasks were incubated at 27°C on a rotary shaker at 210 rpm for 72 hours. One liter of the seed culture was used to inoculate the fermentor and fermentation was carried out for 6 days at 27°C under the following conditions: agitation at 500 rpm, aeration at 350 liters/hour and pH regulation at 7.5 using 1 N acetic acid.

Assay Procedures

Antibiotic activity was monitored by biological and chromatographic methods during the fermentation and the purification procedures. Biological activities were detected by a paper-disc agar diffusion method using *S. cerevisiae* FL200 grown in YP-glycerol medium: yeast extract (Difco) 1%, Neopeptone (Difco) 1%, glycerol 1% and agar 1.5%.

Resolution of the CL307-24 complex was performed by TLC or HPLC. The fractions to be analyzed were applied onto a silica gel 60 plate (Merck, France) and developed using a solvent system composed of $CHCl_3$ - MeOH - 20% NH_4OH (80 : 15 : 5). The CL307-24 compounds were detected by spraying with 50% H_2SO_4 and heating at 150°C.

The HPLC system employed a Gilson pump (model 303), a manometric module (model 802C, Gilson Medical Electronics S.A., France) and a Rheodyne injector (model 7125, Rheodyne Inc., Cotati, CA, U.S.A.). The column effluents were monitored using a Beckman 156 refractive index detector (Beckman Instruments, France). The CL307-24 complex was resolved using a cyano column (25 × 0.6 cm) (SFC, France) with a mobile phase made up with hexane - $CHCl_3$ (56 : 44) with triethylamine (5 mM) at a flow rate of 2 ml/minute.

Compounds CL307-24 I, II, III and IV have the respective Rf values of: 0.63, 0.68, 0.55 and 0.53 for TLC, and the respective K' values of: 2.7, 1.6, 4.2 and 5.15 for HPLC.

Extraction and Purification

The fermentation broth (10 liters) was centrifuged at 3,200 × *g* for 15 minutes. The cake was extracted twice using 1 liter of MeOH each time. The two extracts were pooled with the supernatant, adjusted to pH 3 and applied to a 1 liter Amberlyst A15 (H^+) column (Rohm and Hass, France) which was then washed with water (2 liters). The CL307-24 complex, eluted with 4 liters of 2 N HCl - MeOH (1 : 1), was neutralized with concentrated NaOH and was absorbed on 500 ml of Amberlite XAD-2 (Rohm and Hass, France) in batch conditions with agitation for 1 hour. The resin was then packed into a column and was washed with 1 liter of 50% aqueous MeOH. The active fractions were eluted with 1 liter of MeOH and concentrated under reduced pressure to remove MeOH. The concentrate was extracted twice with 200 ml of $CHCl_3$. The organic layers were pooled and concentrated to near dryness under reduced pressure. The resulting oily extract (about 1 g) was solubilized in 1 ml of $CHCl_3$ and was applied to a 60 g silica gel column.

Elution was carried out with $CHCl_3$ - 1-Propanol (95 : 5) to obtain 3 fractions: 1 (CL307-24 I), 2 (CL307-24 I, II) and 3 (CL307-24 I, II, III, IV) as monitored by TLC. Fraction 1 containing CL307-24 I was dried under reduced pressure. The solid thus obtained was resuspended in a small volume of $CHCl_3$ and was precipitated with MeOH to yield 20 mg of CL307-24 I as a pure amorphous white powder. To obtain pure CL307-24 II, fraction 2 was processed by HPLC using a PRP1 column (30.5 × 0.7 cm) (Hamilton Company, Nevada, U.S.A.) using a mobile phase made with MeOH - 5 mM triethylamine (97 : 3) at a flow rate of 2 ml/minute. CL307-24 III and IV were isolated from fraction 3 by the same HPLC conditions, using a mobile phase of: MeOH - 5 mM triethylamine (95 : 5). The active fractions containing the compounds CL307-24 II, III, IV, respectively were dried under reduced pressure, resuspended in a small volume of $CHCl_3$ and further purified by precipitation with MeOH.

Results and Discussion

Taxonomy of the Producing Strain

The cultural characteristics of strain CL307-24 are shown in Table 1. The strain produced a substrate mycelium which fragmented on all of the media tested and was colored only in GAPY medium. Rare, white aerial mycelia were observed on GAPY, ISP2, ISP3 (oatmeal agar) and nutrient agar media. On CZAPEK's agar medium, aerial mycelia were more abundant but nevertheless remained moderate. Spores were borne in straight to flexuous chains on aerial mycelia (Fig. 1). Spores were ellipsoidal to cylindrical, $0.4 \sim 0.6 \times 1.0 \sim 1.2\,\mu m$, with a smooth sheath-like covering (Fig. 2). Sporangia, flagellated spores and sclerotia were not observed. The presence of *meso*-diaminopimelic acid, arabinose and galactose in whole cell hydrolysates indicated a type IV cell wall[7]. The carbon utilization pattern and other physiological characteristics of strain CL307-24 are shown in Tables 2 and 3, respectively.

Strain CL307-24 was assigned to the genus *Saccharopolyspora* as cited in the BERGEY's Manual of

Table 1. Cultural characteristics of strain CL307-24.

Medium	Mycelial growth	Aerial mycelium	Reverse color	Soluble pigment
GAPY	Abundant-orange	Poor-white	Brown-yellow	None
Yeast extract-malt extract agar (ISP2)	Good-colorless	Poor-white	Brown-yellow	None
Oatmeal agar (ISP3)	Moderate-colorless	Very poor-white	Brown-yellow	None
Inorganic salts starch agar (ISP4)	Moderate-colorless	None	Brown-yellow	None
Glycerol-asparagine agar (ISP5)	Moderate-colorless	None	Brown-yellow	None
Peptone-yeast extract-iron agar (ISP6)	None	—	—	—
Tyrosine agar (ISP7)	Moderate-colorless	None	Brown-yellow	None
Nutrient agar	Poor	Poor-white	Brown-yellow	None
BENNETT's agar	Moderate-colorless	None	Brown-yellow	None
CZAPEK's agar	Good-colorless	Moderate-white	Brown-yellow	Orange-yellow

Fig. 1. Scanning electron micrograph of spore chains of strain CL307-24 grown on GAPY medium incubated for 14 days at 27°C (× 5,000).

Bar represents 10 μm.

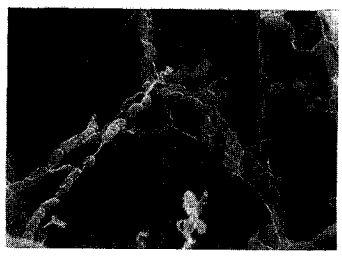


Fig. 2. Transmission electron micrograph of spores of strain CL307-24 grown on GAPY medium incubated for 14 days at 27°C (× 10,000).

Bar represents 0.5 μm.

Table 2. Utilization of carbon sources by strain CL307-24.

Carbon source	Growth	Carbon source	Growth
D-Glucose	++	Salicin	−
D-Fructose	++	Saccharose	+
D-Xylose	−	Raffinose	−
L-Arabinose	++	Lactose	++
D-Galactose	++	Glycerol	++
L-Rhamnose	−	Starch	++
Inositol	−	Cellulose	−
D-Mannitol	−	Chitin (colloidal)	+

++ Good utilization.
+ Poor utilization.
− No utilization.

Table 3. Physiological characteristics of strain CL307-24.

Test	Reaction[a]	Test	Reaction[a]
Temperature[b]:		Hydrolysis of:	
Range	24 to 37°C	Adenine	+
Limit	42°C	Cellulose	−
Optimum	37°C	Chitin	−
NaCl tolerance[b]	10%	Starch	+
pH tolerance[b]	pH 7 to 9	Urease production	+
Anaerobiosis[b]	—	Skim milk	Peptonization and coagulation
Melanin formation	—	Liquefaction of gelatin	+
H_2S production	—	Resistance to lysozyme	−
Nitrate reduction	+		

[a] Observations after incubation for 21 days at 27°C except for the temperature range study.
[b] GAPY medium.

Systematic Bacteriology[8]. Among the seven species recently described in this genus, *S. taberi*[9] does not produce aerial mycelium and *S. hirsuta*[10], *S. erythraea*[11] and *S. spinosa*[12] can be distinguished from strain CL307-24 by their spore surfaces, hairy for *S. hirsuta* and spiny for *S. erythraea* and *S. spinosa*. *S. rectivirgula*[9], *S. gregorii* and *S. hordei*[13] show a smooth spore surface like strain CL307-24. Cultural and physiological characteristics and the carbon utilization pattern of strain CL307-24 were compared to those of the last three species cited. Table 4 records the principal differences which distinguish CL307-24 among these strains.

This comparison indicates that strain CL307-24 is a different species than those already described in the genus *Saccharopolyspora*. Therefore, it seemed appropriate to designate it as *S. aurantiaca* sp. nov., in reference to the color of the mycelium on GAPY medium. The strain CL307-24 was deposited at the collection de Bactéries de l'Institut Pasteur, Paris, France, and was assigned as IP3034.92.

Production of CL307-24 Complex

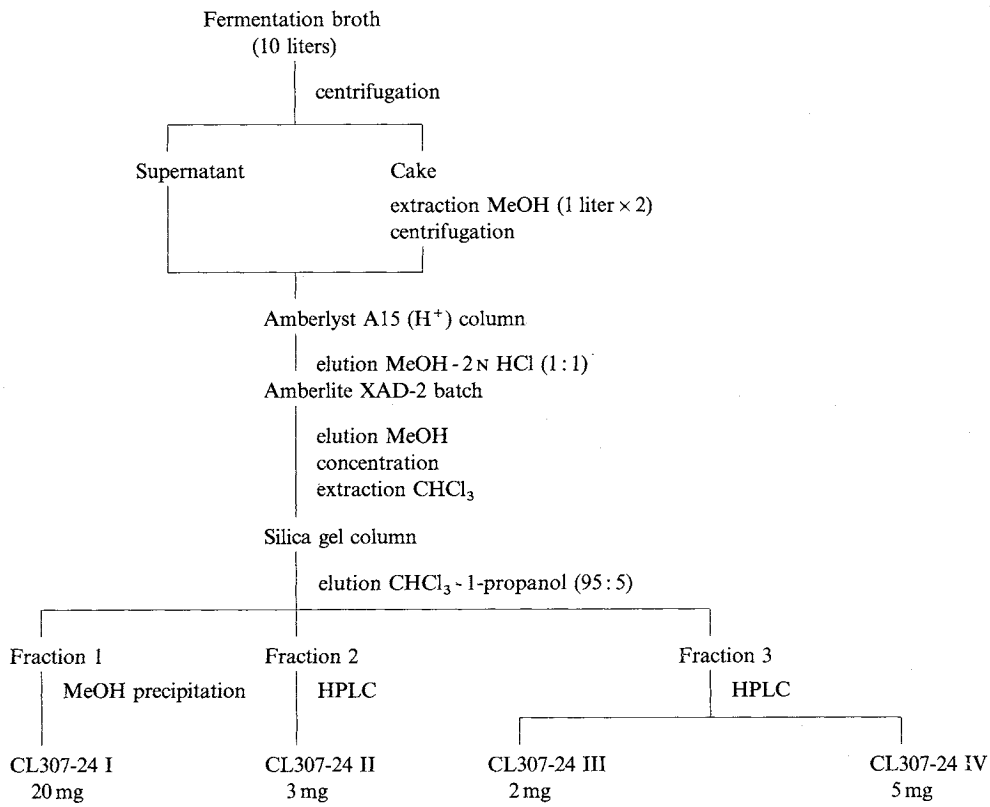
Fermentation of strain CL307-24 was conducted in a 14-liter fermentor containing 10 liters of production medium. The antibiotic activity produced in this medium was monitored using *S. cerevisiae* grown on glycerol as the carbon source. The pH of the culture increased until the threshold of 7.5 was reached on the third day and was thereafter regulated by acetic acid. The pO_2 slowly decreased until the fourth day, then began to increase indicating the end of growth. The antimicrobial product, were first

Table 4. Principal differences among the physiological characteristics of *Saccharopolyspora* spp. and strain CL307-24.

Test	Strain CL307-24	*S. rectivirgula* ATCC 33515	*S. gregorii* NCIMB 12823	*S. hordei* NCIMB 12824
Substrate mycelium[a]	Abundant-orange	Abundant-orange	Moderate-yellow	Poor-colorless
Aerial mycelium[a]	Poor-white	Moderate-pink	Poor-white	None
Soluble pigment[b]	Orange-yellow	None	None	None
Temperature range	24 to 37°C	37 to 60°C	10 to 35°C	20 to 60°C
NaCl tolerance	10%	10%	>13%	10%
Urease production	+	+	−	−
Utilization of:				
D-Xylose	−	+	+	+
L-Rhamnose	−	−	+	+
Mannitol	−	+	+	+
Lactose	+	+	−	−
Starch	+	−	−	+

[a] GAPY medium.
[b] CZAPEK's agar.

Fig. 3. Extraction, isolation and separation of CL307-24 complex.

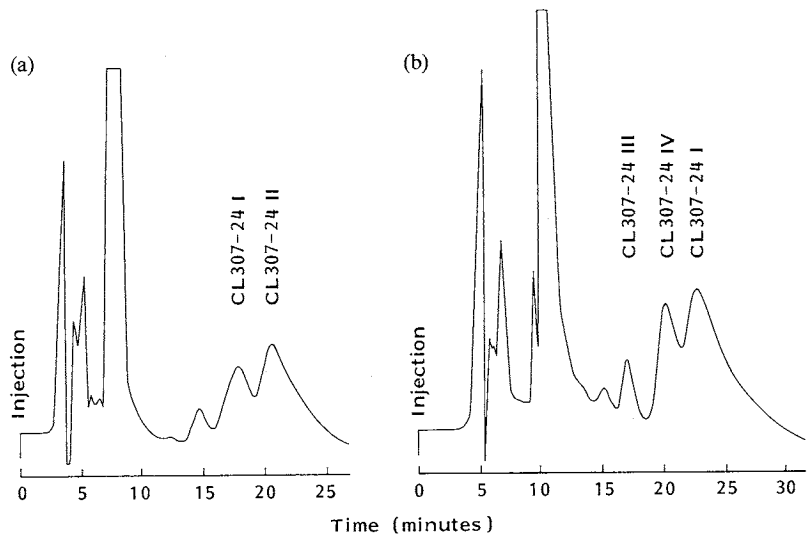


detected in the culture on the fourth day and increased to 20 μg/ml by the sixth day.

Isolation and Purification

The isolation and separation procedures for the CL307-24 complex are outlined in Fig. 3. Purification was based on the different physico-chemical properties of the four components of the CL307-24 complex,

Fig. 4. HPLC chromatogram of fractions 2 and 3 of the CL307-24 complex eluted from the silica gel column.



Column PRP1 (30.5×0.7 cm); mobil phase: MeOH-5 mM triethylamine: (a) (97:3), fraction 2 (Fig. 3), and (b) (95:5), fraction 3 (Fig. 3).

i.e. cationic (A15 (H^+) resin), hydrophobic (XAD-2 resin) and polar properties (silica gel). CL307-24 I, the major active metabolite was first eluted during silica gel purification, and precipitated by MeOH. The remaining minor components were further purified as CL307-24 II, III, IV by HPLC (Fig. 4).

The yields of pure CL307-24 I, II, III and IV from 10 liters of whole broth were 20, 3, 2 and 5 mg, respectively.

The details of the physico-chemical and biological properties of the CL307-24 complex are described in the another paper[2].

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