CHILAPHYLIN, A NEW ANTIBIOTIC PRODUCED BY *STREPTOMYCES MELANOSPOROFACIENS* STRAIN CHILEA

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A soil isolate, designated *Streptomyces melanosporofaciens* strain Chilea, differentiated from the type strain *Streptomyces melanosporofaciens* ISP 5318 mainly by its compact spirals, positive sucrose utilization and different pattern of antibiotics, was found to produce a new antibiotic proposed as chilaphylin. Chilaphylin is a neutral, probably aliphatic compound, active *in vitro* against Gram-positive bacteria including *Mycobacterium*, and different from melanosporin and elaiophylin, the main antibiotics produced by the type strain ISP 5318.

Streptomyces melanosporus, first described by ARCAMONE *et al.*[1] and redescribed as *Streptomyces melanosporofaciens* ISP 5318[2] is known to produce two antibiotics, melanosporin and elaiophylin, different in physico-chemical and biological properties. A microorganism isolated from a Chilean soil, which showed very similar but not identical properties, was designated as *Streptomyces melanosporofaciens* strain Chilea. This strain produces melanosporin, but only traces of elaiophylin. Instead, it produces a new antibiotic which has been denominated chilaphylin, and which is also produced, but to a lesser extent, by strain ISP 5318. This paper describes some characteristics of strain Chilea and of chilaphylin, which differentiates them from the type strain and from known antibiotics, respectively.

Characters of Strain Chilea

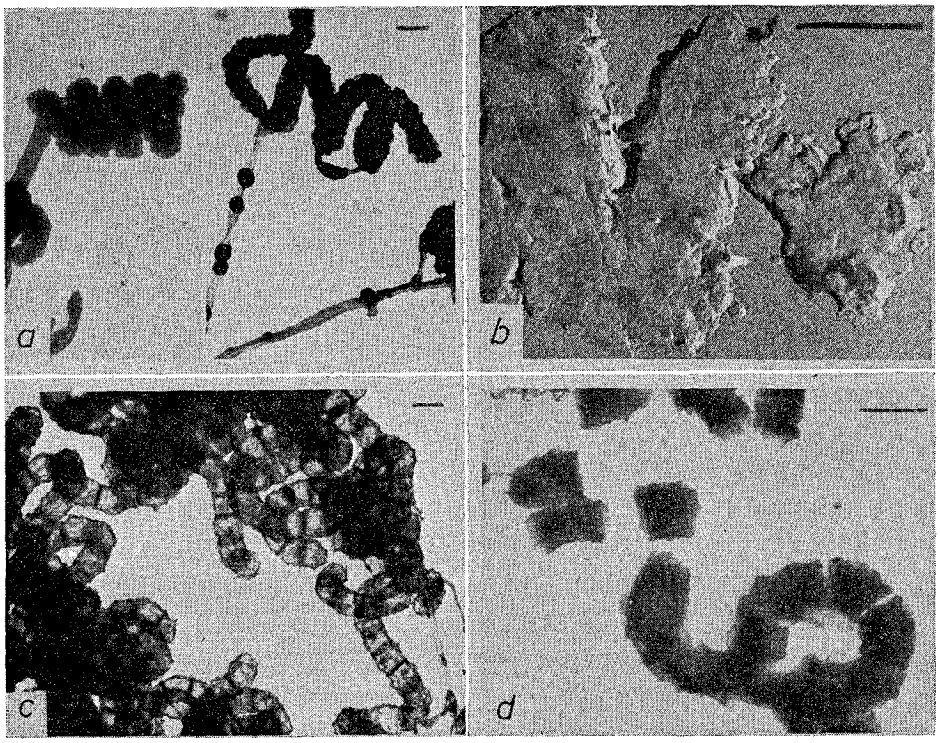
Streptomyces melanosporofaciens strain Chilea was isolated from a soil of northern Chile (Vallenar). The cultural characteristics were studied and compared directly with a culture of *Streptomyces melanosporofaciens* ISP 5318 according to the methods recommended by SHIRLING and GOTTLIEB[3].

Spore chain morphology: Section Spirales. Spores form close spirals with 2~5 turns, falling into the range of "10~50 spores". This morphology is seen on yeast-malt agar, oatmeal agar, starch-salts agar and glycerol-asparagine agar. Spore surface warty rather than rugose[4]. Spore chain morphology is presented in Fig. 1.

Color of colony: Aerial mass in the gray-color series on yeast-malt agar, oatmeal agar, salts-starch agar and glycerol-asparagine agar. Areas of sporulation gray at first, but sometimes they become moist-black in old cultures (aproximately one month), as spore masses coalesce in a liquid exudate on the aerial mycelium.

Fig. 1. Electron micrographs of spore-chains of *Streptomyces melanosporofaciens* strain Chilea.

a) Spirals from a 18 days culture on glycerol-asparagine agar (direct impression) and b) carbon repligraph.
c) Spirals from a 14 days culture on carbon utilization medium with sucrose (direct impression).
d) Loose spores from a 21 days culture on oatmeal agar (direct impression). Marker represents 1 μ.



Reverse side of colony: grayish yellow on yeast-malt agar, light yellow on salts-starch agar and glycerol-asparagine agar.

Color in medium: melanoid pigments are not formed in peptone-yeast iron agar, tyrosine agar or tryptone-yeast broth. No pigment is found in the medium in yeast-malt agar, oatmeal agar or salts-starch agar. In glycerol-asparagine agar a light yellow soluble pigment is produced.

Carbon utilization: D-glucose, D-xylose, *i*-inositol, sucrose, D-mannitol, D-fructose, rhamnose and raffinose are utilized for growth. Only traces of growth are obtained in L-arabinose.

Strain Chilea differs from strain ISP 5318 mainly in the following aspects: Spore chain morphology of strain ISP 5318 consists generally of loops, incomplete spirals or short spirals with no more than 10 spores per chain. Moist-black character of spore masses is frequent and it is observed earlier, usually within the first week of sporulation. Strain ISP 5318 may produce a reddish-brown non-melanoid pigment in tyrosine agar which is not found in strain Chilea. Strain ISP 5318 does not produce a yellow pigment in glycerol-asparagine agar. Strain ISP 5318 does not utilize sucrose or it produces only traces of growth. The pattern of antibiotics of both strains submitted to the same conditions is different, as will be shown.

Production and Isolation of Antibiotics

Parallel cultures of strain Chilea and strain ISP 5318 were done on a rotatory shaker at 30°C (160 rev/min, 5-cm stroke) in 500-ml Erlenmeyer flasks containing 150 ml of a potato-dextrose broth pH 7.0, for a period of 14 days.

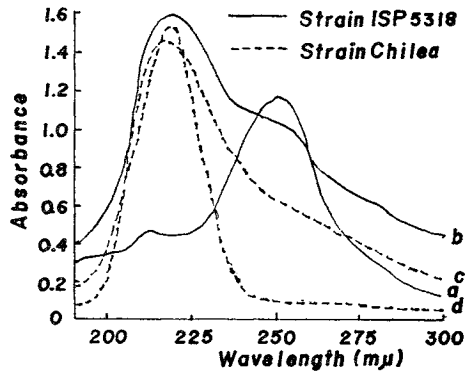
Global antibiotic activity was located by the disc plate assay with *Bacillus subtilis* as test organism, and characterization of the different active fractions was determined by bioautography of paper chromatograms developed in ethyl acetate.

The broth was filtered at pH 7.0 and extracted with ethyl acetate, solvent which was capable to extract selectively a fast moving component (Rf 0.9 in the paper chromatography), detected towards the tenth day of fermentation, approximately. Then a slow moving component (Rf 0.02), which had appeared upon the fourth day of fermentation, was extracted with *n*-butanol from the remaining broth. The same procedure could be followed for mycelium. Butanolic extract was further concentrated at reduced pressure, and the fraction eluted with methanol from Rf 0.10 of a TLC Silica-gel HR Merck (solvent acetone-water, 9 : 1) was found to be melanosporin, the same antibiotic produced by strain ISP 5318, as compared from parallel cultures and from the literature[1,5].

Other active minor fractions from the butanolic extract of strain Chilea were revealed in the TLC: Rf 0.32; Rf 0.67 and Rf 0.80, which could account for the "polyene fractions" mentionned by ARCAMONE *et al*[1].

The fast moving component extracted by ethyl acetate was dried over Na_2SO_4, concentrated at reduced pressure to an oily syrup containing the crude antibiotic, diluted with chloroform and passed through an active acid alumina column. Washing out was first done with chloroform, and then the active fraction was eluted with growing amounts of methanol in chloroform, up to 90 %. The active fraction was concentrated at reduced pressure, passed through a column of Silica-gel S Merck, from which after washing out with chloroform, the antibiotic was eluted with 50 % methanol in chloroform, separating from an inactive fraction absorbing at the same zone of UV, which remained in the column. The active solution was allowed to stand at low temperature (4°C) until white-yellowish, light crystals appeared, which were separated in cold and corresponded to a new antibiotic, which was denominated chilaphylin.

Fig. 2. UV absorption curves of active ethyl acetate extracts redissolved in methanol.
a) and c) correspond to mycelium extracts; b) and d) to broth extracts.



A comparison of the antibiotic production was made between strain Chilea and strain ISP 5318, from the ethyl acetate extracts containing the fast moving components, both for mycelium and broth. From UV spectra of the extracts redissolved in methanol, the different conditions were analyzed (Fig. 2). Even in these conditions, a difference between both strains was observed. The major fraction from broth and mycelium, absorbing at 252 nm, when

purified was shown to be elaiophylin, as compared with a pure sample. Strain Chilea contains only traces of elaiophylin in the mycelium, and nothing in broth. The lower UV absorbing fraction was present in both strains, and when separated from inactive fractions absorbing in the same zone, it was shown to correspond to the new antibiotic chilaphylin. To obviate interferences, the extraction of chilaphylin from strain Chilea was done only from the broth.

Table 1. Antibiotic activity of chilaphylin *in vitro*.

Test organism	Minimal inhibitory concentration (μg/ml)
Staphylococcus aureus ATCC 6538	1
Bacillus subtilis ATCC 6633	1
Bacillus cereus ATCC 11778	10
Bacillus megaterium ATCC 10778	10
Mycobacterium tuberculosis $H_{37}Rv$	1
Salmonella typhosa	>100
Escherichia coli ATCC 26	>100
Alcaligenes faecalis ATCC 4741	>100
Proteus vulgaris ATCC 6380	>100
Torula utilis NRCC 4-900	100
Candida albicans ATCC 10261	100
Trichophyton mentagrophytes LSH 336	>100
Aspergillus niger (NRRL 372)	>100

From a TLC (Silica-gel; acetone-water, 9:1) the different antibiotics produced by both strains can readily be differentiated by revealing with 15% H_3PO_4[6]: melanosporin Rf 0.10, violet-brown on visible light; elaiophylin Rf 0.88, pink; and chilaphylin Rf 0.90 of lemon-yellow color.

Physical and Chemical Properties of Chilaphylin

Chilaphylin is a neutral active compound of lipidic appearance. It crystallizes at low temperatures in form of very fine white-yellowish arborescent star-shaped crystals, melting at room temperature (melting point ~25°C) to a yellowish oil. Refraction index: 1.4688 (20°C). Soluble in chloroform, ether, hexane, benzene, pyridine, ethyl acetate and dimethylsulfoxide, slightly soluble in acetone and lower alcohols, and insoluble in water. Dextrorotatory, $[\alpha]_D^{20}+38°$ (*c* 0.1, chloroform). Its optical rotatory dispersion curve shows a positive COTTON effect (peak~260 nm, λ_0~225 nm). Elemental analysis gave the following results: C 73.85, H 11.66%. It does not contain N, the main difference from melanosporin, and in which it resembles elaiophylin. Molecular weight determinations could not be done for technical reasons.

Ultraviolet maximum varied according to the solvent. Determinations carried out in N_2 atmosphere gave a maximum at 224 nm in heptane, hexane and methanol ($E_{1cm}^{1\%}=225$). In cyclohexane a maximum of 221 nm was observed. It differs importantly from melanosporin ($UV_{max.}$ 234 nm) and from elaiophylin ($UV_{max.}$ 252 nm) in methanol.

Biological Properties

An antibacterial spectrum (broth dilution method) and antifungal spectrum (agar dilution method) of chilaphylin *in vitro* are shown in Table 1. Chilaphylin is active mainly on Gram-positive bacteria, including *Mycobacterium tuberculosis*.

Acute toxicity of chilaphylin *in vivo* was tested in mice averaging 20 g weight, by intraperitoneal injection. The LD_{50} dose was 350 mg/kg.

Discussion

From the characteristics of strain Chilea and type strain ISP 5318 there are differences sufficient only to consider them two distinct varieties of *Streptomyces melanosporofaciens*.

There are some common facts concerning the antibiotic production which relates *S. melanosporofaciens* with *Streptomyces hygroscopicus* var. *azalomyceticus* producing azalomycins B and F[7,8] similar to elaiophylin and melanosporin, respetively. From chemical and biological properties, there is relation of melanosporin to hygrostatin, produced by *Streptomyces hygrostaticus*[5], species from which no description was available. There could be some relation between N-containing, antifungal melanosporin-like antibiotics production and an hygroscopic character of the producing microorganism. More recently, melanosporin has been related[9] to gougeroxymycin and to copiamycin, but these last antibiotics are only antifungal and not active against Gram-positive bacteria as the former.

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