STREPTOVIRUDINS, NEW ANTIBIOTICS WITH ANTIBACTERIAL AND ANTIVIRAL ACTIVITY

I. CULTURE TAXONOMY, FERMENTATION AND PRODUCTION OF STREPTOVIRUDIN COMPLEX

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A new antibiotic complex has been isolated from cultures of *Streptomyces* strain No. JA 10124. On the basis of taxonomic studies, the producing microorganism is described as *Streptomyces griseoflavus* (KRAINSKY, 1914) WAKSMAN *et* HENRICI, 1948, subsp. *thuringiensis* subsp. nov., type strain JA 10124. The antibiotic complex, designated as streptovirudin, was isolated from extracts of both mycelium and culture filtrate. It is a white amorphous material which consists of ten closely related components including streptovirudins A, B, C, D and E. The streptovirudin complex exhibits antibiotic activity against Gram-positive bacteria, mycobacteria, and various DNA- and RNA-viruses.

During the course of our screening program for antiviral antibiotics inhibition of various viruses was found with extracts from *Streptomyces* strain No. JA 10124. The organism was identified as a new subspecies of *Streptomyces griseoflavus* (KRAINSKY, 1914) WAKSMAN and HENRICI, 1948, for which the name *Streptomyces griseoflavus* subsp. *thuringiensis* is proposed. The name "streptovirudin" has been proposed for the antibiotic material isolated from fermentations of JA 10124.[1,2] The antibiotic complex and each of its components appeared to be new and were therefore isolated in pure form. They are active against a variety of Gram-positive bacteria and various DNA- and RNA-viruses. The subject of this communication is the characterization of the producing strain, the fermentative production of the antibiotic complex, its isolation, purification and characterization.

Description of the Producing Strain

The producing microorganism, *Streptomyces* strain JA 10124 was isolated from compost collected near Jena. The taxonomy of this strain has been studied after the recommendations of SHIRLING and GOTTLIEB.[3] Media for the observation of physiological and cultural characteristics were prepared according to SHIRLING and GOTTLIEB,[3] WAKSMAN,[4] and also GAUZE *et al.*[5]

All media were incubated at 28°C for 14~20 days and observed during this time. The colour determinations of the cultures were made with reference to BAUMANN, Farbtonkarte Atlas II recommended by PRAUSER.[6]

Morphological characteristics

Spore chain morphology: Section *Spirales*. Spore chains in the shape of more or less

regular spirals consisting of 2~8 turns, more frequently of 5~6. This morphology can be seen on inorganic salts-starch agar, glycerol-asparagine agar and sucrose-nitrate agar. Spore surface: Spiny.

Table 1. Cultural characteristics of strain JA 10124

Culture medium	Substrate mycelium		Diffusible pigment	Aerial mycelium	
	Growth	Colour on reverse side		Growth	Colour
Oatmeal agar ISP No. 3 SHIRLING and GOTTLIEB[3]	flat, smooth	pale ivory with brownish center Co 7a to Cr 6c, 6t, 7s[6]	none	central area thin powdery	light grey to grey brown Cr 7c to W4
Inorganic salts-starch agar ISP No. 4, SHIRLING and GOTTLIEB[3]	flat	yellowish brown and darker areas Co 6a to 6s	none	velvety with age	white to faint grey W2 to Cr 7c
Glycerol-asparagine agar ISP No. 5, SHIRLING and GOTTLIEB[3]	flat	ivory to brownish-reddish in various shades of colour Cr 7a to Or 6r	none, may become reddish-brown	powdery	white to grey W1 to 2 to Oc 7t
Malt extract-yeast extract agar ISP No. 2 SHIRLING and GOTTLIEB[3]	flat	light brown with brown-violet shining sectors Co 5b, 6b to 6c, R 6r	none	thin velvety	brownish-grey Cr 7t
Tyrosige agar ISP No. 7 SHIRLING and GOTTLIEB[3]	flat	cream-coloured to grey-brown to black-brown Cr 6c to 0 7r	faint brown (may be tinged with red)	velvety	whitish to grey Oc 7c to 7t
Sucrose-nitrate agar. WAKSMAN Medium No. 1[4]	thin, flat, smooth	dark grey-brown 0 6r	none, later may become a brown-red	thin, velvety	whitish to faint grey W1 to Cr 7c
Glucose-asparagine agar, WAKSMAN Medium No. 2[4]	slightly raised	cream-coloured with violet areas Co 6b, R 6r	none	in the central area of the growth powdery to thin velvety	whitish to faint grey 0 7m
Glycerol-calcium malate agar, WAKSMAN Medium No. 7[4]	thin, flat, smooth	transparent colourless, whitish	none	none or thin powdery	white
Glucose-casein digest-yeast-beef agar, WAKSMAN Medium No. 30[4]	wrinkled, waxy	pale brownish, spots with orange or violet tinge Co 6b, 0 7t	none	powdery to thin velvety	whitish to faint grey Cr 7b
Potato-plug, WAKSMAN Medium No. 40[4]	raised, wrinkled	pale brown to medium brown	none	thin velvety	whitish
Sucrose-medium, GAUZE *et al.*, p. 21[5]	heavy, surface, pellicle and flocculent growth at bottom	whitish	none	powdery	white
Glucose-nitrate broth, GAUZE *et al.*, p. 21 +1% Glucose[5]	ring and descending pellicle	whitish	none	traces	white

(continued Table I)

Culture medium	Substrate mycelium		Diffusible pigment	Aerial mycelium	
	Growth	Colour on reverse side		Growth	Colour
Cellulose, GAUZE *et al.*, p. 21[5]	sparse growth appears late	colourless	none	powdery to thin velvety	grey
Skim milk, HABS Medium No. 27[17]	ring and pellicle, waxy	faint brow	absent or may be faint reddish	powdery	white

Incubation of the cultures at 28°C. The observations were recorded after 14 days. The colour determination of the cultures were made with reference to BAUMANNS Farbtonkarte Atlas II (PRAUSER)[6].

Cultural characteristics

The cultural characteristics of strain JA 10124 on various media are presented in Table 1.

Colour of aerial mycelium: In general white, turning to faint grey, at maturity grey, sometimes with a reddish or brownish tinge. Colours specially characteristic on inorganic salts-starch agar, glycerol-asparagine agar, oatmeal agar, malt extract-yeast extract agar.

Colour of reverse side of culture: Cream coloured to brownish on oatmeal agar, and inorganic salts-starch agar; brown on sucrose-nitrate agar and tyrosine agar; reddish colour with violet tinge on glycerol-asparagine agar, malt extract-yeast extract agar, glucose-asparagine agar, glucose-casein digest-yeast-beef agar, glucose-yeast extract agar and the medium 1 of GAUZE *et al.*[5]

Colour in medium: Melanoid pigments are not formed in tyrosine agar (ISP No. 7) or tyrosine agar (WAKSMAN No. 42).[4] In most media no pigment was noted. A faint reddish-brownish pigment appeared in glycerol-asparagine agar, sucrose-nitrate agar and in the tyrosine agar medium.

Physiological characteristics

Physiological properties observed on various media have been summarized in Table 2. So far as the utilization of carbon sources according to the method of PRIDHAM and GOTTLIEB[7] good growth was observed with D-glucose, D-fructose D-arabinose, D-xylose, L-rhamnose, D-mannitol and inositol as shown in Table 3.

These characteristics (as well as those reported in Tables 1, 2 and 3) were compared with the characteristics of known species as described by HÜTTER.[8] According to HÜTTER's treatment, strain JA 10124 is closely related to *Streptomyces griseoflavus*. Strain JA 10124 also bears a resemblance to the species *Streptomyces matensis* MARGALITH *et al.* 1959[9] with respect to the observed reddish pigments and the lack of yellow pigments in vegetative mycelia. HÜTTER[8] considers the latter species as a synonym of *Streptomyces griseoflavus*.

A suggested neotype strain of *Streptomyces griseoflavus* (HÜTTER 1967, 20) is CIFFERI A 28 No. 1118=CBS 409.52=ETH 10249=ISP 5456. This strain also has been described for the International Streptomyces Project (ISP) (SHIRLING and GOTTLIEB.[10] The ISP description conforms generally with that of strain JA 10124 except that the yellow pigmentation of the reverse side of the cultures cited for strain ISP 5456 was not be observed with strain JA 10124. However, according to SHIRLING and GOTTLIEB[11] strains with a yellow colony reverse side lack

Table 2. Physiological characteristics of strain JA 10124

Utilization of malate	± good
Hydrolysis of starch	very good
Coagulation and peptonization of skim milk	+ delayed
Reduction of nitrate	none
Inversion of sucrose	none

Table 3. Carbon utilization of strain JA 10124
Basal medium and method according to PRIDHAM and GOTTLIEB[13]

Good growth	D-Glucose, D-fructose, α-lactose. D-galactose, D-arabinose, D-mannose, D-xylose, L-rhamnose, trehalose, maltose, dextrin, starch, D-mannitol, inositol
Moderate growth	Salicin, sodium acetate, sodium citrate, sodium succinate
Weak growth	Sucrose, aesculin, glycerol
No growth	L-Sorbose, raffinose, inulin, dulcitol, sorbitol

a distinctive substrate pigment. Strain JA 10124 conforms in this respect.

According to the ISP description of *Streptomyces matensis* (SHIRLING and GOTTLIEB),[12] this species shows a reddish and brownish coloured substrate mycelium and diffusible pigments. The production of melanoid pigments was not noted, whereas in the original description MARGALITH *et al.*[9] assign this species to the chromogenic group. With regard to the lack of production of melanoid pigments and the other principal taxonomic characteristics, *Streptomyces matensis* seems to bear a strong resemblance to *Streptomyces griseoflavus* based on comparison of the respective ISP descriptions.

To summarize: Strain JA 10124 identified as a strain of *Streptomyces griseoflavus*. Because of the particular colour of the substrate mycelium—a definite yellow colour never has been observed, but the colour frequently has a reddish tinge— and the particular antibiotic production of streptovirudin we consider strain No. JA 10124 to represent a subspecies of *Streptomyces griseoflavus* and propose the name *Streptomyces griseoflavus* subsp. *thuringiensis* subsp. nov. (The district where the town of Jena is situated is called Thuringia in Latin).

A culture of the type strain has been deposited with the culture collection of the Zentralinstitut für Mikrobiologie und experimentelle Therapie, Jena, DDR, as JA 10124.

Production and Assay

Fermentation Conditions

A soil stock of *Streptomyces griseoflavus* subsp. *thuringiensis*, strain JA 10124, was used to seed 80 ml of inoculum medium in a 500-ml wide-mouth flask. The inoculum medium consisted of 1.5% glucose, 1.5% soy bean meal, 0.03% KH_2PO_4, 0.5% NaCl, 0.1% $CaCO_3$ and tap water. After inoculation the culture was incubated at 28°C on a rotary shaker (180 r.p.m.) for two days. A 5% amount of this seed was used to inoculate the fermentation medium. The fermentation medium consisted of 2% glucose, 1% soy bean meal, 0.5% corn steep liquor, 0.5% NaCl, 0.3% $CaCO_3$, 0.3% sunflower oil or 0.002% Antaphron R (silicon-antifoam agent from VEB Chemiewerk Nünchritz, DDR), pH 6.8 after sterilization. All fermentations were conducted at 28°C, unless otherwise noted. The specified media for the shaken-flask-cultures

were sterilized at 120°C for 35 minutes.

For the preparation of inoculum material for tank fermentations the same inoculum medium was used as described above, and 0.5% of the seed was used to inoculate 20 liters or 150 liters in seed tanks. The seeded medium was agitated (200 r.p.m.) and aerated (1/1/min) at 28°C for 24 hours.

The fermentation medium for tank fermentations (450 liters) was inoculated with 5% of seed from the seed tank. The medium was agitated (350 r.p.m.) and aerated (1/1/min) at 28°C and was harvested on the fourth or fifth day. Antibiotic activity was determined by an agar diffusion cup test[13] with *Bacillus subtilis*, strain ATCC 6633, as test organism.

Antiviral Activity

Vaccinia virus was used as the test material during the course of isolation. Antiviral activity against a number of viruses was determined by plaque inhibition as described by TONEW and TONEW.[14]

Isolation and Purification

Extraction of Streptovirudin Complex from Mycelium

The wet filter cake (about 18 kg from a 450-liter tank fermentation) was extracted twice with 70-liter portions of methanol. The extracts were combined and, after filtration, concentrated *in vacuo* to remove methanol. The resulting suspension was re-extracted twice with 4 liters of 1-butanol. The butanol layer then was concentrated to a volume of 1 liter. The concentrate was held at 4°C overnight to precipitate the streptovirudin complex. The precipitate was collected by filtration and dried to give 19.6 g of crude antibiotic material.

The crude streptovirudin complex (19 g) was suspended in 2 liters of methanol. After filtration the solution was mixed with 40 g of activated carbon. After stirring for 30 minutes the carbon was filtered off and washed with 100 ml of methanol. The carbon then was eluted four times with 1.5-liter portions of methanol-chloroform (1:1) each time. The clarified extracts were combined and concentrated to a volume of 200 ml. The concentrate was allowed to stand overnight at 4°C, and the precipitate (3.2 g of the purified antibiotic) was filtered off. From the mother liquor further material (5.1 g) was obtained after evaporation to dryness.

Extraction of Streptovirudin Complex from Culture Filtrate

A 450-liter amount of culture filtrate was extracted with 80 liters of 1-butanol. The butanol layer then was concentrated *in vacuo* to a volume of 7 liters. The concentrate was allowed to stand overnight at 4°C. The precipitated antibiotic material was filtered off and dried to give 91.4 g of crude streptovirudin complex. Further concentration of the filtrate to a volume of 1 liter afforded about 21 g of similar material. Purification of the crude antibiotic with carbon was performed as described in the previous section.

Purification of Streptovirudin Complex by Column Chromatography

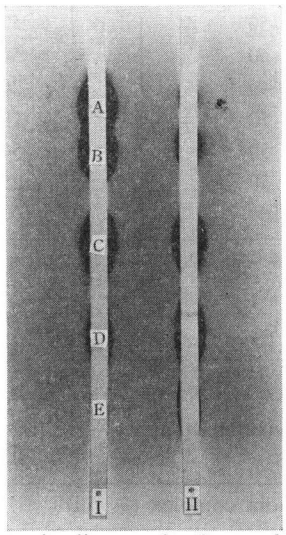
Silicagel (0.05~0.2 mm, Merck) was mixed with chloroform-methanol (1:1) and packed into a column (3×45 cm). Crude streptovirudin complex (250 mg) was dissolved in 10 ml of methanol and, after addition of 10 ml of chloroform, applied on the top of the column. The column was eluted with the chloroform-methanol mixture. Fractions were assayed for anti-

bacterial activity using *B. subtilis*, strain ATCC 6633. Fractions containing bioactive material were combined and evaporated to yield 160 mg of purified streptovirudin complex.

Characterization of the Streptovirudin Complex

As mentioned earlier, preparations of streptovirudin were isolated from extracts of culture filtrates as well as of mycelium. After purification of the crude material with carbon followed by column chromatography, the residues from the bioactive fractions were crystallized from mixtures of methanol and water to give the antibiotic complexes as pure white powders. A typical paper chromatogram of streptovirudin complex is presented in Fig. 1. Two preparations (from mycelium and culture filtrate) have been studied. Chromatograms of either material showed the presence of five bioactive compounds (streptovirudins A, B, C, D and E). Furthermore, the chromatograms shown in Fig. 1 indicate that the preparations from culture filtrate and from extracts of mycelium differ from each other in the content of the components A~E. In further separation studies using Sephadex LH 20 it has been shown that each activity is composed of two different compounds, indicating that the streptovirudin complex consists at present of 10 components. Isolation procedures, chemical properties and biological characterization of the components are described in a separate communication.[15]

Fig. 1. Paper chromatogram of streptovirudin complex



I: Streptovirudin complex from culture filtrate.
II: Streptovirudin complex from mycelium.
Paper: Schleicher & Schüll Nr. 2043 b Mgl.
Solvent system: *n*-propanol-water (1.5:8.5, vol/vol): developed twice, ascending technique. Antibiotics were detected by bioautography with *B. subtilis*, strain ATCC 6633 seeded agar.

The streptovirudin complex is a white, amorphous material which is soluble in methanol and pyridine. It is slightly soluble in water or ethanol and insoluble in chloroform, acetone, benzene, or ethyl acetate. The ultraviolet absorption spectrum of the streptovirudin complex in methanol has a maximum at 212 nm and a shoulder at 258 nm.

The streptovirudin complex was markedly active against representative Gram-positive bacteria. It also was moderately active against mycobacteria, but inactive against Gram-negative bacteria. The antibiotic activity is illustrated in Table 4.

In vitro activity against a number of viruses also was determined.[16] Using an agar-plaque diffusion test the streptovirudin complex was found to be active against vaccinia virus, pseudorabies virus, sheep abortion virus, fowl plague virus, Newcastle disease virus, and Sindbis virus. No activity was observed when tested against columbia SK, Mengo, Coxsackie B1~B5, ECHO type 30 and type 33 viruses, and wild and attenuated types 1, 2, and 3 of polioviruses (Table 5).

In acute toxicity studies in mice the streptovirudin complex was moderately well tolerated

by the oral route. It was, however, more toxic when administered subcutaneously, intravenously and intraperitonealy (Table 6).

Table 4. Antimicrobial activity of streptovirudin complex (Agar diffusion method)

Test organism and strain*	Streptovirudin complex (MIC, μg/ml)	
	from culture filtrate	from mycelium
Bacillus subtilis, SG 119	5	2.5
Bacillus subtilis, ATCC 6633	5	2.5
Micrococcus pyogenes subsp. *aureus*, SG 511	20	10
Bacillus globifer, OH 11	5	2.5
Bacillus mycoides, SG 756	5	2.5
Sarcina lutea, SG 125A	>50	> 50
Escherichia coli mutabile, SG 458	>50	> 50
Mycobacterium phlei, SG 346	40	20
Mycobacterium smegmatis, SG 987	50	10
Saccharomyces cerevisiae, JH 1	50	50
Kloeckera brevis, JH 3	50	25
Penicillium notatum, JP 36	>100	>100

* The epithets and strain designation are those recorded in the culture collection of our institute:
SG VEB Schott & Gen., DDR-69 Jena
JH Jena/Hefen
JP Jena/Pilze
ATCC American Type Culture Collection, Maryland, USA
OH Botanisches Institut Hamburg, BRD

Table 5. Antiviral activity of the streptovirudin complex (plaque diffusion test)

Virus	Streptovirudin complex (μg/ml)			
	250	125	62.5	31.25
Vaccinia	26*	22	18	10
Pseudorabies	30	24	20	16
Sheep abortion	28	23	16	13
Fowl plague	26	18	16	10
Newcastle disease	24	19	16	12
Sindbis	27	19	15	11
Mengo	—	—	—	—

* mm diameter of the inhibition zone of plaque formation.

Table 6. Acute toxicity of streptovirudin complex in mice

Route of administration	LD_{50} (mg/kg)	
	Antibiotic complex from culture filtrate	Antibiotic complex from mycelium
Oral	250	150
Subcutaneous	15	5~10
Intraperitoneal	15~17.5	3.5~7.5
Intravenous	17.5	not measured

The streptovirudin complex was found to be stable at least up to 5 hours at 90°C. No loss of activity was observed on standing for 4 weeks at 37°C.

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