ISOLATION AND CHARACTERIZATION OF NRCS-15, A NEW IRON-CONTAINING ANTIBIOTIC

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(Received for publication July 3, 1973)

A new iron-containing antibiotic named NRCS-15 was isolated from a *Streptomyces* strain. The antibiotic inhibits the growth of Gram-positive bacteria. The antibiotic is similar to the sideromycin group of antibiotics in that it contains iron but it differs in some other characters. Antibiotic NRCS-15 liberated 8 amino and 3 organic acids on hydrolysis. The morphological and physiological characteristics of the producing organism as well as the isolation and properties of the antibiotic are described.

A new iron-containing antibiotic has been isolated from the fermentation broth of *Streptomyces* NRC-S-15 by solvent extraction. It exhibits strong inhibitory activity against Gram-positive bacteria. Although a number of iron-containing antibiotics have been described, the physico-chemical and biological properties of this compound (NRCS-15) indicate it to be a new antibiotic.

This paper deals with the characterization of streptomyces producing strain, the fermentation process, the isolation procedure and the properties of the antibiotic.

Characteristics of the Antibiotic-producing Strain

Streptomyces strain NRC-S-15 was isolated from a sandy soil collected at Riadh, Saudi Arabia. The morphology, cultural characteristics and the physiology of this strain was studied.

1. Morphological properties:

When the organism was grown on various media (Table 1) it formed aerial mycelia which developed into long spirals (Plate 2). The spores were oval with smooth surface lacking spines or hairs (Plate 2).

2. Cultural properties:

Cultural properties on various media[1] are listed in Table 1.

Plate 1. Sporophores of *Streptomyces* NRC-S-15 (×400)

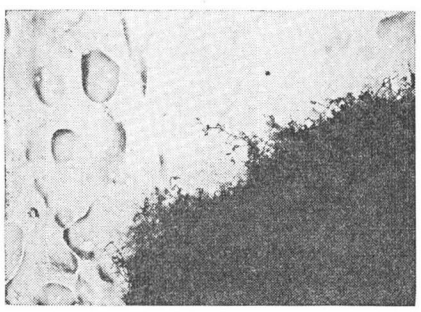


Plate 2. Electron micrograph of spores of *Streptomyces* NRC-S-15 (×16,000)

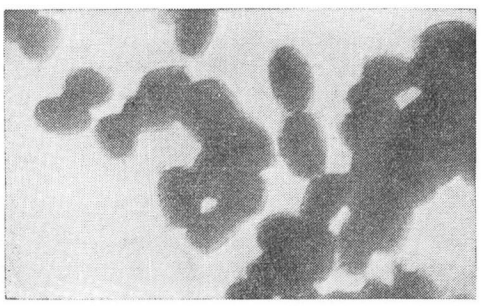


Table 1. Cultural properties of *Streptomyces* strain NRC-S-15 on various media*

Medium	Growth	Aerial mycelium	Soluble pigment
Starch-nitrate agar	deep gray growth	light powdery gray	straw yellow
Sucrose-nitrate agar	light brown~ reddish brown	light gray to deep gray	faint brown
Glucose-nitrate agar	pink growth	powdery gray	pink
Glucose-asparagine agar	purple growth	pale gray	none
Glycerol-nitrate agar	dark brown	white, later becoming gray cottony	none
Glycerol-asparagine agar	purple growth	deep gray	none
Potato plug	reddish brown	deep gray	light brown
Nutrient agar	deep gray	white, later becoming gray	none

* Observations were recorded after 14 days incubation at 28°C

Table 2. Physiological properties of *Streptomyces* NRC-S-15 strain

Production of hydrogen sulfide	−
Tyrosinase reaction	−
Reduction of nitrate	+
Coagulation of skimmed milk	−
Peptonization of skimmed milk	+
Liquefaction of gelatin	+
Cellulytic activity	−

Table 3. Utilization of carbon source by *Streptomyces* NRC-S-15 strain

Utilization	Carbon source
Positive	Glucose, frucose, maltose, lactose, galactose, trehalose, mannitol and starch
Doubtful	Mannose, arabinose, sucrose, sorbose, Melibiose, cellobiose, sorbitol and sodium acetate
Negative	Raffinose, ribose and inulin

3. Physiological properties:

Physiological properties including the utilization of carbon sources[2], are summarized in Tables 2 and 3.

On the basis of these observations, the distinctive features of the strain are as follows: The mature aerial mycelium forms long spirals consisting of chains having smooth-surfaced oval spores. The colour of the vegetative growth on synthetic media is gray to deep gray to reddish brown. A straw yellow or reddish soluble pigment could be detected on various media.

4. Comparison of strain NRC-S-15 with related streptomycetes:

Streptomyces strain NRC-S-15 closely resembles the *flavus* series of streptomycetes based on its cultural characteristics. Consequently it was checked for identity with the known species of this group described by WAKSMAN[3]. In view of this comparison (Table 4), NRC-S-15 strain appears to be most related to this series. However, on the basis of the information available it was not possible to assign it to a specific species.

Production and Isolation

Antibiotic NRCS-15 was produced in shaken flask culture incubated at 28°C on a rotary shaker at 200 r. p. m. The composition of the medium was (g/liter): soluble starch 1.5, glucose 1.0, $NaNO_3$ 0.3, K_2HPO_4 0.12, KCl 0.08, $MgSO_4 \cdot 7H_2O$ 0.06 and $FeSO_4 \cdot 5H_2O$ 0.002. The antibiotic content reached a maximum (120 mg/liter) after 2~3 days.

Table 4. Comparison of strain NRC-S-15 with related *Streptomyces* species

Organism	Morphology on glucose asparagine agar	Spirals	Spore surface	Melanin pigment	Antimicrobial activity
S. aureus	Growth light orange, raised center, hyaline margin. Aerial mycelia light drab.	Closed or open spirals	Spherical to oval	+	+
S. citreus	Growth glossy olive yellow center elevated. Aerial mycelia white to pinkish	Long narrow open spirals	Spherical to oval	−	−
S. alboflavus	Growth restricted much folded, cream coloured with sulfur-yellow surface. No. aerial mycelia. No soluble pigment	Very little tendency to produce spirals	Oval covered by long fine hairs	−	+
S. flaveolus	Aerial mycelia pale gray. soluble gigment yellow green	Short closed and open spirals produced on all media	Oval to elliptical covered with long fine hairs	−	+
S. griseoflavus	Growth citron yellow. Aerial mycelia powdery greenish yellow changing to gray	No spirals produced	Spore oblong covered with short spines	−	+
S. NRC-S-15	Aerial mycelia gray. No soluble pigment	Long monopodially branching chains of spirals	Oval spores with smooth surface	−	+

The broth was harvested and extracted with chloroform at pH 8.0~8.5. The extract was concentrated to a syrupy residue which was purified by dissolving it in a small volume of chloroform. The chloroform solution was chromatographed on an activated charcoal column which was developed with 70 % aqueous ethanol. Active fractions were collected and concentrated *in vacuo* to an oily residue which was dissolved in methanol. An inactive white precipitate was removed by filtration. The methanol solution which contained the active fraction was evaporated until dry. The isolated product was dissolved in chloroform or ethyl acetate and precipitated out of solution with petroleum ether (40~60°C).

Physical and Chemical Properties

Antibiotic NRCS-15 was obtained as an amorphous buff plates that melted at 149~150°C. The results of the elemental analysis were: C 55.82, H 6.33, N 8.08, Cl 2.52 and Fe 1.61 %. As shown in Fig. 1, the ultraviolet absorption spectrum of the antibiotic in 95 % ethanol exhibits a peak at 210 mμ ($(E^{1\%}_{1cm}$ 122.5), in acidic ethanol (95 % ethanol - 0.1 N HCl), at 208 mμ ($E^{1\%}_{1cm}$ 112.3), and in alkaline ethanol (95 % ethanol - 0.1 N NaOH) at 222 mμ ($E^{1\%}_{1cm}$ 1212.5).

The infrared spectrum of antibiotic NRCS-15 (Fig. 2) shows characteristic bands at 3400, 2950, 1660, 1460, 1360, 1280, 1250, 1100, 830 and 750 Cm^{-1}. The R_f values on descending paper chromatograms with different developing solvents are expressed in Fig. 3. Location of the zones occupied by the antibiotic was determined bioautographically using *Bacillus subtilis* as the test

Fig. 1. Ultraviolet spectrum of antibiotic NRCS-15

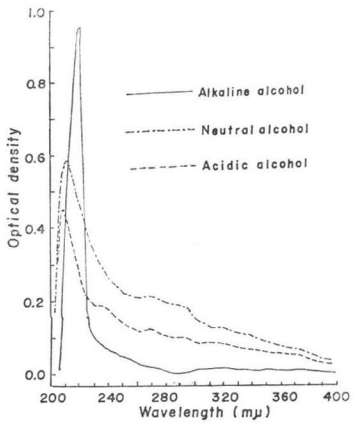
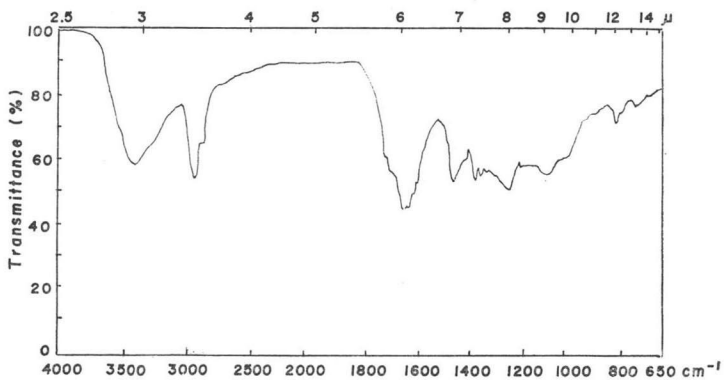


Fig. 2. Infrared spectrum of antibiotic NRCS-15 pelleted in KBr



organism. One definite inhibition zone was always observed.

The antibiotic exhibits an indicator-like property, it is yellow in acid and turns red in alkaline solution. Antibiotic NCRS-15 is soluble in alcohols, esters, acetone and chloroform, slightly soluble in diethyl ether and insoluble in petroleum ether. The antibiotic gives positive results with ninhydrin, biuret, potassium thiocyanate, potassium ferrocyanide and nitration tests, and negative results with acid and alkaline $KMnO_4$, MOLISCH, FEHLING, MILLON, SAKAGUCHI, ferric chloride and iodine solutions.

Eight amino acids and 3 organic acids were detected in the acid hydrolysate (6 N HCl at 105 °C for 24 hours) of the antibiotic. These acids were: lysine, aspartic acid, glutamic acid, threonine, alanine, tryptophan, valine, leucine, oxalic acid, tartaric acid and maleic acid. Tryptophan was detected in the alkaline hydrolysate (concentrated or saturated solution of barium hydroxide at 125~130°C). These products were indentified when chromatographed by paper chromatography using Whatman No. 1 sheets and butanol-acetic acid-water (12 : 3 : 5) as the developing solvent.

Biological Properties

The antimicrobial spectrum of the antibiotic was obtained using agar diffusion and agar

Fig. 3. Behaviour of NRCS-15 when chromatographed with various developing solvents

No.	Solvent used
1	Petroleum ether
2	Diethyl ether
3	Butanol-petroleum ether-H_2O (5 : 2.5 : 5)
4	Ethyl acetate-petroleum ether-H_2O (5 :2.5 : 5)
5	Chloroform-H_2O (1 : 1)
6	Na_2CO_3 (0.5%)
7	Butanol-acetic acid-H_2O (4 : 1 : 5)
8	Na_2HPO_4 (0.5%)
9	Chloroform-petroleum ether-H_2O (5 : 2.5 : 5)
10	Methanol
11	Ethyl acetate-petroleum ether-H_2O (5 : 2.5 : 5)
12	NH_4Cl (3%)
13	Chloroform-ethyl alcohol-A_2O (5 : 2.5 : 5)
14	2N HCl
15	Ethyl acetate
16	Chloroform-ammonia (1 : 1)
17	Ethyl acetate-acetic acid (1 : 1)
18	*n*-Butanol
19	Ethyl acetate-ammonia (1 : 1)
20	Chloroform
21	H_2O
22	Butanol-ethyl alcohol-H_2O (5 : 2.5 : 5)
23	Chloroform-acetic acid (1 : 1)

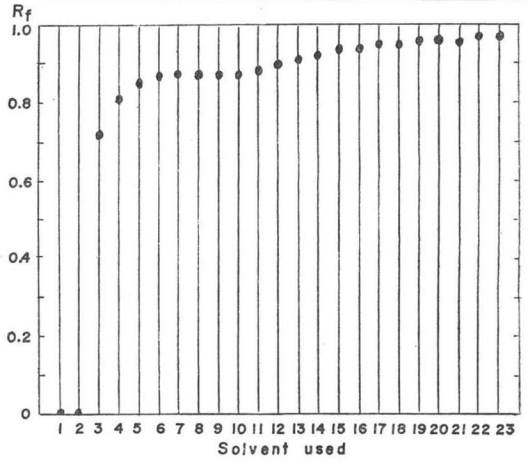


Table 5. Antimicrobial spectrum of NCR-S-15

Agar diffusion method		Agar dilution method*	
Test organism	M. I. C. (mcg/ml)	Test organism	M. I. C. (mcg/ml)
Bacillus subtilis ICC	1.5	*Staphylococcus aureus* FDA 209 P	0.05
Bacillus subtilis 24 G	1.5	*Staphylococcus aureus* Smith	0.025
Bacillus subtilis 19 T	3.12	*Staphylococcus aureus* #193	0.05
Staphylococcus aureus NIHJ	0.8	*Bacillus mycoides*	0.2
Staphylococcus aureus FDA 209 P	<0.18	*Bacillus sphericus*	<0.0003
Saphylococcus aureus T-88	0.8	*Bacillus subtilis*	<0.0003
Bacillus mycoides USSR	12.5	*Escherichia coli* NIHJ	12.5
Escherichia coli NRRL-B-210	>100	*Escherichia coli* Juhl	25
Escherichia coli NICB-8743	>100	*Klebsiella pneumoniae*	6.3
Pseudomonas aeruginosa ATCC 14502	>100	*Pseudomonas aeruginosa*	100
Lactobacillus acidophilus	100	*Proteus vulgaris*	25
Mycobacterium phlei	>100	*Proteus mirabilis*	50
Sarcina lutea	>100		
Saccharomyces cerevisiae NRRL-Y-567	>100		
Candida utilis	>100		
Aspergillus niger	>100		
Aspergillus terreus	>100		

* The agar dilution method was carried out at Bristol Laboratories.

dilution methods of assay (Table 5). NRCS-15 is primarily active against Gram-positive bacteria, weakly against Gram-negative bacteria and not active against yeast and molds.

It is of particular interest to note that the sideramine (ferrioxamine B) failed to antogonize the antimicrobial activity against *Bacillus subtilis* ICC strain of NRCS-15 (Plate 3), ASK-753[4] and ferramidochloromycin (FACM)[5] (iron-containing antibiotics) while it did antagonize the activity of ferrimycin[7] and the antibiotic No. 22765[12].

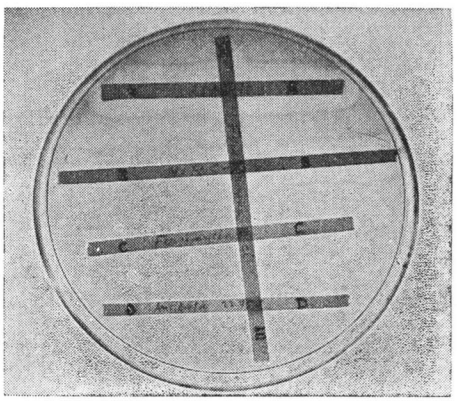
The antibiotic showed *in vivo* activity in mice infected intraperitoneally with *Staphylococcus aureus* Smith at 12.5 mg/kg with toxic manifestation at 60 mg/kg. The subcutaneous LD_{50} was calculated to be 50 mg/kg.

Plate 3. Antimicrobial effects of ferrimycin, Antibiotics NRC-S-15, ASK-753 and 22765 in presence of ferrioxamine B

Test organism: *Bacillus subtilis*, ICC strain

Strip A loaded with ASK-753
〃 B 〃 〃 NRC-S-15
〃 C 〃 〃 Ferrimycin*
〃 D 〃 〃 22765*
〃 E 〃 〃 ferrioxamine B*

* These substances were kindly offered by Dr. H. BICKEL, assistant manager, Ciba Ltd., Switzerland



Discussion

Antibiotic NRCS-15 was isolated from a *Streptomyces* strain which appears to be closely related to the *flavus* series which differs in some morphological, cultural and physiological properties.

The antibiolotic has an indicator like property and in this respect it resembles mycorhodin[8] which have this character but the

two compounds differ in their physicochemical and biological properties.

Antibiotic NRCS-15 is freely soluble in water and most organic solvents, but hardly soluble in diethyl ether and insoluble in petroleum ether. On acid and alkaline hydrolyses the antibiotic yields 8 amino and 3 organic acids which differentiate it from other polypeptide iron-containing antibiotics.

NRCS-15 is unique among the iron-containing peptide antibiotics in that it resembles members of sideromycin antibiotics[7~12] in being a water-soluble polypeptide containing iron. However, it differs from this group in its acid-base character, aromatic nature and chlorine content. Its antimicrobial activity is not antagonized by ferrioxamine B. For these reasons NRCS-15 is considered as a new iron-containing antibiotic.

Acknowledgement

The author wishes to express his gratitude to Dr. MAXWELL GORDON, Director of Research Planning, Bristor Laboratories, Syracuse, New York, for the valuable help which he offered regarding the *in vitro* and *in vivo* evaluation of the antibiotic.

Thanks are alos due to Dr. SAMIR, M. BADR, EL DIN, Microbial Chemistry Laboratory, National Research Center, for his discussion of this manuscript.

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