PRODUCTION OF A NOVEL RED PIGMENT, RUBROLONE, BY *STREPTOMYCES ECHINORUBER* SP. NOV.

I. TAXONOMY, FERMENTATION AND PARTIAL PURIFICATION

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A taxonomic study of *Streptomyces* X-14077 (NRRL 8144), which produces a water-soluble purple-red pigment complex, revealed it to be a new species which has been named *Streptomyces echinoruber* sp. nov. The pigment complex was produced in deep culture fermentation and isolated by solvent extraction and concentration. The major pigment component, rubrolone, has low toxicity and may have potential as a food coloring agent. It appears to be devoid of antibiotic activity.

In the course of our search for non-toxic red pigment of microbial origin for use as food colors, an actinomycete was isolated from a soil sample collected in Argentina. When grown in shake culture on a number of liquid media, this organism produced a beautiful water-soluble, purple-red pigment complex.

This report deals with the taxonomy of this new organism, which we propose to name *Streptomyces echinoruber* sp. nov., and the production, isolation and biological properties of the purified major component, rubrolone, of the pigment complex. A preliminary description of these findings has been reported.[1] Additional studies on the isolation, identification and physicochemical properties of the pigment will be reported in the following paper.[2] Food coloring properties of the pigment will be reported.[3]

1. Taxonomy of the Producing Organism

The organism grows easily in many standard actinomycete media, including the ISP media (Difco) that are recommended by SHIRLING and GOTTLIEB[4] for the description of *Streptomyces* cultures. The characteristics of the aerial growth, spore mass color, color of the reverse substrate mycelium and production of soluble pigment in these various media are described in Table 1. The agar plates were incubated at 28°C, and the results were recorded after 14 days of incubation.

Carbon utilization tests were performed in the ISP-9 medium (Difco). Other physiological tests including sodium chloride tolerance (0, 3, 5, 7, and 10% NaCl), reduction of nitrate, and hydrolysis of casein were carried out by the methods of GORDON and SMITH[5]. Hydrolysis of starch was determined with Actinomyces broth (Difco) containing 0.25% of soluble starch and 2% agar. After incubation the plates were flooded with iodine-KI solution. Gelatin liquefaction was determined according to the method of SKERMAN[6] modified by the use of Actinomyces broth (Difco) instead of nutrient broth. Decomposition of adenine, xanthine, hypoxanthine, and tyrosine were determined by the method of GORDON[7]. The action on skim milk was tested on 10% skim milk powder (Difco) in water.

Table 1. Growth characteristics of strain X-14077 in various agar media

Medium	Characteristics of aerial growth	Spore mass color (a)	Color of reverse substrate mycelium (a)	Soluble pigment (a)
1. ISP–2	Abundant growth; colonies raised, very coarse surface, edges biting into agar. Very good sporulation	ashes (5 *fe*)	burgundy (7 1/2 *pl*)	tomato red (6 1/2 *pe*)
2. ISP–3	Flat colonies, finely coarse surface; about 50% sporulation	fawn (4 *ig*); cedar (6 1/2 *le*) on non-sporulated areas.	rose brown (6 1/2 *ni*)	cedar (6 1/2 *le*)
3. ISP–4	Poor growth and sporulation, finely coarse surface	old wine (7 *ng*)	rose wine (7 *le*)	absent
4. ISP–5	Fair growth; flat colonies, finely coarse surface; variable sporulation	rose beige (4 *ge*)	rose beige (4 *ge*)	absent
5. Thermoactinomyces Fermentation medium (b)	Abundant growth; raised, wrinkled surface, edges slightly biting; good sporulation	(2 *dc*) to covert gray (2 *fe*)	hard to determine due to density of pigment excreted by the culture	redwood (6 *ne*)
6. Tomato paste (c)	Abundant growth; coarse surface. Center of colonies raised; partial sporulation (about 50% of surface)	cloud pink (7 *cb*) to dawn pink (7 *dc*) to pussy willow gray (5 *dc*)	same as in previous medium	barn red (6 *pg*)
7. Glycerol-asparagine (d)	Abundant growth; leathery; center of colonies raised; partial sporulation (about 50%)	natural (3 *dc*) to silver gray (3 *fe*) or ashes (5 *fe*)	same as in Thermoactinomyces medium	coral rose (6 *ic*)
8. Starch casein (e)	Abundant growth; colonies very slightly raised, with smooth edges; good sporulation; sectoring of colonies	natural (3 *dc*) to ashes (5 *fe*)	same as in Thermoactinomyces medium	brick red (6 1/2 *ne*)
9. BENNETT'S medium[14]	Abundant growth; colonies raised and somewhat wrinkled, slightly biting edges; agar cracked. Partial sporulation (about 30%)	natural (3 *dc*) to silver gray (3 *fe*)	cork tan (4 *ie*) to dark brown (4 *pn*)	redwood (6 *ne*)
10. HICKEY and TRESNER medium[15]	Abundant growth; raised, wrinkled, coarse surface, biting edges; partial sporulation (about 50%)	sand gray (3 *cd*) to cloud pink gray (7 *cb*)	same as in Thermoactinomyces medium	brick red (6 1/2 *ne*)
11. Sporulation medium (f)	Abundant growth; wrinkled, coarse surface; edges of colonies biting; poor sporulation (about 5%)	natural (3 *dc*)	same as in Thermoactinomyces medium	cedar red wood (6 *le*)

Table 1. (continued)

(a) The color scheme was taken from the Color Harmony Manual, 4th edition, 1958 (Container Corporation of America, Chicago).

(b) Thermoactinomyces fermentation broth (Difco) to which 1.5% agar was added. This medium is no longer commercially available. The composition is as follows: Tryptone, 0.5%; yeast extract, 0.2%; Soytone, 0.2%; white potato dextrin, 1.0%; D-mannitol, 0.5%; $MgSO_4$, 0.02%; $Fe(NH_4)_2SO_4$, 0.001%; $ZnCl_2$, 2.1 μg/ml; $MnCl_2$, 1.8 μg/ml; $CuSO_4$, 0.2 μg/ml; $Co(NO_3)_2$, 0.5 μg/ml; H_3BO_3, 0.6 μg/ml; pH 7.2.

(c) Glucose, 1.0%; K_2HPO_4, 0.1%; tomato paste, 2.0%; Wilson's Medopeptone, 0.1%; $CaCO_3$, 0.2%; agar, 1.5%; pH 6.8~7.3.

(d) Glycerol, 1.0%; asparagine, 0.1%; K_2HPO_4, 0.1%; agar, 2.0%; pH 7.0. This medium is the same as ISP-5 with the exclusion of the trace salts.

(e) Modified from the medium described by WAKSMAN[13] by the addition of 0.05% $MgSO_4$.

(f) Medium No. 5, American Type Culture Collection Catalogue of Strains, 12th edition, 1976 (American Type Culture Collection, Rockville, Maryland).

Fig. 1. Strain X-14077. Spore chains. 14 days on ISP-3 agar. ×3,000.

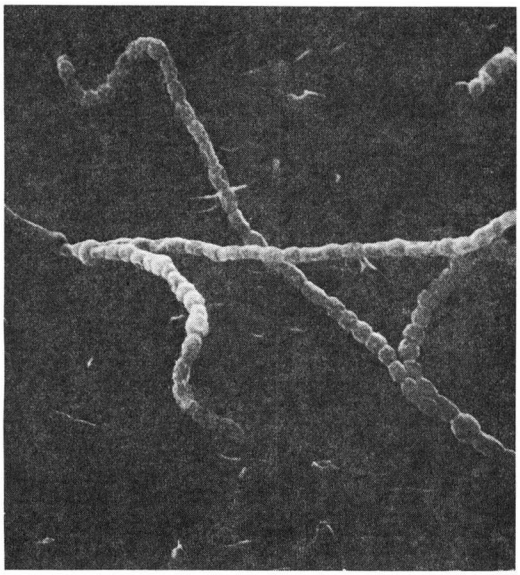
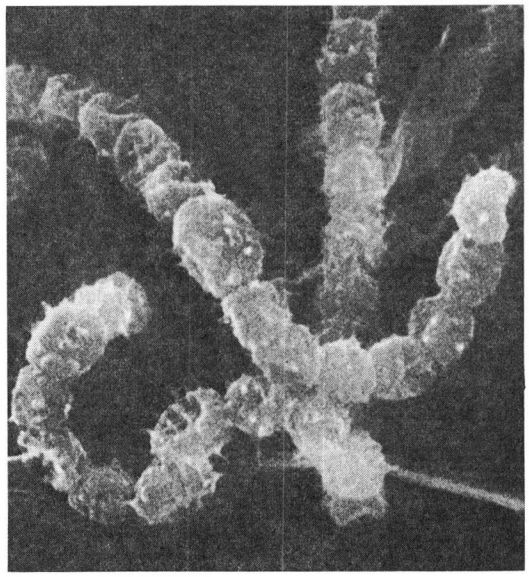


Fig. 2. Strain X-14077. Spore chains. 14 days on ISP-3 agar. ×10,000.



The ability of the culture to grow at various temperatures (10, 20, 24, 28, 36, and 45°C) was determined with tryptone-yeast broth (medium ISP-1, Difco). Incubation was done in a well-regulated water bath with rotary shaking.

With the exceptions indicated below, a 2-day old culture in ISP-1 broth was used as inoculum for the different tests. The seed culture was homogenized, centrifuged, and the pellet resuspended in sterile saline before use as inoculum. In the case of media #5 through #11 (Table 1), the inoculum was taken from a culture grown in Thermoactinomyces Fermentation broth (Difco). Cell wall analysis was performed by the method of BECKER *et al.*[8] Sensitivity to various antibiotics was tested using 6.35-mm commercial antibiotic discs (Baltimore Biological Laboratories, Cockeysville, Md.) with seeded tray of HLR #1 agar (Difco).

Morphological Characteristics.

Strain X-14077 produces a non-fragmenting substrate mycelium and an aerial mycelium forming spore chains. After 11~14 days of incubation at 28°C in various media abundant sporophores can be observed under a microscope with low magnification (Fig. 1). The spore chains have the shape of spirals,

hooks and loops. The spirals are either rudimentary or more tightly coiled, and belong to the *retinaculum apertum* type. Each chain contains more than 10 but less than 50 spores. The spores are globose, measuring from 1.2~1.5 μm by 1.1~1.4 μm, and their surface is spiny (Fig. 2). The cell wall contains the L, L-isomer of diaminopimelic acid. This property, taken together with the above morphological characteristics, show that the organism belongs to the genus *Streptomyces*[9].

The characteristics of the aerial growth, spore mass color, color of reverse mycelium and production of red soluble pigment on various solid media are given in Table 1. A comparison of strain X-14077 with *Streptomyces* species described in BERGEY's Manual[10], NONOMURA's classification[11], and PRIDHAM and LYONS classification[12], shows that the species *S. lucensis*, *S. griseoincarnatus*, and *S. griseorubens* resemble our organism with respect to the gray spore mass color, spiral spore chains, and spiny spore surface. Some similarities are also evident in the pattern of utili-

Table 2. Carbohydrate utilization by the 4 strains included in the study

Utilization of:	Strain			
	X-14077	*S. lucensis*	*S. griseoincarnatus*	*S. griseorubens*
D-Xylose	+	+	+	+
L-Arabinose	+	+	+	+
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Galactose	+	+	+	+
L-Rhamnose	−	−	+	+
D-Mannitol	±	+	+	+
i-Inositol	−	−	±	+
Sucrose	+	+	+	−
Salicin	−	−	±	−
Raffinose	−	−	−	−
Cellulose	−	−	−	−

Table 3. Color of the spore mass and of the reverse substrate mycelium of strain X-14077, *S. lucensis*, *S. griseoincarnatus* and *S. griseorubens* in various media*

Medium	Strain X-14077	*S. lucensis*	*S. griseoincarnatus*	*S. griseorubens*
ISP-2	U: ashes (5 *fe*)	U: ashes (5 *fe*) with white specks	U: off-white to silver gray (3 *fe*)	U: silver gray (3 *fe*) to natural (3 *dc*)
	R: burgundy (7 1/2 *pl*)	R: oak brown (4 *pi*)	R: deep brown (4 *pl*) to oak brown (4 *pi*)	R: cinnamon (3 *le*) to clove brown (3 *ni*) and topaz (3 *ne*)
ISP-3	U: fawn (4 *ig*); cedar (6 1/2 *le*) in unsporulated areas	U: rose beige (4 *ge*) to lead gray (5 *ih*) with white edges	U: shell pink (5 *ba*) to pewter (13 *fe*). Unsporulated areas are tan yellow.	U: pussywillow gray (5 *dc*) and honey gold (2 *ic*) in unsporulated areas
	R: rose brown (6 1/2 *ni*)	R: light beige (3 *ec*)	R: golden brown (3 *pg*) to topaz (3 *ne*)	R: honey gold (2 *ic*)
ISP-4	U: old wine (7 *ng*)	U: ashes (5 *fe*) to white	U: shell pink (5 *ba*)	U: natural (3 *dc*) and silver gray (3 *fe*)
	R: rose wine (7 *le*)	R: sand (2 *ec*) to eggshell (2 *ca*)	R: beaver (4 *li*) to cinnamon (3 *le*)	R: covert gray (2 *fe*)
ISP-5	U: rose beige (4 *ge*)	U: rose beige (4 *ge*) to white	U: white to silver gray (3 *fe*)	U: off-white on maize (2 *ga*) unsporulated areas
	R: rose beige (4 *ge*)	R: natural (3 *dc*) to eggshell (2 *ca*)	R: string (2 *dc*) to covert gray (2 *fe*)	R: maize (2 *ga*)
CZAPEK-DOX	U: rosewood (5 *ge*); cocoa brown (5 *ni*) in unsporulated areas	U: pussywillow gray (5 *dc*)	U: pearl (3 *ba*) to silver gray (3 *fe*)	U: shell tint (3 *ba*)
	R: cocoa brown (5 *ni*)	R: string (2 *dc*)	R: light tan (3 *gc*) to sand (2 *ec*)	R: shell tint (3 *ba*)

* U: upper surface; R: reverse mycelium

Table 4. General morphological and physiological properties of the strains*

Character	Strain X-14077	*S. lucensis*	*S. griseoincarnatus*	*S. griseorubens*
Color of aerial mycelium	gray	gray	gray	gray
Soluble pigment	red	none	none	none
Shape of spore chains	RA	S	S	RA-S
Spore surface	spiny	hairy-spiny	spiny	spiny
Melanin production	−	+	−	−
Starch hydrolysis	+	+	+	+
Nitrate reduction	+	+	+	−
Gelatin liquefaction	+	++	++	+
Casein hydrolysis	+	+	+	−
NaCl tolerance	3~5%	4%	7~10%	7%
Adenine decomposition	+	+	−	−
Xanthine decomposition	−	−	tr.	+
Hypoxanthine decomposition	tr.	tr.	+	+
Tyrosine decomposition	tr.	−	tr.	+
Action on skim milk	no change; final pH, 6.2; pellicle, pink color at top	peptonization; final pH, 6.6; pellicle, brown color at top	slight peptonization; final pH, 6.4; pellicle, tan color at top	slight coagulation; peptonization; final pH, 6.8; pellicle, no pigment**

* Symbols: RA: *retinaculum-apertum* (open loops); S: spira (spirals); tr.: traces.
** Results obtained at 36°C. No growth was observed at 28°C.

zation of various carbon compounds (Table 2). Colors of the growth in various media for the three strains are presented in Table 3. In general terms, growth characteristics of strain X-14077 and of *S. lucensis* are similar. In both cases the colonies are raised, coarse, with well-defined edges. Instead, *S. griseoincarnatus* gives flat colonies with diffuse, feathery edges. Other properties of the four strains are presented in Table 4.

The most striking character of strain X-14077 is the production of the deep cherry-red pigment which freely diffuses into the medium. This pigment is produced in most media tested and it becomes evident after 1 or 2 days of incubation. The other 3 cultures do not produce similar pigment.

Other characteristics in which strain X-14077 differs from one or more of the strains of the other 3 species are the following: shape of spore chains, melanin production, nitrate reduction, NaCl tolerance, the decomposition of purines, and the capacity for growth at the expense of L-rhamnose, *m*-inositol, and sucrose (Tables 3 and 4).

The 4 strains examined in this study show a similar pattern of sensitivity to the antibiotics penicillin, ampicillin, streptomycin, gantrisin, gentamicin, kanamycin, chlortetracycline, chloramphenicol, bacitracin, and streptothricin (Ro 5-4345). Therefore, antibiotic susceptibility has no practical taxonomic value for these microorganisms.

In our opinion, strain X-14077 differs significantly from other species of the genus *Streptomyces*, and therefore we propose the creation of a new species under the name *Streptomyces echinoruber* sp. nov., with strain X-14077 (NRRL 8144) as the holotype.

2. Fermentation and Isolation Procedures

Stock slants of the culture were grown on Bacto Thermoactinomyces fermentation (TAF) medium

No. 0824–01, 24 g/liter plus 2% Bacto-Agar, grown at 28°C for 5 days, by which time sporulation was abundant. A slant was macerated with distilled water to provide fluid inoculum for 500-ml Erlenmeyer flasks containing 75 ml of the desired medium. The flasks were incubated at 28°C on a rotary shaker at 240 rpm for 3 days. This inoculum was used alone, or occasionally combined with about 40 ml of a macerate prepared from the mycelia grown on 20 ml of TAF agar medium cultured 3 days in a 100× 20 mm Petri dish. The combined inoculum was used to seed 12 liters of medium in a 14-liter Chemapec bench-scale fermentor. Typically this fermentor medium was of the following composition, in percent by weight in deionized water:

0.5 N-Z Amine-B (enzyme hydrolysate of animal and milk proteins, Humko-Sheffield Chemical Co., Lyndhurst, N. J.); 0.2 yeast extract (Difco Laboratories, Detroit, Michigan); 0.2 soy protein enzyme hydrolysate (Bacto-Soytone, Difco); 1.0 soluble starch (acid-treated starch, No. 178; Difco Labs); 0.5 mannitol; 1 ml of trace elements solution* per liter of medium; 0.01 antifoam (SAG-4130, trimethyl polysiloxane aqueous emulsion defoamer, Union Carbide Corp.); pH 7.2±0.2.

Incubation was usually at 28°C, with aeration at 4 liters per minute, agitation at 1,200 rpm, and atmospheric pressure. After 66 hours, 11 liters of well-developed growth were transferred to 225 liters of the same fermentor medium described above, in a 400-liter stainless steel fermentor. This fermentor was operated at 28°C, with aeration at 110 liters/min and atmospheric pressure for 42 hours, then at 70 liters/min, agitation at 350 rpm, and at a back pressure of about 0.35 kg/cm^2. An aliquot was sampled periodically for analysis and microbiological control and harvested at 67 hours. For recovery, 4.55 kg of diatomaceous earth (Hyflo Super-Cel, Johns-Manville) were added to the tank contents and the broth was centrifugally filtered on a 102-cm perforated-basket centrifuge using a coarse canvas bag on which a precoat layer of filter-aid had been deposited. The filtrate was extracted 3 times with an equal volume of *n*-butanol. Emulsions formed at each extraction were broken by passage through a Sharples AS–26P centrifuge. Extracts, which contained up to 98% yield of pigment (40 g of pigment A) were pooled and concentrated 20~40 fold under reduced pressure below 50°C in a Rotator wiped film evaporator. A typical concentrate contained 236 g of residue which was then further treated[2] to give pure material.

To follow the amount of pigment produced, the following method of choice was finally adopted: 5 ml of broth supernatant were extracted 1~3 times with 5 ml portions of *n*-butanol with mixing for 15 minutes at room temperature. The *n*-butanol layer was separated by centrifuging for 15 minutes at slow speed. Two aliquots of the pooled butanol phases, one of 20 μl and one of 40 μl, were spotted on a silica gel plate with indicator and the plate developed 10 cm with chloroform - methanol (3: 1) for approximately 40 minutes. The spot at Rf 0.13 was then read by densitometry at 525 nm. Alternatively, the plate could be streaked, developed, then scraped and eluted with methanol, and assayed by absorbance at 525 nm. For quantitative estimations, unknown samples were compared with the best available standard. The $E^{1\%}_{1cm}$ at 525 nm of the purest sample during the fermentation development work was 150, although later preparations were as high as 202.

3. Production and Isolation of Pigment

Strain X-14077 produces one major pigment A (Ro 21–1660) which we have named rubrolone, a minor component B (Ro 21–4958), a third pigment C which has not been obtained pure, and traces of

* Trace elements solution contained, in g/liter, 7.5 $FeSO_4 \cdot 7H_2O$; 7.02 $Fe(NH_4)_2(SO_4)_2$; 4.4 $ZnSO_4 \cdot 7H_2O$; 1.54 $MnSO_4 \cdot H_2O$; 0.314 $CuSO_4 \cdot 5H_2O$; 0.404 $CoCl_2 \cdot 6H_2O$; 0.572 H_3BO_3; water to volume.

several other red compounds. The three pigments A, B and C are readily distinguished by tlc on silica gel F254 plates using chloroform - methanol (3: 1) as a developing solvent. The respective Rf values are A 0.13, B 0.28, and C 0.03.

A titer of 160 mg pigment A per liter has been achieved in 400-liter fermentors, and titers up to 340 mg/liter have been obtained in 14-liter fermentors receiving optimized low aeration (0.05 vol air/vol. broth/min.), high agitation, pH control at 7.2~8.0 and nutrient feeding of concentrated TAF medium and mannitol. Pro Flo (partially defatted cooked cottonseed flour, Trader's Oil Mill Co., Fort Worth, Texas) or O. M. Peptone (soluble meat peptone aqueous concentrate, Oscar Mayer, Madison, Wisconsin) were effective nitrogen substitutes for soytone in total pigment production. Two dextrins, Staley No. 11 and Stadex 60K (A. E. STALEY) were found to be as effective as soluble starch, and less expensive. Sorbose, sorbitol, glycerol and mannose did not support pigment production, while lactose was almost as effective as mannitol. Glucose substitution for mannitol led to the production of a pigment which moved more rapidly on tlc plates than pigment A. A high pigment production in shake flasks was also possible in a medium containing 1.5% debittered dried yeast, 1% cornstarch, 0.1% $CaCO_3$, 0.1% K_2HPO_4 and 3% corn oil, pH 7.0, in addition to the TAF medium.

For recovery, 75~80% of the pigment was removed after a single extraction of broth filtrate with an equal volume of *n*-butanol. The remainder of the pigment could be recovered with a second or third extraction. Such butanol extracts were concentrated under reduced pressure, and further purified by column chromatography, as described separately.[2] Recovery was poor when the broth was extracted with chloroform, carbon tetrachloride, ethyl acetate, methyl isobutyl ketone or hexane.

4. Biological Properties of Pigment A

When tested at a concentration of 1 mg/ml against 19 microorganisms by agar diffusion cup-plate assay, pigment A gave no inhibitory activity. Included in the test organisms were: *Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Klebsiella pneumoniae, Serratia marcescens, Serratia* sp. 101, *Acinetobacter calcoaceticus, Staphylococcus aureus, Sarcina lutea, Bacillus megaterium, Bacillus* sp. E, *Bacillus subtilis, Bacillus* sp. TA, *Mycobacterium phlei, Streptomyces cellulosae, Paecilomyces varioti, Penicillium digitatum, Candida albicans*, and *Saccharomyces cerevisiae*.

The acute mouse toxicity of rubrolone (pigment A, Ro 21-1660/002, lot #3217-122A, with an estimated 80% purity) was found to be $LD_{50}\pm$S. E., mg/kg, >4,000 p. o. and 930±205 i. p. A slightly less pure preparation (Ro 21-1660/l, lot #3217-38AA) gave in mice an LD_{50}, mg/kg of >1,000 p.o. and i.p. At a dose of 250 mg/kg i.p., rubrolone was inactive in the Sarcoma 180 and EHRLICH solid carcinoma experimental mouse models, and also inactive at 250 p.o. in the leukemia L1210 ascites model.

5. Tinctorial Power and Stability

Neutral solutions of one unit of FD&C Red No. 2 (amaranth) appear to be equal to two units of our red pigment. However, at pH 3 and 5 the color intensity of pigment A increases and it becomes equal to that of Red No. 2. The color of unautoclaved and autoclaved solutions at pH 3, 5 and 7 appears stable. At room temperature and pH 6.4 or in 2% citric acid solution, no visible change in color was observed after 1 month. Although pigment A is as stable as Red No. 2 to normal light conditions, it is less stable to strong reducing agents such as ascorbic acid, and it is immediately decolorized in sodium bisulfite

solution. Additional results on tinctorial power will be presented in a forthcoming publication by A. EMODI and H. NEWMARK[3].

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References

1) BERGER, J.; K.E. REICHELT & W. SCHUEP: Certain pyrano-furo-azuleno-pyridines. U.S. Patent 4,057,553, Nov. 8, 1977
2) SCHÜEP, W.; J. F. BLOUNT, T. H. WILLIAMS & A. STEMPEL: Production of a novel red pigment, rubrolone, by *Streptomyces echinoruber* sp. nov. II. Chemistry and structure elucidation. J. Antibiotics 31: 1226~1232, 1978
3) EMODI, A. & H. NEWMARK: to be published
4) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Internat. J. Syst. Bacteriol. 16: 313~340, 1966
5) GORDON, R. E. & M. M. SMITH: Rapidly growing acid-fast bacteria. J. Bacteriol. 66: 41~48, 1953
6) SKERMAN, V. B. D.: A guide to the identification of the genera of bacteria. p. 253 The Williams and Wilkins Co., Baltimore, 1967
7) GORDON, R. E.: The taxonomy of soil bacteria. *In* T. R. G. GRAY & D. PARKINSON (*ed.*), Ecology of Soil Bacteria. pp. 293~321, Liverpool University Press, Liverpool, 1974
8) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
9) WILLIAMS, S. T.; F. L. DAVIES & T. CROSS: Identification methods for microbiologists. Part B, Soc. for Appl. Bacteriol., Tech. Series #2, 1966
10) BUCHANAN, R. E. & N. E. GIBBONS (*ed*): BERGEY's Manual of Determinative Bacteriology, 8th edition. pp. 748~829, The Williams and Wilkins Co., Baltimore, 1974
11) NONOMURA, H.: Key for classification and identification of 458 species of streptomycetes included in ISP. J. Ferment. Technol. 52: 78~92, 1974
12) PRIDHAM, T. G. & A. J. LYONS, Jr.: Progress in clarification of the taxonomic and nomenclatural status of some problem actinomycetes. Dev. Ind. Microbiol. 10: 183~221, 1969
13) WAKSMAN, S. A.: The Actinomycetes. Vol. 2, p. 330, The Williams and Wilkins Co., Baltimore, 1961
14) JONES, K. L.: Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelium is a fluctuating characteristic. J. Bacteriol. 57: 141~145, 1949
15) HICKEY, R. J. & H. D. TRESNER: A cobalt-containing medium for sporulation of streptomyces species. J. Bacteriol. 64: 891~892, 1952