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STUDIES ON XANTHOBACIDIN, A NEW ANTIBIOTIC FROM *BACILLUS SUBTILIS* ACTIVE AGAINST *XANTHOMONAS*[1][2]

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Abstract

An antibiotic with an activity most effective againt *Xanthomonas* was isolated from a soil-born bacterium, which was identified as a strain of *Bacillus subtilis*. The antibiotic is stable and soluble in both water and lower aliphatic alcohols. Based on solubility, stability, antibacterial spectra and other properties, the antibiotic can be distinguished from other known antibiotics produced by *Bacillus*. This antibiotic has been named xanthobacidin.

Introduction

Since tyrothricin, a mixture of gramicidin and tyrocidine, was first isolated from *Bacillus brevis* by Dubos (1939), more than sixty antibiotics produced by *Bacillus* have been reported. Korzybski *et al.* (1967) have written a detail review on this subject.

In the course of a research project designed to isolated the phytopathogen-related antagonists, a soil-born bacillus was obtained. This bacterium produced an antibiotic which was highly effective against *Xanthomonas* and different from other antibiotics previously described. Therefore the organism was characterized and the properties of the active principle against *Xanthomonas* were studied. Based on the results of this study, the antibiotic produced by a strain of *Bacillus subtilis* was identified to be a new antibiotic and named xanthobacidin.

Materials and Methods

Organisms

Xanthomonas oryzae 604 was isolated from the bacterial leaf blight of rice. *X. oryzae* 604^{str} was a streptomycin resistant mutant induced from *X. oryzae*

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604. *Rhizobium phaseoli* was isolated from a nodule of *Phaseolus aureus*. *Micrococcus luteus* was supplied by Sheng-Yu Wu, Dept. of Botany, National Taiwan University. *Streptococcus nonhemolyticus, Staphylococcus aureus* were supplied by Chia-Yu Yang, Taiwan Provincial Junior College of Nursing. *Erwinia carotovora, E. annas, Corynebacterium michiganense, Pseudomonas tolaasi, P. solanaceanum* were supplied by Shih-Tieh Hsu, Dept. of Plant Pathology, National Chung-Hsing University. The following bacteria were obtained from Institute of Botany, Academia Sinica: *Agrobacterium tumefaciens* B6 (from Chi-Cheng Chang), *Sarcina lutea, Mycobacterium pseudotuberculosis* 607, *M. phlei, Bacillus subtilis* pci 617, *B. cereus, Salmonella paratyphi* B, *S. swiseptifer* 2267, *Escherichia coli* NIHJ (from Rong-Yang Wu), *Pseudomonas syringae, Xanthomonas pruni, X. alfalfae, X. vesicatoria, X. citri, X. cyamopsis*, and *X. phaseoli* (from Tsong-Teh Kuo).

Media

Beef extract-peptone (BP) medium contains beef extract, 0.3%; peptone, 0.5%, pH 7.2.

Potato-sucrose (PS) medium contains potato, 200 g; sucrose, 15 g; peptone, 5 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; $Na_2HPO_4 \cdot 12H_2O$, 2 g; and H_2O, 1,000 ml, pH 7.0.

Synthetic medium contains sucrose, 1.5%; $(NH_4)_2HPO_4$, 0.5%; cysteine, 0.01%; KCl, 0.1%; NaCl, 0.1%; $FeCl_3$, 0.001%; $MnCl_2$, 0.0005%; $MgCl_2$, 0.02%, pH 7.5.

Isolation of the antagonistic organism

The antagonistic bacterium, *Bacillus subtilis* N-210, was isolated from soil by double layer plating method. Soil samples were taken from the rice paddy field at Nankang area. *Xanthomonas oryzae* was used as the test organism for screening the antagonistic bacteria.

Preparation of antibiotic

Bacillus subtilis N-210, preserved in PS agar slant, was transfered to liquid PS medium and incubated at 30°C on a rotary shaker for 12 hours. A volume of 0.1 ml of this culture was re-transfered to synthetic liquid medium and grown for 48 hours under the same conditions.

Purification of antibiotic

The cells of 48 hours culture of *B. subtilis* N-210 in synthetic medium were harvested by centrifugation. The antibiotic was precipitated from the supernatant by adjusting its pH to 3.0 with 1.0 N HCl and then collected by centrifugation. This precipitate was dissolved in a solution consisting *n*-propanol : water : ammonium water (6:3:1, v/v/v). The undissolved substance was removed by centrifugation. To the supernatant 0.1 M tris buffer, pH 8.0,

was added to a final concentration of 0.01 M. After propanol and ammonium were removed by rotary evaporation at 40°C, the antibiotic was further purified by Sephadex G-25 column chromatography with 0.01 M tris buffer at pH 8.0 as the eluting buffer. The active fractions from column were pooled and appropriate amount of propanol was added. The antibiotic was then salted out by adding ammonium sulfate to 50% saturation. The propanol extract containing the active antibiotic was collected and dried by evaporation. Pure propanol was added to dissolve the antibiotic. By this step the contaminated salts could be excluded. The antibiotic purified by these procedures has an activity of 50 units (defined below) per μg against *X. oryzae*.

Assay of xanthobacidin

Antibiotic activity was assayed on an agar plate by using the paper disc method. Agar plates were prepared by the double layer method with *Xanthomonas oryzae* 604^{str} as test organism in the presence of 1,000 ppm of streptomycin in the agar. The paper disc (0.6 cm in diameter) was prepared from filter paper (Toppan Shoji Co., Class A).

For each disc 10 μl of antibiotic solution was added with micropipete. Disc was allowed to dry on parpafilm before use. A unit of antibiotic activity in this study was defined as the amount of antibiotic present in the disc which forms a clear zone of 0.1 cm in width around the paper disc after 16 hours of incubation at 30°C.

Characterization of the antagonistic organism

The morphology of the individual cell, endospore, and colony was determined from single cell culture. Properties of the culture in various conditions were also described. The studies of physiological and biochemical properties, including the effects of pH, temperature and salt concentration on growth, the fermentation of carbohydrates and the production of various enzymes were performed according to the methods described by Salle (1954) and Levine (1954).

Results

*Characterization of **Bacillus subtilis** N-210*

This rod shap', spore-forming *B. subtilis* is an aerobic, gram-positive bacterium. The young cells in PS medium grown at 30°C appear either single or double with an average size of 1.1×3.0 micron. The cell stained uniformly with safranin. The cell is motile by means of peritrichous flagella. Endospores are formed in 48 hours in PS medium at 30°C. Sporangia is not swollen. The spore is ellipsoidal, central to paracentral with an average size of

1.03×2.06 micron.

Pellicle is formed on surface of the liquid bacterial culture when it is grown without shaking. Slight sediment was seen in shaking culture. The bacterium grows better in glucose agar than in broth and it grows abundant in potato slant with a brown-yellow color. The bacterium spreads fast on fresh prepared PS plate and gives a dull, flat and rough surface. Moderate growth with echimulate form was seen on agar slant. The bacterium produces slight yellow-green soluble pigment in PS medium and synthetic medium. Citrate can not be ultilized as sole carbon source, while ammonium sulfate but not urea or nitrate can be used as sole nitrogen source.

The bacterium grows normally in 8% NaCl BP broth. At higher NaCl concentration up to 12%, the bacterium can grow but with reduced growth rate which decreases as the NaCl concentration increases. The bacterium grows well at a pH range between 5.0 to 8.0 with the optimum at pH 8.0. Optimum temperature for growth is 30° to 40°. There is acid but no gas from sucrose, glucose and mannitol in fermentation test. There is no acid from lactose or arabinose.

As shown in Table 1, the bacterium hydrolyzes starch, peptonizes milk, produces nitrite from nitrate and forms acetyl-methyl carbinol from glucose-peptone water. It does not liquify gelatin and produces no indole from peptone water. It contains catalase and urease but no oxidase or lecithinase.

Table 1. *Biochemical characteristics of* ***Bacillus subtilis*** *N-210*

Test	Reaction
Gelatin hydrolysis and liquefaction	Negative
Starch hydrolysis	Positive
Peptonization of milk	Positive
Acetyl-methyl-carbinol production (Voges-Proskauer reaction)	Positive
Nitrite from nitrate	Positive
Catalase	Positive
Urease	Positive
Oxidase	Negative
Indole from peptone water	Negative
Lecithinase	Negative
Ultilization of citrate as sole carbon source	Negative

Production of xanthobacidin

Xanthobacidin was isolated from the filtrate of bacterial culture. The amount of antibiotic produced depended on the culture media and growth

conditions used. The antibiotic produced in BP broth is less than that in PS medium or in synthetic medium. The production of xanthobacidin in BP broth was completely inhibited by NaCl at a concentration higher than 6%. The production of antibiotic increased when sucrose or glucose was present in the BP broth. The production of xanthobacidin was directly related to the growth of cells (Figure 1). Spore-formation was not required for the production of xanthobacidin.

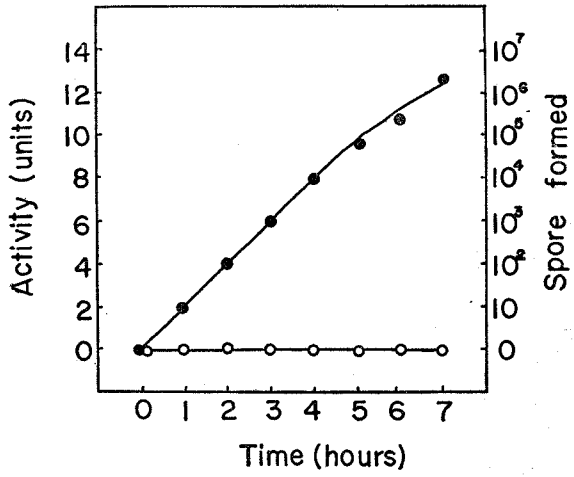

Fig. 1. Production of xanthobacidin. A 16 hour culture of *Bacillus subtilis* N-210 in PS medium was collected and washed twice with fresh PS medium. The cells were transfered to a fresh medium to have a final concentration of 5×10^8 cells/ml and inoculated at 30°C with rotatory shaking. Samples were taken at different period for assay of antibiotic activity and examination of spore formation.
○—○—○: Number of spores formed;
●—●—●: Activity of antibiotic produced.

Properties of xanthobacidin

Purified xanthobacidin exhibited one active spot on paper chromatography in the following solvent systems: (1) distilled water (Rf=0.65); (2) *n*-butanol saturated with water (Rf=0.80); (3) ethyl acetate saturated with water (Rf=0.12); (4) benzene saturated with water (Rf=0).

Xanthobacidin is soluble in water, methanol, 95% alcohol, propanol and butanol but insoluble in acetone, amyl acetate, chloroform, ether, or benzene. The antibiotic in fermentation broth can be precipitated by manipulating the pH value to 3.0. No loss of antibiotic activity is detectable at pH 3.0 or pH 9.0 for a period of two hours at 30°C and by heating in water at 100°C for 10 minutes. The antibiotic is ninhydrin negative and insensitive to papain, trypsin

and pepsin. The antibiotic is dialyzable through cellulose membrane. The antibiotic can not be antagonized by albumin, riboflavin nor affected by EDTA and triton-100.

The antibacterial spectrum is shown in Table 2. Xanthobacidin is rather specific to *Xanthomonas*, *Agrobacterium* and *Rhizobium*. There was no activity against yeast or fungi.

Table 2. *Antibacterial spectrum of the purified xanthobacidin*

Organism	Relative Activity*
Sarcina lutea	0
Micrococcus luteus	32
Streptococcus nonhemolyticus	32
Staphylococcus aureus	32
Mycobacterium pseudotuberculosis 607	0
Mycobacterium phlei	2
Bacillus subtilis pci 617	2
Bacillus cereus	4
Erwinia carotovora	4
Erwinia ananas	4
Corynebacterium michiganense	4
Salmonella paratyphi B	16
Salmonella swiseptifer 2267	32
Escherichia coli NIHJ	32
Pseudomonas tolaasi	2
Pseudomonas solanacearum	2
Pseudomonas syringae	128
Rhizobium phaseoli	1,024
Agrobacterium tumefaciens B6	2,048
Xanthomonas pruni	1,024
Xanthomonas alfalfae	1,024
Xanthomonas vesicatoria	1,024
Xanthomonas citri	1,024
Xanthomonas cyamopsidis	1,024
Xanthomonas phaseoli	4,096
Xanthomonas oryzae	4,096

* Antibiotic was diluted in a series of two-fold dilution. The number given here is the highest dilution factor that still exhibits a bactericidal effect against a given bacterium strain.

Differentiation of xanthobacidin from known antibiotics

Xanthobacidin can be differentiated from 65 known antibiotics from *Bacillus* by means of different solubility, stability, antibacterial spectrum and other

properties. These known antibiotics may have multiple properties that are different from that of xanthobacidin, only one different property between each known antibiotic and xanthobacidin is mentioned here, however.

As shown in Table 3, 4 and 5, biocerin, tyrocidine, gramicidin, gramicidin S, gramicidin J, xanthellin, cerein, tetaine, edeine, licheniformins, subtenolin, brevolin, rhizoctonia factor, polypeptin, nigrin, iturine, esperin, petrin, endosubtilysine, bacilip A and B, and laterosporin A and B can be distinguished from xanthobacidin by their solubility; fungistatin, fungocin, mycosubtilin, datemycin, toximycin, analysine, bulbiformin, simplexin, bacillin, globicin, neocidin, obutin, brevin and fluvomycin can be distinguished from xanthobacidin by their antibacterial spectrum; subtilin, subtilin C, bacilysin, alvein, rhizobacidin, bacitracin, eumycin and bacimethrin can be distinguished from xanthobacidin by their stability.

Polymycins, a group of antibiotics includes the closely related peptides

Table 3. *Previously known antibiotics fron* ***Bacillus*** *which can be differentiated from xanthobacidin by their solubility.*

Antibiotic	Differentiated characteristic	Reference
Biocerin	insoluble in water	Johnson *et al.* (1949).
Tyrocidine	insoluble in water	Dubos and Hotchkiss (1941).
Gramicidine	insoluble in water	Dubos and Hotchkiss (1941).
Gramicidin S	insoluble in water	Winnick *et al.* (1961).
Gramicidin J	insoluble in water	Otani *et al.* (1958).
Xanthellin	insoluble in water	Wachter *et al.* (1951.
Cerein	insoluble in organic solvent	Borowski *et al.* (1955).
Tetaine	insoluble in organic solvent	Borowski *et al.* (1957).
Edeine	insoluble in organic solvent	Kurylo-Borowska (1959).
Licheniformins	insolubie in ethanol	Callow and Hart (1946).
Subtenolin	insoluble in ethanol	Hirschorn *et al.* (1948).
Brevolin	insoluble in butanol	Kawamato and Motomura (1954).
Rhizoctonia factor	insoluble in butanol	Michener and Snell (1949).
Polypeptin	soluble in acetone	Mcleod (1948); Garson *et al.* (1949).
Nigrin	soluble in acetone	Utkin *et al.* (1945).
Iturine	soluble in chloroform	Delcambre (1950).
Esperin	soluble in chloroform	Kochi (1951).
Petrin	soluble in chloroform	Tiffin (1958).
Endosubtilysine	soluble in chloroform	Saint-Rat and Olivier (1946).
Bacilipins	soluble in ether	Newton (1949).
Laterosporins	The solubility in water diminished as the pH increased to 9.0.	Barnes (1949).

Table 4. *Previously known antibiotics from* ***Bacillus*** *which can be differentiated from xanthobacidin by their antibacterial spectrum.*

Antibiotic	Differentiated characteristic	Reference
Fungistatin (Syn-Antibiotic XG)	active mainly against fungi	Hobby *et al.* (1949).
Fungocin	active mainly against fungi	Cercos and Castronovo (1952).
Mycobacillin	active mainly against fungi	Majumdar and Bose (1958).
Mycosubtilin	active mainly against fungi	Walton and Woodruff (1949).
Datemycin	active mainly against fungi	Yajima (1955).
Toximycin	active mainly against fungi	Korzybski *et al.* (1967).
Analysine	active mainly against fungi	Vallee (1945).
Bulbiformin	active mainly against fungi	Vasudeva *et al.* (1958)
Simplexin	active against Gram-positive, Gram-negative and fungi.	Cordon and Haensler (1939); Foster and Woodruff (1946).
Bacillin	active against Gram-positive, Gram-negative and acid-fast bacilli	Foster and Woodruff (1946).
Globicin	active against Gram-positive and acid-fast bacilli.	Quinn (1952).
Neocidin	active against Gram-positive	Tsukamura (1950).
Brevin	active against Gram-positive	Barnes and Newton (1953).
Obutin	active against Gram-positive	Shemyakin and Khokhlov (1953).
Fluvomycin (syn. Vivicil, Riomycin, or Efsiomycin)	active against Gram-positive	Carvajal (1953).

Table 5. *Previously known antibiotics from* ***Bacillus*** *which can be differentiated from xanthobacidin by their stability.*

Antibiotic	Differentiated characteristic	Reference
Subtilin	sensitive to trypsin	Jansen and Hirschmann (1944).
Subtilin C	sensitive to trypsin	Hassall (1948).
Bacilysin	sensitive to trypsin	Newton (1949).
Alvein	sensitive to trypsin	Gilliver *et al.* (1949).
Rhizobacidin	sensitive to pepsin	Korzybski *et al.* (1967).
Bacimethrin	decomposed in acid solution	Tanaka *et al.* (1963).
Eumycin	unstable as pH increased to 8.0	Johnson and Burdon (1946).
Bacitracin (syn. Ayfivin)	unstable as pH increased to 9.0	Johnson *et al.* (1945).

polymyxin A (syn. aerosporin), polymycin B1 and B2, polymycin C, polymycin E1 and E2. polymycin M, circulin A and B, and colistin (syn. colimycin) (Paulus, 1967) can be distinguished from xanthobacidin in that they are all ninhydrin positive.

Pumilin (Bhate, 1955) and megacine (Ivanovics and Alförd, 1954) can be differentiated from xanthobacidin by their non-diffusability in agar. Bacillomycin (Landy *et al.*, 1948), aspergillus factor (Michener and Snell, 1949) and the antibiotic reported by Vuuren *et al.* (1961) are different from xanthobacidin in being non-dialyzable.

Other antibiotics that are different from xanthobacidin are subtilysine (Vallee, 1945), colistatin (Gauze, 1946), and the antibiotic produced by *Bacillus natto* (Ishidate *et al.*, 1949). Subtilysine is of a enzyme nature exhibiting lytic activity against living *E. coli* and *Salmonella exteritidis.* Colistatin is only bacteriostatic but not bactericidal. Antibiotic produced by *B. natto* exhibits marked activity against gram-positive bacteria, especially *Micrococcus pyogenes* var. *aureus.*

Discussion

The isolate that produces the new antibiotic xanthobacidin has been identified as a strain of *B. subtilis* according to the key to the species of genus *Bacillus* in Bergey's Manual of Determinative Bacteriology (7th edition). The characteristics of the isolate, such as the morphology of the vegetative cells, sporangia, and colony as well as the physiological and biochemical properties are similar to those described for *B. subtilis* based on the Bergey's Manual. However, several properties of the isolate, such as the ability to produce soluble yellow-green pigment, the inability to hydrolyze gelatin or to ultilize citrate as the sole carbon source and the absence of acid formation from arabinose in fermentation test, are different from *B. subtilis.*

A large number of antibiotics produced by bacteria belong to peptide. They are usually produced when the cultures have passed through the logarithmic growth phase and begin to sporulate. In these fermentation, the antibiotic production phase is correlated with formation of sporangia and spores, and no antibiotic activity is found in non-sporulating cultures. All of the antibiotic activity (bacitracins, tyrocidins, polymyxins) produced by these sporulating bacteria is associated with the spores and cells, and none apparently is found "free" in the medium (Bodanszky and Perlman, 1969). In the case of *B. subtilis* N-210, the production of xanthobacidin is proportionally related to the growth of cells. The formation of spore is not required for antibiotic production and the antibiotic produced is found mainly in the bacterial filtrate. These properties in addition to a negative ninhydrin test indicates that xanthobacidin apparently is not a peptide antibiotic.

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對枯草菌株 N-210 所生抗生素 Xanthobacidin 的研究

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自土壤中分離的枯草菌株 N-210 爲一抗生素生產菌株，其所生的抗生素對植物病原菌 *Xanthomonas* 屬的細菌具有特別强的抗生作用。根據不同的溶解度，穩定度，抗菌特異性和其他的性質，此抗生素和由 *Bacillus* 菌屬所生的已知抗生素不同，此抗生素乃命名爲 Xanthobacidin。

本文對此菌株及 Xanthobacidin 的性質，抗生素的產生以及純化等方法亦加以說明。