

ISOLATION OF A NEW ANTIBIOTIC, LATEROSPORAMINE  
(STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. XIII<sup>1)</sup>)

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A new antibiotic, named laterosporamine, was isolated from the culture broth of *Bacillus laterosporus* 340-19. The antibiotic is active against Gram-positive and Gram-negative bacteria *in vitro* and *in vivo*. It is a water-soluble basic substance, positive to ninhydrin, SAKAGUCHI's and DRAGENDORFF's reagents. A non-peptidic structure with an approximate empirical formula  $C_{17}H_{35}N_7O_4$  was suggested.

In the course of our screening program for new antibiotics from the genus *Bacillus*, strain 340-19 was found to produce a water-soluble basic antibiotic and a basic acylpeptide antibiotic simultaneously. The water-soluble basic antibiotic active against Gram-positive and Gram-negative bacteria was isolated and named laterosporamine, since the producing organism was identified as a strain of *B. laterosporus* and the antibiotic contained an amine component.

In this paper, the taxonomic characteristics of the producing organism as well as the production, isolation and preliminary characterization of the antibiotic are presented.

#### Taxonomic Characteristics of the Producing Microorganism

The bacterium was isolated from a soil sample of Chaing Mai district, Thailand.

It is a Gram-positive, motile, rod-shaped bacillus ( $0.7\sim 0.8\times 2.5\sim 3.0\ \mu$ ) with rounded ends and produces a dormant endospore ( $0.7\times 1.6\ \mu$ ) which occupies a lateral position in a spindle-shaped, swollen sporangium. After lysis of the sporangium, a parasporal body remains firmly adherent to the spore.

On peptone-beef extract agar, it shows moderate, colorless filiform growth with dull-shining surface, opaque density and soft to butyrous structure. The growth on glucose nutrient agar is thicker than that on nutrient agar, and the surface of the growth becomes wrinkled on aging.

The optimum temperature for growth is about 37°C and the optimum pH is about 6.8~7.6.

The bacterium exhibits weakly facultative anaerobic growth on OF-test with formation of acid but no gas from glucose.

It does not grow in KOSER's and CHRISTENSEN's citrate media or in 5, 7.5 and 10% NaCl broth at 28°C for 5 days.

Gelatin liquefaction is very strong but peptonization of milk is very weak (28°C for 8 days). Starch hydrolysis is not observed at 28°C for 5 days. Nitrite formation from nitrate, urease test, oxidase activity and catalase test are all positive but VOGES-PROSKAUER test (28°C for 1~5 days), indol formation (28°C for 1~7 days) and tyrosinase test are all negative.  $H_2S$  formation on Difco peptone iron agar is negative to negligibly weak.

Mannose, fructose, trehalose and glycerol are attacked with acid but no gas formation.

Glucose and maltose are also attacked but the acid formation is relatively weak. No acid formation was observed from starch, glycogen, inulin, mannitol, sorbitol, salicin,  $\alpha$ -methylglucoside, *m*-inositol, L-arabinose, D-xylose, sucrose and lactose. Negligible amount of acid formation was observed on galactose.

By the criteria of BERGEY'S Manual of Determinative Bacteriology<sup>2)</sup> this bacterium is clearly a *Bacillus laterosporus*. A similar conclusion is obtained by the criteria of WOLF and BERKER.<sup>3)</sup> On the basis of the following characters, this culture is identical with the type strain of *B. laterosporus* ATCC 64: (1) typical parasporal body formation, (2) elliptical spore in central swollen sporangia, (3) Gram-positive, motile, facultative anaerobic rod of middle size, (4) positive activity against gelatin liquefaction; nitrite formation from nitrate; negative activity against starch hydrolysis and VOGES-PROSKAUER-test, (5) acid formation from glucose but not from L-arabinose and D-xylose. The cultural characteristics on agar media are also similar to those of the type strain. Only one difference between them is "acid formation from mannitol", viz the type strain produces but 340-19 does not. Therefore, we decided that this culture is a strain of *B. laterosporus*, and named it *Bacillus laterosporus* No. 340-19.

#### Production and Isolation

Spores of the strain 340-19 were inoculated into 800 ml of a medium consisting of glucose 1.0 %, glycerine 0.5 %, peptone 1.0 %, meat extract 0.5 % and sodium chloride 0.3 %, pH 7.0, in a 2-liter Erlenmeyer flask, which was cultured at 28°C for 24 hours on a rotary shaking machine. The culture was then transferred to a 30-liter jar fermentor containing 15 liters of a medium consisting of glucose 1.0 %, glycerine 0.25 %, peptone 0.25 %, soybean meal 1.0 % and sodium chloride 0.3 %, pH 7.0. Fermentation was carried out at 28°C for 41 hours under aeration of 15 liters per minute and agitation of 250 r.p.m.

The culture broth (ca. 30 liters) was filtered with Hyflo Super-Cel (300 g) at pH 5.0. The filtrate was adjusted to pH 7.0 and passed through an Amberlite IRC-50 (Na) column (2 liters). The column was washed with water and eluted with 0.5 N HCl, being monitored with an *Escherichia coli* assay plate. Active eluate fractions were collected and neutralized with sodium hydroxide to pH 7.0. Activated carbon (Darco G-60, 30 g) was added to the eluate, and the mixture was stirred for half an hour and filtered. The carbon cake was washed with water and eluted three times with 200 ml of 50 % aqueous acetone acidified with hydrochloric acid (pH 2.0).

The extract was evaporated to nearly aqueous solution under reduced pressure, adjusted to pH 8.0 with dilute sodium hydroxide and extracted with *n*-butanol. A basic acylpeptide antibiotic active against only Gram-positive bacteria was in the butanol layer, whereas laterosporamine remained in the aqueous layer. Lyophilization of the aqueous solution gave a crude powder (270 mg) of the antibiotic.

The crude powder was preparatively chromatographed on a filter paper (Toyo Roshi No. 51, 60×60 cm) with *n*-butanol-acetic acid-water (4:1:2) developed continuously by descending manner for 43 hours. The zone of the antibiotic, detected by ninhydrin or SAKAGUCHI reactions in separate tests was cut out and extracted with water. The extract was lyophilized to a residue, which was dissolved in a small amount of methanol and precipitated with acetone, giving laterosporamine hydrochloric acid salt as a hygroscopic colorless powder (100 mg).

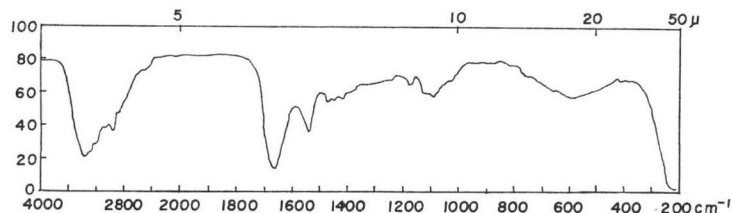
The above preparation showed a single spot on TLC with the following Rf values: Rf *ca.* 0.42 on Avicel with *n*-propanol-pyridine-acetic acid-water (15:10:3:12); Rf *ca.* 0.52 on Silica gel GF with the above solvent; Rf *ca.* 0.14 on Avicel with *n*-butanol-acetic acid-water (4:1:2); and Rf *ca.* 0.04 on Silica gel GF with the above solvent.

### Physical and Chemical Properties

Laterosporamine hydrochloric acid salt is a hygroscopic colorless powder, whose melting point and elemental composition could not be measured because of the hygroscopic nature. Its basic nature was indicated by paper electrophoresis: with buffer solutions of 0.09 M formic acid-2-dimethylaminoethanol buffer pH 4.0 and 0.09 M acetic acid-2-dimethylaminoethanol buffer pH 9.3<sup>1)</sup> at 15 V/cm for one hour, laterosporamine migrated to the cathode. It is soluble in water, methanol and ethanol, but insoluble in acetone, ethyl acetate, chloroform and ether. The antibiotic shows positive reactions with ninhydrin, SAKAGUCHI's and DRAGENDORFF's reagents, and decolorizes potassium permanganate solution.

Laterosporamine hydrochloric acid salt showed  $[\alpha]_D^{21.0^\circ} -11.7 \pm 0.9^\circ$  (*c* 0.583, water). The u.v. spectrum in water showed only end absorption. The i.r. spectrum (KBr) is illustrated in Fig. 1.

Fig. 1. Infrared absorption spectrum of laterosporamine hydrochloric acid salt (KBr).



Laterosporamine forms its reinecke salt as red crystals, m.p. 152~156°C (dec.). Anal. Found: C, 25.35; H, 4.65; N, 24.72; Cr, 10.85; MW, 1257 (osmometry in tetrahydrofuran).  $C_{17}H_{35}N_7O_4 \cdot 3HCr(NH_3)_2(SCN)_4 \cdot 2H_2O$  requires: C, 24.95; H, 4.33; N, 25.09; Cr, 11.18; MW, 1,395.79. The  $^{13}C$  nmr spectrum of the hydrochloric acid salt measured in  $D_2O$  indicated the presence of 17 carbons. However, the exact molecular formula is still uncertain.

Hydrolysis of laterosporamine afforded an amine, identified with spermidine, and a SAKAGUCHI-positive substance,  $C_6H_{13}N_3O$ , whose structure is now under investigation; it did not release any amino acids. Details of the degradative studies will be published elsewhere.

Among the known antibiotics from the genus *Bacillus*, only bacimethrin<sup>5)</sup> and a group of aminoglycoside antibiotics; *i.e.* butirosins A, B and the analogs,<sup>6)</sup> are regarded as water-soluble basic substances with non-peptidic structures. None with the structural characteristics of laterosporamine has been found in the literature. Therefore, we conclude that laterosporamine is a new antibiotic.

### Biological Properties

Laterosporamine exhibits weak inhibitory activities against Gram-positive and Gram-negative bacteria *in vitro*. When the original bacterial suspensions (*ca.*  $10^8 \sim 10^9$  cells/ml) prepared by cultur-

ing in MÜLLER-HINTON Broth (Difco) were used for streaking in measurement of the antimicrobial spectrum, minimal inhibitory concentration was hardly obtained against most bacteria

Table 1. Antimicrobial spectrum of laterosporamine

Test organism	MIC (mcg/ml)*
<i>Bacillus subtilis</i> PCI 219	6.25
<i>Bacillus anthracis</i>	25.0
<i>Staphylococcus aureus</i> FDA 209P JC-1	1.56
<i>Staphylococcus aureus</i> Smith	12.5
<i>Streptococcus pneumoniae</i> Type I	25
<i>Streptococcus pyogenes</i> C-203	50
<i>Escherichia coli</i> NIHJ JC-2	50
<i>Klebsiella pneumoniae</i>	12.5
<i>Salmonella typhimurium</i>	50
<i>Pseudomonas aeruginosa</i>	50

Obtained by two-fold agar dilution method on a modified MÜLLER-HINTON Agar "Nissan."

\* The MICs were obtained by streaking the bacterial suspensions of approximately  $10^8$  cells/ml.

The ED<sub>50</sub> values are listed in Table 2. Acute toxicity was not observed at a dose of 50 mg/kg by intraperitoneal and subcutaneous routes.

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tested. However, when bacterial suspensions of approximately  $10^8$  cells/ml were used for streaking, the MICs listed in Table 1 were obtained.

Laterosporamine exhibited some therapeutic effects, when tested against *Staphylococcus aureus* and *Klebsiella pneumoniae* in mice.

Table 2. Therapeutic effect of laterosporamine administered subcutaneously to ICR mice infected with *Staphylococcus aureus* or *Klebsiella pneumoniae*

	<i>S. aureus</i>	<i>K. pneumoniae</i>
ED <sub>50</sub> * (mg/kg×2)	4.2	13.3

\* The ED<sub>50</sub> is expressed as mg/kg in two subcutaneous doses, given 1 and 5 hours postinfection.