ISOLATION OF A NEW ANTIBIOTIC 333–25, RELATED TO ANTIBIOTIC EM 49

(STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. XI[1])

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A new antibiotic, 333–25, active against Gram-positive and Gram-negative bacteria, was isolated from the culture broth of *Bacillus circulans* 333–25. The antibiotic is a basic acylpeptide containing 2,4-diaminobutyric acid (5), leucine (2), phenylalanine (1) and a fatty acid. It is closely related to antibiotic EM 49, but can be differentiated by chromatographic behaviour.

In the course of screening for new antibiotics from the genus *Bacillus*, two closely related antibiotics were found to be produced by a number of strains of *Bacillus circulans*. Both antibiotics are active against Gram-positive and Gram-negative bacteria. The hydrochlorides of the antibiotics are soluble in water, and the constituents of the antibiotics were identified as 2,4-diaminobutyric acid (Dab), leucine and phenylalanine and fatty acid components. Differentiation between the antibiotics was possible based on mobility on TLC and on analysis of the fatty acid component by GLC.

While our studies were under way, the isolation of antibiotic EM 49[2,3,4] was reported, and more recently the chemical structure of antibiotic EM 49 was published.[5]

One of the two antibiotics isolated by us was identical with antibiotic EM 49 in direct comparison by TLC experiment and even in analysis of fatty acid. However, the other antibiotic, designated antibiotic 333–25, was differentiated from antibiotic EM 49 in mobility on TLC and in analysis of fatty acid by GLC. Therefore, the antibiotic designated as 333–25 is a new antibiotic related to antibiotic EM 49.

In this paper, the taxonomy of the producing strain as well as the production, isolation and preliminary characterization of antibiotic 333–25 will be presented.

Taxonomy of the Producing Strain

The taxonomic characteristics of the strain 333–25, from which the strain was identified as *B. circulans*, is described below.

A. Morphology

1. Vegetative cells (28°C, 1~2 days): The rods, on nutrient agar, are 0.8 μ by 3.5~6 μ with rounded ends. They occur singly or in mass and are motile with peritricous flagella. Gram-positive.

2. Spore and sporangium (28°C, 3 days): Spores, on nutrient agar, are 1.1 μ by 2.0 μ, easily stainable ovals, and located in central to subterminal position. The sporangium is definitely swollen.

B. Cultural Characteristics

1. Nutrient agar colony (37°C, 1~7 days): Circular, convex, entire, shining surface colony. The surface becomes shiny-dull with age and the consistency and optical density turn from translucent and viscid to opaque and gummy with aging.

2. Nutrient agar slant (37°C, 1~7 days): Growth moderate, filiform, creamy-gray color, shining (20 hours) to shiny-dull (over 2 days) surface, viscid to gummy structure and translucent to opaque density. No diffusible pigment was observed.

3. Nutrient broth (30°C, 1~4 days): Moderate, cloud-like growth was observed in the upper layer of the tube (1~2 days). Ring formation and some sediment were present at a later stage.

C. Physiological Characters

1. Relation to oxygen (30°C, 1~5 days)[10]: OF-Test on GPBY-agar stab was oxidative. No growth was observed in deep culture. Acid and gas formation were not observed.

2. Temperature relations (AMS-agar, 1 day): Relative growth on 25°C, 30°C, 37°C, 50°C and 63°C was −, +, ++++, −and−, respectively. Therefore, the optimum temperature lies around 37°C.

3. Citrate utilization (30°C, 1~7 days): No growth on KOSER's synthetic medium[9] but poor growth on CHRISTENSEN's nutrient supplemented medium.[9]

4. Starch hydrolysis (30°C, 1~7 days): Strongly positive.

5. Gelatin stab (28°C, 1~15 days): Growth moderate with slow liquefaction (after 6 days).

6. Litmus milk (30°C, 1~14 days): Rapid peptonization with weak acid formation (2~5 days) and litmus reduction (6~14 days).

7. Nitrate reduction to nitrite (30°C, 1~3 days): Positive.

8. Acetylmethylcarbinol formation (30°C, 1~6 days): Negative.

9. Indole formation (30°C, 1~7 days): Negative.

10. Urease activity (30°C, 1~7 days): Negative.

11. Catalase (cells on nutrient agar, 37°C for 1 day): Positive.

12. Carbohydrate utilization (28°C, 1~6 days): Acid formation was observed from D-xylose, D-galactose, sucrose, maltose, trehalose and starch. Negligible amounts of acid were formed from lactose and glycerol. No acid formation was observed from D-glucose, D-mannose, D-fructose, L-arabinose and inositol.

13. NaCl broth (nutrient broth, 30°C, 1~7 days): No growth was observed in 3.3%, 5% and 7% NaCl broth.

IM-Agar: soluble starch 0.2%, glycerol 0.2%, polypeptone 0.25%, beef extract 0.25%, yeast extract 0.25%, NaCl 0.3%, agar 1.0~1.2% (w/v), pH 6.8.

GPYB-Stab: glucose 1.0%, Polypeptone 0.5%, yeast extract 0.2%, beef extract 0.3%, BCP 0.015%, agar 0.4% (w/v), pH 6.6.

AMS-Medium: glucose 0.7%, casein hydrolyzate 0.5%, yeast extract 0.1%, NaCl 0.3%.

D. Classification

The above observations indicate that this bacillus should be situated in the "circulans complex."[7,8] The one difference between standard strains and our strain is the inability of the latter to produce acids from hexoses, *i.e.* standard strains produce acids from glucose, mannose and fructose but this strain does not. This insufficiency may derive from a lack of enzymes of the hexose monophosphate pathway such as hexose phosphorylase or a lack of permease mechanisms. The difference is insufficient to support the designation of a new species. Therefore, we classified this culture as a strain of the viscid-type circulans,[7,8] and named it *Bacillus circulans* No. 333–25.

Production and Isolation

Spores of the strain 333–25 were inoculated in 800 ml of a medium consisting of glucose 1.0%,

peptone 0.5%, tryptone 0.5%, yeast extract 0.2% and NaCl 0.05% (pH 7.0) in a 2-liter Erlenmeyer flask, and cultured at 28°C for a day on a rotary shaking machine. The culture was then transferred to a 30-liter jar fermentor containing 20 liters of a medium consisting of glucose 1.0%, peptone 0.5%, tryptone 0.5%, casamino acids 1.0% and corn steep liquor 0.4% (pH 7.0). Fermentation was carried out at 28°C for 4 days with aeration at 20 liters per minute and agitation at 350 r.p.m.

Some 60 liters of the culture broth obtained as above were adjusted to pH 3.0 with hydrochloric acid and mixed with 30 liters of methanol and 15 liters of *n*-butanol. After stirring for 30 minutes, the treated broth was filtered. The filtrate was evaporated under reduced pressure to a nearly aqueous solution, which was then adjusted to pH 10.0 with sodium hydroxide and extracted twice with 10 liters of *n*-butanol. About a half volume of ethyl acetate was added to the *n*-butanol extract, and the antibiotic contained was transferred to *ca.* 5 liters of acidified water (pH 2.0 with hydrochloric acid). Two additional transfers with water were conducted. The antibiotic was re-extracted with *n*-butanol from the water solution after adjustment to pH 10.0 with sodium hydroxide. The *n*-butanol solution was then concentrated to a syrup after adjusting to pH 7.0. Addition of ethyl acetate to the concentrate afforded a crude powder of the antibiotic (*ca.* 10 g).

The crude powder was chromatographed on a silica gel plate (Merck, Silica gel GF, thickness 750 μ, 100×20 cm) with chloroform - ethanol - 14% ammoniacal water (4: 7: 2). The separated zone of the antibiotic was detected by a UV-lamp and extracted with acidified (pH 2.0) 50% aqueous methanol. After evaporation of methanol, the antibiotic was extracted with *n*-butanol at pH 10.0. Transfer to acidic water and re-extraction with *n*-butanol were repeated to prevent the carry over of ash-like material derived from the silica gel. The *n*-butanol solution was washed with water and concentrated to a syrup, from which the free base of antibiotic 333–25 was obtained as a colorless amorphous powder by addition of ethyl acetate. From some 10 g of the crude powder, *ca.* 2.0 g of the free base was obtained.

The hydrochloride of the antibiotic was prepared as follows: the free base was dissolved in a small volume of methanol, slightly acidified with hydrochloric acid and precipitated by addition of acetone. Dissolution in methanol and precipitation with acetone was repeated once.

Physical and Chemical Properties

Antibiotic 333–25 (free base) is a colorless amorphous powder, that has no definite melting point and colors gradually above *ca.* 200°C. The hydrochloride melts with decomposition at 215~220°C.

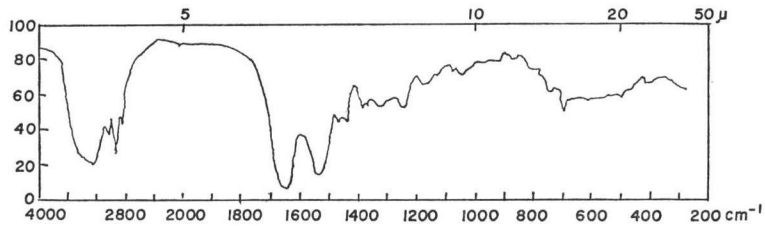
Antibiotic 333–25 gave a single round spot, Rf *ca.* 0.12, on a silica gel plate (Merck, Silica gel GF) with chloroform - ethanol - 14% ammoniacal water (4: 7 :2), whereas antibiotic EM 49 gave an Rf *ca.* 0.18 in this system. On paper electrophoresis carried out with buffer solutions of 0.09 M formic acid - 2-dimethylaminoethanol buffer pH 4.0 and of 0.09 M acetic acid - 2-dimethylaminoethanol buffer pH 9.3[6] at 15 V/cm for one hour, antibiotic 333–25 migrated to the cathode in both buffers.

The hydrochloride is soluble in water and lower alcohols, but insoluble in acetone, ethyl acetate and chloroform. It is positive to ninhydrin reagent but negative to SAKAGUCHI, PAULY, EHRLICH, BENEDICT and ferric chloride reagents.

Elemental analysis gave: Anal Found: C, 54.47; H, 8.07; N, 15.92. This is near to the molecular formula anticipated from the constituents found with the acid hydrolyzate, *i.e.* Dab (5 moles), leucine (2 moles), phenylalanine (1 mole) and a fatty acid ($C_9H_{18}O_3$): $C_{50}H_{87}N_{13}O_{10}\cdot 4H_2O$ requires: C, 54.50; H, 8.63; N, 16.53. Antibiotic 333–25 showed a specific rotation: $[\alpha]_D^{22.0} -65.6 \pm 2.1°$ (*c*,

0.506, 0.5 N HCl), whereas the antibiotic identical with antibiotic EM 49, isolated by us, showed $[\alpha]_D^{22.0}$ −29.8±0.4° (*c*, 0.521, 0.5 N HCl). The ultraviolet absorption spectrum measured with the hydrochloride in methanol showed end absorption and weak maxima: λ_{max}^{MeOH}: 253 nm ($E_{1cm}^{1\%}$ 2), 259 nm ($E_{1cm}^{1\%}$ 2), 265 nm ($E_{1cm}^{1\%}$ 2). The infrared absorption spectrum of the free base, typical of a peptide, is illustrated in Fig. 1.

Fig. 1. Infrared absorption spectrum of antibiotic 333-25 (KBr).



Analysis of the Acid Hydrolyzate

Antibiotic 333–25 was hydrolyzed with constant boiling hydrochloric acid at 110°C for 40 hours. Analysis of the hydrolyzate with an automatic amino acid analyzer, Hitachi KLA-5, gave the following amino acids (μmoles per mg): Dab (4.30), leucine (1.70) and phenylalanine (0.80). This suggested that the constituents on a molar basis are Dab (5 moles), leucine (2 moles) and phenylalanine (1 mole), and that the molecular weight of antibiotic 333–25 is approximately 1,200.

The hydrolyzate was extracted with ethyl ether. When the ethereal extract was methylated and analyzed by G.L.C., several peaks were observed. However, when acid hydrolysis was carried out for shorter time, a main peak appeared. Further studies clarified that the main constituent fatty acid of antibiotic 333–25 is β-hydroxy anteisononanoic acid. The detailed data will be reported in the next publication.[11]

Biological Properties

Antibiotic 333–25 is active against both Gram-positive and Gram-negative bacteria *in vitro* as shown in the antimicrobial spectrum (Table 1) obtained by the usual agar dilution method. When the *in vivo* activity of the antibiotic was tested in mice against systemic injection of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, therapeutic efficacy was observed against *E. coli* and *K. Pneumoniae*, but not against *P. aeruginosa*. The ED_{50} values obtained are listed

Table 1. Antimicrobial spectrum of 333–25

Test organism	MIC (mcg/ml)
Bacillus subtilis PCI 219	6.25
Bacillus anthracis	50
Staphylococcus aureus FDA 209P JC-1	6.25
Staphylococcus aureus Smith	50
Streptococcus pneumoniae type I	>50
Streptococcus pyogenes C-203	50
Escherichia coli NIHJ JC-2	25
Klebsiella pneumoniae	12.5
Salmonella typhimurium	3.13
Pseudomonas aeruginosa	6.25

Obtained by the usual agar dilution method.

Table 2. Therapeutic effect of 333–25 administered subcutaneously to ICR mice infected with *Escherichia coli* or *Klebsiella pneumoniae*

	E. coli	*K. pneumoniae*
ED_{50}^{*} (mg/kg×2)	0.95	0.95

* The ED_{50} is expressed as mg/kg in two subcutaneous doses, given 1 and 5 hours postinfection.

in Table 2. The acute toxicity of this antibiotic was tested in mice, and the following LD_{50} values were observed: 12.5~25 mg/kg (iv), 25~50 mg/kg (ip), 500 mg/kg (sc) and >500 mg/kg (po).

Consideration

As already mentioned, antibiotic 333–25 is closely related to antibiotic EM 49, but was clearly differentiated by direct comparison with TLC and by comparison of their optical rotational activities. Further studies on the constituents, which will be published in the next paper,[11] clarified the difference between the two antibiotics to be shown in the fatty acid constituents and in the chiralities of the constituent amino acids.

Recently, two additional antibiotics, Bu-1880[12] and Y-8495,[13] that are similar to antibiotic EM 49 and antibiotic 333–25 with respect to constituent amino acids have been reported. However, the constituent fatty acid of antibiotic Bu-1880 is the same as the major fatty acid component of antibiotic EM 49, indicating that antibiotic 333–25 differs from antibiotic Bu-1880. Moreover, the ratio of the constituent amino acids of antibiotic Y-8495 is apparently different from that of the above three antibiotics. Therefore, antibiotic 333–25 is concluded to be new.

Acknowledgement

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