ISOLATION OF TWO NEW POLYMYXIN GROUP ANTIBIOTICS (STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. XX[1])

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Two new members of polymyxin group antibiotics, polymyxins S_1 and T_1, were isolated from the culture broths of strains identified as *Bacillus polymyxa* Rs-6 and *Bacillus polymyxa* E-12, respectively. These antibiotics are strongly basic substances, their hydrochloric acid salts are soluble in water and methanol. They are primarily active against Gram-negative bacteria *in vitro* and *in vivo* though polymyxin T_1 exhibits higher activities against Gram-positive bacteria than other polymyxin group antibiotics.

In the course of our screening program for new antibiotics from the genus *Bacillus*[1], two antibiotics primarily active against Gram-negative bacteria were isolated from culture broths of strains numbered Rs-6 and E-12, which were identified as *Bacillus polymyxa*. These antibiotics were named polymyxins S_1 and T_1 respectively, because their physico-chemical and biological properties and the chemical structures, which will be presented in the following reports[2,3], indicated that they are new members of the polymyxin group antibiotics.

This paper deals with the taxonomic characteristics of the producing organisms, the production and isolation of the antibiotics, and the physico-chemical and biological properties.

Taxonomic Characterization of the Producing Microorganisms

The taxonomic characteristics of the strain Rs-6 are described below.

Morphology

(1) Vegetative cells (28°C, 1~2 days): Gram-positive rods on nutrient agar are 0.5~1.0 by 2.5~5.0 μ (0.7 by 3.0~4.0 μ mostly) with rounded end. They occur singly or in a mass and are motile.

(2) Spores and sporangia (28°C, 1~2 days): Spores on nutrient agar are mostly 1.2 by 1.6μ easily stainable, oval, central to subterminal. Sporangia are definitely swollen.

B. Cultural Characteristics

(1) Colony on No. 172 agar medium* (28°C, 1~2 days): Circular (2~4 mm in diameter), convex, entire, smooth, glistening, gummy to slimy, translucent to opaque.

(2) Nutrient agar slant (28°C, 1~12 days): Growth moderate, filiform, whitish gray, surface shiny (at 1 day) changing to dull after 2 days. Butyrous consistency and opaque density. Diffusible and non-diffusible pigments are not observed.

(3) Nutrient broth (28°C, 1~5 days): Uniform, significant growth. No ring or pellicle formation is observed.

C. Physiological Characters

* No. 172 Medium: Soluble starch 2.0%, glucose 1.0%, Casamino acid 0.5%, yeast extract 0.5%, $CaCO_3$ 0.1%, agar 1.2% (w/v), pH 6.8.

(1) Relation to oxygen (28°C, 1~2 days): OF-test on GPYB-agar stab* is facultative anaerobic. Acid and gas is produced from glucose.

(2) Temperature relation (Gly-IM** agar, 1 day): Optimum is approximately 37°C. It does not grow at 50°C.

(3) Citrate utilization (28°C, 1~2 days): No growth on KOSER's synthetic medium.

(4) Starch hydrolysis (28°C, 1~3 days): Positive.

(5) Gelatin stab (28°C, 1~20 days): Slowly liquefied.

(6) Casein hydrolysis (28°C, 1~2 days): Positive.

(7) Litmus milk (28°C, 2~30 days): Peptonized slowly. Coagulation is not observed.

(8) Nitrate reduction to nitrite (28°C, 2~4 days): Negative.

(9) Acetylmethylcarbinol production (28°C, 2 day): Positive.

(10) H_2S formation (28°C, 2 days, Difco peptone iron agar): Negative.

(11) Urease activity (28°C, 1~7 days): Negative.

(12) Catalase (28°C, 1 day): Positive.

(13) Oxidase (28°C, 1 day): Positive.

(14) Carbohydrate cleavage (28°C, 3~11 days): Acid formation was observed from D-ribose, D-glucose, D-mannose, D-galactose, D-fructose, sucrose and salicin. Very weak production of acid was observed from maltose and raffinose. No acid formed from L-arabinose, D-xylose, L-rhamnose, lactose, dextrin, starch, glycogen, inulin, glycerol, inositol, adonitol, mannitol, sorbitol and α-methylglucoside.

(15) NaCl broth (28°C, 2~6 days): No growth in 5 and 10% NaCl broth.

The taxonomic characteristics of the strain E-12 are closely similar to those of the strain Rs-6 described above, except for the following three apparently different physiological characteristics:

1) Litmus milk (28°C, 1~6 days): Coagulated with acid formation.
2) Nitrate reduction to nitrite (28°C, 1~3 days): Positive.
3) H_2S formation (28°C, 1~6 days, Paper strip method): Weakly positive.

The above description indicates that these bacteria should be classified as *Bacillus polymyxa*[4,5]. *Bacillus macerans* differs from Rs-6 and E-12 by a) acetylmethylcarbinol production and b) caseine hydrolysis[4,5]. The descriptions of *B. polymyxa*[4] are very similar to those of Rs-6 and E-12, and there are no significant taxonomic differences between them. Therefore, we concluded that Rs-6 and E-12 are strains of *B. polymyxa*.

The taxonomic study was carried out according to the Manual of Microbiological Method[6] and Identification Method for Microbiologist[7] except where indicated otherwise.

Production and Isolation

The fermentation and isolation of polymyxins S_1 and T_1 were carried out similarly. One example is described here.

Spores of the strain E-12 were inoculated into 120 ml of a medium consisting of glucose 1.0%, peptone 0.5%, meat extract 0.5% and sodium chloride 0.1%, pH 7.0, in a SAKAGUCHI flask. After being cultured for one day at 27°C on a reciprocal shaker, about 4 ml of the culture was transferred into 120 ml of a medium consisting of soluble starch 2.0%, Soytone 3.0%, $MgSO_4 \cdot 7H_2O$ 0.2%, and $CaCO_3$ 1.0%, pH 7.0, in a SAKAGUCHI flask. Fermentation was carried out for one day at 27°C on a reciprocal shaker.

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* GPYB medium: Glucose 1.0%, peptone 0.5%, yeast extract 0.2%, beef extract 0.3%, agar 0.4% (w/v) pH 6.6.

** Gly-IM agar: Glycerol 0.5%, peptone 0.25%, beef extract 0.25%, yeast extract 0 25%, Bacto soytone 0.25%, NaCl 0.3%, agar 1.25% (w/v) pH 6.8.

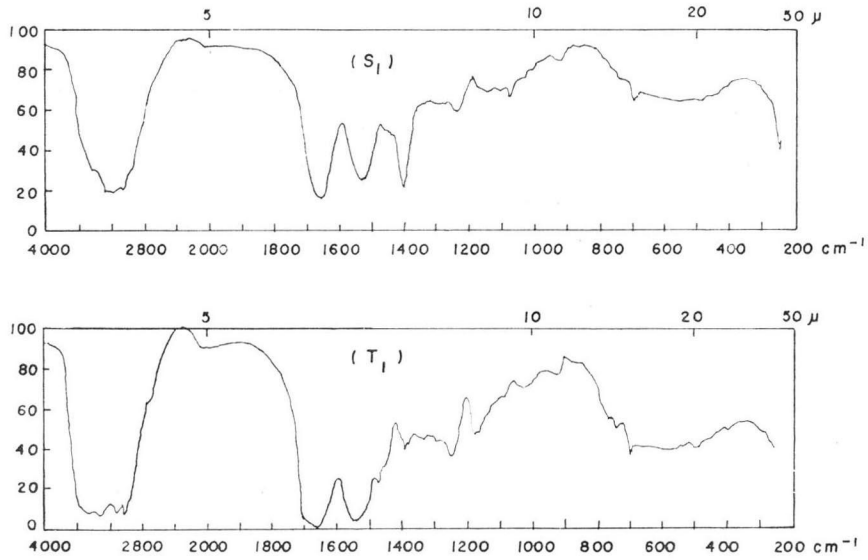
Table 1. Some properties of polymyxins S_1 and T_1

	S_1 tetrahydrochloride	T_1 pentahydrochloride
Rf in PC*	0.45	0.63
TLC**	0.35	0.12
$[\alpha]_D^{25}$ in (water)	$-54.3\pm1.7°$ (c 0.541)	$-78.0\pm1.2°$ (c 1.025)
$\lambda_{H_2O}^{max}$, nm ($E_{1cm}^{1\%}$)	253 (1.4), 259 (1.5), 264 (1.2)	252 (2.3), 258 (2.4), 264 (2.1)

* Toyo Roshi No. 51, *n*-butanol - acetic acid - water (4: 1: 2)

** Pre-coated Silica Gel F-254, Merck, acetone - water - acetic acid - 2 N ammonium hydroxide (15: 5: 1: 2)

Fig. 1. Infrared absorption spectra of polymyxin S_1 tetrahydrochloride and polymyxin T_1 pentahydrochloride (KBr).



About 6.0 liters of the culture broth was adjusted to pH 2.0 with hydrochloric acid and 120 g of Hyflo Super-Cel was added. After heating for 10 minutes at 80°C, it was filtered. The filtrate was mixed with 72 g of oxalic acid and adjusted to pH 7.0 with sodium hydroxide. A colorless precipitate of calcium oxalate was filtered off. The filtrate was then passed through an Amberlite IRC-50 (Na) column (350 ml). The column was washed with water and eluted with 0.5 N HCl. Active eluates as assayed on an *E. coli* assay plate were combined and extracted with butanol at pH 11.0. The butanol solution was washed once with water and concentrated to a residue at pH 4.0 (adjusted with HCl). The residue was washed with acetone, to give a crude powder (1.1 g). The crude powder was dissolved in 50 ml of water, and the solution was made alkaline with sodium hydroxide to above pH 11.0. The free base of the antibiotic separated as a gel-like precipitate. This was collected by centrifugation, washed with water and then dissolved in diluted hydrochloric acid. After lyophilization, the residue was dissolved in a small amount of methanol and precipitated by addition of acetone, giving the hydrochloric acid salt of the antibiotic as a colorless powder (800 mg). For analytical purpose, a portion of the preparation was further purified by preparative paper chromatography on Toyo Roshi No. 51

Table 2. Antimicrobial spectra of polymyxins S_1 and T_1

Test organisms	MIC (mcg/ml)	
	S_1	T_1
Escherichia coli NIHJ JC-2	1.56	3.13
Escherichia coli 80750	0.78	—
Klebsiella pneumoniae	0.78	3.13
Salmonella typhimurium	1.56	3.13
Pseudomonas aeruginosa	6.25	6.25
Bacillus subtilis PCI 219	>50	3.13
Staphylococcus aureus FDA 209P JC-1	>50	12.5
Staphylococcus aureus 80257	>50	25.0
Staphylococcus aureus Smith	>50	12.5
Streptococcus pneumoniae type I	>50	100.0
Streptococcus pyogenes C-203	>50	50.0

Obtained by the usual agar dilution method.

Table 3. Therapeutic effect and toxicity of polymyxins S_1 and T_1 in IRC mice

	ED_{50}* (mg/kg×2)		LD_{50} (mg/kg) ip
	K. pneumoniae	*E. coli*	
Polymyxin S_1	0.19	0.22	25~50
Polymyxin T_1	—	1.00	32.4

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

with *n*-butanol - acetic acid - water (4: 1: 2). The zone of the antibiotic was detected by ninhydrin color, and it was extracted with water. Lyophilization and then precipitation by acetone from methanol solution gave a pure preparation of the hydrochloride of polymyxin T_1 as a colorless powder.

The same procedure of fermentation and isolation about the strain Rs-6 gave a similar result in the preparation of polymyxin S_1, though the production yield was lower: 180 mg of the hydrochloride was obtained from 6.0 liters of the culture broth.

Physical and Chemical Properties

Polymyxin S_1 tetrahydrochloride was obtained as a colorless amorphous powder, which melted with decomposition at 202~213°C.

Anal. Found: C, 47.08; H, 7.22; N, 16.14; Cl, 10.69.
Calcd. for $C_{53}H_{91}N_{15}O_{15}\cdot 4HCl\cdot H_2O$: C, 47.42; H, 7.28; N, 15.65; Cl, 10.57.

Polymyxin T_1 pentahydrochloride was also obtained as a colorless amorphous powder, which melted with decomposition at 220~230°C.

Anal. Found: C, 48.62; H, 7.81; N, 15.73; Cl, 12.70.
Calcd. for $C_{58}H_{102}N_{16}O_{12}\cdot 5HCl\cdot 2H_2O$: C, 48.58; H, 7.81; N, 15.63; Cl, 12.36.

The basic nature of these antibiotics was shown by paper electrophoresis in buffer solutions of pH 9.3 and 4.0. The hydrochlorides were freely soluble in water and methanol, but sparingly soluble or insoluble in acetone, ethyl acetate, chloroform and ether.

Both the preparations showed a single spot on paper chromatograms and on TLC visualized with bioautography on an *E. coli* assay plate or by ninhydrin. The approximate Rf values, the $[\alpha]_D$ values and the absorption maxima of both the antibiotics measured in water are listed in Table 1. The infrared absorption spectra are illustrated in Fig. 1.

Biological Properties

Polymyxin S_1 is active against Gram-negative bacteria *in vitro* but not against Gram-positive bacteria. On the other hand, polymyxin T_1 is active against Gram-negative bacteria and also active against some Gram-positive bacteria as shown in Table 2.

Both polymyxins S_1 and T_1 are also active *in vivo*. The ED_{50} values in mice against *Klebsiella pneumoniae* or *Escherichia coli* are shown in Table 3.

Table 4. Constituent amino acids and fatty acids of polymyxin group antibiotics

Antibiotic	Amino acid						Fatty acid	Literature
	Dab	Thr	Leu	Phe	Ser	Ile		
Polymyxin A_1*	5L, 1D	3L	1D				a-C_9	9-a
Polymyxin A_2	5L, 1D	3L	1D				i-C_8	9-a
Polymyxin M	6L	3L	1D				a-C_9	9-b
Polymyxin K	6L	3L	1D				+**	9-c
Polymyxin B_1	6L	2L	1L	1D			a-C_9	9-d
Polymyxin B_2	6L	2L	1L	1D			i-C_8	9-e
Polymyxin C	+	+		+			+	9-f
Polymyxin P_1	6	3		1			a-C_9	9-g
Polymyxin P_2	6	3		1			i-C_8	9-g
Polymyxin D_1	5L	3L	1D		1D		a-C_9	9-h
Polymyxin D_2	5L	3L	1D		1D		i-C_8	9-h
Polymyxin E_1 (Colistin A)	6L	2L	1L, 1D				a-C_9	9-i
Polymyxin E_2 (Colistin B)	6L	2L	1L, 1D				i-C_8	9-j
Circulin A	6L	2L	1D			1L	a-C_9	9-k
Circulin B	6L	2L	1D			1L	i-C_8	9-l
Polymyxin S_1	5L	3L		1D	1D		a-C_9	
Polymyxin T_1	6L	1L	2L	1D			a-C_9	

The figures give the number of amino acid residues per molecule. The + signs express the presence of the amino acids or unidentified fatty acids.

Dab: 2,4-Diaminobutyric acid. a-C_9: Anteisononanoic acid. i-C_8: Isooctanoic acid.

* Identity with polymyxin M has been suspected.

** The presence of hydroxy fatty acids was stated.

Acute toxicities of both antibiotics seem to be at the same level as other polymyxin group antibiotics. The LD_{50} values in mice by intraperitoneal route are also listed in the Table.

Discussion

Structural studies on polymyxins S_1 and T_1 have elucidated their structures, which will be presented in the following papers[2,3]. The constituents of polymyxin S_1 are 5 moles of L-2,4-diaminobutyric acid, 3 moles of L-threonine, one mole each of D-phenylalanine and D-serine and anteisononanoic acid, and the constituents of polymyxin T_1 are 6 moles of L-2,4-diaminobutyric acid, one mole of L-threonine, 2 moles of L-leucine, one mole of D-phenylalanine and anteisononanoic acid. The structures revealed that both antibiotics belong to the polymyxin group. Many members of polymyxin group antibiotics have been isolated. The structures of most of them have been clarified. The members appeared in literatures are listed with respect to their constituent amino acids and fatty acids in Table 4. By comparison, it is evident that polymyxins S_1 and T_1 are new members of this group of antibiotics.

In most cases of the production of polymyxin group antibiotics, one strain produced two variants, which have the same peptide part and different fatty acids; *i.e.* anteisononanoic acid and isooctanoic acid. In the cases of the strains Rs-6 and E-12, it has been found that the products are almost single entities; they usually contain mainly anteisononanoic acid and only a trace amount of isooctanoic acid. (The constituent fatty acids are analyzed by means of gas chromatography). However, we had found in some production lots from the strain E-12 the content of isooctanoic acid to be as high as about 5%. This meant that the preparation contained 5% of an another antibiotic component which should be called polymyxin T_2. It should be mentioned that the content ratio of fatty acid constituents in an antibiotic complex of acylpeptides somewhat varies with fermentation conditions[8].

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