NEW PEPTIDE ANTIBIOTICS LI-F03, F04, F05, F07, AND F08, PRODUCED BY *BACILLUS POLYMYXA*

I. ISOLATION AND CHARACTERIZATION

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A strain of *Bacillus polymyxa* produced a new peptide antibiotic complex, named LI-F, composed of more than ten components. The components, antibiotics LI-F03, F04, F05, F07, and F08 were isolated from the complex by reversed phase HPLC. They are active against fungi, yeasts, and Gram-positive bacteria. The fast atom bombardment mass spectra revealed that the individual isolated antibiotics are still mixture of two homologous components, being very difficult to separate from each other.

A bacterial strain, L-1129, identified as *Bacillus polymyxa* was found to produce a new antibiotic complex, LI-F. The antibiotic complex was isolated from fermentation broth by chromatography on Amberlite XAD-2. HPLC analysis showed that the complex was composed of at least ten components. Five components, antibiotics LI-F03, F04, F05, F07, and F08, were isolated from the complex and purified by reversed phase HPLC. Chemical characterization of the components indicated that they were peptides. Although the five antibiotics isolated here behaved as a single entity under all conditions tested for HPLC, their fast atom bombardment mass spectra (FAB-MS) indicated that each antibiotic was a mixture of two homologous components. In this paper, we will describe the production, isolation, physico-chemical and biological properties of the components.

Producing Organism

Antibiotics LI-Fs-producing strain, L-1129, was isolated from a soil sample collected in Odawara City, Kanagawa Prefecture. The taxonomic study of the strain was carried out according to the Prokaryotes[1] and Classification and Identification of Microorganisms[2]. The taxonomic characteristics of the strain are given below.

Morphological Characteristics

1) Vegetative cells on nutrient agar (35°C, 1 day). Shape and size, rods; $0.8\sim1.0\times2.0\sim6.0$ μm. Motility, positive. Polymorphism, negative.

2) Spores (35°C, $1\sim3$ days). Shape, elliptical. Position, subterminal. Sporangia, swollen at spore site.

3) Gram-stain, prevailing negative.

Table 1. Physiological characteristics.

Temperature range for growth	15~45°C
pH range for growth	5~7
Relation to oxygen	Facultative anaerobic
OF test (Hugh Leifson method)	Fermentative
Nitrates reduction to nitrites	Positive
Denitrification test	Negative
Voges-Proskauer reaction	Positive
Methyl red test	Negative
Citrate utilization	Growth on KOSER's and CHRISTENSEN's media
Indole production	Negative
H_2S production	Negative
Acetoin production	Positive
Starch hydrolysis	Positive
Casein hydrolysis	Positive
Gelatin stab	Slowly liquefied
Litmus milk	Peptonized slowly
Oxidase reaction	Negative
Catalase reaction	Positive
Urease activity	Negative
NaCl broth	No growth in 5% NaCl broth
Carbohydrate cleavage, acid and gas formations	L-Arabinose, D-glucose, D-xylose, D-mannose, D-fructose, D-galactose, maltose, sucrose, lactose, trehalose, D-mannitol
Neither acid nor gas	D-Sorbitol, inositol, glycerol

Cultural Characteristics

1) Colony on nutrient agar plate (35°C, 1~3 days) and slant (35°C, 1~7 days): Colorless, regular margin, smooth surface. Diffusible pigments were not observed.

2) Nutrient broth (35°C, 1~3 days): Uniform, moderate growth, turbid with sediment.

Physiological Characteristics

The physiological characteristics are summarized in Table 1.

Table 1 indicates that the bacteria should be classified as *B. polymyxa*. Thus, it was concluded that L-1129 was a strain of *B. polymyxa*.

Production

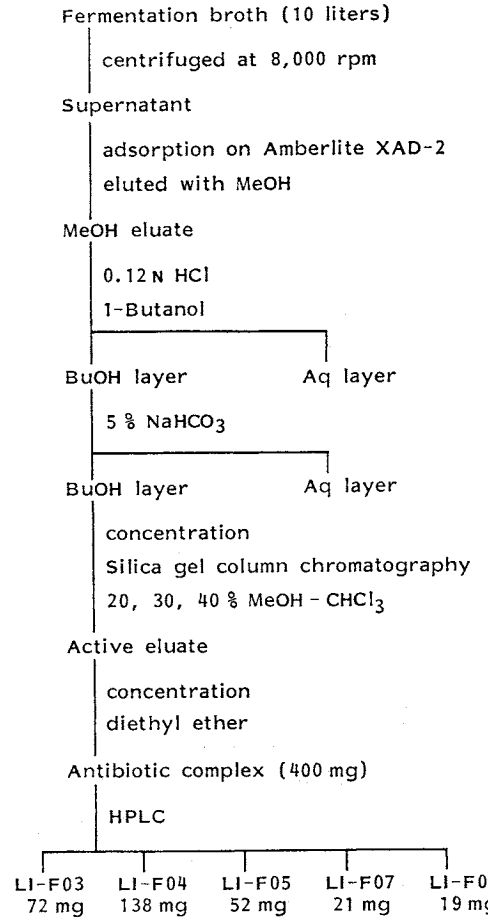
The growth of *B. polymyxa* L-1129 on slant culture was used to inoculate two 500-ml Sakaguchi flasks containing 100 ml of sterile medium of Tripticase soy broth (BBL). The flasks were shaken on a reciprocal shaker at 125 strokes per minute for 24 hours at 30°C. The content of the flasks was transferred to a 20-liter volume of stainless steel tank containing 10 liters of fermentation medium composed of corn meal 4.5%, yeast extracts 0.1%, $(NH_4)_2SO_4$ 0.5% and $CaCO_3$ 0.5%. Fermentation was allowed to proceed at 30°C, at the air flow of 18 liters per minute and with agitation of 400 rpm. Time course of antibiotics production was followed by a cup method using *Staphylococcus aureus* FDA 209P as the test organisms. A peak antibiotic potency of 100 μg/ml was obtained after 22 hours' fermentation.

Isolation and Purification

A flow diagram for the isolation procedure is given in Fig. 1. Activity against *S. aureus* FDA 209P was employed to monitor the isolation of antibiotics LI-Fs from the culture broth of *B.*

polymyxa L-1129. The fermented broth (10 liters) was centrifuged at 8,000 rpm and the supernatant was passed through a column of Amberlite XAD-2. The column was washed with water and eluted with methanol to yield a crude eluate. After concentration *in vacuo*, the eluate was dissolved in 2 liters of 0.12 N HCl and the resulting solution was extracted twice with the same volume of 1-butanol. The 1-butanol layer collected was washed with 5% sodium bicarbonate and the alcohol layer was evaporated *in vacuo* and the oily matter obtained was applied to silica gel column chromatography. After developing with chloroform, the column was eluted by using a methanol step gradient. The active fractions were combined and concentrated *in vacuo* to afford a crude powder. For further purification, the crude powder dissolved in a small amount of methanol was reprecipitated with diethyl ether. Antibiotic complex (400 mg) was obtained as a purified colorless powder. An analytical HPLC chromatogram of the complex showed that it consisted of at least ten components designated as antibiotics LI-F01~LI-F10 (Fig. 2). The positive ion FAB-MS of the complex suggested that it consisted of more than ten components (Fig. 3). In order to isolate each component, the complex was subjected to reversed phase HPLC under the condition as follows: Packing; Cosmosil $5C_{18}$ (Nakarai, 10×250 mm), mobile phase; acetonitrile - water (1 : 1.8, containing 0.05% trifluoroacetic acid), flow rate; 3 ml/minute, detection; UV at 215 nm. By this procedure, the five components, antibiotics LI-F03 (72 mg), F04 (138 mg), F05 (52 mg), F07 (21 mg), and F08 (19 mg), were isolated and purified.

Fig. 1. Isolation procedure of antibiotics LI-F03, F04, F05, F07, and F08.



Physico-chemical Properties

Antibiotics LI-F03, F04, F05, F07, and F08 were obtained as colorless amorphous powder. The antibiotics showed similar solubilities; slightly soluble in water, methanol and acetonitrile, and insoluble in chloroform, acetone, ethyl acetate, diethyl ether and *n*-hexane. The physico-chemical properties of the antibiotics are summarized in Table 2.

The antibiotics showed color reaction similar to one another. Thus, they gave positive reaction to biuret reagent, but were all negative to ninhydrin, Ehrlich, Fehling and Molisch reactions. To Pauly reaction the antibiotic LI-F03 was positive, but others were negative. The IR spectra of the antibiotics are shown in Fig. 4, suggesting that they are peptides. The UV spectrum (Fig. 5) of antibiotic LI-F03 displayed absorption maxima at 224, 278 and 285 nm in methanol, indicating the

Fig. 2. Analytical HPLC chromatogram of antibiotic LI-Fs complex.

Packing: Cosmosil $5C_{18}$ (4.6×150 mm, Nakarai chemicals). Mobile phase: acetonitrile - H_2O (1:2), containing 0.05% TFA. Flow rate: 1 ml/minute. Detection: 215 nm, 0.08 aufs. Chart speed: 5 mm/minute.

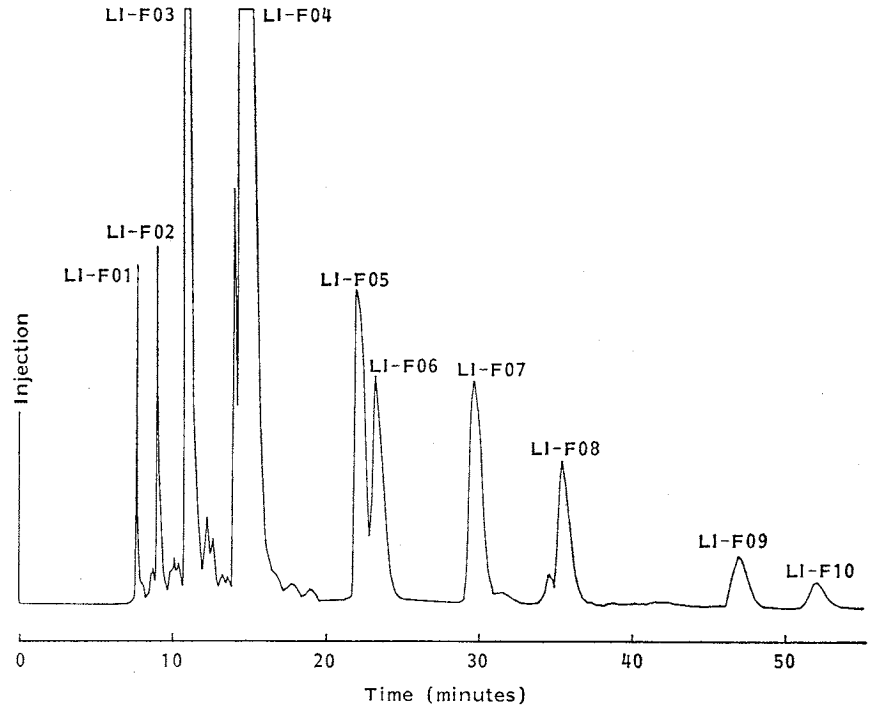
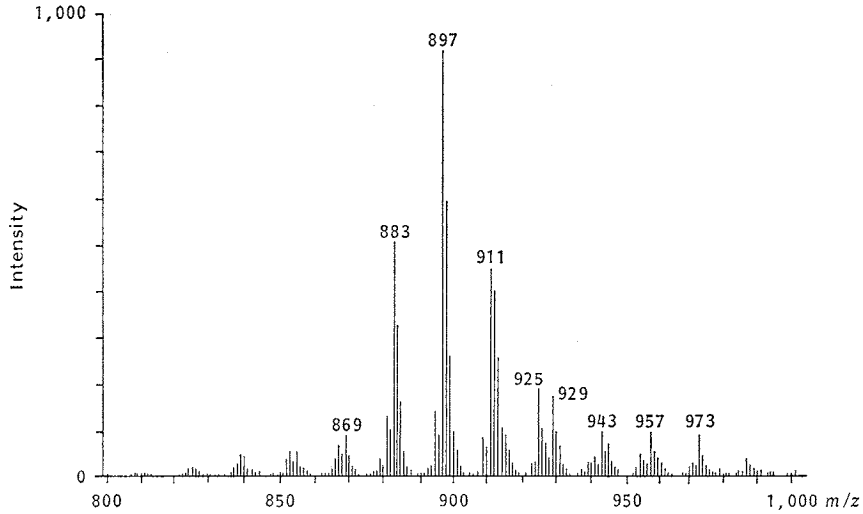


Fig. 3. Positive ion FAB-MS of antibiotic LI-Fs complex.



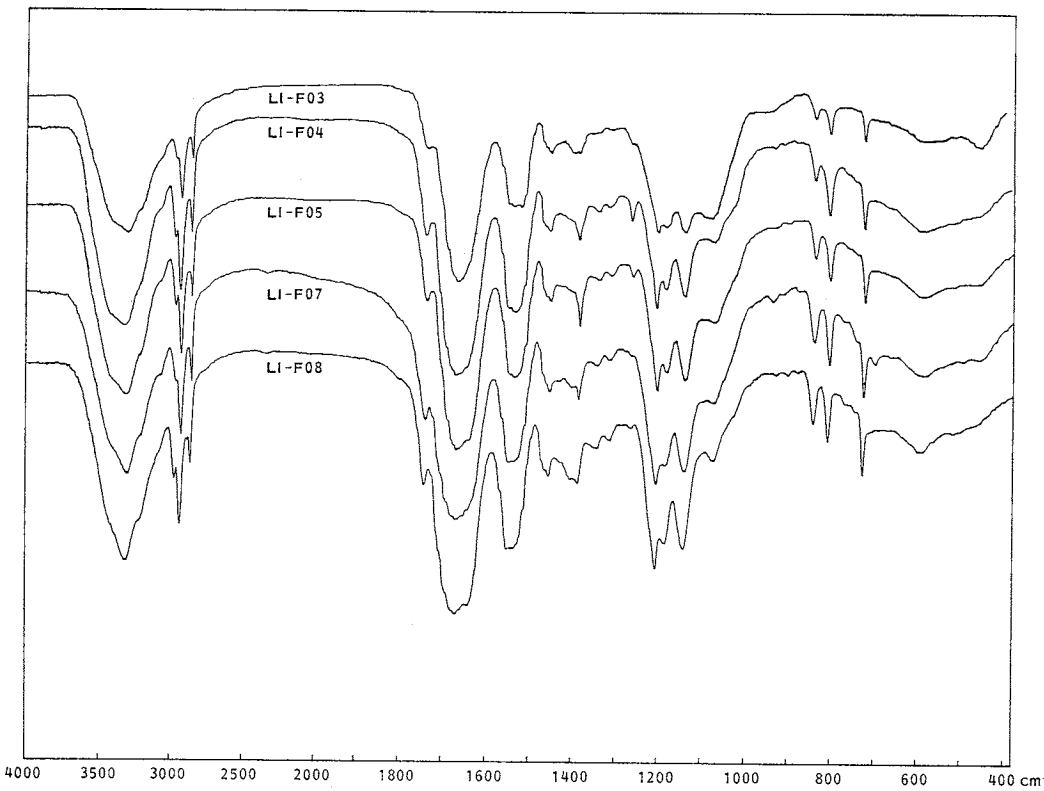
presence of tyrosine residue, while the other antibiotics showed only end absorption. The individual antibiotics were hydrolyzed with 6 N HCl at 105°C for 22 hours and the hydrolysates were applied to automatic amino acid analyzer, Hitachi 835 type. The results are shown in Table 2. All constituent amino acids of antibiotics LI-F03, F04 and F07 were identified, while antibiotic LI-F05 and

Table 2. Physico-chemical properties of antibiotics LI-F03, F04, F05, F07, and F08.

	Antibiotics				
	LI-F03	LI-F04	LI-F05	LI-F07	LI-F08
MP (°C)	229~234	236~239	231~233	235~238	237~238
$[\alpha]_D^{27}$ (c 0.1, MeOH)	+18°	+10°	−1.5°	+14°	+24°
UV λ_{max}^{MeOH} ($E_{1cm}^{0.0125\%}$)	[a]	End	End	End	End
FAB-MS[b] (m/z)	961 (89), 947 (100)	897 (100), 883 (90)	911 (100), 897 (87)	945 (100), 931 (94)	925 (100), 911 (96)
Constituent amino acids[c]	Asp (1.0), Glu (1.0), Thr (3.8), Ala (2.0), Val (2.2), Tyr (1.7)	Asp (1.1), Glu (1.0), Thr (3.8), Ala (2.2), Val (3.8)	Asp (1.0), Glu (1.0), Thr (3.6), Ala (2.0), Val (1.6), Ile (0.9), U_1 (0.7)	Asp (1.3), Glu (1.0), Thr (4.3), Ala (2.4), Val (2.2), Phe (1.5)	Asp (0.9), Glu (1.0), Thr (3.5), Ala (2.0), Ile (1.5), U_2 (1.5)

[a] See Fig. 5. [b] Relative intensity. [c] The molar ratios.

Fig. 4. IR spectra of antibiotics LI-F03, F04, F05, F07, and F08 (KBr).



F08 gave an unidentified amino acid, U_1 and U_2, respectively, in addition to the identified amino acids. Both the unidentified amino acids seem to be identical with each other as judged by their same retention times. Furthermore, the amino acid analytical results revealed that four amino acids, aspartic acid (Asp), glutamic acid (Glu), alanine (Ala) and threonine (Thr), were common to all the antibiotics and their molar ratios in all the antibiotics were identical. Other constituents such as fatty acid were

not found in the hydrolysates.

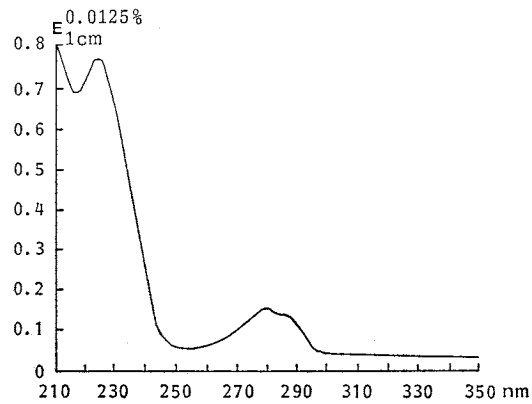
Fig. 6 shows the positive ion FAB-MS of antibiotic LI-F08, giving two intense peaks at *m/z* 911 and 925. Similarly the FAB spectra of other four antibiotics also gave the characteristic homologous peaks, having almost the same intensities, *m/z* 947 and 961 in LI-F03, *m/z* 883 and 897 in F04, *m/z* 897 and 911 in F05, and *m/z* 931 and 945 in F07, described in Table 2. As the mass differences between the homologous peaks are fourteen, it was concluded that these peaks were from the protonated molecular ions and each antibiotic isolated was still a mixture of two components. Significant fragment ion peaks were not observed in all spectra.

Biological Activities

Antimicrobial Activity

The minimal inhibitory concentrations (MICs) of the antibiotics were determined by means of a two-fold serial agar dilution method in Mueller-Hinton media for bacteria, in Sabouraud media for fungi and in yeast morphology media for yeasts. Antibacterial and antifungal spectra of the antibiotics are given in Tables 3 and 4, respectively. These antibiotics were similarly active against Gram-positive bacteria, mycobacteria, and a wide range of fungi and yeasts, whereas no activity was observed on Gram-negative bacteria.

Fig. 5. UV spectrum of antibiotic LI-F03 in MeOH.



Toxicity

The acute toxicity (LD_{50}) of the antibiotics by the intraperitoneal route were determined in *dd*Y mice weighing 20 to 21 g. Graded doses of each antibiotic, suspended in 0.2% carboxymethyl cellulose saline, were administered in five

Table 3. Antibacterial spectra of antibiotics LI-F03, F04, F05, F07, and F08.

Organisms tested	MIC (μg/ml)				
	LI-F03	LI-F04	LI-F05	LI-F07	LI-F08
Bacillus subtilis PCI 219	3.12	6.25	3.12	3.12	3.12
Micrococcus luteus	0.78	1.56	1.56	0.39	0.78
Staphylococcus aureus FDA 209P	1.56	1.56	1.56	0.39	0.78
S. aureus IFM 67	3.12	3.12	1.56	1.56	1.56
S. aureus IFM 74	3.12	3.12	1.56	1.56	1.56
S. aureus Rosa	3.12	3.12	3.12	0.78	1.56
S. aureus Smith	1.56	3.12	1.56	1.56	1.56
S. aureus Yamaguchi	1.56	3.12	1.56	1.56	1.56
S. albus	3.12	3.12	1.56	1.56	1.56
S. citreus	3.12	3.12	3.12	0.78	1.56
Streptococcus faecalis	>100.0	>100.0	12.5	6.25	6.25
Corynebacterium diphtheriae	0.78	1.56	1.56	0.78	1.56
Mycobacterium smegmatis	3.12	6.25	6.25	3.12	6.25
Escherichia coli F1	>100.0	>100.0	>100.0	>100.0	>100.0
Pseudomonas aeruginosa	>100.0	>100.0	>100.0	>100.0	>100.0
Salmonella typhimurium	>100.0	>100.0	>100.0	>100.0	>100.0

Medium: Mueller-Hinton agar. Incubation: 24 hours to 5 days at 37°C, depending on test strain.

Fig. 6. Positive ion FAB-MS of antibiotic LI-F08.

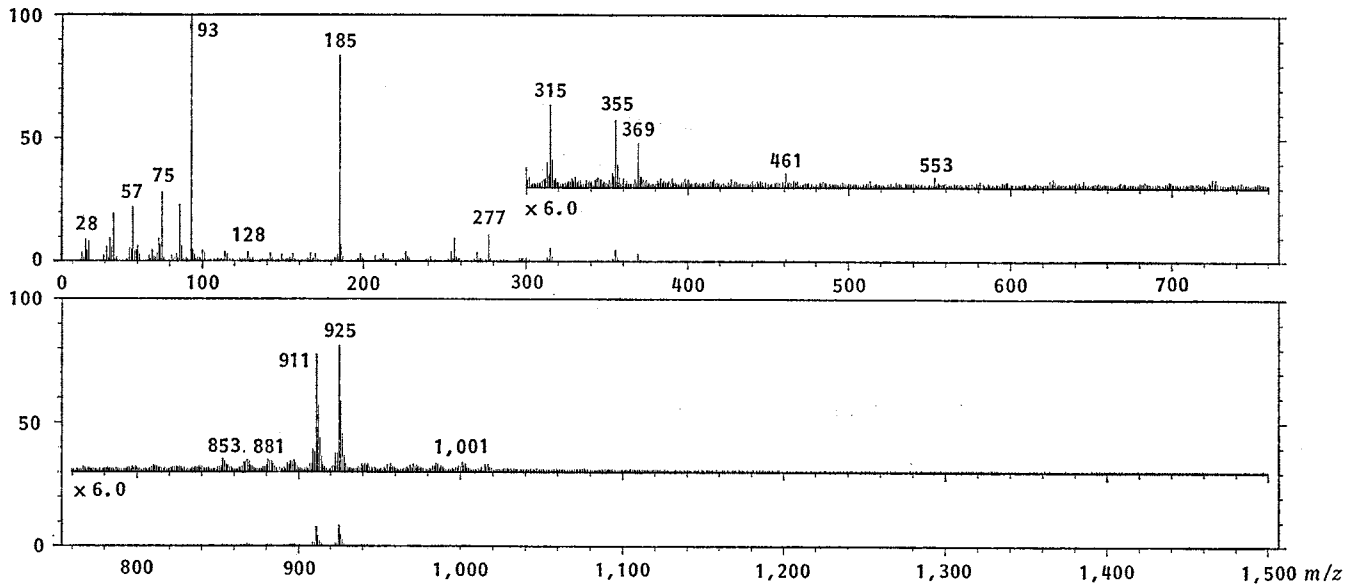


Table 4. Antifungal spectra of antibiotics LI-F03, F04, F05, F07, and F08.

Organisms tested	Medium	MIC (μg/ml)				
		LI-F03	LI-F04	LI-F05	LI-F07	LI-F08
Aspergillus flavus	S	6.25	0.39	<0.20	<0.20	<0.20
A. nidulans	S	6.25	6.25	3.12	6.25	6.25
A. niger	S	6.25	3.12	3.12	12.5	6.25
A. oryzae	S	>100.0	>100.0	12.5	>100.0	>100.0
A. versicolor	S	6.25	6.25	3.12	6.25	3.12
A. fumigatus IFM 25	S	>100.0	>100.0	>100.0	>100.0	>100.0
Microsporum canis	S	100.0	50.0	12.5	100.0	25.0
M. gypseum	S	100.0	100.0	12.5	100.0	25.0
Trichophyton rubrum IFM 40732	S	100.0	100.0	12.5	100.0	25.0
T. rubrum IFM 40733	S	100.0	6.25	6.25	25.0	6.25
T. mentagrophytes IFM 40735	S	100.0	25.0	6.25	25.0	12.5
T. mentagrophytes IFM 40737	S	100.0	50.0	12.5	50.0	12.5
Sporothrix schenckii IFM 40750	S	3.12	3.12	3.12	3.12	3.12
S. schenckii IFM 40751	S	3.12	3.12	3.12	1.56	3.12
Fonsecaea pedrosoi	S	6.25	3.12	3.12	3.12	6.25
Candida albicans IFM 40001	Y	6.25	6.25	3.12	12.5	6.25
C. albicans IFM 40003	Y	50.0	12.5	6.25	50.0	6.25
C. albicans IFM 40005	Y	6.25	6.25	6.25	50.0	12.5
C. albicans IFM 40008	Y	6.25	6.25	6.25	50.0	50.0
C. albicans 7N	Y	>100.0	>100.0	12.5	>100.0	50.0
C. guilliermondii	Y	>100.0	>100.0	12.5	>100.0	>100.0
C. tropicalis	Y	>100.0	12.5	12.5	>100.0	100.0
C. krusei	Y	>100.0	>100.0	100.0	>100.0	>100.0
C. parapsilosis	Y	>100.0	12.5	12.5	>100.0	100.0
C. utilis	Y	>100.0	12.5	6.25	50.0	12.5
Cryptococcus neoformans IFM 40037	Y	1.56	1.56	1.56	1.56	1.56
C. neoformans IFM 40038	Y	3.12	3.12	3.12	1.56	1.56
C. neoformans IFM 40047	Y	3.12	3.12	1.56	1.56	1.56
Geotrichum candidum	Y	>100.0	12.5	12.5	>100.0	>100.0
Torulopsis glabrata	Y	>100.0	>100.0	100.0	>100.0	>100.0
Saccharomyces cerevisiae	Y	>100.0	>100.0	50.0	>100.0	>100.0
Cladosporium fulvum	S	>100.0	6.25	6.25	6.25	3.12
C. sphaerospermum	S	6.25	3.12	3.12	6.25	3.12
Debaryomyces hansenii	S	>100.0	>100.0	>100.0	>100.0	>100.0
Fusarium moniliforme	S	>100.0	6.25	6.25	12.5	6.25
F. oxysporum	S	>100.0	100.0	6.25	6.25	6.25
F. roseum	S	>100.0	100.0	6.25	6.25	6.25
F. solani	S	>100.0	100.0	6.25	100.0	6.25
Gibberella fujikuroi	S	>100.0	100.0	6.25	25.0	6.25
Helminthosporium sesamum	S	6.25	3.12	3.12	3.12	3.12
Penicillium expansum IMF 40619	S	1.56	1.56	3.12	3.12	3.12

Medium: Sabouraud agar (S), yeast morphology agar (Y).
Incubation: 24 hours to 3 days at 37°C, depending on test strain.

mice by a single injection.

The results are given in Table 5.

Table 5. Acute toxicities of intraperitoneally injected antibiotics LI-F03, F04, F05, F07, and F08 in mice.

Antibiotics	LD_{50} (mg/kg)
LI-F03	150~200
LI-F04	150~200
LI-F05	150~200
LI-F07	100~150
LI-F08	100~150

Discussion

The present work represents a family of closely related new peptide antibiotics. A complex of new peptide antibiotics, which was composed of more than ten components, was isolated

from *B. polymyxa* L-1129. The five components, designated as antibiotics LI-F03, F04, F05, F07, and F08, were isolated from the complex by reversed phase HPLC. From comparison of physicochemical properties and antimicrobial activities of the antibiotics with those of peptidic products from bacteria reported, they are easily distinguisable from the polymyxin group[3] including colistin and circulin, thus they are considered to be new peptide antibiotics. However, antibiotic LI-F04, the most abundant component, might be identical with gatavalin[4], because of the identity of their constituent amino acids.

The five antibiotics isolated here behaved as a single entity under various HPLC conditions tested. However, it is obvious by the FAB-MS data that each antibiotic is a mixture of two homologous components. This evidence is also substantiated by the amino acid composition data, which give the calculated molecular weight of more than 1,000 to each antibiotic. Thus, attempts to separate each antibiotic into two components have been performed under various HPLC conditions, but they are unsuccessful till now. Further investigation for the separation and studies on the amino acid sequences of the antibiotics are being continued.

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