PB-5266 A, B AND C, NEW MONOBACTAMS

I. TAXONOMY, FERMENTATION AND ISOLATION

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New monobactams, PB-5266 A, B and C were isolated from the culture filtrate of *Cytophaga johnsonae* PB-5266 by various types of column chromatography and preparative reverse-phase HPLC. PB-5266 A, B and C exhibited weak antibacterial activity against a sensitive mutant of *Escherichia coli* to β-lactam antibiotics.

In recent years several monobactams produced by bacteria have been reported.[1~6] During our screening program developed to detect β-lactam antibiotics from bacteria, we have previously isolated a monobactam named PB-5582 A from *Cytophaga johnsonae*,[7] which was identical with SQ 28,332 isolated from a *Flexibacter* sp.[5] Now we wish to report the isolation of three new monobactams from a strain of *C. johnsonae*. The taxonomic study of the producing strain, fermentation conditions and isolation of three new monobactams PB-5266 A, B and C are presented. The brief description on biological property is also reported.

Taxonomy

The producing organism numbered PB-5266 in our laboratories was isolated from a soil sample collected in Amami-oshima, Kagoshima prefecture, Japan.

The organism is a Gram-negative, non-sporulating rods ($0.3\sim0.5\ \mu\times1.0\sim2.0\ \mu$) with tapered ends. It has no flagella and exhibits a spreading growth due to gliding motility. Sheaths and micro-cysts are not observed. On nutrient agar, it forms circular, entire, translucent and smooth colonies with yellowish salmon color. Soluble pigments are not formed.

The organism, a facultative anaerobe, showed good growth at 28°C but not at 5°C and 37°C. Other physiological characteristics are shown in Table 1. On cleavage of carbohydrate, acid formation was observed from D-glucose, D-galactose, D-mannose, L-rhamnose, D-xylose, D,L-arabinose, D-cellobiose, D-trehalose, sucrose, maltose, lactose, salicin, dextrin and starch, but not from D-fructose, raffinose, D-sorbitol, adonitol, inositol, D-mannitol, dulcitol, glycerol, ethanol and inulin. No gas formation was observed from the above carbohydrates.

From comparison of these characteristics with those of bacteria registered in the 8th Ed. of BERGEY'S Manual of Determinative Bacteriology,[8] the organism should be ascribed to the genus *Cytophaga* or *Flexibacter*. However, the organism attacks chitin and carboxymethylcellulose, but *Flexibacter* does not. Therefore the organism should be classified to the genus *Cytophaga*. Further, by comparison with eight species of the genus, the strain PB-5266 was identified as *C. johnsonae*.

Fermentation

The cell mass on two slant cultures of PB-5266 were suspended in sterilized saline and used to

Table 1. Physiological characteristics of strain PB-5266.

Properties observed	Results	Properties observed	Results
Catalase test	+	Cellulose degradation	−
Oxidase test	+	Phosphatase test	+
OF-test	Oxidative	Indole production	−
Peptonization of milk	+	Voges-Proskauer test	−
Coagulation of milk	−	Methyl red test	−
Haemolysis	−	H_2S production	−
Gelatin liquefaction	+	β-Galactosidase production	+
Chitin degradation	+	Nitrate reduction	−
Starch degradation	+	Denitrification	−
Agar degradation	−	Citrate utilization	−
Degradation of carboxymethylcellulose	+	Sensitivity to O/129	Sensitive

Fig. 1. Isolation of PB-5266 complex.

Broth supernate
1) adsorbed on Amberlite IRA-458 (Cl^-), eluting with 5% NaCl in 50 mM KH_2PO_4
2) adsorbed on charcoal, eluting with 60% acetone
3) chromatography on QAE-Sephadex (Cl^-), eluting stepwise with 0.2 M NaCl and 0.5 M NaCl in 50 mM KH_2PO_4
4) adsorbed on charcoal, eluting with 60% acetone
5) chromatography on cellulose, eluting with CH_3CN - H_2O (75 : 25)
6) chromatography with MCI-GEL CHP-20P, eluting with 10 mM KH_2PO_4
7) adsorbed on charcoal, eluting with 60% acetone
8) chromatography on cellulose, eluting with propanol - H_2O (75 : 25)

PB-5266 Complex

inoculate 20 liters of a medium consisting of fish meal 2.0%, potato starch 1.0%, corn starch 1.0%, KH_2PO_4 0.5%, adjusted to pH 4.5 with H_3PO_4 before sterilization in a 30-liter jar fermentor. Fermentation was carried out at 28°C for 1 day under aeration of 20 liters per minute and agitation of 200 rpm. Antibiotic activity was monitored by disc agar diffusion method using *Escherichia coli* LS-1 (a supersensitive mutant to β-lactam antibiotics) through fermentation and isolation process.

Isolation

A crude preparation of PB-5266 complex containing several active components was obtained from fermentation broths as outlined in Fig. 1.

Since these antibiotics were extremely labile at pH higher than 6.0, the isolation was carried out at pH 4.0~5.0. PB-5266 A, B and C were separated by reversed phase preparative HPLC, giving the respective pure preparations.

Chromatographic Behavior of PB-5266 Components

PB-5266 A, B and C are water soluble, strongly acidic substances showing the same mobility as sulfazecin on paper electrophoresis at pH 4.5. Rf values on cellulose plates and retention volumes in HPLC are shown in Table 2.

Biological Activities

PB-5266 A, B and C similarly exhibited weak antibacterial activity only against *E. coli* LS-1 (a

Table 2. Chromatographic behavior of PB-5266 A, B and C.

	Rf values on cellulose plates*		Retention volumes on HPLC** (ml)
	70% Propanol	70% Acetonitrile	
PB-5266 A	0.41	0.48	3.80
PB-5266 B	0.36	0.41	2.70
PB-5266 C	0.34	0.41	2.56

* Avicel plate (purchased from Funakoshi Co., Ltd.) were used.
** Nucleosil $5C_{18}$ column (4.6×150 mm) with 20 mM KH_2PO_4 were used.

supersensitive mutant to β-lactam antibiotics) by the usual disk agar diffusion method so far as tested.

Experimental

Isolation of PB-5266 A, B and C

The culture broth (144 liters) by jar fermentors was adjusted to pH 4.5 with H_2SO_4 and then centrifuged. The antibiotic substances in the supernate were adsorbed on an Amberlite IRA-458 (Cl^-) (12 liters). The resin was washed with H_2O and eluted with 5% NaCl in 50 mM KH_2PO_4. The active eluates were adsorbed on charcoal (140 g, Wako Chemicals) and eluted with 60% acetone. Concentration and freeze-drying at pH 4.5 gave a crude powder (6.3 g). The powder was charged to a QAE-Sephadex column (500 ml) and eluted with 0.2 M NaCl in 50 mM KH_2PO_4 (2 liters) followed by elution with 0.5 M NaCl in 50 mM KH_2PO_4 (2 liters).

The active eluate in 0.2 M NaCl was desalted by adsorption and elution on charcoal and then freeze-dried to give a brown powder (2.26 g). Following solubilization in 75% acetonitrile, the material was chromatographed on an Avicel cellulose column (Funakoshi Co., Ltd.) with 75% acetonitrile to give a yellow powder (924 mg). This was applied to a column of MCI-GEL CHP-20P and developed with 10 mM KH_2PO_4. The active eluant was desalted on charcoal to give a colorless powder (355 mg), which was then subjected to cellulose chromatography with 75% propanol. Concentration and freeze-drying gave a colorless powder of PB-5266 complex (184 mg).

Active fractions eluted with 0.5 M NaCl were treated as above to step 6 and then rechromatographed on QAE-Sephadex with a linear gradient of 50 mM KH_2PO_4 and 0.5 M NaCl in 50 mM KH_2PO_4. Desalting on charcoal gave an additional preparation of PB-5266 complex (46 mg).

The PB-5266 complex was separated by preparative HPLC on a Nucleosil $5C_{18}$ column (10× 250 mm) with 20 mM KH_2PO_4. Peak fractions were adsorbed on charcoal (at pH 4.0) and extracted with 60% acetone (adjusting pH 4.5 with potassium hydroxide). Concentration and freeze-drying of the extracts at pH 4.5 afforded pure preparations of potassium salts of PB-5266 A (40 mg), B (25 mg) and C (25 mg), respectively.

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