ISOLATION AND CHARACTERIZATION OF AGGLOMERINS A, B, C AND D

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New antibiotics, agglomerins A, B, C and D, were isolated from the culture broth of a bacterial strain identified as *Enterobacter agglomerans*. These antibiotics are acidic in nature and their sodium salts are obtained as colorless crystalline powders, soluble in lower alcohols. All the antibiotics shows characteristic UV maxima at 248 and 298 nm. Molecular formulas: A, $C_{15}H_{21}O_4Na$; B, $C_{17}H_{23}O_4Na$; C, $C_{17}H_{25}O_4Na$ and D, $C_{19}H_{27}O_4Na$; were indicated by elemental analysis and MS. These antibiotics are active against a wide variety of anaerobic bacteria and weakly against aerobic Gram-positive bacteria *in vitro*.

In recent years, an effort for screening for anti-anaerobic antibiotics from actinomycete origin have been made by ŌMURA and his co-workers, resulting in the finding of thiotetromycin, clostomicin, luminamicin and lustromycin[1]. We also have carried out a screening trial for new antibiotics active dominantly against anaerobic bacteria, and isolated some new antibiotics. One of them, named agglomerins, was isolated from the culture broth of a bacterial strain numbered PB-6,042 and identified as *Enterobacter agglomerans*. It was further separated by reversed phase HPLC to four components, A, B, C and D. These antibiotics are mainly active against anaerobic bacteria and weakly active against aerobic Gram-positive bacteria. Their unique structures will be reported in an accompanying paper[2].

In this paper, the taxonomy of the producing organism, the isolation, physico-chemical and biological properties of the antibiotics are presented.

Taxonomy

The producing organism numbered PB-6,042 was isolated from river water collected in Kobe-city, Japan.

Table 1. Physiological characteristics of strain PB-6,042.

Properties observed	Results	Properties observed	Results
Catalase test	+	β-Galactosidase test	+
Oxidase test	−	Urease test	−
OF-test	Fermentative	Deoxyribonuclease test	−
Peptonization of milk	+	Tween 80 esterase test	−
Coagulation of milk	+	Voges-Proskauer test	+
Gelatin liquefaction	+	Methyl red test	−
Esculin hydrolysis	+	Nitrate reduction	+
Indole production	+	Denitrification	−
H_2S production	−	Citrate utilization	+
Arginine dihydrolase test	+	Malonate utilization	+
Lysine decarboxylase test	−	Mole % G+C of DNA	53.0
Ornithine decarboxylase test	−		

The organism is Gram-negative, non-sporulating rods ($0.5 \sim 0.7 \times 2.0 \sim 5.0\,\mu m$) with rounded ends. Motile by three to five peritrichous flagella. On nutrient agar, it forms circular, flat, entire, translucent, glistening and smooth colonies with grayish pale brown color.

The organism, facultatively anaerobic, showed good growth at 28°C. Other physiological characteristics are shown in Table 1. On cleavage of carbohydrates, acid formation with gas was observed from DL-arabinose, D-xylose, D-glucose, D-mannitol and D-melibiose. Acid formation without gas was observed from D-fructose, D-mannose, L-rhamnose, inositol, sucrose and D-raffinose. No acid formation was observed from adonitol, dulcitol, D-sorbitol, lactose, maltose and trehalose.

From comparison of these characteristics with those of bacteria registered in the Vol. 1 of BERGEY's Manual of Systematic Bacteriology[3], the organism should be ascribed to the genus *Enterobacter*. According to further comparison with seven species of the genus, the organism is closely related to *E. agglomerans* biogroup G4.

Fermentation

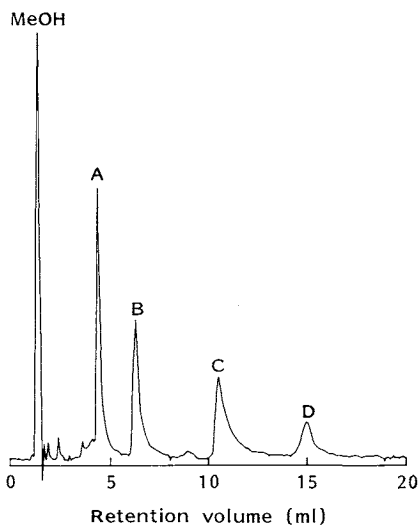
The cell suspension of the strain PB-6,042 was inoculated into 15 liters of a medium consisting of glucose 1.0%, yeast extract 0.5%, $CaCO_3$ 0.7%, pH 7.0, in a 50-liter jar fermenter. Fermentation was carried out for 2 days at 32°C under aeration of $0.3 \sim 1.0$ v/v/m and agitation of $450 \sim 600$ rpm.

Antibiotic activity was monitored by pulp disk agar diffusion method on an assay plate of *Clostridium difficile* ATCC 17857 which was incubated under anaerobic condition throughout this experiment.

Isolation and Purification

The culture broth (30 liters) obtained as above was adjusted to pH 4.0 with dil HCl. Some 6 kg of NaCl and 10 liters of EtOAc were added to the broth, which was vigorously stirred for 30 minutes and then centrifuged. The EtOAc layer separated was washed with 2% aqueous $NaHCO_3$ solution and then water. Evaporation of the solvent gave an oily residue. The residue was applied to a silica gel column (Merck Silica gel 60, 3.6×40 cm), which was washed with $CHCl_3$ and then eluted with $CHCl_3$-

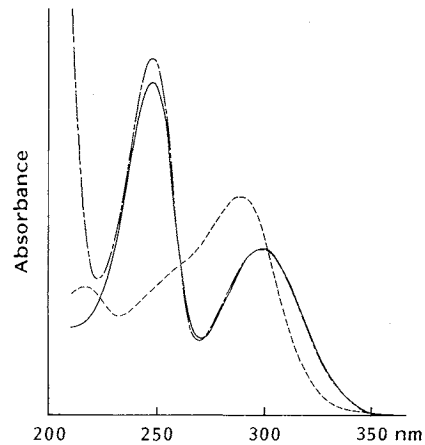
Fig. 1. HPLC of agglomerins A, B, C and D.



Column: Nucleosil 5 C_{18} (4.6×250 mm). Mobil phase: CH_3CN-50 mM phosphate buffer, pH 7.5, containing 50 mM Na_2SO_4 (40:60). Flow rate: 1 ml/minute. Monitored by O.D. at 254 nm.

Fig. 2. UV absorption spectrum of agglomerin A.

——MeOH, —·— dil NaOH-MeOH, – – – dil HCl-MeOH.



MeOH (9:1). The active eluate fraction from the column was, after evaporation of the solvent, applied again to the same column, which was washed with EtOAc and eluted with EtOAc-MeOH (9:1). Evaporation of the active eluate fraction afforded a powder (1.0 g) of agglomerin complex.

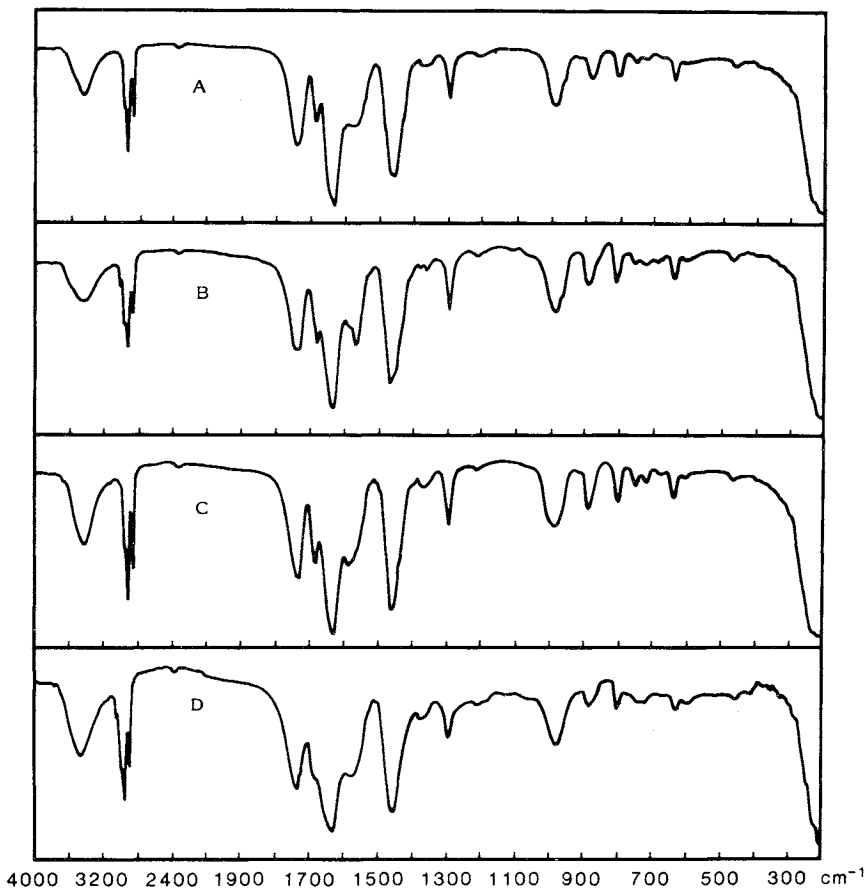
The complex was separated by preparative HPLC using a Nucleosil 10 C_{18} column (2.0 × 25 cm) with CH_3CN-50 mM phosphate buffer, pH 7.5, containing 50 mM Na_2SO_4 (48:52). Components were eluted in the order A, B, C and D. Each peak was collected and evaporated to a nearly aqueous solution, from which the antibiotic was extracted with EtOAc. The extract was washed with water, dried with Na_2SO_4 and then evaporated to give a sodium salt of the antibiotic. From some 500 mg of the above complex, component A (115 mg), B (92 mg), C (74 mg) and D (24 mg) were obtained.

Physico-chemical Properties

Agglomerins A, B, C and D are acidic substances. Their Na salts are obtained as colorless crystalline powders. These are considerably unstable in acidic conditions and convert to inactive products probably due to polymerization. The Na salts are soluble in DMSO, MeOH, EtOH and EtOAc, slightly soluble in $CHCl_3$, but substantially insoluble in diethyl ether, petroleum ether and water.

Agglomerins A, B, C and D are not distinguishable by TLC, showing Rf 0.21 with $CHCl_3$-MeOH (9:1) and Rf 0.39 with EtOAc-MeOH (9:1) on a Merck Silica gel GF plate. These substances are easily

Fig. 3. IR absorption spectra of agglomerins A, B, C and D (KBr).



separable by HPLC as shown in Fig. 1.

The UV absorptions of agglomerins A, B, C and D are substantially identical to each others λ_{max}^{MeOH} nm (ε) 248 (18,842), 298 (9,242); $\lambda_{max}^{dil\ NaOH\text{-}MeOH}$ nm (ε) 248 (20,205), 298 (9,242); $\lambda_{max}^{dil\ HCl\text{-}MeOH}$ nm (ε) 215 (7,640), 285 (12,239) (Fig. 2). The IR spectra of agglomerins A, B, C and D are also quite similar to each others (Fig. 3). CD measurement shows no optical activities of these compounds. MP's and the results of elemental analyses and secondary ion mass spectrometry (SI-MS) are shown in Table 2.

Biological Properties

Agglomerins A, B, C and D are moderately active against anaerobic Gram-positive and Gram-negative bacteria as shown in Table 3. These also shows weak activity against aerobic Gram-positive bacteria (Table 4).

Table 2. MP, elemental analysis and SI-MS of agglomerins A, B, C and D.

	A	B	C	D
MP (°C)	113~115	85~88	125~128	103~106
Molecular formula	$C_{15}H_{21}O_4Na\cdot\frac{1}{4}H_2O$	$C_{17}H_{23}O_4Na\cdot\frac{1}{4}H_2O$	$C_{17}H_{25}O_4Na\cdot\frac{1}{4}H_2O$	$C_{19}H_{27}O_4Na\cdot\frac{1}{4}H_2O$
Anal				
Calcd:	C 61.53, H 7.35, Na 7.86	C 64.05, H 7.37, Na 7.22	C 63.65, H 7.80, Na 7.17	C 65.80, H 7.80, Na 6.63
Found:	C 61.50, H 7.36, Na 7.47	C 63.99, H 7.51, Na 6.91	C 63.90, H 8.16, Na 6.75	C 65.56, H 8.07, Na 6.11
SI-MS (M+Na) (*m/z*)	311	337	339	365

Table 3. Antibacterial spectrum of agglomerins A, B, C and D against anaerobic bacteria.

Test organism	MIC (μg/ml)			
	A	B	C	D
Peptococcus asaccharolyticus ATCC 14963	12.5	6.25	3.13	6.25
P. prevotii ATCC 9321	12.5	6.25	3.13	6.25
Peptostreptococcus micros VPI 5464-1	1.56	1.56	0.78	0.78
Streptococcus constellatus ATCC 27823	3.13	1.56	0.78	1.56
Eubacterium limosum ATCC 8486	6.25	3.13	1.56	3.13
E. aerofaciens ATCC 25986	12.5	6.25	3.13	6.25
Propionibacterium acnes ATCC 11827	3.13	3.13	0.78	1.56
Bifidobacterium adolescentis JCM 1250	12.5	6.25	0.78	6.25
B. bifidum JCM 1122	12.5	12.5	6.25	6.25
B. longum ATCC 15707	6.25	6.25	3.13	3.13
Clostridium perfringens ATCC 13124	12.5	6.25	3.13	3.13
C. difficile ATCC 17857	3.13	1.56	0.78	1.56
Bacteroides fragilis GM 7000	3.13	3.13	3.13	6.25
B. fragilis ATCC 25285	3.13	3.13	1.56	3.13
B. thetaiotaomicron WAL 3304	3.13	3.13	3.13	6.25
B. vulgatus ATCC 29327	3.13	3.13	3.13	12.5
B. melaninogenicus GAI 0413	6.25	6.25	6.25	12.5
Fusobacterium varium ATCC 8501	>100	>100	>100	>100
F. necrophorum ATCC 25286	6.25	12.5	12.5	ND
F. nucleatum ATCC 25586	6.25	12.5	12.5	25
F. mortiferum ATCC 9817	12.5	25	25	25

Inoculum size: One loopful of 10^6 cfu/ml.
Medium: GAM Agar (Nissui).
ND: Not determined.

Table 4. Antibacterial spectrum of agglomerins A, B, C and D against aerobic bacteria.

Test organism	MIC (μg/ml)			
	A	B	C	D
Staphylococcus aureus FDA JC-1	25	12.5	>100	>100
S. aureus Smith	>100	12.5	>100	6.25
Streptococcus pyogenes C-203	25	12.5	12.5	12.5
S. pneumoniae Type 1	25	12.5	12.5	12.5
Escherichia coli NIHJ JC-2	>100	>100	>100	>100
E. coli EC-14	>100	>100	>100	>100
Klebsiella pneumoniae SR1	>100	>100	>100	>100
Proteus vulgaris CN-329	>100	>100	>100	>100
Enterobacter cloacae ATCC 13047	>100	>100	>100	>100
Serratia marcescens ATCC 13880	>100	>100	>100	>100
Pseudomonas aeruginosa ATCC 25619	>100	>100	>100	>100

Experimental

The UV absorption spectra were measured with a Hitachi 323 spectrometer and the IR absorption spectra with a Jasco DS-403G spectrometer, CD spectra with a Jasco J-40C automatic recording spectropolarimeter and SI-MS with a Hitachi M-68 mass spectrometer (Shionogi version of M-80).

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