AUREOBASIDINS, NEW ANTIFUNGAL ANTIBIOTICS

TAXONOMY, FERMENTATION, ISOLATION, AND PROPERTIES

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Aureobasidins A to R were isolated from the fermentation broth of *Aureobasidium pullulans* R106. Aureobasidins are cyclic depsipeptide antibiotics with MW's ranging from 1,070 to 1,148. Aureobasidins showed high *in vitro* antifungal activity against *Candida albicans*.

In the course of our screening for antifungal substances from various microorganisms, new antifungal antibiotics that we named aureobasidins were obtained from the fermentation broth of a strain of *Aureobasidium pullulans*, a species of black yeast.[1,2] The producing organism, strain R106, was isolated from a leaf collected at Tsushima in Nagasaki, Japan.

In this paper we describe the taxonomy of the producing organism, the production and isolation procedures of aureobasidins A to R, and their physico-chemical and biological properties. The structural studies of aureobasidins A to R will be described in the following papers.[3,4]

Materials and Methods

General

FAB-MS were recorded on a Jeol JMS DX-302 spectrometer. Optical rotations were measured with a Jasco DIP-181. The antibiotics were hydrolyzed with 6N HCl at 110°C and the hydrolysates were examined in an amino acid autoanalyzer (Jeol JCL-300).

Detection of Aureobasidins

Aureobasidins were detected by a bioassay, which was carried out by the paper disc diffusion method, based on their *in vitro* antifungal activity against *Candida albicans* TIMM 0136 grown in a medium consisting of yeast nitrogen base (Difco) 0.67% and glucose 2%.

Aureobasidin A concentrations were measured by reversed phase HPLC (Capcell Pak C_{18}, Shiseido) with a solvent of acetonitrile-water (7:3) at the UV absorption of 230 nm.

Measurement of Antifungal Activity

The MICs against fungi were determined by an agar dilution method on Casitone agar medium. Aureobasidins were dissolved in methanol and the solution was diluted with 50% aqueous methanol. A loopful of a fungal cell suspension in sterile water (5×10^7 cells/ml) prepared from the slant culture was streaked on the surface of the agar plates that contained a drug. After incubation of the plates at 30°C

for 3 days, the lowest concentration of the drug causing virtually complete growth inhibition was determined as the MIC.

Results

Taxonomic Studies

The cultural characteristics of strain R106 on various media after incubation for 4, 7 and 14 days at 25°C are shown in Table 1. The temperature permitting growth of the strain was 12.5 to 29°C, and optimum temperature for growth was 23 to 29°C. The strain showed good growth in a vitamin-free medium.

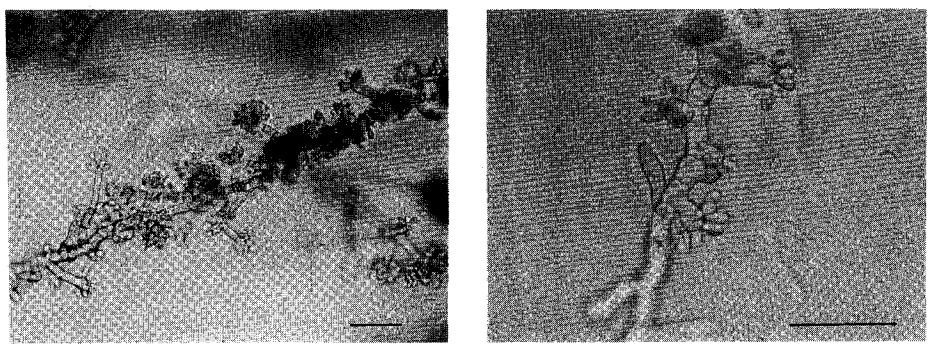
Strain R106 showed good growth on potato-dextrose (PD) agar, CZAPEK agar, and yeast extract-malt extract agar media. The surface of colonies of the strain was usually mucoid or pasty, and were sometimes velvety; with time, the colonies became leathery. The color of colonies was white to creamy or light pink, then changing olive green to light brown or brown, and often finally changing to black with production of dark brown pigments, which were insoluble. Rhizoid-like structures often formed around the colonies. Hyphae sized 2 to 15 μm i.d. elongated into the agar medium without forming aerial mycelia. Blastic

Table 1. Cultural characteristics of *Aureobasidium pullulans* R106.

Medium	Color of colonies after cultivation for			Growth characteristics
	4 days	7 days	14 days	
Yeast extract-malt extract agar	Creamy	Olive green	Light brown	Good growth; chlamydospores are formed
Potato-dextrose (PD) agar	Creamy or light pink	Olive green	Light brown	Good growth; blastic conidia are abundant and form ball-like clusters
CZAPEK agar	Creamy	Brown	Black	Good growth; hyphae are abundant, and are often thick-walled
SABOURAUD-dextrose agar	Creamy or light pink	Olive green	Dark green	Good growth; chlamydospores are formed
Oatmeal agar	Creamy gray	Dark brown	Dark brown	Moderate growth; hyphae are abundant
YpSs agar	Creamy	Light olive green	Olive green	Good growth; hyphae are thin blastic conidia are abundant and form ball-like clusters

Fig. 1. Microscopic features of strain R106.

Grown on PD agar for 21 days. Bar represents 20 μm.



conidia measuring $1\sim5\times2\sim10\,\mu m$, were often formed in an intercalary or terminal position on hyphae like fingers, and sometimes formed ball-like clusters (Fig. 1). Vegetative cells in the early stage of growth were yeast-like, ellipsoidal or lemon-shaped measuring $3\sim5\times8\sim15\,\mu m$ and multiplied by polyblastic budding. The strain formed arthrospores sized $4\sim10\times10\sim25\,\mu m$. No ascospores were observed.

From the mycological characteristics described above, strain R106 was identified as belonging to the genus *Aureobasidium*. Among the known species of *Aureobasidium*,[5~7] strain R106 was most like a strain of *A. pullulans*. So we designated the strain *A. pullulans* R106. The strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with an accession No. of FERM BP-1938.

Fermentation

A loopful of cells of a slant culture of strain R106 was inoculated in 100 ml of a liquid medium composed of yeast nitrogen base (Difco) 0.67% and glucose 2% in a 500-ml Erlenmeyer flask and the flask was shaken at 25°C for 2 days to give a seed culture. The seed culture (1,000 ml) was transferred into a 200-liter fermenter containing 100 liters of a medium consisting of glucose 2%, $(NH_4)_2SO_4$ 0.5%, KH_2PO_4 0.15%, $MgSO_4\cdot7H_2O$ 0.05%, $CaCl_2\cdot2H_2O$ 0.01%, NaCl 0.01%, $FeCl_3\cdot6H_2O$ 0.5 μg/ml and $ZnSO_4\cdot7H_2O$ 0.5 μg/ml. Fermentation was carried out at 25°C for 56 hours with aeration (100 liters/minute) and agitation (100 rpm). To the culture, 20 liters of a medium containing glucose 10%, $(NH_4)_2SO_4$ 2.5%, Polypepton 5%, KH_2PO_4 0.75%, $MgSO_4\cdot7H_2O$ 0.25%, $CaCl_2\cdot2H_2O$ 0.05%, NaCl 0.05%, $FeCl_3\cdot6H_2O$ 2.5 μg/ml and $ZnSO_4\cdot7H_2O$ 2.5 μg/ml was supplemented, and fermentation was further continued at 25°C for 78 hours with aeration (120 liters/minute) and agitation (100 rpm).

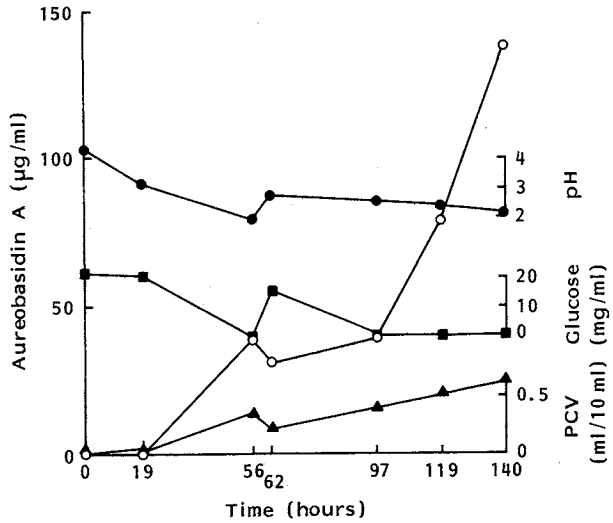
The time course of the production of aureobasidin A is shown in Fig. 2.

Isolation and Purification

The fermentation broth was centrifuged to separate the supernatant and the mycelial cake. The mycelial cake was extracted with 10 liters of ethanol. The ethanol extract was concentrated under reduced pressure

Fig. 2. Time course of production of aureobasidin A by fermentation.

○ Aureobacidin A (μg/ml), ● pH, ■ glucose (mg/ml), ▲ packed cell volume (ml/10 ml).



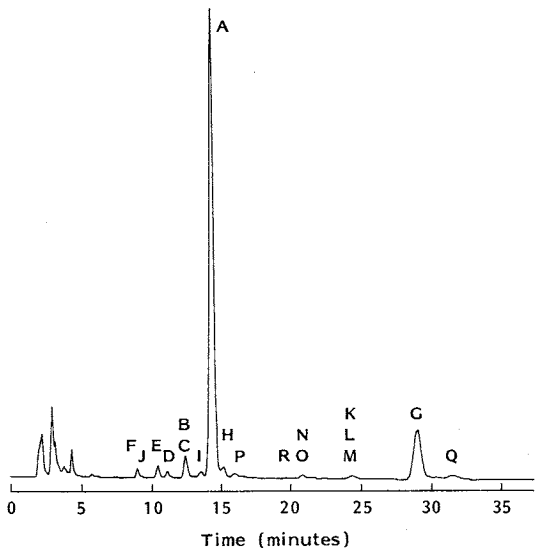
The glucose concentration was determined by the glucose oxidase method.

to remove ethanol and the residue was extracted twice with ethyl acetate. The ethyl acetate extract was concentrated to dryness under reduced pressure, and the residue was dissolved in chloroform. The chloroform solution was put onto a column of silica gel (1.5 liters). After the column was washed with 3 liters of hexane, it was developed and eluted with 6 liters of hexane - 2-propanol (7 : 3). The active fraction eluted with hexane - 2-propanol was condensed under reduced pressure to give 15 g of a residue.

The residue was dissolved in acetonitrile. HPLC analysis of the solution suggested that at least 10 related compounds were contained in the residue (Fig. 3).

The solution was applied on a preparative ODS-silica gel column of Soken Pak/C_{18} (5 cm × 50 cm, 15/30 μm) and developed with acetonitrile - water (7 : 3). The fractions containing aureobasidins A to R were collected separately and concentrated under reduced pressure to give residues of crude aureobasidins A to R. The residues containing A, D, E, F, G, H, I, J, P, Q and R were each dissolved in acetonitrile, applied on a column of Capcell Pak C_{18} (2 × 25 cm) and eluted with acetonitrile - water (7 : 3) to give pure aureobasidins (A: 3,500 mg, D: 96 mg, E: 81 mg, F: 72 mg, G: 1,380 mg, H: 32 mg, I: 60 mg, J: 24 mg, P: 15 mg, Q: 14 mg, and

Fig. 3. HPLC of a mixture of aureobasidins.



Column: Capcell Pak C_{18} (SG 120), 6 × 150 mm. Mobile phase: 75% CH_3CN - 25% H_2O, 1.0 ml/minute. Temperature: 50°C.

Table 2. Physico-chemical properties of aureobasidins A to R.

	A	B	C	D	E	F	G	H	I
FAB-MS (M+H, *m/z*)	1,101	1,087	1,087	1,101	1,117	1,087	1,085	1,071	1,101
Amino acid analysis	Pro aIle Leu Phe	Pro aIle Leu Phe	Pro Val Leu Phe	Pro aIle Leu Phe	Pro aIle Leu Phe	Pro Val aIle Leu Phe	Pro aIle Lue Phe	Pro Val aIle Leu Phe	Pro Leu Phe
$[\alpha]_D^{20}$ (MeOH)	−254.3° (*c* 1.0)	−222.3° (*c* 1.0)	−345.0° (*c* 0.3)	−241.3° (*c* 1.0)	−211.6° (*c* 1.0)	−191.8° (*c* 1.0)	−248.1° (*c* 1.0)	−170.0° (*c* 0.8)	−226.3° (*c* 1.0)

	J	K	L	M	N	O	P	Q	R
FAB-MS (M+H, *m/z*)	1,115	1,071	1,071	1,071	1,083	1,149	1,071	1,133	1,101
Amino acid analysis	Pro aIle Leu Phe	Pro aIle Leu Phe	Pro Val Leu Phe	Pro aIle Leu Phe	Pro aIle Leu Phe	Pro aIle Leu Phe	Pro Val aIle Leu Phe	Pro aIle Leu Phe	Pro aIle Leu Phe
$[\alpha]_D^{20}$ (MeOH)	−217.6° (*c* 1.0)	−228.1° (*c* 0.7)	−229.7° (*c* 0.31)	−210.0° (*c* 1.0)	−205.1° (*c* 1.0)	−256.7° (*c* 1.0)	−215.4° (*c* 0.44)	−272.9° (*c* 1.0)	−247.9° (*c* 1.0)

Pro: Proline, aIle: alloisoleucine, Val: valine, Leu: leucine, Phe: phenylalanine.

Table 3. Antifungal activity of aureobasidins A to R.

Organisms	TIMM No.	MIC (μg/ml)								
		A	B	C	D	E	F	G	H	I
Candida albicans	0136	<0.05	0.10	0.10	0.20	<0.05	0.78	3.12	1.56	<0.05
C. albicans	0171	<0.05	0.10	0.10	0.20	<0.05	0.39	1.56	1.56	<0.05
C. albicans	1768	<0.05	0.78	1.56	0.39	<0.05	0.39	3.12	12.5	0.20
C. kefyr	0301	0.20	0.20	0.20	0.78	0.20	0.39	25	>25	0.20
C. glabrata	1062	0.05	0.20	0.20	1.56	0.20	1.56	>25	>25	0.39
Cryptococcus neoformans	0354	0.78	1.56	1.56	25	3.12	25	>25	>25	25

Organisms	TIMM No.	MIC (μg/ml)								
		J	K	L	M	N	O	P	Q	R
Candida albicans	0136	0.78	1.56	6.25	6.25	1.56	12.5	3.12	12.5	25
C. albicans	0171	0.78	1.56	6.25	12.5	1.56	25	1.56	25	25
C. albicans	1768	12.5	1.56	25	12.5	12.5	>25	25	>25	>25
C. kefyr	0301	3.12	6.25	12.5	25	6.25	>25	3.12	>25	>25
C. glabrata	1062	3.12	>25	>25	>25	12.5	>25	>25	>25	>25
Cryptococcus neoformans	0354	25	>25	>25	>25	>25	>25	>25	>25	>25

R: 18 mg). The residue containing aureobasidins B and C was dissolved in chloroform and chromatographed on a silica gel HPLC column (Nucleosil-5, 10 × 250 mm) with a solvent of hexane - 2-propanol - acetonitrile (85 : 6 : 9) to obtain purified aureobasidins (B: 55 mg, and C: 54 mg). The residue containing aureobasidins K, L and M was purified in the same way by silica gel HPLC column chromatography to obtain pure aureobasidins (K: 107 mg, L: 42 mg, and M: 78 mg). The residue containing aureobasidins N and O was also purified by silica gel HPLC to obtain pure aureobasidins (N: 18 mg, and O: 18 mg).

Physico-chemical Properties

Some properties of aureobasidins A to R are summarized in Table 2. Aureobasidins showed maxima at 258 (ε 3,850) and 264 nm (ε 2,660) in UV absorption spectrum in methanol. Aureobasidins were soluble in ethanol, chloroform, ethyl ether and insoluble in water. Aureobasidins contained phenylalanine, proline, and leucine in common, and contained five different amino acids, including *N*-methylated amino acids. Aureobasidins were cyclic depsipeptide antibiotics containing eight amino acids and a hydroxy acid[3,4] with MW's of 1,070 ~ 1,148.

Biological Properties

The antifungal activity of aureobasidins is shown in Table 3. Aureobasidins showed strong antifungal activity, especially against *C. albicans*. Aureobasidins A, B, C and E showed higher activity than the others.

Aureobasidin A, B, E or G showed no signs of toxicity when administered once to mice intraperitoneally at the dose of 200 mg/kg.

Details of the biological properties of aureobasidin A, including its *in vitro* and *in vivo* antifungal activity, will be described elsewhere.

Discussion

There are some peptide antibiotics with antifungal activity, including the aculeacin/echinocandin family and lipopeptin.[8~12] These antibiotics all contain fatty acids, which are important for their activity. Aureobasidins are new antifungal peptide antibiotics without fatty acids, but which have high activity and low toxicity.

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