ACULEACIN A RESISTANT MUTANTS OF *CANDIDA ALBICANS*

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Mutants of *Candida albicans* resistant to aculeacin A, a yeast cell-wall inhibitor, were isolated after mutagenesis with ultraviolet light. The parental strain was sensitive to 0.1~0.5 μg/ml of the antibiotic. In contrast, the minimum inhibitory concentration for the mutants ranged from 50 to 200 μg/ml. Except for papulocandin, another cell-wall inhibitor, the antibiotic susceptibility of the mutants was similar to the parental strain. The parent strain and the aculeacin resistant mutants exhibited similar morphological changes at subinhibitory levels of aculeacin and had comparable growth rates on complex media. The lipid and sterol content of the parent and the mutants were significantly different. For example, the total lipid content was two-fold higher in the mutant strains. Drug resistance in the mutants was specific for aculeacin and papulocandin and appeared to be associated with alteration in the lipid composition of membranes.

The aculeacin complex of antibiotics, composed of oligopeptides with palmitic acid side chains, was isolated from *Aspergillus aculeatus* by MIZUNO *et al.*[1]. This complex was reported to inhibit fungal cell-wall synthesis in *Saccharomyces cerevisiae*[2]. These antifungal agents are especially active *in vitro* against yeast, including *Candida albicans*, as well as some filamentous fungi. A structurally distinct antifungal agent, papulocandin, also preferentially inhibits yeast cell-wall synthesis[3]. Aculeacin and papulocandin are potentially promising as new therapy for candida infection. In this paper we describe the isolation and characterization of aculeacin A resistant mutants of *Candida albicans*.

Materials and Methods

Culture and Culture Condition

The virulent *Candida albicans* strain B-311 used in this study was obtained from Smith Kline & French culture collection. Cells suspended in 10% glycerol were preserved at −170°C in a liquid nitrogen refrigerator. Stock cultures were grown on Sabouraud-dextrose agar for 48 hours and stored for up to three weeks at 4°C.

Isolation of Aculeacin Resistant Mutants

Cells from a 48-hour slant culture were suspended in 10 ml of saline-Tween 80 and exposed in a Petri dish to ultraviolet light (distance 30 cm) for 60 seconds. The treated population was then incubated in the dark for 2 hours and one ml aliquots transferred into Sabouraud-dextrose broth containing various concentrations of aculeacin A. The cultures were incubated at 30°C for 5 days on a rotary shaker. Cells from culture medium containing 50 μg/ml of aculeacin were streaked on Sabouraud agar. Colonies were isolated after 4~5 days and transferred to media containing 50 μg/ml of aculeacin A. The subcultures of the presumptive resistant mutants were incubated for 7 days and then preserved. Resistance to aculeacin was confirmed after preservation.

Growth Studies

Erlenmeyer flasks (500 ml) containing 100 ml Trypticase Soy Broth were inoculated with one ml of 24 hours culture and incubated at 30°C on a rotary shaker (250 rpm). Cell density was determined by

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measuring the turbidity at 600 nm with a spectrophotometer.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MIC) were determined by a two-fold broth dilution method using a microtiter apparatus. The microtiter wells contained 0.1 ml of Sabouraud-dextrose broth. Each well was inoculated with approximately 10^5 cells/ml and incubated at 30°C. The MIC was defined as the lowest concentration of antibiotic which inhibited visible growth after 48 hours.

Lipid Analysis

Total lipids were extracted from cells with chloroform - methanol (2: 1) and separated using silica gel thin-layer chromatography[4].

Results

Isolation of Aculeacin-resistant *acl*^{R} Mutants

Growth of *Candida albicans* strain B-311 was inhibited by incorporation of 0.5 μg/ml of aculeacin A in agar medium. Seven aculeacin-resistant mutants designated *acl*^{R} 1～7 of the B-311 strain were isolated after UV treatment from media containing 50 μg/ml aculeacin. The spontaneous frequency of aculeacin resistant mutants was approximately 2×10^{-7}. Minimum inhibitory concentrations of aculeacin for the parent and the mutant strains are shown in Table 1. Two distinct colony-morphology types, smooth and rough, were observed when the mutants were grown on media containing 100 μg/ml of aculeacin A (Fig. 1). In the presence of subinhibitory levels of aculeacin A, the *C. albicans* strain B-311 grew as a smooth colony type. Under these conditions, distorted cells containing enlarged vacuoles were prevalent with this strain (Fig. 2 B). Without the addition of aculeacin, no distorted cells were observed. In contrast, *acl*^{R} mutants 1 and 6 formed distorted cells in the presence and absence of aculeacin (Fig. 2 C and D).

The mean generation time for the parent and the mutant strain was identical, 2～2.5 hours in Trypticase Soy Broth.

Antimicrobial Susceptibility

The MIC values of several antifungal agent for *C. albicans* strain B-311, mutants *acl*^{R} 1, 2 and 6 are shown in Table 2. Except for the two glucan synthesis inhibitors, aculeacin A and papulocandin B where the MIC values exceeded 200 μg/

Table 1. Minimum inhibitory concentrations for aculeacin resistant mutants.*

Strain	Aculeacin A (μg/ml)
Parental	0.1～0.5
Mutants *acl*^{R}-1	>200
acl^R-2	>50
acl^R-3	50
acl^R-4	50
acl^R-5	25
acl^R-6	>200
acl^R-7	100

* Determined by microtiter broth dilution method.

Fig. 1. Morphology of aculeacin A resistant mutants *acl*^{R}-1 (A: rough colony type) and *acl*^{R}-2 (B: smooth colony type) on a medium containing 100 μg/ml of aculeacin A.

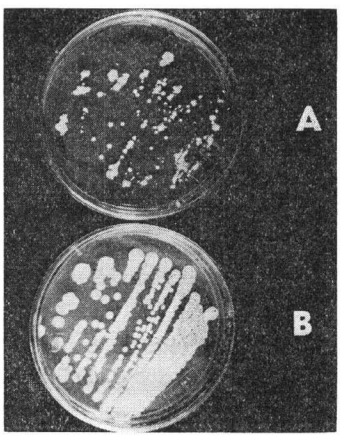

Fig. 2. Microphotographs of *C. albicans* B-311 and *acl*^{R}-1 mutant.
(A) B-311 without aculeacin A. (B) B-311 with subinhibitory level of aculeacin A. (C) *acl*^{R}-1 without aculeacin A. (D) *acl*^{R}-1 with aculeacin A.

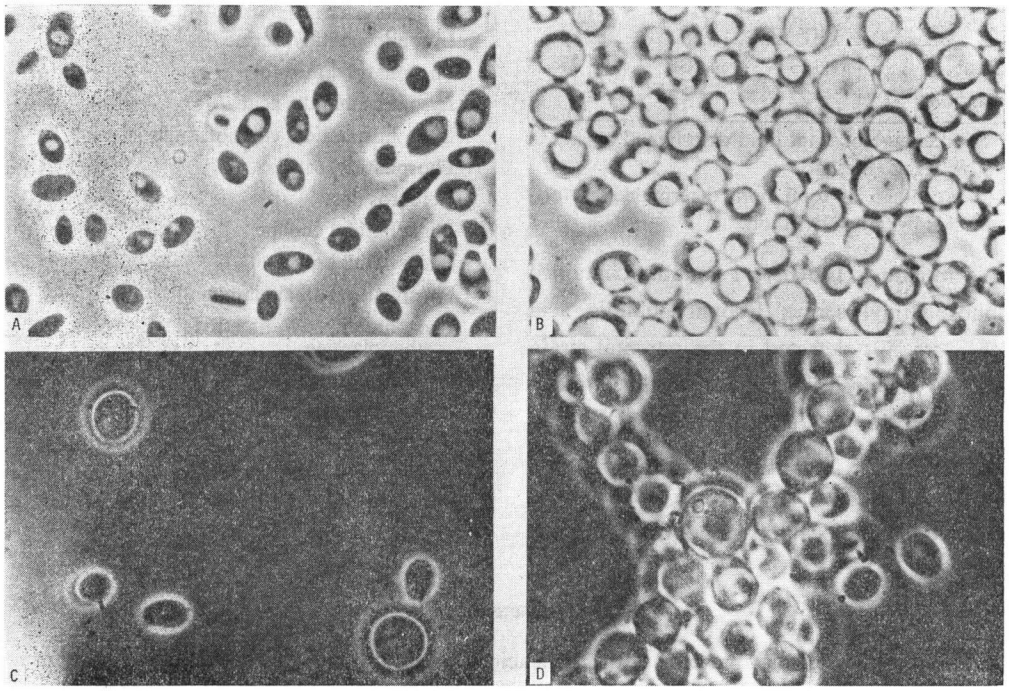

Table 2. Susceptibility of aculeacin resistant mutants to antifungal antibiotics.

Antibiotic	Minimum inhibitory concentration* (μg/ml)			
	Parental	*acl*^{R}-1	*acl*^{R}-2	*acl*^{R}-6
Aculeacin A	0.1~0.5	>200	>50	>200
Papulocandin B	0.1~0.5	>200	>50	>200
Amphotericin B	<0.2	<0.2	<0.2	<0.2
5-Fluorocytosine	~0.5	~0.5	~0.5	~0.5
Cycloheximide	~500	~500	~500	~500
Griseofulvin	>100	>100	>100	>100

* Same as Table 1.

ml, the susceptibility to other antifungal agents was identical to the parental strain.

Biochemical Basis for Aculeacin Resistance

Antibiotic resistance in microbes is commonly attributed to enzymatic inactivation, alterations in cell-permeability or formation of modified target sites. The possible enzymatic inactivation of aculeacin A by *acl*^{R} mutants was investigated with cell suspensions and cell-free extracts. There was no apparent inactivation of the drug with any cell preparation. The possible alteration in cell permeability of *acl*^{R}-1 mutant was investigated by comparing the total lipid composition from a mutant and the parental strain. These data are shown in Table 3. The total lipid content of the *acl*^{R}-1 mutant was higher, roughly two-fold, and the fatty acid content was increased three-fold. Sterol content were also significantly increased

Table 3. Sterol and fatty acid composition of the parent and the *acl*^{R}-1 mutant.

Strain	Total lipid mg/g (dry wt.)	Sterol* (%)	Fatty acids** (%)
Parent	13	38	2
Mutant *acl*^{R}-1	22	49	6

* Percentage of total lipid determined as sterols by T.L.C. method.

** Percentage of total lipid determined as fatty acids by T.L.C. method.

in this mutant. The *acl*^{R}-1 mutant contained lysophosphatidyl serine, lysophosphatidic acid which were not detected in the parental strain (Table 4). The relationship between cell membrane composition and resistance was further supported by the observation that a fatty acid auxotroph of *C. albicans* showed resistance to aculeacin and papulocandin (unpublished report).

Table 4. Polar lipid composition of parent and *acl*^{R} mutant.

Polar lipids*	Parent	Mutant
Lysophosphatidyl serine	−	+
Phosphatidyl inositol	+	+
Lysophosphatidic acid	−	+
Lysophosphatidyl ethanolamine	+	+
Phosphatidyl serine	+	+
Cerebrosides	+	+
Phosphatidyl choline	+	+
Phosphatidyl glycerol	+	+
Phosphatidyl ethanolamine	+	+
Phosphatidyl-*N*,*N*-dimethyl ethanolamine	+	+
Phosphatidic acid	+	+
Phosphatidyl-*N*-methyl ethanolamine	+	+

* Polar lipids detected by silica gel thin-layer chromatography: (+) present, (−) not detected.

Discussion

The antibiotic-resistant patterns of the aculeacin-resistant mutants described here are similar to the patterns previously reported for a papulocandin B-resistant mutant[3]. The papulocandin B-resistant mutant of *C. albicans* was cross resistant with another cell-wall inhibitor, echinocandin. The cross resistance of independently isolated mutants to aculeacin, papulocandin and echinocandin, structurally similar cell-wall inhibitors, suggests a common transport or biochemical site of action for these inhibitors. All of these cell-wall inhibitors cause alterations in the morphological appearance of susceptible strains. MIYATA, *et al.*[5] reported swollen cellular bodies, "drumsticks", with *Schizosaccharomyces pombe*. Similar abnormal alterations in cell morphology were observed with the *C. albicans* B-311 strain and the *acl*^{R} mutants in the presence of subinhibitory levels of aculeacin A. The enlarged cells were observed in the absence of aculeacin A with the mutants, *acl*^{R}-1 and -6. MIYATA *et al.*[5] suggested that the morphological changes were due to a alteration in the cell-wall composition at the site of cell division. The consistent appearance of altered cell types with yeast cell-wall inhibitors further suggest a common site of action.

Glucan synthetase, a target site of aculeacin A, is a membrane bound enzyme[6]. MARRIOTT[7] has reported that the plasma membrane bound mannan-synthetase of *C. albicans* is influenced by its lipid environment. A lipid analysis of *C. albicans* B-311 and *acl*^{R}-1 revealed a change in the content of fatty acids, sterols and phospholipids. An alteration in the membrane lipid composition may account for the aculeacin resistance in these mutants.

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