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# Isolation and Characterization of *Saccharomyces cerevisiae* Mutants Resistant to Aculeacin A

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**Aculeacin A is a lipopeptide that inhibits β-glucan synthesis in yeasts. A number of *Saccharomyces cerevisiae* mutants resistant to this antibiotic were isolated, and four loci (*ACR1*, *ACR2*, *ACR3*, and *ACR4*) whose products are involved in the sensitivity to aculeacin A of yeast cells were defined. Mutants containing mutations in the four loci were also resistant to echinocandin B, another member of this lipopeptide family of antibiotics. In contrast, *acr1*, *acr3*, and *acr4* mutants were resistant to papulacandin B (an antibiotic containing a disaccharide linked to two fatty acid chains that also inhibits β-glucan synthesis), but *acr2* mutants were susceptible to this antibiotic. This result defines common and specific steps in the entry and action of aculeacin A and papulacandin B. The analysis of double mutants revealed an epistatic effect of the *acr2* mutation on the other three mutations. Cell walls of the four different mutants did not show significant alterations in composition with respect to the parental strain, and in vitro glucan synthase activity was also unaffected. However, cell surface hydrophobicity in three of the mutants was considerably decreased with respect to the parental strain.**

β-Glucan is the main structural component of the yeast cell wall, to which it confers its characteristic rigidity (1, 10). A number of antibiotics are inhibitors of β-glucan synthesis in yeast species (8). In their presence, the cell wall structure becomes destabilized and cell lysis occurs unless the medium is protected osmotically. Papulacandin B is an amphiphilic molecule with a hydrophilic moiety consisting of glucose and galactose and a hydrophobic moiety containing two long-chain unsaturated fatty acids (2). It has been shown that papulacandin B specifically reduces the levels of β-glucan in the cell walls of a number of yeasts (2, 8, 36). On the other hand, synthesis of α-glucan (which is a major component of *Schizosaccharomyces pombe* cell walls [5]) is not inhibited by papulacandin B (36). Another family of β-glucan inhibitors comprises aculeacin A, echinocandin B, and cilofungin (LY121019), among others; they contain a cyclopeptide moiety linked to a fatty acid chain. Several studies have demonstrated that in the presence of one of these lipopeptide antibiotics, synthesis of β-glucan is affected in vivo in yeast species such as *Saccharomyces cerevisiae*, *Candida albicans*, and *S. pombe* (12, 20, 21, 28, 33, 37, 38). Indirectly, incorporation of mannoproteins into the walls of yeast cells treated with aculeacin A or papulacandin B is also inhibited (22, 35).

The exact mechanism of action of these antibiotics is not well understood. In vitro, high concentrations of papulacandin B inhibit β-1,3-glucan synthase activity in extracts from *S. pombe* or *Geotrichum candidum* (25, 36), but this antibiotic has been observed to be ineffective on extracts from *S. cerevisiae* (8, 11). Echinocandin B (28) and cilofungin (33, 34) are noncompetitive inhibitors of the glucan synthase activity of *C. albicans*, although in our laboratory neither aculeacin A nor echinocandin B inhibits *S. cerevisiae* β-1,3-glucan synthase activity (11) when a conventional in vitro assay for this enzyme (29, 32) is employed. In *S. cerevisiae* and *C. albicans*, glucan synthase is found at the plasma membrane (24, 29, 32). It is reasonable that in vivo inhibition of β-glucan synthesis might be a consequence of the direct interaction of the antibiotic with the synthase; alternatively, it may be due to some indirect effect. The lipid moiety of the lipopeptide antibiotics plays an important role in their mechanism of action, probably by establishing specific interactions with the membrane (34), and its role in the transport of the antibiotic through the cell wall and/or plasma membrane should also be considered. In this context, it can be noted that although cilofungin differs from aculeacin A and echinocandin B exclusively in the fatty acid moiety (6), cultures of two independent *S. cerevisiae* wild-type strains were resistant to cilofungin at 100 μg/ml but not to aculeacin A or echinocandin B (11).

Ribas et al. (26) have obtained a number of *S. pombe* mutants resistant to papulacandin B; some of these mutants exhibit gross morphological changes and have alterations in the polysaccharide content of the cell wall. In order to better understand the mechanism of action of the lipopeptide antibiotics, we have obtained a collection of *S. cerevisiae* mutants resistant to aculeacin A and have begun to characterize some of them. Although this yeast species differs from others in some aspects of susceptibility to the antibiotics discussed in this article, it has been chosen because a simultaneous biochemical and genetic approach can be easily used with it.

## MATERIALS AND METHODS

**Yeast strains.** *S. cerevisiae* OL1 (*MATα leu2-3 leu2-112 his3-11 his3-15 ura3-251 ura3-337*) (3) was used as the parental strain to isolate aculeacin A-resistant mutants. Strain SEY2101 (*MATa ura3-52 leu2-3 leu2-112 suc2-Δ9*

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*ade2-1*) (9) was used to introduce the *ade2* marker into the OL1 background by conventional mating experiments.

**Growth media.** YPD medium (1% yeast extract, 2% peptone, 2% [wt/vol] glucose) was used as a rich medium. For some experiments, this was supplemented with 1 M sorbitol (YPDS medium). In some genetic experiments, YNB medium without amino acids (Difco) plus 2% (wt/vol) glucose was used as a minimal medium; nutritional requirements were added as indicated in reference 30. Media were solidified with 1.5% (wt/vol) agar. Cultures were incubated at 30°C unless otherwise indicated. In experiments involving fractionation of cell wall polysaccharides, cells grown in YPD medium to the exponential phase were sedimented and resuspended in fresh YPD medium containing a lower concentration of nonradioactive glucose (0.05%, wt/vol) and 0.3 μCi/ml (0.25 Ci/mmol) of [U-$^{14}$C]glucose; incubation was then continued for 4 h more.

**Isolation of aculeacin A-resistant mutants.** About $3 \times 10^8$ cells from an exponentially growing culture of strain OL1 in YPD medium were mutagenized with ethyl methanesulfonate (30) under conditions where about 40% of the cells remained viable. Mutagenized cells were washed twice, resuspended in 10 ml of liquid YPDS medium, and incubated for 15 h at 24°C. Thirteen aliquots of 50 μl ($10^6$ CFU) each were plated separately in solid YPD medium containing 1 M sorbitol plus aculeacin A (15 μg/ml). After 3 days at 24°C, 303 resistant clones were recovered.

**Analysis of susceptibility to antibiotics.** The antibiotics were assayed at the concentrations indicated for each experiment by using stock solutions (10 mg/ml in dimethyl sulfoxide, kept at −20°C). Two different methods were used for analyses with liquid media. For parallel tests of a large number of strains, late-exponential-phase cultures in YPD or YPDS medium ($A_{600}$ = 1.0 to 1.5) were diluted 1/100 in test tubes containing 1 ml of the same medium, the antibiotic (problem tubes) or an equivalent volume of the solvent (control tubes) was added 1 h later, and incubation was continued for 24 h at 30°C. The $A_{600}$ value reached by each culture was measured in a Shimadzu UV-240 spectrophotometer (1-cm light path). The same procedure was used for MIC determinations with twofold serial dilutions of the antibiotic. Alternatively, individual strains were inoculated into fresh YPD or YPDS medium (with the antibiotic or the solvent alone) by using 1/50 volume of late-exponential cultures in the same medium and periodically measuring the $A_{600}$ during exponential growth at 30°C. Qualitative analyses of susceptibility to aculeacin A were also carried out by streaking the colonies to be tested on solid YPD or YPDS medium containing the antibiotic at 10 μg/ml and incubating the plates at 30°C for 72 h.

**Protoplast obtention and regeneration.** Protoplasts were obtained from exponentially growing cultures as described previously (35). They were regenerated at 30°C in liquid YPDS medium containing 0.05% (wt/vol) glucose by initially resuspending them at a protoplast concentration equivalent to that of the original cell culture; antibiotics were added (in some experiments) 1 h after protoplast resuspension, and [U-$^{14}$C]glucose was added 1 h later.

**Wall purification and polysaccharide fractionation.** Cells from exponentially growing cultures were broken mechanically by vortexing in the presence of glass beads (0.5- to 0.6-mm diameter) for eight periods of 30 s each, and walls were purified by repeated washings and differential centrifugation (16). Regenerating protoplasts were broken by resuspension in 10 mM Tris chloride buffer (pH 7.5) and shaking for 10 s with glass beads; the cultures were then washed repeatedly as described above.

Polysaccharides were fractionated from purified walls labelled with [U-$^{14}$C]glucose as described in reference 22. Alkali-insoluble material corresponds to β-glucan (Zymolyase sensitive) plus chitin (Zymolyase resistant), whereas the alkali-soluble material is divided into a Fehling solution-precipitable fraction (mannan) and a nonprecipitable fraction (alkali-soluble β-glucan).

**Determination of β-1,3-glucan synthase activity.** Isolation of membrane fractions and determination of enzyme activity were done as described in reference 32. Aculeacin A, echinocandin B, or papulacandin B (from stock solutions at 10 mg/ml in methanol) was added in some assays; control assays were performed with an equivalent volume of the solvent alone.

**Hydrophobicity assays.** Cell surface hydrophobicity (CSH) in *S. cerevisiae* populations was determined by two methods. The phase partition test (27) was used with cyclohexane in the organic phase. The microsphere hydrophobicity assay was used as described in reference 18. In the latter assay, the percentage of hydrophobic cells was considered the CSH value.

**Yeast genetic techniques.** Crosses between strains, selection of diploids, dissection of tetrads, and determination of nutritional requirements were carried out as described in reference 30.

**Analytical determinations.** Wall sugar was determined by the phenol-sulfuric method (7) with D-glucose as the standard. Protein concentration was measured by the Lowry method (18a).

**Chemicals and reagents.** Radioactive chemicals were from New England Nuclear (Boston, Mass.). UDP-glucose and α-amylase were purchased from Boehringer GmbH (Mannheim, Germany). 2-Mercaptoethanol, EDTA, bovine serum albumin, GTP, ATP, amino acids, nitrogen bases, and polystyrene microspheres were from Sigma Chemical Company (St. Louis, Mo.). Aculeacin A was a gift from Toyo Jozo Co. Ltd., Tagata-gun, Shizuoka-ken, Japan. Papulacandin B was a gift from CIBA-GEIGY, Basel, Switzerland.

## RESULTS

**Characterization of aculeacin A-resistant mutants.** In order to isolate aculeacin A-resistant mutants from wild-type strain OL1, conditions that would also allow the recovery of thermosensitive or lysis mutants or both were used. Of 303 clones isolated, none was dependent on the presence of sorbitol as an osmotic stabilizer in the medium for growth. Five clones were thermosensitive (they did not grow at 37°C), although thermosensitivity did not show meiotic cosegregation with resistance to the antibiotic. Thus, these five mutants were not studied further. Another 44 resistant clones were analyzed for wall polysaccharide composition by a simplified protocol for 2-ml cultures. None of them exhibited gross differences with respect to the parental OL1 strain. Six of these clones were employed for further analyses. These were crossed once with strain SEY2101 and then twice more with the parental OL1 strain and in all cases with selection for resistance to aculeacin A. This phenotype always showed a 2:2 meiotic segregation pattern, a demonstration of the monogenic character of the resistance. The six resulting isolates employed in the next experiments (named LMF101 to LMF106) have the same genotype as OL1, aside from the resistance to aculeacin A. All six are stable in the absence of continued selective pressure.

TABLE 1. Effects of different antibiotics on *S. cerevisiae acr* mutants

| Strain | MIC (μg/ml) of: | | | |
|---|---|---|---|---|
| | Aculeacin A | Papulacandin B | Echinocandin B | Aculeacin A in diploids[a] |
| OL1 | 5 | 2.5 | 10 | 5 |
| LMF101 | 100 | 50 | 50 | 5 |
| LMF102 | 50 | 2.5 | >100 | 5 |
| LMF103 | >100 | 2.5 | >100 | 5 |
| LMF104 | 100 | 2.5 | 75 | 5 |
| LMF105 | 100 | 50 | >100 | 5 |
| LMF106 | 50 | 50 | 50 | 5 |

[a] Diploids were obtained by crossing each mutant strain of the appropriate mating type with the wild-type strain.

Isolates LMF101 to LMF106 of the a mating type were crossed with the parental strain. All of the resulting diploids were sensitive to aculeacin A (Table 1); thus, the resistance mutations are recessive.

Aculeacin A had a variable effect on growth rate depending on the mutant strain and the presence or absence of sorbitol in the growth medium (Fig. 1). LMF101 was the most affected strain; its growth rates with respect to control cultures without the antibiotic were about 45% in YPD medium and 80% in YPDS medium. The growth defect of strain LMF106 in YPD medium plus aculeacin A was also significantly reversed by the osmotic stabilizer (Fig. 1).

The six mutant strains did not exhibit gross morphological alterations with respect to the parental strain. However, cultures of LMF101 and LMF106 in the absence of sorbitol contained a certain number of lysed cells, which were not observed in the presence of the stabilizer. Cultures of the other mutants were free of lysed cells under all growth conditions.

Several other phenotypic traits of the mutant strains were studied (Table 1). The MIC of aculeacin A varied among strains, being at least 10-fold higher than the value of the parental strain. All six mutants were also resistant to echinocandin B. However, while mutants LMF101, LMF105, and LMF106 were also resistant to papulacandin B, strains LMF102, LMF103, and LMF104 were susceptible to this antibiotic.

**Extracellular stability of the antibiotic molecules.** Resistance in the mutants is not caused by extracellular degradation of the antibiotic, since cell-free supernatant fluids obtained from 6-h cultures of the mutants grown in the presence of aculeacin A (5 μg/ml) were still able to inhibit growth of the wild-type strain (11).

**Determination of the number of genetic loci defined by mutants LMF101 to LMF106.** We constructed all possible diploids by mating all possible pairs of mutants; in each of the mating experiments, one of the mutants was *HIS3 ade2* while the other was *his3 ADE2*. The 15 diploids were then sporulated. Both random spore analysis experiments (using the recessive *ade2* marker as the indicator of haploid colonies [see reference 30]) and tetrad analysis indicated that the resistance mutations carried by LMF102, LMF103, and LMF104 affect the same locus or closely linked loci. In fact,

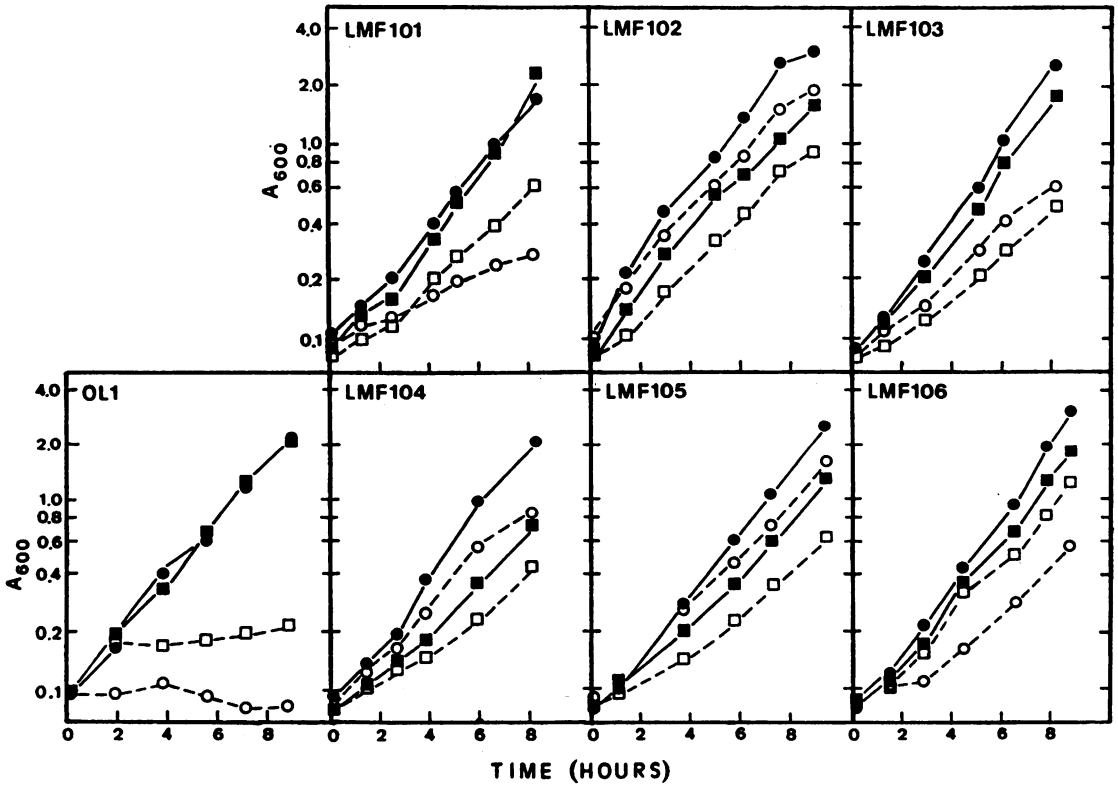


FIG. 1. Growth curves of the parental strain, OL1, and aculeacin A-resistant mutants (LMF101 to LMF106) in YPD (● and ○) or YPDS (■ and □) medium at 30°C in the absence (● and ■) or presence (○ and □) of aculeacin A (10 μg/ml). In treated cultures, the antibiotic was added at time zero.

TABLE 2. Phenotypes of double *acr* mutants

| Strain | Relevant genotype | Growth with papulacandin B (10 μg/ml)[a] | Dependence on osmotic stabilizer[b] |
|---|---|---|---|
| LMF101 | *acr1* | + | + |
| LMF102 | *acr2* | − | − |
| LMF105 | *acr3* | + | − |
| LMF106 | *acr4* | + | + |
| LMF107 | *acr1 acr2* | − | − |
| LMF108 | *acr1 acr3* | + | − |
| LMF109 | *acr1 acr4* | + | + |
| LMF110 | *acr2 acr3* | − | − |
| LMF111 | *acr2 acr4* | − | − |
| LMF112 | *acr3 acr4* | + | − |

[a] Growth was considered to be inhibited when the $A_{600}$ reached in the presence of the antibiotic after 24 h was less than 10% of that in control tubes.
[b] Dependence means that the growth rate of the cultures in the presence of 1 M sorbitol was at least twice that observed in the absence of the stabilizer.

no haploid-sensitive spores could be derived from crosses between these mutants. On the other hand, random spore analysis resulted in a segregation pattern close to 3:1 for the aculeacin A resistance: susceptibility character in crosses among LMF101, LMF105, and LMF106 or between one of these mutants and one from the group consisting of LMF102, LMF103, and LMF104, indicating that these mutants carry independent mutations in separate chromosomes. To confirm this, tetrad analyses of the same crosses resulted in parental ditypes (4:0 segregation pattern for resistance and susceptibility to aculeacin A), nonparental ditypes (2:2), and tetratypes (3:1).

Thus, at least four different genes are defined by the six mutants characterized in this work: *ACR1* (defined by LMF101), *ACR2* (defined by LMF102), *ACR3* (defined by LMF105), and *ACR4* (defined by LMF106).

**Epistatic effects among *acr* mutations.** Double *acr* mutants were recovered from the aculeacin A-resistant spores present in nonparental ditype tetrads obtained by crossing single *acr* mutants. Two differential phenotypic traits (susceptibility to papulacandin B and effect of sorbitol on growth rate) of the double mutants were analyzed (Table 2). The mutation in *acr2* is epistatic to mutations in the other loci, and the *acr3* mutation is also epistatic to *acr1* and *acr4*.

**Cell wall composition in aculeacin A-resistant mutants.** The amount of cell wall sugar was not severely affected in the strains carrying the four *acr* mutations in comparison with the wild type (Table 3). However, the in vivo incorporation of radioactivity into the walls of mutant cells was not affected by the antibiotic, while the level of inhibition was 96% in the wild type. As expected, incorporation into the alkali- and acid-insoluble fraction (β-glucan) was most affected in the wild-type strain. Fractionation of wall polysaccharides from the mutants yielded a pattern not significantly different from that of the wild type, and the presence of aculeacin A in the mutant cultures did not have an important effect on the relative percentages (Table 3).

In vitro β-1,3-glucan synthase activity in the *acr* mutants as well as in the parental strain was measured (Table 4). All of the strains had similar levels of GTP- or ATP-activable glucan synthase activity. Both in the parental and in the mutant strains, aculeacin A and papulacandin B (at concentrations up to 20 μg/ml in the assay mixture) and echinocandin B (up to 40 μg/ml) did not have an inhibitory effect in vitro (11).

**Effect of aculeacin A and papulacandin B on regenerating protoplasts.** Incorporation of radioactivity into cell wall polysaccharides in regenerating protoplasts of the parental and *acr* strains was sensitive to aculeacin A and papulacandin B (30 to 60% of the level of incorporation in the control). The different polysaccharide fractions were affected to similar extents by the antibiotics (11), in accordance with previous observations with wild-type yeasts (22, 35).

**CSH in mutant cells.** CSH values for the parental and mutant strains were determined by the phase partition test (28) and the microsphere hydrophobicity assay (14). Strains with the *acr1*, *acr3*, or *acr4* mutation had CSH values of less than half of the parental value (Table 5). In contrast, the parental strain and the *acr2* mutant had similar CSH values when CSH was measured by the phase partition test; when CSH was determined by the microsphere hydrophobicity assay, *acr2* cells displayed a CSH value intermediate be-

TABLE 3. Polysaccharide fractionation of isolated cell walls from [$^{14}$C]glucose-labelled cultures of wild-type and aculeacin A-resistant strains[a]

| Strain (relevant genotype) | Presence of aculeacin A (10 μg/ml) | Amt of wall sugar/cell (dry wt) (μg/mg)[b] | Wall radioactivity[c] | % of wall radioactivity in: | | | |
|---|---|---|---|---|---|---|---|
| | | | | Alkali-soluble material | | Alkali-insoluble material | |
| | | | | Fehling solution precipitable | Fehling solution nonprecipitable | Acid soluble | Acid insoluble |
| OL1 | − | 44 | 1 | 23 | 48 | 2 | 27 |
| | + | ND | 0.02[d] | | | | |
| LMF101 (*acr1*) | − | 46 | 1 | 29 | 37 | 3 | 31 |
| | + | ND | 0.16 | 28 | 43 | 2 | 27 |
| LMF102 (*acr2*) | − | 56 | 1 | 26 | 36 | 5 | 33 |
| | + | ND | 0.37 | 25 | 53 | 3 | 19 |
| LMF105 (*acr3*) | − | 52 | 1 | 32 | 41 | 1 | 26 |
| | + | ND | 0.85 | 28 | 52 | 5 | 15 |
| LMF106 (*acr4*) | − | 39 | 1 | 23 | 45 | 2 | 30 |
| | + | ND | 0.10 | 21 | 52 | 2 | 25 |

[a] Exponentially growing cultures in YPD medium at 250 μg (dry weight) of cells per ml were labelled with [$^{14}$C]glucose for 4 h as described in Materials and Methods. In the aculeacin A-treated cultures, the antibiotic was added 1 h before the isotope.
[b] Values are the means of results from three independent experiments. ND, not determined.
[c] Radioactivity values (per milliliter of culture) are normalized with respect to those of untreated cultures, which are given the unit value.
[d] Walls were not fractionated because of the small amount of radioactivity incorporated.

TABLE 4. β-Glucan synthase activity in *acr* mutants

| Strain (relevant genotype) | Nucleotide (concn) | Sp act (nmol of glucose incorporated/ min/mg of protein) |
|---|---|---|
| OL1 | None | 6.3 |
| | GTP (0.1 mM) | 20.8 |
| | ATP (4 mM) | 19.0 |
| LMF101 (*acr1*) | None | 9.4 |
| | GTP (0.1 mM) | 16.3 |
| | ATP (4 mM) | 15.7 |
| LMF102 (*acr2*) | None | 8.9 |
| | GTP (0.1 mM) | 21.7 |
| | ATP (4 mM) | 20.3 |
| LMF105 (*acr3*) | None | 7.8 |
| | GTP (0.1 mM) | 15.6 |
| | ATP (4 mM) | 15.9 |
| LMF106 (*acr4*) | None | 6.4 |
| | GTP (0.1 mM) | 16.0 |
| | ATP (4 mM) | 16.9 |

tween that of the parental strain and those of the other three mutants.

**Resistance to aculeacin A and resistance to papulacandin B are not necessarily correlated.** The properties of the four *acr* mutants described above indicate that acquisition of resistance to aculeacin A does not imply resistance to papulacandin B in parallel. That is, some of the physiological functions involved in the action of both antibiotics seem to be common to both drugs, while others are different for each drug. To confirm this, a collection of mutants resistant to papulacandin B (10 μg/ml) was obtained by the same protocol used for aculeacin A. Of 15 independently obtained mutants, 10 were also resistant to aculeacin A, while the other 5 showed the same levels of susceptibility to this antibiotic as the parental OL1 strain.

## DISCUSSION

A mutant of *C. albicans* simultaneously resistant to papulacandin B and echinocandin B had been isolated previously (2), and another *C. albicans* strain obtained as resistant to aculeacin A showed cross-resistance to papulacandin B (19). A collection of *S. pombe* papulacandin B-resistant mutants has been obtained (26), and some of them are still susceptible to aculeacin A. In the present work with *S. cerevisiae* mutants, we have shown that resistance to aculeacin A does not necessarily correlate with resistance to papulacandin B, that is, there are common and specific steps in the mechanisms of entry and action of these antibiotics. The functions defined by *ACR1*, *ACR3*, and *ACR4* are required for cell sensitivity to both families of antibiotics. In turn, the physiological function defined by *ACR2* is necessary for the sensitivity of cells to aculeacin A although not to papulacandin B. Interestingly, double mutants with mutations in *ACR2* and in one of the other three *ACR* loci are also sensitive to papulacandin B; since single mutants with mutations in *ACR1*, *ACR3*, or *ACR4* are resistant to this antibiotic, the ACR2 function must remain unaltered for the effect of papulacandin B to be blocked.

TABLE 5. CSH in wild-type strain and *acr* mutants

| Strain (relevant genotype) | CSH (mean ± SD) determined by: | |
|---|---|---|
| | Phase partition test[a] | Microsphere hydrophobicity assay[b] |
| OL1 | 34.1 ± 11.4 | 31.3 ± 0.8 |
| LMF101 (*acr1*) | 14.9 ± 6.8 | 15.1 ± 2.9 |
| LMF102 (*acr2*) | 33.5 ± 9.3 | 22.2 ± 2.1 |
| LMF105 (*acr3*) | 16.7 ± 5.0 | 14.3 ± 3.7 |
| LMF106 (*acr4*) | 15.3 ± 4.1 | 14.3 ± 1.5 |

[a] Values correspond to the percentage of cell biomass (measured as $A_{600}$) remaining in the cyclohexane phase. They are the means of three independent experiments (each one involving three measurements).

[b] Values correspond to the percentage of cells having three or more microspheres attached. They are the means of three independent experiments.

None of the four mutants exhibits gross alterations in cell wall polysaccharide composition. The mutants contain normal levels of GTP- or ATP-activable β-1,3-glucan synthase activity. This argues against the synthase being directly altered in any of the *acr* mutants. Obviously, the possibility of the existence of more than one activity involved in the synthesis of β-1,3-glucan in *S. cerevisiae*, in a manner similar to chitin synthesis (4, 23, 31), must be comtemplated. Synthesis of β-1,3-glucan probably is a complex process in which many different cellular functions are involved.

Since protoplasts from the four independent *acr* mutants are sensitive to aculeacin A and papulacandin B, the mutations probably affect cell surface structures or functions involved in the accessibility of the antibiotic molecules to the plasma membrane. However, we should not completely discard the alternative possibility that the physiological functions affected by aculeacin A or papulacandin B in regenerating protoplasts do not exactly correspond to those affected in whole cells; in this respect, the inhibitory effect of aculeacin A was much less pronounced on protoplasts than on whole cells in the parental strain. Having considered this, we still favor the suggestion that the *acr* mutations alter entry of the antibiotic molecules into whole cells.

The fact that the three mutants resistant to both aculeacin A and papulacandin B exhibit lower CSH values than the wild-type strain points to the importance of surface charge in the accessibility of the target to the antibiotic molecules. Several observations suggest that changes in yeast cell wall proteins are responsible for the modifications in surface hydrophilicity and hydrophobicity (15, 18). Thus, antibiotic resistance in the mutants described above may derive more from fine changes in the wall mannoprotein pattern that alter CSH than from the overall polysaccharide composition. CSH may participate, together with other mechanisms, in the adherence of *C. albicans* to surfaces in vivo, contributing to the virulence of this yeast species (13, 17). Therefore, isolation of aculeacin A- and papulacandin B-resistant mutants can be a strategy for obtaining *C. albicans* strains with decreased virulence and for studying the basis of pathogenicity in this yeast species.

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