Complementation Analysis of Resistance to 5-Fluorocytosine in *Candida albicans*

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A complementation test was devised to study allelism among the genetic determinants of resistance to 5-fluorocytosine in *Candida albicans*. Complementation was demonstrated in control hybrids produced by crossing a resistant strain that was deficient in cytosine deaminase activity with four other resistant strains deficient in UMP pyrophosphorylase activity. This complementation test was used to test allelism of the resistance determinants present in five clinical isolates. All were found to bear recessive alleles of the locus (FCY1) that determined 5-fluorocytosine resistance associated with low levels of UMP pyrophosphorylase activity.

A survey of various clinical isolates of *Candida albicans* showed that the majority (57%) were susceptible to the antifungal agent 5-fluorocytosine (5-FC) and that a small minority (6%) were highly resistant (1). The remaining isolates in the sample exhibited an intermediate level of resistance and were phenotypically distinct from the susceptible isolates and the highly resistant isolates. A separate survey demonstrated the existence of four classes of resistance to 5-FC (12), two of which appear to have an intermediate level of resistance.

Typical isolates of the kind that displayed intermediate (or partial) resistance gave rise to variants that were highly resistant. The genetic basis of this instability was shown to be a heterozygosity for resistance (written FCYIfcy, where fcy denotes the [recessive] resistance allele). Mitotic segregation (FCYIfcy → fcylfcy + FCYIfCY) yielded a highly resistant segregant and a susceptible cosegregant (14, 15).

The enzymatic basis of resistance to 5-FC in *C. albicans* is poorly understood, and the relevant gene-enzyme relationships are not known. Previous studies showed that some resistant strains were deficient in cytosine deaminase activity (8). Deficient UMP pyrophosphorylase activity was found to be the basis for resistance in five highly resistant and seven partially resistant clinical isolates (15). While resistance due to defective uptake of 5-FC has not been reported in *C. albicans*, a weak resistance resulting from defective uptake has been shown in Saccharomyces cerevisiae (5).

In the present study, we describe a complementation test for 5-FC resistance in *C. albicans*. We used this method to show that, because of complementation, a hybrid formed by spheroplast fusion of a cytosine deaminase-deficient strain with a UMP pyrophosphorylase-deficient strain was less resistant than either (highly resistant) parent. In addition, we show that the resistance determinants present in five independent clinical isolates failed to complement a standard resistance determinant (fcyl) and were therefore taken to be alleles of the gene FCY1.

**MATERIALS AND METHODS**

**Strains.** Clinical isolates QC6, QC12, and QC19 were obtained from Queen Charlotte’s Maternity Hospital, London. Clinical isolates AD5, AD11, AD13, and AD18 have been described previously (15). AD11, AD13, AD18, QC6, and QC12, which were typical type C strains (partially resistant to 5-FC and unstable for resistance [14]), were the strains from which we obtained highly resistant segregants. Methionine-requiring derivatives were isolated from these segregants by the use of UV-induced segregation as previously described (16, 19) to serve as parent strains for hybridization. RAD11, a parent strain used for the test, was the highly resistant, methionine-requiring derivative of the clinical isolate AD11; RQC6 was the analogous derivative of QC6, etc.

The standard fcyl allele present in tester strain 1257 was obtained by de novo mutation in a susceptible strain that bore a useful marker (his). The sequence of isolation was: clinical isolate AD5 (HIS/his FCY1/FCY1) → segregant 85A (his/his FCY1/FCY1) → mutant 1187 (his/his FCY1/FCY1) → segregant 1257 (his/his fcyl/fcy1). In brief, a histidine-requiring segregant (85A) was obtained from the clinical isolate AD5, and the fcyl allele was obtained by a spontaneous mutation that yielded strain 1187. The tester strain 1257 was a spontaneous resistant segregant derived from strain 1187. AD5 was shown previously to have high UMP pyrophosphorylase activity (3.4 U) (15). In contrast, we detected no UMP pyrophosphorylase activity in 1257 (see text).

Tester strain D14, which carried the fcy2 allele, was a lysine-requiring derivative of a 5-FC-resistant segregant obtained from clinical isolate QC19.

**Media.** Minimal medium (MIN) was yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Mich.) (6.7 g/liter) plus glucose (20 g/liter). MFC50 medium was MIN with added 5-FC (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 50 μg/ml. MFU50 medium was MIN with added 5-fluorouracil (Sigma) at a final concentration of 50 μg/ml. Filter-sterilized 5-FC, or 5-fluorouracil, was added to autoclaved MIN. The media were supplemented, as needed, with L-histidine, L-lysine, and L-methionine (final concentration, 40 μg/ml). Bacto-Agar (Difco) was used to solidify the media. The cultures were grown at 37°C unless otherwise indicated.

**Hybridization.** The method of Poulter et al. (9) was used to obtain spheroplasts from parent strains and induce fusion.
Hybrids were selected by spreading fusion mixtures on MIN and incubating the cultures at room temperature.

**Assessment of resistance.** A sample of an overnight culture on 5-FC-free medium was suspended in water, and a portion containing ~2 × 10^6 cells was spread on MFC50 medium supplemented with histidine, lysine, or methionine as necessary; two to four strains were tested per standard (10-cm) petri dish. The cultures were incubated for 2 to 4 days and scored as being susceptible, partially resistant, or highly resistant, as previously described (1, 14).

**Enzyme assays.** Cultures were grown and crude lysates were made as described previously (15). UMP pyrophosphorylase (UMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.9) activity was assayed as described previously (15). Cytosine deaminase (cytosine aminohydrolase, EC 3.5.4.1) activity was assayed by a method similar to that of Hoeprich et al. (4), which determined the rate of conversion of radiolabeled cytosine to uracil. Enzyme activities are expressed in nanomoles per minute per milligram of protein. The substrate for the cytosine deaminase assay was [2-14C]cytosine (specific activity, 54.6 µCi/mol) at an initial concentration of 2.5 mM in the reaction mixture. The radiolabeled cytosine was obtained from Research Products International (Mount Prospect, Ill.) and was dissolved in 1 mM MgCl_2. The reaction (at 37°C) was started by adding the substrate (20 µl) to crude lysate (20 µl); it was stopped by adding 1 M hydrochloric acid solution (40 µl) containing unlabeled cytosine (0.5 mg/ml) and unlabeled uracil (0.5 mg/ml). The acidified mixture was centrifuged, and a sample (10 µl) of supernatant was chromatographed on cellulose thin-layer sheets (Sigmacell type 100 cellulose with 254-nm fluorescent indicator; Sigma); the solvent was n-butanol plus water (86:14, vol/vol). The cytosine and uracil, located by viewing the chromatogram under UV light, showed clear resolution. Cytosine and uracil standards were chromatographed in parallel to the reaction mixtures. The pyrimidine-containing areas of the chromatograms were cut out, and the radioactivity was determined with Aquasol scintillant (New England Nuclear Corp., Boston, Mass.) and a Beckman LS250 scintillation counter. Protein was determined by the Lowry method (7), with bovine serum albumin as the standard. Under the assay conditions described, cytosine was converted to uracil at a constant rate for 30 min.

**DNA determination.** The amount of DNA in samples of cultures was determined by the diphenylamine method of Riggsby et al. (10) with the standards described previously (17, 18).

**RESULTS**

It was first necessary to demonstrate that complementation of resistance determinants could be detected. In two highly resistant parents, if the resistance in one results from low UMP pyrophosphorylase activity and resistance in the other results from low cytosine deaminase activity, it would be expected that a hybrid formed by the fusion of such strains would be less resistant than either parent because of complementation. The cross may be represented as fcy1/fcy1 FCY1/FCY1 FCY2/FCY2 × fcy2/fcy2 FCY1/FCY1 FCY2/FCY2, in which the allele FCY1 determines UMP pyrophosphorylase activity and the allele FCY2 determines cytosine deaminase activity. The recessive alleles fcy1 and fcy2 determine decreases in those respective activities. By this hypothesis, the tetraploid hybrid would have functional alleles (FCY1, FCY2) at both genes and should be susceptible to 5-FC (or at least be less resistant than either parent) because it would have the enzymatic activities necessary to convert 5-FC to 5-fluorouridyl acid, the first key toxic intermediate.

**Tester strains.** Resistance associated with low UMP pyrophosphorylase activity was determined by the allele fcy1 of tester strain 1257 (genotype; fcy1/fcy1 FCY2/FCY2). Although crude lysates prepared from strain 1257 exhibited no UMP pyrophosphorylase activity (<0.1 U), they did exhibit readily detectable cytosine deaminase activity (3.2 U). Resistance associated with low cytosine deaminase activity was determined by the allele fcy2 of tester strain D14 (genotype; FCY1/FCY1 fcy2/ fcy2). Crude lysates prepared from strain D14, on the other hand, exhibited no cytosine deaminase activity (<0.1 U), yet did exhibit readily detectable UMP pyrophosphorylase activity (3.3 U). Strain 1257 was resistant to both 5-FC and 5-fluorouracil, consistent with its low UMP pyrophosphorylase activity. In contrast, strain D14 was resistant to 5-FC but was susceptible to 5-fluorouracil, consistent with its normal level of UMP pyrophosphorylase activity. The origins of the tester strains are described in Materials and Methods.

**Complementation.** Resistant tester D14, which was cytosine deaminase deficient, was crossed with four UMP pyrophosphorylase-deficient resistant strains (1257, RAD11, RAD13, RAD18) (Table 1). Each of the resultant hybrids was found to be either susceptible or only slightly resistant to 5-FC, in marked contrast to the parent strains. Resistance phenotypes of the parents and the hybrid are shown in Fig. 1 for a representative cross. Growth of the large majority of cells of the hybrid was inhibited by 5-FC, in contrast to the vigorous growth of the resistant parent strains. The minority of hybrid cells that grew to form colonies will be discussed below. These results showed that (intergenic) complementation was readily detected.

**TABLE 1. Results of complementation tests and analysis of total cellular DNA**

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Complementation*</th>
<th>DNA (fg/cell)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>D14</td>
<td>34.2, 36.2†</td>
<td>75.3</td>
</tr>
<tr>
<td>1257</td>
<td>33.9, 35.3</td>
<td>75.7</td>
</tr>
<tr>
<td>D14 × 1257</td>
<td>+</td>
<td>82.9, 69.2</td>
</tr>
<tr>
<td>RAD11</td>
<td>37.9</td>
<td>77.5</td>
</tr>
<tr>
<td>RAD11 × D14</td>
<td>+</td>
<td>70.5, 80.5</td>
</tr>
<tr>
<td>RAD11 × 1257</td>
<td>–</td>
<td>77.5</td>
</tr>
<tr>
<td>RAD13</td>
<td>40.3, 35.2</td>
<td>83.1</td>
</tr>
<tr>
<td>RAD13 × D14</td>
<td>+</td>
<td>68.2, 60.7</td>
</tr>
<tr>
<td>RAD13 × 1257</td>
<td>–</td>
<td>83.1</td>
</tr>
<tr>
<td>RAD18</td>
<td>42.8, 34.7</td>
<td>83.0</td>
</tr>
<tr>
<td>RAD18 × D14</td>
<td>+</td>
<td>98.7, 111</td>
</tr>
<tr>
<td>RAD18 × 1257</td>
<td>–</td>
<td>83.0</td>
</tr>
<tr>
<td>QC6</td>
<td>40.1</td>
<td>75.7</td>
</tr>
<tr>
<td>QC6 × 1257</td>
<td>–</td>
<td>75.7</td>
</tr>
<tr>
<td>QC12</td>
<td>37.8</td>
<td>65.9</td>
</tr>
<tr>
<td>QC12 × 1257</td>
<td>–</td>
<td>65.9</td>
</tr>
</tbody>
</table>

* Parent strains and hybrids are listed. For example, parent strains D14 and 1257 were fused to generate hybrid D14 × 1257.

* Complementation (+) is reported for those hybrids that were less resistant to 5-FC than the parent strains (see Fig. 1 for an example). Failure to complement (–) is reported for those hybrids that were as resistant as the parent strains (Fig. 1A and C show typical resistance phenotypes of parent strains).

* Multiple entries indicate results obtained from independent cultures.

* These values for D14 content were reported previously (17).
Alleles of FCY1. As a test for allelism, five resistant strains were crossed with the fcyl tester strain 1257 (Table 1). No complementation was detected; parents and hybrids were highly resistant and indistinguishable in phenotype. The five resistant strains tested were highly resistant segregants derived from partially resistant clinical isolates and were UMP pyrophosphorylase deficient. Activities ranged from 0.02 to 0.2 U among those strains, in contrast with the high activity shown by strain D14 (described above) in the present paper and with similar levels of activity in susceptible strains, as shown previously (15). These results indicated that resistance in each of the strains tested was determined by an fcyl allele.

Confirmation of hybridization. The present complementation tests were based on hybridization by spheroplast fusion. In one series of crosses, a lysine-requiring strain (D14) was fused with methionine-requiring strains. In another series of crosses, a histidine-requiring strain (1257) was fused with methionine-requiring strains. Presumptive hybrids were isolated by selection for prototrophs, on the assumption that hybrids would be prototrophic because of the complementation of the nutritional markers. However, it was possible that reversion of the nutritional marker in a parent strain would generate a prototroph that would be taken to be a hybrid. It was therefore necessary to confirm that the present FCY complementation results were based on hybrids rather than revertants.

Hybrids were distinguished from revertants on the basis of total cellular DNA content. A hybrid was expected to contain an amount of DNA (per cell) equal to the sum of the relevant parental values, whereas a revertant was expected to contain an amount of DNA equal to a parental value. DNA content was determined for all parent strains and hybrids employed in the complementation experiments (Table 1).

The mean of 11 determinations of parental DNA content was 37.1 fg per cell (standard deviation, 2.8; range, 33.9 to 42.8). This mean value is in good agreement with the best estimate of the DNA content in C. albicans (10). The mean of 14 determinations of DNA content of hybrids was 79.8 fg per cell (standard deviation, 16.4; range, 60.7 to 115). It was found that all hybrids contained more DNA (per cell) than did either of the relevant parents or any of the parents used. These results supported origin by hybridization rather than reversion.

However, the wider range of values for DNA content found for hybrids (in comparison with the range found for parent strains) suggests that not all hybrids are derived from the fusion of two cells and their two nuclei, with no subsequent change in chromosomal content (see Discussion).

Instability of hybrids. In the cultures of hybrids displaying complementation, we observed a minority of cells that grew to form colonies on MFC50 medium (Fig. 1). It seemed likely that these colonies resulted from the growth of variants that had become resistant owing to a loss of chromosomes bearing the FCY allele(s). A generalized reduction in ploidy was demonstrated in another study (18) in which acquisition of increased resistance to 5-FC was used to select for a decrease in ploidy in hybrids similar to the present hybrids. Three resistant colonies that arose in the D14 × RAD11 complementation test (Fig. 1) were picked, and the DNA content was determined. The cells in two colonies contained less DNA (35.2 and 38.0 fg per cell) than did the hybrid from which they arose (average value, 75.6 fg per cell) (Table 1). These derivatives contained the diploid amount of DNA. The DNA content of cells in the third colony (52.1 fg per cell) suggested that it was an aneuploid derivative of the hybrid. These results extend previous studies (17, 18) which showed that, in the majority of cases, the origin of recessive variants derived from tetraploid hybrids was the result of a ploidy reduction process rather than a recombination process.

DISCUSSION

Recent studies provided tools for genetic analysis in C. albicans. Two lines of evidence, one biochemical (10) and the other genetic (13, 15, 19), indicated that this asexual yeast is diploid. A parasexual approach to genetic analysis, including mitotic recombination (15, 16, 19), hybridization by spheroplast fusion (2, 6, 9, 11), and reduction of ploidy in hybrids (3, 17, 18), was developed. Genetic complementation was used to select prototrophic hybrids resulting from the fusion of parent strains that bore appropriate nutritional markers (2, 6, 9, 11, 17, 18). Application of genetic methods to a clinically relevant phenotype, resistance to 5-FC, revealed that resistant strains can originate, in the laboratory at least, from strains in which the resistance determinant preexists in the heterozygous state (1, 14, 15). The carriage of resistance determinants in this state, in which they are nearly cryptic in terms of phenotype, is made possible by ploidy. The heterozygotes, which are themselves only slightly resistant, are unstable and give rise to highly resistant homozygotes. The high proportion (37%) of heterozygous strains among randomly chosen clinical isolates (1) suggests that heterozygotes are the likely source of resistant strains in clinical practice.

To distinguish between the resistance determinants at the genetic level, thereby permitting their genetic characterization, we developed a functional test for allelism (i.e., a complementation test) among 5-FC resistance determinants. In the present study, complementation of resistance determinants was studied in hybrids generated from the fusion of...
a cytosine deaminase-deficient tester strain with UMP pyrophosphorylase-deficient strains. In the resulting control hybrids, then, it was possible to demonstrate the occurrence of strong (intergenic) complementation.

Complementation was not detected in hybrids generated by the fusion of a UMP pyrophosphorylase-deficient tester strain with five resistant strains that were also UMP pyrophosphorylase-deficient and were derived from partially resistant clinical isolates. Those results were not predictable on simple theoretical grounds (as was the control demonstration of complementation) because it is not known whether UMP pyrophosphorylase activity is determined by one gene or by more than one gene. In the former case, all resistance determinants would be alleles of one gene, while in the latter case, alleles of several genes might be represented in a sample of resistant strains. While our test clearly indicated that all five clinical resistance determinants were *fcyl* alleles, such a sample is small. Further studies of resistance determinants in greater numbers of clinical isolates and also with determinants generated by mutation will be necessary to ascertain the number of genes that determine UMP pyrophosphorylase activity.

Two factors indicate that this complementation method is based upon hybrids that are less precisely defined in genetic terms than are hybrids in a sexual species: there is a wider range of DNA values (femtograms of DNA per cell) in the hybrids than in the parent strains, and the DNA content in hybrids is unstable, as observed here and in previous studies (17, 18). It is possible that some of the hybrids in the present study were triploid rather than tetraploid, and it is also possible that others were aneuploid. This consideration is important in the case of a negative complementation test because it was shown (above) that a 5-FC-susceptible hybrid (positive complementation) may lose chromosomes to generate a 5-FC-resistant aneuploid. However, generation of resistant forms from complementing hybrids is rare (Fig. 1), and all 5-FC-resistant hybrids contained nearly the expected amount of DNA (Table 1). Although the instability of hybrids must be considered in performing and interpreting complementation tests to avoid instances of false-negative complementation, it does not pose a serious problem.

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LITERATURE CITED