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Flucytosine Resistance Is Restricted to a Single Genetic Clade of *Candida albicans*

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Population studies have revealed that *Candida albicans* can be separated into five major clades, groups I, II, III, SA, and E. Groups SA and E are highly prevalent in South Africa and Europe, respectively, while group II is excluded from the southwestern portion of the United State. In each geographical locale, several clades exist side by side, suggesting little interclade recombination. These results suggest clade-specific phenotypes. In the present study we demonstrate that resistance to flucytosine (5FC MIC ≥ 32 μg/ml), an antifungal used for the treatment of systemic *C. albicans* infections, is restricted to clade I. In addition, while 97% of all strains for which 5FC MICs were ≥0.5 μg per ml were members of group I, only 3% were members of the other groups. 5FC MICs were ≥0.5 μg per ml for 72% of all group I isolates, while 5FC MICs were ≥0.5 μg per ml for only 2% of all non-group I isolates. These results demonstrate for the first time the clade specificity of a clinically relevant trait (5FC resistance) and suggest that while intraclade recombination may be common, interclade recombination is rare.

Individual strains of *Candida albicans* can become drug resistant through repeated exposure in vivo and in vitro (29, 36). However, the acquisition of drug resistance does not appear to afford cells with a permanent advantage, since an increase in the prevalence of drug-resistant strains worldwide has not occurred. In most discussions of drug resistance, we tend to consider all strains to be relatively equal in their capacities to evolve drug resistance and to discuss natural resistance at the species level. The assumption of general strain uniformity, however, has been challenged by the identification of deep-rooted *C. albicans* clades that not only maintain their integrity side by side in the same geographical locale but also exhibit geographical specificity (5, 22, 25). Using Ca3 fingerprinting, Pujol et al. (22) identified three major *C. albicans* clades, groups I, II, and III. Blignaut et al. (5), using the same methodology, demonstrated that in South Africa there was a fourth clade, group SA, in addition to groups I, II, and III. Isolates from group SA, which made up roughly half of all isolates from South Africa, were absent from the original U.S. collection analyzed by Pujol et al. (22), suggesting that group SA was highly prevalent in South Africa. Pujol et al. (25) then identified a fifth clade, group E, which was highly prevalent in Europe. Isolates in this clade were also absent from the original collection of Pujol et al. (25) and made up only 1% of South African isolates. With an expanded collection of North American isolates, Pujol et al. (25) demonstrated that the major clades were indeed groups I, II, and III and that groups SA and E made up only 2 and 1% of the collection, respectively. These population characteristics suggest that members of a particular clade may share clade-specific phenotypic characteristics, including resistance to antifungal drugs. In the study described here we have tested whether clades can differ in their general susceptibilities to flucytosine (5FC).

5FC is a pyrimidine analog that enters cells through the action of a permease and is then converted to 5-fluorouracil. 5-Fluorouracil can interfere with RNA synthesis or can inhibit DNA synthesis. Strains can readily develop resistance to 5FC in vitro by exposure to the drug (34). Indeed, because cells readily develop resistance to 5FC, it is usually administered with amphotericin B (1, 29). To test whether clades differ in their susceptibilities to 5FC, we have analyzed the genetic relatedness of natural isolates identified as resistant to 5FC in large screens and have compared the susceptibilities of isolates from each of the five clades of *C. albicans*. Our results demonstrate that all natural strains of *C. albicans* resistant to 5FC are members of the group I clade and that while the 5FC MIC was ≥0.5 μg per ml for 72% of all group I isolates, the 5FC MIC was ≥0.5 μg per ml for only 2% of all non-group I isolates. These results provide the first genetic evidence for clade-specific drug resistance and imply that while recombination rarely occurs between isolates of different clades, it may occur more frequently between isolates of the same clade.

MATERIALS AND METHODS

***C. albicans* isolates.** Isolates were collected from blood or sterile body fluids from patients at more than 42 different geographical sites participating in the SENTRY surveillance program (19); 164 isolates were from 19 sites in the United States and Canada, 46 isolates were from 11 sites in Europe, 22 isolates were from 4 sites in South America, and 11 isolates were from 3 sites in Turkey and Israel. The isolates were not exposed to any antifungal agent prior to collection. The isolates were initially plated on blood agar and Sabouraud dextrose agar at the original site of collection and were then sent to the University of Iowa Hospitals and Clinics for banking and further analysis. Upon receipt, each isolate was subcultured onto potato dextrose agar (Remel, Lenexa, Kans.) and CHROMagar (Hardy Diagnostics, Santa Maria, Calif.) to assess viability and species homogeneity. The isolates were identified as *C. albicans* with Vitek and API kits (bioMerieux, St. Louis, Mo.). Clonal isolates were stored as water suspensions at ambient temperature.

DNA fingerprinting. All isolates were fingerprinted by Southern blot hybridization with the complex DNA fingerprinting probe Ca3 (2, 13, 23, 28) by methods previously described in detail (30, 31, 32). In brief, DNA was extracted

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from the cells, digested with *Eco*RI, and electrophoresed in a 0.8% agarose gel at 50 V. The DNA was then transferred to a Hybond N^+ membrane (Amersham, Piscataway, N.J.) by capillary blotting, prehybridized with salmon sperm DNA, hybridized overnight with the ^{32}P-labeled Ca3 probe, and autoradiographed.

Computer-assisted cluster analysis. Autoradiogram images were digitized into the DENDRON software database (31). Hybridization patterns were unwarped, processed, and then automatically scanned to identify all bands and to link common bands by using DENDRON software (31). The patterns of all test isolates were then compared in a pairwise fashion, and similarity coefficients (S_{AB}) were computed according to the formula for the Dice coefficient (31, 32). An S_{AB} threshold of 0.7 was selected for determination of groups in cluster analyses (5, 22, 31).

Drug susceptibility testing. Antifungal drug susceptibility testing was performed by the reference broth microdilution method described in National Committee for Clinical Laboratory Standards document M27-A (18). 5FC was obtained from Sigma. Serial dilutions were made in RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid. Microdilution trays containing arrays of 5FC at final dilutions of 0.06 to 128 μg/ml were prepared in a single lot. The trays were stored at −70°C prior to use. Prior to testing, each isolate was passaged once on potato dextrose agar to ensure that it had optimal growth characteristics. One hundred microliters of a suspension of cells that varied in concentration from 0.5×10^3 to 2.5×10^3 cells per ml was added to each well of a microdilution tray. The trays were incubated in air at 35°C, and MIC endpoints were read after 48 h. Drug- and yeast-free controls were included in each tray. Following incubation, the growth in each well was compared visually with that in control wells. The MICs were defined as the lowest concentration resulting in a prominent decrease in turbidity. Quality control of the susceptibility assay was performed by using reference isolates *Candida parapsilosis* ATCC 22091 and *Candida krusei* ATCC 6258 (4, 18). The categories of 5FC susceptibility were interpreted according to the following breakpoints (18, 27): susceptibility, ≤4 μg per ml; intermediate resistance, 8 to 16 μg per ml; resistance, ≥32 μg per ml.

RESULTS

The five major clades of *C. albicans*. We generated a dendrogram that includes a basic collection of 243 *C. albicans* isolates that were DNA fingerprinted with the complex probe Ca3 (Fig. 1). The basic collection included 99 group I isolates, 26 group II isolates, 48 group III isolates, 14 group SA isolates, 17 group E isolates, and 39 outliers (isolates that did not cluster in the five major groups). These isolates were selected from our general collections to represent all major groups and outliers. All of these isolates were DNA fingerprinted and were then analyzed for 5FC susceptibility.

All 5FC-resistant strains are in group I. Of the 243 isolates tested, 9 (3.7%) proved to be 5FC resistant (i.e., MIC ≥ 32 μg per ml). The MIC for one isolate was intermediate (MIC = 16 μg per ml). When these 10 isolates were color coded red and black, respectively, in the dendrogram presented in Fig. 1, it was revealed that they all clustered exclusively in group I.

Ten additional isolates highly resistant to 5FC (MICs ≥ 128 μg per ml) were identified in a separate screen of a collection of 5,208 *C. albicans* isolates (20). These isolates were DNA fingerprinted with Ca3 and added to the dendrogram of the collection of 243 isolates, in which they were color coded blue (Fig. 1). All 10 isolates also clustered exclusively in group I. These results indicate that among natural isolates, 5FC resistance is specific to group I.

In general, resistant strains appeared to be distributed throughout cluster I, suggesting a random distribution (Fig. 1). However, there were four examples of subclusters of two and three 5FC-resistant isolates, defined by an S_{AB} threshold of 0.90.

Group I isolates are in general less susceptible to 5FC. The results presented in Fig. 1 indicate that resistant strains are restricted to group I. However, resistant strains represented only 4% of group I isolates. To test whether group I isolates in general were less susceptible to 5FC than non-group I isolates, a histogram of the MICs for group I and non-group I isolates was generated (Fig. 2). The distributions were significantly different. While the MICs for non-group I isolates were distributed between ≤0.06 and 0.50 μg per ml, with an MIC at which 50% of isolates tested are inhibited (MIC_{50}) of 0.12 μg per ml, the MICs for group I isolates were distributed between 0.12 and ≥128 μg per ml, with an MIC_{50} of 1.00 μg per ml, a value eight times higher than that for non-group I isolates (Fig. 2). When 0.50 μg per ml was used as an arbitrary MIC threshold for strains with decreased 5FC susceptibility, 97% of isolates for which the MIC was ≥0.50 μg per ml proved to be in group I, while the remaining 3% were in groups other than

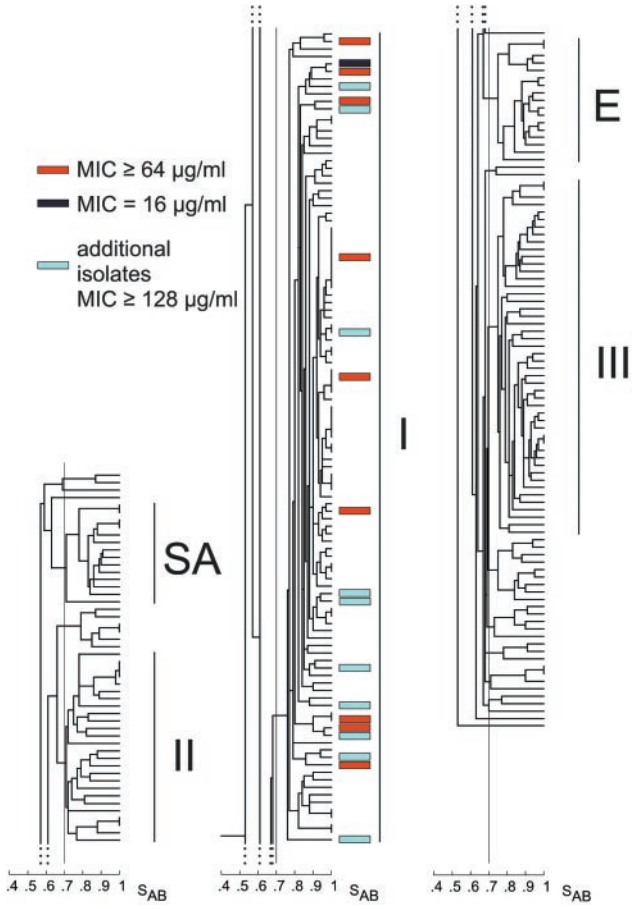

FIG. 1. 5FC resistance is restricted to group I isolates of *C. albicans*, as demonstrated by cluster analysis of a collection of 253 natural *C. albicans* isolates. The five major *C. albicans* clades (groups I, II, III, SA, and E) are shown. For presentation purposes, the dendrogram has been separated into three sections, the top (groups SA and II), middle (group I), and bottom (groups E and III), from left to right, respectively. The red boxes indicate the positions of the 9 isolates resistant to 5FC (MIC ≥ 32 μg/ml) identified among a randomly selected collection of 243 isolates, and the black box indicates the single isolate with intermediate resistance (MIC = 16 μg/ml) identified among those isolates. The blue boxes indicate the positions of the 10 isolates resistant to 5FC (MIC ≥ 128 μg/ml) that were identified in a previous analysis of 5,208 isolates (20). Note that all of those natural isolates that are resistant to 5FC are members of group I.

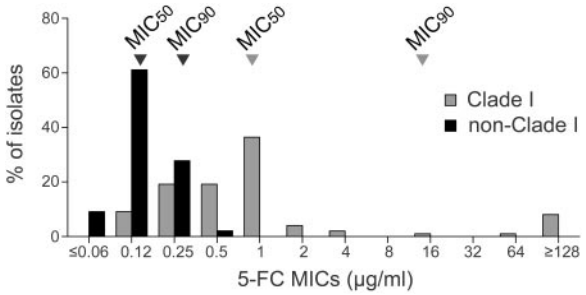

FIG. 2. The 5FC MIC distributions differ between the 99 group I and 144 non-group I isolates. The MIC_{50}s and MIC_{90}s are indicated for each group of isolates; black arrowheads point to the results for non-group I isolates, and gray arrowheads point to the results for group I isolates. The two distributions were significantly different ($P < 0.001$ by both the Kolmogorov-Smirnov and Fisher's exact tests).

group I. The disproportionate concentration of less susceptible isolates in group I is demonstrated in the dendrogram in Fig. 3, in which all isolates for which MICs were ≥0.50 μg per ml are color coded red. All but 3 of the 78 strains for which the MIC was ≥0.50 μg per ml (97%) clustered in group I. Less susceptible isolates made up 72% of group I isolates but only 2% of non-group I isolates.

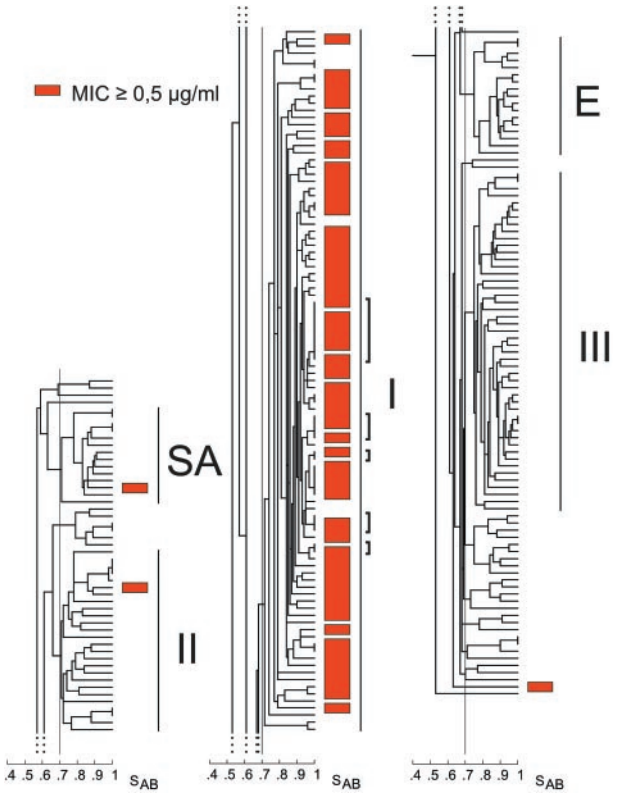

FIG. 3. Group I isolates are, on average, less susceptible to 5FC than non-group I isolates. In a cluster analysis of a collection of 243 *C. albicans* isolates, the positions of isolates for which 5FC MICs were≥0.50 μg/ml are indicated by red boxes. The large majority of isolates (97%) for which MICs were ≥0.50 μg/ml were members of group I. The brackets indicate the five groups of isolates that have identical patterns by fingerprinting with the Ca3 probe and that differ in their susceptibilities to 5FC.

The distribution of less susceptible isolates in the group I cluster appeared to be randomly distributed (Fig. 3). More susceptible isolates did not cocluster. In addition, there were five examples within the group I cluster of isolates with identical patterns by fingerprinting with probe Ca3 (i.e., S_{AB} = 1.00) that differed in their susceptibilities (Fig. 3).

DISCUSSION

Using DNA fingerprinting with the complex probe Ca3, Pujol et al. (22) identified three major clades of *C. albicans*, groups I, II, and III, among a limited collection of 26 unrelated U.S. isolates. Pujol et al. (22) demonstrated that the clustering capacity of the Ca3 fingerprinting method was similar to those of the multilocus enzyme electrophoresis and randomly amplified polymorphic DNA methods, thus verifying its clustering efficacy and the integrity of the three clades (31). Blignaut et al. (5) subsequently demonstrated that in addition to groups I, II, and III, a fourth clade, group SA, accounted for 55% of all isolates from black individuals and 33% of all isolates from white individuals in South Africa. Group SA was absent from the original collection of 26 U.S. isolates of Pujol et al. (22). Pujol et al. (25) subsequently identified a fifth clade, group E, to which 26% of all European isolates belonged. Since groups SA and E each contained only 2% North American isolates and group E contained only 1% South African isolates, groups SA and E were considered geographically specific. Furthermore, Pujol et al. (25) demonstrated that group II was missing from the southwestern United States. Because human populations are mobile and mix, geographical specificity has been interpreted to reflect both differences in nonhuman reservoir populations and phenotypic differences between clades (33). However, because the discoveries of geographical specificity are new, no phenotypic differences have been reported, until now.

In the present study, we have tested whether clades exhibit differences in drug susceptibility by cluster analysis of 5FC-resistant isolates and isolates less susceptible to 5FC. Our results demonstrate that isolates that are naturally 5FC resistant are restricted to group I and that group I isolates are generally less susceptible to 5FC than non-group I isolates. Isolates of group I represent 47% of isolates in North America (25), 20% of isolates in Europe (25), and 19% of isolates in South Africa (5). Therefore, group I represents a major *C. albicans* clade in all of the geographical regions so far studied.

In prior studies, there were already indications that 5FC resistance was not equally distributed among strains. 5FC resistance was associated with, but was not restricted to, one of the two serotypes of *C. albicans*, serotype B (3, 7, 35). However, since the two *C. albicans* serotypes have been shown to be interconvertible within a strain (21), the association with 5FC resistance that was demonstrated was not necessarily genotypic. Prior studies also indicated that 5FC resistance is associated with the absence of the ribosomal IS*1* intron (17). However, this association is not tight, since both group I and group II isolates lack this intron (5, 15, 25), but only group I isolates are resistant. Our results therefore appear to be the first to relate a single bona fide genetic group of *C. albicans* with a

drug resistance phenotype. Our studies, however, did not test whether the average clade I isolate more readily acquires resistance upon exposure to 5FC than non-group I isolates, even though group I isolates are, on average, already less susceptible to the drug. Experiments to test this question are in progress.

Finally, our results provide some insight into the process of recombination in *C. albicans*. Recently, Hull and Johnson (9) identified the mating locus of *C. albicans*, and mating type-dependent fusion has been demonstrated both by complementation (10, 16) and at the cellular level (11, 12). Although these observations provide a formal mechanism for recombination, population studies suggest a clonal population structure (6, 8, 26), suggesting that recombination, at least between clades, is a rare event. The recent observation that clades remain intact side by side in the same geographical locale (5, 22, 25, 33) supports the idea that recombination is rare between isolates of different clades. In the present study we have demonstrated that natural drug resistance and a general decrease in susceptibility are exclusive characteristics of only one of the five clades, lending support to the idea that little recombination occurs between isolates of group I and isolates from the four other clades. On the other hand, decreased 5FC susceptibility appeared to be distributed randomly throughout clade I, suggesting that recombination within the clade may homogenize the characteristic. Two recent findings support this hypothesis. First, homozygous *MTL***a** and *MTL*α strains are relatively frequent in group I (14). Second, acquisition of mating competency due to a spontaneous loss of heterozygosity at the *MTL* locus has been described in *C. albicans* strains (14, 24). Most of the strains for which this phenomenon was observed were from group I, suggesting that *MTL* homozygosity may be generated more frequently in this group. However, one cannot rule out the possibility that a single clone that was originally less susceptible to 5FC and that was prevalent worldwide led to clade I. The latter possibility seems unlikely, given the genetic individuality and deep-rootedness of clade I.

The results presented here have demonstrated clear differences in 5FC susceptibility between clades and suggest that other phenotypic characteristics may differ between clades. The results demonstrate that no single strain represents the entire *C. albicans* species and that the characterization of *C. albicans* for a particular virulence characteristic should therefore include representatives from each clade.

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