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Isolation of *Candida* Species on Media with and without Added Fluconazole Reveals High Variability in Relative Growth Susceptibility Phenotypes

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Mouthwashes from human immunodeficiency virus-positive individuals were sampled for yeasts by direct plating on a differential agar medium with and without added fluconazole and via enrichment broths with and without added fluconazole. The colonies of the yeasts isolated were tested for relative growth in the presence of single concentrations of itraconazole and fluconazole. Among 258 culture plates containing yeasts obtained via different isolation routes from 86 yeast-positive samples, 33 (12.7%) of the plates showed unexpectedly high colony-to-colony variation in relative growth. Intercolony variation was seen in 41 (47.7%) of the 86 isolates when relative growth data were analyzed for all colonies of an isolate tested, regardless of the medium used for isolation. The prevalence of relative growth variability with the azoles was highest for *Candida glabrata* (100% of 13 isolates), followed by *Candida krusei* (60% of 5 isolates) and *Candida albicans* (40% of 53 isolates), and the visual patterns of variability seen in scatter plots of the data showed species specificity. Relative growth phenotypes generally tended to be stable for each yeast colony in subcultures, whether or not the medium used for subculture contained antifungal agents. DNA fingerprinting of stable and variable *C. albicans* isolates showed changes in band patterns detected with the probe Ca3, suggesting that the variability may have resulted from selection of different subtypes of the yeasts during the isolation procedure. These findings suggest that the yeasts isolated from single clinical samples were often not clonal in nature. The relative growth test revealed colony variability more readily than conventional susceptibility testing.

In 1995 we set up a study of the epidemiology of oral *Candida* infection in human immunodeficiency virus (HIV)-positive patients. The study design differed from those of others already published in the intended manner of isolation of yeasts from mouthwash samples. We wished to optimize the detection of minority yeast populations and mixed yeast populations in the samples, and we therefore used the differential medium CHROMagar Candida (CAC) for yeast isolation. To maximize the probability of detection of *Candida glabrata* and *Candida krusei* we plated mouthwash samples on CAC containing fluconazole as well as on fluconazole-free medium, in a manner analogous to that of Patterson et al. (16), and we inoculated broths with and without added fluconazole as enrichment cultures for strains and species of yeasts with reduced susceptibility to this agent (7, 11). The broths were then plated out on the differential medium for isolation of the yeasts that grew after enrichment.

We intended to measure the susceptibilities of yeast isolates to itraconazole and fluconazole, the agents most recently available for the treatment of oropharyngeal *Candida* infections. Johnson et al. (7) found differences in MICs for individual colonies of oral *Candida* isolates from AIDS patients, and we wanted to take account of such possible differences by measuring the susceptibilities of multiple colonies of all yeasts isolated from the same sample with and without exposure to fluconazole and with and without enrichment via broth cultures. To ensure a manageable workload with a very large predicted number of colonies, we needed to measure susceptibility with an abbreviated test system. Relative yeast growth at single concentrations of fluconazole and itraconazole was chosen since this method allows data trends to be visualized in the form of two-dimensional scatter plots (14) and it can broadly distinguish fluconazole-susceptible and fluconazole-resistant isolates in relation to the clinical outcome of fluconazole treatment (2, 14).

Figure 1 illustrates the principle of relative growth measurement with two hypothetical compounds, compounds A and B. The susceptibility phenotype of any yeast isolate to the two agents relative to those of other isolates is reflected by its position in a plot such as that shown in Fig. 1. Yeasts such as isolate 3 in Fig. 1 are barely inhibited by both of the agents at the concentrations tested, while isolates in other positions on the plot are at least partly susceptible to one or both of the agents.

As our study progressed it became apparent that different colonies of the same yeast species isolated from a single mouthwash sample sometimes showed much greater variation in the relative growth tests than we had anticipated, particularly when results were compared for colonies grown on different isolation media. Datum points for the different colonies from a single mouthwash sample, particularly for isolates of *C. glabrata*, often formed remarkably disperse clusters in the scatter plots instead of the relatively tight clusters that had been seen with repeated tests with sets of laboratory strains (13, 14). We have therefore investigated relative growth variability in clones from the same clinical sample to establish the nature and characteristics of this unexpected phenomenon.

MATERIALS AND METHODS

Yeast isolation and identification. Between December 1994 and October 1995, 156 HIV-infected and AIDS patients attending the outpatient clinic of the Institute of Tropical Medicine at Antwerp, Belgium, or hospitalized in the University Hospital of Antwerp were included in an epidemiologic survey after

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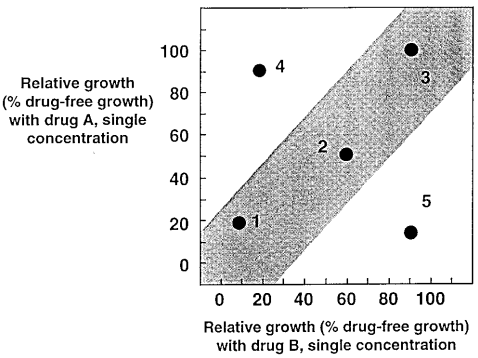

FIG. 1. Principle of relative growth measurement with single concentrations of two antifungal agents, agents A and B. Yeast isolate 1 grows to 20% of the level of growth of the isolate in the drug-free control in the presence of drug A and to 10% of the level of growth of the control in the presence of drug B. The corresponding figures are 50 and 60% for isolate 2, 100 and 90% for isolate 3, 90 and 20% for isolate 4, and 15 and 90% for isolate 5. Isolates 1, 2, and 3 are, relatively susceptible, less susceptible, and relatively resistant to both agents, respectively, while differential susceptibility phenotypes are shown by isolate 4 (resistant to drug A and susceptible to drug B) and isolate 5 (susceptible to drug A and resistant to drug B). The gray zone indicates the approximate area in which isolates would be regarded as showing relative cross-susceptibility or cross-resistance to the two agents tested.

they gave verbal consent. Each patient rinsed his or her mouth with 10 ml of sterile saline and returned the mouthwash to a sterile container. Volumes of 100 μl of each sample were plated onto separate 9-cm petri dishes and the dishes were incubated in air for 48 h. The three media inoculated were CAC (CHROMagar, Paris, France) and CAC with fluconazole (Pfizer Central Research, Sandwich, United Kingdom) added to a concentration of 10 μg/ml (CF10) or 100 μg/ml (CF100). In some experiments mouthwash samples were also plated onto CAC with itraconazole (Janssen Research Foundation, Beerse, Belgium) at 1.0 μg/ml (CIt1.0) and 10 μg/ml (CIt10). Further 100-μl volumes of the mouthwash samples were added to test tubes containing a synthetic broth medium referred to as YNB (yeast nitrogen base [Difco, Detroit, Mich.], 6.7 g/liter; glucose, 10 g/liter; asparagine [Difco], 0.76 g/liter; chloramphenicol, 50 mg/liter; and gentamicin sulfate, 20 mg/liter; sterilized by filtration), the same broth with fluconazole at 10 μg/ml (YBF10), and the same broth with fluconazole at 100 μg/ml (YBF100). The broths were incubated at 37°C for 48 h, and then 100-μl samples were plated onto CAC, CF10, and CF100 for isolation of any yeasts that they contained.

A representative colony of each recovered yeast that showed a distinguishable colony color on any individual isolation plate was identified to the species level. Light green colonies were confirmed as *Candida albicans* by production of germ tubes in fetal calf serum after 3 h at 37°C and the production of chlamydospores on 1% rice cream–1% Tween 80 agar after 48 h at 25°C. Dark green colonies were tested in the same way and also by DNA fingerprinting with the oligonucleotide probe Ca3 to differentiate *Candida dubliniensis* from *C. albicans* (19). Colonies with other colors were identified on the basis of their morphologies on rice cream–Tween 80 agar, and their assimilation patterns were identified with the API ID32C yeast identification system (bioMérieux SA, Marcy-l'Etoile, France). Colonies of the same color on any single isolation plate were assumed to be the same species.

It was possible for the same species in a single mouthwash sample to be cultured by as many as 12 routes of isolation, viz., direct isolation on CAC, CF10, and CF100; growth in YNB followed by isolation on CAC, CF10, and CF100; growth in YBF10 followed by isolation on CAC, CF10, and CF100; and growth in YBF100 followed by isolation on CAC, CF10, and CF100. Because of the complexities arising from these multiple routes of isolation of colonies of a single species from a single species from a single mouthwash sample, the term "isolate" was used to refer to all yeasts of the same species isolated from a single mouthwash sample, regardless of the different routes of isolation, and the term "subisolate" was used to refer to the subset of colonies of that species recovered via a single route from a mouthwash sample. For example, a mouthwash might yield *Candida tropicalis* on CAC, CF10, and CAC after passage through YNB. All the *C. tropicalis* colonies recovered from the three plates constitute one "isolate"; the colonies on each of the three individual plates constitute three "subisolates."

Subcultures of all yeast isolates were made on Sabouraud glucose agar (Oxoid, Basingstoke, United Kingdom) incubated at 37°C for 48 h. For preservation of individual clones, separate yeast colonies were inoculated in 100-fold-diluted casein hydrolysate-yeast extract-glucose (CYG) broth and were grown overnight at 30°C (13). One-tenth of the culture volume of sterile glycerol was added, and the suspensions were stored in small lots at −70°C.

Relative growth and antifungal susceptibility testing. Relative growth testing was done by the method of Odds et al. (14) in microplate cultures with CYG medium (12) with and without fluconazole (15 μg/ml) and itraconazole (0.71 μg/ml). Up to 12 colonies of each subisolate from any individual culture plate were routinely tested. For each colony, a separate inoculum suspension was prepared in diluted CYG broth, and the cell concentration was adjusted for use (14). Each inoculum was added to single wells containing fluconazole and itraconazole and to duplicate drug-free control wells. The turbidity optical density (OD; 405 nm) in the presence of each antifungal agent was expressed as a percentage of the mean turbidity OD of the control wells (all OD values were corrected by subtraction of background OD) to generate the relative growth result for each colony. The percent relative growth data were displayed as clusters on two-dimensional scatter plots.

The susceptibilities of the yeasts to fluconazole and itraconazole was measured in terms of the lowest antifungal concentration that reduced growth below 50% of that of the control (IC_{50}) by the spectrophotometric method of Odds et al. (15). The susceptibility tests were done with both RPMI 1640–2% glucose medium (15) and CYG medium.

Statistical analysis. The extent of variability of clusters of relative growth data was apparent by visual examination of two-dimensional scatter plots of the results. To summarize the clusters seen in the scatter plots for the very many colonies of isolates and subisolates tested, we chose an estimator that represented the area occupied by a cluster of relative growth data. The sum of the variances of the two dimensions, denoted as D^2, was considered to be a good estimator of the total cluster area. The cluster center of gravity had the coordinates x, which was the mean percent relative growth in the presence of itraconazole, and y, which was the mean percent relative growth in the presence of fluconazole.

An average D^2 value of 52 was determined from an experiment in which 14 different yeast subisolates were tested in eight replicates (eight separate inoculum preparations) for measurement of within-run variation of percent relative growth. For symmetrically distributed data, the ratio of D^2 with this reference value follows an F distribution, with degrees of freedom equal to $2 \times (n_i - 1)$ for the numerator and $2 \times (n_r - 1)$ for the denominator, where n_i and n_r are the number of data in the experiment and reference, respectively. Data clusters were regarded as varying significantly more than could be explained by within-run variations when the P value of the F ratio was <0.001. This stringent criterion was chosen since it adequately distinguished clusters that were subjectively judged as highly variable by visual scrutiny of the scatter plots and it minimized the probability of false-positive results when large numbers of separate determinations of D^2 were being made.

DNA fingerprinting of *C. albicans* isolates. Cellular DNA was prepared by the method of Scherer and Stevens (17). The DNA was digested with *Eco*RI (15). Restriction fragments were separated by horizontal electrophoresis in a 0.8% agarose gel overnight at 30 V. DNA was transferred to nitrocellulose membranes by vacuum blotting, and the blots were hybridized with the moderately repetitive oligonucleotide sequence Ca3 (1) (a kind gift of D. R. Soll, University of Iowa), labelled with digoxigenin (5), and developed by means of peroxidase reactivity with a commercially available detection kit used according to the manufacturer's instructions (DIG DNA labeling and detection kit; Boehringer Mannheim, Mannheim, Germany). For presentation of the results of DNA fingerprinting, the gels were scanned into a computer and analyzed with Dendron software (18) to allow image balancing and reproduction of reneighbored lanes from different gels.

RESULTS

Experimental variability of relative growth data. Fourteen yeast colonies, each from a different recent isolate, were set up in eight replicate inoculum cultures in diluted CYG, and the 112 inocula thus obtained were individually tested for percent relative growth with fluconazole and itraconazole in a single test run. Figure 2 presents the results for 8 of the 14 replicate sets of data drawn as a two-dimensional scatter plot. The examples presented in Fig. 2 were chosen to include the colonies that gave the most and the least within-run replicate variation; the subisolates not illustrated in Fig. 2 were excluded only because their relative growth clusters overlapped with those shown. The results indicated that individual colonies differed in the extent of their within-run variations in the relative growth tests. In the worst-case example, *C. glabrata* 123, the range of relative growth in the presence of fluconazole was from 61 to 87% (a difference of 26%) and the range of relative growth in the presence of itraconazole was from 20 to 51% (a difference of 31%).

For the 14 yeasts tested for replicate variation, D^2 between each of the eight points in a relative growth cluster and the

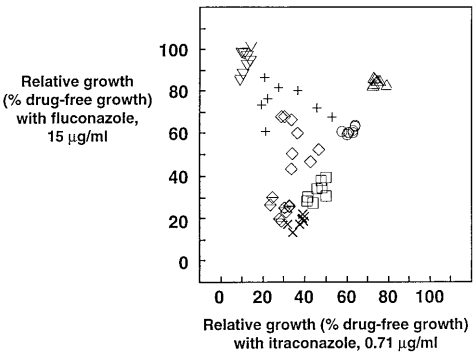

FIG. 2. Within-run variability of relative growth data. Eight replicates of eight different yeast colonies were prepared as separate inoculum cultures, and their growth in the presence of fluconazole (15 μg/ml) or itraconazole (0.71 μg/ml) was measured as a percentage of the drug-free control growth. Symbols for the colonies and their cluster statistic (D^2) are as follows: ×, *C. dubliniensis* 005c ($D^2 = 14$); ▽, *C. krusei* 008c ($D^2 = 56$); ○, *C. glabrata* 011b ($D^2 = 7$); △, *C. glabrata* 013a ($D^2 = 11$); ◇, *C. albicans* 031a ($D^2 = 135$); ⟐, *C. albicans* 047a ($D^2 = 24$); □, *C. dubliniensis* 118b ($D^2 = 89$); +, *C. glabrata* 123b ($D^2 = 236$).

center of gravity of the cluster ranged from 7 to 236, with a mean of 52 and a median of 38. None of the values of D^2 for the clusters resulting from the within-run tests was statistically significantly greater than this average value. For the worst-case within-run variation (*C. glabrata* 123), D^2 was 256 ($P = 0.02$). The data from these experiments indicated a worst-case spread of percent relative growth results with fluconazole and itraconazole of ±15% from the center of gravity on a scatter plot. This spread was attributable to within-run variation.

Yeasts isolated from HIV-positive patients. In total, 134 isolates of different yeast species (at least a single colony from one of the 12 possible routes of isolation) were grown from 99 of the mouthwash samples obtained from 156 HIV-positive patients, indicating an overall yeast positivity rate of 63.5%. Seventy of the yeasts isolated were monocultures of *C. albicans*, two were monocultures of *C. glabrata*, and one was a monoculture of *Candida inconspicua*. The remaining 61 isolates were all grown in mixed cultures containing *C. albicans* together with one other *Candida* sp. (18 samples), two other *Candida* species (7 samples), or three other *Candida* species (1 sample). Most of these isolations were from patients included in a cross-sectional survey of oral *Candida* carriage, and further details of these isolations and the clinical backgrounds of their sources will be reported in a separate publication (18a). All the yeasts isolated grew at least one colony on CAC. For 48 of the yeast isolates, fewer than four colonies in total were obtained from the oral rinse, regardless of the route of isolation; these yeasts were excluded from further analysis. The routes of isolation of the remaining 86 isolates are summarized in Table 1.

The statistics in Table 1 indicate that, as might be anticipated, the number of cultures positive for yeasts tended to be greater for media not containing fluconazole than for cultures in which fluconazole was included and that more isolates grew in media with fluconazole at 10 μg/ml than in those containing fluconazole at 100 μg/ml. However, a notable exception was the YNB enrichment broth (fluconazole-free), which generally gave a lower isolation frequency than the same broth to which fluconazole was added. This difference was particularly notable for isolates of *C. glabrata*, all of which grew after passage in YBF100 subcultured onto CAC, compared with 10 of 12 positive cultures after passage from YBF10 to CAC and only 5 of 12 positive cultures after passage from YNB to CAC.

TABLE 1. Routes of isolation of 86 yeasts included in the study

Yeast species	Total no. of isolates[a]	No. of subisolates obtained on or via the following[b]										
		CAC	CF10	CF100	YNB→CAC	YNB→CF10	YNB→CF100	YBF10→CAC	YBF10→CF10	YBF10→CF100	YBF100→CAC	YBF100→CF10	YBF100→CF100
C. albicans	53	53 (42)	19 (18)	8 (8)	13 (10)	8 (8)	3 (3)	15 (11)	8 (8)	4 (4)	11 (9)	5 (5)	3 (3)
C. dubliniensis	4	4 (4)	0	0	0	0	0	1 (0)	0	0	1 (0)	0	0
C. glabrata	13	13 (12)	13 (13)	8 (7)	6 (5)	5 (5)	6 (6)	11 (8)	12 (7)	10 (6)	13 (8)	13 (8)	10 (3)
C. guilliermondii	1	1 (1)	1 (1)	0	1 (1)	0	0	1 (1)	1 (1)	1 (0)	0	0	0
C. inconspicua	3	3 (3)	3 (3)	0	0	0	0	0	0	0	0	0	0
C. krusei	5	5 (4)	5 (5)	0	2 (2)	2 (2)	1 (1)	4 (2)	4 (2)	2 (0)	4 (2)	4 (2)	2 (1)
C. tropicalis	7	7 (4)	2 (2)	2 (2)	1 (1)	1 (1)	0	4 (3)	3 (3)	1 (1)	2 (1)	1 (0)	0

[a] Four or more colonies.

[b] The abbreviations in subheads indicate whether a subisolate was obtained by direct plating of a mouthwash sample on CAC, CF10, or CF100 or by passage in an enrichment broth (YNB) or in YBF10 or YBF100 followed by plating on the three agar media. Numbers in parentheses are the numbers of subisolates for each route and species for which at least four subisolate colonies were available.

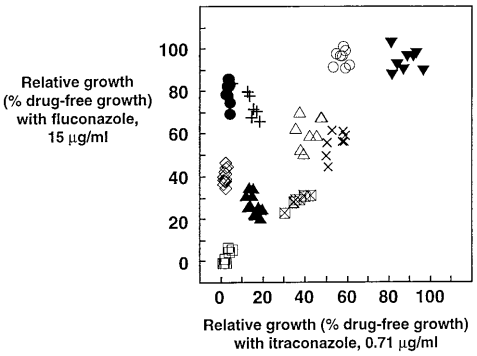

FIG. 3. Two-dimensional relative growth plots for yeast subisolates that showed little colony-to-colony variation. Individual colonies were taken separately from isolation plates inoculated with mouthwash samples and tested for percent relative growth in the presence of fluconazole and itraconazole. Symbols indicate different colonies from the same oral sample. Symbols for patient reference numbers and yeast species (with number of colonies tested, isolation history, and value of D^2 for the cluster of relative growth points in parentheses) are as follows: ○, patient 131, *C. albicans* (n = 8, CF10, D^2 = 22); □, patient 74, *C. guilliermondii* (n = 8, BF10→CF10, D^2 = 13); △, patient 128, *C. glabrata* (n = 8, BF100→CAC, D^2 = 80); ◇, patient 026, *C. inconspicua* (n = 12, CF10, D^2 = 13); +, patient 013, *C. glabrata* (n = 7, CAC, D^2 = 63); ⊠, patient 123, *C. albicans* (n = 8, CF10, D^2 = 24); ×, patient 072, *C. albicans* (n = 8, CF100, D^2 = 51); ●, patient 024, *C. krusei* (n = 9, CF10, D^2 = 29); ▲, patient 009, *C. albicans* (n = 12, CF10, D^2 = 29); ▼, patient 094, *C. glabrata* (n = 8, BF10→CF100, D^2 = 56).

Relative growth of yeast subisolates on fluconazole and itraconazole with different routes of isolation. Relative growth tests done on fluconazole and itraconazole with multiple colonies from single plates (subisolates), when visualized as two-dimensional scatter plots, showed that for most subisolates the cluster of scatter plot points occupied a fairly constrained area, with variation being little more than that seen in tests of within-run variation. However, the data for some subisolates showed a very much wider spread. Examples of scatter plots for 10 nonvariable yeast subisolates are presented in Fig. 3, and examples for 5 hugely variable subisolates are presented in Fig. 4, together with the routes of isolation and cluster size statistics (D^2). The data for isolates represented in Fig. 3 and 4 were chosen not only to show data for subisolates in diverse positions on the scatter plot but also to represent, as far as possible, data for the various *Candida* species tested and the different routes of isolation. The four subisolates whose data are depicted in Fig. 4 all generated values of D^2 significantly greater than the average within-run variation ($P < 0.001$), and they exemplify extreme cases of variation such as that of the *C. albicans* isolate from patient 120 and the *C. glabrata* isolate from patient III.03, for which the relative growth data were spread along the scatter plot from a position (below left) indicating relative susceptibility to fluconazole and itraconazole to a position (above right) indicating relative resistance to these agents.

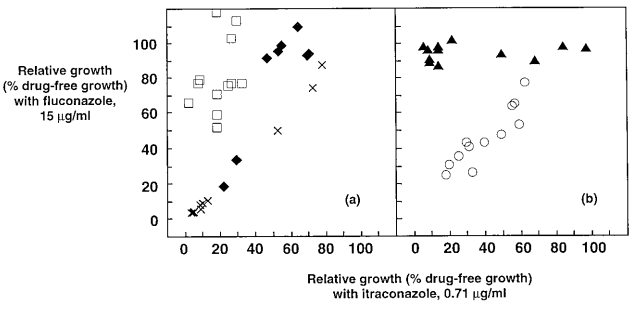

FIG. 4. Relative growth data for yeast subisolates for which there were high levels of variation between results for different colonies. Individual colonies were taken separately from isolation plates inoculated with mouthwash samples and tested for percent relative growth with fluconazole and itraconazole. Symbols indicate different colonies from the same oral sample obtained on a single isolation plate. Symbols for patient reference numbers and yeast species (with numbers of colonies tested, isolation history, and value of D^2 for the cluster of relative growth points in parentheses) are as follows: □, patient I.17, *C. krusei* (n = 12, BF10→CF10, D^2 = 505); ×, patient 120, *C. albicans* (n = 10, CAC, D^2 = 1940); ◆, patient III.03, *C. glabrata* (n = 9, BF10→CF100, D^2 = 1497); ○, patient 039, *C. tropicalis* (n = 12, BF10→CAC, D^2 = 537); ▲, patient III.03, *C. albicans* (n = 12, YNB→CF10, D^2 = 1131). All values of D^2 were significantly greater than the average for within-run variation ($P < 0.001$).

Table 2 summarizes the distribution of highly variable subisolates by yeast species and route of isolation. Data are included in Table 2 only when at least four colonies per subisolate were tested: results for *C. dubliniensis*, *Candida guilliermondii*, and *C. inconspicua* are not given because the numbers of isolates of these species were small and very few examples of relative growth variation of subisolates were found. From Table 2 it can be seen that the tendency to variability in relative growth clusters at the level of subisolates was greatest with *C. krusei* (30.4% of 23 subisolates significantly variable), followed by *C. albicans* (14.7% of 129 subisolates) and *C. glabrata* (6.8% of 88 subisolates), with only one variable subisolate (5.6% of 18 tested) of *Candida tropicalis* being found. If the data in Table 2 are stratified by route of isolation, irrespective of species, isolates enriched by passage through YBF10 had the greatest tendency to give variable relative growth scatter plot clusters (20% of the 55 subisolates grown from this broth showed significant variability, whereas 7.6% of 117 subisolates directly grown on one of the agar media, 18.2% of the 44 subisolates passaged through YNB, and 4.8% of the 42 isolates passaged through YBF100 showed significant variability).

Twenty-five of the mouthwash samples were plated onto CIt1.0 and CIt10. Yeasts from only five samples were isolated on these media (two isolates of *C. albicans* and three isolates of *C. glabrata*). None of the 10 subisolates from these five positive samples showed significant variation in relative growth.

Variation of relative growth profiles in the presence of fluconazole and itraconazole among different yeast isolates. In routine microbiological practice, isolates of the same species cultured from a single oral sample but on different media would often be assumed to represent a single clonal isolate or strain of that species. Tests for relative growth of *Candida* spp. isolated via the various routes used in this study showed that some isolates matched this expectation in terms of their fluconazole-itraconazole susceptibility phenotypes, showing consistent phenotypes with a narrow spread of clusters of relative growth data. Figure 5 provides examples of data for isolates whose cluster statistic, D^2, did not differ significantly from the average value of D^2 obtained from tests of within-run variation. However, many other isolates showed significant, sometimes extreme variations in their relative growth in the presence of fluconazole and itraconazole. Figure 6 provides data for four examples of such variable isolates.

Table 3 summarizes the prevalence of relative growth variation for isolates of different *Candida* species. By far the greatest variation was found for isolates of *C. glabrata*, all 13 of which gave relative growth clusters with D^2 values significantly greater than those expected from within-run variations. Significant relative growth variation was also seen for 40% of the *C. albicans* isolates and three of the five *C. krusei* isolates. By contrast, only one of four *C. dubliniensis* isolates and one of seven *C. tropicalis* isolates showed significant variation in relative growth clusters. The ranges and median values for the

TABLE 2. Statistics for relative growth (fluconazole versus itraconazole) scatter plot clusters of four yeast species, arranged by the isolation histories of the yeast subisolates[a]

Route of isolation	*C. albicans*		*C. glabrata*		*C. krusei*		*C. tropicalis*	
	No.	% Variable	No.	% Variable	No.	% Variable	No.	% Variable
CAC	42	14.3	12	8.3	4	0.0	4	0.0
CF10	18	5.6	13	7.7	5	0.0	2	0.0
CF100	8	0.0	7	0.0	0	0.0	2	0.0
YNB→CAC	10	30.0	5	0.0	2	50.0	1	0.0
YNB→CF10	8	25.0	5	0.0	2	50.0	1	0.0
YNB→CF100	3	0.0	6	0.0	1	100.0	0	0.0
YBF10→CAC	11	36.4	8	12.5	2	50.0	3	0.0
YBF10→CF10	8	12.5	7	14.3	2	50.0	3	33.3
YBF10→CF100	4	0.0	6	16.7	0	0.0	1	0.0
YBF100→CAC	9	11.1	8	12.5	2	50.0	1	0.0
YBF100→CF10	5	20.0	8	0.0	2	50.0	0	0.0
YBF100→CF100	3	0.0	3	0.0	1	0.0	0	0.0

[a] For each species the number of subisolates with each history and the percentage of those isolates that gave variable relative growth scatter plot clusters with a D^2 value significantly greater ($P < 0.001$) than expected by within-run variation are presented. From 4 to 12 colonies of each subisolate were tested.

relative growth centers of gravity and the D^2 statistics in Table 3 convey some of the characteristics of relative growth variation. For *C. glabrata* the values of D^2 were higher than those for other species, indicating that the higher prevalence of relative growth variation in this species was also accompanied by a greater extent of spread of the data cluster (Fig. 6b provides the results for a *C. glabrata* isolate with an extremely wide relative growth cluster). The median value of the center of gravity for this species ($x = 50$; $y = 72$) further shows that the *C. glabrata* isolates were generally among the isolates that were least susceptible to fluconazole and itraconazole by comparison with the susceptibilities of other species. Only the very high median y value of 84 for *C. krusei* isolates exceeded the y value for *C. glabrata*, confirming the known low level of susceptibility of this species to fluconazole.

Among the five isolates for which subisolates were obtained on CIt1.0 and CIt10, inclusion of the relative growth results for these subisolates with those for the other subisolates made no difference to the variable phenotypes of the isolates. One of the two *C. albicans* isolates was nonvariable and one was highly variable, with or without the data from the itraconazole plates included in the analysis; all three *C. glabrata* isolates were highly variable, with or without the results from the itraconazole plates included.

The summary data in Table 3 do not provide full insight into the nature of the variations in relative growth phenotypes. By scrutiny of scatter plots for individual isolates, several definable patterns of variation could be discerned. Of the 21 significantly variable isolates of *C. albicans*, 17 (81%) gave relative growth clusters that were spread along the central diagonal of

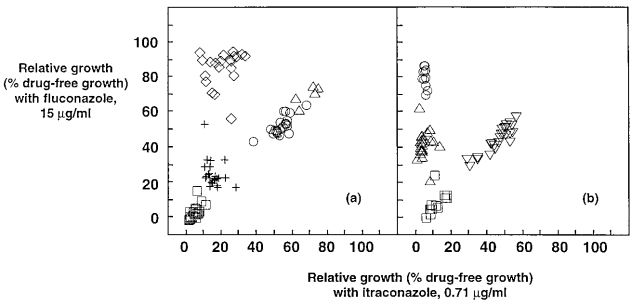

FIG. 5. Relative growth data for isolates of the same yeast species obtained via different isolation routes from the same mouthwash sample: isolates that showed low colony-to-colony variability. (a) All isolates are *C. albicans*. Symbols: ○, patient 126 (26 colonies isolated via CAC, CF10, CF100, and YBF100→CAC); △, patient 099 (5 colonies isolated via CAC and YBF10→CAC); ◇, patient 017 (21 colonies isolated via CAC and CF10); □, patient 075 (26 colonies isolated via CAC, YNB→CAC, and YNB→CF10); +, patient 009 (24 colonies isolated via CAC and CF10). (b) ○, patient 024, *C. krusei* (11 colonies isolated via CAC and CF10); △, patient 026, *C. inconspicua* (24 colonies isolated via CAC and CF10); ◇, patient I.05, *C. glabrata* (16 colonies isolated via CAC and CF10); □, patient 062, *C. albicans* (13 colonies isolated via CAC and CF10); ▽, patient 062, *C. tropicalis* (24 colonies isolated via CAC, CF10, and CF100).

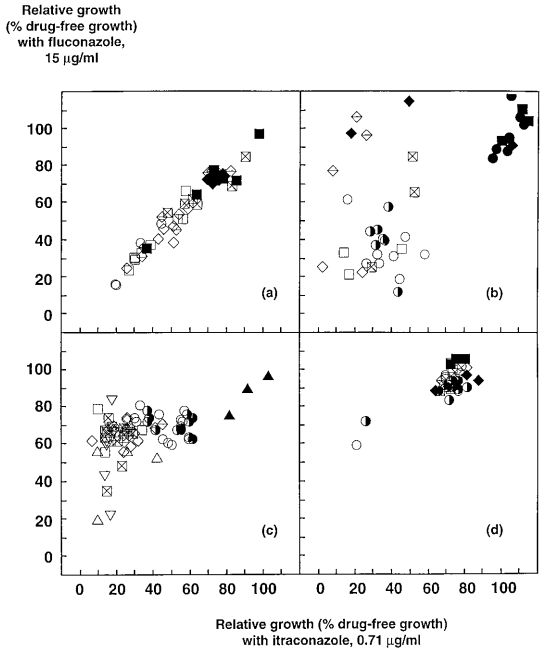

FIG. 6. Relative growth data for isolates of the same yeast species obtained via different isolation routes from the same mouthwash sample: isolates that showed high colony-to-colony variability. (a) Patient 06, *C. albicans* ($n = 52$, $D^2 = 765$; $P < 0.0001$); (b) patient 005, *C. glabrata* ($n = 40$, $D^2 = 2380$; $P < 0.0001$); (c) patient 123, *C. glabrata* ($n = 81$, $D^2 = 599$; $P < 0.0001$); (d) patient 011, *C. glabrata* ($n = 42$, $D^2 = 200$; $P < 0.0001$). In all scatter plots, routes of isolation are indicated by the following symbols: ○, CAC; ◑, CF10; ●, CF100; △, YNB→CAC; ▽, YNB→CF10; ▲, YNB→CF100; □, YBF10→CAC; ⊠, YBF10→CF10; ■, YBF10→CF100; ◇, YBF100→CAC; ⊘, YBF100→CF10; and ◆, YBF100→CF100.

TABLE 3. Occurrence of highly variant relative growth phenotypes among different *Candida* spp.

Species	No. of isolates	Centers of gravity for relative growth clusters[a]				D^2 statistic		No. (%) of isolates for which P was <0.001
		x Range	x Median	y Range	y Median	Range	Median	
C. albicans	53	3–67	29	4–97	31	11–2,444	147	21 (39.6)
C. dubliniensis	4	13–37	25	19–30	25	46–778	511	1 (25.0)
C. glabrata	13	25–77	50	42–96	72	137–2,403	913	13 (100.0)
C. guilliermondii	1	7	7	14	14	341	341	1 (100.0)
C. inconspicua	3	1–13	11	33–55	41	62–347	152	1 (33.0)
C. krusei	5	1–12	8	76–88	84	31–482	169	3 (60.0)
C. tropicalis	7	15–62	40	13–57	42	61–561	114	1 (14.3)

[a] For each isolate the mean percent relative growth with itraconazole (x) and the mean percent relative growth with fluconazole (y) were calculated to determine the centers of gravity of the relative growth clusters (x,y).

the scatter plot, as illustrated in Fig. 4a (patient 120) and Fig. 6a. For some isolates this "diagonal variation" extended along a portion of the diagonal, and for others it extended along almost the whole diagonal. Data clusters showing diagonal variation were occasionally broader than those exemplified in Fig. 4 and 6, but the main direction of the variation along the diagonal was unmistakable. Such a phenotype indicates a pattern of proportional changes in the susceptibilities of isolates to both fluconazole and itraconazole. The same pattern of diagonal variation was noted for the isolates of *C. dubliniensis*, *C. guilliermondii*, and *C. tropicalis* that showed significant relative growth variation. Three of the highly variable *C. albicans* isolates showed "top variation"; that is, their relative growth clusters spread along the top edge of the figure (Fig. 4b, patient III.03). This pattern indicates changes in relative growth in the presence of itraconazole from colony to colony with a stable (but resistant) relative growth in the presence of fluconazole. A single highly variable isolate of *C. albicans* gave a widely spread cluster of relative growth points covering an area similar to that illustrated for a *C. glabrata* isolate in Fig. 6c.

Among the 13 *C. glabrata* isolates, all of which showed significant relative growth variation, the diagonal variation pattern was the least common, being seen with only one isolate. Another isolate (Fig. 6b) showed variation over the entire graphic; this isolate was the only one of any species with this pattern. The most common pattern of variation, seen for 9 of the 13 *C. glabrata* isolates and referred to as the "glabrata pattern," was a broad cluster with two major foci of relative growth points, one around $x = 20$ and $y = 70$ and the second around $x = 80$ and $y = 100$. For five isolates there were fairly even distributions of points at both foci, while for three isolates most points were clustered at the $x = 80$ and $y = 100$ focus, with points at the $x = 20$ and $y = 70$ position for just one or two isolates (Fig. 6d). For the remaining isolate most of the cluster points were arranged around the $x = 20$ and $y = 70$ focus with a single point around $x = 80$ and $y = 100$. The glabrata pattern indicated more variability in relative growth in the presence of itraconazole than in the presence of fluconazole. One *C. glabrata* isolate gave a single but significantly scattered cluster of relative growth points centered around $x = 80$ and $y = 100$, and one other, illustrated in Fig. 6c, showed the glabrata pattern, but with extra relative growth points extending to the bottom left (susceptible) region of the scatter plot.

The three significantly variable isolates of *C. krusei* all showed a similar "left variation" pattern, in which clusters based around a focus at approximately $x = 10$ and $y = 80$ were extended vertically around this point, with minimal variation in the horizontal direction (illustrated in Fig. 4a, patient I.17). This same type of cluster pattern was also seen for the significantly variable isolate of *C. inconspicua*, except that in this case the center of gravity was at $x = 11$ and $y = 33$. Such a pattern of variation indicated stable relative growth in the presence of itraconazole and variable relative growth in the presence of fluconazole.

Relationship between azole resistance, numbers of colonies tested, and the occurrence of the variant relative growth phenotypes. It is evident from comparison of Fig. 5 and 6 that the yeast isolates showing insignificant relative growth variation (Fig. 5) are all represented by fewer datum points than those that showed significant variation (Fig. 6). Moreover, we found no examples of nonvariable yeast isolates that had centers of gravity at the top right (resistant) extreme of the graphic (Fig. 5), yet datum points were almost always found in this region with significantly variable isolates (Fig. 6). There was an obvious association between the numbers of colonies tested, which depended on the numbers of different isolation routes that gave positive cultures, and statistically significant values of D^2. Among 36 yeast isolates, regardless of species, for which fewer than 24 colonies were tested, 8 (22%) showed significant relative growth variability. Among 33 isolates for which 24 to 47 colonies were tested, 16 (48%) showed relative growth variability. Among the 17 isolates for which 48 or more colonies were tested, all 17 (100%) showed significant variability.

The numbers of colonies tested for relative growth per isolate was in turn dependent on the intrinsic azole resistance of the isolate. When few or no colonies were obtained from mouthwash samples inoculated onto media containing fluconazole—a finding indicative of intrinsic fluconazole susceptibility for the isolate—then few subisolates were represented in clusters such as those illustrated in Fig. 5 and 6. By comparison, isolates that grew well when fluconazole was present in the isolation cultures provided a larger number of subisolates and, by definition, a larger number of colonies that were represented in the analysis of whole isolate variability. The symbols used in Fig. 6 to indicate routes of isolation of the yeasts were chosen so that the higher levels of fluconazole exposure appear as darker areas. From this it is apparent that the colonies exposed to those higher fluconazole levels on isolation from the oral samples generally tended to appear in the regions of the relative growth scatter plots indicating less susceptibility. In all cases, the 4 to 12 colonies of each subisolate tested represented an entirely random selection of all the colonies on each plate; in almost all of the cases in which relative growth variation was detected, isolates were cultured in scores to hundreds of colonies on each isolation plate.

Stability of relative growth phenotypes in subculture. The results described so far do not exclude the possibility of artifactual variation in relative growth phenotypes that was evident particularly in the yeast isolates that were recovered after exposure to high fluconazole concentrations in the isolation

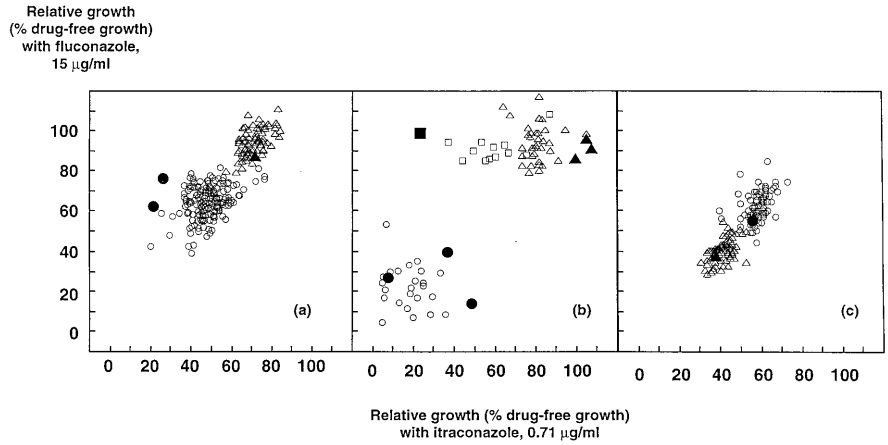

FIG. 7. Relative growth of colonies from subcultures of yeasts grown from mouthwash samples of patients with HIV infection. The relative growth of the original colonies is indicated by the larger filled symbols, and that of colonies from subcultures is indicated by the smaller open symbols. (a) original, first- and second-generation subculture colonies for two colonies from the *C. glabrata* isolate of patient 011 (cf. Fig. 6d). Circles indicate the parents and subcolonies of the two colonies well separated from all other parent colonies in Fig. 6d; triangles indicate one of the parents and two generations of subcultures from the predominant cluster in Fig. 6d. (b) Isolate of *C. glabrata* from patient 005. Filled circles indicate three colonies from the original isolation plates that lay to the bottom left of the scatter plot (cf. Fig. 6b) together with first-generation subcultures from these colonies. The filled and open squares indicate parents and subcultures for a colony that originally appeared at the top left of the scatter plot (Fig. 6b), and the filled and open triangles indicate parents and subcultures for three colonies that originally appeared at the top right of Fig. 6b. (c) Two colonies and their subculture progeny from the *C. albicans* isolated from patient 06 (cf. Fig. 6a).

media and which may have been only temporarily affected by such exposure. To investigate this possibility, subcultures of individual colonies from a selection of isolates with and without variable relative growth phenotypes were made by plating the colonies on CAC. Eight to 12 single colonies of these first-generation subcultures were then tested for relative growth in the presence of fluconazole and itraconazole, and the results were compared with those obtained for the parent colony. A small selection of colonies from highly variable isolates was taken to the second-generation level by subculturing the colony via all possible permutations of direct CAC plating and via YNB enrichment broths with and without fluconazole (10 and 100 μg/ml) and itraconazole (1.0 and 10 μg/ml). From these second-generation plates 80 to 100 individual colonies (8 to 12 per plate) were tested for relative growth in the presence of fluconazole and itraconazole.

In most cases colonies from first- and second-generation subcultures of individual colonies isolated from oral samples showed relative growth phenotypes close to those of the parent clones. In Fig. 7, three examples of scatter plots illustrate how data clusters from colonies in subcultures from highly variable isolates of *C. glabrata* (Fig. 7a and b) and *C. albicans* (Fig. 7c) showed little further variation and remained in almost the same relative growth positions as the parent colonies. This confirmed that the high level of variation in the isolation cultures (exemplified in Fig. 6 and detailed in Table 3) was a real and not an artifactual phenomenon related to the initial isolation process. From six highly variable *C. albicans* isolates, a total of 35 first-generation subcultures were prepared, and only 8 (23%) of these now showed significant variation in relative growth. Four of the first-generation isolates were taken to second-generation subcultures, and only two (both from the same original isolate) showed statistically significant variation in the relative growth clusters, even though these were generated from at least 80 individual colonies from the many subcultures with and without exposure to itraconazole and fluconazole. Figure 7c presents the results for one isolate for which variation reached the level of statistical significance in second-generation subcultures, yet visually the degree of variation, despite the large number of datum points, was less than that for the original isolate (Fig. 6a). A similar reduction in variability in subcultures was also apparent for *C. glabrata* isolates. From seven isolates, all highly variable at the time of isolation, 53 first-generation subcultures were prepared, and significant variability was seen for only 8 (15%) of these. Seven colonies from first-generation subcultures were taken to the level of the second generation in subcultures with all permutations of broth and agar media as described above. Four of the seven colonies showed statistically significant variability, but again, this was reduced in extent compared with the original isolations (see, e.g., Fig. 7a and b).

DNA fingerprints of variable and nonvariable isolates of *C. albicans*. To investigate whether relative growth variability correlated with changes apparent at the DNA level, DNA from five *C. albicans* isolates was fingerprinted by Southern blotting and hybridization with the moderately repetitive sequence Ca3. For each isolate two or three clones were selected on the basis of their relative growth phenotypes, and two colonies from first-generation subcultures of each clone were fingerprinted to check the reproducibility and stability of the fingerprints from each clone. (These tests could be applied only to *C. albicans* isolates since similar probes for other *Candida* spp. were not available.)

The results are presented in Fig. 8, in which the computer program Dendron was used to build neighbored images of digitized band patterns from different experiments adjusted to the same length by reference to a global standard band pattern (first lane of Fig. 8). For all isolates there was no difference between the band patterns of paired colonies taken from the same subculture. For three isolates the parent clones had shown different relative growth phenotypes, and for these three isolates considerable differences in the DNA fingerprints of the clones were found (Fig. 8). Comparison of the fingerprints for colonies 4 and 11 from patient 07 shows band switching at about 6.5, 4.1, 3.0, and 2.25 kb. Colonies 1 and 3 from patient III.03 showed changes above 7.9 kb and at about 6.6, 3.3, 3.2, and 2.3 kb. The three colonies from patient 06 showed DNA band pattern differences above 7.9 kb and at about 5.4

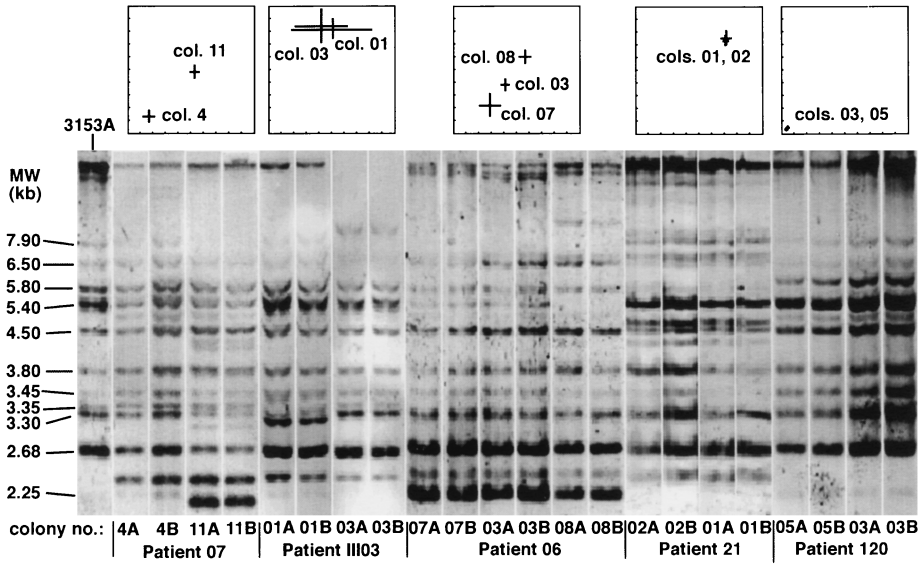

FIG. 8. DNA fingerprints obtained by Southern blotting with the probe Ca3 from different colonies of *C. albicans* isolated from the same oral sample. Band positions and intensities were digitized, reconstructed, and neighbored with the aid of the computer program Dendron by reference to a global standard band pattern obtained from the reference strain, *C. albicans* 3153A (leftmost lane). For each parent colony, two colonies from a first-generation subculture (designated with the suffixes A and B) were used as sources of DNA to control for internal reproducibility of the method. Each pair of lanes, starting from the second lane from the left, therefore represents duplicate DNA preparations from the same parent isolate. The patient reference numbers and the colony numbers of the samples tested are presented below the appropriate lanes. Above the lanes the relative growth phenotype of the colony is indicated by a miniature drawing of the original scatter plot. The vertical and horizontal lines drawn for each colony indicate the standard deviation for the relative growth of eight first-generation colonies obtained from the designated parent colony, and the point of intersection indicates the center of gravity for the cluster.

kb. By contrast, the fingerprints of isolates from patients 21 and 120, in which the parent clones had the same relative growth phenotype, showed far less variation: a difference in band intensity was apparent only at about 3.45 kb for colonies 1 and 2 from patient 21.

Similarity coefficients (S_{AB}s) (18) calculated by the Dendron program for the colonies illustrated in Fig. 8 showed close similarity (S_{AB}s, ≥ 0.9) for all replicate pairs of each DNA preparation and S_{AB}s of < 0.8 between the fingerprints for isolates from each of the five patients. For the different colonies from each single patient, S_{AB} values indicated considerable dissimilarity for patient 07 (S_{AB}, 0.72) and patient III.03 (S_{AB}, 0.53) and less dissimilarity between the different colonies from each of the other three patients (S_{AB}s, ≥ 0.81).

IC_{50}s and percent relative growth. The sometimes extreme differences in relative growth phenotype exhibited by isolates of *Candida* spp. were seldom reflected by differences in the IC_{50}s of fluconazole and itraconazole. Table 4 compares relative growth data with the IC_{50}s of the two agents in broth microdilution tests with RPMI 1640–2% glucose medium—derived from the reference method of the National Committee for Clinical Laboratory Standards (6)—and CYG medium (as used in the relative growth tests). In most cases the results obtained with the two media showed good agreement (Table 4). *C. albicans* isolated from patient 06 was the outstanding exception. Despite the considerable differences in relative growth data for different colonies of each isolate, the IC_{50}s of itraconazole and fluconazole were consistent within a 2-dilution range of variation for each isolate, with only occasional exceptions (Table 4).

Scrutiny of the dose-response curves for representative isolates (Fig. 9) indicates how the differences between relative growth results and the similarities between IC_{50}s arose. For the two colonies from the *C. glabrata* isolate from patient 066 (Fig. 9a and b) the relative growth data differed considerably for both fluconazole and itraconazole, and the concentrations of these agents at which growth was inhibited below 50% of the control growth differed by 3 or 4 dilution steps. However, with the *C. albicans* isolate from patient 120 (Fig. 9c and d) similar evident differences in the dose-response curves and hence the relative growth values were not reflected in substantial differences in IC_{50}s since growth was reduced below 50% of the control growth at similar concentrations: the extent to which growth was reduced once the 50% level was reached varied considerably. Growth of *C. albicans* colony 1.02 (Fig. 9) was not reduced to the baseline level at any concentration of fluconazole or itraconazole tested. However, amphotericin B and flucytosine tested in parallel with the azole antifungal agents did reduce growth to the baseline level, confirming that the result with itraconazole and fluconazole was not an artifact derived from, for example, inappropriate background correction of the OD readings. The CYG medium used for relative growth tests gave rise to more frequent trailing growth of the type exemplified in Fig. 9c and d than did RPMI 1640–2% glucose (data not shown).

Correlations between high relative growth variability and clinical status. The occurrence of high relative growth variability in any species isolated from a mouthwash sample was compared with epidemiologic factors recorded for the patient from whom the sample was obtained. No significant association was found between the prevalence of variable yeast isolates and the sex, age, smoking habits, CD4 lymphocyte count, geographical origin, presence of oral symptoms, or recent antifungal agent use among the patients (data not shown).

DISCUSSION

It is customary in routine medical microbiology practice to regard any colony of a single microbial species obtained from a single sample as representative of all colonies of that species

TABLE 4. IC_{50}s of fluconazole and itraconazole in two broth media for highly variant isolates of *C. albicans* and *C. glabrata*

Species	Patient no.	Colony no.	% Relative growth in the presence of the following:		IC_{50} (μg/ml) for isolates grown in RPMI 1640 and the following:		IC_{50} (μg/ml) for isolates grown in CYG medium and the following:	
			Fluconazole	Itraconazole	Fluconazole	Itraconazole	Fluconazole	Itraconazole
C. albicans	06	1″.08	65	50	0.25	≤0.016	>64	>16
		1.04	27	29	0.25	≤0.016	>64	>16
		3′.03	36	37	0.25	<0.016	>64	>16
C. albicans	120	1.02	37	42	0.5	0.25	1	≤0.016
		1.03	5	5	0.25	≤0.016	0.5	≤0.016
		1.05	4	6	4	≤0.016	0.25	≤0.016
C. albicans	128	2.08	23	21	0.063	0.063	2	≤0.016
		3.03	23	20	0.063	0.063	1	0.063
		4.04	44	45	0.063	≤0.016	32	8
C. albicans	III.03	1.05	81	12	32	0.25	64	0.5
		2″.08	90	15	32	0.5	64	0.5
		2°.02	90	81	32	0.25	64	0.5
C. glabrata	005	1″.02	28	11	>64	8	64	0.5
		3.01	93	74	>64	>16	>64	2
C. glabrata	008	1.06	68	35	>64	>16	64	1
		3.01	84	82	>64	>16	>64	>16
C. glabrata	013	1″.01	92	54	>64	4	>64	2
		1″.02	50	37	32	2	64	2
		2.05	52	31	>64	4	64	1
C. glabrata	066	1′.03	46	34	16	1	16	0.5
		2″.01	94	67	>64	4	>64	2
		3′.03	99	96	>64	2	>64	8

isolated from that sample, i.e., that the colonies are derived from a clonal population. This study has shown that such an assumption is not always justified when *Candida* spp. are isolated, since some isolates show a tendency toward variability, as evidenced by their relative growth phenotypes in tests with single concentrations of itraconazole and fluconazole. High degrees of variability in relative growth phenotype on any single isolation plate were encountered only in a minority of instances when an oral sample was plated on agar with or without the addition of fluconazole; however, passage of a sample through a broth medium containing fluconazole at 10 or 100 μg/ml greatly increased the variability of the relative growth phenotypes on each isolation plate (Table 2).

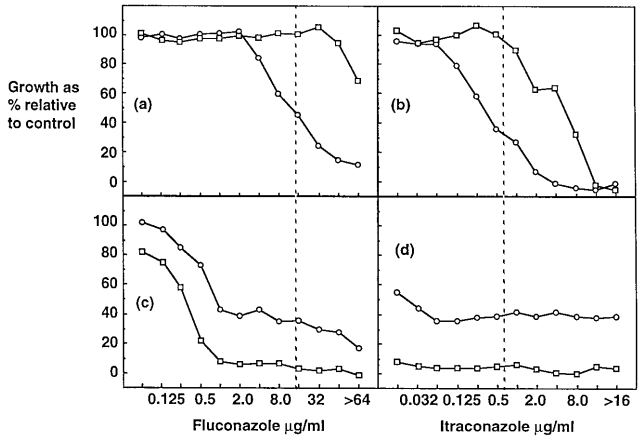

FIG. 9. Examples of dose-response curves for fluconazole and itraconazole against isolates of *C. albicans* and *C. glabrata* with highly variable relative growth phenotypes tested in CYG medium. The vertical dashed lines indicate the concentrations of fluconazole and itraconazole used in relative growth tests. (a and b) *C. glabrata* colonies 1′.03 (circles) and 3′.03 (squares) from patient 066; (c and d) *C. albicans* colonies 1.02 (circles) and 1.05 (squares) from patient 120.

When relative growth data for colonies representing all the different isolation routes used to culture an individual species from a mouthwash sample were combined, the prevalence of high variability within the putative single isolate was extremely high: 100% for *C. glabrata* isolates, 60% for *C. krusei* isolates, and 40% for *C. albicans* isolates (Table 3). We therefore hypothesize that the population of yeasts in the mouths of many of the patients sampled was polyclonal and that the use of different isolation media, particularly the inclusion and noninclusion of an antifungal agent, enhanced the selection of colonies with different susceptibility phenotypes. It is also possible that the stress of transfer of yeasts from a human host to a laboratory culture environment, further augmented by exposure to antifungal agents, induces changes in some of the cell populations that appear as relative growth variability. However, the fact that most phenotypic variability was seen between colonies that had been isolated indirectly after passage in fluconazole-containing broths rather than between isolates obtained by direct plating in agar cultures argues more for a process of selection of existing yeast subpopulations by enrichment rather than for adaptive changes in cells within the original yeast population in the clinical sample.

When variability is the quantity being measured, it is impor-

tant that the measurement system be as precise as possible. In the present case, relative growth measurement lacks ideally high precision; however, the evidence presented nevertheless supports our conclusion that some isolates of *Candida* spp. are indeed able to show high variability in their susceptibilities to antifungal agents. The positions of individual colonies in relative growth scatter plots were generally retained through one and two subcultures, even though a battery of subculture media, solid and liquid and with and without the addition of fluconazole and itraconazole, was used for second-generation subcultures (Fig. 7). Similarly, eightfold replica testing of individual colonies (Fig. 2) never revealed a level of relative growth variability of the order seen with highly variable isolates and subisolates (Fig. 4 and 6). Moreover, the patterns of variation in relative growth exhibited a species-related trend, with predominantly diagonal and top variations in *C. albicans*, glabrata-type variation in *C. glabrata*, and left variation in *C. krusei*, as described in the Results. These observations reinforce the conclusion that the variations described were real and reproducible, within the limits of the test; they tended to show species specificity and were not random and possibly artifactual variations.

Variation in antifungal susceptibility within individual *Candida* isolates has been previously described in other publications. Dellion et al. (4) reported mixtures of susceptible and resistant *C. albicans* colonies in single isolations, although they did not state the extent of susceptibility differences that they observed. Johnson et al. (7) measured fluconazole MICs for 15 *C. albicans* colonies isolated from HIV-positive subjects; in one instance they found a 32-fold range of variation in MICs and concluded that a sweep inoculum of yeasts was preferable to random selection of a single colony to ensure that resistant colonies of an isolate would not be missed. We endorse that recommendation. Cartledge et al. (2) evaluated the fluconazole and itraconazole susceptibilities of *Candida* spp. from oral samples by the same relative growth method used in our study. They acknowledged the possibility of relative growth variations between colonies and described 2 *C. albicans* isolates (of 104 isolates) whose colonies gave widely different relative growth results. They had therefore presumably encountered the same variability as we now describe.

Le Guennec et al. (9) also found intercolony variations in *C. albicans* isolates from mouthwashes taken from AIDS patients. They also used multilocus enzyme electrophoresis to characterize the genetic diversity of the isolates and found small variations between colonies isolated from the same sample. Our present findings, particularly the DNA fingerprints (Fig. 8), agree with those of Le Guennec et al. (9), even though the methods used in the two studies were entirely different. Moreover, our results with the susceptibility phenotypes further suggest that nonclonal populations of yeasts may be found in oral samples even for species other than *C. albicans.*

Our results with IC_{50} measurements (Table 4) indicate that MICs may sometimes underestimate the extent of intraisolate variability. The implications of our findings for routine susceptibility testing with azole antifungal agents are not entirely clear, since currently recommended methods have been developed with minimization of variation as a high-priority requirement. Indeed, we chose the IC_{50} determination rather than the IC_{80} endpoint recommended by the National Committee for Clinical Laboratory Standards (6) to minimize variability in the results obtained in repeat tests with the same isolates (15). Use of inocula from multiple colonies of an isolate (6, 7) can also be expected to minimize the appearance of variability in azole MICs. The present study suggests that the source of variability in susceptibility to azole antifungal agents may partly be from the behavior of the yeasts themselves, in addition to any experimental sources of error. Our relative growth method has been shown to provide information that correlates with the clinical outcome of treatment with azole antifungal agents (2, 14). It makes practical the testing of large numbers of colonies from single clinical isolates of yeasts and it appears to enhance detection of the inherent variability in antifungal susceptibility of an isolate. We suggest that our findings merit corroboration and extension by others to determine the clinical and epidemiologic significance of the variability phenomenon.

The molecular basis of relative growth variability is unknown. Our DNA fingerprints with variable *C. albicans* isolates indicate easily detected changes in the DNA patterns associated with changes in the relative growth phenotype. We were unable to extend these observations to other *Candida* spp., but the results for *C. albicans* alone (Fig. 8) indicate that detectable genetic differences accompany the phenomenon, although these reach the level of low S_{AB} values only in a minority of cases. One possible explanation for the variability would be that the isolates undergo "phenotypic switching," which has been extensively investigated by Soll (21, 22). Soll lists variations in antifungal susceptibility as one consequence of switching (21, 22), but this aspect has not been described in detail. The extent of DNA changes seen in Fig. 8 is greater than has previously been associated with the phenotypic switching phenomenon. Indeed, studies of the microevolution of *C. albicans* isolates in longitudinal studies of *Candida*-positive individuals showed DNA fingerprint changes that were barely detectable or that were undetectable at the level of hybridization with the Ca3 probe (10, 20). The relative phenotypic stability of our subcultures also argues against a switching type of mechanism, since we would expect this to appear in relative growth scatter plots as a bidirectional shuttling of datum points around two or more foci instead of a homogeneous single cluster. We did in fact note such an apparent phenomenon for just one isolate of *C. albicans*, but that was the only example that ever suggested a process of possible phenotypic switching.

The phenomena that we have described in some ways resemble the process called "adaptive resistance" in *Pseudomonas aeruginosa* (3, 8). When populations of this bacterium are briefly exposed to aminoglycoside antibiotics they acquire transient resistance to the antibiotic that is lost with subculture on antibiotic-free medium. A similar but much slower effect has also been noted in a case of urinary tract infection caused by *C. glabrata* in which an isolate with clinically induced resistance to fluconazole reverted to susceptibility after repeated subculture on fluconazole-free medium (23). This type of variation, however, suggests susceptibility changes induced by exposure to an inhibitory agent that revert when the agent is removed. We feel that our results make it more likely that the variability that we have demonstrated mainly reflects selection of existing differences in the original yeast population since further changes were not seen in subcultures.

Whatever the mechanism underlying the variability in relative growth in the presence of fluconazole and itraconazole that we have found in this study, it is clear that the combined stresses of transfer from a host environment to a laboratory medium and exposure to an antifungal agent in the medium are the factors leading to the detectability of the variable phenotypes. Our experiments were done with fluconazole additions to the isolation media. We chose this particular agent because it had been used before (11) and since (16) we began our study as an agent whose addition to isolation media allowed for the detection of azole-resistant yeasts in clinical material. We did not expect to encounter relative growth variability, and our use of itraconazole in isolation media was

therefore begun too late to provide sufficient results for evaluation of the potential of this azole antifungal agent to provoke the variability phenomenon. If, as we suspect, the *Candida* populations infecting the mouths of HIV-positive individuals contain isolates with mixtures of azole susceptibility phenotypes, then it is likely that any azole antifungal agent included in an isolation medium will select out population differences that can be revealed in the form of relative growth variation. Since events such as phenotypic switching and induction of adaptive resistance are also known for some *Candida* isolates, then variations in susceptibility might be an extremely complex phenomenon to be investigated and resolved experimentally.

Although we found no association between the occurrence of variation and the clinical or social parameters of the patients studied, we consider a prospective study of the variability phenomenon to be necessary before any firm conclusions can be drawn concerning its clinical relevance. When a yeast is isolated in the manner used in our present study, it is exposed to a different chemical environment (the growth medium) and to an antifungal agent. It is apparent from our results that the antifungal agent played an important role in causing the yeasts to display variable relative growth phenotypes, and it might be expected that exposure to such agents in vivo might similarly lead to changes in the susceptibility of the population.

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