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Switching of *Candida albicans* during Successive Episodes of Recurrent Vaginitis

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Strain relatedness and switching were monitored in *Candida albicans* strains isolated from different body locations through three episodes of recurrent vulvovaginal candidiasis separated by two treatment-latency periods in a single patient. Strain relatedness was assessed by comparing Southern blot hybridization patterns with the relatively immobile mid-repeat sequence Ca3. The following conclusions are demonstrated. (i) Three different strains of *C. albicans* colonized the mouth, the area under the breasts, and the vulvovaginal, anal, and rectal regions, respectively, at the time of the first infection. (ii) The same strain of *C. albicans* was responsible for the three vaginal infections. (iii) Switching of colony phenotype occurred with each new vaginal infection. (iv) Enrichment of drug-resistant switch phenotypes (assessed in vitro) was unlikely the basis for the changes in the switch phenotypes of the strain found in the vulvovaginal, anal, and rectal areas after treatment of the first infection with clotrimazole. (v) The same strain of *C. albicans* was responsible for the recurrent increases in mouth colonization and was distinct from the recurrent vaginal strain. The results of this case study demonstrate the need for further detailed analyses of full-body mycofloras, strain relatedness, switching repertoires, and changes in drug susceptibility during successive episodes of recurrent vulvovaginal candidiasis.

Recurrence of vaginal candidiasis after termination of treatment in women with no apparent predisposing condition has been reported to be as high as 30% within 60 days (3). Although it seems likely that women suffering from frequent episodes of vaginal candidiasis may share unidentified predisposing conditions (18, 19), it is also possible that infecting strains of *Candida albicans* may share pathogenic characteristics. Surprisingly, very little is known about the epidemiology and pathogenicity of strains involved in recurrent vaginal candidiasis. Indeed, several fundamental questions pertaining to the infecting yeast in recurrent vaginitis remain unanswered, including (i) the source of the reinfecting strain, (ii) the relatedness of the reinfecting strain to strains in other body locations, (iii) the relatedness of strains in sequential episodes, (iv) the susceptibility of recurrent strains to the antifungal drugs used in therapy, and (v) the involvement of high-frequency switching (1, 13, 16, 17, 21, 21a, 22, 24) in recurrence. With the development of rapid methods for testing strain relatedness by Southern blot hybridization to species-specific mid-repeat genomic sequences (14, 22, 24; J. B. Hicks, M. J. McEachern, E. P. Rustchenko-Bulgac, J. Schmid, and D. R. Soll, submitted for publication) and the capacity to assess high-frequency switching at the site of infection (22, 24), several of these questions can now be explored.

Here, we present the results of a case study of a patient with recurrent yeast vaginitis. During three consecutive episodes of vaginal candidiasis, separated by two treatment-latency interphases, we monitored colonization in 12 to 18 body locations. It is demonstrated that the strain of *C. albicans* which continuously colonized the mouth differed from the strain which continuously colonized the vaginal and anal areas and that the same strain of *C. albicans* was responsible for the three episodes of yeast vaginitis. This strain switched colony phenotype between episodes. Surprisingly, the new switch phenotype which predominated after the first drug treatment did not exhibit increased resistance to that drug, suggesting that drug therapy accounted for selective pressure, but that some pathogenic characteristic other than drug resistance (measured in vitro) led to enrichment of the new switch phenotype.

MATERIALS AND METHODS

Isolation of *C. albicans* from body locations. Swabs of all body locations but deep rectum (stool) were obtained by rubbing a wet Culturette (C8852-1; American Scientific Products, McGaw Park, Ill.) several times across the sample area. For deep rectum, a fecal sample was removed on the tip of a gloved finger and the sample was transferred to the Culturette. Each Culturette tip was swirled rigorously in 1.0 ml of double-distilled water, and 0.1 ml was plated on each of five culture plates (10-cm-diameter petri dishes) containing *Candida* agar (amino acid-rich medium of Lee et al. [7] supplemented with 70 μg of arginine per ml, 9 μM zinc [2], and 2% agar). Cultures were incubated for 7 to 9 days at 25°C prior to assessment of colony number and morphology. Colony number is presented as the sum for five plates.

Characterization of switching. Cells from select colonies of primary cultures were replated on fresh *Candida* agar plates at a density of roughly 40 colonies per plate. The frequencies of variant phenotypes and types were scored, and the cells were photographed through a Wild stereo dissection microscope (Wild Heerbrugg Ltd., Heerbrugg, Switzerland).

Species typing. All isolates were initially typed by their sugar assimilation pattern with the commercial kit (6) purchased from API Analytab Products (Plainview, N.Y.). All yeast isolates were typed as *C. albicans*, and this was confirmed by hybridization to the mid-repeat sequence Ca3 (22, 24; Hicks et al., submitted).

Southern blot hybridization. To assess strain relatedness,

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TABLE 1. History of colonization during recurrent infections

Body location sampled	No. of colonies (clone)[a]				
	Day 0 (clotrimazole) (vaginal infection 1)	Day 29 (interphase)	Day 87 (butoconazole) (vaginal infection 2)	Day 101 (interphase)	Day 119 (vaginal infection 3)
Cheek	0	1 (Mo11)	5 (Mo15)	0	16 (Mo25)
Saliva	1	4 (Mo10)	1 (Mo17)		9 (Mo28)
Back of tongue	93 (Mo5)	6 (Mo12)	12 (Mo18)	2 (Mo22)	11 (Mo24)
Nasal canal		0	0	0	
Ear canal		0	0	0	
Armpit	0	0	0	0	
Under breasts	3 (Mo1)	0	0	0	
Nipple		0	0	0	
Navel			0	0	
Groin	13 (Mo6)	1 (Mo13)	1 (Mo16)		0
Vulvar region	678 (Mo4)	0	474 (Mo21)	0	2,000 (Mo26)
Vaginal wall	2,870 (Mo3)	0	786 (Mo19)	0	3,000 (Mo29)
Vaginal pool[b]	7,460 (Mo2)	0	376 (Mo20)	0	
Anus	46 (Mo8)	0	62 (Mo14)	0	13 (Mo23)
Deep rectum (stool)	16 (Mo7)	0	0	0	2 (Mo27)
Back of knee		0	0	0	
Between toes	1 (Mo9)		0	0	

[a] The number of colonies obtained by plating original samples on five agar plates (see Materials and Methods) is presented for each body location. The clone of each original sample which was used for further experimentation is also shown. Clotrimazole treatment was initiated on day zero, and butoconazole treatment was initiated on day 87. In each case, the drug was administered for 7 days.

[b] Observation of yeast cells in the vaginal canal in wet mounts obtained from the vaginal pool at the time of examination yielded the following results: yeast cells were present at day 0, absent at day 29, present at day 87, absent at day 101, and present at day 119.

Southern blot hybridization of total cellular DNA was performed with the *C. albicans*-specific mid-repeat sequence Ca3 (22, 24; Hicks et al., submitted). DNA was isolated from a particular clone by a modification (22) of the method of Cryer et al. (4). In brief, deproteinized, spooled DNA was dissolved in water and digested with *Eco*RI enzyme (New England BioLabs, Inc., Beverly, Mass.). Fragments were separated on a 0.7% agarose gel and transferred to GeneScreen Plus (Dupont, NEN Research Products, Boston, Mass.). Southern blot hybridization using nick-translated Ca3 (12) was performed according to procedures previously described (22).

Testing strains for susceptibility to common antifungal agents. A clone of each isolate was grown in the amino acid-rich medium of Lee et al. (7) supplemented with 70 μg of arginine per ml and 9 μM zinc (2) to mid-log phase. A 0.01-ml sample of cells (~10^5 cells) was inoculated onto each of a series of wells in a microtiter plate. Each well contained 0.25 ml of *Candida* agar plus an increasing concentration of each antifungal agent. Clotrimazole was tested in the range of 0.125 to 2.25 μg/ml in increments of 0.125 μg/ml (a total of 18 wells). Butoconazole was tested in the range of 1.0 to 25 μg/ml in increments of 2 μg/ml (a total of 13 wells). Both drugs were dissolved in dimethylformamide before addition to agar. Controls with dimethylformamide lacking drugs were performed; dimethylformamide alone had no effect on growth. Growth inhibition was assessed after 5 days of incubation at 25°C. The concentrations causing roughly a 50 and 100% inhibition of growth were scored. Each experiment was performed three times, and the means and standard deviations were calculated.

RESULTS

Dynamics of infection and colonization. The history of infection and colonization is presented in Table 1. At the time of the first infection, extreme *Candida* colonization was evident in the vulvar and vaginal areas, heavy colonization was evident in the anal region, and low colonization was evident in the rectal area, mouth, and area under the breasts. The patient was treated with clotrimazole cream for 7 days. At 22 days after termination of treatment, the vaginal and anal areas were free of yeast cells and only low colonization was evident in the mouth. At the time of the second infection (87 days after the first infection), extreme colonization was again evident in the vulvar and vaginal areas, heavy colonization was evident in the anal area, and low colonization was evident in the mouth. The patient was then treated with butoconazole cream for 7 days. At 7 days after termination of treatment, only low colonization was evident in the mouth. At the time of the third infection (32 days after the second infection), heavy colonization was again evident in the vulvar and vaginal areas, and low colonization was evident in the anal, rectal, and mouth areas.

Colony morphologies and switching repertoires of isolated strains. At the time of the first infection, the major colony phenotypes differed between the isolates from (i) the vulva, vagina, anus, and rectum, (ii) the mouth, and (iii) the area under the breasts. Cells from the vulvar, vaginal, anal, and rectal isolates (Mo7, Mo8, Mo6, Mo3, Mo2, and Mo4) generated a medium-sized smooth white colony, with little or no hyphae penetrating the agar peripheral to the colony (Fig. 1A) even after 9 days of incubation. Anal and rectal isolates were contaminated with bacteria and appeared slightly smaller than the vulvar and vaginal isolates on first and second platings (Fig. 1B), but when plated independently of bacteria, they were identical. Cells from the mouth isolate (Mo5) generated a medium-sized, smooth white colony which exhibited peripheral mycelia in the agar bordering the colony after 7 to 9 days of incubation (Fig. 1C). Cells from the isolate from under the breasts (Mo1) generated a medium-sized smooth white colony with no peripheral mycelia, which was just slightly larger than the vulvar, vaginal, anal, and rectal isolates (Fig. 1D). All of the isolates switched at relatively high frequencies to variant colony

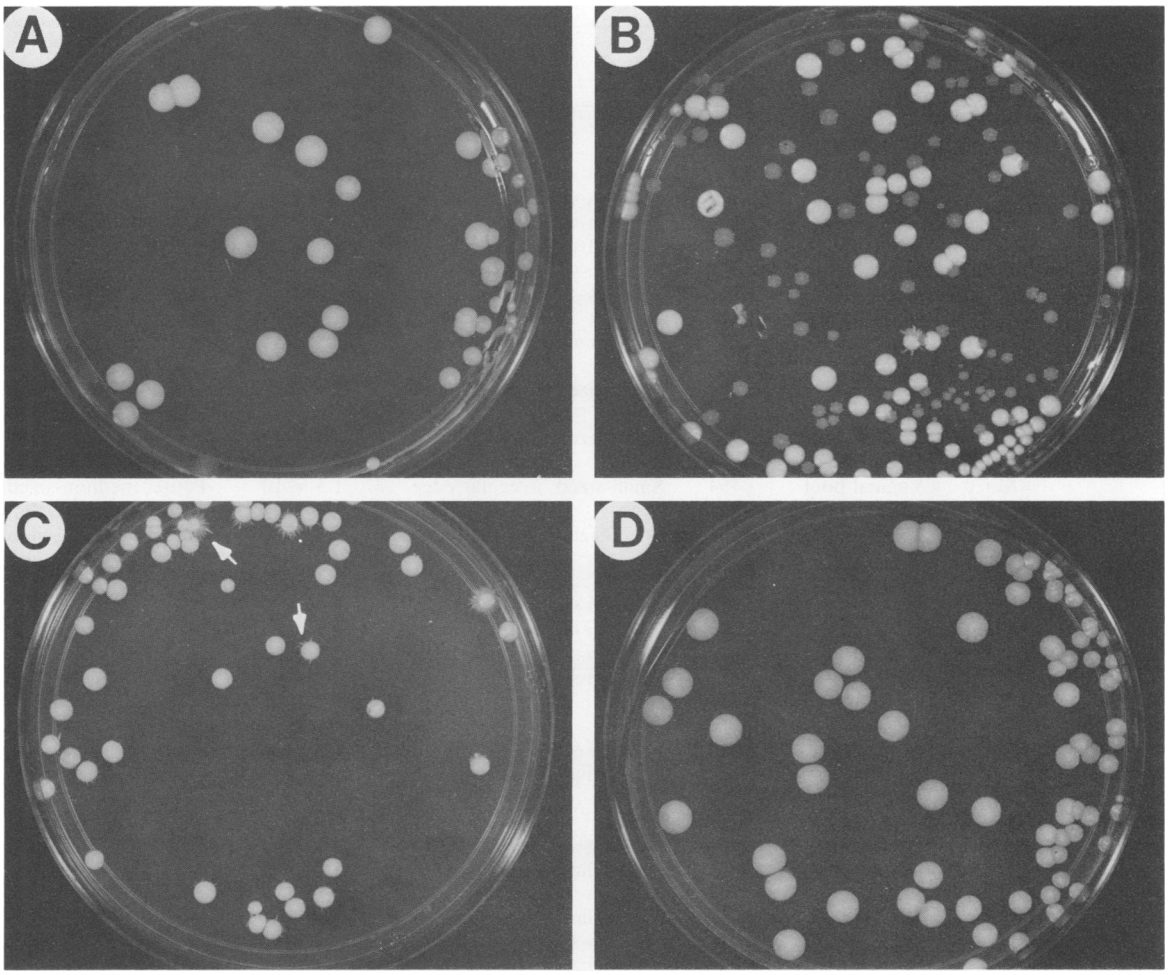

FIG. 1. Representative colony phenotypes of isolates at time of first infection. (A) Vaginal wall (Mo3 origin); (B) deep rectum (stool) (Mo7 origin); (C) back of the tongue (Mo5 origin); (D) under the breasts (Mo1 origin). Note that the colonies from the back of the tongue are beginning to exhibit mycelial periphery in the agar bordering the colony (examples noted by arrows). Pictures are from the second plating.

morphologies and exhibited roughly similar repertoires of variant colony phenotypes (Table 2). Examples of the switching repertoires of the vulvar, vaginal, anal, and rectal strains are presented in Fig. 2A to D; examples of the mouth strain are presented in Fig. 2E to H; and examples of the strain found under the breasts are presented in Fig. 2I and L. All isolates typed as *C. albicans* by sugar assimilation patterns.

Demonstration of at least three infecting strains by Southern blot hybridization with the mid-repeat sequence Ca3. To assess the relatedness between the various isolates at the time of the first infection, Southern blot hybridization patterns of *Eco*RI digests of total cellular DNA probed with the cloned mid-repeat sequence Ca3 were compared. Ca3 represents a cloned *Eco*RI fragment which hybridized to at least 15 fragments of *Eco*RI-digested genomic *C. albicans* DNA but which exhibited little or no hybridization to the genomic DNA of *Candida tropicalis* (see Fig. 4, lane 10), *Candida krusei*, and *Torulopsis glabrata* (24; Hicks et al., submitted). Intensity varied between bands, presumably as a result of differences in copy number in the genome. The probe has been demonstrated to be relatively stable within each strain of *C. albicans* examined, generating the same Southern blot hybridization pattern in subclones separated by more than 100 combined generations in strain 3153A and in strain B9 (WO-1) (Hicks et al., submitted) and in subclones of a single strain exhibiting different switch phenotypes at the same site of infection (22). However, hybridization patterns do differ between strains, making Ca3 a useful probe for assessing strain relatedness. The patterns for laboratory strain 3153A and the systemic isolate WO-1 differ in both the positions of bands and the number of genomic repeats (Hicks et al., submitted; also, see Fig. 4, lanes 11 and 12, respectively). It has also been demonstrated that the patterns of only two of nine independently isolated vaginal strains were similar, but when analyzed in parallel, lanes exhibited a single band difference (22). The Southern blot hybridization patterns of *Eco*RI-digested DNA of 10 commensal strains, each isolated from the mouth of a different healthy individual, were compared. All patterns differed (Fig. 3). In an analysis of seven isolates from systemic infections, all patterns again differed (J. Schmid and D. R. Soll, unpublished observations). Therefore, if the Ca3 Southern blot hybridization patterns of two isolates exhibit significant differences, one can be reasonably sure that the two strains are unrelated. If we assume that there are at least 10 strains equally distributed in nature which are distinguishable by different hybridization patterns, then the probability of isolating the same

TABLE 2. Switching frequencies and phenotypes of isolates taken during the three infections

General body location and infection no.	Clonal isolate[a]	Body location sampled	No. of colonies[b]	Predominant phenotype	Variant frequency	Variant phenotypes
Vulva, vagina, anal, and rectal area						
1	Mo7	Anus	1,899	Medium-sized, smooth white	2.1×10^{-3}	Petite
	Mo8	Rectum	6,959	Medium-sized, smooth white	2.7×10^{-3}	Petite; ultrapetite; large-sized
	Mo6	Groin	834	Medium-sized, smooth white	1.4×10^{-2}	Petite; ultrapetite
	Mo3	Vaginal wall	4,324	Medium-sized, smooth white	2.6×10^{-2}	Petite; ultrapetite
	Mo2	Vaginal pool	1,717	Medium-sized, smooth white	3.4×10^{-2}	Petite
	Mo4	Vulva	5,413	Medium-sized, smooth white	1.0×10^{-2}	Petite; ultrapetite
2	Mo14	Anus	678	Small-sized, irregular edge	7.5×10^{-2}	Medium-sized, smooth white; petite; ultrapetite
	Mo16	Groin	641	Small-sized, irregular edge	1.1×10^{-1}	Medium-sized, smooth white; petite; ultrapetite
	Mo19	Vaginal wall	764	Small-sized, irregular edge	1.3×10^{-1}	Petite; medium-sized, smooth white; ultrapetite; star
	Mo20	Vaginal pool	794	Small-sized, irregular edge	1.5×10^{-1}	Petite; medium-sized, smooth white; ultrapetite; star
	Mo21	Vulva	753	Small-sized, irregular edge	1.2×10^{-1}	Medium-sized, smooth white; petite; ultrapetite; star
3	Mo23	Anus	5,804	Medium-sized, smooth white	7.9×10^{-3}	Petite; ultrapetite
	Mo27	Rectum	5,674	Medium-sized, smooth white	4.9×10^{-3}	Petite; ultrapetite
	Mo29	Vaginal wall	2,888	Medium-sized, smooth white	2.8×10^{-3}	Petite; ultrapetite
	Mo26	Vulva	3,958	Medium-sized, smooth white	3.3×10^{-3}	Petite; ultrapetite
Mouth						
1	Mo5	Back of tongue	4,453	Medium-sized, smooth white, myceliated periphery	1.5×10^{-2}	Petite; irregular wrinkled; ultrapetite; large-sized; small star
2	Mo18	Back of tongue	794	Medium-sized, smooth white, myceliated periphery	1.3×10^{-3}	Petite
	Mo15	Cheek	789	Medium-sized, smooth white, myceliated periphery	2.5×10^{-3}	Petite
	Mo17	Saliva	776	Medium-sized, smooth white, myceliated periphery	1.3×10^{-3}	Petite
3	Mo24	Back of tongue	6,394	Medium-sized, smooth white, myceliated periphery	7.0×10^{-3}	Petite; ultrapetite; large-sized
	Mo25	Cheek	3,753	Medium-sized, smooth white, myceliated periphery	4.0×10^{-3}	Petite; ultrapetite
	Mo28	Saliva	5,942	Medium-sized, smooth white, myceliated periphery	1.3×10^{-2}	Petite; ultrapetite
Under breasts, 1	Mo1	Under breasts	1,401	Medium-sized, smooth white	1.3×10^{-2}	Petite; star; mycelial burst

[a] Clonal isolate is a representative strain cloned from the original isolate and used for analyses of switching.
[b] Number of colonies plated from the clonal isolate for the assessment of switching frequency.

strain twice in a row under nonselective conditions is 0.1×0.1, or 0.01, and the probability of isolating it three times in a row is $0.1 \times 0.1 \times 0.1$, or 0.001. Therefore, if two isolates cloned from different body locations at the same time or from the same body location at different times exhibit the same pattern, it is highly likely that they represent the same infecting strain.

Southern blot hybridization patterns with Ca3 are presented for *Eco*RI-digested DNA of isolate Mo1 from under the breasts (Fig. 4, lane 1), isolate Mo5 from the back of the tongue (lane 5), vulvar and vaginal isolates Mo2, Mo3, and Mo4 (lanes 2, 3, and 4, respectively), groin isolate Mo6 (lane 6), rectal and anal isolates Mo7 and Mo8 (lanes 7 and 9, respectively), and isolate Mo9 from between toes (lane 8). Three distinct patterns were evident for the nine isolates: pattern 1, Mo1; pattern 2, Mo5; pattern 3, Mo3, Mo4, Mo6, Mo7, Mo8, and Mo9. These groupings suggest that at the time of the first vaginal infection, three distinct strains of *C. albicans* colonized the patient at separate body locations: one strain colonized the mouth, a second colonized under the breasts, and a third colonized the vulvar and vaginal regions, rectal, and anal regions, and between toes. The differentiation of strains according to the Southern blot hybridization patterns with Ca3 corresponded to the separation based on major colony phenotypes of the initial isolates.

New colony phenotype but same strain in the second vaginal infection. The patient was treated with vaginal application of clotrimazole for a 7-day period following presentation of the first infection and was examined 22 days after termination of treatment. At this time, the patient was asymptomatic. Low colonization was demonstrated in mouth samples and one colony was obtained from the groin sample (Table 1). No

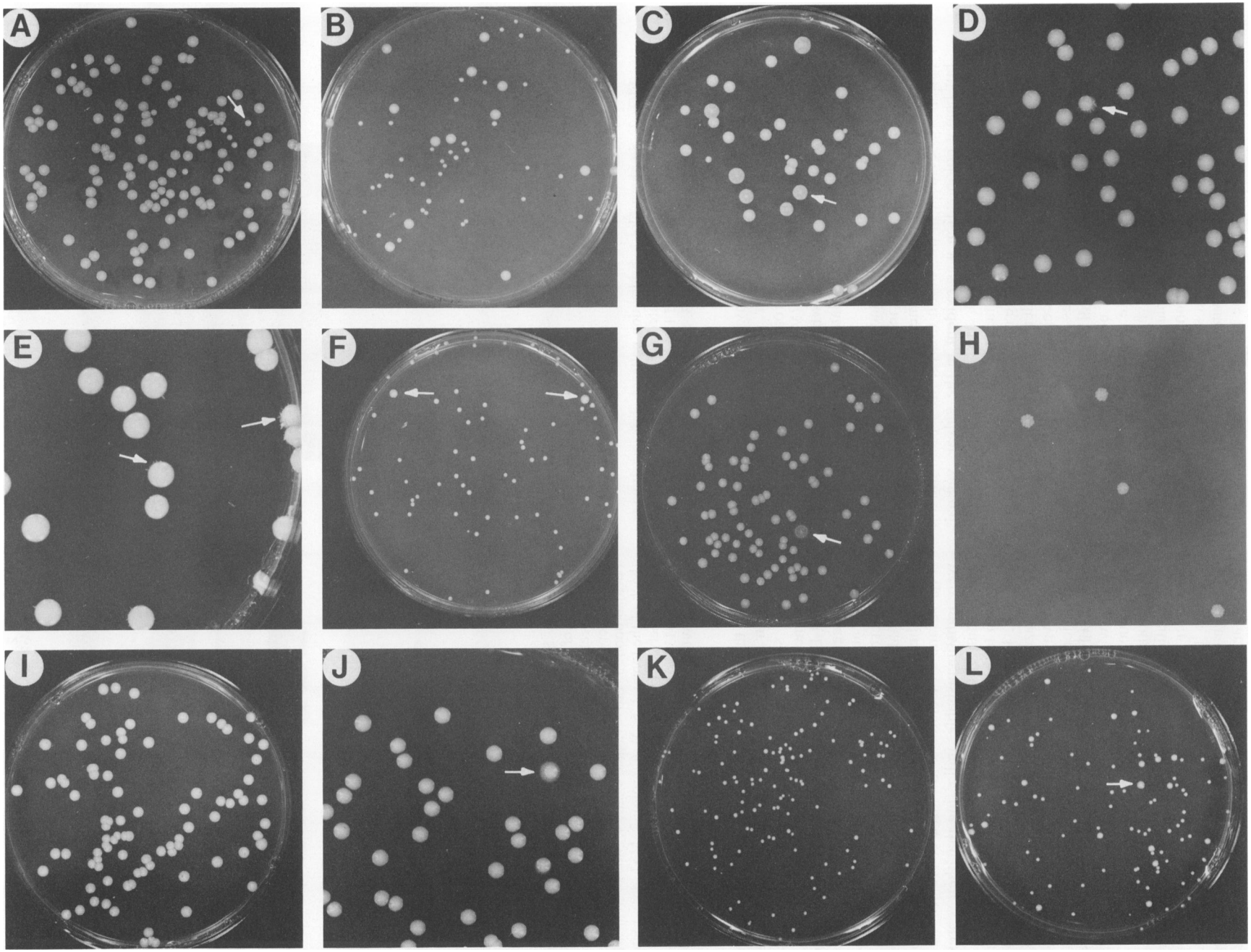

FIG. 2. Examples of switch phenotypes in the three infecting strains at the time of the first infection. (A to D) Vulvar, vaginal, anal, and rectal isolates; (E to H) mouth isolates; (I to L) isolates from under the breasts. (A) A Mo2 clone with a high frequency of petite colonies (arrow); (B) plating of a petite colony from Mo3 with a high proportion of medium-sized, smooth white colonies; (C) a Mo4 producing a large mottled phenotype (arrow); (D) a Mo2 clone exhibiting heavily myceliated colonies (arrow); (E) Mo5 producing peripheral mycelia (arrows) after 7 days; (F) a Mo5 clone exhibiting a petite phenotype (note switches to medium-sized, smooth white colonies [arrows]); (G) a Mo5 clone with a switch to irregular-edge, wrinkled colony (arrow); (H) a small star variant of Mo5; (I) colony morphology and size homogeneity of strain Mo1; (J) a Mo1 clone with a colony exhibiting very heavy peripheral myceliation (arrow); (K) a Mo1 clone exhibiting a petite phenotype; (L) a Mo1 petite clone switching back to a small-sized phenotype (arrow). All colonies were photographed after 7 days of incubation at 25°C.

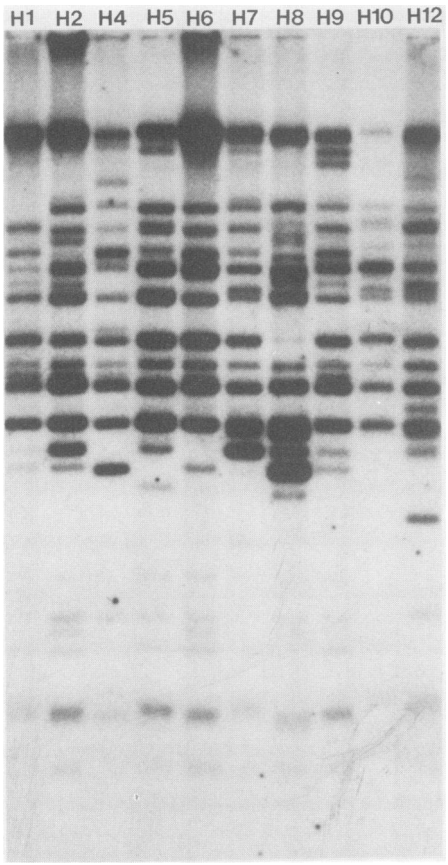

FIG. 3. Southern blot hybridization patterns showing strain relatedness of 10 independently isolated strains from the mouths of healthy individuals. *Eco*RI-digested DNA was hybridized with the moderately repetitive sequence Ca3.

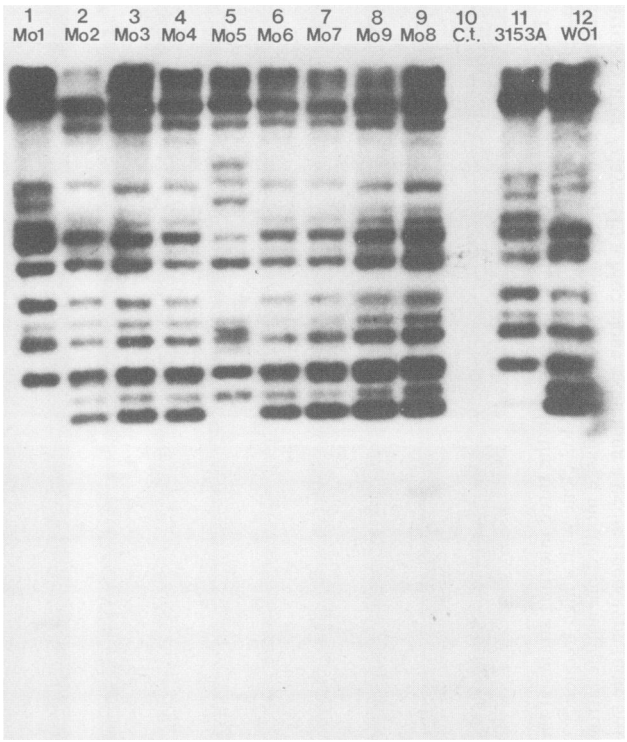

FIG. 4. Southern blot hybridization patterns showing strain relatedness of isolates at the time of the first infection. *Eco*RI digests of whole-cell DNA were hybridized with Ca3, a relatively nonmobile mid-repeat sequence. Lanes: 1, Mo1 (under the breasts); 2, Mo2 (vaginal pool); 3, Mo3 (vaginal wall); 4, Mo4 (vulvar area); 5, Mo5 (back of the tongue); 6, Mo6 (groin); 7, Mo7 (deep rectum [stool]); 8, Mo9 (between toes); 9, Mo8 (anus); 10, *Candida tropicalis* (C.t.) ATCC 34139; 11, *C. albicans* 3153A (laboratory strain); 12, *C. albicans* WO-1.

colonization was evident in any of the other tested body locations, including the vulvar, vaginal, anal, and rectal regions. After 9 days, isolates from the saliva (Mo10), cheek (Mo11), and back of the tongue (Mo12) formed colonies with heavy peripheral myceliation which were identical to the colonies formed by mouth isolates at the time of the first infection (Fig. 1C).

The patient presented a second vaginal infection roughly 87 days after the first vaginal infection and 80 days after cessation of clotrimazole treatment (Table 1). Low colonization was evident in the mouth (Mo15, Mo17, and Mo18), but heavy colonization was evident in the vulvar, vaginal, and anal regions (Mo19, Mo20, Mo21, and Mo14). Mouth isolates again exhibited the same colony phenotype as the original mouth isolate at the time of the first infection (Mo5), forming mycelia in the agar bordering the colony after 7 days (Fig. 5A to D) and a heavy mycelial periphery after 9 days. In marked contrast, the isolates from the vulvar, vaginal, and anal regions exhibited a small colony phenotype (Fig. 5E to H) with a slightly irregular edge when viewed at high magnification, a phenotype quite distinct from the predominant medium-sized, smooth white phenotype in vulvar, vaginal, anal, and rectal samples at the time of the first infection (Fig. 6). All of the strains isolated from the vulvar, vaginal, anal, and rectal regions switched at high frequency (Table 2); all exhibited a high switching frequency to a larger smooth white colony phenotype similar to the original vulvar, vaginal, anal, and rectal strains (Fig. 2B). The difference between the predominant phenotypes of the strains infecting the vulvar, vaginal, anal, and rectal regions at the time of the first and second infections suggested that reinfection was due to a new strain. To test this possibility, *Eco*RI digests of total cell DNA from Mo3 (vaginal wall, first infection) and Mo19 (vaginal wall, second infection) were probed with the mid-repeat sequence Ca3 by Southern blot hybridization and the patterns were compared. The hybridization pattern of the vaginal wall isolate Mo3 (Fig. 7, lane 1), isolated at the time of the first infection, and the vaginal wall isolate Mo19 (Fig. 7, lane 2), isolated at the time of the second infection, were indistinguishable, indicating that they represented the same strain of *C. albicans*.

Return to the original colony phenotype in the third vaginal infection. The patient was treated with vaginal application of butoconazole for a 7-day period following presentation of the second vaginal infection and was examined 7 days after termination of treatment. Low colonization was demonstrated only in the sample from the back of the tongue (Table 1). The two colonies obtained in the sample from the back of the tongue sample exhibited the same myceliated periphery as previous mouth isolates.

The patient presented a third vaginal infection roughly 32 days after the second and 25 days after cessation of butoconazole treatment (Table 1). Medium colonization was evident in mouth isolates, heavy colonization was evident in the vulvar and vaginal isolates, and low colonization was evident in the anal and rectal isolates (Table 1). The mouth

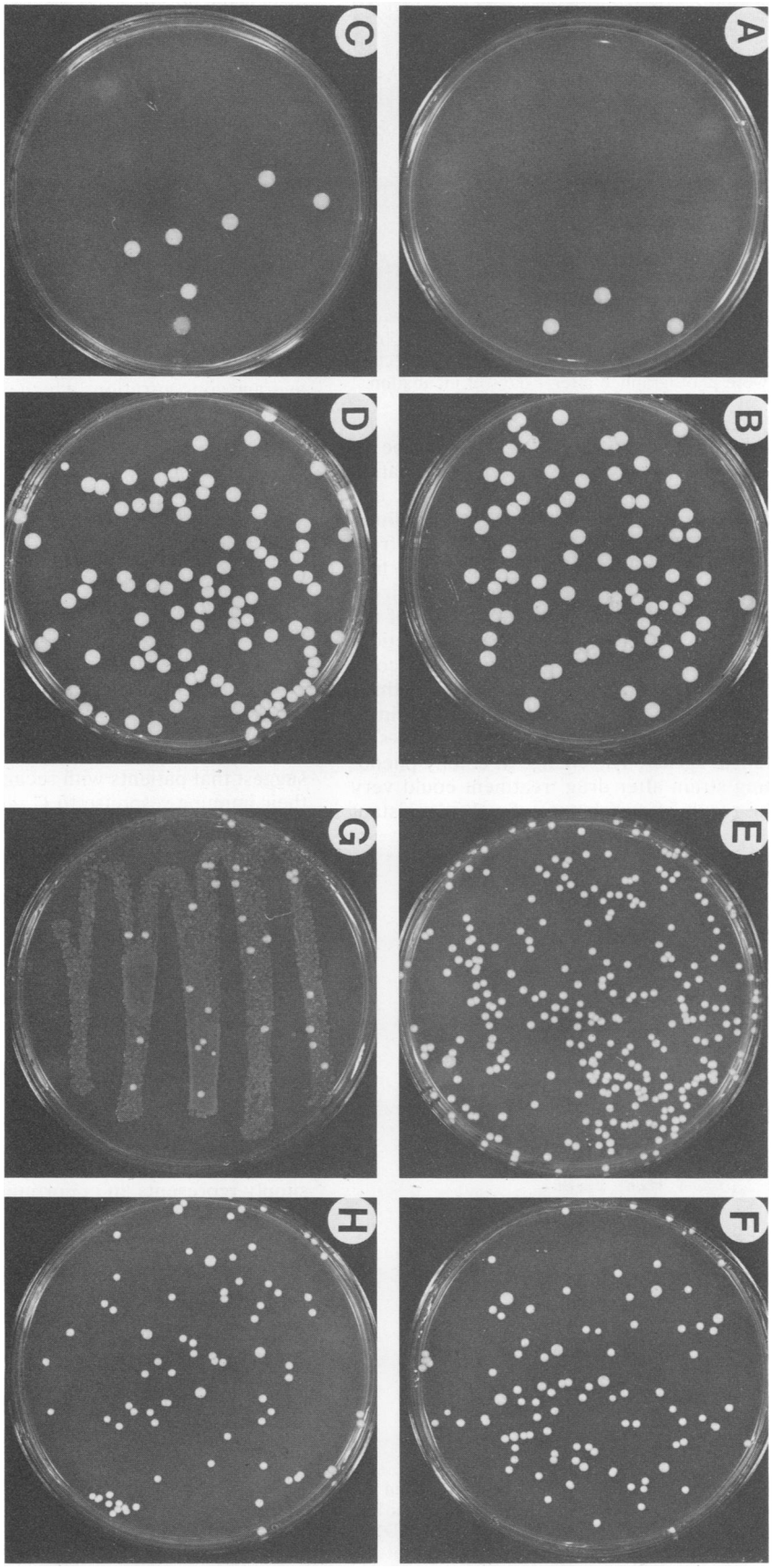

FIG. 5. Colony phenotypes of isolates at time of second infection. (A) Original plating of cheek isolates (Mo15 origin); (B) secondary plating of Mo15; (C) original plating of isolate from the back of the tongue (Mo18 origin); (D) secondary plating of Mo18; (E) original plating of vaginal wall isolate (origin of Mo19); (F) secondary plating of Mo20; (G) original plating of anal isolate (origin of Mo14); (H) secondary plating of Mo14. Note the single bacterial colony (gray) in panel C and the heavy bacterial background carpet in the streak on the plate in panel G.

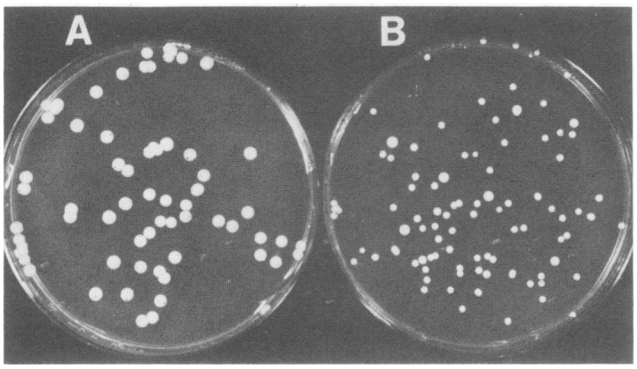

FIG. 6. Comparison of colony morphology of the isolates from the first vaginal infection (Mo3) (A) and the second vaginal infection (Mo19) (B). Plates were photographed after 7 days of incubation.

isolates again exhibited the unique colony phenotype of previous mouth isolates, with peripheral hypha formation after 9 days of incubation. The vulvar, vaginal, anal, and rectal colony phenotypes returned to the original medium-sized, smooth white morphology of the strain isolated from the vulvar, vaginal, anal, and rectal samples of the first infection. To test the relatedness of the medium-sized, smooth white vulvar, vaginal, anal, and rectal strain of the third infection with those of the first and second infection, Southern blot hybridization patterns with Ca3 were compared (Fig. 7). The patterns were identical for all three isolates, indicating that they represented the same strain.

Susceptibility of the vaginal wall isolates to imidazoles as well as other antifungal agents. The change in colony phenotype of the infecting strain after drug treatment could very well have been the result of enrichment of a drug-resistant

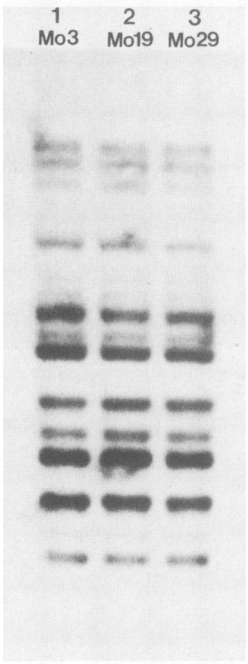

FIG. 7. Southern blot hybridization patterns showing strain relatedness of vaginal isolates from the first (Mo3), second (Mo19), and third (Mo29) vaginal infection. *Eco*RI digests of whole-cell DNA were hybridized with Ca3, a relatively nonmobile mid-repeat sequence.

switch phenotype (17). To test this possibility, clones Mo3 (vaginal wall, first infection) and Mo19 (vaginal wall, second infection) were tested for susceptibility to clotrimazole by using an agar well assay system (see Materials and Methods). The average concentration (for three experiments) of clotrimazole which inhibited growth of Mo3 by roughly 50% after 5 days at 25°C was 1.5 (± standard deviation [SD], 0.0) μg/ml, and the average concentration which completely inhibited growth was 1.75 (SD, ± 0.35) μg/ml. Surprisingly, the average concentration (for three experiments) which inhibited growth of Mo19 by 50% was 0.31 (SD, ± 0.09) μg/ml, and the average concentration which completely inhibited growth was 1.20 (SD, ± 0.28) μg/ml. Therefore, Mo19 was slightly more susceptible, not less susceptible, to clotrimazole than Mo3 was. On the other hand, Mo29 was slightly less susceptible than Mo19 was to butoconazole. The average concentrations which caused 50 and 100% inhibition for Mo29 after 5 days at 25°C were 9.25 (SD, ± 1.8) and 19.5 (SD, ± 2.1) μg/ml, respectively, and for Mo19, 5.2 (SD, ± 4.6) and 12.0 (SD, ± 4.5) μg/ml, respectively.

DISCUSSION

A significant proportion of vulvovaginal candidiasis patients have more than one episode within a year (18, 19). Although it is likely that these patients possess some predisposing condition which accounts for recurrence, the majority of these patients appear to be relatively healthy in most other respects and do not exhibit a disproportionate level of high risk factors, such as diabetes, oral contraception, corticosteroid treatment, hormone therapy, or pregnancy (e.g., see reference 20). Results have been obtained which suggest that patients with recurrent vaginitis are defective in their immune response to *C. albicans* (26), but direct proof for this hypothesis has been difficult to obtain (5). In addition, it has been demonstrated that increased adhesion of *C. albicans* to vaginal epithelium is probably not involved (25).

In addition to knowing very little about the predisposing physiological factors involved in recurrent vulvovaginal candidiasis in otherwise healthy patients, even less is known about the microbiology of the infecting strains (18, 19). It has been assumed that the source of the reinfecting strain is the gastrointestinal tract (8), but until recently, methods for testing strain relatedness were based on physiological, rather than genetic, tests (e.g., the biotyping methods of Odds and co-workers [9]). The lack of attention paid to the infecting organism in recurrent vulvovaginal candidiasis may stem from the assumption that the basis for recurrence resides solely in the physiology of the host and that *C. albicans* simply represents an opportunistic pathogen, residing in the host as a commensal. Indeed, biotyping of vaginal *C. albicans* strains in 1,082 nonpregnant patients visiting genitourinary clinics in Leicester and Loughborough, England, indicated only a low level of association of particular biotype groups with the level of disease (11).

In the present case study, we have employed newly developed Southern blot hybridization methods to assess strain relatedness and plating methods to assess the frequency of switching and the repertoire of switch phenotypes to test (i) whether the same strain colonizes all body locations at the time of a vaginal infection, (ii) whether the same strain is responsible for recurrent infections of the vagina, and (iii) whether switching occurs in the infecting strain between successive episodes of vulvovaginal candidiasis and imidazole treatment. It was first demonstrated that the isolates from 12 body locations at the time of the first

infection could be separated into three groups according to the Southern blot hybridization pattern with probe Ca3, original colony phenotype, and switching repertoire. The demonstrated stability of the Ca3 Southern blot hybridization pattern within a strain over many generations (Hicks et al., submitted) supports the conclusion that the three categories delineated by Southern blot patterns represent at least three genetically distinct strains, and the difference in colony phenotypes and switching repertoires reinforce this conclusion. Since there appear to be far more than 10 Ca3 patterns in commensal strains which have been isolated from healthy individuals as well as in pathogenic strains from patients with candidiasis, the probability of isolating two independent strains in a row with identical hybridization patterns and band intensities is at most 1 in 100. Therefore, it is likely that the isolates from the groin, vulvar region, vaginal wall, vaginal pool, anus, rectum, and between toes, all of which were isolated at the same time and exhibited identical patterns, probably represent the same infecting strain. The strain isolated from the mouth was distinct from the strain from the vulvovaginal, anal, and rectal areas, and neither strain was cloned from the alternative body region. It should also be noted that at the time of the first vaginal infection, there was increased colonization not only in the rectal region, which represented the same strain as in the genital locations, but also in the mouth region, which represented a different strain.

Vaginal application of clotrimazole after presentation of the first infection suppressed colonization at the three original colonized locations. The patient presented with her second vaginal infection 2.5 months after termination of clotrimazole treatment. Increased colonization was also observed at that time in the mouth. The strain isolated from the vagina at the time of the second infection exhibited the same Southern blot hybridization pattern with Ca3 as the strain isolated from the vagina at the time of the first infection, indicating that the same strain was responsible for the recurrent infection. However, the phenotype of the strain isolated at the time of the second vaginal infection was that of a small colony with irregular edges, which differed markedly from the phenotype of the original infecting strain. This small irregular-edge phenotype exhibited an extremely high frequency of switching, with a repertoire of switch phenotypes which included the original medium-sized, smooth white colony phenotype. Surprisingly, the small irregular-edge phenotype did not exhibit increased resistance to clotrimazole when assayed in vitro. Therefore, recolonization with the second phenotype did not appear to be due to enrichment of a drug-resistant switch phenotype. This does not exclude the possibility that clotrimazole treatment killed off the predominant medium-sized, smooth white phenotype and that the minor small irregular-edge phenotype survived because of a phenotypic characteristic which allowed it to escape the drug by entering a body location poorly penetrated by the drug or because it differentiated to a drug-resistant state not observed or tested in vitro.

Vaginal application of butoconazole after presentation of the second infection suppressed colonization once again in the mouth and at the site of infection. The patient then presented with a third infection 22 days after termination of butoconazole treatment. Increased colonization was also observed once again in the mouth. To our surprise, the recurrent strain in the vulvovaginal, anal, and rectal regions had now changed back to a medium-sized, smooth white colony phenotype similar to that at the time of the first infection. In addition, the Southern blot hybridization pattern with Ca3 indicated that the vulvovaginal isolate was still the same strain as the one in the first and second infection.

The results presented in this case study indicate the following. (i) Different strains colonized three different body locations. (ii) The same strain was responsible for the three episodes of vulvovaginal candidiasis. (iii) Changes in the predominant phenotype occurred between each episode. (iv) The same mouth strain persisted through fluctuations in colonization due to imidazole treatment. In a report by Skorepova and Hauck (15), it was demonstrated that in one patient treated with nystatin and in another patient treated with ketoconazole, recurrent isolates exhibited biotypes which differed from those of the isolates prior to drug treatment (10). Biotype changes were accompanied by changes in drug susceptibility. These studies were performed prior to the development of DNA probes for assessing relatedness and therefore depended upon physiological differences which could have been due to high-frequency switching, rather than genetically different strains. Therefore, the methods described in this report, which combine the use of genetic probes for assessing strain relatedness, analyses of switching repertoires, and drug susceptibility tests, must be applied to a number of similar patients exhibiting recurrent *Candida* infections to test the generality of the conclusions from this one case study.

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