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Most Frequent Scenario for Recurrent *Candida* Vaginitis Is Strain Maintenance with "Substrain Shuffling": Demonstration by Sequential DNA Fingerprinting with Probes Ca3, C1, and CARE2

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The following three basic scenarios have emerged for the genetic relatedness of strains in recurrent vaginal candidiasis: strain maintenance without genetic variation, strain maintenance with minor genetic variation, and strain replacement. To test the frequency of each of the three scenarios, the genetic relatedness of *Candida albicans* isolates from each of 18 patients with recurrent infections was assessed by sequential DNA fingerprinting with the following three probes: the Ca3 probe; the C1 probe, a subfragment of the Ca3 probe which hybridizes to hypervariable genomic fragments; and the unrelated CARE2 probe. In each of the 18 patients with recurrent infections, the same strain was responsible for sequential infections, suggesting that the predominant scenario is strain maintenance. However, in 56% of these patients, the strain exhibited minor genetic variations in sequential infections. These changes were not found to be progressive. Rather, the changes suggest that substrains of an established infecting strain are shuffled in sequential infections. Results are also presented that in 45% of patients with recurrent infections, oral and vulvovaginal isolates were identical, in 35% they were highly related but not identical, and in 20% they were unrelated. These results differ markedly from those for commensal isolates simultaneously cultured from the oral cavity and vulvovaginal region of healthy individuals. Finally, it is demonstrated that in all eight cases in which *C. albicans* was isolated from both the male sexual partner of the patient with a recurrent infection and the patient, an isolate from the male partner was identical or highly related to the vulvovaginal strain. These results demonstrate that in patients with recurrent vulvovaginitis, a single strain usually dominates both in the different body locations of the patient and in the male partner and that it is maintained through sequential infections. However, in patients with recurrent infections, different substrains of the established clone dominate in an apparently random fashion, a process that we refer to as "substrain shuffling."

A significant proportion of women suffer from several episodes of vaginal candidiasis within a 1-year period, and in many cases, recurrent infections occur immediately after the cessation of therapy (8, 16, 22). Although drug therapy usually suppresses symptoms and appears to eradicate vaginal colonization, the rapidity of reinfection in many cases suggests that the assumed predisposing condition of the host persists and an infectious strain of yeast has found immediate access to the treated vaginal canal. It has been a general assumption that the source of the reinfecting yeast is a strain carried in another body location such as the anorectal region (8, 9) or a strain carried by the patient's sexual partner (3). In the first genetic analysis of sequential isolates from a single recurrent vaginitis patient performed by DNA fingerprinting, it was demonstrated that a single strain was responsible for three sequential episodes over a 119-day period (19). Subsequent studies of the genetic relatedness of the strains responsible for recurrent infections revealed three general scenarios for reinfection. Stein et al. (21), using a ribosomal fingerprinting probe, demonstrated that a single strain of *Candida albicans* usually persisted through sequential episodes of candidiasis but that strain replacement sometimes occurred. Vazquez et al. (22), using electrophoretic karyotyping, demonstrated that 8 of 10 patients maintained a single strain of *C. albicans* through sequential episodes but that 2 patients underwent apparent strain replacement, and Mercure et al. (8), using the moderately repetitive fingerprinting probe 27A, demonstrated that 24 of 28 patients maintained a single strain but that 4 patients underwent strain replacement. Finally, Schröppel et al. (14), using the moderately repetitive fingerprinting probe Ca3, demonstrated strain maintenance in one sequence of isolates responsible for recurrent infections and strain replacement in a second sequence. They also presented evidence that in the case of maintenance, the C1 subfragment sequences of the strain underwent continuous microevolution. The three possible scenarios which therefore emerge for recurrent vaginitis are the following: (i) maintenance of a genetically stable strain, (ii) maintenance of a strain which is undergoing microevolution, and (iii) strain replacement.

To determine the generality of the microevolution of strains during recurrent episodes of vulvovaginal candidiasis, we have performed a detailed genetic analysis of recurrent strains in 18 patients, using three probes to assess genetic relatedness and strain evolution. Multiple isolates from patients with recurrent infections were sequentially fingerprinted with (i) the complex Ca3 probe (10, 13, 19), (ii) the hypervariable C1 subfragment of the Ca3 probe (1, 7), and (iii) the unrelated CARE2 probe (5).

MATERIALS AND METHODS

Collection of isolates. The women who were enrolled in the study presented with symptoms of recurrent vulvovaginal candidiasis at one of two family practice clinics in the Ann Arbor, Mich., area. Symptoms included inflamed vulva and/or vaginal mucosa, light to heavy vaginal discharge in the case of vaginal infection,

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and the presence of vaginal *C. albicans* by plating on Sabouraud's agar and typing of the species by determining the sugar assimilation pattern with the commercially available kit API 20C (Analytab Products, Plainview, N.Y.). Recurrence was defined as two or more infections in a 6-month period. In select cases, the male partners of the women participated. Specimens were collected from vulvovaginitis patients by gently rubbing individual sterile Dacron swabs across the surface of the vulva, vaginal canal, rectum, and back of the tongue. In some cases, specimens were obtained from urine or stool samples. Specimens were collected from the back of the tongue, urine, stool, and, in some cases, semen of the male sexual partner. Isolates identified as *C. albicans* by their sugar assimilation patterns were transferred to brain heart infusion agar slants and were subsequently fingerprinted. Patients with recurrences were treated with a 7-day regimen of terconazole (Terazol.7; Ortho Pharmaceuticals, Raritan, N.J.). Each patient was referred to as a recurrence patient (RP) and was distinguished by a number (e.g., RP39). Isolates were defined by the RP, body location, and day of isolation. Body locations included tongue (t), stool (st), vulva (vu), and vaginal canal (va). Therefore, a stool sample obtained from patient RP39 on day 42 would be labeled RP39st(42), and an isolate from the vaginal canal of patient RP6 on day 239 would be labeled RP6va(239).

DNA fingerprinting. Southern blot hybridization was performed as described previously (13). Briefly, cells from each isolate were plated at low density on agar containing the medium described by Lee et al. (6) supplemented with arginine and zinc (2). Cells from single clonal colonies were transferred to YPD broth (2% glucose, 2% Bacto Peptone, 1% yeast extract) and were grown to the late log phase. The DNA of each isolate was prepared by the method of Scherer and Stevens (11). The DNA of each isolate was digested with *Eco*RI, separated on a 0.8% agarose gel, and sequentially hybridized with the complex Ca3 probe (1, 10, 19), the hypervariable C1 subfragment of Ca3 (1, 7, 14), and the complex probe CARE2 (5) by previously described methods (12, 19). CARE2 was a generous gift from Brent Lasker of the Centers for Disease Control and Prevention, Atlanta, Ga. In the sequential hybridization regimen, a gel was stained with ethidium bromide to assess loading, washed, transferred to a nitrocellulose membrane, and first hybridized with the random primer-labeled Ca3 probe. The blot was exposed to XAR-S film (Eastman Kodak Co., Rochester, N.Y.) with a Cronex Lightning-Plus intensifying screen (Du Pont Co., Wilmington, Del.). The blot was stripped of the probe by heating to 80°C for 15 min in 1 mM EDTA and was then rehybridized with the random primer-labeled C1 fragment of Ca3. The blot was exposed to XAR-S film, stripped, reprobed with the random primer-labeled CARE2 probe, and exposed a final time to XAR-S film.

Analysis of fingerprinting patterns. Ca3 and CARE2 hybridization patterns were relatively complex and were therefore analyzed by using the Dendron software package, version 2.0 (7). Since the C1 hybridization patterns were relatively simple and represented a subset of bands in the Ca3 pattern, the C1 patterns were not analyzed with the Dendron software. Autoradiographic images of Ca3 and CARE2 patterns were digitized into the Dendron data file with a Scanjet IIcx flatbed scanner (Hewlett-Packard Co., Palo Alto, Calif.) equipped with the transparency option. Linear and nonlinear distortions in gel images were removed, when necessary, by the "unwarping" option of Dendron. After the gel image was processed, the lanes were automatically identified and scanned and bands were automatically identified and assigned an intensity class from 0 (no band) to 3 (highest intensity). The similarity coefficient (S_{AB}), based on band positions and intensity (13), was computed for every pair of isolates in the study. Dendrograms based on S_{AB}s were generated for selected strains by the unweighted pair method (15). The average S_{AB} between Ca3 patterns of 20 isolates, each from a different patient, was 0.70 ± 0.10, and the average S_{AB} between CARE2 patterns of the same 20 isolates was 0.47 ± 0.13. These independent values were considered measures of strain unrelatedness in this particular study (13).

Neighboring lanes. To visually compare lanes not in sequence in the same gel or on different gels, the neighboring function of the Dendron software was used. Gels were unwarped by removing "smiles," "frowns," and other nonlinear distortions and were normalized to a global standard in the Dendron database. Nontandem lanes of the same gel or lanes from different gels were then windowed and juxtaposed.

RESULTS

Sample collection. Thirty women suffering from symptoms of recurrent vaginal candidiasis and, in several cases, their male partners were recruited into the longitudinal study. Samples were obtained from the vulva, vaginal canal, stool, and tongues of women when they presented with sequential episodes and prior to treatment, while samples were obtained from the stool and tongues of their partners and, in select cases, from the urine and semen of the partners. One hundred sixty-one isolates were typed as *C. albicans* by sugar assimilation patterns and were serially fingerprinted with the Ca3, C1, and CARE2 probes. One hundred fifty-nine of these in turn were typed as *C. albicans* by hybridization with the species-specific Ca3 and CARE2 probes. Of the 30 patients with recurrent infection, two or more sequential isolates from the vulvovaginal region were obtained from 18 patients. The present study focuses on the recurrent isolates from these latter patients.

Probes Ca3 and CARE2 provide independent measures of genetic relatedness. The primary goal of the study was to assess quantitatively the genetic relatedness of *C. albicans* isolates obtained from sequential episodes of recurrent vaginitis. To accomplish this, we sequentially hybridized *Eco*RI-digested DNA of each isolate with the probes Ca3, C1, and CARE2. The complex Ca3 probe is species specific (10, 19, 20) and generates a pattern of 10 to 20 bands, which includes invariant, moderately variable, and hypervariable bands (1, 13). The hypervariable bands in the Ca3 pattern hybridize primarily with the C1 subfragment of the probe (1, 7), which contains the repeat element RPS1 (4, 7). Therefore, Ca3 provides a complex pattern for assessing genetic relatedness through computer-assisted computation of an S_{AB} (13), while the C1 fragment provides a significantly reduced pattern which can reflect microevolution in a clonal population (7). Like Ca3, CARE2 generates a complex pattern when it is used as a fingerprinting probe (5). Since it does not hybridize to Ca3 (6a), it provides an independent method for assessing genetic relatedness through the computation of an S_{AB}. The relatedness of the Ca3 and C1 patterns and the independence of the CARE2 pattern are demonstrated in Fig. 1. In this comparison, the Ca3, C1, and CARE2 patterns are compared for laboratory strain 3153A, isolates obtained from the stool [RP39st(42)] and the tongue [RP39t(42)] of patient RP39 on the same day, and a stool isolate [RP42st(31)] obtained from RP42 on day 31. These clinical isolates were selected for this comparison because they exhibit Ca3 patterns similar to, but not identical to, that of laboratory strain 3153A and demonstrate the resolving powers of the three probes. The Ca3 hybridization patterns of the two isolates from RP39 differed in the position of a high-molecular-weight band (Fig. 1A). The difference was apparent in the C1 hybridization patterns, demonstrating that the difference was due to changes in a hypervariable C1-containing fragment of the same strain. The Ca3 patterns of RP39st(42) and RP39t(42) differed from the Ca3 patterns of both 3153A and RP42st(31) by the positions and intensities of at least five bands in each case (Fig. 1A). Some, but not all, of these differences involved C1-containing fragments. The CARE2 hybridization patterns of RP39st(42) and RP39t(42) were identical, demonstrating that in some cases hypervariability because of reorganization of C1-containing fragments is not reflected in the more stable CARE2 pattern. However, CARE2 discriminated between the RP39(42) isolates and both the 3153A and RP42 isolate. The CARE2 pattern of strain 3153A differed from those of both the RP39 isolates by at least eight bands and that of the RP42 isolate by roughly the same number of bands, and the CARE2 patterns of the RP39 isolates differed from that of the RP42 pattern by roughly five bands.

To prove that the Ca3 and CARE2 patterns provide independent but similar measures of genetic relatedness and unrelatedness, we selected five pairs of isolates, each pair representing sequential vaginal isolates from one of five different patients. The 10 isolates were first fingerprinted with Ca3, and the S_{AB}s between all possible pairs were determined. A dendrogram which was based on these S_{AB}s was then generated (Fig. 2A). Each pair was highly related, with S_{AB}s of ≥0.92. In the two cases in which isolates were obtained from the same individual at the same time (RP41, RP42), the S_{AB}s were 1.0, but in all other cases, in which isolates were obtained from the

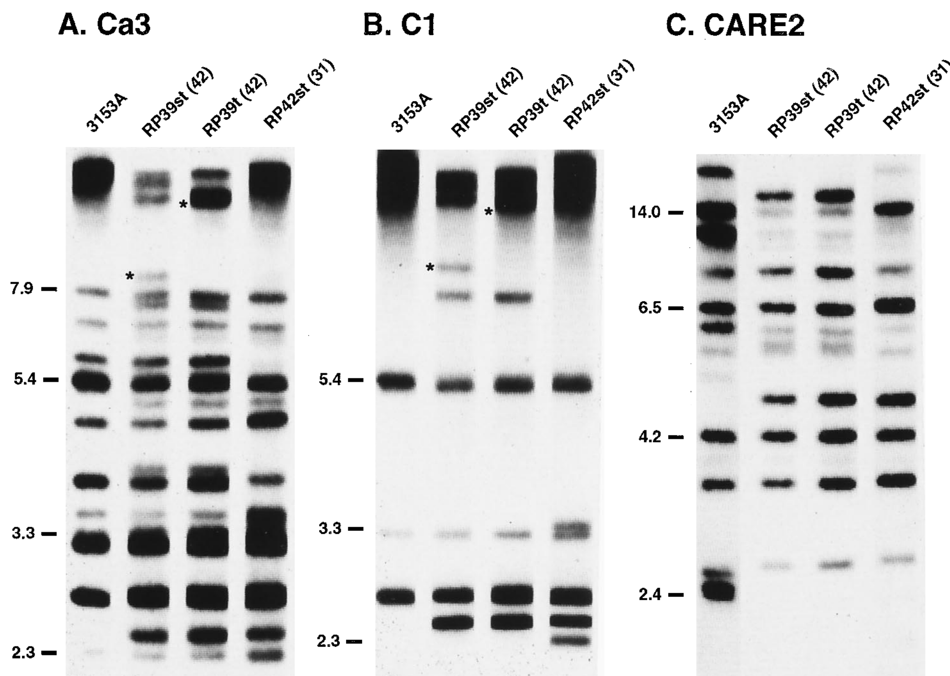

FIG. 1. Comparison of the patterns of hybridization of *Eco*RI-digested DNA and the species-specific probes Ca3 (A), C1 (B), and CARE2 (C). C1 is a subfragment of Ca3 (1, 7), and CARE2 is an unrelated repetitive sequence (5). The fingerprinted *C. albicans* strains included the common laboratory strain 3153A and a stool [RP39st(42)] and a tongue [RP39t(42)] isolate cultured from RP39 on the same day (day 42) of the surveillance, and a stool isolate [RP42st(31)] cultured from RP42 on day 31 of surveillance. Although highly similar, the patterns of RP39st(42) and RP39t(42) differ by a single C1 band in both the Ca3 and C1 patterns. The different band positions are noted with asterisks. Note that although Ca3 and C1 distinguish RP39st(42) and RP39t(42) by a single band, CARE2 does not distinguish the two. More importantly, the bands in the Ca3-C1 patterns do not overlap the bands in the CARE2 patterns. Molecular sizes are presented in kilobases at the left of each panel. The lanes presented in each panel were not run in the sequences presented and were therefore "neighbored" by the neighboring program of the Dendron software package (see Materials and Methods).

same individual at different times, there were slight differences, resulting in S_{AB}s ranging between 0.92 and 0.98. The S_{AB}s computed between clusters including two or more pairs varied between 0.73 and 0.86. The minor differences between pairs from the same individual isolated at different times were primarily due to variability in high-molecular-weight C1 fragment bands (data not shown). The same five pairs of isolates were then fingerprinted with the CARE2 probe, and a dendrogram which was based on the S_{AB}s between all possible pairs of the 10 isolates was generated (Fig. 2B). Just as in the case of the Ca3-based dendrogram, each pair formed a highly related cluster, although in the case of CARE2, each pair had an S_{AB} of 1.0. The S_{AB}s computed between clusters of two or more pairs varied between 0.26 and 0.64. Therefore, Ca3 and CARE2 both clustered each highly related pair of isolates. However, Ca3 fingerprinting was more sensitive in discriminating the microevolutionary changes occurring within clonal populations over time at a much higher rate than CARE2 because of the hypervariability of the C1 fragments.

To demonstrate that Ca3 and CARE2 also assess unrelatedness in a similar fashion, 10 unrelated isolates from 10 different individuals were first fingerprinted with Ca3, and the S_{AB}s between all possible pairs were computed. A dendrogram based on these S_{AB}s was generated (Fig. 2C). No clusters of highly related isolates ($S_{AB} > 0.92$) formed. The mean S_{AB} for the 10 strains was 0.74 ± 0.11. The 10 isolates were then fingerprinted with the CARE2 probe, and a dendrogram based on the computed S_{AB}s between all pairs was generated (Fig. 2D). As was the case with Ca3 fingerprinting, no clusters of highly related strains formed. The mean S_{AB} for the 10 strains was 0.61 ± 0.15. Together, these results demonstrate that although the Ca3 and CARE2 probes are unrelated, they provide independent but similar assessments of relatedness and unrelatedness.

The major scenario of recurrent vaginitis is strain maintenance with or without minor variation. We previously reported that multiple isolates (9 to 12) cultured at the same time from each of six single-episode vulvovaginal infections were identical or highly related, demonstrating that in each case the infecting population was clonal (7). Here, we have compared isolates from sequential episodes of recurrent vulvovaginal candidiasis in order to test the frequencies of strain maintenance, strain evolution, and strain replacement. All of the 18 sets of sequential isolates in this analysis represented cases of strain maintenance. The S_{AB}s of the Ca3 patterns between sequential isolates from each patient ranged between 0.87 and 1.00; the mean S_{AB} was 0.97 ± 0.03 (Table 1). The relatedness of isolates from all tested body locations of the 18 RPs over the entire period of surveillance in each case is diagrammed in Fig. 3. In the diagrams, genetically identical isolates (i.e., with S_{AB}s of 1.0) are connected by solid lines, genetically similar but nonidentical isolates (i.e., with S_{AB}s of 0.87 to 0.99) are connected by hatched lines, and genetically unrelated isolates (i.e., with S_{AB}s of <0.87) do not have connecting lines. RP20 provides an example of strain maintenance without genetic change in the vulvovaginal region (Fig. 3G). The patient presented with symptomatic vaginitis and vulvovaginal colonization five times in a 337-day period. The vulvovaginal isolates RP20vu(0), RP20va(134), RP20va(184), RP20va(227), and RP20va(337) collected over the 337 days of surveillance retained the same Ca3 patterns (Fig. 4A). Even the hypervariable high-molecular-weight C1 subfragment bands remained constant through the

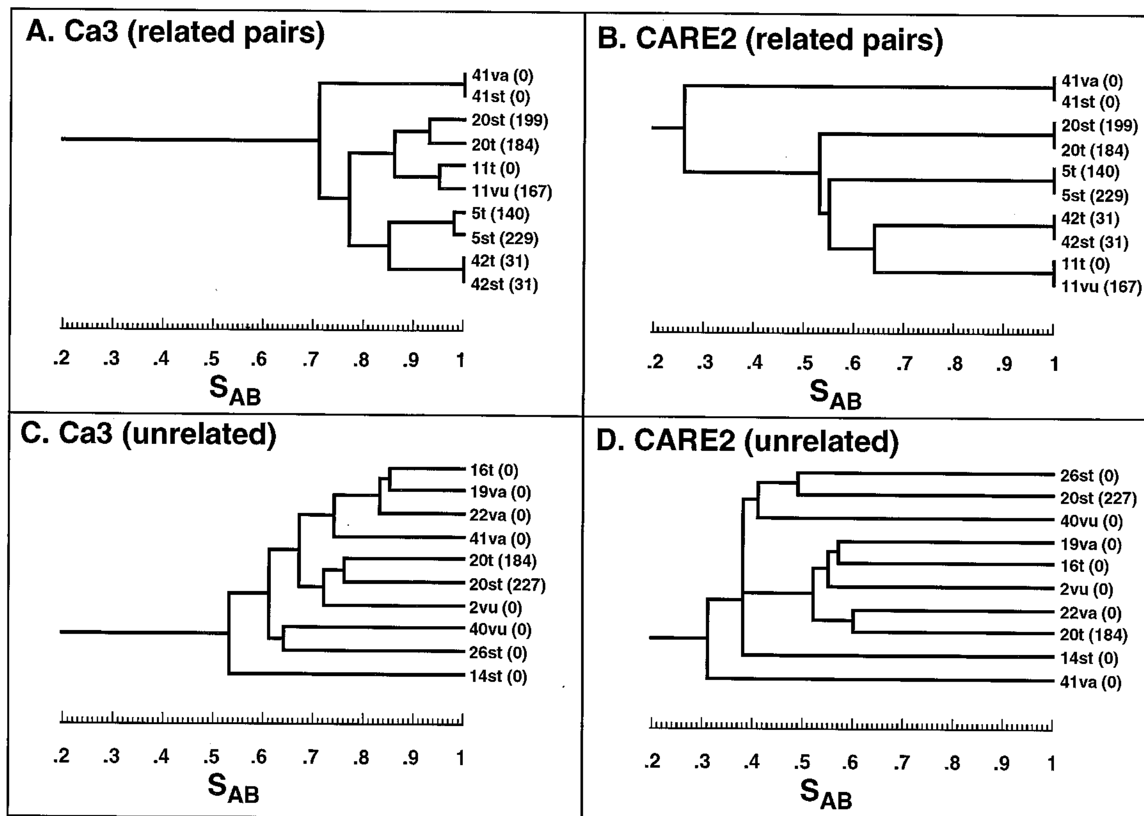

FIG. 2. A comparison of the capacity of Ca3 and CARE2 to identify highly similar isolates and distinguish unrelated isolates. In the former case, *Eco*RI-digested DNAs from five pairs of highly related isolates were probed with either Ca3 or CARE2, and dendrograms based on S_{AB} values were generated for each probe (A and B, respectively). In the latter case, *Eco*RI-digested DNA from 10 unrelated isolates were probed with either Ca3 or CARE2, and dendrograms based on the S_{AB} values were generated for each probe (C and D, respectively). Both probes clustered each related pair (A and B, respectively), and both probes clustered none of the unrelated strains (C and D, respectively).

337 days of surveillance (Fig. 4B). However, patient RP20 carried a completely unrelated strain, strain RP20t(134), in the oral cavity at day 134 (Fig. 4A). The Ca3 pattern of this oral strain differed from that of the vulvovaginal strain by more than 10 band positions (Fig. 4A). RP20t(184) differed from RP20t(134) by several band differences, all in the C1 pattern (Fig. 4B). The S_{AB} between RP20t(134) and RP20t(184) was 0.92. The patterns of the isolates from the oral cavity after 184 days remained constant and differed from those of the corresponding vaginal strains, with an average S_{AB} of 0.75 (Fig. 3 to 5). The CARE2 patterns of the sequential vulvovaginal and oral isolates from RP20 supported the interpretations made from the Ca3 and C1 patterns (Fig. 4C). The CARE2 patterns of the five sequential vulvovaginal isolates of RP20 were identical, but they differed from the CARE2 pattern of isolate RP20t(134) by approximately 10 bands (Fig. 4C). The CARE2 patterns of the five oral isolates were also identical, again demonstrating that the Ca3 and C1 patterns are more prone than CARE2 patterns to microevolutionary change. In Fig. 5A and B, dendrograms which are based on the S_{AB}s computed from the Ca3 and CARE2 patterns, respectively, were generated for all pairs of RP20 isolates. It is clear that the patterns generated by both probes separate into a major vulvovaginal cluster, which also contains the strains from the two late stool samples, strains RP20st(227) and RP20st(337), and a major oral cluster, which also contains the strain from the early stool sample, RP20st(199). The isolates from patients RP5, RP38, RP1, RP10, RP42, RP11, RP13, RP28, and RP40 also provide

TABLE 1. S_{AB}s computed for consecutive vulvovaginal isolates[a]

Patient	S_{AB} (Ca3)	$\bar{x} \pm$ SD	S_{AB} (CARE2)	$\bar{x} \pm$ SD
RP4	1.00, 1.00, 1.00, 0.98	1.00 ± 0.01	1.00, 1.00, 1.00, 1.00	1.00 ± 0.00
RP6	1.00, 0.98, 1.00, 0.98, 1.00, 0.98	0.99 ± 0.01	1.00, 0.97, 1.00, 0.97, 1.00, 0.97	0.99 ± 0.02
RP18	0.98, 1.00	0.99	0.89, 1.00	0.95
RP20	0.97, 0.95, 0.96, 0.96	0.96 ± 0.01	1.00, 1.00, 1.00, 1.00	1.00 ± 0.00
RP5	1.00	1.00	1.00	1.00
RP1	1.00	1.00	1.00	1.00
RP43	1.00	1.00	1.00	1.00
RP42	1.00	1.00	1.00	1.00
RP10	0.91, 0.91	0.91	1.00, 1.00	1.00
RP39	0.95	0.95	1.00	1.00
RP9	0.96	0.96	1.00	1.00
RP13	1.00	1.00	1.00	1.00
RP11	0.98	0.98	1.00	1.00
RP38	1.00	1.00	1.00	1.00
RP8	0.87	0.87	0.69	0.69
RP40	1.00	1.00	0.89	0.89
RP19	0.90, 0.96, 0.96	0.94 ± 0.03	0.81, 0.88, 0.97	0.89 ± 0.08
RP28	1.00	1.00	1.00	1.00

[a] When only one S_{AB} is presented, it means that there were only two sequential isolates from that patient. Sequential S_{AB}s were computed for more than two sequential isolates and are presented in temporal order of isolation. The mean ± standard deviation S_{AB}s for Ca3 and CARE2 were 0.97 ± 0.03 (n = 33) and 0.97 ± 0.07 (n = 33), respectively.

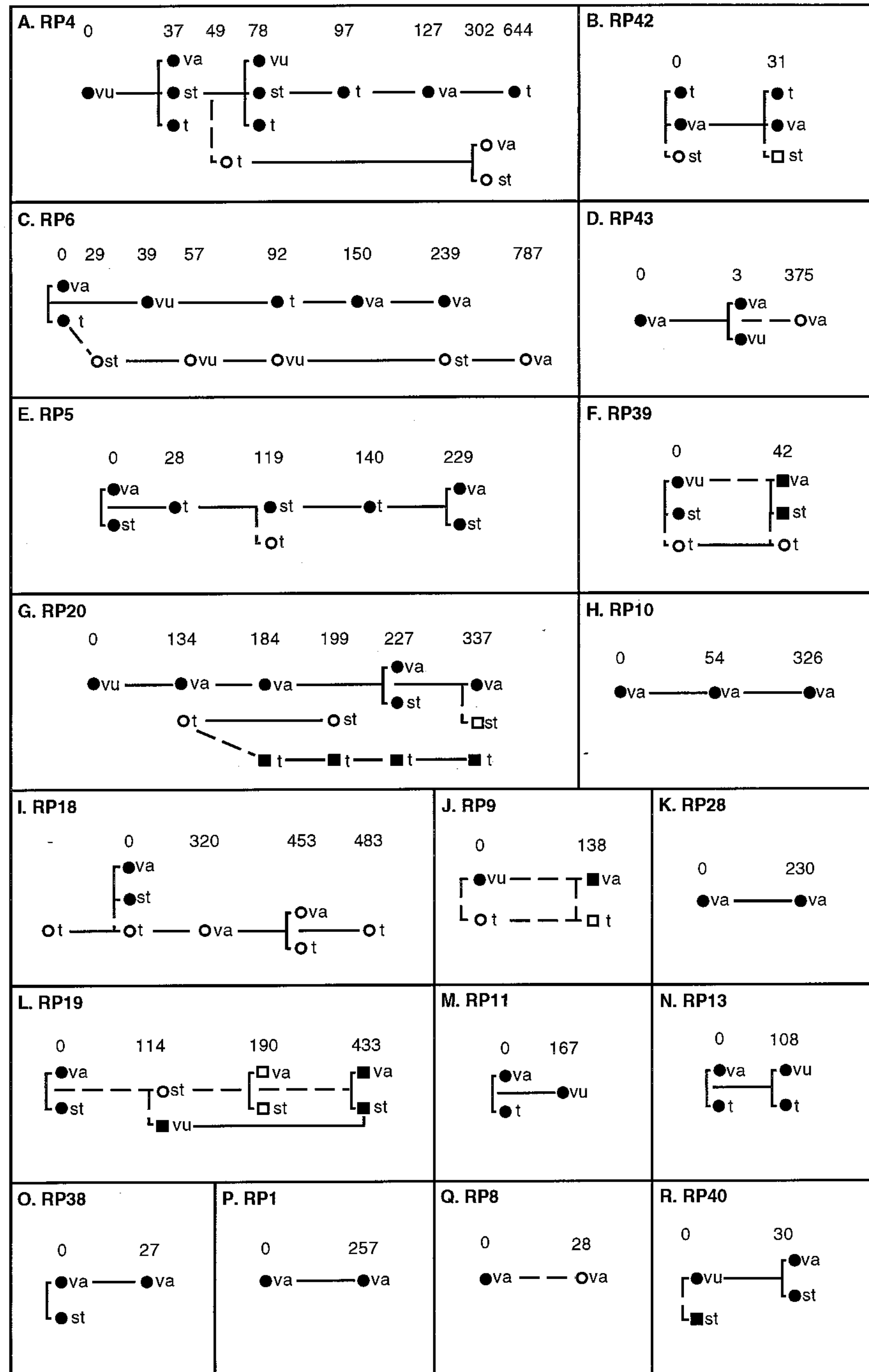

FIG. 3. Diagrams of the relatedness of isolates from each of 18 recurrent vaginitis patients. Isolates are presented in temporal order, with the number of days of isolation during each surveillance period given at the top of each panel. Continuous lines and identical symbols denote the complete identity of the Ca3 and C1 patterns, dashed lines and different symbols denote related patterns which are highly similar but nonidentical, and no connection and different symbols denote unrelatedness. vu, vulva isolate; va, vaginal canal isolate; st, stool isolate; t, tongue isolate.

similar examples of sequential vulvovaginal isolates which maintained fixed Ca3 patterns over periods ranging between 27 and 326 days of surveillance (Fig. 3). In all of these cases, the CARE2 patterns of the vulvovaginal strains were also invariant, verifying the results obtained by Ca3 fingerprinting.

Of the 18 sets of sequential vulvovaginal isolates, 9 sets included isolates with identical Ca3, C1, and CARE2 patterns. Each of the remaining nine sets of isolates, although highly similar, contained one or more isolates with minor variations in the Ca3 and C1 patterns. The isolates from RP19 provide an example of sequential isolates with minor variations in both C1 and non-C1 fragments of the Ca3 pattern (Fig. 3L and 6A). The Ca3 pattern of isolates RP19va(0) differed from that of the subsequent vulvovaginal isolate RP19va(190) by the presence

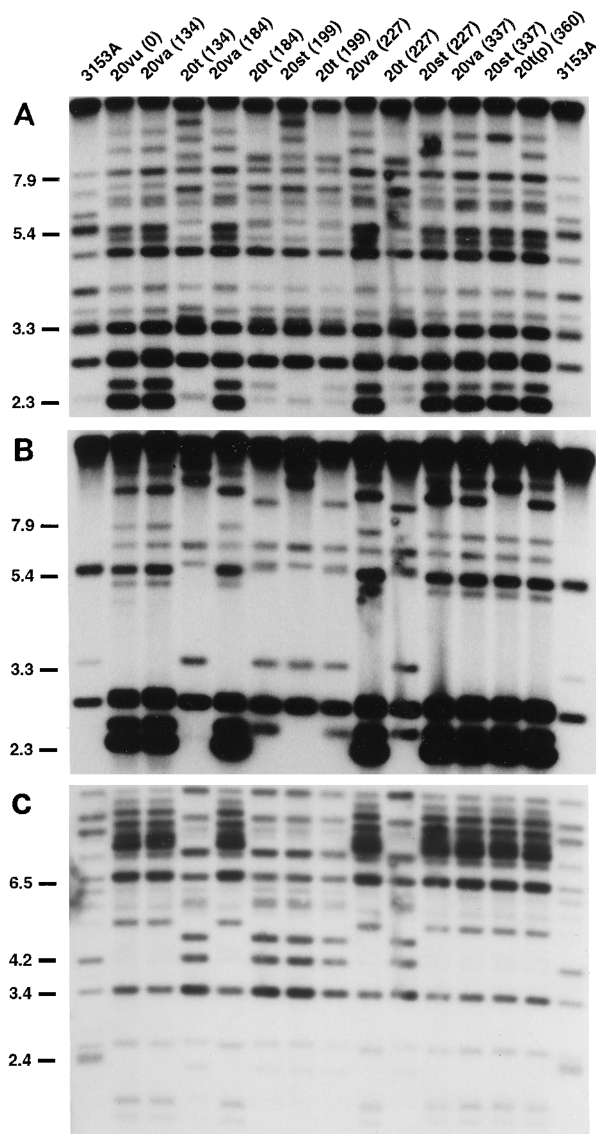

FIG. 4. Ca3 (A), C1 (B), and CARE2 (C) hybridization patterns of isolates from RP20. Total genomic DNA from each isolate was digested with *Eco*RI, and Southern blots were sequentially hybridized with the Ca3, C1, and CARE2 probes. Molecular sizes are presented in kilobases to the left of each hybridization pattern. Isolates from RP20 are presented as follows: 20, body location (day of isolation). 3153A, laboratory strain used as a standard. vu, vulva; va, vaginal canal; st, stool; t, tongue; (p), partner.

or absence of one band and the relative intensities of two bands. The RP19va(0) pattern contained a band at approximately 6.0 kb which was absent from the RP19va(114) pattern (Fig. 6A). This band represented a C1 fragment (Fig. 6B). The two patterns also differed in the intensities of two bands at approximately 2.5 and 4.8 kb. Repeat gels demonstrated that these intensity differences were reproducible. The former band represents a C1 fragment, but the latter does not (Fig. 6B). The patterns of the subsequent vulvovaginal isolates RP19va(190) and RP19va(433) were highly similar.

In the RP19 collection of isolates, every vulvovaginal isolate was accompanied by a stool isolate. For every pair, the Ca3 pattern of the stool isolate was identical or almost identical to that of the vulvovaginal isolate (Fig. 3L and 6A). The Ca3 patterns of the vulvovaginal-stool isolates at 0, 114, and 433 days were identical, while those of the isolates at 119 days differed by the intensity of a single band (Fig. 6A). The reduced C1 patterns of each pair at 0, 114, and 433 days were identical, and the lowest-molecular-weight band differed in intensity between the isolates at 190 days (Fig. 6B). The CARE2 patterns of each pair were identical (Fig. 6C). The dendrograms describing the Ca3 and CARE2 pattern relationships of all vulvovaginal and stool isolates in the RP19 collection are presented in Fig. 5C and D, respectively.

Changes in the C1 pattern of sequential isolates are not progressive. Although the Ca3-C1 patterns of the sequential vulvovaginal isolates of RP19 changed between the first and second isolates and the second and third isolates, the changes were not obviously progressive. For instance, the dramatic decrease in the intensity of the 2.5-kb C1 band between the isolates obtained on day 0 and those obtained on day 114 was followed by a subsequent increase in intensity between 114 and 190 days (Fig. 6A). This may reflect a progressive change if the basic mechanism of C1 variability involves deletion and duplication of the *Eco*RI fragment(s) composing the 2.5-kb C1 fragment. However, the sequential Ca3 patterns of several additional collections of isolates strongly suggest that in many cases variation in successive strains represents shuffling of apparently stable subgenotypes (substrains) in the infecting clonal population. The sequential vulvovaginal isolates from RP6 demonstrate the shuffling scenario (Fig. 7A). Two C1 genotypes, genotypes 1 and 2, were represented in sequential isolates in the following order: 1-1-2-2-1-1-2. Genotypes 1 and 2 differed by a single C1 band (Fig. 7A) and a single CARE2 band. In RP19, sequential vulvovaginal strains exhibited three Ca3 genotypes, genotypes 1, 2, and 3, and were represented in sequential isolates in the following order: 1-2-3-2 (Fig. 7B). In several cases, shuffling of genotypes also was observed in oral isolates. In the case of RP4, two genotypes, genotypes 1 and 2, were represented in the following order: 1-2-1-1-1 (Fig. 7C). In the case of RP5, two genotypes, genotypes 1 and 2, were represented in the following order: 1-2-1 (Fig. 7D).

The remaining six sets of sequential vulvovaginal isolates in which there was genotypic variability (RP4, RP43, RP18, RP39, RP9, and RP8) included three sets in which isolates were obtained at only two time points (RP39, RP9, and RP8), making it impossible to assess whether genetically distinct substrains reappeared in sequence. In the case of RP43, a variant phenotype appeared in the last of three samples (Fig. 3D). However, in the RP4 and RP18 sets, the second genotype which appeared in the sequence of vulvovaginal isolates was identical to those of earlier isolates from the tongue, in the former case, or from the stool, in the latter case (Fig. 3A and I, respectively). In the former case, the CARE2 patterns did not discriminate between isolates, while in the latter case, the CARE2 patterns supported the differences between isolates determined by the Ca3 patterns. Therefore, of the nine sets of sequential vulvovaginal isolates in which there was one or more minor changes in genotype, four sets included late variants which were identical either to an earlier vaginal isolate or to an earlier isolate from another body location.

Genetic relatedness of vulvovaginal isolates and isolates from male sexual partners. For 8 of the 18 patients with recurrent vulvovaginal candidiasis, *C. albicans* was obtained from the male sexual partner at the surveillance period. The level of relatedness of each isolate from a partner was based on a comparison of its Ca3 pattern and that of a vulvovaginal isolate cultured from the female partner closest to the time of sampling of the male. The S_{AB}s of isolates from the male and female partners are presented in Table 2. In all of the eight pairs of partners, the Ca3 patterns of one or more of the

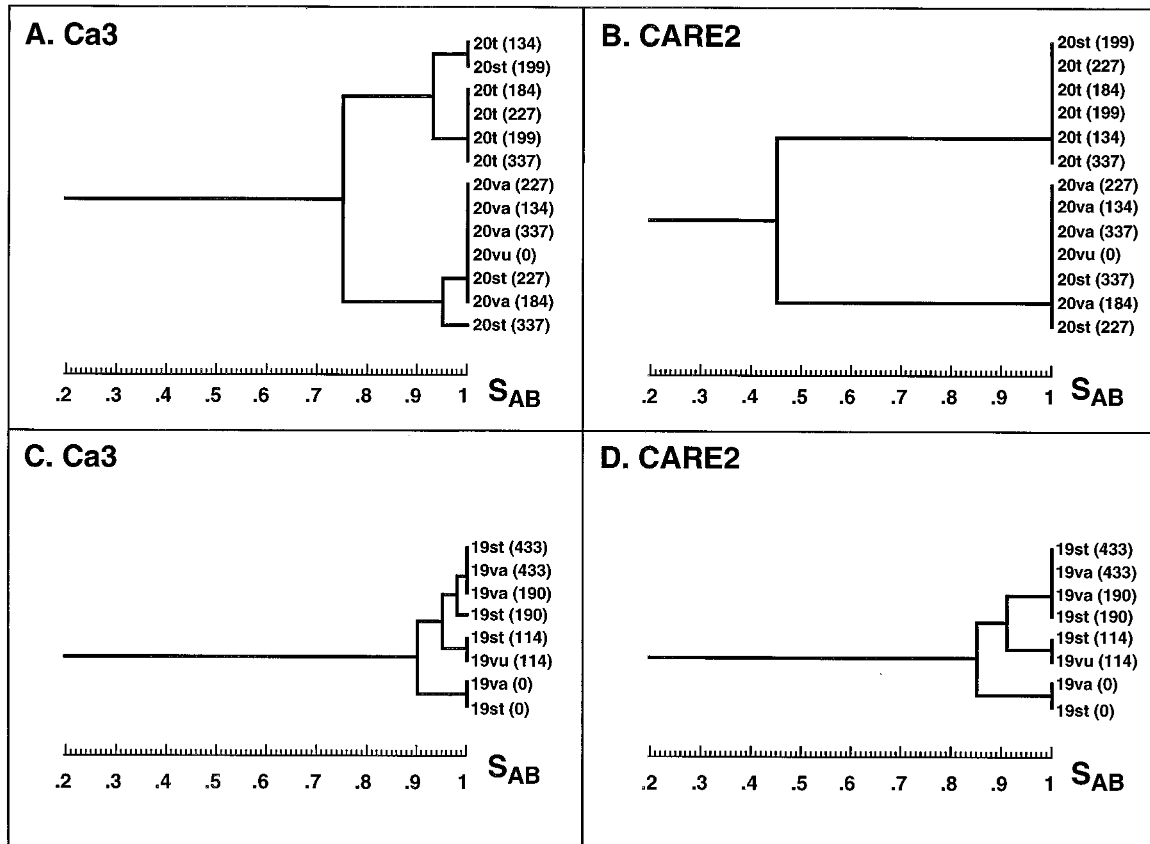

FIG. 5. Dendrograms based on the computed S_{AB}s between the isolates of RP20 fingerprinted with DNA probes Ca3 (A) and CARE2 (B) and the isolates of RP19 fingerprinted with probes Ca3 (C) and CARE2 (D). Note that both probes separate the RP20 isolates into two clusters made up primarily of tongue isolates in one cluster and vulvovaginal isolates in the second cluster and that both probes generate a single cluster for all RP19 isolates. Isolates of RP20 and RP19 are coded for anatomical location (day of isolation). vu, vulva; va, vaginal canal; st, stool; t, tongue.

isolates from the male partner were highly similar ($S_{AB} \geq 0.88$) or identical ($S_{AB} = 1.00$) to that of the corresponding vulvovaginal isolate. For the male partners of RP5 and RP38, one of two or more isolates were unrelated (Table 2). In the case of RP5, the stool sample of the male partner contained a yeast species other than *C. albicans* (i.e., there was no hybridization with the *C. albicans*-specific Ca3 probe), and in the case of RP38, the S_{AB} between the isolate from the semen sample of the male partner and the vaginal isolate of the female partner was 0.70, a measure of unrelatedness.

Genetic relatedness of vulvovaginal, oral, and stool isolates. For 10 of the patients with recurrent vulvovaginal candidiasis, tongue and vulvovaginal isolates were simultaneously obtained in culture at one or more sampling times. The total number of simultaneously cultured samples was 20, and of these, 45% contained isolates that were identical, 35% contained isolates that were highly similar, and 20% contained isolates that were unrelated (Table 3). For 10 of the 18 patients, stool and vulvovaginal samples were simultaneously cultured at one or more sampling times. The total number of simultaneously cultured samples was 20, and of these, 70% contained isolates that were identical, 30% contained isolates that were highly similar, and 0% contained isolates that were unrelated (Table 3). Stool and oral cavity samples from five individuals were simultaneously cultured. The total number of simultaneously cultured samples was 10, and of these, 20% contained isolates that were identical, 70% contained isolates that were highly similar, and 10% contained isolates that were unrelated (Table 3).

DISCUSSION

The three basic scenarios for the genetic relatedness of sequential isolates. The combined results of a number of studies (8, 14, 17, 21, 22) suggested that there are three basic scenarios for the genetic relatedness of isolates obtained from patients with sequential episodes of recurrent yeast vaginitis. In the first scenario, the same strain is responsible for each recurrent episode and remains genetically invariant, within the resolving power of the genetic fingerprinting method that is used. In the second scenario, the same strain is responsible for recurrent episodes, but small variations occur in the genotype, suggesting that the infecting strain undergoes progressive microevolution. In the third scenario, the infecting strain is replaced by an unrelated strain in a subsequent episode. Unfortunately, most of the previous studies, including our earliest one (17), did not use methods which could discriminate minor genetic variations within a strain and, in some cases, did not use fingerprinting methods which had been adequately characterized for their capacity to resolve genetic distances between strains. Therefore, in most of these studies, strain maintenance with microevolution was not discriminated, and in some cases, it was not clear whether different strains really represented high-frequency changes in the same strain. We therefore performed the study described here to assess the frequencies of the three basic scenarios using a fingerprinting system which has been carefully characterized for its capacity to discriminate between unrelated strains and for its capacity

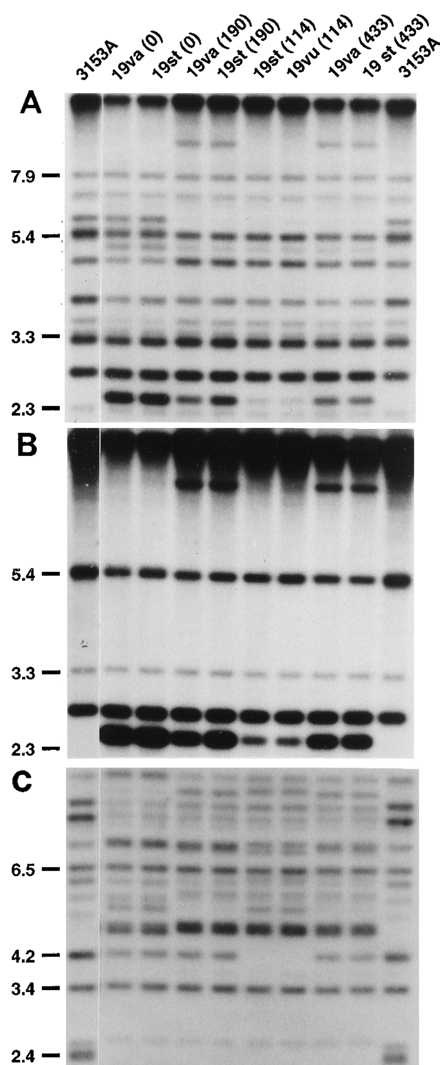

FIG. 6. Ca3 (A), C1 (B), and CARE2 (C) hybridization patterns of isolates from RP19. Total genomic DNA from each isolate was digested with *Eco*RI, and Southern blots were sequentially hybridized with Ca3, C1, and CARE2. Molecular sizes are given in kilobases to the left of each panel. Isolates of RP19 are presented as follows: 19, body location (day of isolation); 3153A, laboratory strain used as standard. vu, vulva; va, vaginal canal; st, stool.

to resolve minor genetic variations within a strain (7, 12–14). We have also used a second, independent system to verify the general measures of relatedness and unrelatedness obtained with the first system.

We have found that the major scenarios for the relatedness of isolates from sequential episodes are strain maintenance with minor change and strain maintenance without minor change. Because the Ca3 probe and the C1 subfragment probe identify changes in hypervariable regions of the genome of *C. albicans* more readily than the CARE2 probe does, the proportion of isolates from sequential episodes with minor changes was 56% when Ca3 and C1 were used as fingerprinting probes and 28% when CARE2 was used as a fingerprinting probe. Although we found no instances of strain replacement in sequential episodes among the 18 patients in the present study using fingerprinting with unrelated probes for verification, we and others have demonstrated that although this represents a minor scenario, it indeed occurs. Of the three earlier cases of recurrent vaginitis that we analyzed with either the Ca3 probe alone (17) or the Ca3 probe in combination with the C1 probe (14), one involved bona fide strain replacement. Of five sets of recurrent isolates, Stein et al. (21), using Southern blot hybridization with a ribosomal probe, presented evidence suggesting that one involved strain replacement. Mercure et al. (8), using Southern blot hybridization with the 27A probe, which is an independent isolate of a Ca3-related sequence (11), found that 14% of recurrences were due to recolonization with a different strain. Finally, Vazquez et al. (22), comparing karyotypic patterns resolved by pulsed-field gel electrophoresis, presented evidence suggesting that differences in 2 of 10 sets of sequential isolates involved strain replacement. Although the majority of these studies did not use methods which provided quantitative measurements of relatedness and unrelatedness, one can still suggest a rough estimate of 80 versus 20% for the proportion of relapses in which the same strain, with or without minor genetic variation, is involved versus the proportion of relapses in which a strain is replaced with an unrelated strain. The present study suggests that this estimated proportion of strain replacement may be an overestimate.

Substrain shuffling rather than progressive microevolution may account for minor variations in genotype in sequential isolates. The second objective of the present study was to obtain a better picture of strain evolution in recurrent infections involving a single strain. In a previous study, we found that the infecting strain of a vulvovaginitis patient changed its genotype through reorganization of high-molecular-weight C1-containing fragments in recurrent infections (14). In each of the three sequential infections analyzed, an isolate from the vaginal wall, vaginal pool, and vulva area were simultaneously cultured and fingerprinted with the Ca3 probe. For each set of isolates collected at a single time point, the Ca3 patterns were identical. However, the common genotype of each set differed from that of each of the other two sets. This result suggested that the genotype of the majority of yeast cells in each infection was relatively homogeneous, but that the genotype underwent progressive microevolution and variant phenotypes were selected after drug therapy in each successive infection. This interpretation in turn suggested that colonizing populations should contain minority variant genotypes. In an analysis of the genetic homogeneity of commensal and infecting populations of the mouth and vagina with both the Ca3 and the C1 probes, we demonstrated that colonizing populations were clonal but did contain a minority of cells exhibiting variant genotypes (7). In that study, 9 to 14 independent isolates from each of nine colonizing populations of *C. albicans* were analyzed. The independent isolates of each of the nine sets were either identical or highly similar, demonstrating the clonal nature of the colonizing populations. The average proportion of cells in each population exhibiting a predominant Ca3 pattern was 85%, which can be viewed as the average level of homogeneity. The average proportion exhibiting a minor, highly related variant genotype was 15%. Minor variant genotypes in each set differed from the major genotype by no more than one band difference in the Ca3 pattern, suggesting that they represented new variants. These results demonstrated that infecting populations underwent microevolution and that variants with minor alterations in C1-containing genomic sequences could be resolved by Southern blots probed with Ca3 or C1. These results supported the hypothesis that microevolution occurred in infecting clonal populations and that minor variants were selected in recurrent infections following drug therapy.

As noted, the major changes observed in the Ca3 patterns of minor variants in each clonal population were primarily due to the reorganization of a single C1-containing fragment. The C1

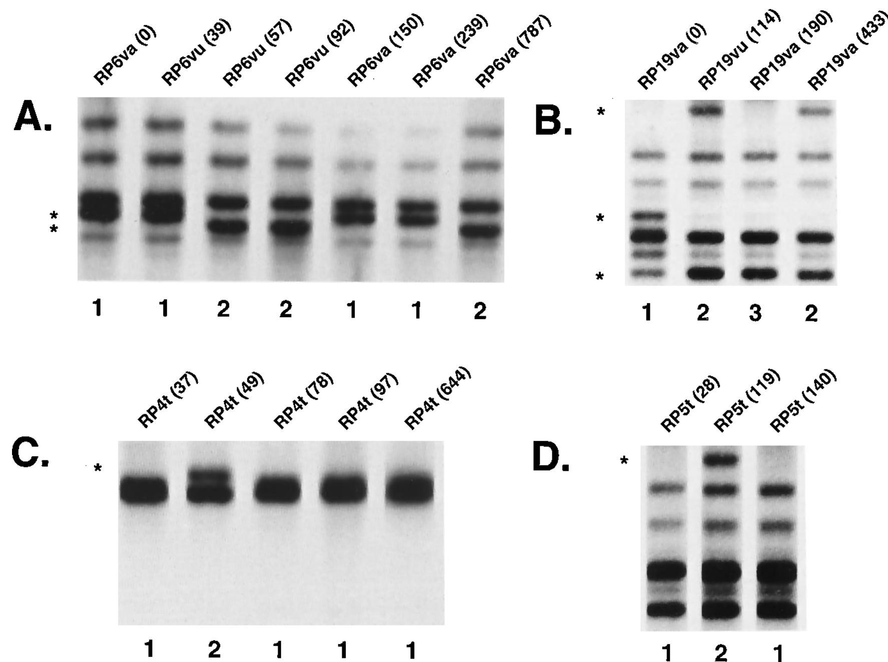

FIG. 7. Examples of "substrain shuffling" for vulvovaginal isolates of RP6 (A) and RP19 (B) and oral isolates of RP4 (C) and RP5 (D). Only the high molecular weights of either the Ca3 patterns (B and D) or the C1 pattern (A and C) are presented. Band position changes are noted by asterisks to the right of the patterns. Patterns are labeled under each lane in the temporal order in which they appear. vu, vulva; va, vaginal canal; t, tongue. The lanes presented in each panel were not run in the sequence presented and were therefore neighbored by the neighboring program of the Dendron software package.

subfragment of Ca3 has been sequenced and has been shown (7) to contain the repeat sequence RPS1, which in turn contains three copies of the repetitive element COM29 (4). The variations in C1-containing fragments are most likely due to duplication or deletions of RPS1 at a given genomic site (9a).

We therefore expected to find in this expanded study of recurrence isolates that in cases in which minor variations in the C1 pattern were observed in three or more sequential isolates, there would be progressive changes in the patterns. In other words, the predominant genotype of an infecting population in a prior infection would be replaced in the subsequent infection by a substrain which represented a minor genotype in the prior infection, and that it would in turn be replaced in the subsequent infection by a substrain representing a minor genotype in the second infection, in other words, a variant of its own genotype. Therefore, pattern changes should show continuity. Surprisingly, this was not observed. Instead, we have observed several cases of apparent shuffling of a set number of patterns, demonstrated most poignantly in the case of RP6. For seven sequential infections, there was shuffling back and forth between two patterns (patterns 1 and 2) in the order 1-1-2-2-1-1-2. Patterns 1 and 2 differed by one C1 band, and one could therefore argue that the mechanism for deletion and duplication of RPS1 in the variable C1 fragment was restricted to two alternative states and, therefore, that the variation between genotypes 1 and 2 in the seven episodes is in fact sequential. However, the CARE2 patterns of the same sequential isolates suggest that this latter explanation is not plausible. Minor variants 1 and 2, distinguished by differences in a single C1 band, also exhibited an alteration in a single band in the CARE2 pattern, generating the same alternating genetic pattern, namely, 1-1-2-2-1-1-2. Since variability of C1 and that of CARE2 appear to be independent events, it is highly unlikely that sets of repeat sequences recombine in a coordinate and reversible fashion, in each case between two alternative states. It is far more likely that genotypes 1 and 2 represent two relatively stable subgenotypes in the infecting population and that we are, in fact, observing a shuffling process between the two subgenotypes in sequential infections, which we refer to as "substrain shuffling." Interestingly, sequential tongue isolates from RP6 exhibited only genotype 1 and sequential stool isolates exhibited only genotype 2. It appears that the alternative genotypes may be firmly entrenched in alternative reservoirs, which may provide random sources of recurrent vaginal infections. There is no question that microevolution, which is distinguishable primarily by changes in the C1 pattern, and in some cases, changes in the CARE2 pattern, occurs in clonal

TABLE 2. Relatedness of vulvovaginal and partner isolates

Isolate	Location of partner's strain	Day of partner strain isolation	S_{AB} for pair
RP42va(0)	Oral	9	0.96
RP11va(0)	Oral	220	1.0
RP5va(0)	Oral	28	0.92
RP5va(229)	Oral	171	0.92
	Stool	171	0.0[a]
RP20va(337)	Oral	360	0.97
RP40vu(30)	Urine	36	0.94
	Stool	36	1.0
RP10va(0)	Stool	25	0.95
Rp39vu(0)	Stool	26	0.97
RP39va(42)	Stool	46	0.98
RP38va(0)	Oral	3	0.88
RP38va(27)	Semen	27	0.70
	Urine	370	0.93
	Stool	370	0.93

[a] The S_{AB} was 0.0 because the partner strain was a species other than *C. albicans* and therefore did not hybridize with any of the species-specific probes (Ca3, C1, CARE2).

TABLE 3. Genetic relatedness of vulvovaginal, oral, and stool isolates

Patient	Relatedness[a]								
	Oral/vulvovaginal			Stool/vulvovaginal			Stool/oral		
	Ident.	Highly sim.	Unrel.	Ident.	Highly sim.	Unrel.	Ident.	Highly Sim.	Unrel.
RP4	2			3					
RP42	2				2			2	
RP6	1	1			1				
RP43									
RP18	1	1		1				1	
RP39		2		2				2	
RP20			4	1	1		1	1	1
RP10									
RP5		1		2			1	1	
RP9		2							
RP28									
RP19				3	1				
RP11	1								
RP13	2								
RP38				1					
RP1									
RP8									
RP40				1	1				
Percent	45	35	20	70	30		20	70	10

[a] Numbers refer to the number of cases in which isolates were simultaneously obtained in cultures of samples from the two noted anatomical locations. Relatedness was assessed by the computed S_{AB}s. An S_{AB} of 1.00 represented identical strains (Ident.), an S_{AB} of 0.90 to 0.99 represented highly similar (but nonidentical) strains (Highly sim.), and an S_{AB} of less than 0.90 represented unrelated strains (Unrel.). Percent refers to proportion of cases in the different categories.

populations colonizing an individual, but it now seems less likely that progressive changes accompany sequential episodes of yeast vulvovaginitis.

The relationship of vulvovaginal isolates to isolates from other body locales. We previously demonstrated (18) that of 11 healthy women simultaneously carrying oral and vulvovaginal *Candida* spp., 7 (64%) carried unrelated strains in the two body locations, while 4 (36%) carried highly similar but nonidentical substrains. In no case were strains from the two body locations identical. In contrast, all (100%) pairs of simultaneous isolates from the vulva and vaginal canal were identical in the same study (18). These results suggested that in a majority of cases oral and vulvovaginal strains were unrelated and that in a minority of cases different strains or substrains of a general infecting clone were more highly adapted to alternative body locations, and in a commensal situation, the strains or substrains maintained their apparent independence. However, here we have found that of 20 simultaneous oral and vulvovaginal isolates from 10 RPs, 20% were unrelated, 35% were highly similar but nonidentical, and 45% were identical. Therefore, while simultaneous oral and vaginal commensal isolates from healthy individuals rarely appear to be identical, 45% of simultaneous isolates from RPs are identical. This difference has two alternative explanations. First, the pathogen load at the site of a vulvovaginal infection may be so great at times that the infecting strain colonizes additional body locations because of sheer number. Alternatively, if an infecting strain is eradicated from the vulvovaginal region by drug therapy, it may be recolonized by a substrain from another body location, hence the higher degree of genetic similarity between oral and vulvovaginal isolates in recurrent infections.

The relatedness of vulvovaginal isolates and isolates from the male partner. We previously found that in 8 of 10 cases in which *C. albicans* was simultaneously isolated from the vulvovaginal region of patients with a single episode of vaginitis and their male sexual partners, the isolates were either identical or highly similar but not identical (12). In a subsequent study (14) of two RPs, we demonstrated in one case that oral and glans penis isolates of the male partner were highly similar or identical to the vulvovaginal isolates of the patient. In a second RP we demonstrated that the male partner carried the same strain in his glans penis that the RP initially carried in her vulvovaginal region and an initially unrelated strain in his mouth which replaced the original vulvovaginal strain in a recurrence episode (14). Here, we have found that in all eight cases in which isolates were available from the male sexual partner, one or more of these isolates were either identical or highly similar to the vulvovaginal isolates from the patient. Together, these results demonstrate that in the majority of cases of both patients with single episodes of infection and patients with recurrent infections, the healthy male partners carry the same general strain responsible for the vaginal infection. It should be noted that this strain does not usually result in an infection in the male partner, demonstrating that the same strain is capable of being both a commensal organism and a pathogen. However, because of the number of cases in which the commensal substrain from the male differs from the infecting substrain from the female by, on average, one C1 band, we cannot rule out associated phenotypic differences. It is not clear if cocarriage of the same strain by sexual partners is a peculiarity of vaginitis patients or if cocarriage is also a characteristic of the commensal strains of healthy male and healthy female sexual partners as well. This latter possibility is being tested.

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