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Genetic Similarity and Maintenance of *Candida albicans* Strains from a Group of AIDS Patients, Demonstrated by DNA Fingerprinting

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By using the computer-assisted Dendron system to analyze the patterns of Southern blots probed with the repetitive sequence Ca3, we have compared oral isolates of *Candida* spp. from a group of 11 nonhospitalized patients with AIDS suffering from recurrent episodes of oral thrush in Leicester, England, with oral isolates from a group of control individuals. Genetic diversity among the AIDS strains was significantly reduced compared with that of control strains. In addition, the same strains persisted through recurrent infections in patients with AIDS. Although AIDS strains were genetically less diverse than either control strains or oral commensal strains analyzed in previous studies, the majority did not form a genetically distinct group. The results of this study suggest that in the majority of patients with AIDS in this group from Leicester, original commensal strains were replaced, replacement occurred early in the manifestation of AIDS, and replacement occurred only once.

Oral defense mechanisms are impaired in patients with AIDS, and such patients are therefore susceptible to infection by a number of opportunistic organisms, including *Candida albicans* (2, 4, 20). Since *C. albicans* is a commensal strain in the oral cavities of healthy individuals (5), it has been assumed that commensal strains are the agents of subsequent infections in compromised hosts (8, 17, 18). In support of this assumption, it was recently reported that oral isolates of *C. albicans* from patients with AIDS were similar to oral isolates from healthy individuals not only for a number of phenotypic characteristics but also in the diversity of restriction fragment length polymorphisms and in the frequency of occurrence of a 3.7-kb versus a 4.2-kb *Eco*RI fragment of *C. albicans* DNA encoding 25S rRNA (19). However, a number of other studies have indicated that strains infecting patients with AIDS differ from commensal strains in the frequency of the serotype B phenotype (for a review, see reference 6) as well as phenotypes discriminated by other serological markers (1).

If the strains of *C. albicans* infecting the oral cavities of patients with AIDS were identical to those present as commensal strains in the patients' mouths prior to infection with human immunodeficiency virus, then the level of genetic diversity among AIDS and commensal isolates should be equal. The resolving power of the methods used by Whelan et al. (19) may not have been great enough to test this point. In their study, they found that among isolates from both healthy patients and those with AIDS, 51% of all isolates exhibited identical restriction fragment length polymorphism patterns, and Southern blot hybridization with the 25S rRNA probe resolved only two alternative bands.

Recently, computer-assisted methods were developed for assessing genetic relationships among *C. albicans* isolates by calculating similarity coefficients (S_{AB}) between complex patterns generated by Southern blot hybridization with the fingerprinting probe Ca3. These methods have been demonstrated to provide a high degree of resolution for assessing genetic relationships within and between groups of strains (10, 12, 14). By using the method in a preliminary analysis of nine nonhospitalized patients with AIDS who suffered from oral thrush in Leicester, England, we found an unusually low level of genetic diversity among the *Candida* isolates obtained from them (6). In the present study, we have extended this analysis to include additional patients and sequential isolates from half of the patients, and we have compared the strains infecting these patients with a group of commensal isolates obtained during the same time period from nonhospitalized patients at the same hospital in Leicester. The results demonstrate that, at least in this group of patients with AIDS, there is a significant reduction in genetic diversity among the infecting strains, and, as others have found (2a, 19), the same strains persist through recurrent infections in the patients. Although there was a marked decrease in genetic diversity among the AIDS isolates, a comparison of these strains with control isolates and both commensal and pathogenic isolates from previous studies demonstrated that the AIDS isolates did not form a genetically distinct group. The results suggest that in most patients with AIDS in the group from Leicester, there was replacement of the original oral commensal strains, replacement occurred early in the manifestation of AIDS, and replacement probably occurred only once.

MATERIALS AND METHODS

Sampling procedures and stock maintenance. Sterile swabs were drawn over the posterior palates and tonsils of both control patients and patients with AIDS. Swabs were then transported in Stuart's transport medium (Medical Wire and Equipment, Ltd., Corsham, Wiltshire, England) to a microbiology laboratory, where the samples were plated on Sabouraud agar. Slants of each isolate were then mailed to Iowa City, Iowa, for fingerprinting. All patients with AIDS suf-

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fered from recurrent episodes of oral candidiasis and were treated as outpatients. Samples from control patients who were treated in the asthma clinic of the same hospital were taken once in the same time period as those from the patients with AIDS, and control patients exhibited no signs of oral thrush at the time of sampling. Patients with AIDS were all male, and the average age at the time of sampling was 35 ± 7 years; the control patients were 37% male and 63% female, and the average age at the time of sampling was 49 ± 12 years. Patients with AIDS were coded A1 to A11, and control isolates were coded COD plus clinic number.

DNA fingerprinting, calculation of S_{AB}, and genesis of dendrograms. The methods of Schmid et al. (10) were employed. In brief, cells from each storage streak were grown in YPD medium (2% dextrose, 2% Bacto Peptone, 1% yeast extract) to early stationary phase, and DNA was prepared by the method of Scherer and Stevens (9). DNA was measured by ethidium bromide dot quantitation, digested with *Eco*RI enzyme, separated on an 0.8% (wt/vol) agarose gel, transferred to nitrocellulose membrane, and hybridized with the nick-translated probe Ca3 (7, 10, 13, 15, 16). Washed hybridization membranes were pressed against XAR-S film (Eastman Kodak Co., Rochester, N.Y.) with a Cronex Lightning-Plus intensifying screen (Dupont Co., Wilmington, Del.) in a lightproof box.

Southern blots of the different isolates were compared by using the Dendron computer program, which has been described in previous communications (10, 14). The method for calculating the similarity coefficient (S_{AB}) for the patterns of strains A and B has been previously described (10). If the patterns are identical, the S_{AB} is equal to 1.00. If one or more bands differ in size and/or intensity, there is a gradation of S_{AB} from 0.99 to 0.01, with decreasing similarity. An S_{AB} of 0.00 indicates that no bands in two patterns are of the same size or that one strain does not hybridize with Ca3.

Dendrograms based on S_{AB}s were generated through the Dendron program (10) by the unweighted pair group method (11). In dendrogram construction, the program first searches for the two strains with the highest S_{AB} and groups them into a unit with a branching point corresponding to their S_{AB}. The program then searches again for the strain-strain or strain-unit pair with the highest S_{AB} and connects it. The process continues until all isolates are connected. It should be noted that a branch point connecting a unit and a strain is determined by the average S_{AB} between each member of the unit and another strain or unit, and is therefore not as accurate a measure of S_{AB} as that determined between individual strains.

Mathematical model of relationships among genetically unseparated groups of isolates. If isolates from two groups, A and B, containing n_A and n_B isolates, respectively, are not genetically separated, the probability that an isolate in group A will be most closely related to another isolate in group A [$p_{(A\text{-}A)}$] is given by the formula $p_{(A\text{-}A)} = (n_A - 1) \times (n_A - 1 + n_B)^{-1}$, and the probability that a strain in group B will be most closely related to another isolate in group B [$p_{(B\text{-}B)}$] is $p(B\text{-}B) = (n_B - 1) \times (n_B - 1 + n_A)^{-1}$.

The expected number of cases in which strains of one group will be most closely related to another member of that group is $p_{(A\text{-}A)} \times n_A$ for group A and $p_{(B\text{-}B)} \times n_B$ for group B. If there is no genetic separation between groups A and B, the total expected number of cases (N_m) in which members of the two groups are more closely related to another member of their own group than to members of the other group is $N_m = p_{(A\text{-}A)} \times n_A + p_{(B\text{-}B)} \times n_B)$, and the frequency of these events [$p_{(A\text{-}A,B\text{-}B)}$] is computed by the formula $p_{(A\text{-}A,B\text{-}B)} = N_m \times (n_A + n_B)^{-1}$.

Digitizing and "neighboring" with Dendron. In the automated Dendron system, gels are digitized into the Dendron data file in a Macintosh II computer with a Sharp scanner. The digitized gels can then be processed and the lanes can be rearranged through a "neighboring" program in the Dendron software package.

Species typing by sugar assimilation patterns. Select isolates were typed for species by sugar assimilation patterns with a commercial kit (API 20C) purchased from Analytab Products (Plainview, N.Y.). Typing was performed twice.

RESULTS

Fingerprinting with Ca3. The Ca3 probe hybridizes to between 15 and 25 fragments of *Eco*RI-digested *C. albicans* DNA and to all but one *C. albicans* chromosome (7, 10, 15). We previously demonstrated that Ca3 fulfills the basic requirements of an effective fingerprinting probe, generating stable Southern blot patterns within a strain over hundreds of generations but generating different patterns for the majority of unrelated strains (10). For a fingerprinting probe to be effective, patterns must be highly reproducible, and this is demonstrated within a single blot for the laboratory strains 3153A and AIDS isolates A7-881 and A3-881 (Fig. 1A) and between repeat blots for laboratory strains 3153A and LS-O and AIDS strains A8-891, A11-891, A9-891, A10-891, A5-892, A1-891, and A4-891 (Fig. 1A and B). Repeat samples of the same strain represent independent DNA preparations.

Relatedness of *Candida* strains in the Leicester AIDS group. In two previous studies (10, 14), we demonstrated that the average S_{AB} between unrelated oral commensal strains of *C. albicans* was 0.69 ± 0.08 (n = 10) and 0.67 ± 0.11 (n = 18), respectively. The 16 control isolates in the study reported here conformed to this level of unrelatedness (Fig. 2A). The S_{AB}s ranged between 0.50 and 0.89, and the average S_{AB} was 0.65, with a standard deviation of ±0.08. Only two pairs of control isolates (COD67 and COD33; COD72 and COD99) had S_{AB}s higher than 0.85. The relationships between 11 *Candida* isolates, each from a different patient with AIDS, differed markedly from those of control isolates. The S_{AB}s ranged between 0.0 and 1.0 (Fig. 2B), and the average S_{AB} was 0.51, with a standard deviation of 0.36, 4.5 times the standard deviation for control isolates. This expanded standard deviation resulted primarily from an extremely unrelated cluster of isolates from patients A1 and A3 (Fig. 2B). Isolates from these two patients exhibited Southern blot hybridization patterns which included only four to six primarily faint bands (Fig. 1), suggesting that they represented a species other than *C. albicans*. This result was confirmed by the sugar assimilation patterns of these strains, which did not conform precisely to the patterns of any known *Candida* species. When the average S_{AB} was computed for the *C. albicans* strains from the remaining nine patients with AIDS, the standard deviation decreased to 0.121 but the average S_{AB} increased to 0.75.

In contrast to the low level of clustering at S_{AB}s ≥ 0.85 observed among the control isolates (Fig. 2A), there was significant clustering among the AIDS isolates (Fig. 2B). Isolates from five different patients with AIDS (A4, A5, A7, A9, and A10) clustered in a large group in the middle of the dendrogram in Fig. 2B, and isolates from four additional patients with AIDS clustered in two additional groups (A2 and A6; A1 and A3). Therefore, while only 4 of the 16 control isolates (25%) clustered, 9 of the 11 AIDS isolates

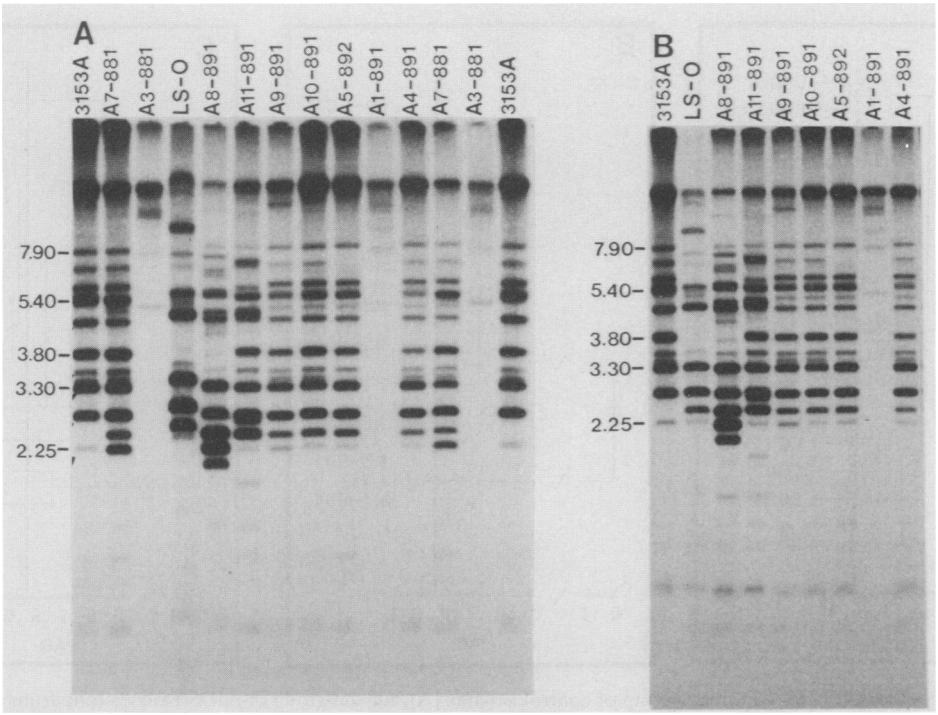

FIG. 1. Repeat Southern blots of AIDS isolates probed with Ca3, demonstrating reproducibility of patterns between lanes in a single blot and between lanes in different blots. 3153A, reference laboratory strain; LS-O, laboratory strain Odds; A7-881, A3-881, A8-891, A11-891, A9-891, A10-891, A5-892, A1-891, and A4-891, isolates from patients A7, A3, A8, A11, A9, A10, A5, A1, and A4, respectively. Repeat blots included independently isolated DNA in order to demonstrate the reproducibility of a pattern for a single strain. Estimated molecular sizes, in kilobases, for select bands of the 3153A pattern are presented to the left of each blot. Dates of isolation are provided in Table 1.

clustered. To test the significance of this difference, a z test was applied to the percentage of comparisons within each group yielding S_{AB}s ≥ 0.85 between isolates from different individuals. The difference was found to be significant, with $P < 0.001$.

Some isolates from individual patients with AIDS exhibited apparently identical Ca3 hybridization patterns (e.g., A2-901 and A6-901), while in other cases (e.g., A4-891, A7-881, and A9-891) the patterns were similar but nonidentical, differing by the presence or absence of one or two bands or the intensities of one to three bands. By using a neighboring program in the Dendron software package, the digitized fingerprints of laboratory strain 3153A and isolates A4-891, A7-881, and A9-891 have been positioned in neighboring lanes for comparison (Fig. 3). In the dendrogram in Fig. 2B, isolates A4-891 and A7-881 are connected at an S_{AB} of 0.88. In the neighbored patterns in Fig. 3 (lanes 2 and 3), A4-891 exhibits reduced intensity of two medium-molecular-weight bands and one low-molecular-weight band compared with A7-881. Strains A7-881 and A9-891 are connected at an S_{AB} of 0.88 (Fig. 2b). A9-891 possesses a high-molecular-weight band that A7-881 lacks, but the intensity of one of its low-molecular-weight bands is reduced compared with that of A7-881 (Fig. 3, lanes 3 and 4). A4-891 and A9-891 are connected at an S_{AB} of 0.94 (Fig. 2b). A4-891 lacks a high-molecular-weight band which is present in A9-891 (Fig. 3, lanes 2 and 4).

Sequential isolates from the same patients with AIDS are highly similar. For 6 of the 11 patients with AIDS, multiple *Candida* isolates were obtained over periods ranging from approximately 1 month to 1 year (Table 1 and Fig. 2C). In five of the six cases, the S_{AB}s were 0.89 or greater. However, in two of the six cases (A3-881 and A3-901), the S_{AB} was only 0.75. However, this low S_{AB} is misleading since, as mentioned previously, the Ca3 hybridization patterns for these isolates, as well as for A1-891, contained very few bands (Fig. 1) resulting in substantive decreases in the S_{AB} with slight differences in pattern. Therefore, in all cases sequential isolates from the same individual were highly similar. By using the neighboring program in the Dendron package, the digitized fingerprints of strain 3153A and isolates A5-881, A5-891, and A5-901 have been positioned in neighboring lanes for comparison in Fig. 4. The average S_{AB} among sequential isolates in this case was 0.95 ± 0.03 (Table 1). The patterns of A5-881, A5-891, and A5-901 corresponded for both band position and band intensities, even in the case of A5-901, which was underloaded in the original gel (Fig. 4).

To demonstrate further the similarity of sequential isolates, we searched the similarity matrix in the Dendron data file containing the S_{AB}s between all fingerprinted isolates in this study for the number of cases in which an isolate from a patient with AIDS had as its closest related counterpart another isolate from the same patient. Of the 16 isolates, which were obtained by multiple samplings from the same patients, 10 had an isolate from the same patient as its closest counterpart. In a z test in which this value was compared with the value expected if all AIDS isolates randomly associated with all patients with AIDS, the null hypothesis was rejected by $P < 0.005$.

Similarity between AIDS and control isolates. In order to assess the relationships between AIDS and control isolates, a combined dendrogram was generated (Fig. 5). Only a single strain from any one patient with AIDS was incorporated. Three "loose" clusters (with S_{AB}s ≥ 0.72) contained exclusively 10 of the 16 control isolates (COD33 and COD67;

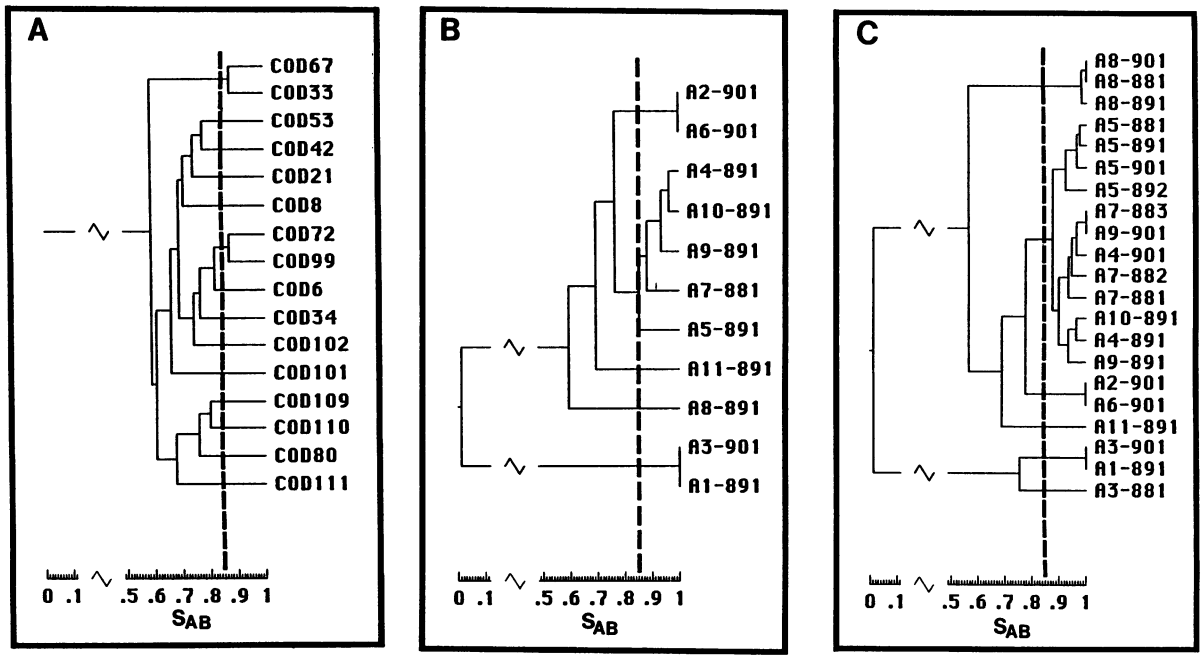

FIG. 2. Dendrograms based on S_{AB}s for the group of control isolates (A), for the group of AIDS isolates (one from each patient) (B), and for all AIDS isolates (C). A dashed line is drawn at $S_{AB} = 0.85$ to denote an arbitrary threshold for closely related clusters of isolates.

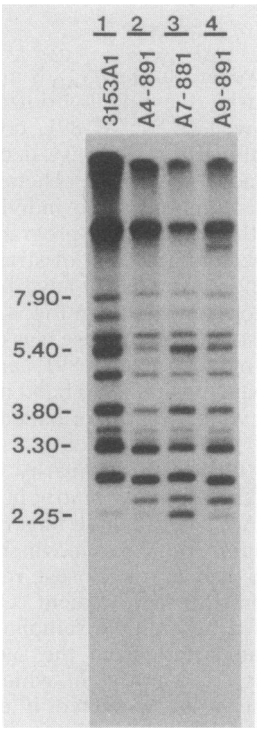

FIG. 3. Comparison of the highly similar Ca3 fingerprinting patterns for isolates A4-891, A7-881, and A9-891. Because the original blot did not have the three test patterns as neighbors, the gel image was digitized into the Dendron data file, and through a neighboring program, the lanes were placed alongside each other. No changes other than neighboring were performed. Estimated molecular sizes, in kilobases, for select bands of the 3153A pattern are presented to the left of the blot.

COD72, COD99, COD6, COD34, and COD102; COD109, COD110, and COD80). Three tight clusters (with S_{AB}s $\geq$ 0.85) contained exclusively 8 of the 11 AIDS isolates (A4-891, A10-891, A9-891, and A7-881; A2-901 and A6-901; A3-901 and A1-891).

Of the 11 AIDS isolates, only 3 (A11-891, A5-891 and A8-891) had control isolates (COD8, COD21, and COD111)

TABLE 1. Dates of isolation and the S_{AB}s for sequential isolates from each patient with AIDS

Patient	Isolate	Date of isolation (mo/day/yr)	S_{AB} between isolates (mean ± SD)[a]
A1	A1-891	03/08/89	—
A2	A2-901	01/29/90	—
A3	A3-881	12/06/88	0.75
	A3-901	01/01/90	
A4	A4-891	03/08/89	0.96
	A4-901	01/01/90	
A5	A5-881	11/29/88	0.95 ± 0.03
	A5-891	02/20/89	
	A5-892	03/08/89	
	A5-901	01/01/90	
A6	A6-901	02/06/90	—
A7	A7-881	08/15/88	0.94 ± 0.01
	A7-882	11/08/88	
	A7-883	12/06/88	
A8	A8-881	11/15/88	0.99 ± 0.01
	A8-891	01/13/89	
	A8-901	01/01/90	
A9	A9-891	03/08/89	0.89
	A9-901	01/01/90	
A10	A10-891	03/08/89	—
A11	A11-891	02/17/89	—

[a] Standard deviations were calculated only when more than two isolates were compared. The mean S_{AB} between all isolates was 0.91 ± 0.09. Dashes represent single-isolate situations.

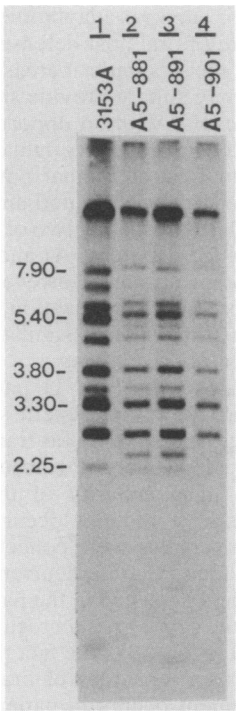

FIG. 4. Comparison of Ca3 fingerprinting patterns for sequential isolates from patient A5. A5-881, A5-891, and A5-901 were isolated on 29 November 1988, 20 February 1989, and 1 January 1990, respectively. Lanes were neighbored as in Fig. 3. Estimated molecular sizes, in kilobases, for select bands of the 3153A pattern are presented to the left of the blot.

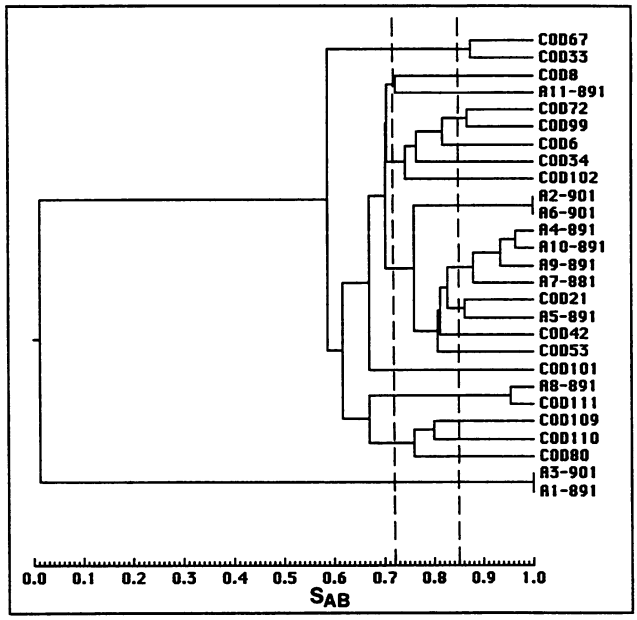

FIG. 5. Combined dendrogram of AIDS isolates (one per patient) and control isolates in this study. Dashed lines are drawn at S_{AB} = 0.72 and S_{AB} = 0.85 to denote arbitrary thresholds for "loose" and "tight" clustering, respectively. In constructing this combined dendrogram, A5-891 was connected to its most similar counterpart, COD21, and this unit was in turn connected to the unit containing A4-891, A10-891, A9-891, and A7-881 at an S_{AB} of 0.82. Because branching points are determined by the average S_{AB} between units, the S_{AB} between A5-892 and the four other similar AIDS isolates, A4-891, A10-891, A9-891, and A7-881, deduced from the branch point in this composite dendrogram differs slightly from the S_{AB} deduced from the branch point in the dendrogram generated exclusively for AIDS isolates (Fig. 2B).

as their most similar counterparts, while 8 had AIDS isolates as their most similar counterparts (Fig. 5). Of the 16 control isolates, only 5 (COD8, COD21, COD42, COD53, and COD111) had AIDS isolates or clusters containing predominantly AIDS isolates as their most similar counterparts, while 11 had control isolates or clusters containing predominantly control isolates (Fig. 5). When we determined the closest matches in a matrix of similarity values of the combined AIDS and control isolates, 20 were most closely related to members of their own group. However, in a z test the difference between the observed associations and random association was only of a marginal significance ($P < 0.05$ and $P < 0.10$, using one-sided and two-sided percentage points of the normal distribution, respectively).

Finally, we compared the 11 AIDS and 16 control isolates with 87 additional oral isolates which had been processed by Dendron in previous studies and stored in the Dendron data file. These additional oral isolates were from (i) healthy individuals, (ii) immunocompetent patients with oral lesions, (iii) immunosuppressed hospitalized patients, and (iv) additional patients with AIDS who were from Iowa. Since the combined dendrogram was quite extensive, containing 114 individual isolates, only a portion of it is shown in Fig. 6. Five of the 11 Leicester AIDS isolates (A7-881, A10-891, A4-891, A9-891, and A5-891) and 1 of the 16 Leicester control isolates were contained within a single large cluster with S_{AB}s $\geq$ 0.85, which also included 16 additional oral isolates (Fig. 6). This represented by far the largest single cluster in the composite dendrogram, including 19% of all compared strains. The Leicester AIDS isolates were members of this highly similar group more often than their commensal counterparts or isolates from oral lesions of individuals not suffering from AIDS. While 45% of the Leicester AIDS isolates were members of this group, only 18% (10 of 57) of additional commensal isolates, 14% (6/43) of oral lesion isolates, and 6% (1 of 16) of Leicester control isolates were members. A z test demonstrated that the percentage of isolates in the cluster was significantly higher among AIDS isolates than among commensal or oral lesion isolates ($P < 0.05$).

Four of the remaining six AIDS isolates not in this cluster distributed randomly in the composite dendrogram. However, the two related isolates which exhibited dramatically reduced banding patterns (A1-891 and A3-881) remained dissimilar to all of the other isolates in the composite dendrograms, with S_{AB}s close to zero between this group and the 112 other oral isolates in the dendrogram.

DISCUSSION

We have employed the probe Ca3 and the computer-assisted Dendron system to examine genetic diversity among oral isolates of *Candida* spp. from patients with AIDS and from control individuals from whom samples were taken during the same time period in the same hospital in Leicester. In a previous characterization of the Ca3 probe, we demonstrated that it effectively discriminates, in more than 98% of all comparisons, between any two randomly selected strains (10) and therefore provides the degree of resolution

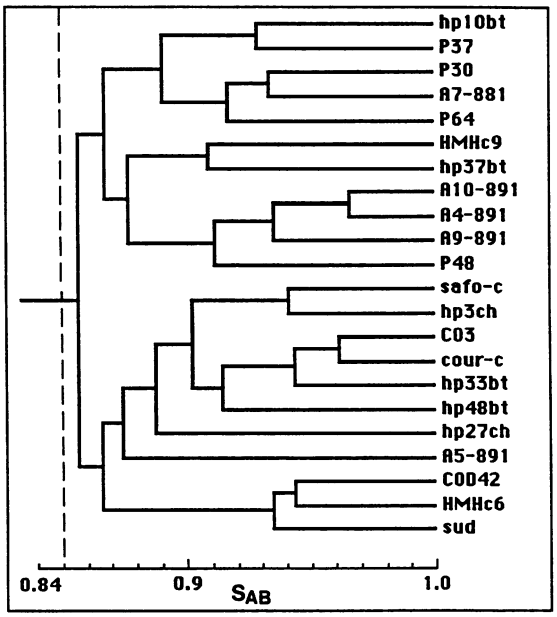

FIG. 6. Cluster of highly similar isolates windowed from a much larger combined dendrogram of 114 oral isolates, including the 11 Leicester AIDS strains, the 16 Leicester control strains, and 87 commensal and pathogenic strains from the oral cavity, analyzed in previous studies and stored in the Dendron data file. This cluster of isolates with S_{AB}s of more than 0.85 includes five highly similar Leicester AIDS strains (A7-881, A10-891, A4-891, A9-891, and A5-891), one Leicester control strain (COD42), nine oral commensal strains from healthy individuals in the Iowa City, Iowa, area (hp10bt, HMHc9, hp37bt, hp3ch, hp33bt, hp48bt, hp27ch, HMHc6, and CO3) and seven oral pathogens (P37, P30, P 64, P48 safo-c, cour-c, and sud). P64 was isolated from a patient with AIDS in Iowa City.

necessary to assess genetic diversity within and between AIDS and control isolates.

We have found that 5 of 11 patients with AIDS in Leicester were infected with genetically similar strains of *Candida* spp. and that 4 of the remaining 6 patients were infected with strains which separated into two genetically similar pairs. The genetic diversity within this set of AIDS strains was significantly lower than that of control strains. In addition, the AIDS isolates and the control isolates in this study formed genetically distinct groups. Both the difference in genetic diversity and the apparent genetic separation of the two groups strongly suggest that strain replacements occurred in the majority of patients with AIDS, a conclusion also made by Brawner and Cutler (1) on the basis of serological comparison of isolates from immunologically compromised patients (including patients with AIDS) and healthy individuals in the United States. However, our results also support the view of Whelan et al. (19) that *Candida* isolates from patients with AIDS do not represent a unique genetic group. Indeed, 5 of the 11 patients with AIDS in Leicester (45%) were infected with strains which clustered in a highly similar group of commensal and pathogenic strains in a composite dendrogram of 114 oral isolates. In contrast, only 1 of the 16 control individuals (6%) carried a commensal strain which was a member of this group. These strains which infect patients suffering from AIDS may therefore not have traits which provide them with selective advantages only in patients with AIDS but rather may have traits that lead to their superior adaptation to the oral cavity in general. The failure of oral host defenses in patients with AIDS, indicated by the dramatic increase in *Candida* sp. carriage (3, 17, 18), may simply provide strains more highly adapted to the oral cavity with an opportunity to compete with and subsequently replace the original oral commensal strains. Isolates from four additional patients with AIDS were found to be randomly distributed among other pathogenic and commensal isolates. Only two of the AIDS isolates from Leicester, those from patients A1 and A3, were clearly dissimilar from all other strains. However, both their dramatically reduced levels of hybridization with the species-specific Ca3 probe and their sugar assimilation patterns place them outside the species *C. albicans*.

The fact that isolates from 9 of 11 patients with AIDS clustered into three groups of high genetic similarity suggests that infection-causing isolates may be transmitted between patients with AIDS. However, if transmission is indeed responsible for the high similarity of the AIDS isolates assessed in this study, it did not occur during the time interval in which the samples were collected since we, like others (2a, 19), have found that in recurrent episodes of oral thrush, the same strains persisted in the patients with AIDS. The maintenance of the same strain in recurrent vaginitis has also been demonstrated (6a, 13). The fact that related strains persist through recurrent episodes of oral thrush suggests that if strain replacement occurs in patients with AIDS, it usually does so only once and probably very early after the initial manifestation of AIDS.

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