JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1999, p. 1035–1044 Vol. 37, No. 4
0095-1137/99/$04.00+0
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Development and Characterization of Complex DNA Fingerprinting Probes for the Infectious Yeast *Candida dubliniensis*

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Received 9 November 1998/Returned for modification 30 November 1998/Accepted 17 December 1998

Using a strategy to clone large genomic sequences containing repetitive elements from the infectious yeast *Candida dubliniensis*, the three unrelated sequences Cd1, Cd24, and Cd25, with respective molecular sizes of 15,500, 10,000, and 16,000 bp, were cloned and analyzed for their efficacy as DNA fingerprinting probes. Each generated a complex Southern blot hybridization pattern with endonuclease-digested genomic DNA. Cd1 generated an extremely variable pattern that contained all of the bands of the pattern generated by the repeat element RPS of *Candida albicans*. We demonstrated that Cd1 does not contain RPS but does contain a repeat element associated with RPS throughout the *C. dubliniensis* genome. The Cd1 pattern was the least stable over time both in vitro and in vivo and for that reason proved most effective in assessing microevolution. Cd24, which did not exhibit microevolution in vitro, was highly variable in vivo, suggesting in vivo-dependent microevolution. Cd25 was deemed the best probe for broad epidemiological studies, since it was the most stable over time, was the only truly *C. dubliniensis*-specific probe of the three, generated the most complex pattern, was distributed throughout all *C. dubliniensis* chromosomes, and separated a worldwide collection of 57 *C. dubliniensis* isolates into two distinct groups. The presence of a species-specific repetitive element in Cd25 adds weight to the already substantial evidence that *C. dubliniensis* represents a bona fide species.

Candida albicans is the most common yeast species carried as a commensal by healthy individuals (42) and the most common species isolated from yeast infections (21, 44). The next most common species include *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, and *Candida krusei* (9, 25). All of these species are readily distinguishable from *C. albicans* both phenotypically and genotypically. However, over the years there have been an ever-increasing number of reports of atypical strains of *C. albicans* that have been distinguished primarily by unusual fingerprint patterns (3, 18, 19, 22, 27, 45). Based upon phenotypic and genotypic differences, Coleman, Sullivan, and coworkers in Dublin, Ireland, were the first to argue that a majority of these atypical *C. albicans* isolates represented a separate species, which they named *Candida dubliniensis* (46).

Because strains of the putative species *C. dubliniensis* exhibited the two major phenotypic characteristics used to identify *C. albicans*, chlamydospore and true hypha formation (46), and because they hybridized with putative *C. albicans*-specific fingerprinting probes (3, 22, 45, 46), there has been some hesitation in considering them members of a bona fide species separate from *C. albicans*. However, the fact that several DNA typing methods, including species-specific probes (3, 22, 45, 46), multilocus enzyme electrophoresis (3, 12, 27, 47), randomly amplified polymorphic DNA (45, 46), electrophoretic karyotyping (46), oligonucleotide fingerprinting probes (45, 46), microsatellite DNA analysis (20), and restriction fragment length polymorphism without probes (46), discriminated these strains from typical *C. albicans* strains suggested that they indeed represent a separate species. For this reason, several methods based on phenotype have recently been developed to distinguish isolates of *C. dubliniensis* from isolates of *C. albicans*, including growth at 42°C (7, 33, 46, 47), β-glucosidase activity (3, 33, 47), colony color on CHROMagar (7, 33), and fluorescence after growth on methyl blue-Sabouraud agar (33). However, none of these methods appear to definitively identify all *C. dubliniensis* isolates.

If *C. dubliniensis* is a bona fide species, it should contain species-specific repetitive elements dispersed throughout the genome, as has been demonstrated for *C. albicans* (28, 31, 40), *C. tropicalis* (11, 41), *C. glabrata* (15), *C. krusei* (4) and *C. parapsilosis* (7a). Demonstration of such species-specific sequences in the *C. dubliniensis* genome would not only reinforce the arguments by Sullivan and Coleman (48) that it is a bona fide species but also provide the basis for a DNA fingerprinting system (39). We therefore screened for and cloned three DNA fingerprinting probes that included moderately repetitive elements. One of them, Cd25, not only proved to be effective in broad epidemiological studies but also contained one or more *C. dubliniensis*-specific sequences dispersed throughout the genome, supporting the argument that *C. dubliniensis* is a distinct species.

MATERIALS AND METHODS

Strains and growth conditions. The *C. dubliniensis* strains used in this study are listed in Table 1. Each isolate was stored at room temperature on a YPD agar slant (2% glucose, 2% Bacto Peptone, 1% yeast extract, 2% agar) in a capped tube. For DNA extraction, the cells were transferred to an Erlenmeyer flask containing YPD medium, grown for 24 h at 25°C, and harvested. To assess the stability of Southern blot hybridization patterns over many generations, cells from a single clone were inoculated into YPD medium at an initial concentration of 5×10^4 per ml and grown to stationary phase (~24 h at 37°C). Stationary-phase cells were diluted in fresh medium, and the process was repeated. After 200 generations, the cells were harvested and plated on agar at low density and cells from nine colonies of each strain were removed and stored on YPD slants in capped tubes for further analysis.

Cloning of genomic fragments containing moderately repetitive sequences. Genomic DNA from *C. dubliniensis* d1558 (Table 1) was used to construct a λ phage genomic library as previously described for *C. albicans* (16, 28, 31, 40, 41). Briefly, the library was constructed in phage λ EMBL3 according to established protocols (29) with 9- to 23-kb fragments from a *Sau*3AI partial digest of genomic DNA size fractionated in a sucrose gradient (16). The library was amplified in *Escherichia coli* P2-392 and plated at a density of 10,000 plaques per

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TABLE 1. History of *C. dubliniensis* isolates used in the characterization of the cloned fingerprinting probes

Patient	Isolate	Date isolated (mo-day-yr)	Body location (pathology[a])	Place of collection[b]
1	P30	10-26-93	Oral (HIV$^+$)	Lausanne, Switzerland
	P5	11-23-92		
2	P14	03-12-93	Oral (HIV$^+$)	Lausanne, Switzerland
	Co4	09-26-93		
3	Co5	09-29-93	Oral (HIV$^+$)	Lausanne, Switzerland
4	d959	1986	Oral (DA)	Valencia, Spain
	d961	1986		
5	d1046	1986	Oral (DA)	Valencia, Spain
6	d1003	1986	Oral (DA)	Valencia, Spain
7	d950	1986	Oral (DA)	Madrid, Spain
8	d952	1986	Oral (DA)	Madrid, Spain
9	d895	06-12-85	Sputum (Hem)	RFH, London, UK
	d872	06-18-85	Fecal (Hem)	
	d879	06-19-85	Oral (Hem)	
10	d457	11-26-85	Fecal (Hem)	RFH, London, UK
	d251	11-26-85		
11	d456	1986	(Hem)	RFH, London, UK
12	d681	12-04-86	Oral (Hem)	RFH, London, UK
	d710	04-14-86	Oral (Hem)	
	d675	04-22-86	Fecal (Hem	
	d632	04-24-86	Oral (Hem)	
13	d88029	1988	Oral (HIV$^+$)	ULMS, Leicester, UK
	d90006	1990		
14	d89014	1989	Oral (HIV$^+$)	ULMS, Leicester, UK
	d90013	1990	Oral (HIV$^+$)	
	d90015	1990	Oral (HIV$^+$)	
	d90033	1990	Oral (HIV$^+$)	
15	d78008	1978	Oral (Healthy)	ULMS, Leicester, UK
16	d81060	1981	Oral (Healthy)	ULMS, Leicester, UK
17	d88014	1988	Oral (HIV$^+$)	ULMS, Leicester, UK
18	d931111	1993	Oral (HIV$^+$)	CWHCT, London, UK
19	d931113	1993	Oral (HIV$^+$)	CWHCT, London, UK
20	d11	1985	Oral (ICU)	Bangor, UK
21	d514	1985	Vaginal (ICU)	Bangor, UK
22	d82006	1982	Oral (Dental)	USDS, Sheffield, UK
23	d73089	1973	Oral (Cardiac)	LGI, Leeds, UK
24	d75043	1975	Oral (Diabetes)	LGI, Leeds, UK
25	d75004	1975	Oral (Diabetes)	LGI, Leeds, UK
26	d920710	1992	Oral (HIV$^+$)	UCL, Brussels, Belgium
27	d930713	1993	Oral (HIV$^+$)	UCL, Brussels, Belgium
28	d932634	1994	Oral (GU)	GC, Turnhout, Belgium
29	d940613	1994	Oral (GU)	GC, Antwerp, Belgium
30	ANSA3	94–95	Oral (HIV$^+$)	UHA, Antwerp, Belgium
31	ANSA5	95–95	Oral (HIV$^+$)	UHA, Antwerp, Belgium
32	ANSA9	94–95	Oral (HIV$^+$)	UHA, Antwerp, Belgium
33	ANSA26	94–95	Oral (HIV$^+$)	UHA, Antwerp, Belgium
34	ANSA27	94–95	Oral (HIV$^+$)	UHA, Antwerp, Belgium
35	ANSA28	94–95	Oral (HIV$^+$)	UHA, Antwerp, Belgium
36	d930936	1993	Oral (HIV$^+$)	JWU, Frankfurt, Germany
37	d930953	1993	Oral (HIV$^+$)	JWU, Frankfurt, Germany
38	d931021	1993	Oral (HIV$^+$)	JWU, Frankfurt, Germany
39	B71507	1992	Oral (HIV$^+$)	TCDH, Dublin, Ireland
40	NCPF3949	88–94	Oral (HIV$^+$)	TCDH, Dublin, Ireland
41	B69819-1	1992	Oral (HIV$^+$)	HSL, Paris, France
42	d1419-2	1993	Oral (HIV$^+$)	CMI, Montpellier, France
43	d1558	1993	Oral (HIV$^+$)	CMI, Montpellier, France
44	d941841	1994	Oral (Hem)	UH, Nijmegen, Netherlands
45	d930822	1993	Oral (HIV$^+$)	VAH, San Antonio, Tex.
46	d109534	05-30-95	(Cancer)	MH, Bakersfield, Calif.
47	d1425638	12-19-95	(Cancer)	BIMC, New York, N.Y.
48	d81217	1981	Oral (NA)	BMC, Houston, Tex.
49	d126423	05-23-95	(Cancer)	UIHC, Iowa City, Iowa
50	10-25-94	Oral (HIV$^+$)		
	d166	04-02-95	Asymptomatic	
	d167	04-02-95	Asymptomatic	
	d168	04-02-95	Asymptomatic	
	d169	04-02-95	Asymptomatic	
	d170	04-02-95	Asymptomatic	
	d171	04-02-95	Asymptomatic	
	d173	04-02-95	Asymptomatic	UIHC, Iowa City, Iowa
	d174	06-21-95	Asymptomatic	
	d176	06-21-95	Asymptomatic	
	d178	10-04-95	Oral thrush	
	d180	10-04-95	Oral thrush	
	d181	10-04-95	Oral thrush	
	d182	10-04-95	Oral thrush	
	d184	10-04-95	Oral thrush	
51	d930664	1992	Oral (HIV$^+$)	LSP, Québec, Canada
52	d930666	1992	Oral (HIV$^+$)	LSP, Québec, Canada
53	M1	89–92	Oral (HIV$^+$)	FH, Victoria, Australia
54	M2	89–92	Oral (HIV$^+$)	FH, Victoria, Australia
55	M3	89–92	Oral (HIV$^+$)	FH, Victoria, Australia
56	M4	89–92	Oral (HIV$^+$)	FH, Victoria, Australia
57	M6	89–92	Oral (HIV$^+$)	FH, Victoria, Australia

[a] HIV$^+$, HIV positive; DA, drug addict; Hem, hematology; ICU, intensive care unit; GU, gynecology unit; NA, not available.
[b] RFH, Royal Free Hospital; ULMS, University of Leicester Medical School; CWHCT, Chelsea & Westminster Health Care Trust; USDS, University of Sheffield Dental School; LGI, Leeds General Infirmary; UCL, Université Catholique de Louvain; GC, Gynecology clinic; UHA, University Hospital of Antwerp; JWU, Johann Wolfgang Universität; TCDH, Trinity College Dental Hospital; HSL, Hôpital Saint Louis; CMI Clinique des Maladies Infectieuses; UH, University Hospital; VAH, VA Hospital; MH, Mercy Hospital; BIMC, Beth Israel Medical Center; BMC, Baylor Medical Center; UIHC, University of Iowa Hospital and Clinics; LSP, Laboratoire de Santé Publique; FH, Fairfield Hospital; UK, United Kingdom.

150-mm-diameter petri dish. Duplicate nitrocellulose filters were prepared from each plate (29) and incubated at 65°C for 20 min in a solution containing 1% bovine serum albumin, 7% sodium dodecyl sulfate (SDS), 0.5 M NaH_2PO_4 (pH 7.0), and 1 mM EDTA (6). One filter of each set (filter A) was hybridized overnight with a total of 10^6 cpm of random primer-labeled [^{32}P]dCTP-*Sau*3AI-*Taq*I-digested genomic DNA of *C. dubliniensis* per ml. The second filter (filter B) was hybridized overnight with 10^6 cpm of random primer-labeled [^{32}P]dCTP-ribosomal DNA of *C. albicans* per ml (43). The filters were washed first with a solution containing 5% SDS, 40 mM NaH_2PO_4, and 1 mM EDTA for 20 min and then with a solution containing 1% SDS, 40 mM NaH_2PO_4, and 1 mM EDTA for 20 min. The final filters were autoradiographed. Filter A was compared to filter B to eliminate plaques that contained ribosomal inserts. Clones that exhibited the more intense levels of hybridization expected of repeat sequences (16) and that did not hybridize to the ribosomal probe in filter B were selected, rescreened under the same conditions, and plaque purified (29). Since all clones selected as putative DNA fingerprinting probes in the first screen generated similar hybridization patterns with *Eco*RI-digested DNA from the same test strains of *C. dubliniensis*, they were deemed to represent the same family of repeats. To obtain additional unrelated clones from the library, a second screen was conducted under less stringent conditions. In the second screen, a representative sequence that had been selected in the first screen, Cd1, was used to probe a duplicate filter to exclude homologous clones. For the second screen, Cd1 was subcloned into the pGEM-3Zf(+) plasmid vector. Prehybridization (7 h at 65°C) and hybridization (overnight at 65°C) of the filters were conducted in a solution of 50 mM NaH_2PO_4 (pH 7.5), 50 mM EDTA, 0.9 M NaCl, 5% dextran sulfate, 150 μg of sheared denatured salmon sperm DNA per ml, and 0.3% SDS. The filters were washed at 45°C with a solution containing 0.3 M NaCl, 0.03 M sodium citrate (pH 7.0), and 0.2% SDS. In the second screen, clones hybridizing to Cd1 or ribosomal DNA were excluded.

Southern blot hybridization and computer-assisted analysis. Southern blot hybridization was performed as previously described (11, 15, 16, 30, 32, 34, 40). Three μg of genomic DNA from each isolate was digested with *Eco*RI (4 U/μg of DNA) for 16 h at 37°C. The digested DNA was electrophoresed overnight at 45 V in a 0.65% agarose gel for Cd1 and a 0.8% agarose gel for the other clones. DNA was transferred to a nylon membrane by capillary blotting (17). Digested DNA of reference strain M6 (Table 1) was run in the far-right lane of each gel to facilitate computer-assisted analysis. The membrane was hybridized with a randomly primed ^{32}P-labeled probe and autoradiographed as previously described (16, 32). Southern blots were then stripped of the initial radiolabeled probe by incubating them first in a solution of 0.4 M sodium hydroxide for 30 min at 45°C and then in a solution of 0.015 M NaCl, 0.0015 M sodium citrate, 0.2 M Tris-HCl (pH 7.5), and 0.1% (wt/vol) SDS for 15 min at room temperature.

To analyze gel patterns, the autoradiograms were digitized into the data file of the DENDRON software program version 2.0 (Solltech, Iowa City, Iowa) with a Scanjet IIcx flatbed scanner (Hewlett-Packard, Palo Alto, Calif.). Distortions in the gels were removed with the unwarping option of DENDRON, and the lanes and bands were automatically identified. Southern blot hybridization patterns were compared through a similarity coefficient (S_{AB}) based on band position

alone for every pair of patterns (isolates) according to the formula $S_{AB} = 2E/(2E + a + b)$, where E is the number of bands shared by strains A and B, a is the number of bands unique to A, and b is the number of bands unique to B. An S_{AB} of 1.00 represented identical patterns, an S_{AB} of 0.0 represented patterns with no correlate bands, and S_{AB}s ranging from 0.01 to 0.99 represented patterns with increasing proportions of bands at the same positions. Dendrograms based on S_{AB} values were automatically generated by the DENDRON program based on the unweighted-pair group method (35).

CHEF electrophoresis. Chromosomes were separated by contour-clamped homogeneous electric field (CHEF) electrophoresis. To generate spheroplasts, strains of *C. dubliniensis* and strain 3153A of *C. albicans* were grown overnight in YPD medium at 25°C. The cells were pelleted by centrifugation and washed twice with sterile water and once with 1 M sorbitol. The cells were then incubated for 30 min at room temperature in a solution containing 1 M sorbitol, 25 mM EDTA, and 50 mM dithiothreitol, harvested, washed with 1 M sorbitol, and resuspended at a concentration of 10^9 per ml in a solution containing 1 M sorbitol, 0.1 M sodium citrate (pH 5.8), 10 mM EDTA, and 0.4 mg of Zymolyase 100T (Seikagaku America, Rockville, Md.)/ml at 37°C for 30 min. The cells were then pelleted, washed twice with a solution containing 1 M sorbitol and 250 mM EDTA, and resuspended in that solution at a final density of 10^9 per ml. Agarose plugs were made by mixing in equal proportions the suspension of spheroplasts and a solution of 1% agarose (Low Melt Preparative Grade; Bio-Rad Laboratories, Hercules, Calif.) containing 10 mM Tris-HCl (pH 8.0) and 0.1 M EDTA. This mixture was poured into plug molds for the CHEF mapper (Bio-Rad Laboratories). The plugs were incubated in a solution containing 0.5 M EDTA, 1% Sarkosyl, and 5 mg of proteinase K/ml for 72 h at 37°C, washed five times with a solution containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and finally stored at 4°C. The plugs were loaded into wells of a 1% (wt/vol) agarose gel containing 22.5 mM Tris-HCl (pH 8.3), 22.5 mM boric acid, and 1 mM EDTA. The electrophoretic conditions providing the best separation of chromosomes were a multistate protocol run at 14°C at an angle of 120° and at a gradient of 4.5 V/cm. The pulse intervals were 120 s for the first 24 h and 240 s for the final 36 h. Following electrophoresis, the gel was stained with ethidium bromide, photographed, Southern blotted, and incubated with a particular DNA probe (29).

RESULTS

Cloning putative DNA fingerprinting probes. A library was constructed in λEMBL3 from a *Sau*3AI partial digest of genomic DNA from *C. dubliniensis* d1558 (Table 1). Digestion products ranged between 9 and 23 kb. The library was plated on five dishes, generating approximately 10,000 plaques per dish. The library on each plate was transferred to duplicate nitrocellulose filters and hybridized in parallel with either radiolabeled *C. dubliniensis* genomic DNA or a radiolabeled ribosomal DNA probe of *C. albicans*. Under nonsaturating conditions, clones containing repeat sequences generated stronger signals than clones containing unique sequences. This difference was the basis of the screen (8, 11, 15, 16, 28, 31, 38). Twenty clones which hybridized intensively with genomic DNA, but not with the ribosomal probe, were selected for further analysis. The screen was repeated to verify that each clone contained a nonribosomal repeat sequence. The 20 clones were plaque purified, labeled, and used to probe Southern blots of *Eco*RI-digested DNA of three unrelated test strains of *C. dubliniensis* to assess the effectiveness of the clones as fingerprinting probes. Of the original 20 clones tested, 19 (95%) generated complex Southern blot hybridization patterns that varied among the three test strains. However, all of the 19 clones generated similar patterns for a particular strain, suggesting that the clones contained one or more common sequences. To assess the relatedness of the 19 clones, each was digested with *Sal*I and *Eco*RI, electrophoresed in a 0.8% (wt/vol) agarose gel for 4 h, and stained with ethidium bromide. Seventeen different digestion patterns were generated, which suggested that 17 of the 19 related clones originated from different genomic sites or represented partial overlaps at the same sites. One representative of this family, Cd1, with an estimated size of 15,500 bp, was selected for further analysis.

To obtain additional, unrelated probes, a second screen was performed under the same conditions as the first, except that hybridization of the nitrocellulose membranes was performed at lower stringency. To exclude sequences homologous to Cd1, the Cd1 fragment was cloned into the pGEM-3Zf(+) plasmid vector and used to probe an additional filter in the screen. Of approximately 100,000 plaques, 100 displayed a signal of relatively high intensity and did not hybridize with either ribosomal DNA or the Cd1 plasmid. Of these, 26 were selected and analyzed. Fifteen generated complex polymorphic patterns when used to probe the three test strains of *C. dubliniensis*. Two general patterns were generated by the 15 probes, suggesting that two additional families of probes containing repetitive sequences were represented. One representative clone from each family, Cd24 and Cd25, were selected for further analysis. The estimated sizes of Cd24 and Cd25 were 10,000 and 16,000 bp, respectively.

Genetic variability displayed by the three selected probes. To test the effectiveness of the three cloned probes and cross-hybridization with *C. albicans* DNA, each clone was used to probe Southern blots of nine unrelated test isolates of *C. dubliniensis* and *C. albicans* 3153A (Fig. 1). The Cd1 probe generated between 9 and 15 bands ranging from 4.6 to 31 kb for each of the nine test strains (Fig. 1A). Cd1 generated significantly different patterns for all nine test strains, even the ones originating from patients treated in the same hospital (e.g., d88029 and d90015, and M3 and M4) (Fig. 1A). Cd1 generated two monomorphic bands (i.e., bands at the same molecular weight in the nine test strains) at 4.6 and 5.3 kb (Fig. 1A), as well as several moderately variable bands and several highly variable bands. In the dendrogram generated for Cd1 in Fig. 1A, the two pairs of isolates with the highest S_{AB}s (0.77 in both cases) were Co4 and M3, and Co5 and M4. The isolates in each pair were from different countries. The Cd1 probe generated three bands with *Eco*RI-digested DNA of *C. albicans* 3153A, a weak one at 31 kb and a weak one and a moderately intense one below 4.6 kb (Fig. 1A).

The Cd24 probe also generated significantly different patterns for all nine test strains (Fig. 1B). Cd24 generated four monomorphic bands, a few moderately variable bands, and several highly variable bands. In the dendrogram generated for Cd24 (Fig. 1B), the pair of isolates with the highest S_{AB} (0.89) was P30 and Co4. The Cd24 probe generated five moderately intense bands and two very weak bands with DNA of *C. albicans* 3153A (Fig. 1B).

The Cd25 probe also generated significantly different patterns for all of the nine test strains (Fig. 1C). Cd25 generated the most complex pattern of the three tested probes. The patterns contained 10 monomorphic bands as well as several moderately variable bands and several highly variable bands. In the dendrogram generated for Cd25 (Fig. 1C), the pair of isolates with the highest S_{AB} (0.98) was P30 and Co4, the same pair exhibiting the highest S_{AB} in the Cd24 dendrogram (Fig. 1B). Cd25 was the only probe of the three with no significant hybridization to DNA of *C. albicans* 3153A (Fig. 1C).

The patterns generated by Cd1, Cd24, and Cd25 were distinctly different. None of the monomorphic or highly variable bands were common, supporting the conclusion that the three probes were unrelated.

The relationship of Cd1 and the *C. albicans* repetitive element RPS. Two of the three probes, Cd1 and Cd24, hybridized with *Eco*RI-digested DNA of *C. albicans* 3153A (Fig. 1A and B). Since the *C. albicans* fingerprinting probes Ca3 (32) and 27A (31) have been demonstrated to cross-hybridize with *C. dubliniensis* DNA (3, 46, 47), we tested whether any of the three *C. dubliniensis* probes identified the same genomic fragments as Ca3 and 27A. The gel shown in Fig. 1 was reprobed with the C1 fragment of Ca3 (1, 14), which contains a portion

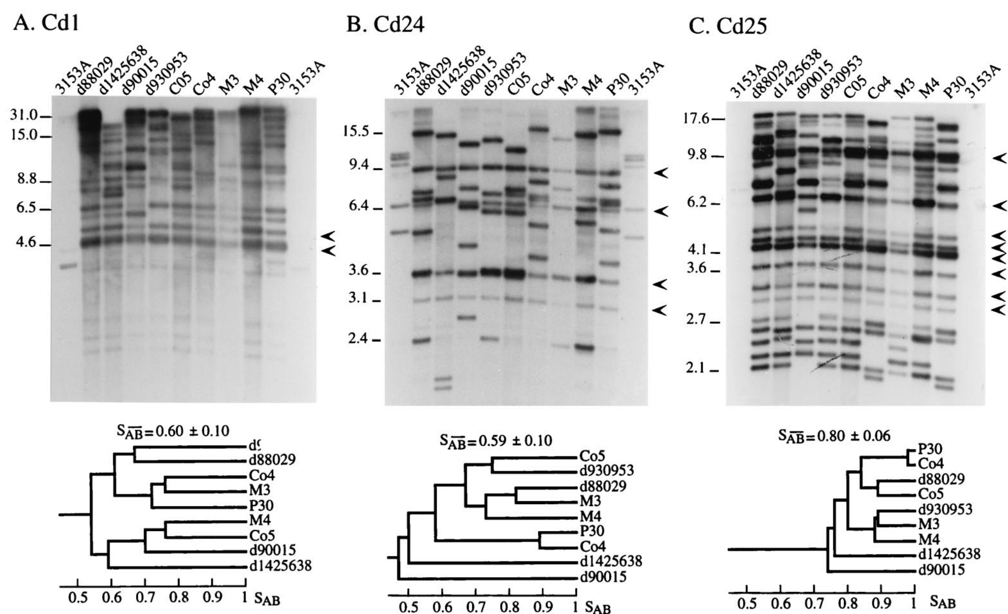

FIG. 1. Southern blot hybridization patterns and resulting dendrograms of *Eco*RI-digested DNA of nine *C. dubliniensis* isolates and *C. albicans* 3153A probed with Cd1(A), Cd24 (B), and Cd25 (C). The DNA was electrophoresed in a 0.65% agarose gel, and the Southern blot was sequentially hybridized with the three probes. The arrowheads to the right of each gel represent prominent invariant (monomorphic) bands. Key molecular sizes are presented in kilobases to the left of each gel. The origins of these test isolates are presented in Table 1. A dendrogram (shown below each gel) for each set of hybridization patterns was generated from the similarity coefficient (S_{AB}) computed for all possible pairs of the nine unrelated *C. dubliniensis* isolates. Since the M3 lane was underloaded, analyses of that pattern were performed on autoradiograms exposed for longer periods. The average S_{AB} is indicated at the top of each dendrogram.

of the *C. albicans* repetitive sequence RPS (13), with a cloned RPS element, RPS39 (27a), and with the probe 27A (31), which also contains an RPS element (27a). RPS39 has 99% homology with other published RPS sequences (27a). The probes C1, RPS39, and 27A all generated the same pattern for each of the nine *C. dubliniensis* test strains (Fig. 2B, C, and D, respectively). This pattern contained approximately 80% of the bands present in the respective Cd1 patterns (Fig. 2A).

While probes C1, RPS39, and 27A generated patterns of four to eight bands when hybridized to *Eco*RI-digested *C. albicans* DNA (Fig. 2A, B, and C, respectively), Cd1 generated only one moderately intense band and two very weak bands (Fig. 2A), none of which corresponded to the bands generated by C1, RPS39, and 27A. These results suggest that Cd1 contains a *C. dubliniensis*-specific sequence dispersed throughout the *C. dubliniensis* genome in close association with an RPS-like element but that the Cd1 probe itself does not contain an RPS sequence.

Cross-verification of the probes Cd1, Cd24, and Cd25. Since the three *C. dubliniensis* probes represent unrelated genomic fragments, they can be considered unrelated fingerprinting methods and can, therefore, be used to cross-verify efficacy by cluster analysis (11, 15, 26, 39, 49). Cd25 generated the most complex Southern blot hybridization patterns and the highest average S_{AB} when used as a probe to fingerprint the nine test isolates (Fig. 1A). It grouped isolates P30 and Co4 into the most related cluster, grouped M3 and M4 into a moderately related cluster, and identified d1425638 and d90015 as the most unrelated isolates in the collection. Cd24 also grouped isolates P30 and Co4 into the most related cluster, grouped M3 and M4 into a moderately related cluster, and identified d1425638 and d90015 as the most unrelated isolates in the collection. This was not the case for Cd1. Cd1 did not group P30 and Co4 into the most related cluster, did not group M3 and M4 in a moderately related cluster, and did not identify

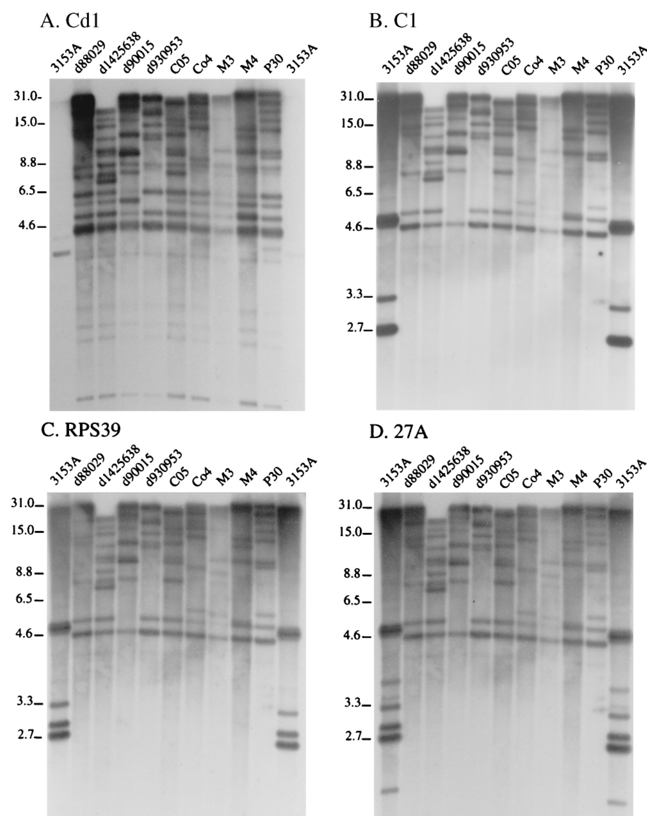

FIG. 2. Hybridization patterns of the nine test isolates of *C. dubliniensis* and *C. albicans* 3153A probed with the Cd1 probe (A), the C1 fragment of the *C. albicans* probe Ca3 (B) (1), the RPS39 element of *C. albicans* (C) (27a), and the *C. albicans* probe 27A (30). The origins of the test isolates are presented in Table 1. Molecular sizes are presented in kilobases to the left of each set of hybridization patterns.

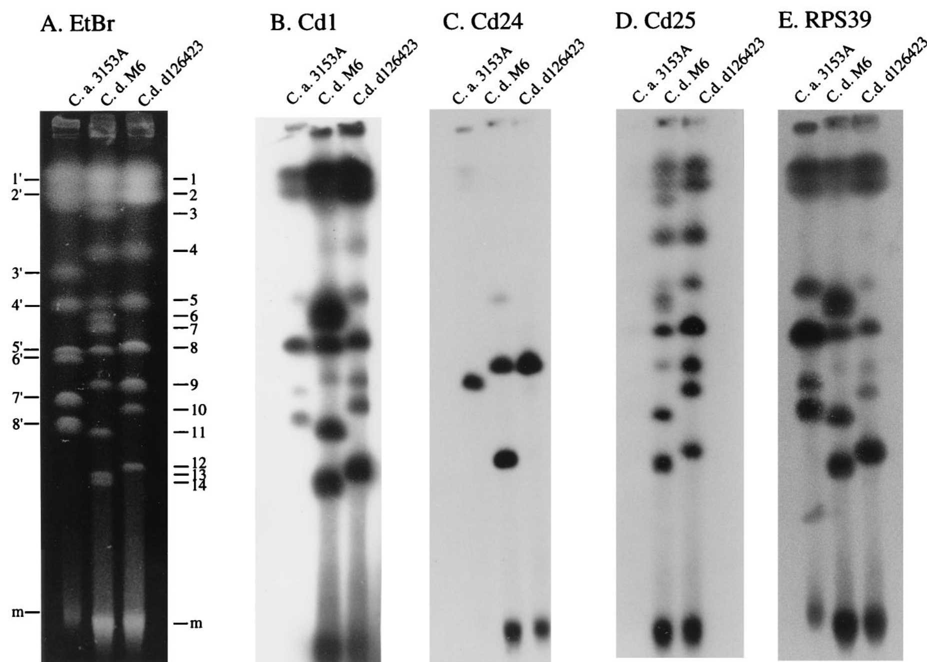

FIG. 3. Hybridization of CHEF-separated chromosomes of *C. dubliniensis* (C.d.) and *C. albicans* (C.a.) with the *C. dubliniensis* probes Cd1, Cd24, and Cd25 and the *C. albicans* repeat element RPS39. Chromosomes of *C. albicans* 3153A and *C. dubliniensis* M6 and d126423 were separated by CHEF, and the gel was stained with ethidium bromide (EtBr) (A). The gels were then Southern blotted and probed with Cd1(B), Cd24 (C), Cd25 (D), and RPS39 (E). *C. albicans* bands are numbered to the left of the EtBr-stained image, and *C. dubliniensis* bands are numbered to the right of the EtBr-stained image. m, minichromosomal band.

d1425638 and d90015 as the most unrelated isolates. These results demonstrate that relative parity exists between Cd25 and Cd24 as fingerprinting probes, but not between either of these probes and Cd1.

The distribution of sequences homologous to Cd1, Cd24, and Cd25 in the *C. dubliniensis* genome. Twelve chromosomal bands and one minichromosomal band were separated by CHEF from *C. dubliniensis* M6, and eight chromosomal bands and one minichromosomal band were separated from *C. dubliniensis* d126423 (Fig. 3A). Eight chromosomal bands and one minichromosomal band were separated from *C. albicans* 3153A (Fig. 3A). Cd1 hybridized to all chromosomal and minichromosomal bands of both *C. dubliniensis* strains (Fig. 3B). The degree of hybridization varied substantially among chromosomal bands. Cd1 also hybridized to all *C. albicans* chromosomal bands except band 3 (Fig. 1B). RPS39 also hybridized to all chromosomal bands of the two *C. dubliniensis* strains except band 4 and hybridized to all *C. albicans* chromosomal bands except band three (Fig. 3E). Moreover, the relative differences between the Cd1 hybridization intensities of the *C. dubliniensis* bands (Fig. 3B) were the same when RPS39 was used as a probe (Fig. 3E). These results demonstrate that Cd1 sequences are dispersed throughout the *C. dubliniensis* genome and are associated in all or a majority of cases with RPS sequences.

Cd24, on the other hand, hybridized to only two chromosomal bands and the minichromosomal band of *C. dubliniensis* M6 and to only one chromosomal band and the minichromosomal band of *C. dubliniensis* d126423 (Fig. 3C). Cd24 also hybridized to one chromosomal band of *C. albicans* 3153A (Fig. 3C). Therefore, although Cd24 generates a complex Southern blot hybridization pattern with *Eco*RI-digested *C. dubliniensis* DNA, it is distributed on only one to two chromosomes.

Cd25 hybridized to all chromosomal bands and the minichromosomal band of the two *C. dubliniensis* strains (Fig. 3D). Cd25, therefore, is distributed on all *C. dubliniensis* chromosomes. It hybridized to no *C. albicans* chromosomal bands (Fig. 3D), supporting the conclusion that Cd25 is the only *C. dubliniensis*-specific probe of the three clones analyzed.

Species specificity of Cd1, Cd24, and Cd25. Each of the three clones was used to probe Southern blots of *Eco*RI-digested DNA of 14 related yeast species (Fig. 4). Cd1 hybridized strongly with *C. dubliniensis* DNA, very weakly with *C. albicans* DNA, and not at all with the DNA of the remaining 12 species (Fig. 4A). Cd24 hybridized strongly with *C. dubliniensis* DNA, weakly with *C. albicans* DNA, and not at all with DNA of the remaining 12 species (Fig. 4B). Cd25 hybridized strongly with *C. dubliniensis* DNA but not at all with DNA of the remaining 13 species (Fig. 4C). When the *C. albicans* repeat element RPS39 was used to probe the same blot, it hybridized strongly with *C. dubliniensis* and *C. albicans* DNA, but not at all with DNA from the remaining 12 species (data not shown). These results demonstrate that Cd1 and Cd24 are *C. dubliniensis* and *C. albicans* specific, and that Cd25 is *C. dubliniensis* specific.

Stability of the DNA fingerprint patterns generated by Cd1, Cd24, and Cd25. The stability of the patterns generated by the three *C. dubliniensis* probes was first tested in vitro for three unrelated strains grown from single colonies for 200 generations. Cells from each of the final growth cultures were plated on agar, and cells from nine colonies of each strain as well as from each original clone were analyzed by Southern blot hybridization with the probes Cd1, Cd24, and Cd25. Stability proved to be both strain and probe related. The nine clones of strain d1419-2 obtained after 200 generations exhibited no differences from each other or from the original clone when fingerprinted with any of the three probes (Fig. 5A). The nine clones of strain B71507 exhibited no differences from each other or from the original clone with probes Cd24 or Cd25 but

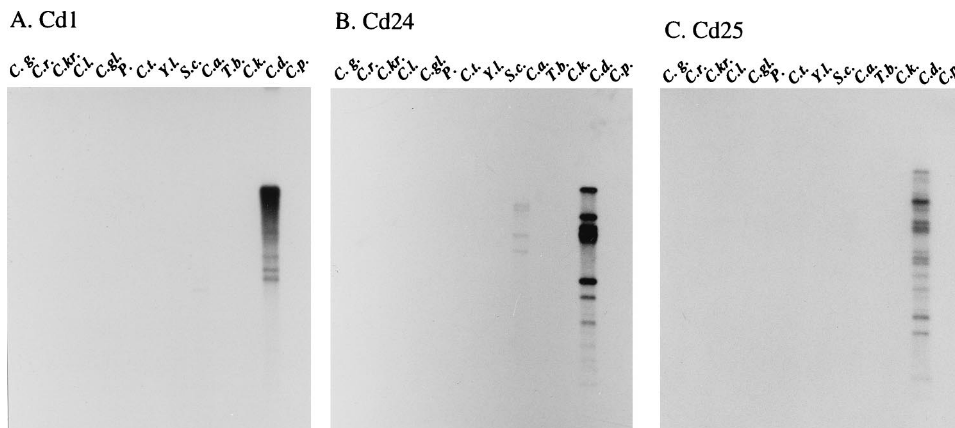

FIG. 4. Species specificity of the three *C. dubliniensis* probes. *Eco*RI-digested DNA of 14 different yeast species was sequentially probed with Cd1 (A), Cd24 (B), Cd25 (C), and the *C. albicans* repeat element RPS39 (D). Lanes: *C.g.*, *Candida guillermondii*; *C.r.*, *Candida rugosa*; *C.kr.*, *C. krusei*; *C.l.*, *Candida lusitaniae*; *C.gl.*, *C. glabrata*; *P.*, *Pichia* sp.; *C.t.*, *C. tropicalis*; *Y.l.*, *Yarrowia lipolytica*; *S.c.*, *Saccharomyces cerevisiae*; *C.a.*, *C. albicans*; *T.b.*, *Trichosporon beigelii*; *C.k.*, *Candida kefyr*; *C.d.*, *C. dubliniensis*; *C.p.*, *C. parapsilosis*.

displayed two patterns with probe Cd1 (patterns 1 and 2), one of which (pattern 1) was the same as that of the original clone (Fig. 5B). Pattern 1 differed from pattern 2 by an 8.8-kb band present in the latter but not in the former. The nine clones of strain Co5 exhibited no differences from each other or from the original clone with probe Cd24. However, the nine clones displayed three patterns (patterns 1, 2, and 3) when probed with Cd25 (Fig. 5C). One of the patterns (pattern 1) was the same as that of the original clone (Fig. 5C). The nine clones of strain Co5 exhibited eight different patterns (patterns 2 through 9) when probed with Cd1. All of these patterns differed from that of the original clone (pattern 1 [Fig. 5C]). These results demonstrate that Cd24 provides the most stable pattern, Cd25 provides the second most stable pattern, and Cd1 provides the least stable pattern over time in vitro. These results also demonstrate that strain d1419-2 has the most stable genotype and strain Co5 has the least stable genotype in vitro.

To examine the patterns generated by the three probes in vivo, 15 *C. dubliniensis* isolates were recovered from the oral cavity of a human immunodeficiency virus (HIV)-positive pa-

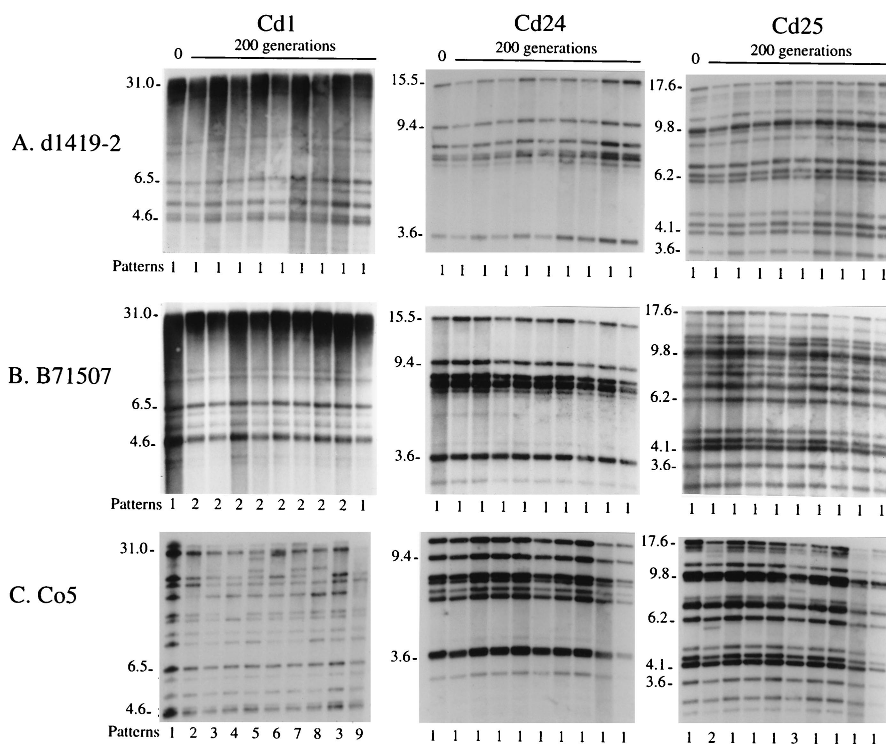

FIG. 5. In vitro analysis of the stability of the patterns generated by *C. dubliniensis* probes Cd1, Cd24, and Cd25. Southern blots of *Eco*RI-digested DNA from clones of *C. dubliniensis* d1419-2 (A), B71507 (B), and Co5 (C), at zero hours (0) and after 200 generations, were probed with Cd1, Cd24, and Cd25. Nine individual clones of each strain were selected randomly for analysis at 200 generations. Variant patterns are numbered at the bottom of each blot. Key molecular sizes in kilobases are presented to the left of each blot.

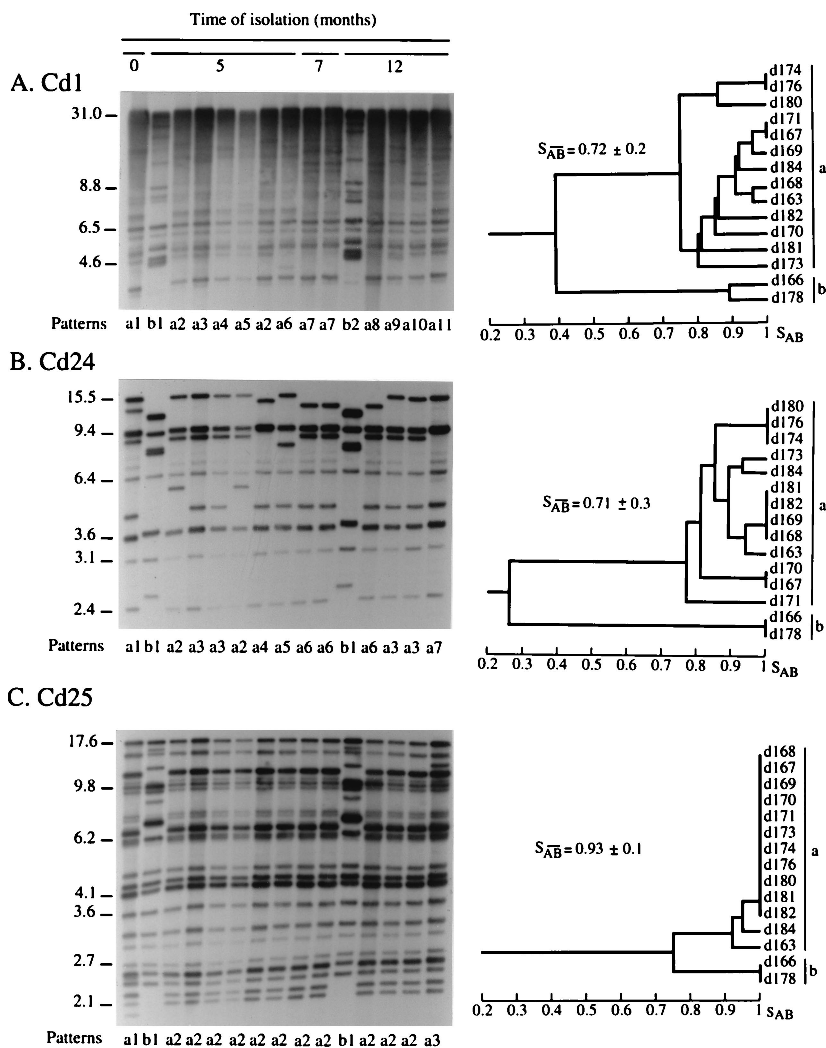

FIG. 6. The variability of probe-generated patterns in a population colonizing an HIV-positive individual over time. *Eco*RI-digested DNA from multiple isolates from patient 50 (Table 1) were probed with Cd1 (A), Cd24 (B), and Cd25 (C). The isolates were obtained at 0, 5, 7, and 12 months. The patient presented with oral thrush at 12 months. Variations in patterns are indicated at the bottom of each blot. This patient was infected by two different strains displaying the general genotypes a and b. Changes in the patterns are numbered in chronological order of occurrence. Molecular sizes are presented in kilobases to the left of the gels. On the right side of each hybridized Southern blot the representative dendrogram is generated. The average S_{AB} is presented at the top of each dendrogram.

tient at the University of Iowa Hospitals and Clinics over a 12-month period (Table 1, patient 50). One isolate was obtained at time zero, seven isolates were obtained at 5 months, two isolates were obtained at 7 months, and five isolates were obtained at 12 months. The last isolates, collected at 12 months, were obtained when the patient presented with his first episode of oral thrush. All isolates were analyzed by Southern blot hybridization with probes Cd1 (Fig. 6A), Cd24 (Fig. 6B), and Cd25 (Fig. 6C). All three probes separated the collection of 15 isolates into two groups distinguishable by the general fingerprint patterns. The first, group a, was composed of 13 isolates, and the second, group b, was composed of 2 isolates. The separation into two groups is evident in the dendrograms generated from the hybridization patterns of each probe (Fig. 6). The node separating group a and b isolates occurred at S_{AB}s of 0.39, 0.27, and 0.75 for probes Cd1, Cd24, and Cd25, respectively (Fig. 6). These node S_{AB}s are all below the average S_{AB} for unrelated isolates (Fig. 1). Probe Cd1 distinguished differences between the two b isolates, but probes Cd24 and Cd25 did not (Fig. 6). It should be noted that the time interval between collection of the two b isolates was 7 months (Fig. 6). Variability was observed in group a isolates with all three probes (Fig. 6). However, the least variability occurred in the Cd25 patterns. For the 13 isolates in group a, Cd1 distinguished 11 patterns, Cd24 distinguished 7 patterns, and Cd25 distinguished 3 patterns (Fig. 6, see the pattern analysis at the bottom of each gel). These results demonstrate that Cd25 provides the most stable pattern in vivo and in vitro, while Cd1 and Cd24, on the other hand, distinguish greater variability within a strain over time and are, therefore, superior for assessing microevolution.

Analysis of a broad collection of *C. dubliniensis* isolates with the probe Cd25. Because Cd25 was deemed the best probe for broad epidemiological studies, it was used to analyze the relatedness of 57 independent *C. dubliniensis* isolates collected in 11 countries (Table 1). Among the 57 isolates, Cd25 discriminated 53 different patterns (Fig. 7). Of the four pairs of identical isolates, three pairs consisted of isolates collected from

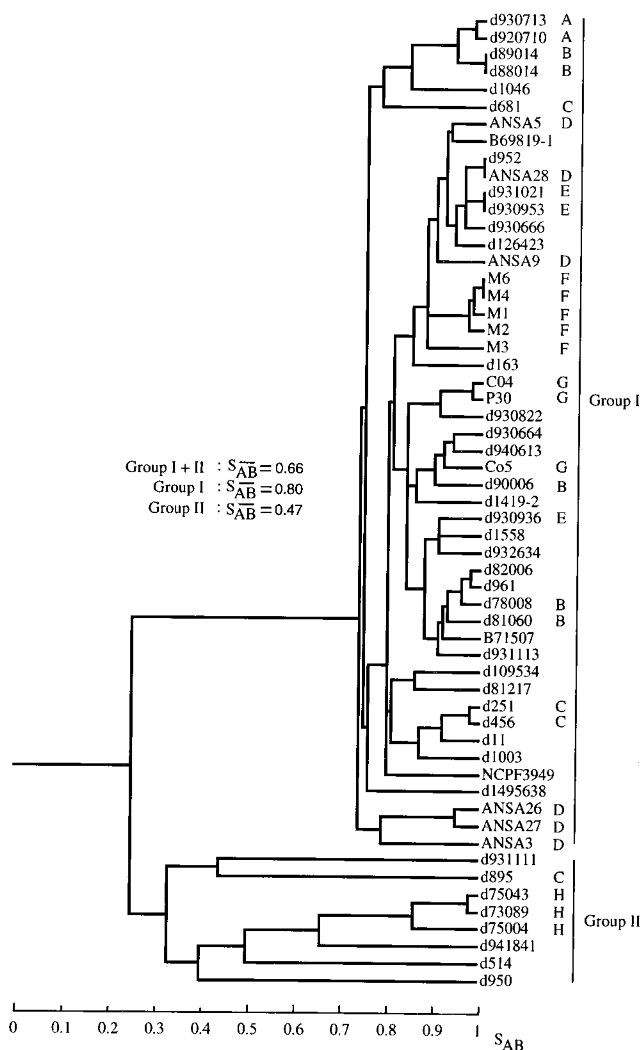

FIG. 7. Dendrograms generated from the similarity coefficients (S_{AB}s) computed for all pairs of 57 unrelated isolates collected worldwide and fingerprinted with Cd25. The origins of the isolates are presented in Table 1. The letters (A through H) to the right of the dendrogram indicate the hospitals of origin in cases where several isolates, each from a different individual, were collected from the same hospital: A, Brussels, Belgium; B, Leicester, United Kingdom; C, London, United Kingdom; D, Antwerp, Belgium; E, Frankfurt, Germany; F, Victoria, Australia; G, Lausanne, Switzerland; H, Leeds, United Kingdom. The two main clusters are delineated to the right of the dendrogram (groups I and II). The average S_{AB}s for each group, and for the total collection, are presented in the middle of the dendrogram.

different patients in the same hospitals, suggesting that each represented nosocomial strains endemic to the respective hospitals (23, 24). Furthermore, of 31 isolates collected from eight hospitals (Fig. 7, A through H), 67% grouped in clusters that contained only strains from a single hospital, suggesting the presence of nosocomial strains endemic to the respective hospitals. Only one pair of identical isolates in the entire collection, d952 and ANSA28 (Fig. 7), was derived from individuals in different hospitals and countries (Table 1).

The isolates in this broad collection separated into two general groups, group I and group II (Fig. 7). The two groups were separated by a node at an S_{AB} of 0.24 (Fig. 7). Group I, which contained 49 isolates, had an average S_{AB} of 0.80. Group I isolates shared several common, or monomorphic, bands (Fig. 8). In contrast, Group II, which contained eight isolates, had a relatively low average S_{AB} of 0.47 (Fig. 7). This low average S_{AB} suggests a high level of diversity within the group. The difference between the two groups is evident in the representative patterns in Fig. 8. While group I patterns were similar, group II patterns differed markedly not only from group I patterns but also from each other. Group II isolates lacked, for the most part, the monomorphic bands described in the original comparison of the three probes (Fig. 1 and 5), which, by chance, was performed with only group I isolates.

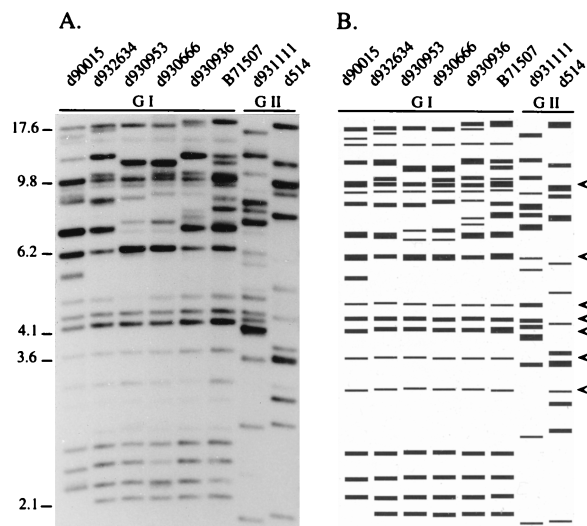

FIG. 8. Examples of Southern blot hybridization patterns of group I and group II isolates probed with Cd25. Hybridization patterns are presented in A, and a model generated by the DENDRON program is presented in B. Molecular sizes are presented in kilobases to the left of the hybridization pattern. The arrowheads to the right of the model indicate the prominent invariant bands shared by group I isolates but not by group II isolates.

Analysis of isolates from the same individuals with the probe Cd25. In the worldwide collection analyzed in this study, multiple isolates were obtained from nine individuals. When these multiple isolates were added to the dendrogram in Fig. 7, in all cases but one the isolates from the same individual clustered at an S_{AB} of 0.95 to 1.00 (data not shown).

DISCUSSION

At the phenotypic level of analysis, a number of traits are readily distinguishable between the majority of *C. albicans* and *C. dubliniensis* isolates (48). Perhaps the most definitive is the inability of *C. dubliniensis* isolates to express β-glucosidase (3, 33, 47). However, conclusive differences must be assessed at the genetic level before one would feel comfortable separating a set of atypical *C. albicans* isolates with common phenotypes into a bona fide new species, especially in view of the capacity of *C. albicans* and related species to change their phenotypes in so pleiotropic a fashion through reversible high-frequency phenotypic switching (36). The first clues that genetic differences also existed between typical and atypical *C. albicans* isolates came from reports that the latter exhibited diminished hybridization patterns with the related probes 27A (46, 47) and Ca3 (22) and that atypical strains could be readily distinguished from other species, including *C. albicans*, by restriction fragment length polymorphisms generated from *Hin*fI-digested DNA (46), randomly amplified polymorphic DNA analysis (46), hybridization to oligonucleotides homologous to microsatellites (45, 46), PCR products of primers homologous to CARE-2 (18, 19), electrophoretic karyotypes (2, 46), multilo-

cus enzyme electrophoresis (3, 12, 27, 47), and sequencing ribosomal DNA (46).

***C. dubliniensis*-specific repeat elements support its status as a species.** We have cloned and characterized complex DNA fingerprinting probes from *C. albicans* (28, 40), *C. glabrata* (15), *C. tropicalis* (11), and *Aspergillus fumigatus* (8) and have found in all cases that they contain repetitive, species-specific sequences. We hypothesized that the repetitive sequences harbored by these probes have evolved rapidly enough to be species specific and, therefore, to be good indicators of speciation. Here, we have cloned one complex probe, Cd25, that is homologous to sequences dispersed throughout the *C. dubliniensis* genome and produces no detectable signal when used to probe 13 related yeast species, including *C. albicans*. The evolution of this specific sequence in a subgroup of atypical *C. albicans* strains with common phenotypic traits supports the argument by Coleman and Sullivan (48) that *C. dubliniensis* represents a bona fide species. However, by the same argument, we have presented contradictory evidence that this subgroup of atypical *C. albicans* isolates also contains dispersed homologs of the *C. albicans*-specific RPS repetitive element (5, 10). These apparently contradictory results are accommodated by a relatively straightforward explanation. *C. albicans* and *C. dubliniensis* both may have evolved from a common ancestor that possessed RPS elements. After separation, *C. dubliniensis* acquired the repeat element in Cd25 that is dispersed throughout its genome. Therefore, *C. albicans* and *C. dubliniensis* are closely enough related to share a repetitive element (RPS) not found in any other *Candida* species but distant enough not to share the repetitive element in Cd25.

The potential of Cd1, Cd24, and Cd25 as *C. dubliniensis* DNA fingerprinting probes. Although all three probes generated complex Southern blot hybridization patterns, they were not equal in their capacities to discriminate among isolates. Cd1 produced far more variable patterns between moderately related and unrelated isolates and did not achieve parity with the other two probes in a cluster analysis of nine test isolates. The pattern generated by Cd1 was also far less stable both in vitro and in vivo than those generated by the other two probes. Because of its decreased stability, Cd1 is less effective in clustering moderately related isolates and therefore will not perform well in broad epidemiological studies. On the other hand, Cd1 is superior to both Cd24 and Cd25 in discriminating microevolutionary changes in clonal populations. These characteristics are similar to those noted for the *C. albicans* probe 27A and the C1 fragment of Ca3, both of which are composed predominately of RPS sequences (27a). Curiously, Cd24 did not discriminate microevolution in the three clones grown for 200 generations in vitro but was an excellent indicator of microevolution in one clonal population of *C. dubliniensis* monitored over a 12-month period in an HIV-positive patient. These results suggest either that the infecting strain in the HIV-positive patient had gone through far more than 200 generations or that variations in the Cd24 pattern are induced by in vivo conditions.

Although Cd24 and Cd25 achieved parity in the cluster analysis, Cd24 generated the least complex pattern of the three probes and exhibited the highest level of hybridization with *C. albicans* DNA. Cd24 was also the least dispersed sequence in the *C. dubliniensis* genome. Cd25, therefore, was selected as the probe of choice for broad epidemiological studies. It generated the most stable pattern over time for clonal populations in vitro and in vivo, produced the most complex pattern, produced the pattern with the greatest number of monomorphic bands for group I isolates, and produced the pattern with the most highly resolved bands of the three probes. Cd25 was also the only *C. dubliniensis*-specific probe. However, as noted, Cd25 was the least effective of the three probes in discriminating microevolution within an infecting strain.

Analysis of a broad collection of *C. dubliniensis* isolates. In an analysis by Southern blot hybridization with the Cd25 probe of 57 isolates collected in 11 countries, two groups were identified, group I and group II. Group I comprised 86% of all isolates and exhibited an average S_{AB} of 0.80, which was relatively high given the complexity of the pattern generated by Cd25. Group II comprised 14% of all isolates and exhibited an average S_{AB} of 0.47, which was relatively low. The lower S_{AB} suggests that the isolates in group II are relatively unrelated. The greater degree of variability among group II isolates suggests either that they have a higher frequency of Cd25 sequence reorganization than group I isolates or that group I represents a younger and therefore more homogeneous subgroup of *C. dubliniensis* that has rapidly become predominant worldwide. The very low node value that separates groups I and II (S_{AB} = 0.24) suggests reproductive isolation between the two groups.

Of the eight group II isolates, six (75%) were collected in the United Kingdom and the remaining two were collected in Spain and the Netherlands. Since the isolates from the United Kingdom comprised only 30% of the test collection, it would appear that group II isolates may be disproportionately concentrated in the United Kingdom. Further analysis of a larger number of *C. dubliniensis* isolates obtained worldwide is now being performed to verify the separation of *C. dubliniensis* into groups I and II described above.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DE10758 and AI2392 from the National Institutes of Health.

We are indebted to F. Odds of Janssen Pharmaceutics; Patrick Boerlin of the Institute of Microbiology at Lausanne, Switzerland; M. McCullough of the School of Dental Science at Melbourne, Australia; and M. Pfaller of the University of Iowa for many of the isolates used in this study.

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