Multiple *Candida* Strains in the Course of a Single Systemic Infection

DAVID R. SOLL,^{1*} MARGARET STAEBELL,¹ CAROL LANGTIMM,¹ MICHAEL PFALLER,² JAMES HICKS,³
AND T. V. GOPALA RAO¹

Departments of Biology¹ and Pathology,² University of Iowa, Iowa City, Iowa 52242, and Department of Molecular Biology, Scripps Institute, La Jolla, California 92037³

Received 16 November 1987/Accepted 13 April 1988

Species and strain variabilities have been monitored during the history of a prolonged *Candida* infection in a single compromised bone marrow transplant patient by analyzing sugar assimilation patterns, high-frequency switching repertoires, and Southern blot hybridization patterns with two cloned mid-repeat sequences (Ca3 and Ca7) which are species specific for *Candida albicans* and one cloned mid-repeat sequence (Ct13-8) which is species specific for *Candida tropicalis*. Evidence is presented that during the course of this infection (i) two strains of *C. albicans* and three strains of *C. tropicalis* were distinguished by their switching repertoires, Southern blot hybridization patterns, and sugar assimilation patterns; (ii) the three *C. tropicalis* strains were in a high-frequency mode of switching; (iii) two *C. tropicalis* strains coexisted in the blood and three *C. tropicalis* strains coexisted in the throat at different times during the history of the infection; (iv) amphotericin B treatment selectively removed one of two *C. tropicalis* strains coexisting in the blood and this strain exhibited greater susceptibility to amphotericin B in vitro (the remaining strain was subsequently removed from the blood by flucytosine treatment); and (v) both the strain removed from the blood by amphotericin B and the strain removed from the blood by flucytosine reappeared several days later at another site of infection. It is demonstrated for the first time that *C. tropicalis* is capable of high-frequency switching of colony morphology just as *C. albicans* is, that there is more than one strain-specific switching repertoire in *C. tropicalis*, and that a *C. tropicalis* mid-repeat sequence can be used for discriminating species and assessing strain relatedness, as previously demonstrated for *C. albicans* mid-repeat sequences.

Although therapeutic strategies are usually based on the assumption that a single *Candida* strain is responsible for a particular infection (2), it has been demonstrated that in some cases more than one strain can colonize the same individual (4, 8-10, 19) and different switch phenotypes of the same strain can cohabit the same site of infection (10, 18). With the development of a number of methods to discriminate between species, strains, and the switch phenotypes of a single strain (1, 5, 6, 8-12, 14-16, 18, 19), it is now feasible to monitor the temporal complexity of infecting yeasts at different body locations during the course of an infection.

In the present study, we have assessed the temporal variabilities of species, strains, and switch phenotypes during the course of a prolonged *Candida* infection in a compromised host. Two cohabiting species, *Candida albicans* and *Candida tropicalis*, were distinguished by Southern blot hybridization to the species-specific mid-repeat sequences Ca3 and Ca7 (18) for *C. albicans* and Ct13-8 for *C. tropicalis*. By comparison of the patterns of Southern blot hybridization and by an analysis of the switching repertoires distinguished by colony morphology (15, 18), two distinct strains of *C. albicans* and three distinct strains of *C. tropicalis* were identified. The three *C. tropicalis* strains were all in a high-frequency mode of switching, which is the first demonstration of switching in this species. Two strains of *C. tropicalis* cohabited the blood early in the infection, and three strains of *C. tropicalis* and one strain of *C. albicans* cohabited the throat after cessation of oral clotrimazole treatment. Amphotericin B treatment appeared to selectively remove one of the two *C. tropicalis* strains cohabiting

the blood, and that strain exhibited greater susceptibility to amphotericin B in vitro than the persisting strain. Although the level of species and strain variance observed in this case study does not occur in all systemic infections (D. R. Soll, manuscript in preparation), the results of this study provide us with a view of just how complex the spatial and temporal variabilities of species, strains, and switch phenotypes can be during the history of a single systemic infection and demonstrate the usefulness of using switching repertoires combined with Southern blot hybridization patterns to cloned species-specific mid-repeat sequences (18) for discriminating between species and between strains within a species.

MATERIALS AND METHODS

Strain isolation and characterization. Isolates removed from different body locations were plated directly onto blood agar (BA; tryptic soy agar with 5% sheep blood [GIBCO Laboratories, Madison, Wis.]; 15 g of Peptone 140, 5 g of Peptone 110, 5 g of NaCl, 15 g of agar, 5% defibrinated sheep blood per liter of H₂O [pH 7.3]), Sabouraud glucose agar (SGA [Difco Laboratories, Detroit, Mich.]; 10 g of neopeptone, 40 g of glucose, 15 g of agar per liter of H₂O [pH 5.0]), or brain heart infusion agar (BHIA; Difco; 200 g of infusion from calf brain, 250 g of infusion from beef heart, 10 g of Proteose Peptone, 2 g of glucose, 5 g of NaCl, 2.5 g of Na₂HPO₄, 15 g of agar per liter of H₂O [pH 7.4]). In all cases, individual colonies formed on the agar surface. Colonies were photographed and then clonally plated on *Candida* agar (nutrient composition of the amino-acid-rich medium of Lee et al. [7] supplemented with arginine [70 µg/ml] and zinc [9 µM] [15]) in 10-cm-diameter petri dishes at roughly 40 CFU per plate. Cultures were incubated for 7

* Corresponding author.

days or longer at 25°C for an assessment of colony morphology. Cells were in turn removed from primary colonies on *Candida* agar and replated on fresh *Candida* agar to assess switching frequencies and the repertoire of switching phenotypes. Primary colonies on original agar were labeled according to location and numbered (e.g., the three colonies from the urine sample which grew on BA were labeled Ur-1, Ur-2, and Ur-3). Each original colony received a unique number. The order of numbers did not represent the order in which isolates were sampled from the patient.

Species typing by sugar assimilation pattern. Selected clones from each isolate were typed for species by examining the pattern of sugar assimilation with commercial kits (6) obtained from Analytab Products (Plainview, N.Y.).

Southern blot hybridization. To assess strain relatedness, as well as to discriminate between the species *C. albicans* and *C. tropicalis*, three species-specific probes were used: Ca3 (formerly referred to as JH3) (18) and Ca7 (formerly referred to as JH7) (18), which are species specific to *C. albicans*; and Ct13-8, which is species specific to *C. tropicalis*. The effective use of the *C. albicans* probes was demonstrated in a previous report (18), and the methods for their isolation as well as their characterization will be presented in a separate communication (J. B. Hicks, M. J. McEachern, E. Bulgac, J. Schmid, and D. R. Soll, submitted for publication). In brief, a lambda or plasmid vector pUC19 library of whole-cell DNA of either *C. albicans* or *C. tropicalis* was plated and transferred to nitrocellulose. Each library was hybridized with ³²P-labeled nick-translated probes made from whole-cell DNA of the same strain. Roughly 10% of the plaques exhibited intense signals, suggesting that they contained cloned DNA segments present in multiple copies. Colonies or plaques showing intense signals were rescreened with separate probes of ribosomal DNA from *Saccharomyces cerevisiae*, mitochondrial DNA obtained from W. S. Riggsby, and whole-cell DNA. After secondary screening, DNA minipreps were made of nonribosomal, nonmitochondrial repeat sequences, and the nick-translated DNA was used to probe *Eco*RI-digested whole-cell DNA for copy number and Southern blot hybridization patterns. The three probes contained single *Eco*RI restriction fragments isolated from *C. albicans* 3153A in the case of Ca3 and Ca7 and *C. tropicalis* ATCC 34139 in the case of Ct13-8. DNA was isolated from *C. albicans* 3153A, *C. albicans* WO-1, *C. tropicalis* ATCC 34139, and selected strains from the patient by a modification (18) of the method of Cryer et al. (3). Each strain was cloned and grown in Lee medium (7) supplemented with zinc (17) to mid-log phase at 25°C before extraction. Deproteinized, spooled DNA was dissolved in water, and 1 µg of DNA was cut with 10 U of *Eco*RI enzyme (New England BioLabs, Inc., Beverly, Mass.) at 37°C for 1 h. Fragments were separated on 0.7% agarose gels, stained with ethidium bromide, destained, and transferred to GeneScreen Plus hybridization membranes (New England Nuclear Corp., Boston, Mass.) for Southern blot hybridization by the procedure described previously (18). Probes Ca3, Ca7, and Ct13-8 were nick translated by the method of Rigby et al. (13) and then hybridized and autoradiographed by methods previously described (18).

Testing clones for susceptibility to amphotericin B. To find a concentration of amphotericin B which affected growth kinetics but did not completely inhibit cell multiplication, cells of laboratory strain 3153A were grown at 25°C in suspension cultures containing Lee medium (7) supplemented with arginine and zinc (17) and containing amphotericin B at concentrations ranging from 1×10^{-3} to 5×10^{-1}

µg/ml. Amphotericin B at 5×10^{-2} µg/ml increased the average generation time but allowed the cell concentration to reach roughly 90% of the untreated stationary-phase level in strain 3153A (data not shown). This concentration was used to compare the susceptibilities of the test clones removed from the patient. Cells from a clonal colony on agar were inoculated at low density into liquid nutrient medium at 25°C, grown to mid-log phase, and then diluted into two 125-ml Erlenmeyer flasks containing 25 ml of fresh medium at 25°C. The control flask contained growth medium alone; the test flask contained growth medium plus 5×10^{-2} µg of amphotericin B per ml. Growth kinetics in the flask containing amphotericin B and in the control flask were compared by taking samples at time intervals and, after briefly sonicating the samples, counting the number of spheres per milliliter with a hemacytometer.

RESULTS

Brief history of the patient. The patient was a 29-year-old female with acute nonlymphoblastic leukemia who received a bone marrow transplant 13 November 1985. Before transplantation, the patient was treated with antibiotics (isoniazid, ethambutol, rifampin, trimethoprim-sulfamethoxazole, and oral clotrimazole). The patient began treatment on 29 November 1985 with a 1-mg test dose of amphotericin B, which was increased to 35 mg/day on 30 November 1985. Skin blisters appeared on 28 November 1985 but disappeared after initiation of amphotericin B treatment. Because of continued blood colonization after initiation of amphotericin B treatment, flucytosine treatment was initiated on 6 December 1985 at a level in serum of 46 µg/ml. The latter treatment was discontinued on 19 December 1985 because of side effects. Amphotericin B treatment was continued through 9 January 1986. The patient began self-treatment with clotrimazole lozenges before 26 November 1985. This treatment was discontinued on 12 December 1985 by the patient's volition. Sampling from different body locations began before 26 November 1985. The dates and body locations for *Candida* isolation and the regimes for antifungal treatment are diagrammed in Fig. 1.

Colony morphologies during the course of infection. A minimum estimate of the number of strains or switch phenotypes colonizing the patient during the course of the infection was made by comparing colony morphologies (15, 16, 18; D. R. Soll, in M. M. Howe and D. E. Berg, ed., *Mobile DNA*, in press; D. R. Soll, B. Slutsky, S. Mackenzie, C. Langtimm, and M. Staebell, in I. Mackenzie and C. Squier, ed., *Diseases of the Oral Mucosa*, in press). In Table 1, the dates of initial isolation, the original body locations, the numbers of colonies on the original plates, and the approximate phenotypes on the original plates are presented. Even in these original cultures, which included a number of different nutrient agars (BHIA, BA, and SGA), there was a suggestion that phenotypic differences existed between isolates from different body locations (e.g., compare urine isolate of 26 November 1985 and skin blister isolate of 29 November 1985, both on BA [Table 1]) and between colonies in the same isolate (e.g., the blood isolate of 1 December 1985 [Table 1]). To obtain reliable and comparable colony phenotypes, individual colonies from the original agar cultures were recloned on *Candida* agar and incubated at 25°C by methods previously described in detail (15, 16; Soll et al., in press). The individual colonies which were replated are listed in Table 1, together with the major phenotypes of the resultant colonies on *Candida* agar. It is

FIG. 1. Diagram of the dates of isolation, major colony phenotypes on *Candida* agar, and antifungal drug regimens. The dates are presented only when *Candida* colonization was detected. The dashed line represents time between a low test dose and a high therapeutic dose of amphotericin B (see text). 5-FC, Flucytosine.

immediately clear that four major phenotypes could be unambiguously distinguished on *Candida* agar: (i) smooth white (Fig. 2A), which exhibited a smooth, unmottled white colony morphology; (ii) irregular edge (Fig. 2B), which exhibited mottling at the colony periphery; (iii) fuzzy (Fig.

2C), which exhibited a dark, flat center, a bumpy intermediate band, and an extremely fuzzy outer band composed of aerial mycelia; and (iv) bumpy halo (Fig. 2D), which exhibited a bumpy center region and a silky, hyphal perimeter. These four defined colony phenotypes on *Candida* agar will subsequently be referred to as SW, IE, F, and BH, respectively.

A clear pattern of temporal and spatial changes in the colony morphologies of isolates accompanied the course of infection (Fig. 1 and Table 1). Three smooth yeast colonies with light center regions were first obtained on BA with samples from the urine of the patient taken on 26 November (Fig. 3A). When one colony (Ur-1) was replated on *Candida* agar, all 11,950 colonies exhibited the same SW phenotype (Fig. 3B). Two yeast colonies with irregular edges were obtained on SGA with samples from the blood of the patient taken on 28 November (Fig. 4A). When one colony (B-6) was replated on *Candida* agar, the major phenotype of 8,450 colonies was a less pronounced but distinguishable IE (Fig. 2B and 4B). Two irregularly shaped colonies were obtained on BA with samples from skin blisters taken on 29 November (Fig. 5A). When both colonies (Sk-1 and Sk-2) were plated on *Candida* agar, the major phenotype of 8,675 colonies of Sk-1 and 11,200 colonies of Sk-2 was F (Fig. 2C and 5C). Thirty-two colonies were obtained on SGA with samples from the blood of the patient taken on 1 December. Twenty-six colonies exhibited the phenotype of the blood isolates of 28 November, and six exhibited a fuzzy phenotype (Fig. 5B). When an irregular edge colony (B-4) was replated on *Candida* agar, the major phenotype of 11,275 colonies was IE (Fig. 2B and 4B). When a fuzzy colony (B-1) was replated, the major phenotype of 11,875 colonies was F (Fig. 2C and 5C). Subsequent yeast isolates were obtained from the blood of the patient taken on 2, 5, 6, and 7 December. In all cases, the colonies replated on *Candida* agar exhibited the IE phenotype only. The absence of the F phenotype in the blood isolates correlated with the establishment of therapeutic doses of amphotericin B (Fig. 1). Blood samples taken after 7 December were negative for yeast cells, which correlated with flucytosine treatment (Fig. 1).

TABLE 1. Numbers and morphologies of colonies initially plated, clones replated on *Candida* agar, and major colony morphologies on *Candida* agar

Date of isolation	Body location	No. of original colonies	Original colony morphology (agar) ^a	Replated clone ^b	Major colony morphology on <i>Candida</i> agar ^c
26 Nov.	Urine	3	Smooth white (BA)	Ur-1	SW
28 Nov.	Blood	2	Irregular (SGA)	B-6	IE
29 Nov.	Skin Blister	2	Irregular (BA)	Sk-1	F
				Sk-2	F
1 Dec.	Blood	32	26 Irregular	B-4	IE
			6 Fuzzy (SGA)	B-1	F
2 Dec.	Blood	1	Smooth white (SGA)	B-8	IE
5 Dec.	Blood	5	Irregular (SGA)	B-9	IE
				B-10	IE
6 Dec.	Blood	2	Irregular (SGA)	B-12	IE
7 Dec.	Blood	1	Irregular (BHIA)	B-13	IE
22 Jan.	Throat	9	6 Smooth white, 3 wrinkled (SGA)	Th-15	SW
				Th-14	BH
28 Jan.	Throat	49	Various (BA)	Th-3	IE
				Th-4	IE
				Th-7	IE
				Th-5	F
				Th-1	BH
				Th-2	BH
				Th-6	BH
28 Jan.	Stools	7	Fuzzy (SGA)	St-1	BH
				St-2	BH
29 Jan.	Throat	1	Smooth white (BA)	Th-8	SW

^a The original samples from the patient were plated on different agar, making colony morphology assessment unreliable. Therefore, select clones were replated on *Candida* agar as described in Materials and Methods. Examples of colony morphologies are presented in Fig. 2, and a temporal diagram of data is presented in Fig. 1.

^b Clones represent single colonies picked from original plates.

^c The number of colonies of each replated clone assessed on *Candida* agar ranged from 3,000 to 12,000.

FIG. 2. Examples of the four major colony phenotypes on *Candida* agar exhibited by original clones. (A) SW; (B) IE (arrow indicates rough border); (C) F; (D) BH.

No yeast isolates were obtained from throat cultures made before 22 January, while the patient was being treated with oral clotrimazole. On 14 January, the patient stopped self-administration of the drug, and on 22 January, nine yeast colonies were obtained on SGA with samples taken from the throat. Six colonies were smooth, and three were wrinkled (Fig. 6A). When a smooth colony (Th-15) was replated, all of the 10,250 colonies exhibited the SW phenotype. When a wrinkled colony (Th-14) was replated on *Candida* agar, the majority of 7,550 cells exhibited the BH phenotype (Fig. 2D). Forty-nine yeast colonies were obtained on BA on 28 January. Seven colonies (Th-1 to Th-7) were replated on *Candida* agar (Table 1). Three (Th-3, Th-4, and Th-7) exhibited the IE phenotype, one (Th-5) exhibited the F phenotype, and three (Th-1, Th-2, and Th-6) exhibited the BH phenotype. A stool sample taken on the same day as the second throat culture also yielded seven colonies which exhibited a fuzzy morphology on SGA and a BH morphology on *Candida* agar (Table 1). Finally, one smooth white colony was obtained on BA with samples from the throat taken on 29 January. This colony exhibited the SW phenotype on *Candida* agar (Table 1).

Separation of species and strains. Differences in colony morphology on *Candida* agar indicated that there were at least four phenotypes during the course of infection in this single patient, but these differences cannot be used to discriminate between species or between strains within a species. Indeed, the common laboratory strain *C. albicans* 3153A has been demonstrated to include in its switching repertoire at least six distinct colony morphologies (15), and *C. albicans* WO-1 includes five distinct colony morphologies (16). Two methods were used to identify the species of the

isolates. First, clones derived from the SW colonies Ur-1 and Th-8; clones from the IE colonies B-6, B-4, B-9, and B-12; clones from the F colonies Sk-1 and B-1; and clones from the BH colonies Th-1 and St-1 were tested for sugar assimilation with the API 20C strip for yeast identification (6). Ur-1 and Th-8 were the only clones which were typed as *C. albicans*; B-6, B-4, B-9, B-12, Sk-1, B-1, Th-1, and St-1 were typed as *C. tropicalis*. In method 2, species were identified by hybridization to cloned genomic mid-repeat sequences which are species specific. The mid-repeat sequence clones Ca3 and Ca7 are specific to *C. albicans* and hybridize to between 15 and 20 *Eco*RI digest fragments on the Southern blot (18). In Fig. 7A and B, the hybridization patterns with Ca3 and Ca7, respectively, are presented for *Eco*RI-digested DNA of *C. albicans* 3153A (lane 1) and WO-1 (lane 2). In both cases, roughly 15 to 20 hybridization bands are evident, but the patterns are distinctly different. In contrast, there was very little hybridization of Ca3 and Ca7 to *Eco*RI-digested DNA of *C. tropicalis* ATCC 34139 (Fig. 7A and B, respectively, lanes 3). In the case of Ca3, there were only two faint bands on the Southern blot (Fig. 7A, lane 3), and in the case of Ca7, there was absolutely no demonstrable hybridization (Fig. 7B, lane 3). Of the clones Ur-1, B-6, B-13, Sk-1, B-1, Th-14, and Th-8, tested for hybridization to Ca3 and Ca7, only Ur-1 (Fig. 7A and B, lanes 4) and Th-8 (lanes 10) DNA exhibited intense hybridization patterns, indicating that these strains were *C. albicans*. Both of these strains exhibited an SW colony phenotype and were typed as *C. albicans* by sugar assimilation patterns. The IE clones B-6 and B-13 (Fig. 7A and B, lanes 5 and 6, respectively), the two F clones Sk-1 and B-1 (lanes 7 and 8), and the BH clone Th-14 (lane 9) exhibited negligible hybridization to

FIG. 3. Examples of the SW *C. albicans* clones. (A) Colony morphology on BA; (B) colonies on *Candida* agar (examples were taken from Ur-1, but similar morphologies on *Candida* agar were obtained for Th-15 and Th-8).

Ca3 and no hybridization to Ca7. All of these strains were typed as *C. tropicalis* by sugar assimilation patterns.

The mid-repeat sequence Ct13-8 is specific to *C. tropicalis* and hybridizes to between 20 and 30 *Eco*RI digest fragments on a Southern blot. In Fig. 8, Southern blot hybridization patterns are presented for Ct13-8 hybridization to *Eco*RI digests of *C. albicans* 3153A DNA (lane 1), *C. albicans* WO-1 DNA (lane 2), and *C. tropicalis* ATCC 34139 DNA (lane 3). Absolutely no hybridization was evident with *C. albicans* DNA (Fig. 8, lanes 1 and 2), and a complex hybridization pattern was evident with *C. tropicalis* (lane 3). Of the clones Ur-1, B-6, Sk-1, B-1, B-4, B-13, Th-14, Th-8, and St-1, tested for hybridization to Ct13-8, only the SW strains Ur-1 (Fig. 8, lane 4) and Th-8 (lane 11) exhibited no hybridization. The IE clones B-6, B-4, and B-13 (Fig. 8, lanes 5, 8, and 9, respectively), the two F clones, Sk-1 and B-1 (lanes 6 and 7, respectively), and the BH clones Th-14 and St-1 (lanes 10 and 12, respectively) exhibited intense hybridization patterns, indicating that these clones were *C. tropicalis*.

Hybridization to species-specific mid-repeat sequences not only provides a method for distinguishing between species but also allows assessment of strain relatedness through comparison of Southern blot hybridization patterns (18). The mid-repeat sequence Ca3 is relatively nonmobile (18; Soll, in press), maintaining the same pattern through extended periods of cell multiplication. The patterns for the two *C. albicans* isolates Ur-1 (Fig. 7A, lane 4) and Th-8 (Fig. 7A, lane 10) differed dramatically, suggesting that these isolates represent distinctly different strains. Since Ca7 is highly mobile (18; Soll, in press), its pattern of hybridization changes rapidly, and therefore it is not as effective a probe for testing strain relatedness. The pattern of Ct13-8 is also relatively stable (Hicks and Soll, unpublished observations) and therefore useful for assessing strain relatedness in *C. tropicalis*. The independently isolated IE clones B-6, B-4,

and B-14 (Fig. 8, lanes 5, 8, and 9, respectively) exhibited identical Southern blot hybridization patterns with Ct13-8. The independently isolated F clones Sk-1 and B-1 (Fig. 8, lanes 6 and 7, respectively) exhibited identical patterns, but this pattern differed markedly from the IE pattern. Finally, the independently isolated BH clones Th-14 and St-1 (Fig. 8, lanes 10 and 12) exhibited similar patterns, and again, this general pattern differed from both the IE pattern and the F pattern. It should be noted that the patterns of Th-14 and St-1 differed by the addition of three bands in the St-1 pattern (see Discussion).

The sugar assimilation tests and Southern blot hybridization analyses demonstrated that during the course of infection, at least two *C. albicans* and three *C. tropicalis* strains colonized the patient.

High-frequency switching in individual strains. The extreme colony phenotypes of the original *C. tropicalis* strains (Table 1 and Fig. 2) suggested that this species may also possess high-frequency switching systems analogous to those demonstrated in *C. albicans* (15, 16, 18; Soll, in press; Soll et al., in press). Switching of colony morphology was therefore assessed in all of the individual strains.

(i) **SW isolates.** In the initial plating of *C. albicans* colonies Ur-1 and Th-8, all 11,950 colonies of the former and 10,250 colonies of the latter exhibited the SW phenotype (Fig. 2A and 3B). In a secondary plating, all 6,600 Ur-1 colonies and 6,020 Th-8 colonies again exhibited the SW phenotype. Therefore, both Ur-1 and Th-8 are either not switching or switching at frequencies below 7×10^{-5} .

(ii) **IE isolates.** *C. tropicalis* colonies B-6, B-4, B-8, B-9, B-10, B-12, B-13, Th-3, Th-4, and Th-7 showed the same IE morphology in the majority of colonies on *Candida* agar (Fig. 2B). However, all strains also produced variant colonies at frequencies ranging from roughly 10^{-3} to 10^{-4} (Table 2). Variant phenotypes included a fuzzy myceliated phenotype (Fig. 4D and E), colonies with fuzzy myceliated sectors

FIG. 4. Examples of the switching repertoire of the IE *C. tropicalis* clones. (A) IE phenotype of the original B-6 colony on SGA; (B) B-6 plated on *Candida* agar (white lines indicate irregular projections); (C) colony exhibiting smooth sectors (ss) and fuzzy myceliated sectors (fs); (D) fuzzy myceliated colony; (E) switch from IE to a fuzzy phenotype (f); (F) IE colony with a fuzzy myceliated sector and a smooth sector; (G) myceliated-edge colony; (H) switch from a myceliated edge phenotype back to an IE phenotype (ie).

FIG. 5. Examples of the switching repertoire of F *C. tropicalis* clones. (A) Original colony of Sk-1 on BA; (B) the mixture of IE (ie) and F (f) colonies from the blood sample taken on 1 December 1985 plated on SGA; (C) an F colony phenotype on *Candida* agar; (D) low magnification of a plate of young F colonies; (E) switch from F (f) to smooth white with myceliated edge [sw(m)]; (F) F colony (f) with a sector that is smooth white with myceliated edge; (G) wrinkled edge; (H) switch from wrinkled edge (wr) to a smooth white colony (s); (I) switch from F to mottled tiny.

(Fig. 4F), a starch white phenotype, a myceliated-edge phenotype (Fig. 4G and H), a petite white phenotype, a grey phenotype, and a small wrinkled phenotype. When colonies exhibiting the predominant phenotype IE were replated, the majority of colonies retained the IE phenotype. When variant colonies were replated, the majority of colonies retained

the variant phenotype and a minority again exhibited other phenotypes within the phenotypic repertoire of these strains. Switching frequencies of variant strains ranged from 10^{-2} to 10^{-4} . Reversibility (switching back to the original IE phenotype) was observed in almost all variants.

(iii) **F isolates.** *C. tropicalis* strains Sk-1, Sk-2, B-1, and

FIG. 6. Examples of the switching repertoire of BH *C. tropicalis* clones. (A) Original colony of Th-1 on BA; (B) BH colony with a broad mycelial halo; (C) a BH colony with narrow rim which has lost the bumpy texture; (D) lower magnification of BH colonies with broad mycelial halos; (E) lower magnification of BH colonies with narrow halos; (F) flower (note similarity to BH phenotype on BA); (G) switch from flower (fl) to smooth ring (s-r); (H) switch from flower to a fuzzy phenotype (f); (I) snowflake.

FIG. 7. Southern blot hybridization patterns of *Eco*RI-digested DNA probed with Ca3, a relatively nonmobile mid-repeat sequence isolated from *C. albicans* (A), and Ca7, a highly mobile mid-repeat sequence isolated from *C. albicans* (B). Lanes 1, *C. albicans* 3153A; 2, *C. albicans* WO-1; 3, *C. tropicalis* ATCC 34139; 4, Ur-1; 5, B-6; 6, B-13; 7, Sk-1; 8, B-1; 9, Th-14; 10, Th-8. Note that Ca3 and Ca7 were relatively specific to *C. albicans*. Ca3 hybridized weakly to no more than two *Eco*RI fragments of *C. tropicalis* from the B-6, B-13, Sk-1, B-1, and Th-14 isolates. Ca7 exhibited no discernible hybridization to DNA of these strains. All of these latter strains were typed as *C. tropicalis* by sugar assimilation patterns.

Th-5 showed the same F morphology (Fig. 2C) in the majority of colonies on *Candida* agar (Table 2). However, all strains also produced variant colonies at frequencies ranging from less than 1×10^{-4} to 4×10^{-4} (Table 2). Variant phenotypes included a smooth white colony with a heavy edge of mycelia penetrating the agar (Fig. 5E and F), a wrinkled-edge phenotype (Fig. 5G and H), a smooth white phenotype (Fig. 5H), and a petite phenotype. When variant colonies were replated, the majority of colonies retained the variant phenotype and a minority again exhibited other phenotypes within the phenotypic repertoire of these strains. Switching frequencies of variant strains ranged from roughly 10^{-2} to less than 10^{-4} , and reversibility was observed in almost all variants.

(iv) **BH isolates.** *C. tropicalis* strains Th-14, Th-1, Th-2, Th-6, St-1, and St-2 showed the same BH morphology (Fig. 2D) in the majority of colonies on *Candida* agar (Table 2). However, all strains also produced variant colonies at frequencies ranging from 4×10^{-4} to 3.3×10^{-2} (Table 2). Variant phenotypes included a bumpy colony with no halo of mycelia in the agar, a starch white phenotype, a flower phenotype (Fig. 6F, G, and H), a smooth ring phenotype (Fig. 6G), and a snowflake phenotype (Fig. 6I). There was also an indication that two BH phenotypes existed, one with a wide halo (Fig. 6D) and one with a narrow halo (Fig. 6E), but the difference was too slight to be added to the frequency of switching. When variant colonies were in turn plated, the majority of colonies retained the variant phenotype and a minority again exhibited other phenotypes within the switching repertoire of these strains. Switching frequencies of

FIG. 8. Southern blot hybridization patterns of *Eco*RI-digested DNA probed with *Eco*RI fragment Ct13-8, a mid-repeat sequence isolated from *C. tropicalis*. Lanes: 1, *C. albicans* 3153A; 2, *C. albicans* WO-1; 3, *C. tropicalis*; 4, Ur-1; 5, B-6; 6, Sk-1; 7, B-1; 8, B-4; 9, B-13; 10, Th-14; 11, Th-8; 12, St-1. Note that fragment Ct13-8 was specific to *C. tropicalis* and did not hybridize to *C. albicans* 3153A and WO-1 or isolates Ur-1 and Th-8.

TABLE 2. Analysis of switching frequencies and the phenotypic repertoires of the three *C. tropicalis* strains

Phenotype and clone	No. of colonies			Frequency of variant colonies ^a
	Total	Identical ^b	Variant ^c	
IE				
B-6	8,450	8,437	14	2×10^{-3}
B-4	11,275	11,272	3	3×10^{-4}
B-8	11,250	11,248	2	2×10^{-4}
B-9	11,850	11,848	2	2×10^{-4}
B-10	9,825	9,821	4	4×10^{-4}
B-12	7,625	7,617	8	1×10^{-3}
B-13	8,850	8,849	1	1×10^{-3}
Th-3	4,600	4,600	0	$<2 \times 10^{-4}$
Th-4	4,140	4,140	0	$<2 \times 10^{-4}$
Th-7	3,999	3,996	1	3×10^{-4}
F				
Sk-1	9,675	9,671	4	4×10^{-4}
Sk-2	11,200	11,200	0	$<1 \times 10^{-4}$
B-1	11,875	11,870	5	4×10^{-4}
Th-5	9,400	9,398	2	2×10^{-4}
BH				
Th-14	7,500	7,307	241	3×10^{-2}
Th-1	5,200	5,197	2	4×10^{-4}
Th-2	4,100	4,094	6	1×10^{-3}
Th-6	5,000	4,966	15	3×10^{-3}
St-1	4,600	4,564	36	8×10^{-3}
St-2	3,100	3,085	4	1×10^{-3}

^a The overall frequencies of variant colonies for IE, F, and BH strains were 4×10^{-4} , 3×10^{-4} , and 1×10^{-2} , respectively.

^b Identical colonies are those which exhibit the same morphology as the parent strain.

^c The variant phenotypes emanating from the IE phenotype included fuzzy myceliated, starch white, myceliated edge, petite white, grey, and small wrinkled. Examples of some of these phenotypes are presented in Fig. 4. The variant phenotypes emanating from the F phenotype included a smooth white colony with mycelia penetrating the agar, wrinkled, smooth white, and petite. Examples of some of these phenotypes are presented in Fig. 5. The variant phenotypes emanating from the BH phenotype included bumpy with no peripheral mycelia, starch white, flower, smooth ring, and snowflake. Examples of some of these phenotypes are presented in Fig. 6. In the BH repertoire, there were indications of BH phenotypes with halos of different widths, but the differences were too slight to be incorporated into an evaluation of switching frequencies.

variant strains ranged from 6×10^{-2} to 3×10^{-3} , and reversibility was again observed in almost all variants.

This assessment of colony morphology demonstrates that all of the *C. tropicalis* strains exhibited a relatively high frequency of switching, that the three different strains exhibited strain-specific switching repertoires, and that switching in these *C. tropicalis* strains exhibits several of the characteristics of high-frequency switching in the related species *C. albicans*. These characteristics include a limited number of colony morphologies (switch phenotypes), heritability of the variant phenotypes, and a high frequency of interconversion and reversibility (Soll, in press). In relation to the course of infection, it should be noted that one switch phenotype predominated for each strain (Table 1).

Differences in amphotericin B susceptibilities. The two *C. tropicalis* strains that were IE and F cohabited the blood when therapeutic doses of amphotericin B were established (Fig. 1). Soon afterwards, the F strain was removed from the blood, but the IE strain persisted, suggesting that the IE strain was more resistant than the F strain to amphotericin B. To test this possibility, the growth kinetics of B-4, an IE clone, and B-1, an F clone, were compared in growth medium containing 5×10^{-2} μ g of amphotericin B per ml, a concentration which slows growth but does not inhibit cell division in *C. albicans* 3153A (see Materials and Methods). In the absence of amphotericin B (Fig. 9A), both B-4 and B-1 multiplied with the same generation time and reached the same number of spheres per milliliter at stationary phase as *C. albicans* 3153A. In the presence of amphotericin B, the F strain multiplied with a longer generation time than the IE strain in repeat experiments (Fig. 9B and C), demonstrating the predicted difference in susceptibilities.

DISCUSSION

Odds and Abbott found that 3% of vaginal isolates contain more than one strain of *C. albicans* (distinguished by biotyping [11]) and 1% contain more than a single *Candida* species (10). In immunocompromised patients undergoing chemotherapy, they found that 12% of oral isolates contained more than one biotype and 20% contained more than one *Candida* species (10). Hopfer and co-workers have also demonstrated that four *Candida* species could be isolated from multiple sites before and after death of a leukemia patient (4). In our analysis of *Candida* isolates from a single bone marrow transplant patient, we have isolated at least two strains of *C.*

FIG. 9. Differential susceptibilities of clones B-4 (IE *C. tropicalis*) and B-1 (F *C. tropicalis*) to amphotericin B. Growth of B-4 (○) and B-1 (●) in the absence of amphotericin B (A) and in the presence of 5×10^{-2} μ g of amphotericin B per ml (B and C).

albicans and three strains of *C. tropicalis*. In addition, we have obtained a hint of the temporal and spatial dynamics of colonization associated with the regimen of drug therapy during the course of infection. Our results, together with those of previous studies, indicate the need for species and strain identification as well as for assessment of the differential susceptibilities of multiple strains to drug therapy during the course of a systemic infection.

Identification of multiple species and strains during the course of infection. By assessing sugar assimilation patterns (5, 6), we found that Ur-1, isolated from the urine, and Th-15 and Th-8, isolated from the throat, could be typed as *C. albicans* and that all other strains could be typed as *C. tropicalis*. This assessment was confirmed for Ur-1 and Th-8 by specific DNA hybridization to the *C. albicans*-specific mid-repeat sequences Ca3 and Ca7 and by the absence of DNA hybridization to the *C. tropicalis*-specific mid-repeat sequence Ct13-8. In contrast, selected strains (B-6, B-13, Sk-1, B-1, and Th-14) which were typed as *C. tropicalis* by sugar assimilation patterns did not exhibit any DNA hybridization to either Ca3 or Ca7 but did exhibit DNA hybridization to Ct13-8.

Within the strains which were typed as *C. tropicalis*, there were three clearly distinct colony morphologies exhibited on *Candida* agar: IE, F, and BH. The IE phenotype was dominant in the blood from 28 November to 7 December 1985 and then appeared in the throat on 28 January 1986. All tested IE colonies were typed as *C. tropicalis* by sugar assimilation patterns, and two, B-6 and B-13, isolated at the beginning and end of the period of blood infection, exhibited similar banding patterns when they were probed with the cloned gene fragment Ct13-8 by Southern blot hybridization. All IE clones also exhibited similar switching repertoires, reinforcing the conclusion that they represented a single infecting strain. The second *C. tropicalis* strain, which was F, appeared first in skin blisters, then in the blood, and finally in the throat. All the tested F colonies again were typed as *C. tropicalis* by sugar assimilation patterns, and two of these, Sk-1 and B-1, exhibited similar banding patterns when they were probed with fragment Ct13-8 by Southern blot hybridization. All F clones exhibited similar switching repertoires, reinforcing the conclusion that they also represented a single infecting strain. The Southern blot hybridization pattern with Ct13-8 and the switching repertoire of the F clones were distinctly different from those of IE clones, indicating that the IE and F phenotypes represented different strains of *C. tropicalis*. The third *C. tropicalis* strain, which was BH, appeared first in the throat and then in the stools. All the tested BH colonies were typed as *C. tropicalis* by sugar assimilation patterns, and two of these clones, Th-14 and St-1, exhibited similar, but not identical, patterns when they were probed with fragment Ct13-8 by Southern blot hybridization. All BH clones exhibited similar switching repertoires, indicating that they represented a single strain. The switching repertoire and general Southern blot hybridization pattern with Ct13-8 were distinctly different from those of the IE clones and the F clones, indicating that the BH phenotype represented a different strain. However, the Ct13-8 Southern blot hybridization patterns of Th-14 and St-1 differed by the addition of three bands to the pattern of the latter isolate. The rest of the pattern, including roughly 21 bands, was similar, suggesting that Ct13-8 transposition or rearrangement occurred in the BH strain during the course of this infection. Differential mobility of the *C. albicans* mid-repeat sequence Ca7 has previously been demonstrated (Soll, in press). In *C. albicans* 3153A, Ca7 is

relatively nonmobile, but in *C. albicans* WO-1, it is highly mobile (Soll, in press).

Treatment with antifungal agents and multiple strains. The temporal and spatial dynamics of the various strains during the course of this infection appeared to be closely related to the regimen of drug treatment. Although the blood of the patient was monitored for *Candida* colonization before 28 November 1985, no colonies were detected. On 28 November 1985, IE cells were detected in blood sample, and on 29 November 1985, F cells were detected in skin blisters. On 1 December 1985, both IE and F cells were detected in the same blood sample. On 30 November 1985, a therapeutic dose of amphotericin B was administered intravenously. By 1 December 1985, the skin blisters began to heal, and on 2 December 1985, F cells were no longer detectable in the blood. We assume that amphotericin B treatment was responsible for blister healing and selective removal of F cells from the blood. The difference in the susceptibilities of F and IE cells to 5×10^{-2} μ g of amphotericin B per ml in vitro is consistent with this interpretation. IE cells persisted in the blood through 6 December 1985, at which time the patient began to receive flucytosine intravenously in addition to amphotericin B. Soon after flucytosine treatment, IE cells could not be detected in the blood, suggesting that flucytosine was effective in removal. However, removal from the blood did not represent removal of either F or IE cells from the body, since both reappeared in the throat in addition to BH and SW cells on 28 January 1986, after the patient stopped oral clotrimazole treatment.

Switching in the *C. tropicalis* strains. Besides species and strain variabilities, *C. albicans* has been demonstrated to possess the potential for intrastrain variability through high-frequency switching (15, 16, 18; Soll, in press; Soll et al., in press). Surprisingly, the *C. albicans* clones Ur-1, Th-15, and Th-8, isolated from this patient, all exhibited the SW phenotype and negligible levels of spontaneous switching in culture. In contrast, all three of the *C. tropicalis* strains isolated from this patient exhibited a high frequency of switching, and the repertoires of switching were strain specific. This represents the first demonstration of switching in *C. tropicalis* strains and suggests that high-frequency switching may represent a general characteristic of infectious *Candida* species. However, there was no indication of cohabitation by multiple-switch phenotypes of the same strain in situ during the course of this infection, even when the same strain was isolated from different body locations or repeatedly isolated from the same body location. In contrast, multiple-switch phenotypes of the same strains were isolated from the same site of infection in roughly a third of patients with vaginal candidiasis (18).

ACKNOWLEDGMENTS

We are indebted to Noel Schroeder and Kimberly Drollinger for assistance in preparing the manuscript.

This research was funded in part by Public Health Service grant AI 23922 from the National Institutes of Health and grants from the Iowa High Tech. Council and from the Cecil J. Rusley Memorial Fund to D.R.S. and by Public Health Service grant GM 37914 from the National Institutes of Health to J.H.

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