INFECTION AND IMMUNITY, Oct. 1995, p. 4078–4083 Vol. 63, No. 10
0019-9567/95/$04.00+0

Candida albicans Cell Walls Contain the Fluorescent Cross-Linking Amino Acid Dityrosine

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Received 22 May 1995/Returned for modification 26 June 1995/Accepted 14 July 1995

Several clinical and laboratory isolates of *Candida albicans* have a natural blue surface fluorescence when cultured and observed with sensitive optics. The localization and color of the fluorescence are similar to those of the natural fluorescence of sporulated *Saccharomyces cerevisiae* which is caused by the generation and surface deposition of the cross-linking amino acid dityrosine. In *S. cerevisiae*, dityrosine production results from the direct action of at least two genes and is responsible for resistance of the ascospores to lytic enzymes and physicochemical trauma. Among the criteria for the identification of dityrosine is pH sensitivity of the fluorescence intensity and a highly characteristic shift of the fluorescence excitation maximum with a change in pH. Video microscopy of whole *Candida* organisms revealed the characteristic dityrosine intensity maximum at pH ~10 and the intensity minimum at pH ~2. Separation of an acid hydrolysate of *Candida* cell walls by reverse-phase high-performance liquid chromatography revealed a fluorescence peak that coelutes with the reagent dityrosine. At pH ~10, this peak has a fluorescence excitation maximum of 320 to 325 nm, while at pH ~2, the excitation maximum is 285 to 290 nm. This excitation maximum shift and the observed emission maximum of ~410 nm are characteristic of dityrosine. Two separate strains of *C. albicans* were injected intraperitoneally into mice and harvested at 24 h. Blue surface fluorescence was observed, suggesting that dityrosine generation occurs in vivo as well as in vitro. This is the first report of the presence of dityrosine in a human fungal pathogen.

Candida albicans and several other *Candida* species are major pathogens in hospitalized, immunocompromised patients (4). The mechanisms allowing *C. albicans* to colonize mucosal surfaces and then invade the bloodstream and visceral organs have long been under intense investigation (32), with a large number of putative virulence factors proposed as contributing to pathogenicity (15). Studies of the *Candida* cell surface (36) have been of particular interest because of its importance in antigenicity (11, 39), in adherence to epithelial and endothelial cells (11), and in interactions with the host immune system (24, 38, 40, 41) and because the mechanisms of cell wall synthesis are critical for our understanding of morphogenesis and for development of new antifungal agents (25). The *Candida* cell wall is composed principally of mannan (linked with protein to form mannoprotein), α-glucan, β-glucan, and chitin, which are complex polymers of mannose, glucose, and *N*-acetylglucosamine, respectively. The content and distribution of these components vary with cell age, culture conditions, and morphology (25, 36). The linkages between the glucan, chitin, and mannoprotein are poorly understood but presumably play a critical role in maintaining the integrity of the organism. Dityrosine, a relatively rare amino acid, is well known as a cross-linking agent that is essential for the resistance of several species, including *Saccharomyces cerevisiae* (6, 10), to adverse environmental conditions. Dityrosine is located in the outermost ascospore layer of *S. cerevisiae* (9) and is the product of a carefully controlled developmental process (6).

In this report, we describe recent observations which indicate that under certain conditions the cell wall of *C. albicans* contains dityrosine. The implications of the presence of dityrosine in the *Candida* cell wall, and possible mechanisms for its formation, are discussed.

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MATERIALS AND METHODS

Strains, media, and growth conditions. A serotype B *C. albicans* strain, originally derived from a clinical specimen and used in previous studies in our laboratory (17, 41), was used for the bulk of these experiments. Additional *C. albicans* strains were obtained from the following sources. B311 was a generous gift from Jeffrey Jones (University of Wisconsin School of Medicine, Madison) (39). Strain 64 was a gift from Alan Sugar (Boston University School of Medicine, Boston, Mass.) (43). A recently isolated clinical strain was also used. *C. albicans* was passaged on Sabouraud agar plates at room temperature. For growth and/or germination of *C. albicans* or *S. cerevisiae* in liquid culture, various media were used, including RPMI 1640 (BioWhittaker, Walkersville, Md.), YPD (1% yeast extract [Difco Laboratories, Detroit, Mich.], 2% peptone [Difco], 2% glucose [2]), and SD synthetic minimal media (0.67% Bacto-yeast nitrogen base without amino acids [Difco] plus 2% glucose with and without supplementary amino acids [2]). Growth of yeast forms in liquid medium was monitored by measuring A_{640}. For quantitation of dityrosine content, RPMI 1640 was seeded with 8×10^6 yeast cells per ml from a 48-h Sabouraud agar plate and rotated for 4 h at 150 rpm at 37°C.

Diploid strains of *S. cerevisiae* (XD18 and XD16 [34]) were a generous gift of Christine Bulawa (Myco Pharmaceuticals, Cambridge, Mass.). These strains were grown at 28°C in YPD to the stationary phase and sporulated by suspension in 1% potassium acetate and rotation (150 to 200 rpm) at 28°C for 3 to 7 days.

Isolation of *C. albicans* cell walls and *S. cerevisiae* ascospore walls. *S. cerevisiae* ascospores and *C. albicans* blastoconidia and germ tubes were disrupted by vortexing with nitric acid-etched 0.5-mm-diameter glass beads in a round-bottom glass test tube. Cells and beads were vortexed for 60 s and then cooled for 60 s in an ice water bath. This was repeated for a total of 8 to 10 cycles, and efficiency of cell disruption was monitored by phase-contrast microscopy. Cell and ascospore walls were pelleted at $600 \times g$ for 10 min, washed 10 times with ice-cold distilled water, and frozen at −20°C until needed. Cell walls were lyophilized and stored at −20°C.

Amino acid hydrolysis. *C. albicans* and *S. cerevisiae* cell walls were hydrolyzed in 6 N HCl at 110°C for 20 h in vacuum hydrolysis tubes (Pierce, Rockford, Ill.) under N_2. Insoluble material was removed by centrifugation, and the supernatant was removed by drying in a vacuum.

Synthesis of authentic dityrosine. Dityrosine was synthesized by oxidation of

L-tyrosine with horseradish peroxidase as previously described (1) and semipurified by CP-11 cellulose phosphate and Dowex 50-X8 column chromatography. Final purification was performed by reverse-phase high-performance liquid chromatography (HPLC) as described below.

Isolation and purification of dityrosine from *C. albicans* and *S. cerevisiae* by HPLC. For most analyses, a 1090 high-performance liquid chromatograph (Hewlett-Packard, Palo Alto, Calif.) equipped with a diode array absorption detector and a fluorescence detector (Hewlett-Packard) was used to isolate and purify dityrosine. HPLC was performed on a Waters μBondapack reverse-phase C_{18} column (3.9 by 300 mm, 10-μm particle size [Millipore Corp., Milford, Mass.]) with acetonitrile-isopropanol (1:1, vol/vol) in 0.1% trifluoroacetic acid (TFA) at 1 ml/min. A gradient was initially developed by increasing the acetonitrile-isopropanol concentration from 0 to 20% over 15 min and then increasing the acetonitrile-isopropanol concentration to 100% over 10 min and completed by returning to 0.1% TFA in 5 min. In other experiments, isocratic elution with 5% (vol/vol) acetonitrile in 0.01 M TFA at 1 ml/min was used. Fractions were collected by fluorescence (excitation wavelength, 285 nm; emission wavelength, 410 nm) or absorption detection of peaks with a Foxy 200 programmable fraction collector (ISCO, Inc., Lincoln, Nebr.). Fluorometric analysis of synthesized dityrosine and of various fractions from organisms was performed on a Perkin-Elmer 650 10-S fluorescence spectrophotometer.

In selected experiments, a Waters Nova-Pak C_{18} reverse-phase column (3.9 by 150 mm; 4-μm-particle size) was used and fluorescence detection was performed with excitation and emission wavelengths of 285 and 425 nm, respectively. Hydrolysates were separated under isocratic conditions with 5% (vol/vol) acetonitrile in 0.01 M TFA.

Dityrosine concentration was determined on the basis of a molar extinction coefficient of 5,400 cm^{-1} M^{-1} (284 nm [0.02 N HCl]) (28).

Microscopy. Fluorescence observations were made with either an Olympus IMT-2 inverted microscope or a Nikon Diaphot inverted microscope (modified to provide all-quartz optics), both equipped with 100-W mercury arc lamps. Most observations were made with an Olympus 100× UV (1.3 NA) objective, although for video microscopy experiments a Nikon 40× CF fluor (0.8 to 1.3 NA) objective was used. For the Olympus microscope, a standard Olympus IMT2-DMU filter cube, used for 4′,6-diamidino-2-phenylindole (DAPI) and Calcofluor observations (UG-1 exciter filter, DM400 dichroic mirror, L420 emission filter), was employed, while for the bulk of our observations, we used the Nikon microscope with an Omega Optical (Brattleboro, Vt.) filter cube equipped with custom filters (330WB80 exciter filter, 400DCLP dichroic mirror, 400EFLP emission filter). For most observations, a drop of organisms was mixed on a glass slide with a drop of 25% ammonia (thus alkalizing the suspension for maximum fluorescence intensity) and a cover slip was added. The cells were photographed with Ektachrome 400 film (Eastman Kodak, Rochester, N.Y.).

Digital imaging. Digital imaging studies were performed with a Biological Detection Systems (Pittsburgh, Pa.) imaging board and software (512 by 474 pixels with eight-bit resolution) mounted in a MacIIfx microcomputer (Apple, Cupertino, Calif.). Image noise reduction was accomplished by averaging usually 16 frames per image. Images were acquired with a Dage-MTI (Michigan City, Ind.) 66SIT camera equipped with manual gain and black level. Camera linearity was established in preliminary experiments with a graded series of neutral-density filters. For comparison of fluorescence intensities among multiple images, the camera was operated in the manual mode. Specimens were mounted in a Dvorak-Stotler (Gaithersburg, Md.) chamber in an air curtain temperature-controlled enclosure. Hard copy output of the digital images was accomplished via a UP-870MD video printer (Sony Corp.) that allows printing of images at a resolution level of 256 Gy.

Mouse inoculations. We inoculated by intraperitoneal injection two pairs of male ICR mice (25 to 26 g; Harlan Sprague Dawley, Indianapolis, Ind.) at 10^8 yeast cells per mouse with either our type B laboratory strain or strain 64, a highly virulent strain used for antifungal antibiotic testing (43). The yeast cells were incubated for 24 h on Sabouraud agar, washed once with phosphate-buffered saline, and diluted with phosphate-buffered saline to 10^9/ml before injection into the mice. At 24 h following inoculation, we sacrificed the animals and lavaged their peritoneal cavities. Fluorescence observations were made as described above.

RESULTS

***C. albicans* has natural blue fluorescence similar to that of sporulated *S. cerevisiae*.** When cultured under certain laboratory conditions and observed with appropriate combinations of filters and fluorescence objectives, our type B laboratory strain of *C. albicans* (originally derived from a clinical specimen and used for a series of studies [17, 38, 40, 41]) has a blue fluorescence along the surface and the bud scars of yeast and germinated cells (Fig. 1). Conditions that promote this on germinated cells also cause visible fluorescence of hyphal cross-walls (Fig. 2), although fluorescence of the interior of either form of *C. albicans* has not been observed. We have also observed blue surface fluorescence with other strains of *C. albicans*, including B311 (39) and 64 (43) and a freshly isolated clinical strain. The blue fluorescence is similar in color and localization to the surface fluorescence reported from ascospores of *S. cerevisiae* (10). For comparison, we sporulated wild-type *S. cerevisiae* XD18 and its mutant progeny XD16 (previously shown to be blue fluorescence positive and negative, respectively [34]) and observed them under conditions identical to those used to demonstrate *C. albicans* fluorescence. Wild-type strain XD18 ascospores had a blue surface fluorescence identical in color to that of *C. albicans* (Fig. 3), while, as expected, mutant XD16 ascospores had no visible fluorescence.

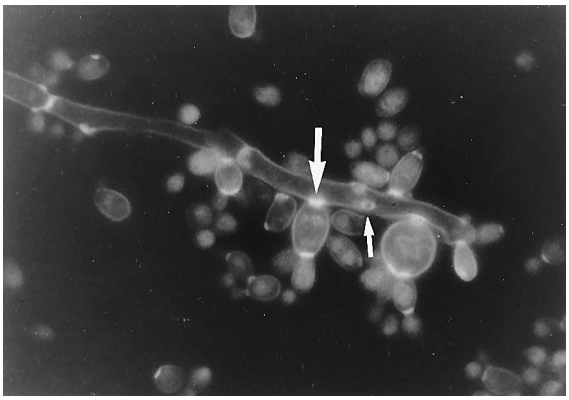

FIG. 1. *C. albicans* incubated overnight in RPMI 1640 at room temperature. Note the fluorescent bud scar (small arrow) and the budding site (large arrow). When directly observed, the internal fluorescence was a pale brown-yellow distinctly different from the blue of the surface.

Video microscopy reveals pH sensitivity of natural fluorescence. Previous work has established that dityrosine in the outer layer of the ascospore wall (10) accounts for the surface fluorescence of sporulated *S. cerevisiae*. Among the criteria used to identify dityrosine is the characteristic pH sensitivity of the fluorescence intensity. Purified reagent dityrosine has a fluorescence intensity maximum at pH ≥10 with a pK_a of ~7.1. When germinated *C. albicans* was imaged at pH ~10 with a low light level-sensitive video camera, the blue surface and septal fluorescence were clearly visible (Fig. 4B). Acidification of the buffer to pH ~2 resulted in nearly complete abolition of

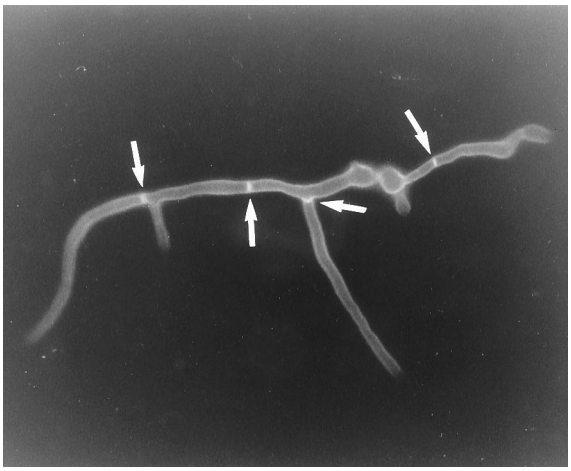

FIG. 2. *C. albicans* cultured in RPMI 1640 for 4 h at 37°C. Note the fluorescent septae (arrows).

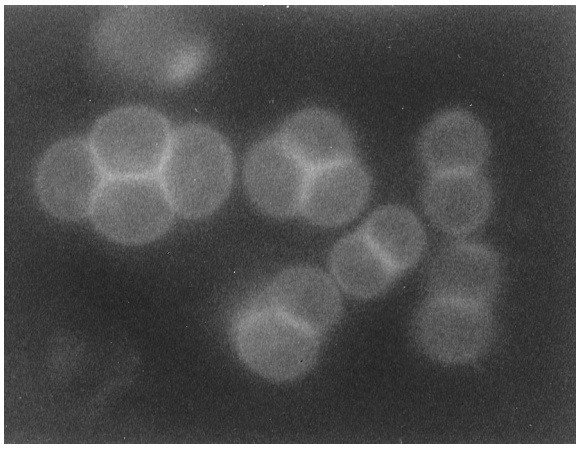

FIG. 3. Sporulated *S. cerevisiae* XD-18. Note the similarity to *C. albicans* of the surface-only fluorescence of the ascospores.

detectable surface and septal fluorescence (Fig. 4C), which was substantially restored by realkalinization of the buffer (Fig. 4D). Dityrosine is readily photobleached so that complete restoration of fluorescence is generally not possible. The residual fluorescence seen in the pH 2 photo (Fig. 4C) was in the interior of the cell and was pale brown-yellow when directly observed through the microscope. Note the significant difference in the apparent size of the organisms when only interior non-dityrosine autofluorescence is visible (pH 2) compared with that of cells observed at pH 10. This emphasizes the surface-only localization of the blue fluorescence. The video camera was operated in the manual mode in the linear range of the instrument; thus, these observations are quantitatively comparable.

Biochemical identification of dityrosine as a natural fluorescent compound. The identical blue color and the pH sensitivity of the in vitro fluorescence of *C. albicans* suggested that dityrosine formation on the *Candida* surface might explain our observations. For biochemical analysis and comparisons, we synthesized dityrosine from L-tyrosine by the horseradish peroxidase-H_2O_2 method (1), since dityrosine is not readily available commercially. Following cellulose phosphate and Dowex 50 chromatography, we further purified this material by reverse-phase HPLC. At pHs 2 and 10, dityrosine has a broad emission maximum centered on 410 to 420 nm. The excitation maximum has a characteristic shift, with a 320- to 325-nm maximum at pH 10 and a 285- to 290-nm maximum at pH 2. Monitoring by determination of A_{214} and by fluorescence detection (excitation at 285 nm, emission at 410 nm), we analyzed peaks obtained from HPLC by fluorometric scanning and confirmed the identity of dityrosine by the expected excitation-emission scans. We thus had a sensitive and specific means for comparing with purified dityrosine the identity of the fluorescent compound of *C. albicans*. Cell walls from *C. albicans* cells and *S. cerevisiae* ascospores were prepared by vortexing intact organisms with glass beads, centrifugation, and extensive washes. The isolated cell walls retained the visible blue fluorescence. The purified cell walls were hydrolyzed and dried in vacuo by standard methods for amino acid analysis (see Materials and Methods for details), and the hydrolysates were separated by reverse-phase HPLC. The retention time of the fluorescent peak from the *S. cerevisiae* hydrolysate was essentially identical to that of the synthesized dityrosine, while the suspected dityrosine peak from the *Candida* hydrolysate had a slightly longer retention time in some analytical runs. We hydrolyzed in parallel samples of synthesized dityrosine, *Candida* cell walls, and a third sample containing exactly the same amounts of dityrosine and *Candida* cell walls as the two previous samples. HPLC was monitored by determining A_{214} and by fluorescence detection (excitation at 285 nm, emission at 410 nm). As demonstrated in Fig. 5, the retention time of the coinjected sample was identical to that of the peak from *Candida* cell walls and the integrated area of the suspected dityrosine peak from the spiked sample was nearly equivalent to the sums of the integrated dityrosine peaks from the first two samples. Following pooling of the appropriate peaks from a large number of analytical HPLC separations, we confirmed the identity of dityrosine by fluorometric scanning, which demonstrated the characteristic shift of the excitation maximum with a change in pH (Fig. 6A) and the expected emission maximum (Fig. 6B). We also demonstrated that the fluorescence intensity of the *Candida* peak is pH sensitive (data not shown).

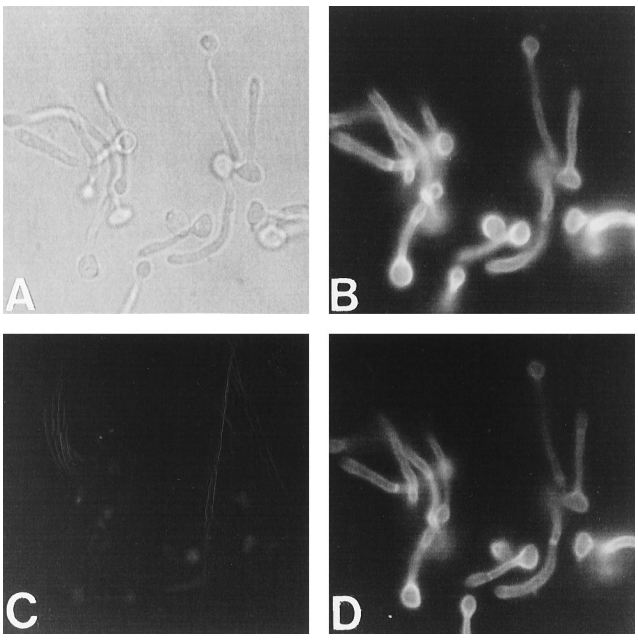

FIG. 4. Digital imaging video microscopy of *C. albicans* fluorescence. A single field was successively imaged for bright-field microscopy (A) and at pHs ~10 (B) ~2 (C), and ~10 (D).

Dityrosine content was determined for dried cell walls from *C. albicans* germinated in RPMI 1640 for 4 h at 37°C. More than 90% of the inoculum germinated. The mean cell wall dityrosine content (± the standard error of the mean) was 258 ± 94 pmol/mg of dry weight ($n = 2$).

Natural surface fluorescence of *C. albicans* recovered from murine intraperitoneal infections. To test whether our in vitro demonstration of dityrosine production has any relevance to the ability of *C. albicans* to produce dityrosine during an infection, we inoculated by intraperitoneal injection two pairs of male ICR mice (Harlan Sprague Dawley) at 10^8 yeast cells per mouse with either our type B laboratory strain or strain 64, a highly virulent strain used for antifungal antibiotic testing in animal models (43). At 24 h, we sacrificed the animals and lavaged their peritoneal cavities. Blue surface-fluorescent organisms, most of which had germinated, were obtained from all of the mice, indicating that, at least for this set of conditions, dityrosine expression by *C. albicans* is not simply an in vitro phenomenon.

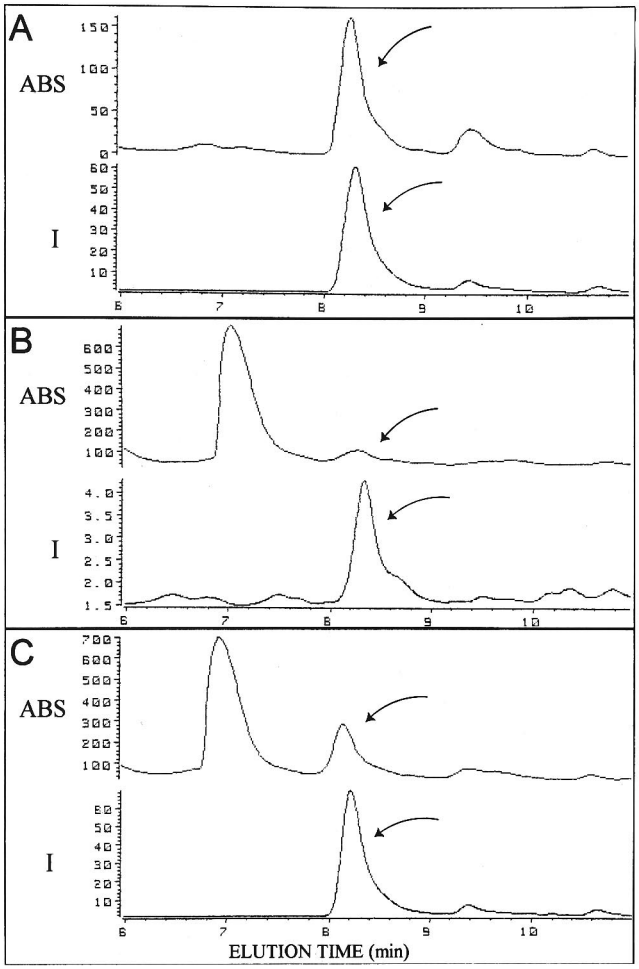

FIG. 5. Chromatographs of reverse-phase HPLC separations of a dityrosine standard (A), hydrolyzed *C. albicans* cell walls (B), and hydrolyzed *C. albicans* cell walls spiked with the dityrosine standard (C). Separations were monitored by determining A_{214} (ABS) and fluorescence (I; excitation at 285 nm, emission at 410 nm). The arrows point to dityrosine peaks.

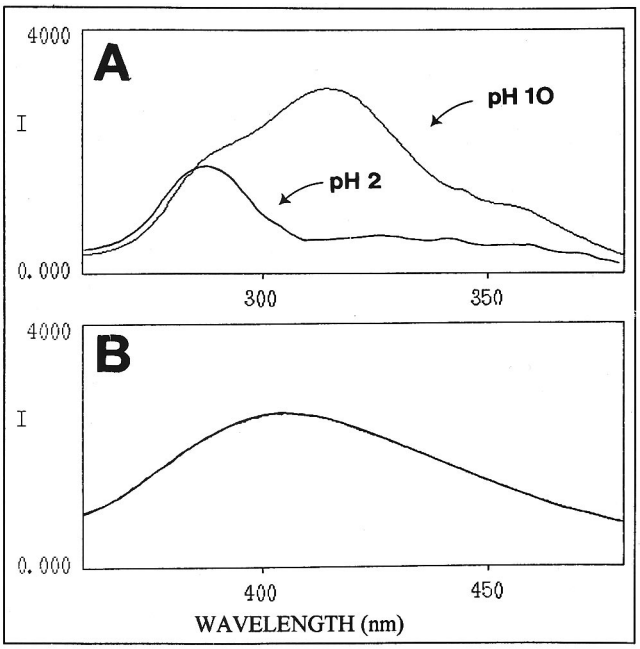

FIG. 6. Fluorometric scans of an HPLC-separated *C. albicans* hydrolysate peak with a retention time consistent with that of dityrosine. (A) Excitation scan performed with monitoring of emission at 410 nm. Note the characteristic shift of the excitation maximum with a change in pH. (B) Emission scan performed with excitation set at 320 nm. As expected, the scan pattern revealed an emission maximum of ~410 nm, which is characteristic of dityrosine. This emission pattern was essentially the same at pHs 2 and 10. I, fluorescence intensity.

DISCUSSION

This is the first report of the presence of dityrosine in a human fungal pathogen. Dityrosine has been detected in the spore coat of the uncommon human pathogen *Bacillus subtilis* (35) and on the cell surface of *Staphylococcus aureus* (33), but no further studies about dityrosine in those pathogenic bacteria have been described. Dityrosine is an intensely fluorescent compound first reported as an in vitro reaction product of horseradish peroxidase and hydrogen peroxide; subsequently, it has also been reported as a product of ionizing radiation, metal-catalyzed oxidation, tetranitromethane, and ozone (1). Dityrosine is believed to be formed by *ortho,ortho* (to the hydroxyl groups) cross-linkage of two tyrosyl radicals (10) (Fig. 7) that confers resistance to acid cleavage (resistant to 6 N HCl at 110°C for 24 h) and by proteases (including trypsin, chymotrypsin, and pronase [1]). Cross-links can be formed between existing tyrosine residues in structural and nonstructural proteins, as well as between soluble tyrosine residues (1). Naturally occurring dityrosine was first identified in hydrolysates of resilin, a structural protein found in the cuticle of locusts (1). It has also been identified in the exoskeletons of several species of nematodes (19, 20, 29), from chicken aorta elastin, and from certain bovine, rat, and cat connective tissues (1). The dityrosine cross-links are believed to be partially responsible for the elastic and insoluble qualities of these proteins (1). Dityrosine has been detected in humans in only trace amounts in proteins from lens cataracts (21), formed possibly by UV light exposure. In vitro, the myeloperoxidase of human neutrophil- and monocyte-derived macrophages can catalyze dityrosine formation from soluble tyrosine (26) and from tyrosine residues in proteins (27, 46), although dityrosine formation by human phagocytes in vivo has yet to be reported.

Work in two separate biological systems suggests that generation of dityrosine in vivo is important for protection of those

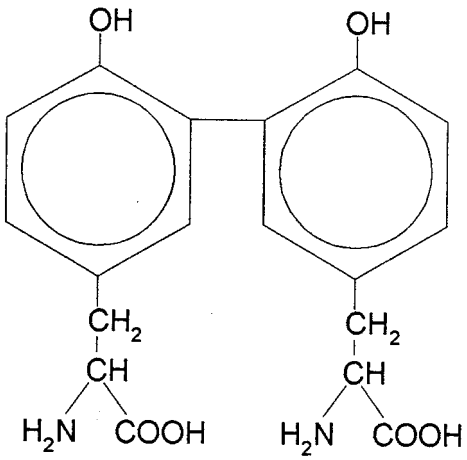

FIG. 7. Structure of dityrosine.

species from adverse environmental events. Dityrosine formation by sea urchin embryos is a carefully controlled developmental process catalyzed by an ovoperoxidase and H_2O_2 that is thought to be critical for protection of developing embryos from proteolysis and physical trauma (16, 31). A dityrosine content of only 5 to 8 residues per 10,000 amino acid residues is sufficient to make the organism resistant to disruption (31). The most extensively characterized biological system for dityrosine generation is that of sporulating *S. cerevisiae*. During periods of nitrogen and carbon starvation, diploid MAT**a**/MATα *S. cerevisiae* undergoes meiosis and ascospore formation. The developing ascospores add a third wall layer, rich in chitosan and chitin (8), and an outer, fourth layer composed of a peptidoglycan-like macromolecule (9) highly enriched in dityrosine and with lesser amounts of several other amino acids. The dityrosine content of *S. cerevisiae* ascospores is by far the highest reported for a natural biological product (31). Exposure of dityrosine-containing ascospores of *S. cerevisiae* to pronase, pepsin, trypsin, glusulase, or zymolyase does not kill the ascospores or alter ascospore morphology as monitored by electron microscopy (9, 34). Compared with wild-type vegetative cells (which have no dityrosine) or dityrosine-negative mutant ascospores, wild-type ascospores containing dityrosine in the outer ascospore layer are (i) 30- to 100-fold more resistant to heating at 55.5°C, (ii) 100-fold more resistant to ether, and (iii) extremely resistant to degradation by glusulase (β-glucuronidase-sulfatase enzymes from snail gut) (6).

Dityrosine production in *S. cerevisiae* ascospores requires the direct action of at least two sporulation-specific genes, designated *DIT1* and *DIT2* (6), and the activity of the general housekeeping gene *CPR1* (7). *DIT1* converts L-tyrosine to a soluble precursor which is covalently cross-linked by *DIT2* to form a dityrosine-containing precursor (7). *CPR1*, a non-sporulation-specific P-450 reductase necessary for ergosterol synthesis, is also absolutely necessary for dityrosine generation. The dityrosine-containing precursor is soluble and is presumably transported to and incorporated into the cell wall (7) to form an insoluble macromolecule with an incompletely defined structure (9).

By using cloned *S. cerevisiae DIT1* and *DIT2* to probe Southern blots of *C. albicans* genomic DNA digested with a variety of restriction endonucleases, we have identified hybridizing sequences at very high stringency which likely are highly homologous with both of the *S. cerevisiae* genes (37a). Many *C. albicans* genes have been identified and cloned by complementation of *S. cerevisiae* mutants and/or homology with *S. cerevisiae* genes, including several involved in cell wall synthesis and remodeling (3, 5, 12–14, 23, 42). Thus, identification of homologs of *DIT1* and *DIT2* in *C. albicans* would not be surprising. The specific locations and function of dityrosine in the *Candida* cell wall are unknown. The blue fluorescence of *Candida* bud scars and hyphal septae is interesting because those structures are enriched in chitin, which is also found in a wall layer near the plasma membrane and throughout the cell wall (22, 45). A direct covalent glycosidic link between chitin and β-(1,6)-glucan has been reported in *C. albicans* (44), while amino acids, particularly lysine and citrulline, have been implicated in the linkage of glucan and chitin in *Schizophyllum commune* (37). Covalent bridges of an undefined nature between mannoprotein and chitin (30) and mannoprotein and glucan (18) have been suggested for *C. albicans*. Incorporation of dityrosine into a highly cross-linked, insoluble macromolecule by *S. cerevisiae* occurs via a nonribosomal mechanism (7, 9), suggesting that, like synthesis of bacterial peptidoglycans, specific enzymes may catalyze the addition of each amino acid. The presence of such a mechanism of dityrosine formation and incorporation in *C. albicans* would provide a previously undescribed means for modifying the *Candida* cell wall. Currently, we are pursuing studies to help define the molecular biologic basis of dityrosine generation in *C. albicans.*

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-28408 from the National Institute of Allergy and Infectious Diseases (E.H.S.) and by the Austrian Fonds zur Förderung der Wissenschaftlichen Forschung, project S5809-MOB (P.B.).

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