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Phosphatase Activity Among *Candida* Species and Other Yeasts Isolated from Clinical Material

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A group of 277 yeasts isolated from burned children and 14 reference strains were tested for phosphatase activity by using phenolphthalein phosphate substrates. Phosphatase activity was widely distributed among various species and strains representing seven genera. *Candida albicans*, which was the most common yeast isolated from clinical material, was notably absent in producing the enzyme, whereas *Candida tropicalis* was the most consistent, strong, and rapidly active phosphatase-producing organism. The characteristic enzyme activity of a selected isolate of *C. tropicalis* was demonstrated in the presence of concentrations of inorganic phosphate which inhibited enzyme activity of other species. The greater enzyme activity of *C. tropicalis* was not related to more rapid or greater cell growth or decrease in the pH of culture media. Extracellular constitutive heat-labile acid phosphatase was found in broth filtrates of *C. tropicalis*, *C. krusei*, and a strain of *Staphylococcus aureus*.

Acid and alkaline phosphatases have been demonstrated cytologically in a variety of bacteria and yeasts (3). Phosphatase production has also been useful in the identification or biotyping of members of the family *Micrococcaceae* (1). Acid phosphatase activity has been correlated with the virulence of *Staphylococcus aureus* (2) and has also been studied in connection with virulence of *Candida albicans* (7). Very little is known about phosphatase activity of other *Candida* species and related yeasts isolated from human clinical specimens.

In this hospital, studies in progress deal with the ecology and epidemiology of yeasts in a population of burned children. During routine procedures to identify these organisms, studies were also conducted to determine the phosphatase activities of *C. albicans* and other species of yeasts.

MATERIALS AND METHODS

Isolation. Yeasts were primarily isolated from burned children using various swab collection methods previously reported (9, 10). Snyder agar and Littman Oxgall agar (BBL) were used to isolate yeasts. These media were incubated aerobically at 35 C for 3 days. Representative colonies were transfered to Sabouraud 4% dextrose (SD) broth (BBL) and further streaked on the SD agar plates, if necessary, to purify the cultures. The cultures were maintained on SD agar slants.

Identification. Clinical isolates were identified according to the procedures of Dolan and Ihrke (6) and Webb et al. (12). One culture each of *C. albicans*, *C. parapsilosis*, and *C. tropicalis* was obtained from the Department of Dermatology, University of Texas Medical Branch. One culture each of *C. albicans*, *Saccharomyces ellipsoideus*, *Schizosaccharomyces octosporus*, and *Geotrichum candidum* was obtained from the midwest Culture Service, Terre Haute, Ind. One culture each *Candida stellatoidea*, *C. tropicalis*, *C. pseudotropicalis*, *C. krusei*, *C. parapsilosis*, and *C. guilliermondii* was obtained from the Texas State Health Department, Austin (courtesy of Carl D. Heather).

Phosphatase activity. Screening tests to detect enzyme activity were conducted in SD broth. One-per cent solutions of phenolphthalein diphosphate or phenolphthalein monophosphate (PMP), obtained from Sigma Chemical Co., St. Louis, Mo., were prepared in distilled water and sterilized by filtration with 0.45-μm disposable filter units (BBL). The substrates were added to 5-ml quantities of SD broth to give a final concentration of 0.02% phenolphthalein diphosphate or PMP. Yeast strains were inoculated into the broths with a loop from 24-h SD broth cultures of each organism. The substrate containing broths were incubated at 35 C. At 24 and 48 h, the tubes were mixed on a vortex, and 1 ml was removed and tested for phosphatase activity by adding one drop of ammonium hydroxide to 1 ml of broth (5). The color intensity of the reaction was scored at 1+ to 4+, which ranged from a pale pink hue to a deep red. Enzyme activity, using the PMP substrate, was also compared in Mycophil broth at pH 7.0 (BBL), in Mycophil broth at pH 4.7, in Sabouraud 4% maltose

broth (BBL), and in SD broth. Enzyme activity was detected with ammonium hydroxide at 24-, 48-, and 72-h intervals. Phosphatase activity was examined in Sabouraud maltose broth with PMP substrate in the presence of gradient concentrations of KH_2PO_4 (11).

Comparative quantitative phosphatase activity was measured in conjunction with viable cell counts and changes in medium pH as a function of growth by using SD broth. Two broths with 0.01% PMP and two SD broths without substrate were inoculated with 10^4 viable cells per ml (colony-forming units) of test cultures. One set of broths with and without PMP was incubated at 30 C, and the other set was incubated at 37 C. All tubes were removed from the incubator at 24 and 48 h. Colony-forming units per milliliter of cultures in one tube incubated at 30 and 37 C was determined by spreading samples diluted in saline on SD agar plates. The pH of the broths was taken potentiometrically. The remainder of the broths containing PMP were centrifuged at 5,000 rpm for 10 min. One milliliter of broth supernatant was removed and mixed with 5 ml of 0.1 N NaOH. The quantity of free phenolphthalein released from the substrate was measured at 550 nm in 0.5-inch (approximately 1.27 cm) standardized tubes using the Bausch and Lomb Spectronic 20 colorimeter. The results were compared with a standard concentration gradient of phenolphthalein. Constitutive phosphatase production was detected in culture filtrates of SD broth without PMP. Broth filtrate (0.5 ml) was added to a sterile tube containing 0.5 ml of SD broth containing 0.02% PMP to give of final substrate concentration of 0.01% per ml (pH 5.6). One sample of each filtrate was boiled for 15 min as a control. All tubes were placed in a 37 C waterbath for 60 min at which time 5 ml of 0.1 N NaOH was added to each tube. Enzyme activity was again determined by the quantity of phenolphthalein released. A standard curve was also prepared to measure acid phosphatase by preparing gradient concentrations of acid phosphatase from 1 U/ml to 0.05 U/ml (Sigma) in SD broth. In these latter experiments, an endemic strain of *S. aureus*, phage type 84/85 (9) was tested with the yeasts. Trypticase soy glucose broth (BBL) was used in place of SD broth for testing the bacterium.

RESULTS AND DISCUSSION

A group of 277 yeasts isolated from burned children together with 14 named reference strains were screened for phosphatase activity (Table 1). Species of seven generic groups were represented, including seven species of the genus *Candida*. Phosphatase activity was observed in a variety of the strains but each of 200 *C. albicans* strains was negative after 24 h of incubation. All of the *C. tropicalis* strains were positive at 24 or 48 h and produced intense red (3+ to 4+) reactions. All of the other yeasts which were phosphatase positive at 24 h produced weaker positive reactions judged to be 1+ to 2+.

Fifty-two strains of candida were further tested for phosphatase activity in four broth media (Table 2). Some variations occurred among some species with regard to phosphatase activity detected in each medium, but *C. tropicalis* produced 3+ to 4+ reactions in all of the media after 24 h of incubation.

Further studies with selected strains of yeast species showed that the addition of gradient amounts of KH_2PO_4 to phosphatase test broth was the least inhibitory to *C. tropicalis* enzyme activity (Table 3). With this organism, phosphatase activity occurred in the presence of KH_2PO_4 concentrations 50 times greater than those which completely inhibited the enzyme activity of other candida species.

A comparative quantitative assay of phosphatase activity with six yeasts and one strain of *S. aureus* (Table 4) revealed that *C. tropicalis* was the most potent phosphatase active organism tested at both 30 and 37 C. The greater enzyme activity of this organism was not related to greater growth than the other strains or a more rapid and lower decrease in the pH of the culture media at either 30 or 37 C.

Phosphatase activity was detected in the substrate-free broth filtrate of *C. tropicalis*, *C. krusei*, and *S. aureus* (Table 5) with the *C. tropicalis* broth filtrate having the most enzyme activity. Among the three organisms with activity, the amounts of phosphatase present were less than 0.05 U/ml of broth based on an acid phosphatase standard. All enzyme activity was destroyed in these broth filtrates by boiling.

Chattaway, Odds, and Barlow (4) demonstrated alkaline and acid phosphatases in *C. albicans* and indicated that since washed whole cells served as a source of acid phosphatase, the enzyme was associated with or near the cell

TABLE 1. *Preliminary survey of phosphatase activity among yeasts*[a]

Organism tested	No. of strains tested	No. of strains positive	
		24 h	48 h
Candida species			
C. albicans	200	0	5
C. stellatoidea	7	0	1
C. parapsilosis	12	4	10
C. tropicalis	32	31	32
C. pesudotropicalis	6	2	3
C. krusei	7	2	4
C. quillermondii	6	1	3
Torulopsis glabrata	4	1	3
Trichosporon cutaneum	9	2	4
Geotrichum candidum	2	0	0
Saccharomyces cerevisiae	3	2	3
Saccharomyces ellipsoideus	1	0	1
Saccharomyces octosporus	1	0	0
Rhodotorula species	1	0	1

[a] Sabouraud dextrose broth containing 0.02% phenolphthalein diphosphate incubated at 35 C.

TABLE 2. *Comparative phosphatase activity of Candida species in various broth media*[a]

Species tested	Strains tested	Sabouraud broth						Mycophil broth					
		Dextrose			Maltose			pH 7.0			pH 4.7		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
C. albicans	16	0	0	0	0	0	13	0	0	0	0	0	7
C. stellatoidea	3	0	0	2	0	0	3	0	0	3	0	0	3
C. tropicalis	18	18	[c]	—	18	—	—	18	—	—	18	—	—
C. pseudotropicalis	3	1	1	2	0	2	2	1	1	1	1	1	3
C. parapsilosis	6	1	1	4	0	1	5	0	2	6	0	4	6
C. guillermondii	3	0	1	2	0	2	2	0	2	2	0	2	2
C. krusei	3	1	2	3	0	3	4	1	2	3	1	2	3

[a] Broths contained 0.02% phenolphthalein monophosphate incubated at 35 C.
[b] Number of strains positive at 24, 48, or 72 h of incubation.
[c] No further tests were done.

TABLE 3. *Comparative phosphatase activity of selected Candida species in the presence of monobasic potassium phosphate*[a]

Species and strain	KH_2PO_4 (μM/ml)														
	0			1			5			10			50		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
C. albicans TSHD	0[b]	0	3+	0	0	0	0	0	0	0	0	0	0	0	0
C. tropicalis TSHD	3+	4+	4+	2+	4+	4+	2+	4+	4+	1+	4+	4+	0	1+	4+
C. parapsilosis UTMB	0	2+	3+	0	0	0	0	0	0	0	0	0	0	0	0
C. pseudotropicalis TSHD	1+	3+	4+	0	2+	3+	0	1+	2+	0	0	0	0	0	0
C. krusei TSDH	2+	4+	4+	1+	2+	4+	0	1+	0	0	0	0	0	0	0

[a] Sabouraud dextrose broth containing 0.02% phenolphthalein monophosphate was incubated at 35 C and tested for activity at 24, 48, and 72 h. The initial pH of broth without added KH_2PO_4 was 5.6 and ranged from pH 5.6 to pH 5.2 with the addition of 50 μM KH_2PO_4 per ml.
[b] Intensity of color reaction 0, negative; 1+ to 4+, positive.

TABLE 4. *Relationships between growth and phosphatase activity of yeasts and Staphylococcus aureus*

Strain tested[a]	37 C						30 C					
	24 h			48 h			24 h			48 h		
	Log 10 CFU[b]/ml	Broth pH	Enzyme activity	Log 10 CFU/ml	Broth pH	Enzyme activity	Log 10 CFU/ml	Broth pH	Enzyme activity	Log 10 CFU/ml	Broth pH	Enzyme activity
C. tropicalis TSDH	6.68	5.1	0.0	6.90	4.7	66.0	7.04	4.7	48.0	7.41	4.5	57.0
C. pseudotropicalis	7.00	4.2	18.0	7.20	4.0	19.0	7.25	4.1	23.0	7.63	3.8	26.0
C. krusei TSHD	7.25	4.8	28.0	7.41	4.3	29.0	7.84	5.0	31.0	7.04	4.3	31.0
C. albicans UTMB	7.10	5.0	0.0	6.70	4.7	2.0	7.17	5.0	0.0	7.38	4.4	0.0
C. parapsilosis TSHD	6.77	5.3	2.0	7.25	4.9	3.8	7.44	5.3	2.3	7.67	4.8	10.0
S. cerevisiae MCS[c]	—	—	—	—	—	—	7.77	4.9	13.0	7.25	4.4	31.0
S. aureus	8.63	5.7	28.0	9.52	5.6	29.0	9.20	6.1	25.0	9.14	5.4	23.0

[a] Yeasts were grown in Sabouraud dextrose broth with initial pH of 5.7. *S. aureus* was grown in Trypticase soy glucose broth (initial pH 7.2). Enzyme activity was expressed as percentage of phenolphthalein released from 0.01% PMP substrate.
[b] CFU, colony-forming units.
[c] This strain grew poorly at 37 C and tests were not conducted at this temperature.

TABLE 5. *Constitutive phosphatase activity in broth filtrates of yeasts and Staphylococcus aureus*

Strains tested	Enzyme activity[a]			
	37 C		30 C	
	24 h	48 h	24 h	48 h
Candida tropicalis	3.9	9.9	3.2	7.9
C. pseudotropicalis	0.0	0.0	0.0	2.0
C. krusei	1.4	5.0	1.4	3.2
C. albicans	0.0	0.0	0.0	0.0
C. parapsilosis	0.0	0.0	0.0	0.0
Saccharomyces cerevisiae	[b]	—	0.0	0.0
Staphylococcus aureus	0.0	2.0	1.0	1.4

[a] Expressed as percent of phenolphthalein released from 0.01% PMP.

[b] Not tested.

wall. The acid phosphatases of *C. tropicalis*, or other yeasts for that matter, appear to be fundamentally different from that of *C. albicans* with respect to the extra-cellular activity of the enzyme in actively growing cultures. Phenolphthalein phosphate substrates were used in this study because of their potential colorimetric diagnostic applications, but additional work in progress indicates that the acid phosphatase enzyme of *C. tropicalis* acts upon *p*-nitrophenyl phosphate substrate (unpublished data).

In view of the well established methods which are available and used successfully for the identification of clinically isolated yeasts, the phosphatase test is not proposed here as a routinely useful diagnostic test but could be a simple and rapid optional method for the separation of *C. albicans* from other closely related species or for the identification of *C. tropicalis*.

Since candida species other than *C. albicans* may cause infection, particularly in the compromised host (8), the demonstration of acid phosphatases in several yeasts which are not commonly pathogenic in humans may also indicate that such enzymes can not be associated with virulence as in the case of *C. albicans* (4, 7) but could be useful to study in comparison to *C. albicans*.

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