JOURNAL OF CLINICAL MICROBIOLOGY, June 2001, p. 2089–2097 Vol. 39, No. 6
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.6.2089–2097.2001

Distinguishing *Candida* Species by β-*N*-Acetylhexosaminidase Activity

KYOKO NIIMI,[1] MAXWELL G. SHEPHERD,[2] AND RICHARD D. CANNON[1]*

Department of Oral Sciences and Orthodontics, School of Dentistry, University of Otago,[1] and Zenith Technology Ltd.,[2] Dunedin, New Zealand

Received 12 December 2000/Returned for modification 29 January 2001/Accepted 22 March 2001

A variety of fungi produce the hydrolytic enzyme β-*N*-acetylhexosaminidase (HexNAcase), which can be readily detected in assays by using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as a substrate. In the present study we developed a microtiter plate-based HexNAcase assay for distinguishing *Candida albicans* and *Candida dubliniensis* strains from other yeast species. HexNAcase activity was detected in 89 of 92 (97%) *C. albicans* strains and 4 of 4 *C. dubliniensis* strains but not in 28 strains of eight other *Candida* species, 4 *Saccharomyces cerevisiae* strains, or 2 *Cryptococcus neoformans* strains. The HexNAcase activity in *C. albicans* and *C. dubliniensis* was strain specific. All except three clinical *C. albicans* isolates among the *C. albicans* strains tested produced enzyme activity within 24 h. These strains did produce enzyme activity, however, after a prolonged incubation period. For two of these atypical strains, genomic DNA at the *C. albicans HEX1* gene locus, which encodes HexNAcase, showed nucleotide differences from the sequence of control strains. Among the other *Candida* species tested, only *C. dubliniensis* had a DNA sequence that hybridized with the *HEX1* probe under low-stringency conditions. The microtiter plate-based assay used in the present study for the detection of HexNAcase activity is a simple, relatively inexpensive method useful for the presumptive identification of *C. albicans* and *C. dubliniensis*.

Candida albicans is the yeast species most frequently isolated from human clinical specimens and causes a spectrum of superficial and systemic infections (15). The superficial infections such as oral candidiasis affect a large proportion of the population including neonates and elderly individuals, and candidal vaginitis afflicts more than 30% of all women (15). Systemic candidiasis usually occurs in immunocompromised individuals and can be life-threatening. The incidence of *Candida* infections has increased in recent years due to growing numbers of human immunodeficiency virus-positive people and patients with artificially suppressed immune systems. There has also been a recent change in the species distribution of *Candida* isolates causing infections. Although there are geography- and infection-related variations, the trend is for a larger proportion of infections to be caused by the innately azole antifungal-resistant *Candida glabrata* and *Candida krusei* species (19, 20). Thus, from a therapeutic point of view it is important to be able to rapidly determine the species of clinical yeast isolates.

C. albicans produces a hydrolytic enzyme, previously termed chitobiase or β-*N*-acetylglucosaminidase, that acts on the chitin oligomers *N,N′*-diacetylchitobiose and *N,N′,N′′*-triacetylchitotriose (13). The enzyme also cleaves the chromogenic substrates *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNP-GlcNAc) and *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide to release *p*-nitrophenol (pNP) and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide to release 4-methylumbelliferone (13, 33). Since the enzyme has broad substrate specificity, it is more correctly termed an *N*-acetylhexosaminidase (HexNAcase [4, 6]).

HexNAcase is produced by a range of organisms including bacteria, fungi, and mammalian cells. Among fungal species, HexNAcase is produced mainly by filamentous fungi such as *Aspergillus* species (1, 12, 22), *Penicillium oxalicum* (34), *Mucor fragiles* (35), *Sclerotinia fructigena* (21), or *Neurospora crassa* (23); and the enzyme plays an important role in autolysis of fungal cell walls in these molds. Although there has been no systematic study of HexNAcase activity by *Candida* species, preliminary reports have shown no or low enzyme activity in yeast species other than *C. albicans* (7, 10, 14). HexNAcase production by *C. albicans* is induced by growth on the enzyme product *N*-acetylglucosamine (GlcNAc) (33), and *C. albicans* can utilize GlcNAc as the sole carbon or nitrogen source. *C. albicans* HexNAcase is a secreted enzyme, and 30% of the total enzyme activity can be recovered from the culture medium of yeast cells after 5 h of growth on GlcNAc (14). These features suggest that a major function of *C. albicans* HexNAcase is in a nutrient-scavenging pathway that may give the cells a growth advantage (14). Indeed, HexNAcase was identified as a virulence factor for *C. albicans* since a HexNAcase-deficient mutant (EOB4) of strain ATCC 10261 was less pathogenic than the parental strain in a mouse infection model (8).

The observation that HexNAcase production among yeast appears to be specific for *C. albicans* and that *C. albicans* secretes this enzyme and can grow on GlcNAc as the sole carbon source led us to investigate whether these features could be used to devise a microtiter plate-based *C. albicans* identification assay.

MATERIALS AND METHODS

Strains and growth conditions. *Candida, Saccharomyces cerevisiae*, and *Cryptococcus neoformans* strains were routinely maintained on YPD agar (yeast extract, 1%; Bacto Peptone, 2%; glucose, 2%; agar, 2%). The sources of the *C. albicans* strains are listed in Table 1, and the sources of non-*C. albicans Candida, S. cerevisiae*, and *C. neoformans* strains are listed in Table 2.

* Corresponding author. Mailing address: Department of Oral Sciences and Orthodontics, University of Otago, P.O. Box 647, Dunedin, New Zealand. Phone: 64 3 479 7081. Fax: 64 3 479 0673. E-mail: richard.cannon@stonebow.otago.ac.nz.

TABLE 1. *C. albicans* laboratory strains and clinical isolates used in the study

Strain	Characteristic or source
A72	A reference strain for HexNAcase assay (4, 14); A. Cassone, Instituto Superiore di Sanita, Rome, Italy
ATCC 10231 and ATCC 10261	American Type Culture Collection, Manassas, Va.
CA2	A. Cassone, Instituto Superiore di Sanita, Rome, Italy
CAI4	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*; W. Fonzi, University of California, Irvine
C-14-1, C-13-2, D27, D57, D68, D106, D156, D259, D273, and D294	Clinical isolates; School of Dentistry, University of Otago, Dunedin, New Zealand
EOB4	HexNAcase-negative mutant (8)
IAM12201	Formerly *C. stellatoidea*; Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan
I1 to I47	Clinical isolates; J. Schmid, Massey University, Palmerston North, New Zealand
MEN	Heterozygous met$^-$, 5FC^r; W. L. Whelan, University of Cambridge, Cambridge, United Kingdom
R-2477	Clinical isolate; Health Science Center at San Antonio, The University of Texas, San Antonio
SGY-243	*ade2*/*ade2*, Δ*ura3*::*ADE2*/Δ*ura3*::*ADE2*; R. Kelly, Squibb Institute for Medical Research, Princeton, N.J.
TIMM1309	Clinical isolate, formerly *C. stellatoidea*; Institute of Medical Mycology, Teikyo University, Tokyo, Japan
TIMM1369 and TIMM3163 to TIMM3166	Clinical isolates; Institute of Medical Mycology, Teikyo University, Tokyo, Japan
1713D2	Heterozygous *HEX1* disruptant (Δ*hex1*::*URA3*/*HEX1*); R. D. Cannon, School of Dentistry, University of Otago, Dunedin, New Zealand
MN2, MN6, and MN14 to MN16	Clinical isolates (10)
MN34	Isolate from healthy individual (10)
93-1591 to 93-2067	Clinical isolates; Health Science Center at San Antonio, The University of Texas, San Antonio

HexNAcase assay of cell-free enzyme activity. Cell-free HexNAcase was measured as described by Niimi et al. (14). Yeast cells were grown in YPD medium at 30°C for 16 h. The cells were harvested (by centrifugation at 3,000 × *g* for 5 min at 4°C), washed twice in sterile water, resuspended at an optical density at 600 nm (OD_{600}) of 1.0 to 2.0; and starved by incubation at 30°C for 3 h with shaking (200 rpm). The cells were harvested, resuspended at an OD_{600} of 1.0 in salts-biotin-yeast nitrogen base medium (14) containing either glucose or GlcNAc as the carbon source (25 mM), and incubated at 30°C for 3 h. The cells were then washed twice and resuspended in 0.5 ml of assay buffer (0.1 M citric acid, KOH [pH 4.0]). Extracts of the glucose- or GlcNAc-grown yeast cells were obtained by vortexing them with glass beads and centrifuging the samples (8,000 × *g* for 5 min at 4°C) to remove cell walls. The HexNAcase activities of the supernatants, which corresponded to cell-associated (cytoplasmic and periplasmic) enzyme activity, were measured with pNP-GlcNAc as the substrate, as described previously (14). The protein concentrations of the cell extracts were determined by the method of Bradford (3) with a Bio-Rad protein assay kit.

Microtiter plate-based HexNAcase assay with intact cells. The HexNAcase activities of intact cells (which comprise periplasmic, wall-associated, and secreted enzyme activities) were measured by a microtiter plate-based assay. Yeast cells from YPD agar plates were suspended in sterile distilled water to concentrations of 1×10^7 to 5×10^7 cells/ml. These cell suspensions were diluted in filter-sterilized buffered YNB-GlcNAc medium (yeast nitrogen base, 0.67% [wt/vol]; 0.1 M citric acid-KOH [pH 4.5]; 5 mM GlcNAc) to a concentration of 2×10^4 cells/ml. The YNB-GlcNAc medium in microtiter plate wells (150 μl per well) was inoculated with the diluted cells (5 μl) to a density of 100 cells per microtiter plate well, and the plate was incubated at 35°C for 24 h. For ura$^-$ strains SGY-243, 1713D2, and CAI4, uracil was added to the buffered medium (50 μg ml^{-1}). Leucine and histidine (50 μg ml^{-1} each) were added to the growth medium for *S. cerevisiae* AH22. Each strain was tested in at least quadruplicate incubations. After 24 h of incubation, 50 μl of pNP-GlcNAc dissolved in YNB-buffered medium was added to the cells to give a final concentration of 0.5 mM, and the cells were incubated at 35°C for a further 4 h. The HexNAcase reaction was terminated by adding 50 μl of 1.6 M NaOH to each microtiter plate well, and the amount of pNP liberated from pNP-GlcNAc was measured at A_{405} ($\varepsilon = 16.0 \times 10^3$ liter mol^{-1} cm^{-1}) with a microtiter plate reader (Bio Kinetics Reader EL 340; Bio-Tek Instruments). The growth yield of cultures was determined by measuring the A_{405} prior to termination of the HexNAcase assay, and the cell density was subtracted from the A_{405} of the enzyme assay. Specific enzyme activity was expressed as nanomoles of pNP minute^{-1} (100 cells [initial inoculum size])$^{-1}$, unless stated otherwise.

GlcNAc assimilation test. *C. albicans* strains and other *Candida* species that did not grow on GlcNAc within 24 h of incubation were tested for GlcNAc assimilation. The cells were inoculated at a density of 10^3 cells ml^{-1} in YNB-buffered medium supplemented with 50 mM glucose or 50 mM GlcNAc and were incubated at 30°C with shaking for 7 days. Cell growth in glucose- or GlcNAc-containing medium was monitored every 24 h, and after 7 days of incubation the HexNAcase activity of the culture (150 μl) was measured by the microtiter plate-based assay.

Southern blotting and hybridization. The methods of Scherer and Stevens (26) or Kingsman et al. (11) were used to isolate genomic DNA from *Candida* or *S. cerevisiae* cells, respectively. Genomic DNA was restricted with *Eco*RI, electrophoresed through 0.8% (wt/vol) agarose, Southern blotted onto Hybond-N$^+$ nylon membranes (Amersham, Little Chalfont, United Kingdom), and hybridized with a *HEX1* probe as described by Sambrook et al. (25). A 2.1-kb DNA fragment of the *HEX1* gene was amplified by PCR with plasmid pRC1708 (4) as a template and the primers CD6 (5′-TTTGGTTGTGCAATGTG-3′) and CD9 (5′-CACGTAGATACAGAAAG-3′). This DNA fragment contained the entire *HEX1*-coding region (except the first 37 bp of the open reading frame) and 452 bp of the 3′ noncoding region. The *HEX1* probe was radiolabeled with [^{32}P]dCTP and hybridized with the Southern blots under either high- or low-stringency conditions (25).

Growth on CHROMagar Candida. CHROMagar Candida (CHROMagar Company, Paris, France) agar plates were prepared according to the manufacturer's instructions. *C. albicans* strains that did not produce HexNAcase activities under the standard conditions and *Candida dubliniensis* and *Candida rugosa* strains were streaked onto CHROMagar Candida plates, and the plates were incubated at 35°C for 48 h. Colony colors were compared to those for reference yeast strains.

RESULTS

Induction of cell-associated HexNAcase activity in selected strains. Induction of HexNAcase activity by growth on GlcNAc was measured in cell extracts (comprising cytoplasmic and periplasmic enzyme activities) of several yeast strains by a standard assay (14). HexNAcase activity was detected in all *C. albicans* strains tested when the strains were grown on glucose, and specific enzyme activity was increased by up to 300-fold when the cells were grown on GlcNAc (Table 3). There was strain variation in this induced HexNAcase activity. Reference strain A72 (from which HexNAcase has been purified and the

TABLE 2. Non-*C. albicans Candida, C. neoformans*, and *S. cerevisiae* strains

Species	Strain(s)	Characteristic or source
C. dubliniensis	CD36, CD41, CD43, CD57	Clinical isolates; D. C. Coleman, School of Dental Science, University of Dublin, Dublin, Republic of Ireland
C. glabrata	CBS138	Schimmelcultures, Baarn, The Netherlands
	CBS2175	Schimmelcultures, Baarn, The Netherlands
	850821	ESR[a]
	850920	ESR
C. guilliermondii	IFO0838	Institute for Fermentation, Osaka, Japan
	85.739	ESR
	85.791	ESR
	89.140	ESR
C. kefyr	Colindale	ESR
	78.1161	ESR
	78.256	ESR
	82.656	ESR
	B2455	ESR
C. krusei	IFO0011	Institute for Fermentation, Osaka, Japan
	89.102	ESR
	89.221	ESR
	90.147	ESR
	B2399	ESR
C. lusitaniae	TIMM1668	Institute of Medical Mycology, Teikyo University, Tokyo, Japan
	TIMM3482	Institute of Medical Mycology, Teikyo University, Tokyo, Japan
C. parapsilosis	MCC499	ESR
	90.111	ESR
	90.454	ESR
	90.463	ESR
	90.493	ESR
C. rugosa	TIMM0307	Institute of Medical Mycology, Teikyo University, Tokyo, Japan
	TIMM3489	Institute of Medical Mycology, Teikyo University, Tokyo, Japan
C. tropicalis	IFO0618	Institute for Fermentation, Osaka, Japan
	820567	ESR
	820738	ESR
C. neoformans	ATCC 90112	New Zealand Reference Culture Collection, ESR, Wellington, New Zealand (NZRM 3396)
	ATCC 90113	New Zealand Reference Culture Collection, ESR, Wellington, New Zealand (NZRM 3397)
S. cerevisiae	AH22	G. R. Fink, Massachussetts Institute of Technology, Cambridge, Mass. (*mat***a**, *leu2-3, leu2-112, his4-519, can1*)
	y55	J. E. Haber, Brandeis University, Waltham, Mass. (HO, *gal3, MAL1, SUC1*)
	2180A	P. A. Sullivan, Massey University, Palmerston North, New Zealand
	DYC	School of Dentistry, University of Otago, Dunedin, New Zealand

[a] ESR, Institute of Environmental Science and Research Health, New Zealand Center for Disease Control, Wellington, New Zealand.

gene encoding HexNAcase, *HEX1*, has been cloned [4]) and clinical isolate I33 produced high levels of enzyme activity while strains I1, ATCC 10261, and SGY-243 produced relatively low levels of activity. *C. dubliniensis* strains behaved similarly to *C. albicans* strains in respect to HexNAcase activity. There were low levels of activity in cells grown on glucose and high levels of activity in cells grown on GlcNAc. The only other *Candida* species to display HexNAcase activity was *Candida tropicalis*. The HexNAcase activities of *C. tropicalis* cells grown on glucose were similar to those of *C. albicans* or *C. dubliniensis* cells grown on glucose. Unlike these species, however, the enzyme activities of *C. tropicalis* cells grown on GlcNAc were only three to four times higher than those of cells grown on glucose. The HexNAcase activity secreted into the culture medium (extracellular enzyme activity) was also measured for these strains. *C. albicans* A72 and *C. dubliniensis* strains CD36 and CD41 grown on GlcNAc secreted, on average, 28, 20, and 12% of total enzyme activity into the medium, respectively. No HexNAcase activity was detected in the culture supernatants of any of the other yeast species tested, including *C. tropicalis*. GlcNAc supported the growth of all yeast species apart from *Candida glabrata* and *S. cerevisiae*.

Having established that *C. albicans* cells could grow on GlcNAc as the sole carbon source and that under these conditions cells secreted high levels of HexNAcase into the culture medium, we proceeded to develop a microtiter plate-based enzyme assay that would detect HexNAcase activity after growth of cells on GlcNAc.

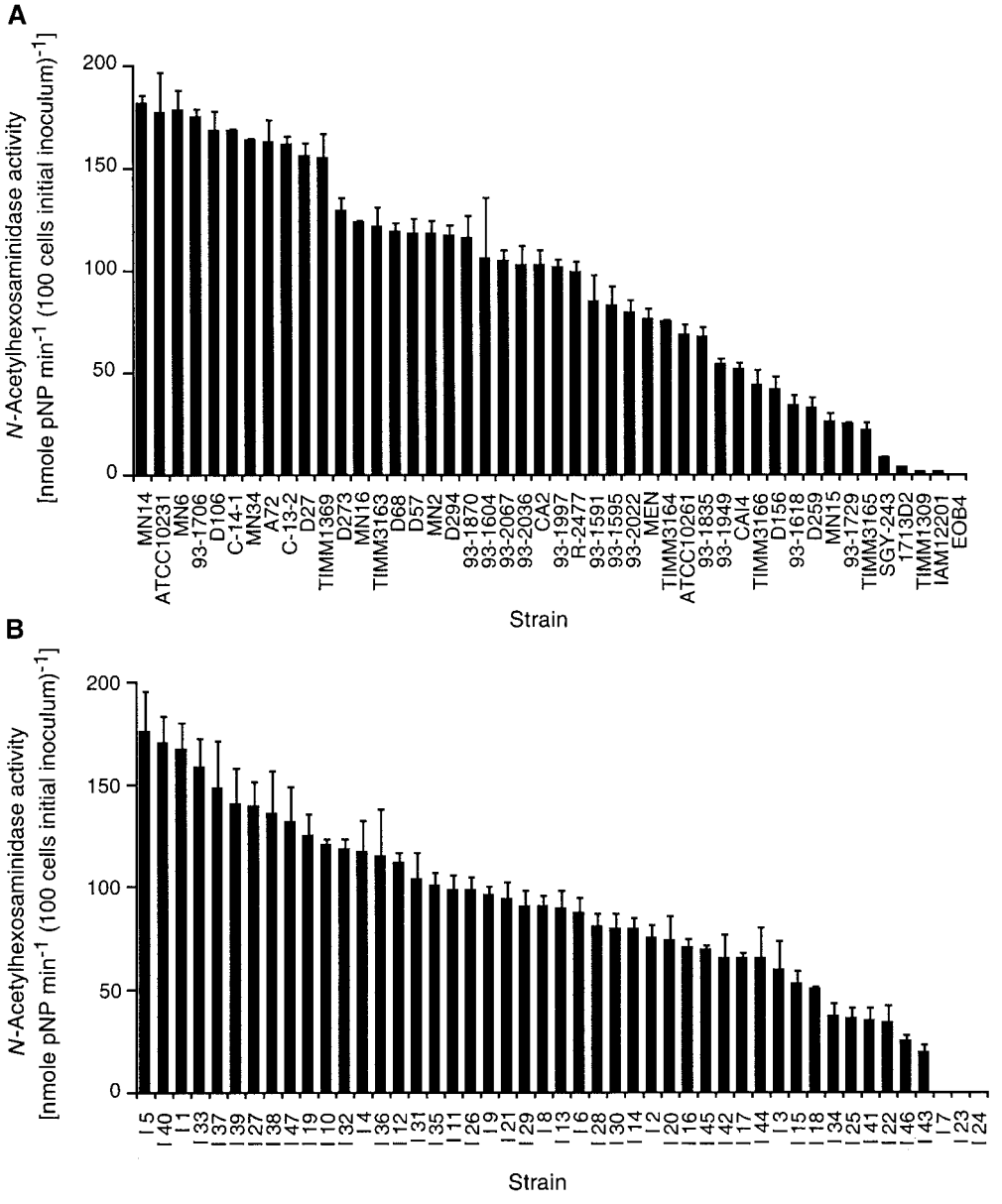

FIG. 1. Strain-specific variations in the HexNAcase activities of *C. albicans* laboratory strains and clinical isolates (A) and in a set of partially characterized *C. albicans* clinical isolates (B) (28). HexNAcase activity was measured by the microtiter plate-based assay. The results are the means ± standard deviations of quadruplicate assays.

HexNAcase microtiter plate-based assay. HexNAcase activity was measured after growth on GlcNAc rather than at the time of growth, as the growth of some *C. albicans* strains was found to be sensitive to the substrate pNP-GlcNAc. The GlcNAc concentration in the growth medium, growth incubation temperature, incubation volume, and initial inoculum size were varied in order to maximize the HexNAcase activity for *C. albicans* A72 obtained after 24 h of incubation. These conditions, detailed in the Materials and Methods section, were then used to measure HexNAcase activity in a range of yeast strains.

HexNAcase activity in *C. albicans* laboratory strains and clinical isolates. There was great strain variation in the HexNAcase activities of *C. albicans* laboratory strains and clinical isolates, with most strains possessing less activity than reference strain A72 (Fig. 1A and B). There was no correlation between HexNAcase activity and whether the strains were laboratory strains or clinical isolates. Clinical isolates I7, I23, and I24 did not produce enzyme activity under the standard assay conditions (Fig. 1B), but these cells did not grow well during the assay. HexNAcase-deficient mutant EOB4 derived from ATCC 10261 (8) also did not produce detectable enzyme activity, as expected (Fig. 1A). Heterozygous *HEX1* disruptant 1713D2 produced slightly less than half the HexNAcase activity of parental strain SGY-243 (Fig. 1A). Both SGY-243 and 1713D2 are ura$^-$ auxotrophs and did not grow well within the 24-h growth period on uracil-supplemented YNB-buffered medium. Another two strains that produced low levels of enzyme

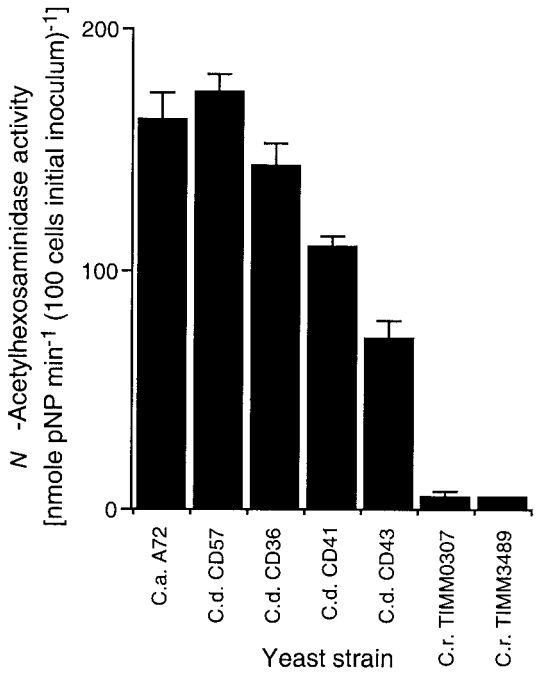

FIG. 2. HexNAcase activities of non-*C. albicans Candida* species. HexNAcase activity was measured by the microtiter plate-based assay. *C. a., Candida albicans* (positive control); *C. d., Candida dubliniensis*; *C. r., Candida rugosa*. The results are the means ± standard deviations of quadruplicate assays.

activity, TIMM1309 and IAM12201, were formerly classified as *Candida stellatoidea* and had been reclassified as *C. albicans* (Fig. 1A). These strains also did not grow well under the assay conditions.

HexNAcase activity in other *Candida* species and in *S. cerevisiae*. Among *Candida* species, *C. dubliniensis* was the only species, other than *C. albicans*, that produced a high level of HexNAcase activity (Fig. 2). The enzyme activities produced by intact cells of the four strains tested were similar to or slightly lower than that for *C. albicans* A72. This was in contrast to the high levels of activity of cell-free extracts (Table 3), which may reflect the smaller proportion of total enzyme activity secreted by intact *C. dubliniensis* strains. *C. rugosa* produced a very low level of enzyme activity: 0.3 to 3.3% of the level of enzyme activity produced by strain A72. *C. tropicalis* did not produce detectable enzyme activity by the microtiter plate-based assay, even though a low level of enzyme activity had been detected in cell-free extracts (Table 3). This is consistent with the finding that no extracellular enzyme activity was detected in *C. tropicalis* culture supernatants. None of the other *Candida* species, *C. neoformans*, or *S. cerevisiae* strains produced detectable enzyme activity under the assay conditions used. All yeast species apart from *C. glabrata, Candida kefyr*, and *S. cerevisiae* grew on GlcNAc during the assay.

GlcNAc assimilation and HexNAcase production in *C. albicans* HexNAcase$^-$ strains. The microtiter plate-based assay failed to identify three *C. albicans* clinical isolates, isolates I7, I23, and I24, by their HexNAcase production. The possibility that this was due to lack of growth during the assay was investigated by undertaking a GlcNAc assimilation test with a prolonged incubation period. Strain I7 started to grow on GlcNAc after 5 days of incubation and eventually produced a low level of HexNAcase activity (Fig. 3). Strain I23 grew on glucose and produced enzyme activity after 1 week of incubation. This strain, however, neither utilized GlcNAc as a carbon source nor produced HexNAcase activity within the 7 days of incubation. I24 did not grow for the first 24 h on either glucose or GlcNAc. However, by 48 h the growth reached the stationary phase and the strain produced a moderate level of HexNAcase activity (Fig. 3). The GlcNAc assimilations of the *C. glabrata* and *C. kefyr* strains, which did not grow on GlcNAc within the 24 h of the microtiter plate-based assay, were also tested. None of the *C. glabrata* strains tested utilized GlcNAc as a sole carbon source. *C. kefyr* showed mixed results; three of the five strains did not utilize GlcNAc, and the other two strains grew slightly on GlcNAc but had no detectable HexNAcase activity.

TABLE 3. HexNAcase activities of cell extracts from *Candida* species and *S. cerevisiae*[a]

Species	Strain	HexNAcase activity (mmol of pNP min^{-1} [mg protein]$^{-1}$)	
		Uninduced (glucose)	Induced (GlcNAc)
C. albicans	A72	0.047	6.66[b]
	ATCC 10261	0.023	1.47
	SGY-243	0.050	3.82
	I1	0.069	3.65
	I17	0.060	6.23
	I33	0.038	12.46
	I44	0.062	5.37
C. dubliniensis	CD36	0.029	14.22
	CD41	0.031	17.88
C. glabrata	CBS138	0	0[c]
C. guilliermondii	85791	0	0
C. krusei	B2399	0	0
C. parapsilosis	MCC499	0	0
C. tropicalis	820567	0.040	0.149
	820738	0.021	0.137
	IFO0618	0.059	0.119
S. cerevisiae	AH22	0	0[c]
	y55	0	0[c]
	2180	0	0[c]
	DYC	0	0[a]

[a] Yeast cells were grown in the presence of either glucose (25 mM; uninduced) or GlcNAc (25 mM; induced) before cell disruption, and the HexNAcase activity was measured by using pNP-GlcNAC as the substrate. The results are the means of two determinations with separate batches of cells, which did not vary by more than 20%.

[b] The value for this enzyme activity is the mean of six determinations, and the standard deviation was ±1.97.

[c] The yeasts did not grow on GlcNAc.

Southern blot analysis of *HEX1* gene in *C. albicans* and other *Candida* species. A 2.1-kb DNA fragment containing the *C. albicans HEX1*-coding region (and an *Eco*RI site) was used as a probe to confirm the restriction fragment profile of the *HEX1* gene in *C. albicans* strains and to detect DNA sequences similar to *HEX1* in other *Candida* species. The probe is predicted to hybridize with two *Eco*RI-digested genomic DNA fragments

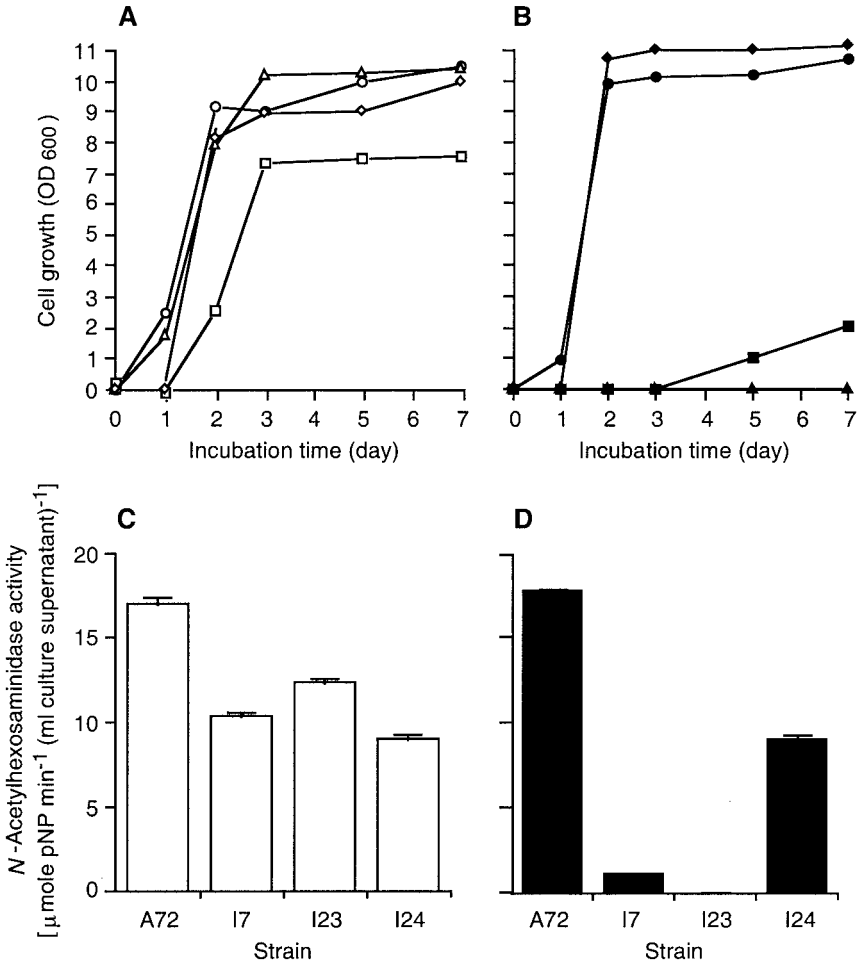

FIG. 3. Growth and HexNAcase activities of *C. albicans* clinical isolates I7, I23, and I24. Cells were grown on glucose (25 mM; A and C) or GlcNAc (25 mM; B and D). Cell growth was measured by monitoring the OD_{600} (A and B). The HexNAcase activities of the culture supernatants were measured at day 7 (C and D). The results are the means ± standard deviations of quadruplicate assays. The strains tested were A72 (positive control; ○, ●), I7 (□, ■), I23 (△, ▲), and I24 (◇, ◆).

(2.9 and 5.2 kb [4]). The probe hybridized with fragments of the expected size for strains A72 and ATCC 10261 (Fig. 4). The band of 1.8 kb for *C. albicans* ATCC 10261 and I7 could represent a fragment length polymorphism due to a point mutation resulting in allelic variation. At distance 1.8 kb downstream of the *Eco*RI site present in *HEX1* (and in the probe), there is a match of five of six nucleotide positions to an *Eco*RI restriction site in the *C. albicans* SC5314 DNA sequence (http://www.sequence.stanford.edu/group/candida/). This position is in the 5.2-kb *Eco*RI fragment. A point mutation at this location to form an *Eco*RI site in one allele would give one fragment of 1.8 kb and one of 5.2 kb (in addition to the two copies of the 2.9-kb fragment) that hybridize with the probe. Genomic DNA isolated from strains I7 and I23, neither of which produced any enzyme activity under the standard microtiter plate-based assay conditions, showed banding patterns different from those of the reference laboratory strains (Fig. 4). Another clinical strain, I24, which produced HexNAcase activity after 48 h showed the expected *HEX1* hybridization pattern (Fig. 4). All the strains of *C. dubliniensis* tested had DNA fragments that hybridized with the *HEX1* probe under low-stringency conditions, but the sizes of those fragments (1.0 and 6.2 kb) were different from those of fragments from *C. albicans*. Although a low level of enzyme activity was detected in cell-free extracts of *C. tropicalis* (Table 3) and in microtiter plate-based assays with *C. rugosa* (Fig. 2), no DNA sequence similar to *HEX1* was detected in these species even under low-stringency conditions. None of the other *Candida* species tested (*C. glabrata, Candida guilliermondii, C. kefyr, Candida krusei, Candida lusitaniae*, and *Candida parapsilosis*) or *S. cerevisiae* contained DNA that hybridized with the *HEX1* probe. The hybridization of the *HEX1* probe with genomic DNA from clinical isolates I7, I23, and I24 under high-stringency conditions confirmed that they are *C. albicans* strains.

Growth on CHROMagar Candida. CHROMagar Candida is a proprietary agar that contains undisclosed chromogenic substrates for metabolic enzymes that color growing yeast colonies and that enables certain species to be differentiated (16). HexNAcase-positive *C. albicans* and *C. dubliniensis* strains gave green or blue-green colonies on CHROMagar Candida, and these multiply subcultured isolates could not be reliably differentiated. Interestingly, *C. albicans* HexNAcase$^-$ mutant

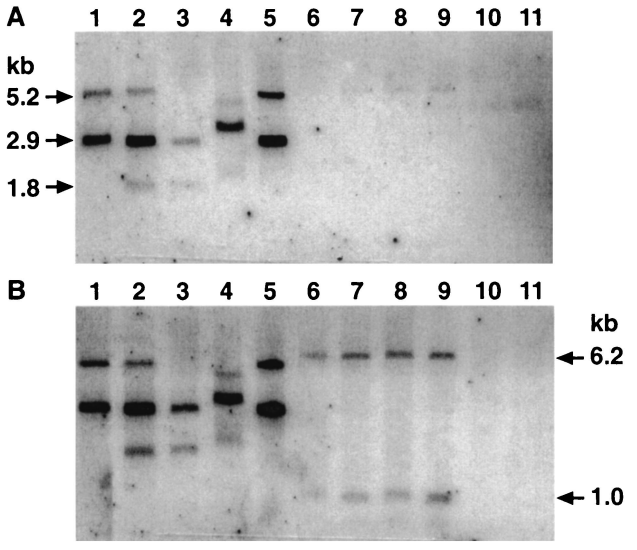

FIG. 4. Southern blot analysis of the *HEX1* gene locus in *Candida* species. Genomic DNA from *Candida* strains was restricted with *Eco*RI, electrophoresed through 0.8% agarose gels, vacuum blotted onto nitrocellulose membranes, and hybridized with a [^{32}P]dCTP-labeled *HEX1* DNA probe. Autoradiograms of Southern blots after hybridization under: high-stringency conditions (A) and low-stringency conditions (B) are shown. Lanes: 1, *C. albicans* A72; 2, *C. albicans* ATCC 10261; 3, *C. albicans* I7; 4, *C. albicans* I23; 5, *C. albicans* I24; 6, *C. dubliniensis* CD36; 7, *C. dubliniensis* CD41; 8, *C. dubliniensis* CD43; 9, *C. dubliniensis* CD57; 10, *C. rugosa* TIMM0307; 11, *C. rugosa* TIMM3489.

EOB4 grew as white (uncolored) colonies on CHROMagar Candida. Clinical *C. albicans* isolate I24, which could utilize GlcNAc but which produced detectable HexNAcase only after 48 h of incubation, grew as green colonies on CHROMagar Candida and was indistinguishable from *C. albicans* A72. I23 formed bluish colonies within the color range for *C. albicans* strains, whereas I7 formed deep blue colonies distinct from the colors of the colonies of the other *C. albicans* strains. The two strains of *C. rugosa* that gave very low levels of HexNAcase activity in the microtiter plate-based assay could be distinguished from *C. albicans* on CHROMagar Candida by a blue-purple colony color.

DISCUSSION

The HexNAcase assay with the substrate pNP-GlcNAc has been used to detect enzyme activity in various fungal species including *Aspergillus niger* (9), *Tremella fuciformis* (30), *S. fructigena* (21), and *C. albicans* (14, 33). Although it is not as sensitive as the methylumbelliferyl assay, it is robust and does not require a UV spectrophotometer. HexNAcase activity was not detected in cell extracts of *C. krusei, C. parapsilosis, C. glabrata, C. guilliermondii*, or *S. cerevisiae*. The absence of detectable HexNAcase activity correlated with a lack of a DNA sequence similar to that of *C. albicans HEX1* in these species. Although a small amount of HexNAcase activity was detected in *C. tropicalis* strains (about 2% of the activity of *C. albicans* A72 cells grown on GlcNAc), this species possessed no DNA sequence similar to the *HEX1* sequence. Kamiyama et al. (10) also detected HexNAcase activity in *C. tropicalis* with the API ZYM system (API SYSTEM S.A., Montalieu Vercieu, France). However, this detection system often gives false-positive results for HexNAcase activity (M. Niimi, University of Otago, personal communication). Therefore, the HexNAcase assay result may be due to the nonspecific activity of another enzyme(s) which fortuitously hydrolyzes the substrate pNP-GlcNAc. Alternatively, the gene in *C. tropicalis* has a low level of nucleotide similarity to *C. albicans HEX1* and cannot be detected under the low-stringency hybridization conditions used. In the present study, *S. cerevisiae* was unable to utilize GlcNAc as a sole carbon source. Uptake of GlcNAc has been observed in pathogenic yeast species including *C. albicans*, but it has not been observed in *S. cerevisiae* (29); therefore, the lack of GlcNAc utilization may be explained by the absence of a GlcNAc uptake system in this species.

GlcNAc, the final product of chitin oligomer degradation by *N*-acetylhexosaminidase, is known to induce HexNAcase synthesis in *C. albicans* (4, 5). Therefore, this amino sugar was added to the semidefined YNB-buffered medium as a carbon source to selectively enable *C. albicans* cells to grow and induce HexNAcase synthesis simultaneously. The microtiter plate-based HexNAcase assay was successfully used to distinguish all *C. dubliniensis* and 89 of 92 (97%) *C. albicans* strains (excluding HexNAcase-deficient mutant EOB4) from 28 strains of eight other *Candida* species. The three clinical *C. albicans* isolates that gave no HexNAcase activity in the microtiter plate-based assay were investigated further. A previous study reported that all *C. albicans* strains tested utilized GlcNAc as a sole carbon source (10). This was not the case, however, for clinical isolate I23. This strain may lack the enzyme(s) involved in GlcNAc uptake or metabolism, resulting in impaired HexNAcase synthesis. Strain I23 did grow on glucose and yielded HexNAcase activity after 7 days of incubation. Although HexNAcase is produced only at a low constitutive rate when cells are grown on glucose, HexNAcase could be secreted and accumulate in the culture medium during a 7-day incubation, as the extracellular enzyme activity has been shown to be stable (13, 14). I23 also possessed *Eco*RI restriction fragments of genomic DNA that hybridized with the *HEX1* probe but whose sizes were different from those for typical *C. albicans* strains, suggesting that there are nucleotide changes, possibly insertions or deletions, at the *HEX1* gene locus. I7 and I24 were found to be HexNAcase-positive strains, as these strains eventually utilized GlcNAc and produced enzyme activity. Strain I24 had DNA bands that hybridized with the *HEX1* probe of the same size as reference strain A72; therefore, it may be that I24 has a low growth rate due to deficiencies in sugar uptake or metabolism. Strain I7 may also possess at the *HEX1* gene locus nucleotides different from those of A72 although these changes are less striking than those for I23. The DNA fingerprints of clinical isolates I1 through I47 have been analyzed by using the moderately repetitive sequence Ca3 to create a dendrogram showing the genetic relationships between *C. albicans* strains (28). Strains I7, I23, and I24 (strains hp56an, hp36bt, and wo-1c, respectively, in the paper of Schmid et al. [28]) fell into three different groups of genetically related strains, but none of these groups was the major cluster of genetically similar strains of *C. albicans* that is highly prevalent in multiple geographic regions, patient types, and types of infection (27). Thus, these three isolates are atypical *C. albicans* strains, and this is reflected in their GlcNAc metabolism.

The microtiter plate-based HexNAcase assay results indicated that *C. albicans* strains are generally able to utilize GlcNAc and produce HexNAcase but that a very few strains are deficient in GlcNAc assimilation or lack a functional *HEX1* gene. The species-specific production of distinctive metabolic enzymes is the basis of a number of *C. albicans* identification systems with chromogenic substrates, such as CHROMagar Candida, Fluoroplate, or umbelliferyl- or pNP-labeled galactosaminide (UAG or the *Candida albicans* screen, test, respectively). These systems do not, however, give 100% specificity in the identification of *C. albicans* (2, 16, 17, 18, 24), as there are always *C. albicans* strains that are defective in the production of nonessential metabolic enzymes. Although primary cultures of *C. dubliniensis* grown on CHROMagar Candida can be distinguished from *C. albicans, C. dubliniensis* can lose the ability to form dark green colonies on this medium after subculture or storage (31). The best interspecies discrimination is obtained when CHROMagar Candida is used as a primary screen. HexNAcase assay results, in contrast, are unaffected by the amount of subculturing. The microtiter plate-based assay has the additional advantages over chromogenic agar of being less expensive and enabling analysis of many isolates in duplicate or quadruplicate simultaneously. The *Candida albicans* screen test uses two chromogenic substrates, pNP-*N*-acetyl-β-D-galactosaminide and L-proline β-naphthylamide (aminopeptidase substrate), to identify *C. albicans* isolates (18). Although this test takes 90 min, the *C. albicans* isolates must be precultured for 18 to 24 h on agar before the test can be performed (18). Our system used a single substrate and incorporated a selective prescreen of growth on GlcNAc as the carbon source. Preliminary experiments indicate that rather than using an inoculum of 100 cells per microtiter well, as in the present controlled study, inoculation of wells straight from a yeast colony identified on a primary screen with a toothpick increases the simplicity of the assay and gives the same degrees of sensitivity and specificity (unpublished data). Use of a higher inoculum density in either microtiter plate wells or tubes could result in a quicker test, but this would lose the advantage of the selective growth prescreen.

C. dubliniensis grew well on GlcNAc as the carbon source, produced high levels of HexNAcase activity under the standard induction conditions, and possessed DNA sequences which showed a low degree of homology to those of the *C. albicans HEX1* gene. *C. dubliniensis* is a newly identified species within the genus *Candida* (31, 32). It produces germ tubes and abundant chlamydospores and was judged to be significantly different from the other *Candida* species on the basis of genetic analyses. Although the karyotype and DNA sequence homology distinguishes *C. dubliniensis* from *C. albicans, C. dubliniensis* is most closely related to *C. albicans* (32). Thus, the liberation of pNP from pNP-GlcNAc in the HexNAcase assay by *C. dubliniensis* has possibly occurred by a catalytic reaction of an enzyme similar to *C. albicans* HexNAcase. The results of the present study further support the fact that *C. dubliniensis* is closely related to *C. albicans* in terms of HexNAcase production and possession of a *HEX1*-like sequence. A small amount of pNP was liberated from pNP-GlcNAc by *C. rugosa* in the microtiter plate-based assay, although no *C. rugosa* DNA fragments hybridized with the *HEX1* gene probe under low-stringency conditions. Again, the enzyme activity of *C. rugosa* may be due to the nonspecific reaction of another enzyme(s), as was seen in *C. tropicalis*, or the gene in *C. rugosa* has a very low level of nucleotide sequence similarity to the *C. albicans HEX1* sequence and the enzyme has different optimum conditions. The possibility of the false identification of *C. rugosa* as *C. albicans* or *C. dubliniensis* by the microtiter plate-based assay is removed by using a cutoff of greater than two times the level for the negative control (no cells added) for positive results. Alternatively, a more specific *C. albicans* identification test could be based on PCR amplification of the *C. albicans HEX1* gene.

The microtiter plate-based assay for the detection of HexNAcase activity demonstrated in the present study is a simple, reliable, and inexpensive method. It could be used in clinical laboratories as a system for the presumptive identification of the medically important yeast species *C. albicans* and *C. dubliniensis*.

ACKNOWLEDGMENTS

We are indebted to the persons listed in the Tables 1 and 2 for providing the yeast strains. We gratefully thank Rachel Baker for technical assistance.

This work was supported by a University of Otago research grant.

REFERENCES

1. **Bahl, O. P., and K. M. L. Agrawal.** 1969. Glycosidases of *Aspergillus niger*. J. Biol. Chem. **244:**2970–2978.
2. **Beighton, D., R. Ludford, D. T. Clark, S. R. Brailsford, C. L. Pankhurst, G. F. Tinsley, J. Fiske, D. Lewis, B. Daly, N. Khalifa, V. Marren, and E. Lynch.** 1995. Use of CHROMagar Candida medium for isolation of yeasts from dental samples. J. Clin. Microbiol. **33:**3025–3027.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Cannon, R. D., K. Niimi, H. F. Jenkinson, and M. G. Shepherd.** 1994. Molecular cloning and expression of the *Candida albicans* β-*N*-acetylglucosaminidase (*HEX1*) gene. J. Bacteriol. **176:**2640–2647.
5. **Gopal, P., P. A. Sullivan, and M. G. Shepherd.** 1982. Enzymes of *N*-acetylglucosamine metabolism during germ-tube formation in *Candida albicans*. J. Gen. Microbiol. **128:**2319–2326.
6. **Horsch, M., C. Mayer, U. Sennhauser, and D. M. Rast.** 1997. β-*N*-Acetylhexoasminidase: a target for the design of antifungal agents. Pharmacol. Ther. **76:**187–218.
7. **Iwamoto, T., T. Sasaki, H. Iio, and H. Naka.** 1989. Production of β-*N*-acetylglucosaminidase by *Aspergillus niger* and localization of the enzyme. Hakkokogaku **67:**433–437.
8. **Jenkinson, H. F., and M. G. Shepherd.** 1987. A mutant of *Candida albicans* deficient in β-*N*-acetylglucosaminidase (chitobiase). J. Gen. Microbiol. **133:** 2097–2106.
9. **Jones C. S., and D. J. Kosman.** 1980. Purification, properties, kinetics, and mechanism of β-*N*-acetylglucosamidase from *Aspergillus niger*. J. Biol. Chem. **255:**11861–11869.
10. **Kamiyama, A., M. Niimi, M. Tokunaga, and H. Nakayama.** 1989. Adansonian study of *Candida albicans*: intraspecific homogeneity excepting *C. stellatoidea* strains. J. Med. Vet. Mycol. **27:**229–241.
11. **Kingsman, A. J., L. Clarke, R. K. Mortimer, and J. Carbon.** 1979. Replication in *Saccharomyces cerevisiae* of plasmid pBR313 carrying DNA from the yeast *trp1* region. Gene **7:**141–152.
12. **Mega, T., T. Ikenaka, and Y. Matsushima.** 1970. Studies on *N*-acetyl-β-D-glucosaminidase of *Aspergillus oryzae*. I. Purification and characterization of *N*-acetyl-β-D-glucosaminidase obtained from Takadiastase. J. Biochem. **68:** 109–117.
13. **Molloy, C., R. D. Cannon, P. A. Sullivan, and M. G. Shepherd.** 1994. Purification and characterization of two forms of *N*-acetylglucosaminidase from *Candida albicans* showing widely different outer chain glycosylation. Microbiology **140:**1543–1553.
14. **Niimi, K., M. Niimi, M. G. Shepherd, and R. D. Cannon.** 1997. Regulation of *N*-acetylglucosaminidase production in *Candida albicans*. Arch. Microbiol. **168:**464–472.
15. **Odds, F. C.** 1988. *Candida* and candidosis, 2nd ed. Baillière Tindall, London, United Kingdom.
16. **Odds, F. C., and R. Bernaerts.** 1994. CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important *Candida* species. J. Clin. Microbiol. **32:**1923–1929.
17. **Perry, J. L., and G. R. Miller.** 1987. Umbelliferyl-labeled galactosaminide as

an aid in identification of *Candida albicans*. J. Clin. Microbiol. **25:**2424–2425.
18. **Perry, J. L., G. R. Miller, and D. L. Carr.** 1990. Rapid, colorimetric identification of *Candida albicans*. J. Clin. Microbiol. **28:**614–615.
19. **Pfaller, M. A., R. N. Jones, G. V. Doern, H. S. Sader, R. J. Hollis, and S. A. Messer.** 1998. International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and antifungal susceptibilities of isolates collected in 1997 in the United States, Canada, and South America for the SENTRY Program. J. Clin. Microbiol. **36:**1886–1889.
20. **Pfaller, M. A., S. A. Messer, R. J. Hollis, R. N. Jones, G. Y. Doern, M. E. Brandt, and R. A. Hajjeh.** 1999. Trends in species distribution and susceptibility to fluconazole among blood stream isolates of *Candida* species in the United States. Diagn. Microbiol. Infect. Dis. **33:**217–222.
21. **Reyes, F., and R. J. Byrde.** 1973. Partial purification and properties of a β-*N*-acetylglucosaminidase from the fungus *Sclerotinia fructigena*. Biochem. J. **131:**381–388.
22. **Reyes, F., J. Calatayud, C. Vazquez, and M. J. Martínez.** 1989. β-*N*-acetylglucosaminidase from *Aspergillus nidulans* which degrades chitin oligomers during autolysis. FEMS Microbiol. Lett. **53:**83–87.
23. **Reyes, F., R. Lahoz, and A. V. Moreno.** 1981. Synthesis of 1,3-β-glucanase and β-*N*-acetylglucosaminidase during autolysis of *Neurospora crassa*. J. Gen. Microbiol. **126:**347–353.
24. **Rousselle, P., A.-M. Freydiere, P.-J. Couillerot, H. de Montclos, and Y. Gille.** 1994. Rapid identification of *Candida albicans* by using Albicans ID and Fluoroplate agar plates. J. Clin. Microbiol. **32:**3034–3036.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Scherer, S., and D. A. Stevens.** 1987. Application of DNA typing methods to epidemiology and taxonomy of *Candida* species. J. Clin. Microbiol. **25:**675–679.
27. **Schmid, J., S. Herd, P. R. Hunter, R. D. Cannon, M. S. M. Yasin, S. Samad, M. Carr, D. Parr, W. McKinney, M. Schousboe, B. Harris, R. Ikram, M. Harris, A. Restrepo, G. Hoyos, and K. P. Singh.** 1999. Evidence for a general purpose genotype in *Candida albicans*, highly prevalent in multiple geographic regions, patient types and types of infection. Microbiology **145:**2405–2413.
28. **Schmid, J., P. R. Hunter, G. C. White, A. K. Nand, and R. D. Cannon.** 1995. Physiological traits associated with success of *Candida albicans* strains as commensal colonizers and pathogens. J. Clin. Microbiol. **33:**2920–2926.
29. **Singh, B., and A. Datta.** 1979. Regulation of *N*-acetylglucosamine uptake in yeast. Biochem. Biophys. Acta **557:**248–258.
30. **Sone, Y., and A. Misaki.** 1978. Purification and characterization of β-D-mannosidase and β-*N*-acetyl-D-hexosaminidase of *Tremella fuciformis*. J. Biochem. **83:**1135–1144.
31. **Sullivan, D., and D. Coleman.** 1998. *Candida dubliniensis*: characteristics and identification. J. Clin. Microbiol. **36:**329–334.
32. **Sullivan, D. J., T. J. Westerneng, K. A. Haynes, D. E. Bennett, and D. C. Coleman.** 1995. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology **141:**1507–1521.
33. **Sullivan, P. A., N. J. McHugh, L. K. Romana, and M. G. Shepherd.** 1984. The secretion of *N*-acetylglucosaminidase during germ-tube formation in *Candida albicans*. J. Gen. Microbiol. **130:**2213–2218.
34. **Yamamoto, K., K. M. Lee, H. Kumagai, and T. Tochikura.** 1985. Purification and characterization of β-*N*-acetylhexosaminidase from *Penicillium oxalicum*. Agric. Biol. Chem. **49:**611–619.
35. **Yamamoto, K., Y. Tsuji, S. Matsushita, H. Kumagai, and T. Tochikura.** 1986. Purification and properties of β-*N*-acetylhexosaminidase from *Mucor fragilis* grown in bovine blood. Appl. Environ. Microbiol. **51:**1019–1023.