Mycoscience 42: 535–541, 2001

Early death at medium acidification and survival after low pH adaptation in *Cryptococcus neoformans*

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Received 22 June 2001
Accepted for publication 9 October 2001

When *Cryptococcus neoformans* was grown in yeast nitrogen base (YNB) supplemented with 0.5% glucose, the medium was acidified to below pH 3 during the exponential growth phase, which caused early growth-phase death in susceptible strains. Even in resistant strains, 30–70% cells died if incubated for 2 d in YNB supplemented with 1.5% glucose, whereas the remaining cells survived long. Two types of fatal alterations have been observed in dead cells. In the first type, release of cytoplasm occurred through weakened parts of the cell wall; structures attached to cell walls of dead cells were shown to be rich in proteins by FITC staining, indicating their cytoplasmic origin. In the second type, cells shrank distinctly with no sign of wall rupture. The shrinkage may be due to dysfunction of the plasma membrane at low pH. The mechanism of cell survival in medium below pH 3 was also examined. Aniline blue alone, or calcofluor together with methylene blue, allowed cell wall glucan or chitin and dead cell cytoplasm to be stained simultaneously. In the later stages of incubation, cells showing bright staining for cell wall glucan and chitin emerged. These changes in cell wall synthesis could be considered as an adaptation mechanism to acidification of the medium, because such cells survived longer than cells showing no change in the cell wall staining pattern.

Key Words——cell wall; *Cryptococcus neoformans*; death; low pH; stress response.

Yeast nitrogen base supplemented with 0.5% glucose is a standard medium for culture of yeast cells. During experiments on the basidiomycetous yeast pathogen *Cryptococcus neoformans* (Sanfelice) Vuill., we found that cells died early during the exponential-phase growth (unpublished data, see Takeo et al., 1995). Except for *Schizosaccharomyces pombe* Lindner (Johnson, 1967) and *Schwanniomyces occidentalis* Klöcker (Johnson et al., 1994), early death in YNB has not been reported in detail, *C. neoformans* in particular. How a pathogenic yeast dies and how it adapts and survives under unfavorable conditions is an important theme in the field of medical mycology, because it may lead to an understanding of a way to control the pathogen. It will also contribute to the understanding of yeast physiology, although the response of yeast cells to a certain stress may be different from species to species. We report here details of the early death of *C. neoformans* in YNB and the existence of an adaptation mechanism to low pH, leading to survival of the yeast cells.

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Materials and Methods

Strains and growth *Cryptococcus neoformans* strains IFM (Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University) 40038, 45940, 47846, 47848, 49165 and 50754 were used, all of which were serotype A. All strains except IFM 47848 were used after single-colony isolation. Cells were precultivated on potato-dextrose-agar slants at 24°C for 1 d, then cultivated in yeast nitrogen base medium (Difco) supplemented with 0.5% glucose (YNB 0.5%G) at 30°C with rotary shaking at 100 rpm, unless otherwise noted. YNB supplemented with 0.25% (YNB 0.25%G), 0.3% (YNB 0.3%G), 1% (YNB 1%G) and 1.5% glucose (YNB 1.5%G) were also used. In control experiments, cells were grown in YNB 0.5%G supplemented with MOPS buffer (Wako, Osaka, Japan), pH 7 (YNB pH 7 0.5%G), or in rich YPG medium (1% yeast-extract (Difco), 1% polypeptone (Wako) and 1% glucose).

OD_{660} was measured as the indicator of cell density in cultures using a spectrophotometer (Shimadzu UV-1200). pH indicator sticks (Macherey-Nagel pH-Fix 0.0–6.0 and pH-Fix 4.5–10.0) were used for time sequential measurement of medium pH. Final pH of the medium was measured with Horiba pH meter model F-22 after removing the cells by centrifugation at 3,000 rpm

for 10 min.

Measurement of survival rates by vital staining and by colony-forming ability To measure their survival rate, cells were stained with 2×10^{-4} M methylene blue and observed under the light microscope. Cells stained deep blue were considered as dead cells, while cells hardly stained or stained light purple were counted as viable cells.

In examining the colony-forming ability of *C. neoformans*, the problem was encountered that cell capsules made colonies sticky, making exact colony counts difficult. After cell numbers were counted using an improved Neubauer type hemocytometer, about 100 cells were plated on a YPG agar plate, which supported the growth of relatively thin-capsuled cells. Plates were incubated at 25°C, and colonies were counted after 3, 4 and 5 d (Ohkusu et al., 2001).

Fluorescence staining To detect cell wall glucan, cells were washed with 50 mM phosphate buffer (pH 7.4) containing saline, then stained with 0.5 mg/ml of aniline blue (Wako) in the same buffer at room temperature for 5 min. To detect cell wall chitin, cells were washed with water, then stained with 10 μg/ml of fluorescent brightener 28 (Sigma) (abbreviated as calcofluor) for a few min, and then washed twice with water. To detect proteins, cells were stained with 30 μg/ml of fluorescein isothiocyanate (FITC) (Sigma) in 0.5 M $NaHCO_3$ for a few min after washing with 0.5 M $NaHCO_3$. Fluorescence was observed under an Olympus fluorescence microscope model BX60 or BH2. Aniline blue- or calcofluor-stained samples were observed using a 330–385-nm excitation filter, a 400-nm dichroic mirror and a 420-nm barrier filter. FITC-stained samples were observed using a 530–550-nm excitation filter, a 570-nm dichroic mirror, and a 590-nm barrier filter.

Results

Roughly a half of cells died quickly when grown in YNB supplemented with 0.3 or 1.5% of glucose All strains grew normally in YNB 0.3%G until the mid-exponential phase. Then cells of susceptible strains IFM 40038 and IFM 45940 started to die, and within 2 d roughly a half (30–60%) of the cells died, as determined by the methylene blue vital staining and colony-forming ability. Death rate as estimated by methylene blue staining roughly corresponded to that estimated by colony-forming ability (data not shown). Cells died before entering the stationary phase, as many dead cells showed buds. In the other strains, less than 1% of cells died after cultivation for 2 d in YNB 0.3%G. When grown in YNB 0.5%G, cells of the two susceptible strains died completely within 2 d (Table 1). In strain IFM 50754, roughly 30% of cells died within 2 d. In the other strains, only a few cells died cultivation for 2 d or 4 d. However, when grown in YNB 1.5%G, roughly a half of the cells died quickly in the four resistant strains (Table 1). Changes in the proportion of dead cells, OD and pH of cultures are shown in Fig. 1 and Tables 1, 2.

The pH of cultures incubated for 2 d decreased sharply, falling below pH 3 even in YNB 0.25%G (Table 2). It should be noted that the pH of the cultures of resistant strains was similarly low.

Cells of all strains examined were long-lived when YNB 0.5%G was buffered to pH 6 (data not shown). Cell walls in living cells are shown in Figs. 2 and 3. Final OD of buffered YNB cultures was between 7 and 10, not much different from unbuffered ones. Thus, in spite of

Table 1. Stability of *C. neoformans* as living cells after incubation for 2 to 6 d in YNB medium.

Strain	Glucose (%)	Proportion of dead cells (%)[a]			
		2d	3d	4d	6d
IFM 40038	0.25	20.0±5.7[b]	24.7±4.1	31.0±3.1	
	0.5	100.0±0			
IFM 45940	0.3	6.0±3.7	20.7±5.7	30.3±0.9	
	0.5	100.0±0			
IFM 50754	0.5		29.0±3.5	27.7±1.5	38.3±2.5
	1.0	35.3±5.2	35.3±1.7	39.3±1.7	
	1.5	50.3±3.3	56.3±3.3	41.7±2.1	57.8±4.0
IFM 47846	0.5	1.7±0.6	4.0±1.0		1.7±0.6
	1.0	35.0±2.2	37.6±2.9	38.3±0.5	
	1.5	44.0±2.5	37.7±3.3	41.7±2.1	46.7±5.0
IFM 47848	0.5	<1.0	<1.0		2.7±0.6
	1.0	28.0±1.6	33.0±0.8	51.0±0.8	
	1.5	29.3±1.3	38.3±3.4	38.7±1.3	41.7±4.8
IFM 49165	0.5	<1.0	<1.0		2.0±1.0
	1.0	36.0±2.5	33.0±0.8	53.0±1.6	
	1.5	45.3±1.3	37.3±0.9	42.3±1.7	54.7±5.9

[a] Determined by methylene blue staining.
[b] Average±standard deviation.

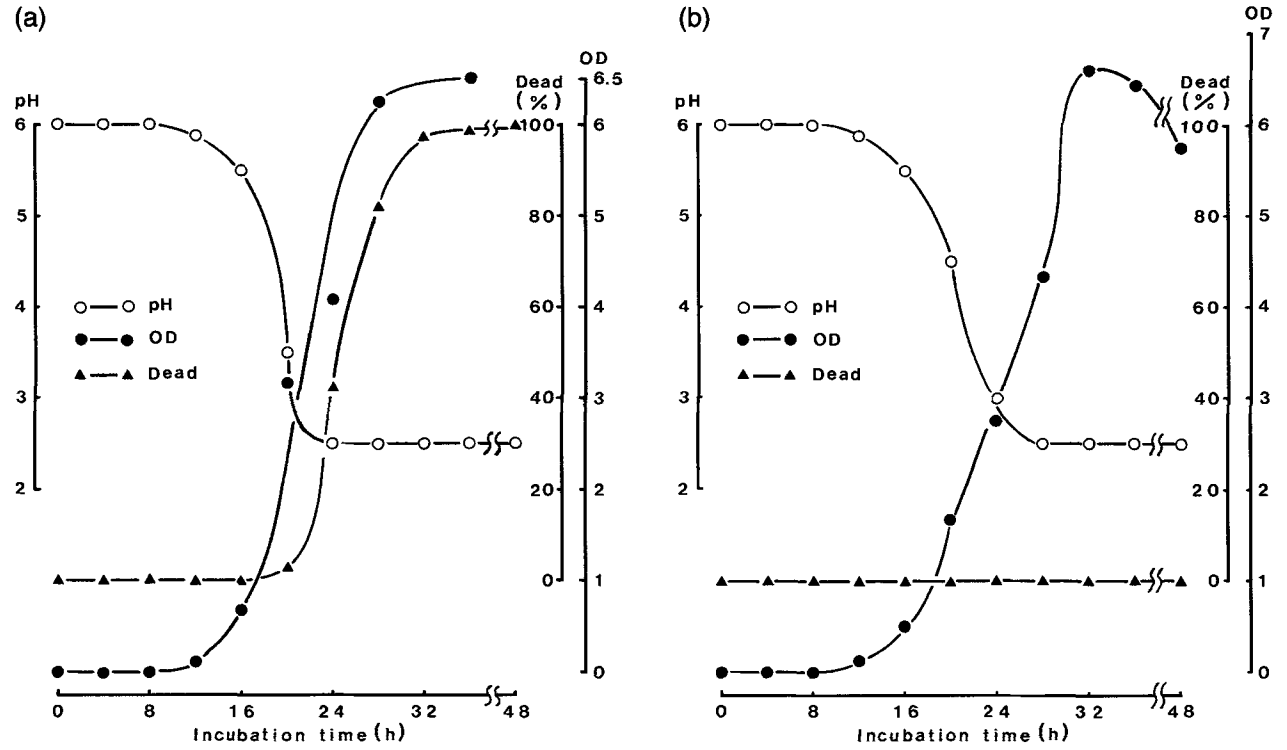

Fig. 1. Growth rate (OD_{660}), death rate (%) and pH of medium during cultivation in YNB 0.5%G. (a) Susceptible strain (IFM 45940). (b) Resistant strain (IFM 47848).

Table 2. pH of *C. neoformans* cultures in YNB supplemented with glucose.

Strain	Glucose (%)[a]				
	0.25	0.3	0.5	1.0[b]	1.5
IFM 40038	2.83	2.71	2.73		
IFM 45940	2.80	2.70	2.65		
IFM 50754	2.75	2.63	2.50	2.27	2.16
IFM 47846			2.61	2.40	2.36
IFM 47848			2.57	2.26	2.28

[a, b] The cells were incubated for 2 d in YNB containing the indicated glucose concentration, except for the column [b] where the cells were grown for 3 d.

the low pH in YNB 0.5%G after 1 d of cultivation, cell numbers were near to those of the stationary phase in both resistant and susceptible strains, regardless of death rates.

Generally speaking, surviving cells became larger. The fact that the survival of a part of the cells was sustainable (Table 1) suggests the existence of an adaptation mechanism which protects *C. neoformans* cells from death in an acidic environment. This suggestion was verified by the findings described in the last section of Results.

Alterations in cell morphology The manner of cell death in YNB supplemented with glucose was an interesting question. Characteristic alterations of cell morphology were observed during the course of this early-phase cell death, which seemed to be an important key to this question. Thus, these alterations in cell shape were scrutinized, after vital staining with methylene blue. First, strain IFM 50754 grown in YNB 0.5%G was examined. Methylene blue-stained cells often exhibited small structures attached to the cell wall. The attachment was not observed in living, i.e., methylene blue-unstained, cells. These features suggested that the weakest parts of the cell wall ruptured, releasing cytoplasmic structures (Fig. 2a). To confirm this, cells were stained vitally with FITC to trace the path of cytoplasmic protein after rupture. Only the cell walls were stained in exponential phase cells grown in YPG or YNB pH 6 0.5%G, indicating that the plasma membrane of living cells was an efficient barrier for FITC passage. In acidic conditions, however, both intracellular contents and attached structures of "bursted" cells were stained with FITC (Fig. 2b). Thus, release of cytoplasm from a small portion of the cell wall was clearly traced (Fig. 2b). Most

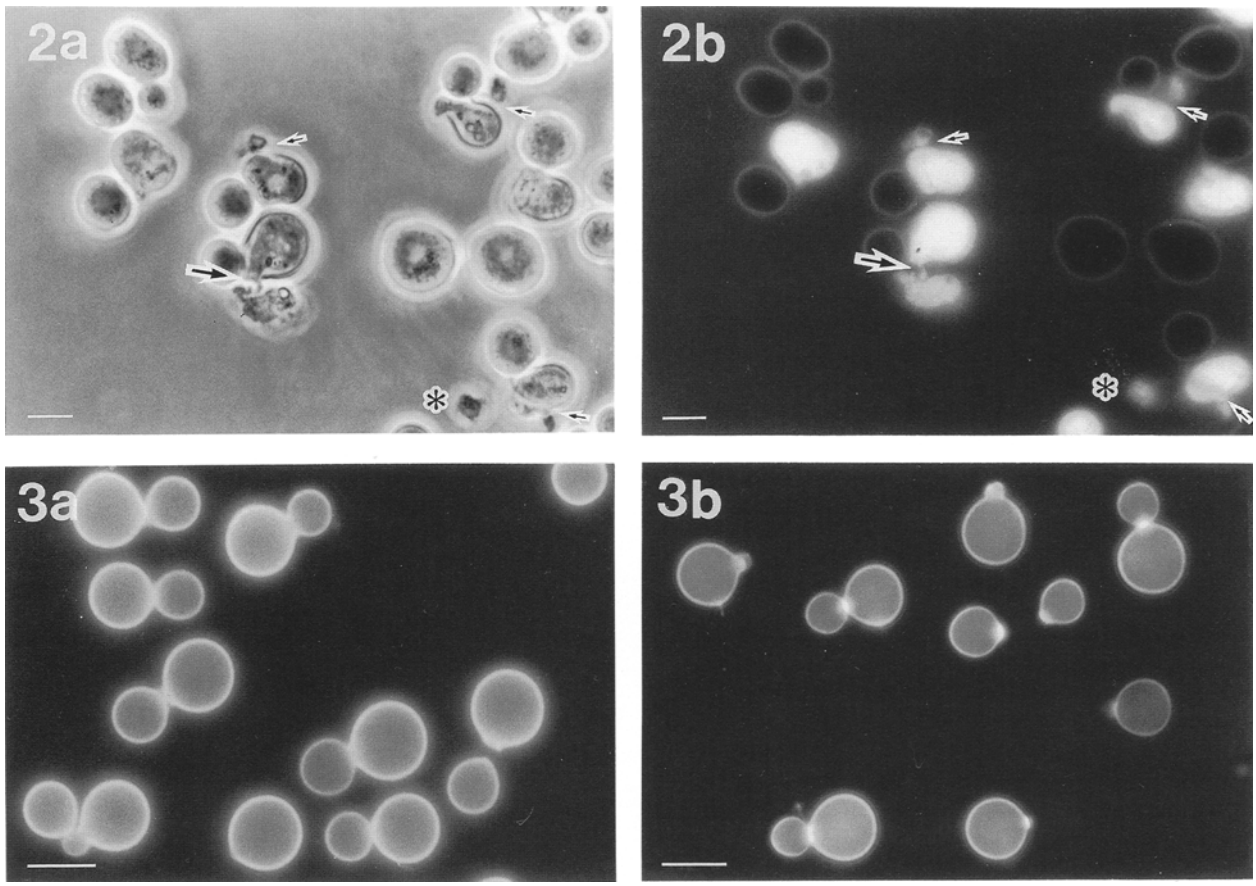

Fig. 2. *Cryptococcus neoformans* strain IFM 50754 grown in YNB 0.5%G for 30 h, when one-third of cells died. Cells were stained with FITC without fixation. Note structures attached to cell walls (small arrows). Note also an isolated structure (*), indicating attachment of structures to cells was not permanent. a. Phase contrast microscopy. b. Fluorescence microscopy. Structures were attached to dead cells only. The connection between cytoplasm of dead cells and attached structures is clearly visible (large arrows). Scale bar = 5 μm.

Fig. 3. Rapidly growing cells stained with aniline blue (a) or with calcofluor (b). a. *C. neoformans* strain IFM 47846 grown in YNB pH7 0.5%G overnight at 30°C. The whole cell wall was stained. b. *C. neoformans* IFM 45940 grown in YNB pH7 0.5%G overnight at 30°C. The whole cell wall was stained clearly. Bud scars were usually indistinct, but one scar per cell was sometimes observable. Scale bar = 5 μm.

cells revealed the remnants produced by the release of cytoplasm. On continuous observation under a light microscope, the moment of bursting was observed several times. Such bursting was also observed when strains IFM 47846, IFM 47848 and IFM 49165 were grown in YNB 1.5%G for 1.5 d.

Strains IFM 40038 and IFM 45940 showed no signs of bursting under similar scrutiny. Dead cell populations with or without buds were analyzed during the early phase, when roughly 30% cells died (Table 3). The proportion of dead cells having small buds became much higher. Thus, cells with small buds appeared to be a susceptible part of the cell population, as clearly shown in Table 3. Cells undergoing cell division appeared also to be susceptible, judging from the rather high proportion of pairs of dead cells.

Rapidly growing cells stained with aniline blue The fluorescent dye aniline blue is known to specifically stain cell wall glucan (Sengbusch et al., 1983). It stained brightly the whole cell walls of *Saccharomyces cerevisiae* Meyen ex E. C. Hansen and *Candida albicans* (Robin) Berkhout (figure not shown). Compared with these ascomycetous yeasts, the cell wall of living *C. neoformans* grown either in YPG, YNB pH7 0.5%G or YNB 0.5%G was less distinctly stained (Fig. 3a). The same was true in typical stationary-phase cells grown in YPG or YNB pH7 0.5%G.

The plasma membrane appeared to be an efficient barrier against aniline blue, since cytoplasm of living cells was not stained. However, when cells were killed by heat, cytoplasm took up the dye and stained blue under normal light, and brightly blue under UV excitation (figure not shown, see Figs. 4, 5). The death rate estimated by cytoplasmic staining with aniline blue roughly corresponded to that estimated by methylene blue staining (data not shown). The correspondence of these methods was also ascertained using a cell population in the later stages of YNB 0.5%G incubation (data not shown).

Table 3. Morphological characteristics of dead (100%) and living cells (100%), as determined by methylene blue staining[a].

	Budding cells					
	Single	Tiny[b]	Small[c]	Medium[d]	Large[e]	Two[f]
IFM 40038						
Dead cells	6±1[g]	9±1	28±5	20±1	9±7	29±3
Living cells	36±8	3±2	12±6	10±1	9±2	32±6
IFM 45940						
Dead cells	5±3	13±3	35±10	8±2	2±1	37±5
Living cells	46±7	9±1	8±5	6±3	11±1	21±10

[a] Cells were grown in YNB 0.5%G for 26 h, when roughly 30% cells were dead in both strains.
[b] Tiny bud taking a pimple form; see Ohkusu et al., 2001.
[c] Small bud with round tip.
[d] 1/8–1/4 of the diameter of the mother.
[e] 1/4–1/2 of the diameter of the mother.
[f] Mother with attached daughter cell (bud having a diameter larger than one half of the mother was regarded as a daughter cell).
[g] Average±standard deviation

Thus, aniline blue could be used simultaneously both for cell wall glucan staining and for determination of living and dead cells.

Rapidly growing cells stained with calcofluor The fluorescent dye calcofluor shows high affinity for cell wall chitin (Pringle, 1991). As larger amounts of chitin can be found in primary septa and chitin rings in *S. cerevisiae* and *C. albicans*, calcofluor stained only the bud scars (figure not shown). However, the staining in *C. neoformans* wall occurred almost uniformly and it was rather strong (Fig. 3b). This was also true when cells were grown in YPG or YNB 0.5%G buffered to pH 6, indicating that the whole cell wall of *C. neoformans* contains considerable chitin. Dead cytoplasm took up little calcofluor. Thus living cells and cells killed by heat did not show appreciable differences in staining pattern. To ascertain whether there is a relationship between wall staining and death, cells were stained with calcofluor and methylene blue simultaneously. Dead cells were stained in the wall by calcofluor and also in cytoplasm by methylene blue.

Bright staining of surviving cells by both aniline blue and calcofluor indicates changes in cell wall synthesis In the later stages of incubation in YNB supplemented with glucose, aniline blue staining revealed interesting alterations as shown in Figs. 4, 5. (1) Cells having rather weakly stained walls often took up the dye in cytoplasm, indicating death. (2) Some cells fluoresced brightly over the whole cell wall. These did not take up the dye in cytoplasm, indicating survival. (3) Tiny and small buds often fluoresced brightly. When only small portion of mother cells stained brightly, this was almost always the budding site (Figs. 4, 5, arrows). Thus, synthesis of cell wall glucan appeared to be stimulated in surviving cells.

Susceptible and resistant strains were grown in YNB 0.3%G and YNB 1.5%G, respectively, for 1.5 d, at which time roughly a half of the cells had died, and one sample was stained with aniline blue, while another was stained with calcofluor and methylene blue. In all cases, cells whose walls were brightly stained by aniline blue or calcofluor were mostly (more than 80%) living as judged by non-staining of the cytoplasm (Figs. 4–6). In contrast, cells having weakly stained cell walls were mostly (more than 80%) dead, showing cytoplasm brightly stained by aniline blue or methylene blue.

Discussion

Early death of *C. neoformans* grown in unbuffered YNB YNB supplemented with 0.5% glucose is a chemically-defined medium and widely used in yeast cultivation. However, it caused early-growth-phase death (early death) in *C. neoformans*. Early death in YNB was first reported in the fission yeast *S. pombe* (Johnson, 1967). It is ascribed to unbalanced growth, and death preferentially occurs at division due to inadequate septal wall formation (Johnson et al., 1994). In the budding yeast *S. occidentalis*, YNB with 1% glucose caused death of more than 50% of the cells (Johnson et al., 1994). Death was suggested to be due to plasma membrane dysfunction.

The pH of *C. neoformans* cultures was very low (2–2.5) when death occurred, as in the case of *S. occidentalis* (Johnson et al., 1994). Buffered YNB did not elicit early death. Because *C. neoformans* has been reported to grow normally in the pH range of 4–7.5 (Howard, 1961), acidification of YNB medium should play the principal role in the early death. YNB 0.5%G also induced early death in other pathogenic and non-pathogenic yeast species (unpublished). *Candida albicans* was very resistant, but most species examined showed similar characteristic to *C. neoformans*: considerable proportion of cells died in susceptible strains, but few cells died in resistant strains.

Malformation of the cell wall at low pH leading to localized rupture In the later stages of incubation in YNB

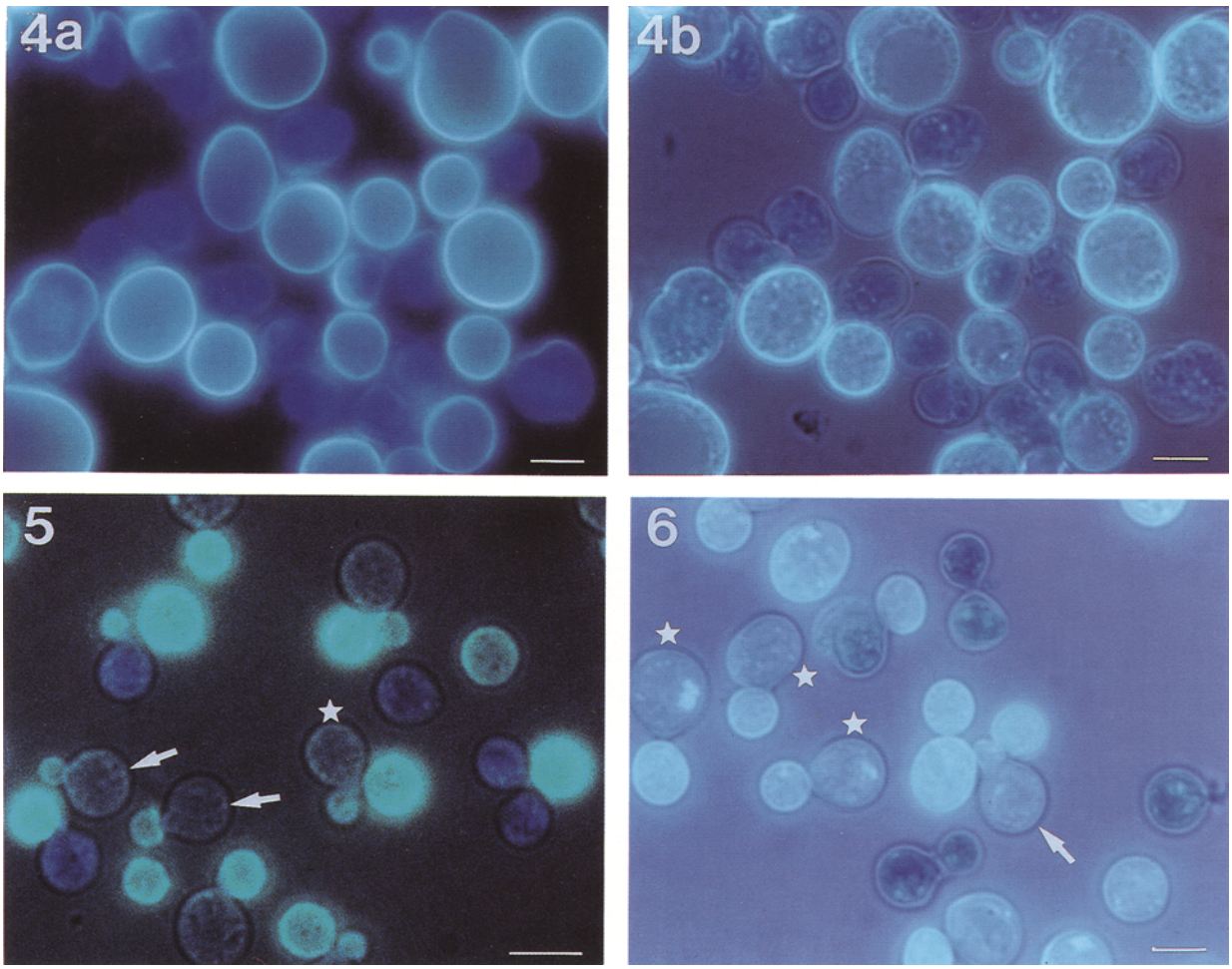

Fig. 4. *Cryptococcus neoformans* strain IFM 47846 grown in YNB 1.5%G for 30 h, when one-third of cells died. Cells were stained with aniline blue. Note that cells with brilliant cell wall staining did not take up the dye into the cytoplasm, showing these cells were alive. In contrast, cells with weakly stained cell wall were dead, as indicated by stain in the cytoplasm. a. Fluorescence microscopy with UV irradiation. b. Fluorescence microscopy with UV irradiation and also with visible light, so that cytoplasm was also visible. Scale bar=5 μm.

Fig. 5. The same as in Fig. 4b but with *C. neoformans* strain IFM 45940 grown in YNB 0.3%G. Here and in Fig. 6, note also pairs of cells in which the mother walls were relatively weakly stained while the daughter walls were brightly stained (stars). Some mothers produced buds with brightly stained walls (arrows). Scale bar=5 μm.

Fig. 6. *Cryptococcus neoformans* strain IFM 47846 grown in YNB 1.5%G for 30 h, when one-third of cells died. Cells were stained with calcofluor and methylene blue. The same features as in Fig. 5 were visible. Scale bar=5 μm.

0.5%G, cytoplasmic structures were released from specific regions of the cell wall in strain IFM 50754 (see Fig. 2). FITC revealed that the structures attached to dead cell walls were rich in proteins (Fig. 2b), indicating their cytoplasmic origin. This attachment was observed in as many as half of dead cells, indicating that the attachment was rather stable. This is due to the rapid and strong precipitation of cytoplasmic proteins caused by the low pH of the medium. The rupture was common in *C. neoformans* strains undergoing early death.

Possible dysfunction of the cell wall and the plasma membrane at low pH All cells of IFM 40038 and IFM 45940 died, with shrinkage, within 2 d of cultivation in YNB 0.5%G. In these cells, however, no remnants of rupture were found even after prolonged observation. The appreciable release of cytoplasm shown in Fig. 2 did not occur in these strains. Homeostasis in cytoplasm of living yeasts appears to be kept well at low pH, since cytoplasmic pH of living yeast cells is known to be relatively constant under various external pH values of 3.5–12 (Imai and Ohno, 1995). Thus, the cell wall or the plasma membrane should be the sites affected seriously by low pH.

Growing cell walls normally maintain a delicate balance between wall softening by lytic enzymes and addition of new wall structures. The emergence of a bud partially breaks or softens the pre-existing mother walls and may cause the cell wall to be very labile. Even in these regions, the cell wall should be able to sustain the balance between lysis and growth in the normal range of

environmental pH. At low pH of 2–2.5, however, this balance may be disturbed, resulting in slow leakage of cytoplasmic material without cell wall rupture.

The second possibility is a fatal dysfunction of the plasma membrane at low pH. The plasma membrane H^+-ATPase is known to be activated at low pH of the medium to compensate for the entrance of protons into the cell (Carmelo et al., 1997; Kotyk et al., 1999). It is also conceivable that exposure of the outer surface of the plasma membrane to low pH can physically stress the plasma membrane. The sum of such stresses may ultimately lead to cell death.

Cells showing enhanced cell wall synthesis were more resistant to both types of medium acidification-induced death The present paper revealed that in *C. neoformans* grown either in YNB 0.3%G (susceptible strains) or YNB 1.5%G (resistant strains) roughly a half of the cells died quickly, whereas the others survived long, although most strains used were isolated from single colony to make cells genetically homogeneous. This fact suggested that *C. neoformans* has an adaptation mechanism to low pH. In line with this expectation, the cells appearing in the late stages of incubation showed changes in cell wall synthesis, as indicated by the bright staining with aniline blue and calcofluor. This increase in staining probably represents increased synthesis of both chitin and glucan. Thus, modifications of cell wall structure may play an important role in the adaptation to low pH. Adapted cells with stronger cell walls survived longer when grown in YNB 0.3%G (susceptible strains) or YNB 1.5%G (resistant strains). It is worthy of note that adaptation to low pH prevented cells not only from rupture but also from the second type of death characterized by shrinkage without rupture. Similar adaptation was also observed in some basidiomycetous yeasts examined, but to lesser extents (unpublished).

Cryptococcus neoformans usually grows in the outer environment. It has commonly been isolated from pigeon droppings, the pH of which is weakly alkaline (Walter and Yee, 1968). Some strains of *C. neoformans*, however, have the capacity to grow also in completely different conditions, e.g., inside animal bodies. Many killing enzymes have pH optima of 4–5, and similar values have been reported in phagocytic vacuoles. Thus, adaptation to low pH may play a role in pathogenicity of *C. neoformans.*

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