Rapid and extensive vacuolation of the budding yeasts *Saccharomyces cerevisiae* and *Candida albicans* by amphotericin B

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The effect of amphotericin B (AMPH) on vacuolation in the budding yeasts *Saccharomyces cerevisiae* and *Candida albicans* was studied. The minimum inhibitory concentration of AMPH for growth of *S. cerevisiae* and *C. albicans* was 1 μg/ml. In untreated control cultures, mature cells had large central vacuoles in the exponential phase, which hampered the detection of vacuolation effect. Small buds in untreated exponential phase cells, however, only rarely showed vacuoles under the light microscope. Treatment with 0.2 μg/ml of AMPH for 20-30 min induced extensive vacuolation not only in mothers but also buds of *S. cerevisiae*. Extensive vacuolation lasted 4 h or more, and growth rate of the cells was much reduced for 8 h or more. Vacuolation itself was not fatal: on removal of the drug most cells gradually recovered from vacuolation and eventually multiplied. A similar effect of AMPH was also observed in *C. albicans* but at a higher concentration (0.5 μg/ml).

Amphotericin B (AMPH) is one of the most widely used antifungal antibiotics. Thus, its mode of action has been studied extensively. The primary site of attack is well established to be the plasma membrane. AMPH specifically binds with plasma membrane sterols, especially ergosterol, permeabilizes the plasma membrane for H^+ and K^+ ions, resulting in deenergetization of the plasma membrane and leakage of K^+ ions from the cell, and shows fungi-static activity (reviewed in: Bolard, 1986; Brajtburg et al., 1990; Kerridge and Whelan, 1984). However, at higher concentrations, AMPH is known to elicit various additional effects on fungal and mammalian cells, e.g., leakage of larger substances, fatal dysfunction of the plasma membrane, oxidation-dependent lethal effects, uptake of DNA and other large substances, stimulation of immune responses, and activation of macrophages to kill *Candida albicans* (Robin) Berkhout (Brajtburg et al., 1985; Kumer et al., 1974; Sullivan et al., 1992). The mechanisms of these diverse effects of AMPH remain to be elucidated.

We recently found a novel effect of AMPH. In a fission yeast *Schizosaccharomyces pombe* Lindner, this drug induces extensive vacuolation (Takeo et al., 1993). This finding, however, is not necessarily relevant to usual budding yeasts, because the fission yeast has a widely different mode of growth from usual budding yeasts. Growth in *S. pombe* occurs only at the

tip, and the cell divides after sufficient elongation by binary fission (Mitchison, 1970; Mitchison and Nurse, 1985). Furthermore, *S. pombe* usually has no large vacuoles. In contrast, budding yeasts multiply by budding, and divide unequally, the mother being larger than the bud (Hartwell and Unger, 1977). The larger mother cells usually have large central vacuoles even when cells are rapidly growing. Due to different modes of cell growth, and because fission yeasts represent only a minor subset of yeasts, we studied the response to AMPH of budding yeasts, *Saccharomyces cerevisiae* Hansen and a pathogenic yeast *C. albicans*, to learn if the antibiotic also induces vacuolation in these organisms.

Materials and Methods

Saccharomyces cerevisiae IFM 40210 (ATCC 7753) and *C. albicans* IFM 40009 (ATCC 48130) were grown at 28°C with shaking to exponential phase (OD_{660}, 0.5-2) in EMM2 supplemented with 1% glucose and 0.5% yeast extract (Takeo et al., 1993). Then the cells were suspended in the same fresh medium (OD, 0.02-0.1) and incubated as above. After 30 min, AMPH in dimethyl sulfoxide was added to a final concentration of 0.1-4 μg/ml, followed by incubation as above, usually for 1-2 h. AMPH, 1-5 mg, was stored in small tubes in a deep-freezer after calibration. The final DMSO concentration was always less than 1%. In separate control experiments, it was confirmed that the presence of 1% DMSO in the growth medium did not induce either vacuolation or cell death after 4 h of incubation. An aliquot of the solution

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was sequentially withdrawn to determine viability by vital staining, and/or to check colony-forming ability and structural alterations of yeast cells. For vital staining, 10 μl of cell suspension was put on a slide glass, and 20 μl of 3×10^{-4} M methylene blue (Merk) in water was added. To examine colony-forming ability, the cells were plated on YPG agar (1.5%), containing 4% glucose, 1% peptone and 1% yeast-extract, and incubated at 28°C for 3-4 days. For recovery experiments, *S. cerevisiae* was first treated with 0.2 μg/ml of AMPH for 30-40 min. Then cells were centrifuged at 6,000 rpm for 2 min. After discarding supernatant, 0.2 ml of fresh YPG medium was added, and 0.1 ml of cell suspension was incubated on a YPG agar plate at 27°C.

Vacuolation of the mother and the bud parts was defined as the existence of vacuoles larger than a half of the smaller diameter of the ellipsoidal mother and bud parts, respectively. Thus, the operational definition was

dependent on cell size.

Photomicrographs were taken using a Nikon Optiphot phase-contrast microscope equipped with a Nikon Plan 100 BM bright field lens.

Results and Discussion

First, untreated and rapidly growing yeast cells were observed as a control. Most mature cells had large central vacuoles, which hampered the detection of the vacuolation effect of AMPH. Furthermore, the presence or absence of large vacuoles itself was not always clear, especially when cells were grown on rich media, such as YPG medium. This was probably because the high optical density of the cytoplasm obscured dark vacuoles. However, growing small buds in budding cells rarely contained vacuoles visible with the optical methods we employed (see Materials and Methods) (Fig. 1). Thus, we
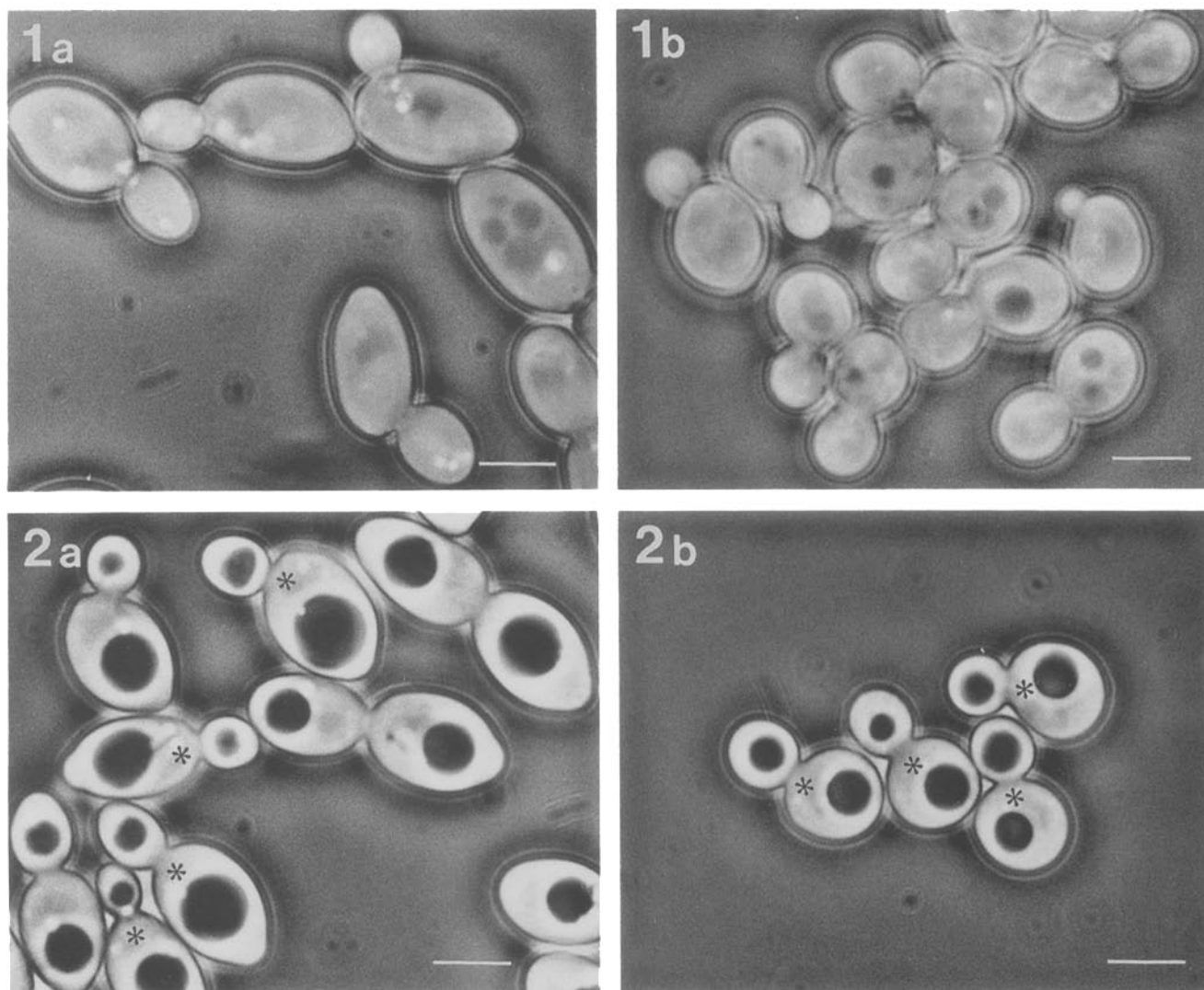

Fig. 1. Untreated control cells grown in EMM2+0.5% yeast extract at 27°C to mid-exponential phase, and suspended in fresh EMM2+0.5% yeast extract for 2 h. (a) *S. cerevisiae*. (b) *C. albicans*. The bar in all the figures indicates 5 μm.
Fig. 2. (a) *S. cerevisiae* cells after treatment with 0.2 μg/ml of AMPH for 40 min. (b) *C. albicans* cells with 0.5 μg/ml of AMPH for 40 min. Note extensive vacuolation both in mother and in small buds (*).

Table 1. Typical time course of AMPH-induced extensive vacuolation in S. cerevisiae small buds (%).*,**

The amount of AMPH (µg/ml)	OD_{660nm} of cells	Incubation time (min)		
		40	40	40
		20	30	40
0.2	0.18	20	70	80
0.5	0.27	30	80	87

* Small buds having large vacuoles (larger than a half of the diameter of bud) were counted as positive. In untreated control cells they were always smaller than 1%.

** Because of rapid induction of vacuolation, the numbers shown here are rough estimates.

observed mature cells to check increase in the size of central vacuoles, and small buds to obtain convincing evidence of vacuolation.

The minimum inhibitory concentration of AMPH for growth of both S. cerevisiae and C. albicans was 1 µg/ml. In S. cerevisiae, vacuolation of mature cells occurred after AMPH treatment at a concentration of 0.1 µg/ml, but the effect was neither extensive nor persistent. Most of vacuolated cells gradually recovered, although a few cells became more extensively vacuolated with time. At 0.2 µg/ml, the vacuolation effect was rapid and extensive. Most cells, including small buds, were extensively vacuolated after the treatment for 30 min (Fig. 2, Table 1). Another important alteration of the cells was that the vacuoles came to show a distinct contrast from other parts of cytoplasm so that they could be observed very clearly. They remained extensively vacuolated for 4 h. Cells did not show appreciable growth for 6 h, judging from the nearly constant value of optical density (Fig. 3), although some cells gradually
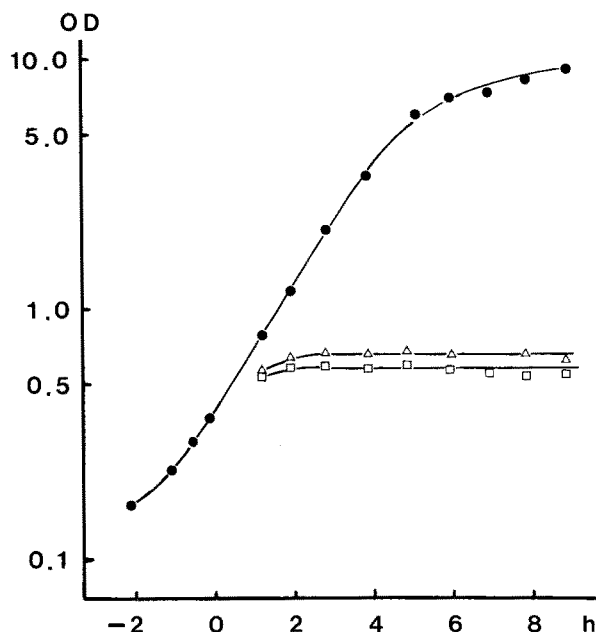
recovered from vacuolation and eventually multiplied.

Recovery experiments were done. Large vacuoles gradually lost their distinct contrast, and finally retained the usual contrast in the majority of cells (80-95%) after 2 h of incubation. At this time, large vacuoles disappeared in most small buds (not shown).

Rapid and extensive vacuolation was also observed in C. albicans, but at a higher AMPH concentration (0.5 µg/ml) and to a lesser extent than that of S. cerevisiae. After 30 min of treatment with 0.5 µg/ml of AMPH, 40% of small buds were extensively vacuolated (Fig. 2b).

Vacuolation is generally regarded as a manifestation of low cellular activities. It is possible that this is also the case in AMPH-induced vacuolation as follows. The AMPH treatment may have led to leakage of H^+ ions, and the resulting deenergetized plasma membrane could give reduced energy-dependent transportation of ions and nutrients (Bolard, 1986; Kerridge and Whelan, 1984). The extensive vacuolation of budding yeasts occurred after the dysfunction of the plasma membrane, judging from the time course of the vacuolation. According to this hypothesis, vacuolated cells must be low in cellular activities. On the other hand, the dysfunction of extensively vacuolated cells was not always serious: cells took up methylene blue very slowly and only weakly at vital staining; and most cells after 30 min of treatment at
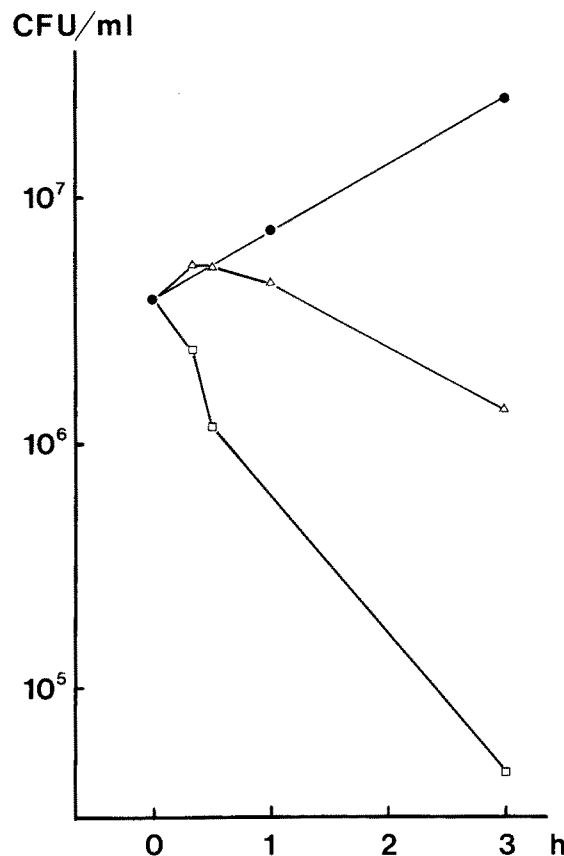

Fig. 3. Time course of optical density of S. cerevisiae without (●) or with AMPH treatment (△, 0.2 µg/ml; □, 0.5 µg/ml).

Fig. 4. Colony-forming units of S. cerevisiae without (●) or with AMPH treatment (△, 0.2 µg/ml; □, 0.5 µg/ml).

0.2 μg/ml AMPH retained colony-forming ability (Fig. 4).

Induction of rapid and extensive vacuolation appeared to be a characteristic of polyene macrolide antibiotics, such as nystatin, filipin, and pimalicin (Yarita et al., 1993). This should not be regarded as a simple result of inhibition of growth, but as an active process of yeast cells in response to disturbance of cell activities due to the effect of polyenes, because other types of antifungal agents generally do not elicit extensive vacuolation (Yarita et al., 1993, and unpublished observation).

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