Transport and regulatory characteristics of the yeast bicarbonate transporter homolog Bor1p

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Jennings ML, Howren TR, Cui J, Winters M, Hannigan R. Transport and regulatory characteristics of the yeast bicarbonate transporter homolog Bor1p. Am J Physiol Cell Physiol 293: C468–C476, 2007. First published April 25, 2007; doi:10.1152/ajpcell.00286.2005.—The functional properties of the Saccharomyces cerevisiae bicarbonate transporter homolog Bor1p (YNL275wp) were characterized by measuring boron (H$_3$BO$_3$, Na$^+$, and Cl$^-$) effluxes. Neither Na$^+$ nor Cl$^-$ appears to be a transported substrate for Bor1p. Uphill efflux of boron mediated by Bor1p was demonstrated directly by loading cells with boron and resuspending in a low-boron medium. Cells with intact BOR1, but not the deletant strain, transport boron outward until the intracellular concentration is sevenfold lower than that in the medium. Boron efflux through Bor1p is a saturable function of intracellular boron (apparent $K_m$ $\sim$1–2 mM). The extracellular pH dependences of boron distribution and efflux indicate that uphill efflux is driven by the inward H$^+$ gradient. Addition of 30 mM HCO$_3^-$ does not affect boron efflux by Bor1p, indicating that HCO$_3^-$ does not participate in Bor1p function. Functional Bor1p is present in cells grown in medium with no added boron, and overnight growth in 10 mM H$_3$BO$_3$ causes only a small increase in the levels of functional Bor1p and in BOR1 mRNA. The fact that Bor1p is expressed when there is no need for boron extrusion and is not strongly induced in the presence of growth-inhibitory boron concentrations is surprising if the main physiological function of yeast Bor1p is boron efflux. A possible role in vacuolar morphology in the deletant strain.

Boron: SLC4; YNL275w

THE BICARBONATE TRANSPORTER gene family known as SLC4 (28) includes Na$^+$-independent Cl$^-$/HCO$_3^-$ exchangers, electrogenic and electroneutral Na$^+$-HCO$_3^-$ cotransporters, and Na$^+$-dependent anion exchangers (1, 18, 26). The functions of these transporters have been characterized extensively in mammals and in some invertebrates (27). In addition, the genomes of plants and fungi (but not prokaryotes) contain open reading frames (ORFs) that are members of SLC4 but whose functions are, for the most part, not well established. An exception is the Arabidopsis thaliana SLC4 ORF known as BOR1, which was shown by Takano et al. (32) to be necessary for boron$^+$ [B(OH)$_3$/B(OH)$_4^-$] loading of xylem. A. thaliana Bor1p functions as a pathway for uphill boric acid efflux from pericycle cells into root stellar apoplasm (32), thereby increasing the concentration of boron in xylem. In Arabidopsis, boron levels do not strongly affect BOR1 gene transcription; the level of functional transporter, however, is regulated by stimulation of endocytosis and degradation of Bor1p when boron levels are high (31).

In their study of Arabidopsis BOR1, Takano et al. (32) found that a homologous Saccharomyces cerevisiae ORF, YNL275w (BOR1), is responsible for the ability of yeast to maintain a low cellular boron content following incubation for 1 h in medium containing 0.5 mM boric acid. More recently Nozawa et al. (20) showed that BOR1 deletant strains are more susceptible than wild-type strains to growth inhibition by boric acid and that overexpression of BOR1 produces further protection against boric acid toxicity. Very recently, Takano et al. (30) published the first boron transport measurements in yeast, showing that efflux is slower in the deletant strain than in the wild-type strain.

Other functional information about Bor1p comes from the work of Zhao and Reithmeier (37), who expressed S. cerevisiae Bor1p with a COOH-terminal tag and found that the protein is targeted to the plasma membrane and is not a glycoprotein. They also found that Bor1p can bind stilbenedisulfonates and that this binding can be inhibited by monovalent inorganic anions, but not by sulfate or phosphate. A recent study by Decker and Wickner (10) showed that S. cerevisiae Bor1p-green fluorescent protein (GFP) localizes preferentially to the vacuole and that cells lacking Bor1p have fragmented vacuoles. These findings suggest a role for yeast Bor1p in the trafficking of proteins needed for vacuole fusion.

A separate SLC4 member (human SLC4A11), previously known as BTR1 (24) and renamed NaBC1, was shown recently by Park et al. (23) to be an electrogenic Na$^+$-borate [B(OH)$_4^-$] cotransporter. In the absence of boric acid, NaBC1 acts as a conductive pathway for Na$^+$ and H$^+$/OH$^-$ ions. Park et al. (23) further showed that boric acid and/or borate, at low concentrations (<0.5 mM), activates the MAP kinase pathway and stimulates mammalian cell growth. Higher boric acid/borate

1 For brevity, the word boron and its symbol B is used to denote boric acid [H$_3$BO$_3$; B(OH)$_3$]. Whenever boric acid is present, its conjugate base borate [H$_2$BO$_3^-$; B(OH)$_4^-$] will also be present in proportions dictated by the pK$_a$ (9.25) and the pH. When specific chemical species are discussed, the terms boric acid and borate are used, but otherwise, boron is used to mean boric acid/borate. We assume that the only chemical form of boron present is boric acid/borate, i.e., that boric acid is not chemically transformed to other compounds.

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2 Following the work of Takano et al. (32), the ORF YNL275w has been designated BOR1 in the Saccharomyces Genome Database and National Institutes of Health database. Accordingly, BOR1 is used in this article to refer to this gene, and Bor1p is used to refer to the protein.

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concentrations inhibit growth. Overexpression of NaBC1 causes both the stimulatory and inhibitory effects of boron to take place at lower concentrations, indicating that NaBC1 uses the inward Na\(^+\) gradient to drive an uphill influx of borate (23).

The experiments described in this article were designed to test several functional characteristics of boron transport through \textit{S. cerevisiae} Bor1p, including the driving force for uphill transport, the possible role of bicarbonate, the saturability of efflux, the regulation of the transporter by boron in the growth medium, and the proposed effect of Bor1p on vacuolar morphology. We demonstrate directly that Bor1p mediates a saturable uphill boron efflux, with characteristics consistent with a bicarbonate-independent exchange of extracellular H\(^+\) for intracellular H\(_2\)BO\(_3\). Functional Bor1p is constitutively expressed in cells grown in rich medium without added boron, and functional protein as well as BOR1 mRNA are increased only slightly during growth in boron concentrations that significantly inhibit growth. The lack of major induction results in only a limited ability of native Bor1p to increase the resistance of yeast to growth inhibition by boric acid.

**MATERIALS AND METHODS**

\textbf{Materials, strains, and growth conditions.} Unless otherwise indicated, all media, salts, buffers, and other chemicals were obtained from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO). Boric acid transport experiments were performed on Euroscarf strains 1169 and 6808, purchased from Invitrogen (Carlsbad, CA). Both strains were derived from strain BY4741 (\textit{hrr004 ura3-100 his3-11,15 ade2-1} \textit{kan1} \textit{a}; \textit{Saccharomyces cerevisiae}), which is deleted in several Na\(^+\)-dependent transporters including the sodium-coupled borate transporter Na\(_{BC1}\) (24). Boric acid transport experiments were performed on Euroscarf strains 1169 and 6808, buffered with 50 mM potassium citrate, pH 5.2–5.3, at 30°C to initiate efflux, and intracellular boron content of cold-washed and unwashed cells. This estimate of boron content was also performed by inductively coupled plasma mass spectrometry (ICP-MS), using a PerkinElmer Sciex DRC II Dynamic Reaction Cell ICP-MS (13). Results using the two methods were indistinguishable (see Fig. 2A).

For investigation of the effect of bicarbonate on boron distribution, cells from overnight cultures were incubated 1 h at 30°C in YPD medium containing 2 or 4 mM boron, buffered with 50 mM potassium citrate and 10 mM Bis-Tris (pH 6.2) or 50 mM MES-Tris (pH 7.0). Comparisons were made of boron contents of cells in two parallel 10-ml suspensions. In one of the suspensions, KHCO\(_3\) was added from a 1 M stock to a final concentration of 29 mM, and the suspension was bubbled (50–100 ml/min) with a mixture of air, O\(_2\), and CO\(_2\) to keep the extracellular pH at 6.2 ± 0.1 (70% CO\(_2\)) or 7.0 ± 0.1 (12% CO\(_2\)) and the O\(_2\) between 21 and 30%. The extracellular pH in the CO\(_2\)/HCO\(_3\)-supplemented suspensions drifted downward very slowly, and no addition of base was needed to keep the pH in the stated range during the incubation. In the other suspension, no addition of KHCO\(_3\) was made, and the suspension was bubbled with air to minimize the accumulation of CO\(_2\) and HCO\(_3\). The extracellular pH of the air-bubbled suspension was monitored continuously, and additions of KOH were made periodically to keep the pH with 0.1 unit of the initial pH. After 1 h, aliquots of both suspensions were diluted 10-fold in ice-cold distilled water and centrifuged immediately, and the cellular boron contents were determined as described above.

Cell water was calculated from the wet weight of the cell pellet, with the assumption that the cell pellet contains 0.78 g total water/g dry weight measured in several Na\(^+\) efflux pathways (2).

Preparation of plasmid pG1T containing epitope-tagged BOR1. Plasmid pG1T was a generous gift from Robert Farley (University of Southern California). The sole \textit{EcoRI} site in pG1T, near the Gal11 promoter, was removed by site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA), and an \textit{EcoRI} site was introduced into the multiple cloning site. The BOR1 ORF was amplified from genomic DNA (from strain FY282) using PCR with primers located 226 bases upstream of the translation start site and 88 bases downstream of the stop codon. The S' end of each primer contained sequences for restriction sites (\textit{EcoRI} and \textit{NheI}), which were used to insert the PCR product into the multiple cloning site of pG1T. The final construct (pG1T/BOR1), including hemagglutinin sequences between the initiator methionine and the remaining BOR1 ORF, was verified by sequencing.

**Boric acid/borate influx.** Overnight cultures were centrifuged and resuspended in YPD (same volume as the original culture) buffered with 5 mM citrate, titrated to pH 5.2 with KOH, plus various concentrations of B(OH)\(_3\) to initiate influx, and incubated with shaking at 30°C. At various times, aliquots were diluted >10-fold in ice-cold distilled water and centrifuged immediately (2 min, 4,000 rpm), and the supernatant was removed completely. Cell pellets were resuspended in 50 or 100 \(\mu\)l of water and heated for 30 min at 100°C (32). Solids were centrifuged in a microfuge, and 25 \(\mu\)l of supernatant were assayed for total boron colorimetrically by the curcumin reaction (Texas A&M Technical Bulletin; \textit{http://www-odp.tamu.edu/publications/notes/15_f_chem2.htm}). Boron content is represented as millimoles per liter of cell water. Interference in the assay by other components of the cellular extract was not significant, as indicated by the fact that late time points in influx experiments in low-boron media showed negligible boron contents. As an additional confirmation of the validity of the assay, cellular boron contents were also measured on some of the samples by inductively coupled plasma mass spectrometry (ICP-MS). RT-PCR. RNA was extracted from yeast with Trizol reagent (Invitrogen) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (ABgene, Rochester, NY) primed with random hexamers. Real-time PCR was performed using a MyIQ thermal cycler (Bio-Rad, Hercules, CA) with primers obtained from Integrated DNA Technologies (Coralville, IA). Primers for amplifying BOR1 were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and had the sequences ACT-TGGCTGGCATAAGAACC (forward) and ATTCCTAGAACCCCGCTGAT (reverse). Control PCR reactions verified the absence of genomic DNA.

**Differential interference contrast and FM 4-64 microscopy.** Fluorescent dye FM4-64 [N-(3-triethylammoniumpropyl)-4-(p-dihethylaminophenylhexatrienyl) pyridinium dibromide; Synapto red] was purchased from Calbiochem (La Jolla, CA). Cells were labeled with

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FM4-64 essentially as described by Vida and Emr (34). Overnight cultures of control and BOR1 deletant strains were centrifuged and resuspended in YPD at absorbance (600 nm) of 20, and FM 4-64 was added to a final concentration of 32 μM from a 16 mM stock in DMSO. Cells were incubated with dye for 15 min, centrifuged, resuspended in YPD, and incubated for 1 h at 30°C to allow the dye to be internalized and traffic to the vacuole (34) and was then put on ice before slide preparation. The suspension (2 μl), either in YPD or after centrifugation and resuspension in water, was applied to glass slides coated with polylysine, covered with an 18 × 18-mm coverslip, sealed with nail polish, and examined immediately using a Zeiss Axiovert 200M microscope with a ×100 objective. Final images (see Fig. 8) were prepared from raw micrographs using NIH ImageJ software.

RESULTS

Lack of 22Na+ or 36Cl− flux mediated by Bor1p. Because many members of the bicarbonate transporter family (SLC4) transport Cl−, Na+, or both (1, 18, 26), it is of interest to test these ions as potential substrates of Bor1p. Overexpression of BOR1 in the deletant strain did not increase the influx of either 22Na+ or 36Cl− (Fig. 1). Overexpression of BOR1 in strain B31, which is deficient in several Na+ efflux pathways (2), also did not stimulate 22Na+ influx (Fig. 1C). We also examined 36Cl− influx at concentrations of 0.01–1 mM, which are lower than those that have been investigated previously in yeast (9, 14, 17). Influxes at low concentration revealed the existence of a high-affinity transporter for 36Cl−, which are described separately (Jennings ML and Cui J, unpublished observations). The high-affinity Cl− flux was present in both the wild-type and the BOR1 deletant strain (Fig. 1B) and was therefore not catalyzed by Bor1p. We conclude that neither Na+ nor Cl− is transported by Bor1p under the conditions examined.

Steady-state distribution of boron and time course of influx. Figure 2B shows the time course of increase of total cellular boron for cells incubated at 30°C in YPD plus 4 mM H3BO3, buffered at pH 5.2 with 5 mM Na-citrate. In the BOR1 deletant strain, the time course of influx was indistinguishable from a single exponential, with a steady-state cellular boron content that was slightly lower (per unit cell water) than the extracellular concentration. The steady-state boron distribution ratio ([B]in/[B]out) was 0.7–0.8. This distribution ratio may not be significantly different from unity, considering that after 60–90 min, the influx had only reached about 95% of equilibrium, and there were minor losses (probably 5–10%, but difficult to measure) of boron during the wash before analysis. Given these considerations, a measured distribution ratio of 0.7–0.8 in the deletant strain is very close to that expected for a passive distribution. Our data indicate that, to a first approximation, boron is neither extruded from nor concentrated in the cells of the BOR1 deletant strain.

It should be pointed out that the boron distribution ratio in the BOR1 deletant (Fig. 2) was significantly lower than those reported very recently by Takano et al. (30) in the same strain. For example, Takano et al. (30) found that at an extracellular boron concentration of 10 mM, the cellular boron was 19 mmol/kg wet wt, which was over 30 mmol per liter of intracellular water. The difference between our data and those of Takano et al. (30) is not likely to be caused by the analytical method; curcumin and ICP-MS give the same values for boron concentration in hot water extracts of yeast (Fig. 2A).

In strains with wild-type BOR1, cellular boron reached a steady-state level more rapidly than in the deletant strain (Fig. 2B), and the steady-state level was far lower than that expected for a passive distribution. Figure 2C shows the steady-state boron distribution ratio as a function of extracellular concentration. As expected for a passive distribution, the symmetrical boron distribution in the deletant strain was independent of concentration over the range studied. In contrast, for cells with wild-type BOR1, the distribution ratio [B]in/[B]out became progressively larger at higher concentrations; at 16 mM boron, the two strains had similar intracellular boron contents, as expected if Bor1p-mediated efflux is a saturable function of the intracellular boron concentration. The concentration depen-
In contrast to the deletant strain, the time course of boron efflux from the wild-type strain was exponential only at the lowest concentrations (Fig. 3B). At cellular boron concentrations above \( \sim 1 \text{ mM} \), the semilogarithmic slope was progressively steeper as the intracellular concentration decreased, indicating that efflux saturates at high boron concentrations. Instantaneous effluxes at each intracellular boron concentration are plotted in Fig. 3C. Efflux was described reasonably well by a hyperbolic (Michaelis-Menten) function plus a nonsaturable component, the magnitude of which was derived independently from the fluxes in the knockout strain (Fig. 3A). The maximal efflux for the saturable component was 0.7 mmol-liter cell water\(^{-1}\cdot \text{min}^{-1}\), and the half-maximal intracellular boron concentration was roughly 1–2 mM.

**Direct demonstration of uphill boron efflux.** The steady-state distribution and efflux rates of boron in wild-type strain were certainly consistent with the idea that Bor1p mediates an uphill boron efflux. A direct demonstration of active boron transport requires loading of cells with a high boron concentration and showing that Bor1p can transport boron outward against a gradient. In the experiment shown in Fig. 4, cells were pre-

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**Fig. 2.** A: comparison of cellular boron contents by the curcumin method and inductively coupled plasma mass spectrometry (ICP-MS). Yeast cells were incubated in YPD medium (1% Difco yeast extract, 2% Bacto-peptone, 2% glucose) containing various concentrations of \( \text{H}_3\text{BO}_3 \). Cells were centrifuged, resuspended in YPD, incubated for various times, and then centrifuged to stop the boron efflux. Cells were then heated for 30 min at 100°C in 50 or 100 μl of water. Boron content of the extract was determined using either the curcumin method or ICP-MS (see MATERIALS AND METHODS). B: time course of increase in cellular boron in S. cerevisiae strains with wild-type (●) or deleted BOR1 (▲). Cells from overnight cultures were suspended in YPD plus 4 mM \( \text{B(OH)}_3 \) and incubated at 30°C. Suspension was diluted 10-fold in cold water at the indicated times and centrifuged immediately, and total cellular boron was determined using the curcumin method. Error bars represent range of data for 2 determinations on a single cell preparation. C: steady-state boron distribution ratios (cell water:extracellular medium) in wild-type (●) or BOR1 deletant strain (▲) following incubation in YPD containing 5 mM citrate, pH 5.0–5.3, at 30°C for 60–90 min in the presence of 1, 2, 4, 8, or 16 mM \( \text{H}_3\text{BO}_3 \). Error bars represent range of data for 2 experiments (1 and 16 mM boron) or SD for 3–4 experiments (2, 4, or 8 mM boron). [B], boron concentration.

**Fig. 3.** Saturable boron efflux mediated by Bor1p. A: time course of efflux of boron from cells with deleted BOR1 (strain 1169). Cells were loaded with boron by incubation in YPD plus 2, 4, or 16 mM \( \text{B(OH)}_3 \) for 1.5 h at 30°C. Efflux was measured in YPD with no added boron, pH 5.0–5.5, at 30°C. Efflux was measured as in A. C: efflux of boron from cells with wild-type BOR1 (strain 6808). Cells were loaded with boron by incubation in YPD plus 4, 8, or 16 mM \( \text{B(OH)}_3 \) for 1.5 h at 30°C. Efflux was measured as in A. C: efflux of boron from cells with wild-type BOR1 (strain 6808). Data points were derived from the efflux measurements in Fig. 4B and other similar experiments (see MATERIALS AND METHODS). Open circles represent efflux from cells that had been grown overnight in YPD plus 10 mM \( \text{H}_3\text{BO}_3 \); filled symbols represent efflux from cells that had been exposed to 4, 8, 10, or 16 mM \( \text{H}_3\text{BO}_3 \) for 60–90 min before the efflux was measured. The dashed line represents the diffusive efflux measured in the deletant strain (from data in A). The curve through the data represents a Michaelis-Menten function added to the diffusive efflux represented by the dashed line. The Michaelis-Menten parameters for the curve were \( V_{\text{max}} = 0.67 \text{ mmol-liter cell water}^{-1} \cdot \text{min}^{-1} \) and \( K_m = 1.8 \text{ mmol-liter cell water} \).
the steady-state boron distribution was not affected by the presence of 25 mM HCO$_3^-$ (with sufficient CO$_2$ to be in equilibrium with HCO$_3^-$ at the stated pH). Moreover, net boron efflux into a low-boron (10$^{-5}$ M) medium at pH 6.35 was not affected by 29 mM HCO$_3^-$ (Fig. 5C). The fact that there was no detectable effect of ~30 mM HCO$_3^-$ in the extracellular medium (which must also increase intracellular CO$_2$ and HCO$_3^-$) suggests that bicarbonate does not interfere with boron transport (see DISCUSSION).

**Lack of induction of BOR1 by growth in media containing H$_2$BO$_3$**. The rate of boric acid efflux was not strongly affected by the duration of exposure of cells to boric acid. The filled symbols in Fig. 3C refer to cells that had been acutely loaded

loaded in YPD medium containing 40 mM H$_2$BO$_3$, centrifuged, and resuspended in medium containing 2 mM H$_2$BO$_3$, and the time courses of intracellular and extracellular boron concentrations were measured. In the deletant strain, intracellular boron concentration dropped progressively from an initial value of over 20 mM to a final value very close to the extracellular concentration, as expected if boron transport in these cells is mainly by way of passive diffusion. In the wild-type strain, the boron concentration dropped to a value far below the extracellular level, demonstrating directly that Bor1p catalyzed an uphill efflux of boron, resulting in an inward gradient of about sevenfold. The final boron distribution in cells preloaded in a 40 mM boron medium and then resuspended in 2 mM boron was indistinguishable from that of cells in 2 mM boron with no preexposure to high boron concentration (Fig. 2).

**Extracellular pH dependence of efflux suggests H$^+$ antiport.** The rate of efflux of boron from cells with intact BOR1 was dependent on the extracellular pH (Fig. 5A). Efflux was more rapid at pH 5.5 than at pH 6.5–7, consistent with the idea that the uphill boron efflux is driven by the inward H$^+$ gradient. Figure 5B shows that the steady-state distribution of boron was more symmetrical at higher pH, again consistent with the idea that the uphill boron efflux is driven by an influx of H$^+$. We attempted to detect H$^+$ influx associated with boron efflux, but the background rate of H$^+$ efflux was too high to detect an decrement associated with boron transport.

**Lack of effect of bicarbonate on boron distribution and efflux.** Because Bor1p is a member of the bicarbonate transporter family, it was of interest to determine whether bicarbonate itself is either transported by Bor1p or affects boron transport. Tracer (H$^{14}$CO$_3^-$) bicarbonate transport measurements are possible in principle, but only with extremely high pH on both sides of the membrane (see Ref. 36); these conditions are not compatible with a study of intact yeast. It is, however, possible to vary CO$_2$/HCO$_3^-$ at constant extracellular pH and measure the effect on boron distribution and transport. Figure 5B shows that at extracellular pH of either 6.2 or 7.0,
with H$_3$BO$_3$ for 1–1.5 h. Data indicated by open symbols were derived from experiments on cells that had been grown overnight in 10 mM H$_3$BO$_3$. This concentration retarded growth somewhat (see below) but allowed growth of cells in sufficient quantities for transport assays. The boron efflux at each intracellular concentration was indistinguishable in cells loaded overnight from that in cells exposed to boron for 1–1.5 h. This finding is illustrated further in Fig. 6A, which shows the time course of boron efflux from parallel cultures, each of which was loaded with boron by incubation in 10 mM H$_3$BO$_3$. One of the cultures was incubated with 10 mM H$_3$BO$_3$ overnight, and the other was incubated for 1 h. There may have been a slight increase in the rate of efflux in the cells that had been exposed to boron overnight, but the effect was small. Overnight incubation at an even higher boron concentration, 40 mM, also did not increase the rate of boron efflux compared with cells loaded for only 1.6 h (Fig. 6A, open symbols).

Figure 6B shows the relative amounts of BOR1 mRNA, as estimated by real-time RT-PCR on extract of cells from the same cultures as those used in the transport experiments in Fig. 6A. There may have been a slight stimulation of mRNA levels by incubation in 10 mM H$_3$BO$_3$, but, as was observed with transport, the effect was small. Therefore, expression of neither BOR1 mRNA nor functional Bor1p is strongly stimulated by growth in media containing concentrations of H$_3$BO$_3$ sufficient to cause a severalfold inhibition of cumulative growth in overnight cultures (see below).

**Effect of H$_3$BO$_3$ on growth of *S. cerevisiae*.** Although $\sim 10^{-5}$ M H$_3$BO$_3$ is included in many synthetic culture media for *S. cerevisiae* (29), it is not clear whether boron is absolutely essential for yeast growth. Boron, however, is a significant nutrient for *S. cerevisiae*, because the addition of boric acid to nominally boron-free synthetic medium increases cell number by $\sim 50\%$ in overnight cultures (3). The half-maximal effect of boron on growth is in the low micromolar range (3). Using both the curcumin assay and ICP-MS, we estimated that the boron concentration in the YPD medium we used was $1.5 \times 10^{-5}$ M. The boron concentration in normal YPD medium is therefore adequate for optimum growth.

At concentrations much higher than those normally found in growth medium, boric acid is known to be toxic to yeast (21, 22). If Bor1p is able to keep the cellular boron levels low in the presence of potentially toxic extracellular concentrations of boron, then the BOR1 deletant strain should be more susceptible than wild-type strains to growth inhibition by boron. Figure 7A shows that wild-type and BOR1 deletant strains had similar sensitivity to growth inhibition by H$_3$BO$_3$. Although expression of native Bor1 did not strongly protect yeast from
growth inhibition by H$_2$BO$_3$, overexpression of Bor1p under control of the GAL1 promoter did protect (Fig. 7B), as expected from the work of Nozawa et al. (20).

**Vacuolar morphology in BOR1 deletant strain.** Decker and Wickner (10) recently showed that the majority of cells in a BOR1 deletant strain have fragmented vacuoles that lack several proteins normally associated with vacuoles. This finding suggests that Bor1p has a subcellular function that is distinct from its role as a plasma membrane transport protein.

To investigate further a possible role of Bor1p in vacuolar dynamics, we used FM 4-64 fluorescence (10, 34) to examine vacuolar morphology in control and BOR1 deletant strains. For micrographs shown in Fig. 8, A and C, slides were prepared directly from suspensions in YPD. In these micrographs, the FM 4-64 fluorescence in most cells was mainly in one to three medium-sized vacuoles, very similar to the FM 4-64 pattern originally shown by Vida and Emr (34). Vacuolar morphologies in YPD were similar in BOR1 deletant and wild-type strains (Fig. 8, A and C), although there appeared to be slightly more cells with multiple vacuoles in the deletant strain. Because of the heterogeneity of vacuolar morphology in both wild-type and deletant strains, it is difficult to quantify the difference between the two strains in YPD medium.

It is known that suspending either *Schizosaccharomyces pombe* (4, 6) or *Saccharomyces cerevisiae* (35) in hyposmotic medium causes a rapid fusion of vacuoles into a single large organelle in most cells. Figure 8, B and D, shows vacuolar morphology of control and deletant strains within 30 min following resuspension in distilled water. The morphology is clearly distinct from that observed in YPD medium, with many cells showing single large vacuoles. Blind scoring of full fields indicated that the most common morphology in both strains was the single large vacuole but that the deletant strain had a higher percentage of cells (~25%) with multiple vacuoles than did the wild-type strain (~10%).

To ensure that the deletant strain used for microscopy was authentic, we measured cellular boron contents (following 60 min of incubation in 2 mM extracellular boron in YPD) in aliquots of the same cultures used for microscopy. As expected, the intracellular boron concentration in the contents of the wild-type strain were sevenfold lower than in the deletant strain. Our data indicate that there does appear to be an abnormal vacuolar morphology or a vacuolar fusion defect in the BOR1 deletant strain used, but the abnormality is not nearly as pronounced as that described by Decker and Wickner (10).

**DISCUSSION**

*Active efflux of boron driven by electrochemical H$^+$ gradient.* The transport experiments described provide direct demonstration of uphill boron efflux mediated by *S. cerevisiae* Bor1p (Fig. 4). The most likely mechanism for this efflux is a coupled exchange of intracellular H$_3$BO$_3$ for extracellular H$^+$, as indicated by the fact that the steady-state boron gradient was steeper at more acid extracellular pH and net efflux was accelerated by lowering the extracellular pH (Fig. 5). In glucose-fed *S. cerevisiae* in the presence of extracellular K$^+$ (in our experiments as K-citrate buffer), the cytoplasmic pH is near neutrality (7.0 ± 0.5) over a wide range (pH 4–8) of extracellular pH values (5, 25). It is reasonable to assume, therefore, that the cytoplasmic pH in our experiments was

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*Fig. 8.* Vacuole morphology in wild-type and BOR1 deletant strains. Overnight cultures in YPD were labeled with FM4-64 as described by Vida and Emr (34) (see MATERIALS AND METHODS). Slides were prepared of cells either in YPD (A and C) or within 30 min of suspension in water (B and D). Images at left of each panel are differential interference contrast images, and images at right are FM4-64 fluorescence.
neutral and that the inward H\(^+\) gradient decreased as the extracellular pH was raised. Accordingly, the observed effect of extracellular pH on both the boron efflux and the steady-state distribution (Fig. 5) are consistent with the idea that Bor1p catalyzes a 1:1 exchange of H\(^+\) for H\(_3\)BO\(_3\). A coupled H\(^+\)/H\(_2\)BO\(_3\) exchange through Bor1p is certainly consistent with the fact that other members of the bicarbonate transporter family are coupled exchangers or cotransporters (1, 18, 26). It is also worth noting that at least one member of the family, the anion exchanger AE1, can transport H\(^+\) under some circumstances (16, 19).

**Lack of apparent interaction with bicarbonate.** We found that there was no effect of 25–30 mM HCO\(_3^-\) on boron distribution or H\(_3\)BO\(_3\) efflux mediated by Bor1p in the extracellular pH range 6.2–7.0 (Fig. 5). It has been known for many years that bubbling a yeast suspension with CO\(_2\) causes major buildup of intracellular HCO\(_3^-\) (5, 7). Therefore, intracellular and extracellular HCO\(_3^-\), CO\(_2\), and H\(_2\)CO\(_3\) concentrations were all elevated in the experiments in Fig. 5, with no detectable effect on either boron distribution or efflux. The simplest interpretation of this finding is that HCO\(_3^-\) is not a substrate for yeast Bor1p. This is perhaps not surprising, given that H\(_3\)BO\(_3\) is transported outward by Bor1p and that, under most conditions, there is an outward bicarbonate gradient in yeast [cytoplasmic pH higher than extracellular pH (5, 25)]. Accordingly, Bor1p clearly does not catalyze an exchange of HCO\(_3^-\) for H\(_2\)BO\(_3\).

Takano et al. (32) measured the effect of neutral pH and HCO\(_3^-\) on boron distribution in wild-type yeast. In agreement with our data, they found that the boron distribution at neutral pH is much more symmetrical than at acid pH. They also found that addition of 30 mM NaHCO\(_3\) doubles the boron contents of yeast cells incubated in a medium containing 0.5 mM H\(_3\)BO\(_3\) and buffered at pH 7 with MES-Tris. This result is clearly different from those of the experiment shown in Fig. 5, in which we found no effect of a similar HCO\(_3^-\) concentration. The final pH (after 1 h of incubation) of the suspensions used by Takano et al. (32) was not specified; it is possible that pH differences caused by the presence or absence of HCO\(_3^-\) could contribute to the observed effect of 30 mM HCO\(_3^-\) on boron distribution.

**Other transport pathways for H\(_3\)BO\(_3\).** The present data can be explained nearly quantitatively without postulating any boron transport pathways other than efflux through Bor1p and passive diffusion across the lipid bilayer. The rate constant for boron efflux in the deletant strain is \(\sim 0.05/\text{min} (\text{Fig. } 3)\). For a cell with a volume-to-surface ratio of \(\sim 0.8 \times 10^{-24} \text{ cm}^2\) (29), this rate constant corresponds to a permeability coefficient of \(\sim 6 \times 10^{-8} \text{ cm/s}\). This value is toward the lower end of the range (9.5 \(\times 10^{-9}\) to \(7 \times 10^{-6} \text{ cm/s}\)) determined by Dordas and Brown (11) for the passive permeability of lipid bilayer membranes to B(OH)\(_3\). This permeability coefficient is about fivefold lower than the H\(_2\)BO\(_3\) permeability of squash root plasma membrane vesicles, where proteins of the aquaporin family may contribute to the H\(_2\)BO\(_3\) flux (12). In S. cerevisiae the passive H\(_2\)BO\(_3\) permeability is sufficiently low that there is no reason to postulate a role for aquaporins, and the recent data of Nozawa et al. (20) indicate that neither aquaporin AQP1 nor AQP2 has a measurable effect on boron distribution.

Nozawa et al. (20) also showed that deletion of the urea transporter DUR3 causes a decrease in the cellular boron level, suggesting that this transporter can drive boron into the cell or inhibit the efflux driven by Bor1p. Under the conditions we used, we did not find any evidence for uphill inward transport of boron; further work is needed to determine the mechanism by which DUR1 deletion causes a decrease in cellular boron.

**Role of Bor1p in protecting yeast from boron toxicity.** Our data on the effects of boron on growth are similar to those of Takano et al. (30), although they are presented in a somewhat different way. Our data are given as growth rates in exponential cultures, whereas Takano et al. (30) plotted cumulative growth after 11 h. Takano et al. found that cumulative growth of the deletant strain in 5 mM boron is 25% lower than that of the wild-type strain. In terms of growth rate of an exponential culture, this amounts to a difference of about 15%. We did not observe a detectable difference between wild-type and deletant strains regarding the effects of boron on growth rates, but it is possible that an effect of 15% would be within the error of our measurements.

Nozawa et al. (20) showed that in medium containing 90 mM boron, the deletion strain has about 10% more cellular boron than the wild-type strain (20). We did not examine concentrations this high, but a 10% difference is certainly compatible with the data we obtained at lower concentrations (Fig. 2). At high boron concentrations, the efflux driven by Bor1p is sufficient to cause a significant difference in growth on plates (20), although the cellular boron levels are decreased by only 10%.

Although it is quite clear from this study and the work of Takano et al. (32) that Bor1p catalyzes the uphill extrusion of boron across the plasma membrane of S. cerevisiae, it is also true that some of the characteristics of yeast boric acid transport described presently would not be expected if the main purpose of Bor1p were to protect cells from the toxic effects of boric acid/borate. Bor1p is constitutively expressed in cells grown in YPD medium, which contains boric acid at concentrations far below toxic levels, and the BOR1 gene is not induced by exposure of cell to boron (Fig. 6; see also Ref. 30). If the purpose of Bor1p were to extrude boric acid as a detoxification mechanism, then it would be surprising that the protein is produced under conditions in which it would not be needed and not strongly induced by conditions that would seem to require it.

**Oxynion transport by Bor1p?** Zhao and Reithmeier (37) showed that a variety of inorganic anions, including some oxynions, can compete for stilbenedisulfonate binding to Bor1p, and it is possible that Bor1p is a H\(^+\)-coupled exchanger that can extrude not only boric acid but other inorganic species. Boron is a metalloid, along with silicon, germanium, arsenic, antimony, tellurium, and polonium. S. cerevisiae has separate mechanisms for arsenic and antimony resistance that are unrelated to BOR1 (33). Arsenite exposure has an approximately twofold stimulatory effect on transcription of BOR1 (15), and it is possible that Bor1p can transport arsenite or arsenate.

There are many other possible oxides or oxyanions that could show that a variety of inorganic anions, including some oxynions, can compete for stilbenedisulfonate binding to Bor1p, and it is possible that Bor1p is a H\(^+\)-coupled exchanger that can extrude not only boric acid but other inorganic species. Boron is a metalloid, along with silicon, germanium, arsenic, antimony, tellurium, and polonium. S. cerevisiae has separate mechanisms for arsenic and antimony resistance that are unrelated to BOR1 (33). Arsenite exposure has an approximately twofold stimulatory effect on transcription of BOR1 (15), and it is possible that Bor1p can transport arsenite or arsenate. There are many other possible oxides or oxyanions that could be substrates. In addition to determining the boron content of the control and BOR1 deletant strains, the cellular contents of 20 other metals and metalloids was determined by ICP-MS (data not shown). There were no consistent differences in the cellular contents of any of these elements in the deletant and control strains under the conditions tested. A systematic study using media containing specific additions of other metalloids is
necessary to determine whether there are other substrates for Bor1p.

Possible effect on vacuolar morphology. The micrographs in Fig. 8 show that vacuolar morphology, in either YPD or hyposmotic medium, is similar in control and BOR1 deletion strains. There are slightly more cells with multiple vacuoles in the deletion strain, but the difference is much less dramatic than that found by Decker and Wickner (10), who showed that 95% of BOR1 deletion cells have fragmented vacuoles. We have no explanation for the difference between the two studies, and further work is needed to determine the extent of the vacuolar abnormality in BOR1 deletion strains under various growth conditions.

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