

# Vacuolar H<sup>+</sup>-ATPase Protects *Saccharomyces cerevisiae* Cells against Ethanol-Induced Oxidative and Cell Wall Stresses

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## ABSTRACT

During fermentation, increased ethanol concentration is a major stress for yeast cells. Vacuolar H<sup>+</sup>-ATPase (V-ATPase), which plays an important role in the maintenance of intracellular pH homeostasis through vacuolar acidification, has been shown to be required for tolerance to straight-chain alcohols, including ethanol. Since ethanol is known to increase membrane permeability to protons, which then promotes intracellular acidification, it is possible that the V-ATPase is required for recovery from alcohol-induced intracellular acidification. In this study, we show that the effects of straight-chain alcohols on membrane permeabilization and acidification of the cytosol and vacuole are strongly dependent on their lipophilicity. These findings suggest that the membrane-permeabilizing effect of straight-chain alcohols induces cytosolic and vacuolar acidification in a lipophilicity-dependent manner. Surprisingly, after ethanol challenge, the cytosolic pH in  $\Delta vma2$  and  $\Delta vma3$  mutants lacking V-ATPase activity was similar to that of the wild-type strain. It is therefore unlikely that the ethanol-sensitive phenotype of *vma* mutants resulted from severe cytosolic acidification. Interestingly, the *vma* mutants exposed to ethanol exhibited a delay in cell wall remodeling and a significant increase in intracellular reactive oxygen species (ROS). These findings suggest a role for V-ATPase in the regulation of the cell wall stress response and the prevention of endogenous oxidative stress in response to ethanol.

## IMPORTANCE

The yeast *Saccharomyces cerevisiae* has been widely used in the alcoholic fermentation industry. Among the environmental stresses that yeast cells encounter during the process of alcoholic fermentation, ethanol is a major stress factor that inhibits yeast growth and viability, eventually leading to fermentation arrest. This study provides evidence for the molecular mechanisms of ethanol tolerance, which is a desirable characteristic for yeast strains used in alcoholic fermentation. The results revealed that straight-chain alcohols induced cytosolic and vacuolar acidification through their membrane-permeabilizing effects. Contrary to expectations, a role for V-ATPase in the regulation of the cell wall stress response and the prevention of endogenous oxidative stress, but not in the maintenance of intracellular pH, seems to be important for protecting yeast cells against ethanol stress. These findings will expand our understanding of the mechanisms of ethanol tolerance and provide promising clues for the development of ethanol-tolerant yeast strains.

The budding yeast *Saccharomyces cerevisiae* has been widely used in industrial fermentation, such as in the production of alcoholic beverages and ethanol fuel. During fermentation, yeast cells encounter several environmental stresses, such as increased alcohol concentration, high osmolarity, and temperature fluctuations. Among these, ethanol is a major stress factor that affects the vitality and viability of yeast cells, thereby leading to an arrest of the fermentation process (1). High ethanol concentrations are known to influence a number of metabolic processes, including the inhibition of glucose and amino acid transport, denaturation of the key glycolytic enzymes pyruvate kinase and hexokinase, and increased membrane permeability (1).

Cellular membranes, especially the plasma membrane, have been suggested to be the main target of ethanol stress in yeast cells (2). Ethanol and other short-chain alcohols are believed to disturb the cellular membrane through the association of their aliphatic chains with the hydrophobic interior of membranes, thereby affecting membrane permeability and stability (3). The loss of membrane integrity decreases the cell's ability to maintain a concentration gradient across the plasma membrane, which is important for coupled transport of several metabolites, such as amino acids and ions (4, 5). Since the budding yeast normally lives in a slightly acidic environment with a high proton concentration, the increase

in membrane permeability caused by alcohol may lead to an increased passive influx of protons across the membrane, thereby inducing intracellular acidification (6). Compounds with high lipophilicity, usually expressed in terms of the log octanol-water partition coefficient ( $\log P_{ow}$ ), are known to possess a high affinity for the cellular membrane (7). In addition, the toxicities of alcohols have been shown to be correlated with their lipophilicity (3, 8–10). For instance, alcohols with high lipophilicity, such as 1-octanol ( $\log P_{ow} = 3.07$ ), were more toxic to yeast cells than those with low lipophilicity, such as ethanol ( $\log P_{ow} = -0.30$ ) (8).

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Taken together, these results indicate it is possible that alcohols with high lipophilicity trigger a high degree of membrane permeabilization, leading to greater proton influx and a significant decrease in intracellular pH. In agreement with this idea, Leão and Van Uden (6) reported that upon exposure to alkanols, i.e., ethanol, isopropanol, propanol, and butanol, the extracellular pH increased in a concentration- and lipophilicity-dependent manner, possibly as a consequence of the increased proton influx. However, since the alterations in intracellular pH in response to alcohols with different lipophilicities have not been determined, the correlation between the lipophilicity of alcohols and their effects on membrane permeability and intracellular pH remains unclear.

A number of genes associated with vacuolar H<sup>+</sup>-ATPase (V-ATPase), including genes encoding structural components of V-ATPase and genes involved in the assembly of V-ATPase, have been shown to be required for tolerance not only to ethanol but also to other alcohols (9, 10). The yeast V-ATPase is a multisubunit proton pump composed of a peripheral V<sub>1</sub> ATP-hydrolytic domain and an integral V<sub>0</sub> proton-translocating domain. The V-ATPase plays a vital role in the maintenance of intracellular pH homeostasis through proton translocation across the vacuolar membrane driven by ATP hydrolysis, resulting in vacuolar acidification (11). In addition to the V-ATPase, the plasma membrane H<sup>+</sup>-ATPases, which are composed of the essential Pma1p and its homolog, the nonessential Pma2p, are also known to function in pumping protons out of the cell to control the intracellular pH balance (12, 13). The activities of both V-ATPase and plasma membrane H<sup>+</sup>-ATPase Pma1p are induced under acidic pH conditions, possibly in response to cytosolic acidification (14, 15). The *vma* mutants lacking V-ATPase activity exhibit a more-alkaline vacuolar pH than the wild-type strain (16) and are unable to grow at pH values of >7 and <4 (17, 18). Moreover, a loss of V-ATPase activity triggers ubiquitination and endocytosis of Pma1p, possibly to balance overall pH homeostasis (16, 19). These phenotypes of the *vma* mutants suggest that the V-ATPase and the plasma membrane H<sup>+</sup>-ATPases together play an interdependent role in the regulation of intracellular pH homeostasis. In addition to the expected phenotypes of the *vma* mutants, which are a direct consequence of impaired intracellular pH regulation, these mutants also exhibited other pleiotropic phenotypes, including sensitivity to a variety of oxidants, such as H<sub>2</sub>O<sub>2</sub> (20), sensitivity to transition metals, such as copper and zinc (21, 22), poor growth under conditions of both high and low concentrations of calcium and iron (18, 23, 24), poor growth on nonfermentable carbon sources (25), and defective vacuolar morphology and vacuolar protein sorting (26, 27). Some of these phenotypes can be easily explained by an increased accumulation of reactive oxygen species (ROS) or defective vacuolar function, important for storage and sequestration of several metabolites and toxins in these mutants (20, 25). Based on these previous observations, it is possible that the V-ATPase, possibly in collaboration with the plasma membrane H<sup>+</sup>-ATPase, is required for recovery from cytosolic acidification or other harmful effects caused by alcohols. However, the roles of these proton pumps during alcohol stress remain unclear.

In this report, we show a clear correlation between the lipophilicity of straight-chain alcohols and their effects on membrane permeability and intracellular pH alteration, suggesting that the alcohol-induced cytosolic and vacuolar acidification may result from increased membrane permeability. However, the ethanol-sensitive phenotype of *vma* mutants lacking V-ATPase activity is

likely due to elevated intracellular ROS levels and a defect in the response to cell wall damage caused by ethanol rather than the increased cytosolic acidification.

## MATERIALS AND METHODS

**Strains and culture conditions.** The yeast strains used in this study were the BY4742 wild-type strain (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and the congeneric *Δvma2::kanMX*, *Δvma3::kanMX*, and *Δpma2::kanMX* strains. All strains were obtained from Open Biosystems (Huntsville, AL). YPD (1% yeast extract, 2% peptone, 2% dextrose) and SD (2% dextrose and 0.67% yeast nitrogen base without amino acids) media supplemented with appropriate nutrients were prepared as described previously (28) and adjusted to pH 6.0 with the optional addition of 200 mg/liter G418 (Geneticin; Sigma-Aldrich).

**Growth assay.** A serial-dilution spot test was used to examine alcohol sensitivity. Exponential-phase cells were diluted to an optical density at 600 nm (OD<sub>600</sub>) of 1.0 (approximately 10<sup>7</sup> cells/ml) and then serially diluted 10-fold. Aliquots of 3 μl were spotted onto freshly prepared YPD agar plates containing 18% methanol, 10% ethanol, 7% 1-propanol, 3% 1-butanol, and 100 μg/ml calcofluor white and incubated at 30°C for 3 days. For growth under anaerobic conditions, the inoculated plates were incubated at 30°C for 3 to 4 days in an AnaeroPack rectangular jar (Mitsubishi Gas Chemical Co., Tokyo, Japan) containing an AnaeroPack working as an oxygen absorber-CO<sub>2</sub> generator (Mitsubishi Gas Chemical Co.). To test amphotericin B (AmB) sensitivity, log-phase cultures were inoculated into YPD medium containing 0.5 or 1 μM AmB to an OD<sub>600</sub> of 0.1 and incubated with shaking at 30°C. Cell growth was monitored at 2-h intervals for 24 h by measuring the OD<sub>600</sub>.

**Sytox green uptake.** Yeast membrane permeabilization was monitored by measuring Sytox green uptake, as described previously (29). Briefly, log-phase cells were harvested and resuspended in 50 mM morpholineethanesulfonic acid (MES)-NaOH buffer (pH 5.5) containing 1 μM Sytox green (Invitrogen) to an OD<sub>600</sub> of 1. The Sytox green fluorescence was measured with a SpectraMax M3 microplate reader (Molecular Devices) at an excitation wavelength of 488 nm and an emission wavelength of 544 nm. The relative fluorescence units were expressed as a percentage of the fluorescence values of the wild-type strain grown in YPD medium.

**Measurements of cytosolic and vacuolar pH.** Cytosolic pH was measured using pHluorin (30), a pH-sensitive green fluorescent protein (GFP) expressed from plasmid pYES-P<sub>ACT1</sub>-pHluorin under the control of the *ACT1* promoter (a generous gift from Gertien J. Smits, University of Amsterdam, the Netherlands), according to a method described previously (16), with some modifications. Briefly, yeast cells were transformed with this plasmid, and transformants were selected in SD medium lacking uracil (SD-Ura) and confirmed by fluorescence microscopy (30). Yeast cells grown to log phase in the specified medium were harvested, washed, and resuspended at 1 g of wet cell mass/ml in glucose-free medium. Fluorescence intensities at excitation wavelengths of 390 and 470 nm were measured at a constant emission wavelength of 512 nm by the SpectraMax M3 microplate reader (Molecular Devices). Calibration of fluorescence with pH was performed for each strain and included buffers titrated to different pH values within a range of 4.5 to 8.0, as described previously (16).

Vacuolar pH was measured according to a previously described method (31). Yeast cells grown to log phase in the specified medium were harvested, washed, resuspended in the same culture medium containing 50 μM 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Sigma), and incubated at 30°C for 30 min. Fluorescence intensities at excitation wavelengths of 450 and 490 nm were measured at a constant emission wavelength of 535 nm by the SpectraMax M3 microplate reader (Molecular Devices). The vacuolar pH was estimated from the calibration curve for each strain, which was constructed by incubating the yeast strain in buffers titrated to different pH values within a range of 4.0 to 8.0, as described previously (31).

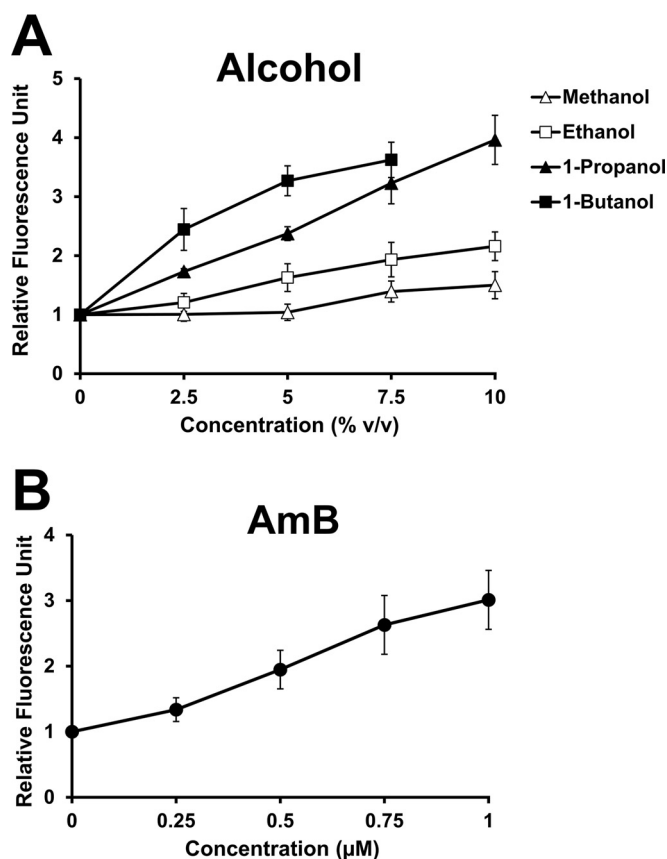
**Zymolyase susceptibility test.** Susceptibility to Zymolyase was carried out according to the method of Ovalle et al. (32), with some modifications. Log-phase cells were diluted to an  $OD_{600}$  of 0.5 in TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 7.5]) containing 100  $\mu$ g/ml (1 U) Zymolyase 20T (Zymo Research). Changes in the  $OD_{600}$  was monitored at 15-min intervals for 2 h.

**Measurement of intracellular ROS levels.** The oxidant-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma) was used to determine the level of intracellular ROS as described previously (33). Briefly, exponential-phase cells were treated with 10  $\mu$ M DCFH-DA in culture medium for 1 h. The cells were then harvested, washed twice in ice-cold phosphate-buffered saline, resuspended in phosphate-buffered saline, and disrupted with glass beads. After being centrifuged at  $13,000 \times g$  for 10 min at 4°C, the supernatant was collected and used for the measurement of fluorescence intensity at an excitation wavelength of 504 nm and an emission wavelength of 524 nm by the Spectra-Max M3 microplate reader (Molecular Devices). The fluorescence intensity value was normalized to the protein level in the supernatant.

## RESULTS

**Effects of straight-chain alcohols on yeast cell growth and membrane permeability are correlated with their lipophilicity.** To determine the effects of straight-chain alcohols with different lipophilicities expressed in terms of  $\log P_{ow}$  values on yeast cells, we examined the growth of the wild-type strain (BY4742) on YPD agar plates containing methanol ( $\log P_{ow}$ , -0.74), ethanol ( $\log P_{ow}$ , -0.3), 1-propanol ( $\log P_{ow}$ , 0.25), or 1-butanol ( $\log P_{ow}$ , 0.84), which have similar structures and chemical properties but different aliphatic chain lengths, by using a spot susceptibility test. The wild-type strain was able to grow in the presence of methanol, ethanol, 1-propanol, and 1-butanol at concentrations of up to 20%, 15%, 7.5%, and 5%, respectively (see Fig. S1 in the supplemental material), showing a clear correlation between yeast cell growth and lipophilicity ( $\log P_{ow}$  value) of straight-chain alcohols.

Since it has been suggested that an effect of ethanol on enhancing membrane permeability is probably due to an insertion of its hydrophobic portion into the hydrophobic interior of the biological membrane (34), it is possible that highly lipophilic alcohols with high affinity to the cellular membrane may have high membrane-permeabilizing effects. To explore the relationship between the lipophilicity of straight-chain alcohols and their effects on membrane permeability, we determined the membrane permeability of the wild-type strain exposed to increasing concentrations of methanol, ethanol, 1-propanol, and 1-butanol by using the Sytox green uptake assay. Sytox green is a high-affinity nucleic acid-staining fluorescent dye that can penetrate the plasma membrane only when the membrane integrity is compromised (29). Our results revealed that the fluorescence intensities of cells treated with straight-chain alcohols increased with increasing concentrations of (in decreasing order) 1-butanol, 1-propanol, ethanol, and methanol, indicating a strong dependence of fluorescence intensity on the lipophilicity and concentration of a straight-chain alcohol (Fig. 1A). To determine whether increased fluorescence intensities of the cells treated with alcohols correlate with a known instance of increased membrane permeability, we performed this assay in the wild-type strain challenged with amphotericin B (AmB), a polyene antifungal drug that, together with ergosterol, forms an ion-permeable self-assembling transmembrane pore (35), and we found that the fluorescence intensities increased with increasing concentrations of AmB (Fig. 1B). Taken together, these results suggest that the membrane-permeabilizing

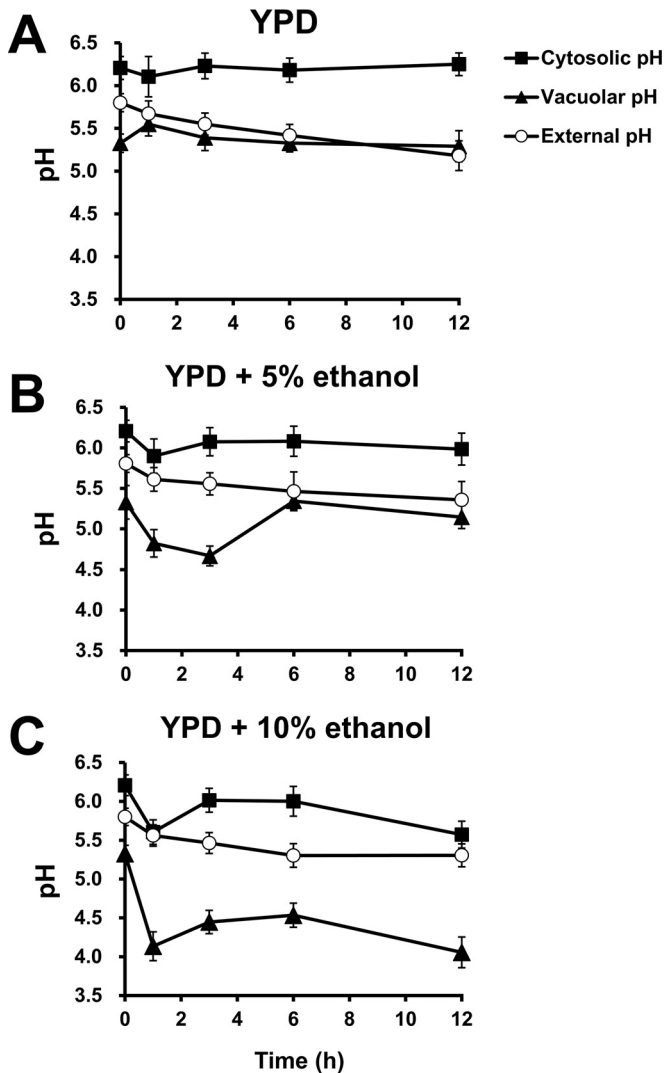


**FIG 1** Sytox green uptake by yeast cells exposed to straight-chain alcohols and amphotericin B (AmB). The exponentially growing wild-type cells (BY 4742) were incubated in YPD medium containing the indicated concentrations of methanol ( $\Delta$ ), ethanol ( $\square$ ), 1-propanol ( $\blacktriangle$ ), or 1-butanol ( $\blacksquare$ ) (A) or of AmB ( $\bullet$ ) (B) at 30°C for 1 h. The cells with an  $OD_{600}$  of 1 loaded with Sytox green were immediately used for fluorescence measurement at an excitation wavelength of 544 nm and an emission wavelength of 488 nm. The fluorescence intensities of the treated cells were normalized to that of the untreated control and plotted as relative fluorescence units (RFU). All experiments were carried out in triplicate. The error bars indicate the standard deviations (SD). v/v, vol/vol.

effects of straight-chain alcohols are correlated with their lipophilicity.

**Degrees of cytosolic and vacuolar acidification induced by straight-chain alcohols are dependent on their lipophilicity.** It has been shown that straight-chain alcohols induce passive fluxes of ions, including protons, across the plasma membrane due to increased membrane permeability (6), which may disturb the intracellular pH. To investigate the dynamic changes in intracellular and extracellular pHs in response to ethanol, time courses of the changes in cytosolic, vacuolar, and external pHs of the wild-type strain were monitored. The cytosolic and vacuolar pH values of the wild-type cells precultivated to exponential phase in YPD medium for 12 h were 6.2 and 5.3, respectively, while the external pH of the preculture before a shift to fresh medium was 5.2. We first investigated the pH fluctuations under normal conditions by measuring the cytosolic, vacuolar, and external pH values of wild-type cells grown in YPD medium adjusted to pH 5.8 (Fig. 2A). After a shift to fresh YPD medium, the cytosolic pH of wild-type cells was maintained at approximately 6.1 to 6.2 throughout an





**FIG 2** Dynamic changes in cytosolic, vacuolar, and external pHs of yeast cells during ethanol exposure. Cytosolic, vacuolar, and external pH values of the wild-type cells (BY4742) treated with 0% (A), 5% (B), or 10% ethanol (C) were measured. For the cytosolic pH (■), log-phase cells transformed with pYES-*P<sub>ACT1</sub>*-pHluorin were harvested at the indicated times and used for the measurement of fluorescence intensity. For the vacuolar pH (▲), the exponentially growing cells without the plasmid were harvested at the indicated times and loaded with BCECF-AM prior to the measurement of fluorescence intensity. The extracellular pH (○) at the indicated times was also monitored. All experiments were carried out in triplicate. The error bars indicate the SD.

incubation period of 12 h, whereas the vacuolar pH of this strain increased to 5.6 within 1 h after a shift and dropped to approximately 5.3 after incubation for >3 h (Fig. 2A). On the other hand, the external pH gradually dropped to 5.2 during a 12-h cultivation (Fig. 2A).

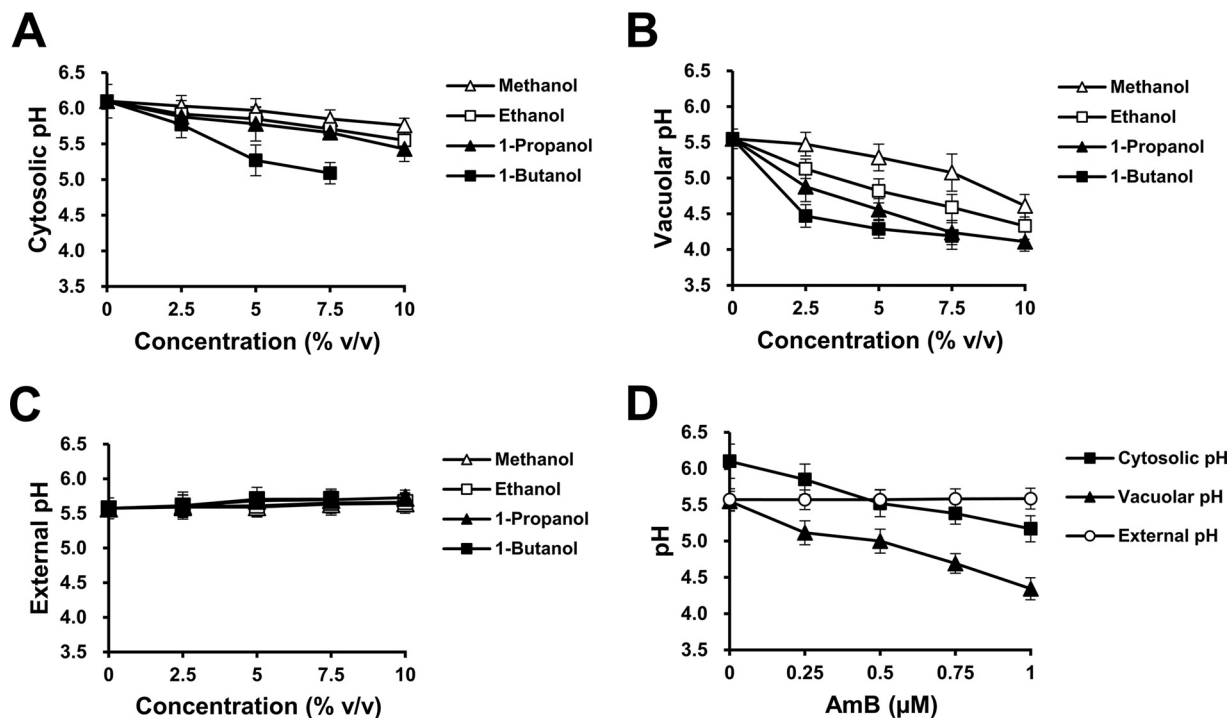
To investigate the effect of ethanol on pH fluctuations, the cytosolic, vacuolar, and external pH values of wild-type cells challenged with 5% and 10% ethanol were determined (Fig. 2B and C). The cytosolic pH of the wild-type strain challenged with 5% ethanol dropped to 5.9 within 1 h and was maintained at 6.0 to 6.1 after exposure for >3 h, whereas during exposure to 10% ethanol, it strikingly dropped to 5.6 within the first hour, increased to 6.0 during 3 to 6 h of incubation, and declined again to 5.6 after a 12-h

exposure (Fig. 2B and C). After the wild-type strain was challenged with 5% ethanol, the vacuolar pH decreased to 4.7 within 3 h and increased to 5.1 to 5.3 after exposure for >6 h (Fig. 2B). When wild-type cells were treated with 10% ethanol, the vacuolar pH remarkably dropped to 4.1 within 1 h, was maintained at 4.5 during 3 to 6 h of incubation, and fell to 4.1 after a 12-h exposure (Fig. 2C). With regard to the external pH, when cells were treated with 5% or 10% ethanol, it gradually decreased to 5.4 or 5.3, respectively, during a 12-h exposure (Fig. 2B and C). These results suggest a dose-dependent effect of ethanol on cytosolic acidification, which seems to be recovered by vacuolar acidification and proton extrusion from cells.

To determine the effect of straight-chain alcohols with different lipophilicity values on intracellular pH disturbance, we measured the cytosolic, vacuolar, and external pH values of the wild-type strain after 1 h of exposure to various concentrations (0 to 10%) of methanol, ethanol, 1-propanol, and 1-butanol. Our results revealed that the acidification of the cytosol and vacuole was enhanced in a concentration- and lipophilicity-dependent manner (Fig. 3A and B). Meanwhile, during short-term exposure, the external pH of wild-type cells grown in the presence or absence of straight-chain alcohols was not significantly altered (Fig. 3C). To determine whether the effect of straight-chain alcohols on cytosolic and vacuolar acidification correlates with increased membrane permeability, the cytosolic, vacuolar, and external pH values of the wild-type strain challenged with 0 to 1  $\mu$ M AmB for 1 h were measured. Similar to the treatments with straight-chain alcohols, the cytosolic and vacuolar pHs decreased with increasing concentrations of AmB while the external pH was not affected (Fig. 3D). Taken together, these results suggest that the increased membrane permeability caused by straight-chain alcohols induces cytosolic and vacuolar acidification in a concentration- and lipophilicity-dependent manner.

**Cytosolic acidification does not account for the ethanol-sensitive phenotype of the *vma* mutants lacking V-ATPase activity.** The V-ATPase and plasma membrane H<sup>+</sup>-ATPase functioning in pumping protons into the vacuole and out of cells, respectively, are known to play a pivotal role in the maintenance of cytosolic pH homeostasis (16). Previously, a number of mutants lacking V-ATPase activity have been shown to be sensitive to alcohols, including ethanol (9, 10). It is therefore possible that the V-ATPase and/or the plasma membrane H<sup>+</sup>-ATPase is required for recovery from cytosolic acidification as a consequence of increased membrane permeability induced by alcohols. To confirm the importance of V-ATPase for alcohol tolerance, the  $\Delta$ *vma2* and  $\Delta$ *vma3* mutants, lacking the V<sub>0</sub> and V<sub>1</sub> subunits of the V-ATPase, respectively, were used. To investigate the role of plasma membrane H<sup>+</sup>-ATPase, we used the  $\Delta$ *pma2* mutant, lacking the minor plasma membrane H<sup>+</sup>-ATPase, because *PMA1*, which encodes the major plasma membrane H<sup>+</sup>-ATPase, is an essential gene, precluding the construction of such a deletion mutant. We examined the growth of these mutants in the presence and absence of various straight-chain alcohols and found that, compared to the wild-type strain, the  $\Delta$ *vma2* and  $\Delta$ *vma3* mutants, but not the  $\Delta$ *pma2* mutant, were more sensitive to 18% methanol, 10% ethanol, 7% 1-propanol, and 3% 1-butanol (Fig. 4). In addition, only the *vma* mutants were also more sensitive to AmB than the wild-type strain (see Fig. S2 in the supplemental material).

To investigate whether the ethanol-sensitive phenotype of the *vma* mutants is a consequence of severe cytosolic acidification due



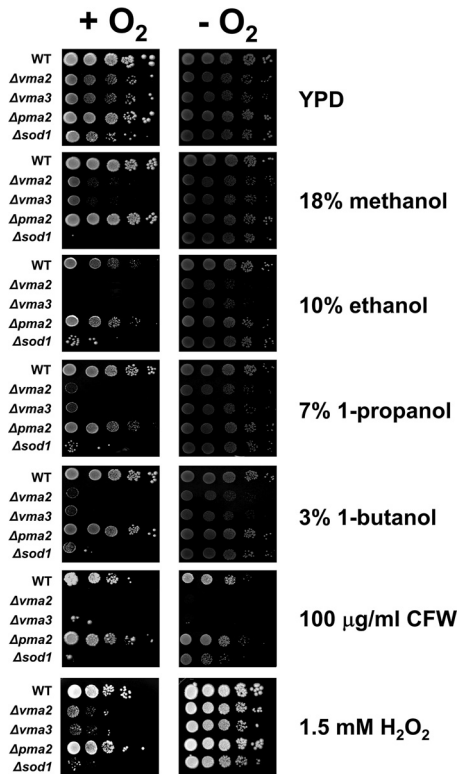
**FIG 3** Changes in cytosolic, vacuolar, and external pH in response to straight-chain alcohols and AmB in the wild-type strain. Cytosolic, vacuolar, and external pH values of the wild-type cells (BY4742) incubated in YPD medium containing 0 to 10% methanol ( $\Delta$ ), ethanol ( $\square$ ), 1-propanol ( $\blacktriangle$ ), or 1-butanol ( $\blacksquare$ ) (A to C) or 0 to 1  $\mu$ M AmB (D) at 30°C for 1 h were measured. The log-phase wild-type cells transformed with pYES- $P_{ACT1}$ -pHluorin were used for the measurement of cytosolic pH (A), whereas log-phase wild-type cells without the plasmid were used for the measurement of vacuolar pH (B) and external pH (C). All experiments were carried out in triplicate. The error bars indicate the SD.

to the lack of an ability to pump excess protons from the cytosol into the vacuole, we monitored the cytosolic, vacuolar, and external pH values of the  $\Delta vma2$  and  $\Delta vma3$  mutants after challenge with ethanol or AmB for 1 h. As expected, under all conditions, the vacuolar pH of the *vma* mutants was more alkaline than that of the wild-type strain (Fig. 5B and E). For the external pH, no significant differences were observed between the wild-type and mutant strains (Fig. 5C and F). In contrast to the wild-type strain, the cytosolic pH of *vma* mutants was not significantly changed in response to ethanol or AmB (Fig. 5A and D). Furthermore, although the cytosolic pH of *vma* mutants was lower than that of the wild-type strain when they were grown in YPD medium, it was similar to that of the wild-type strain under conditions of 10% ethanol or 0.5  $\mu$ M AmB and even more alkaline than the wild-type strain when treated with 1  $\mu$ M AmB (Fig. 5A and D). However, it was still possible that the effect of alcohol on cytosolic acidification in *vma* mutants might occur after long-term exposure; therefore, we measured the cytosolic pH of these mutants treated with ethanol or AmB for 12 h. As with short-term exposure, the cytosolic pH of *vma* mutants was not altered by long-term exposure to ethanol or AmB and was at levels similar to those of the wild-type strain when high concentrations were used (see Fig. S3A and D in the supplemental material). Based on our observations, it is unlikely that the sensitivity of *vma* mutants to ethanol results from severe cytosolic acidification.

**Loss of V-ATPase activity leads to delayed cell wall remodeling in response to ethanol stress.** It has been reported that several cell wall-related genes are important for ethanol tolerance and that cell wall composition is remodeled in response to ethanol

stress (9, 10, 36, 37). To test whether the ethanol-sensitive phenotype of the *vma* mutants is a consequence of a defect in cell wall remodeling, we first examined the aerobic growth of the  $\Delta vma2$  and  $\Delta vma3$  mutants in the presence of the cell wall-perturbing agent calcofluor white. The  $\Delta vma2$  and  $\Delta vma3$  mutants were highly sensitive to 100  $\mu$ g/ml calcofluor white (Fig. 4), suggesting impaired cell wall stress responses in these mutants. To investigate remodeling of the cell wall in response to ethanol stress, the wild-type,  $\Delta vma2$ , and  $\Delta vma3$  cells challenged with 5% and 10% ethanol for up to 12 h were examined for sensitivity to Zymolyase, a cell wall-degrading enzyme whose major activities are  $\beta$ -1,3-glucanase and  $\beta$ -1,3-glucan laminaripentaohydrolase. Zymolyase resistance (as determined by measuring the optical density of the cell suspensions) of wild-type cells increased with increasing ethanol concentration and exposure time to ethanol (Fig. 6), suggesting the concentration- and exposure time-dependent regulation of cell wall remodeling in response to ethanol. On the other hand, the *vma* mutants were more sensitive to Zymolyase than the wild-type strain after short-term exposure to ethanol for 1 h but displayed increased resistance to Zymolyase to wild-type levels after exposure to ethanol for  $\geq 3$  h (Fig. 6). Since the *vma* mutants exhibited a delay, but not a complete loss, of cell wall remodeling in response to cell wall stress caused by ethanol, it seems that the defect in the cell wall stress response may not be a major cause of ethanol sensitivity resulting from a loss of V-ATPase activity.

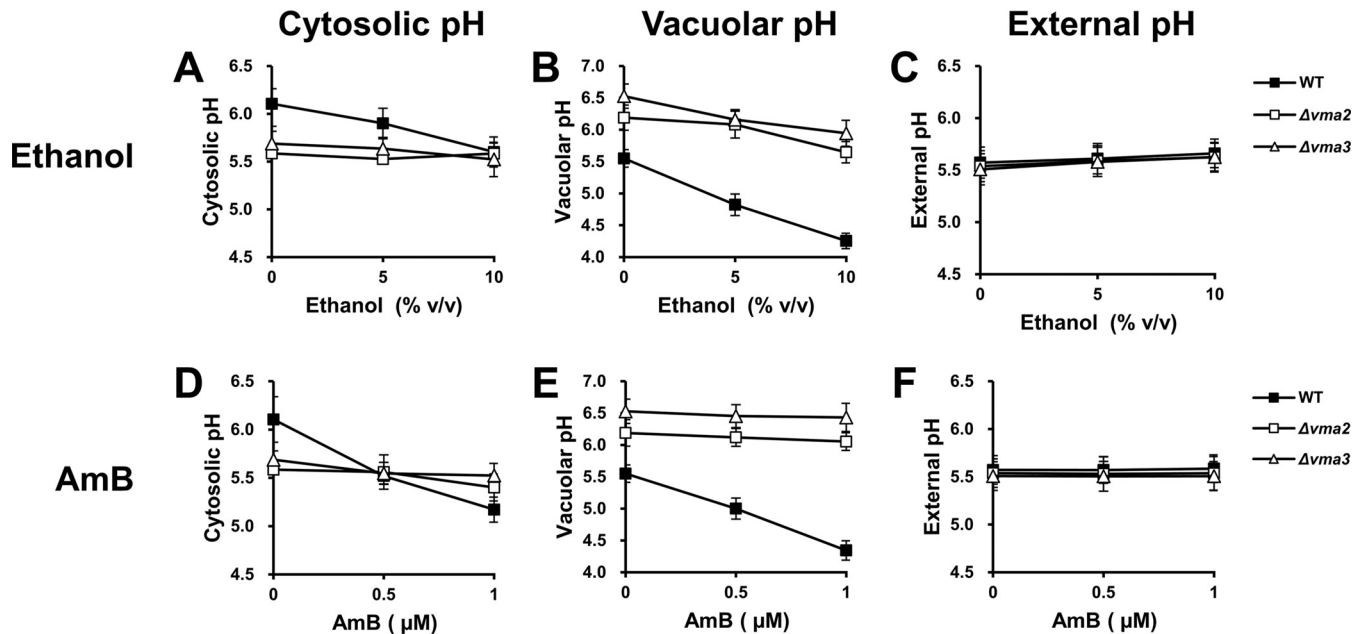
**V-ATPase is important for protecting yeast cells against ethanol-induced endogenous oxidative stress.** Previously, it was demonstrated that the loss of V-ATPase function leads to chronic oxidative stress and that the level of intracellular reactive oxygen



**FIG 4** V-ATPase is important for tolerance to not only straight-chain alcohols but also cell wall and oxidative stresses. Wild-type,  $\Delta vma2$ ,  $\Delta vma3$ ,  $\Delta pma2$ , and  $\Delta sod1$  cells grown to log phase in YPD broth were serially diluted 10-fold from an initial  $OD_{600}$  of 0.1. Aliquots (3  $\mu$ l) were spotted on the YPD agar plate containing 18% methanol, 10% ethanol, 7% 1-propanol, 3% 1-butanol, 100  $\mu$ g/ml calcofluor white (CFW), or 1.5 mM  $H_2O_2$  and incubated at 30°C under aerobic (+ $O_2$ ) and anaerobic (- $O_2$ ) conditions for 3 days.

species (ROS) of yeast cells increases during exposure to ethanol (20, 38). To address whether the sensitivity of *vma* mutants to ethanol stress results from an elevated endogenous oxidative stress induced by ethanol, we examined the growth of the wild-type,  $\Delta vma2$ ,  $\Delta vma3$ , and  $\Delta sod1$  strains (the last lacking cytoplasmic Cu/Zn-superoxide dismutase, required for the elimination of cytosolic ROS) on YPD agar plates supplemented with straight-chain alcohols, calcofluor white, or  $H_2O_2$  under aerobic and anaerobic conditions. When grown aerobically, the  $\Delta sod1$  mutant was sensitive to not only  $H_2O_2$  but also all straight-chain alcohols examined and calcofluor white, and the growth defect of this mutant was restored by cultivation under anaerobic conditions (Fig. 4). These findings suggest that in addition to ethanol, the other straight-chain alcohols and calcofluor white are capable of inducing endogenous oxidative stress, whereas in the *vma* mutants, only alcohol sensitivity but not calcofluor white sensitivity was suppressed under anaerobic conditions (Fig. 4). These results suggest that in the *vma* mutants lacking V-ATPase activity, the alcohol sensitivity may result from elevated endogenous oxidative stress induced by straight-chain alcohols, whereas the calcofluor white-sensitive phenotype is caused by oxidative-stress-independent pathways.

To further investigate the role of V-ATPase in the protection of yeast cells against ethanol-induced oxidative stress, we measured intracellular ROS levels in the wild-type,  $\Delta vma2$ , and  $\Delta vma3$  strains after exposure to ethanol, AmB, or  $H_2O_2$  for 1 h. Our results showed that ROS production was significantly enhanced in response to not only  $H_2O_2$  and ethanol but also AmB (Fig. 7), suggesting that increased membrane permeability may induce endogenous oxidative stress. Furthermore, the *vma* mutants exhibited higher intracellular ROS levels than the wild-type strain under both stress and nonstress conditions (Fig. 7), raising the possibility that the loss of V-ATPase function may result in a defect in the



**FIG 5** Effects of straight-chain alcohols and AmB on cytosolic, vacuolar, and external pH in the mutants lacking V-ATPase. Cytosolic, vacuolar, and external pH values of the wild-type (BY 4742) (■),  $\Delta vma2$  (□), and  $\Delta vma3$  (△) cells incubated in YPD medium containing 0, 5, or 10% ethanol (A to C) or 0, 0.5 or 1  $\mu$ M AmB (D to F) at 30°C for 1 h were measured. Log-phase cells transformed with pYES- $P_{ACT1}$ -pHluorin were used for the measurement of cytosolic pH (A and D), whereas log-phase cells without the plasmid were used for the measurement of vacuolar pH (B and E) and external pH (C and F). All experiments were carried out in triplicate. The error bars indicate the SD.

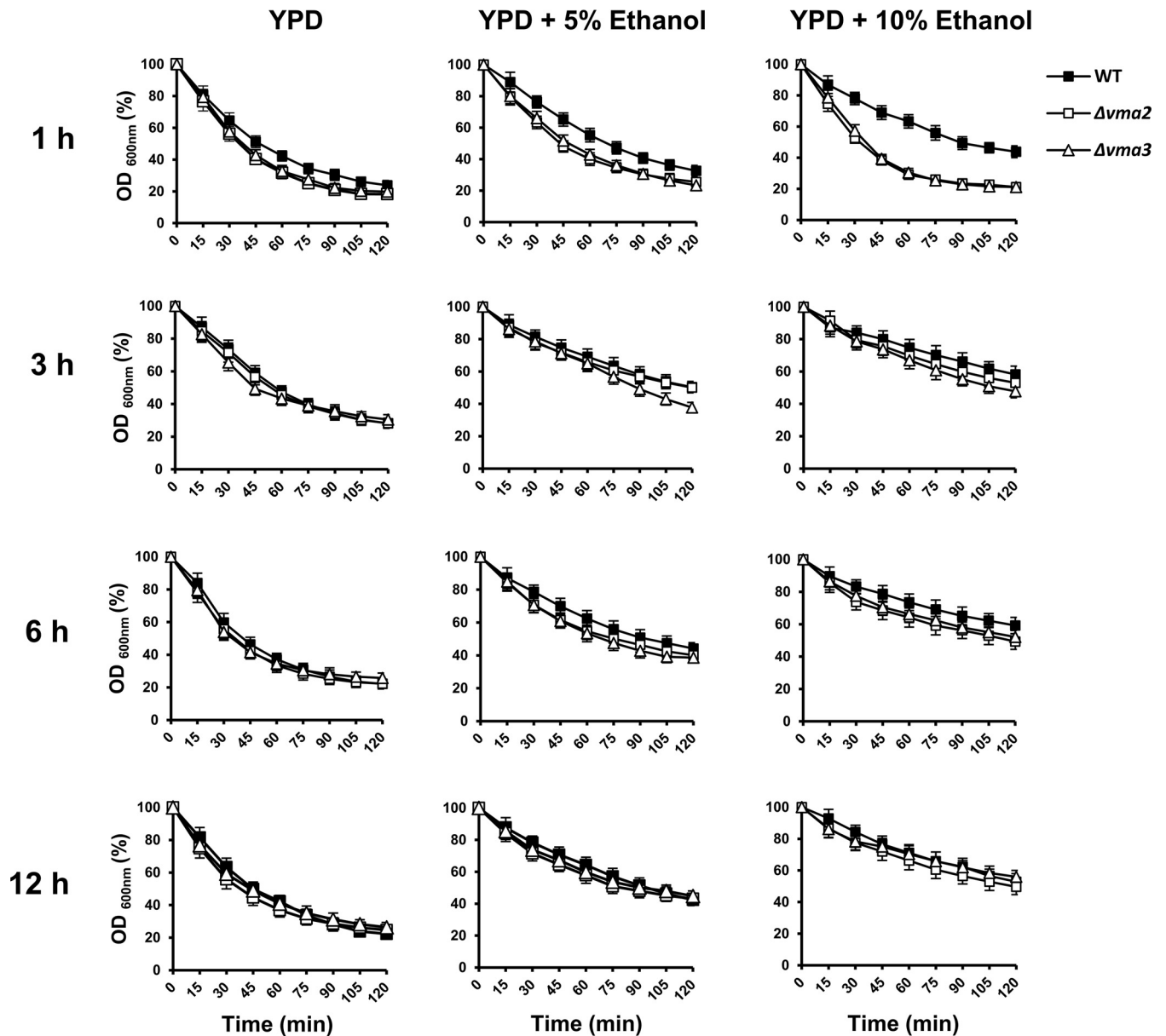


FIG 6 Role of V-ATPase in the early response to ethanol-induced cell wall stress. The exponentially growing wild-type (BY4742) (■),  $\Delta vma2$  (□), and  $\Delta vma3$  (△) cells were incubated in YPD medium lacking ethanol or in YPD medium containing 5% or 10% ethanol at 30°C for 1 and 3 h. Cells were harvested and adjusted to an  $OD_{600}$  of 0.5 in TE buffer containing 100  $\mu\text{g/ml}$  (1 U) Zymolyase 20T. Susceptibility to Zymolyase was monitored by measuring the  $OD_{600}$  at the indicated times and expressed as a percentage of  $OD_{600}$  relative to that at 0 min. All experiments were carried out in triplicate. The error bars indicate the SD.

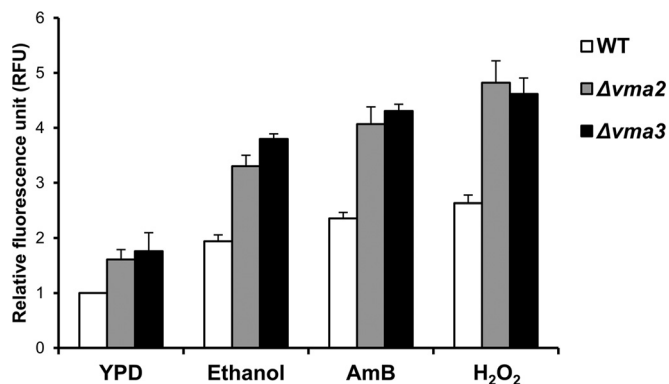
regulation of intracellular redox homeostasis, which is potentially disturbed by the membrane-permeabilizing effect of ethanol.

## DISCUSSION

In this study, a clear correlation between the lipophilicity of straight-chain alcohols and their effects on growth inhibition and membrane permeabilization was demonstrated. The straight-chain alcohols, such as ethanol, have been proposed to disturb the integrity of the cellular membrane through partitioning into a lipid bilayer with their aliphatic chains associated with the fatty acyl chains of phospholipids (39). It is therefore likely that the straight-chain alcohols with long aliphatic chains will be deeply

inserted into the hydrophobic interior of the membrane lipid bilayer, thereby causing a high degree of membrane permeabilization. Since in our experiments the pH of fresh YPD medium (pH = 5.8) was more acidic than the cytosolic pH of the precultivated wild-type cells (pH = 6.2), it is plausible that acidification of the cytosol and vacuole after alcohol challenge was caused by the passive influx of protons into alcohol-permeabilized cells. In agreement with the effect of straight-chain alcohols on membrane permeabilization, the degrees of cytosolic and vacuolar acidification were strictly dependent on both the concentration and lipophilicity of straight-chain alcohols. Our findings are consistent with the previous work of Leão and Van Uden (6), who showed that upon exposure to alkanols, i.e., eth-





**FIG 7** Loss of V-ATPase activity promotes ROS accumulation. Exponentially growing wild-type (BY4742) (white bars),  $\Delta vma2$  (gray bars), and  $\Delta vma3$  (black bars) cells loaded with DCFH-DA were incubated in YPD medium containing 10% ethanol, 1  $\mu$ M AmB, or 2 mM H<sub>2</sub>O<sub>2</sub> at 30°C for 1 h. The fluorescence intensity of each sample was normalized to that of the untreated wild-type cells and expressed as relative fluorescence units (RFU). All experiments were carried out in triplicate. The error bars indicate the SD.

anol, isopropanol, propanol, and butanol, the extracellular pH increased in a concentration- and lipophilicity-dependent manner, possibly due to increased proton influx.

Since the V-ATPase is involved in the maintenance of intracellular pH homeostasis through its role in pumping excessive protons into the vacuole (11), it has been thought that almost all phenotypes of the *vma* mutants lacking V-ATPase activity are caused by a highly acidified cytosol, which would inhibit cell function. Contrary to this common notion, our results revealed that the alcohol-sensitive phenotype of the *vma* mutants was not caused by severe cytosolic acidification but rather by elevated levels of endogenous oxidative stress. The generation of ROS by the mitochondrial electron transport chain is frequently the major cause of endogenous oxidative stress in eukaryotic cells (40). However, this does not account for ROS accumulation in the  $\Delta vma2$  mutant, because a loss of mitochondrial DNA did not improve the growth of this mutant or its resistance to the oxidative stress-inducing agent H<sub>2</sub>O<sub>2</sub> (20). The mechanism of oxidative stress induction in the mutant lacking V-ATPase activity is still unclear. Recently, Diab and Kane (41) showed that the  $\Delta vma2$  mutant accumulated a higher level of intracellular iron than the wild-type strain, in spite of the upregulation of the iron regulon, which is induced by the activated Aft1p and Aft2p transcription factors in response to iron deprivation (42). Excessive iron has a high potential to induce oxidative stress through its promotion of a Fenton reaction, which converts H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub> into toxic radicals, i.e., hydroxyl radicals and superoxide anions, respectively (43). It is therefore possible that the increased intracellular iron levels may be one of the causes of oxidative stress in the mutant lacking V-ATPase activity. Although the *vma* mutants did not display acute severe cytosolic acidification in response to straight-chain alcohols, the possibility that the increased oxidative stress in these mutants was a consequence of chronic cytosolic acidification could not be excluded. It is well known that the structures of cellular macromolecules, such as protein and DNA, are disturbed under acidic pH conditions, leading to dysfunctional organelles and molecules, especially those required for important cellular functions, including the oxidative defense system (44). Hence, the chronic oxidative stress in the mutant lacking V-ATPase activity

may be induced by the enhancement of cellular damage and/or reduction of cellular antioxidant activity resulting from cytosolic acidification. If this is the case, the *vma* mutants associated with cellular dysfunction may be unable to promptly respond to alcohol-induced oxidative stress, leading to high-level accumulation of intracellular ROS and eventually growth inhibition.

It is known that in response to cell wall stress, the cell surface sensors, i.e., Wsc1-3p, Mid2p, and Mtl1p, transmit stress signals via Rho1p GTPase to the cell wall integrity signaling pathway, resulting in the increased expression of several cell wall biosynthesis genes, such as *FKS2* and *CRH1*, which encode the catalytic subunits of 1,3- $\beta$ -glucan synthase and chitin transglycosylase, respectively. The structural components and networks of the yeast cell wall are thereby remodeled in order to maintain cell wall integrity and function (45). Endocytosis and recycling of cell wall sensors from endosomes back to the plasma membrane have been shown to play an important role in regulation of the cell wall integrity pathway (46–48). Similar to the mislocalization of plasma membrane H<sup>+</sup>-ATPase Pma1p in the *vma* mutants (16), the deletion of *VMA* genes has recently been reported to result in mislocalization of Wsc1p from the plasma membrane to the vacuole (49), suggesting a role for V-ATPase in the maintenance of yeast cell wall integrity through the endocytic recycling of Wsc1p. It is therefore possible that the defect in cell wall remodeling upon ethanol exposure in the *vma* mutants may be a consequence of the mislocalization of cell wall stress sensor(s), at least Wsc1p, which potentially leads to a reduced sensing efficiency for ethanol-induced cell wall stress. Since our results revealed that the *vma* mutants displayed only a delay, and not a complete loss, of cell wall remodeling in response to ethanol, it is likely that the cell wall stress sensors remaining at the cell surface (if any) retain the ability to sense cell wall stress, although longer detection times seem to be required.

In conclusion, although straight-chain alcohols induced cytosolic and vacuolar acidification through their effects on increasing membrane permeability, the ethanol sensitivity of mutants lacking V-ATPase activity, which is generally involved in the maintenance of intracellular pH homeostasis, seemed to result from increased endogenous oxidative stress and a defect in cell wall remodeling.

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