รายงานวิจัยฉบับสมบูรณ์

โครงการ

ผลกระทบของโลหะหนักชนิดต่าง ๆ ต่อการเปลี่ยนแปลงค่า pH ภายในเซลล์และระดับความเครียดออกซิเดทีฟในยีสต์ *Saccharomyces cerevisiae*

โดย ดร. ชูวงศ์ เอื้อสุขอารี

มิถุนายน 2555

สัญญาเลขที่ MRG5380030

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

รหัสโครงการ : MRG5380030

ชื่อโครงการ : ผลกระทบของโลหะหนักชนิดต่าง ๆ ต่อการเปลี่ยนแปลงค่า pH ภายในเซลล์และระดับความเครียดออกซิเดทีฟในยีสต์ *Saccharomyces cerevisiae*

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ระยะเวลาโครงการ : 2 ปี (มิ.ย. 2553 - มิ.ย. 2555)

แม้ว่าที่ผ่านมามีการศึกษาความเป็นพิษของโลหะหนักต่อเซลล์ประเภทยูแคริโอตอย่างกว้างขวาง แต่กลไกการตอบสนองต่อโลหะหนักในระดับเซลล์ยังไม่เป็นที่ชัดเจน จากการศึกษาก่อนหน้านี้พบว่า H^+-ATPase ที่แวคิวโอล (V-ATPase) ซึ่งทำหน้าที่หลักในการรักษาสมดุลของ pH ภายในเซลล์มีบทบาทในการทนทานต่อแคดเมียมของเซลล์ด้วย ดังนั้นจึงเป็นไปได้ว่าโลหะหนัก เช่น แคดเมียม อาจก่อให้เกิดความเป็นกรดภายในเซลล์ และเซลล์จำเป็นต้องใช้ V-ATPase ในการปรับสมดุลของ pH ภายในเซลล์ จากผลการศึกษาของผู้วิจัยพบว่ายีสต์สายพันธุ์กลายที่ขาด V-ATPase (สายพันธุ์กลาย *vma*) นอกจากจะไวต่อแคดเมียมแล้วยังไวต่อโคบอลต์ นิกเกิล และสังกะสี ในขณะที่สายพันธุ์กลาย Δ*pma2* ที่ขาดเอนไซม์ H^+-ATPase ที่เยื่อหุ้มเซลล์ (P-ATPase) ไม่แสดงความไวต่อโลหะหนักเหล่านี้ จากการศึกษาบทบาทของ V-ATPase และ P-ATPase ในการแพร่ของสารผ่านเยื่อหุ้มเซลล์และการรักษาสมดุลของ pH พบว่าโลหะหนักส่งผลให้อัตราการแพร่ของสารผ่านเยื่อหุ้มเซลล์เพิ่มมากขึ้นและก่อให้เกิดการเปลี่ยนแปลงของค่า pH ทั้งภายในและภายนอกเซลล์ในสายพันธุ์ธรรมชาติ อย่างไรก็ตามแม้ว่าสายพันธุ์กลาย *vma* จะมีอัตราการแพร่ของสารผ่านเยื่อหุ้มเซลล์คล้ายกับสายพันธุ์ธรรมชาติ แต่ค่า pH ที่แวคิวโอลและไซโตพลาสซึมของสายพันธุ์กลาย *vma* กลับสูงกว่าสายพันธุ์ธรรมชาติทั้งในสภาวะที่มีและไม่มีโลหะหนัก ผลการทดลองนี้แสดงให้เห็นว่าค่า pH ที่ไซโตพลาสซึมไม่ใช่ปัจจัยที่ทำให้สายพันธุ์กลาย *vma* ไวต่อโลหะหนัก เนื่องจากที่ผ่านมามีรายงานว่าการทำงานของ V-ATPase จำเป็นต่อการป้องกันการเกิดความเครียดออกซิเดชั่นด้วย ดังนั้นผู้วิจัยจึงได้ศึกษาบทบาทของ V-ATPase ในการปกป้องเซลล์จากความเครียดออกซิเดชั่นที่เกิดจากโลหะหนัก พบว่าความไวต่อโลหะหนักและ H_2O_2ของสายพันธุ์กลาย *vma* ถูกยับยั้งภายใต้สภาวะที่ไม่มีออกซิเจน และระดับ ROS ของสายพันธุ์กลาย *vma* ในสภาวะปกติมีค่าสูงกว่าสายพันธุ์ธรรมชาติ แม้ว่าระดับ ROS ของสายพันธุ์กลาย *vma* มีค่าสูงขึ้นเมื่อสัมผัสกับโคบอลต์ นิกเกิลและสังกะสี แต่ปริมาณการสะสมก็มีค่าใกล้เคียงกับสายพันธุ์ธรรมชาติ ดังนั้นผลการศึกษานี้จึงบ่งชี้ให้เห็นว่าบทบาทของ V-ATPase ในการทนทานต่อโลหะหนักอาจไม่ได้มีเพียงการปกป้องเซลล์จากความเครียดออกซิเดชันที่เกิดจากโลหะหนักเพียงเท่านั้น

คำหลัก : V-ATPase, โลหะหนัก, ค่า pH ภายในเซลล์, ความเครียดออกซิเดทีฟ, *Saccharomyces cerevisiae*

Abstract

Project Code : MRG5380030

Project Title : Effects of heavy metals on intracellular pH alteration and oxidative stress level in *Saccharomyces cerevisiae*

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Project Period : 2 years (June 2010 – June 2012)

Although the toxicity of heavy metals to eukaryotic cells has been extensively studied, the mechanisms of cellular response to heavy metals have not been clearly understood. In a previous study, it was found that vacuolar H^+-ATPase (V-ATPase) is required for Cd tolerance. Since V-ATPase is known to play an important role in maintaining intracellular pH homeostasis through vacuolar acidification, it may be possible that some metals, such as Cd, induce intracellular acidification, thereby requiring V-ATPase to cope. It was found that the *vma* mutants lacking subunits of V-ATPase, but not the *Δpma2* mutant lacking minor plasma membrane H^+-ATPase (P-ATPase), were sensitive to not only Cd but also Co, Ni, and Zn. To investigate the role of V-ATPase and P-ATPase in heavy metal tolerance, the effects of heavy metals on membrane permeability and pH homeostasis were examined. The results showed that heavy metals induced an increase in membrane permeability and alteration of both intracellular and extracellular pHs in the wild-type strain. Although the *vma* mutants exhibited similar membrane permeability to that of the wild-type strain, the vacuolar pHs and cytosolic pHs of the *vma* mutants were more alkaline than those of the wild-type strain; in both the presence, or absence, of heavy metals. These results suggest that the cytosolic pH does not account for metal sensitivity in the *vma* mutants. Since V-ATPase has been shown to be required for preventing oxidative stress, the additional role of V-ATPase in protecting cells from oxidative stress induced by heavy metal was examined. It was found that the sensitivity to all metals and the H_2O_2 of the *vma* mutants was suppressed under anaerobic conditions and the reactive oxygen species (ROS) levels of the *vma* mutants were higher than in the wild-type strain. Although, after exposure to Co, Ni, and Zn, the *vma* mutants exhibited increased ROS accumulation, the levels were similar to those of the wild-type strain. The results suggest that the roles of V-ATPase in metal tolerance may do more than protect cells from oxidative stress caused by metals.

Keywords : V-ATPase, heavy metal, intracellular pH, oxidative stress, *Saccharomyces cerevisiae*

EXECUTIVE SUMMARY

In this study, we determined the growth of the *S. cerevisiae* wild-type strain, the *Δvma2* and

Δvma3 mutants lacking V_0 and V_1 subunit of the vacuolar H^+-ATPase, respectively, and the *Δpma2* mutant lacking the minor plasma membrane H^+-ATPases in the presence of four kinds of heavy metals, i.e. $CdCl_2$, $CoCl_2$, $NiCl_2$, and $ZnCl_2$. We found that the *Δvma2* and *Δvma3* mutants, but not the *Δpma2* mutant, were sensitive to all heavy metals tested, indicating that the vacuolar H^+-ATPase is involved in tolerance to these heavy metals. In order to address the role of V-ATPase in heavy metal tolerance, the impacts of heavy metals on membrane permeability, pH homeostasis, and ROS induction were tested. We found that the membrane permeability of the *Δvma2* and *Δvma3* mutants after exposure to Cd, Co, and Ni, except for Zn, were similar to those of the wild-type strain. These results suggested that the sensitivity to heavy metals of the *vma* mutants was not resulted from the effect on membrane permeability. However, our results revealed that heavy metals induced alteration of both intracellular and extracellular pHs. The vacuolar pHs of the *vma* mutants were more alkaline than that of the wild-type strain under both normal and heavy metal stress conditions. These results indicated that a loss of V-ATPase activity to translocate protons into vacuole causes an alkaline vacuole. Unexpectedly, the cytosolic pHs of the *vma* mutants were more alkaline than those of the wild-type strain, suggesting the important role of other H^+-ATPase such as Pma1p in maintenance of intracellular pH in the yeast strain lacking V-ATPase activity and the cytosolic pH does not account for the sensitivity to heavy metals in the *vma* mutants. Since heavy metal has been reported to induce the chronic oxidative stress in the yeast cell, the accumulation of endogenous oxidative stress might be the primary cause of heavy metal toxicities. We found that the sensitivity to heavy metal stress of the *vma* mutants was suppressed under anaerobic condition, in which ROS production is unable to be activated. However, ROS accumulation in the *vma* mutants under Cd exposure was higher than the wild-type and *Δpma2* strains. This suggests that V-ATPase may contribute to ROS defense during heavy metal stress, leading to reduced adverse physiological effects caused by Cd, but not Co, Ni and Zn, in the wild-type cells. Conclusively, this study showed the evidences for the effects of heavy metal on membrane permeability, intracellular pH homeostasis, and ROS generation in yeast cells. Variations and inconsistencies among each heavy metal treatment and yeast strains may be due to the fact that the effects of heavy metals on eukaryotic cells are complicated. Therefore, the undefined roles of

V-ATPase in tolerance to heavy metal should be further investigated, especially the roles in ion homeostasis and ROS generation.

INTRODUCTION

All living organisms are often exposed to heavy metals since the origin of life. Many heavy metals are non-essential, while some serve as essential elements; nevertheless, they are toxic in excessive quantities. Deposition of heavy metals in the environment has dramatically increased during the last century as a result of many massive industrial activities (Waisberg *et al.*, 2003). The contamination of heavy metals widely used in the industries such as zinc, nickel, cobalt, and cadmium poses considerable threats to the environment and human health.

Nickel plays a role as cofactor in ureases in microorganisms and plants, whereas cobalt is essential component of cobalamin (vitamin B12). However, excessive cellular accumulation of these metals is toxic and causes mutagenic and carcinogenic effects to living organisms (Dunnick *et al.*, 1995; Salnikow & Kasprzak, 2005). On the other hand, some metals such as cadmium are not essential for cellular metabolism and very toxic even at low concentrations. Cadmium is known to cause DNA damage and oxidative stress to cells and Itai-itai disease and renal abnormalities in human (Nogawa *et al.*, 2004).

In eukaryotes, many complex survival mechanisms are used to avoid the toxicity of heavy metals. These include the efflux pump to transport the excessive heavy metal out of the cells, and chelating agents such as glutathione and metallothioneins to chelate and sequester metal ions (Tamás & Martinoia, 2006). In addition, vacuole plays an important role in metal detoxification as reservoir of neutralized heavy metals (Ramsay & Gadd, 1997). Despite the extensive studies of heavy metal detoxification, the role of vacuolar function in metal detoxification is not fully elucidated.

Previously, it has been shown that, in *Saccharomyces cerevisiae*, vacuolar H^+-ATPase functioning in maintaining of intracellular pH homeostasis is required for cadmium tolerance (our unpublished data). V-ATPase is a highly conserved enzyme whose major function is to translocate the excessive proton from cytoplasm into vacuole by using the energy of ATP hydrolysis (Kane, 2006). Based on this idea, it may be possible that heavy metals such as cadmium cause a decrease in

cytosolic pH and V-ATPase may be required for recovery from cytosolic acidification. In addition, the defect in of V-ATPase function results in a diverse range of un expected consequences such as sensitivity to elevated calcium concentration (Nelson & Nelson, 1990), and inability to grow on medium containing non-fermentable carbon source (Supek *et al.*, 1994). Furthermore, it has been shown recently that a loss of V-ATPase activity leads to an increase of endogenous oxidative stress (Milgrom *et al.*, 2007) and metals is known to induce intracellular oxidative stress through increasing the generation of reactive oxygen species (Lopez *et al.*, 2006; Valko *et al.*, 2005). Therefore, it is possible that V-ATPase may also play a role in protecting cells against oxidative stress caused by metals.

In this study, we investigated the role of V-ATPase in regulating intracellular pH homeostasis and inhibiting endogenous oxidative stress during under metal stresses in *S. cerevisiae*.

MATERIALS AND METHODS

Strains and media

The yeast *Saccharomyces cerevisiae* strains used in this study were the wild-type BY4742 strain (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and its isogenic strains the *Δvma2::kanMX*, *Δvma3::kanMX*, *Δpma2::kanMX* mutant and *Δsod1::kanMX* mutant. Culture media used in this study was YPD media composed of 1% yeast extract (Bio Basic Inc), 2% peptone (Bio Basic Inc), and 2% glucose (Bio Basic Inc).

Growth assay

The yeast wild-type (BY4742) strain and the *Δvma2*, *Δvma3*, and *Δpma2* mutants were inoculated into YPD broth media and incubated with shaking at 200 rpm at 30 ºC for 12 hours. The overnight cultures were then diluted to an OD_{600} = 1.0 and serially diluted 10-fold to 10^{-5}. 3 µl of aliquots were spotted onto YPD agar and YPD agar containing 10 µM, 30 µM, and 50 µM $CdCl_2$; 1 mM, 2mM, and 3 mM $CoCl_2$ and $NiCl_2$; 1 mM, 3 mM, and 5 mM $ZnCl_2$. The pH values of the media were adjusted to 6.0 before use. The YPD agar plates were incubated at 30 ºC for 3 days. For the growth assay under anaerobic condition, the YPD agar plates were incubated in CO_2-enriched, oxygen-depleted culture jars at 30 ºC for 5 days.

Measurement of cytosolic pH

The overnight pre-cultivated yeast wild-type (BY4742) in YPD media was transferred into new YPD broth media at an OD_{600} of 0.1, then incubated with shaking at 200 rpm 30 ºC for 12 hours. The cells were harvested by centrifugation at 4,000 rpm for 10 minutes. The cell pellet was washed twice with 4 ºC sterile deionized water and was resuspended in 4 ºC 67 mM phosphate citrate buffer titrated to different pH values within a range of 4.0-8.0. The cell density was measured by

spectrophotometer at the wavelength of 600 nm. An OD_{600} of approximately 0.8 of the yeast culture was incubated in the presence of 10 µM of 5(6)-carboxyfluorescein diacetate and 5 µM amphotericin B at 35 ºC for 60 minutes. Fluorescence intensities at excitation wavelength of 490 and 435 nm were measured at a constant emission wavelength of 520 nm by a spectrofluorometer (Jasco FP-6200). The calibration curve was constructed by plotting ratio of fluorescence intensities at excitation wavelengths of 490 and 435 nm, and was used for the estimation of cytosolic pH (Bracey *et al.*, 1998; Liu *et al.*, 2003).

To determine cytosolic pH of the yeast wild-type cells in response to heavy metals and oxidative stresses, the wild-type (BY 4742) strain precultivated overnight in YPD medium was transferred into new YPD broth and YPD broth containing 10 µM, 30 µM, and 50 µM $CdCl_2$; 1 mM, 2mM, and 3 mM $CoCl_2$ and $NiCl_2$; 1 mM, 3 mM, and 5 mM $ZnCl_2$. The pH values of the media were adjusted to 6.0 before use. The cultures were incubated with shaking at 200 rpm 30 ºC for 12 hours. The cell pellet was washed twice with 4 ºC sterile deionized water and was resuspended in 4 ºC 67 mM phosphate citrate buffer of pH 4. The cell density was measured by spectrophotometer at the wavelength of 600 nm. An OD_{600} of approximately 0.8 of the yeast culture was incubated in the presence of 10 µM of 5(6)-carboxyfluorescein diacetate at 35 ºC for 60 minutes. Fluorescence intensities at excitation wavelengths of 490 and 435 nm were measured at a constant emission wavelength of 520 nm by a spectrofluorometer (Jasco FP-6200). The cytosolics were estimated from calibration curve. To determine cytosolic pH of the yeast mutants in response to heavy metals and oxidative stresses, the *Δvma2*, *Δvma3*, and *Δpma2* mutants precultivated overnight in YPD media was inoculated into new YPD broth and YPD broth media containing 30 µM $CdCl_2$, 2mM $CoCl_2$ and $NiCl_2$, 3 mM $ZnCl_2$. The pH values of the media were adjusted to 6.0 before use. The cultures were incubated with shaking at 200 rpm 30 ºC for 12 hours. The cytosolic pH was measured as mentioned above.

Measurement of vacuolar pH

The wild-type (BY 4742) strain precultivated overnight in YPD media was washed three times with sterile deionized water prior to inoculation into new YPD broth media at an OD_{600} of 0.1 and incubated with shaking at 200 rpm 30 ºC for 12 hours. The cells were then harvested by centrifugation at 4,000 rpm for 10 minutes. The 4×10^7 yeast cells were resuspended in YPD media containing 50 mM MES, 50 mM HEPES, 50 mM KCl, 50 mM NaCl, 0.2 mM ammonium acetate, 10 mM NaN_3, 10 mM 2-deoxyglucose, 50 mM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), titrated to 9 different pH values ranging from 4.0 – 8.0. Cells were then incubated in 50 µM BCECF-acetoxymethyl ester at 30 ºC for 20-30 minutes and immediately used for measurement of fluorescence intensity by automated microplate reader (Spectramax M3 Molecular device). The fluorescence intensities at excitation wavelengths of 450 and 490 nm was measured at a constant emission wavelength of 535 nm by automated microplate reader (Spectramax M3 Molecular device). The calibration curve was constructed by plotting ratio of fluorescence intensities at excitation wavelengths of 450 and 490 nm, and was used for the estimation of vacuolar pH (Martinez-Munoz & Kane, 2008; Padilla-López & Pearce, 2006; Plant *et al.*, 1999).

To determine vacuolar pH of yeast cells in response to heavy metals and oxidative stresses, the wild-type (BY4742) strain were precultivated overnight in YPD media were washed three times with sterile distilled deionized, and then were inoculated into new YPD broth media and YPD broth media containing 10 µM, 30 µM, and 50 µM $CdCl_2$; 1 mM, 2 mM, and 3 mM $CoCl_2$ and $NiCl_2$; 1 mM, 3 mM, and 5 mM $ZnCl_2$. The pH values of the media were adjusted to 6.0. Then the cultures were incubated with shaking at 200 rpm 30 ºC for 12 hours. The cells were harvested by centrifugation at 4,000 rpm for 10 minutes. The 4×10^7 cells were resuspended in the same media prior to incubation in 50 µM BCECF-acetoxymethyl ester at 30 ºC for 20-30 minutes and immediately used for measurement of fluorescence intensity by automated microplate reader (Spectramax M3 Molecular device). The fluorescence intensities at excitation wavelengths of 450 and 490 nm were measured at a constant emission wavelength of 535 nm by automated microplate reader. The vacuolar pHs were estimated from calibration curve. To determine vacuolar pH of the yeast mutants in response to

heavy metals and oxidative stresses, the *Δvma2*, *Δvma3*, and the *Δpma2* mutants were precultivated overnight in YPD media were washed three times with sterile deionized water before inoculated into new YPD broth media and YPD broth media containing 30 µM $CdCl_2$, 2 mM $CoCl_2$ and $NiCl_2$, and 3 mM $ZnCl_2$. The pH values of the media were adjusted to 6.0 before use. The vacuolar pH was measured as mentioned above.

Measurement of Media pH

To determine media pH of yeast cells in response to heavy metals and oxidative stresses, the wild-type (BY4742) strain precultivated overnight in YPD medium was transferred into new YPD broth and YPD broth containing 10 µM, 30 µM, and 50 µM $CdCl_2$; 1 mM, 2 mM, and 3 mM $CoCl_2$ and $NiCl_2$; 1 mM, 3 mM, and 5 mM $ZnCl_2$. The pH values of the media were adjusted to 6.0 before use. Then the cultures were incubated with shaking at 200 rpm 30 ºC for 12 hours. The cells were harvested by centrifugation at 4,000 rpm for 10 minutes. The supernatant was transferred to the new sterile tube and measured the pH by pH meter (Denver Model 215). In the case of the mutant strains, the *Δvma2*, *Δvma3,* and the *Δpma2* mutants were determined the media pH after exposure to 30 µM $CdCl_2$, 2 mM $CoCl_2$ and $NiCl_2$, 3 mM and $ZnCl_2$ for 12 hours as the method mentioned above.

Determination of membrane permeability

To determine the membrane permeability, after exposure to heavy metals and H_2O_2, the wild-type (BY 4742) strain was precultivated overnight in YPD media was inoculated into new YPD broth and YPD broth containing 30 µM $CdCl_2$, 2 mM $CoCl_2$ and $NiCl_2$, 3 mM $ZnCl_2$, and 1 mM H_2O_2 and incubated with shaking at 200 rpm 30 ºC for 12 hours. The pH values of the media were adjusted to 6.0 before use. The cells were then washed twice with 50 mM MES-NaOH buffer pH of 5.5, and inoculated in the same buffer to give an OD_{600} of 1. The 90 µl of cell suspension was transferred into 96-well plates, and 10 µl of SYTOX green was added to the final concentration of 1 µM. The SYTOX green uptake was accessed by the measurement of fluorescence intensity at

excitation wavelength of 544 nm, and emission wavelength of 488 nm by automated microplate reader (Spectramax M3 Molecular device) (Zakrzewska *et al.*, 2007). In the case of the mutant strains, the *Δvma2*, *Δvma3*, and the *Δpma2* mutants were determined the fluorescence intensities value after exposure to 30 µM $CdCl_2$, 2 mM $CoCl_2$ and $NiCl_2$, 3 mM $ZnCl_2$, and 1.0 mM H_2O_2 for 12 hours as the method mentioned above.

Determination of reactive oxygen species (ROS) production

To determine ROS level of yeast cells in response to heavy metals and oxidative stresses, the yeast wild-type (BY4742) strain and the *Δvma2*, *Δvma3*, and *Δpma2* mutant strains were precultivated overnight in YPD media was inoculated into new YPD broth and YPD broth containing 30 µM $CdCl_2$, 2 mM $CoCl_2$ and $NiCl_2$, 3 mM $ZnCl_2$, and 1.0 mM H_2O_2 and incubated with shaking at 200 rpm 30 ºC for 12 hours. Then yeast cells were harvested, resuspended in 5 mL 100 mM potassium phosphate buffer (pH 7.4) containing 10 µM DCFDA, and incubated at 30°C for 30 minutes. Cell were harvested, washed in distilled water and after resuspended in 700 µL H_2O, yeast cells were disrupted with glass beads. To collect the supernatant, samples were centrifuge at 5,000 rpm for 5 minutes. Fluorescence intensities at excitation wavelengths of 490 nm were measured at a constant emission wavelength of 524 nm by automated microplate reader (Spectramax M3 Molecular device). The value of fluorescence intensities were normalized as to the protein in the mixture.

RESULTS

Effect of heavy metals on growth of yeast cells

To determine the role of vacuolar H^+-ATPase and plasma membrane H^+-ATPase in tolerance to heavy metals, the growth of the *Δvma2* and *Δvma3* mutants lacking V_0 and V_1 subunit of the vacuolar H^+-ATPase, respectively, and the *Δpma2* mutant lacking the minor plasma membrane H^+-ATPases, was examined in YPD media containing 10, 30, and 50 μM $CdCl_2$; 1, 2, and 3 mM $CoCl_2$ and $NiCl_2$; and 1, 3, and 5 mM $ZnCl_2$. Our results revealed that the *Δvma2* and *Δvma3* mutants, but not the *Δpma2* mutant, were sensitive to all heavy metals tested (Fig. 1). These results suggested that both V_0 and V_1 subunits of V-ATPase contribute to tolerance to heavy metal stresses.

Effect of heavy metals on yeast membrane permeability

The heavy metal ions containing positive charges are supposed to interact with the negative charges of the cell components, including the negatively charged phosphate group of phospholipid in cellular membrane (Valko *et al.*, 2005). It is therefore possible that the plasma membrane may be the target site of heavy metals and its permeability is disturbed by heavy metals. To test this hypothesis, membrane permeability of the yeast cells grown in the presence of heavy metals was determined by using SYTOX green uptake assay. SYTOX green is a high affinity nucleic acid staining dye that can easily penetrate the plasma membrane only when the membrane integrity is compromised. In this study, the yeast cells treated with amphotericin B, a polyene antifungal drug that forms self-assembly transmembrane pore with ergosterol (Matsuoka & Murata, 2002), were used as control. The relative fluorescence unit (RFU) of the wild-type strain grown in YPD media containing 0.5 μM amphotericin B was increased to 5.2 after 12-hour exposure (Fig 2). Similarly, the RFUs of the wild-type strain were significantly increased to 4.1, 5.7, and 4.0 after exposure to 30 μM $CdCl_2$, 2 mM $CoCl_2$, and 2 mM $NiCl_2$, respectively, and relatively increased to 1.7 after exposure to 3 mM $ZnCl_2$ (Fig 2). These results indicated that heavy metals increased membrane permeability, which

may lead to a proton influx. Similar to those of the wild-type strain, the RFUs of the *Δpma2* mutant lacking the minor plasma membrane were significantly increased from 1.8 to 5.2, 4.8, 3.3, and 3.6, after exposure to amphotericin B, Cd, Co, and Ni, respectively, and slightly increased to 2.1 after exposure to Zn (Fig 2). In case of the *vma* mutants, the RFU values of the *Δvma2* and the *Δvma3* mutants exposed to amphotericin B were significantly increased after 12-hour exposure (5.1, and 6.0, respectively). The RFU values of the *Δvma2* and the *Δvma3* mutants were also drastically increased after exposure to 30 μM $CdCl_2$ (4.7, and 5.9, respectively), 2 mM $CoCl_2$ (4.7, and 3.1, respectively), 2 mM $NiCl_2$ (5.2, and 3.0, respectively), and 3 mM $ZnCl_2$ (3.0, and 5.0, respectively) (Fig 2). These result revealed that the membrane permeabilities of the *Δvma2* and the *Δvma3* mutants after exposure to Cd, Co, and Ni, except for Zn, was similar to those of the wild-type strain. Based on these observations, it is likely that the sensitivity to heavy metals of the *vma* mutants is not resulted from a defect in membrane permeability.

Effect of heavy metal on intracellular and extracellular pH alteration in the wild-type strain

The loss of membrane integrity is known to lead to increased passive influx of protons across the plasma membrane (Sikkema *et al.*, 1995), which may cause a decrease of cytosolic pH. To investigate the effect of heavy metals on pH alteration, we examined the vacuolar pH, cytosolic pH, and media pH of the wild-type cells grown in YPD media containing of 10 μM, 30 μM, or 50 μM $CdCl_2$, 1 mM, 2 mM, or 3 mM $CoCl_2$ and $NiCl_2$, and 1 mM, 3 mM, or 5 mM $ZnCl_2$, after 12-hour exposure. We found that the cytosolic pHs were decreased with increasing concentrations of all heavy metals tested. The cytosolic pHs of the wild-type cells were dropped from the initial pH before a shift of 5.6 to 5.2, 5.2, 5.4, and 5.5, after exposure to 50 μM $CdCl_2$, 3 mM $CoCl_2$, 3 mM $NiCl_2$, and 5 mM $ZnCl_2$ for 12 hours, respectively (Fig. 3). On the other hand, the vacuolar pHs and media pHs were increased with increasing concentration of heavy metals. The vacuolar pHs were raised from the initial pH before a shift of 5.5 to 6.8, 6.5, 6.9, and 6.6, after exposure 50 μM $CdCl_2$, 3 mM $CoCl_2$, 3 mM $NiCl_2$, and 5 mM $ZnCl_2$ for 12 hours, respectively (Fig. 4). While the media pH of the yeast

cells were 5.6, 5.6, 5.5, and 5.4, after exposure 50 μM $CdCl_2$, 3 mM $CoCl_2$, 3 mM $NiCl_2$, and 5 mM $ZnCl_2$, respectively, which were more alkaline than that of cells grown in YPD medium (5.2) without metal supplementation (Fig. 5).

Intracellular pH of the *vma* mutants during heavy metal stresses

Since heavy metals induced increase of membrane permeability and alteration of both intracellular and extracellular pHs (Fig. 2 - 5). It is possible that the sensitivity to heavy metals of the *vma* mutants lacking V-ATPase activity is a consequence of a defect in maintaining intracellular pH homeostasis. To investigate the role of V-ATPase in maintaining intracellular pH during heavy metal stresses, we measured the cytosolic pHs, vacuolar pHs, and media pHs of the *Δvma2*, *Δvma3*, and *Δpma2* mutants after exposure to various concentraitons of heavy metals for 12 hours. We found that the vacuolar pHs of the wild-type and *Δpma2* strains grown in the absence of heavy metals were 5.4 and 5.3, respectively, whereas the vacuolar pHs of the *Δvma2* and *Δvma3* strains were 6.9 and 6.9, respectively (Fig. 6). These results indicated that a loss of V-ATPase activity to translocate protons into vacuole causes an alkaline vacuole. After exposure to Cd, Co, and Ni, the vacuolar pHs of the *Δpma2* strain were raised to approximately 6.7, similar to those of the wild-type strain (Fig. 6). In contrast, the vacuolar pHs of the *Δvma2* and *Δvma3* strains were not significantly changed after 12-hour exposure to Cd, and slightly increased after exposure to Co and Ni (Fig. 6). On the other hand, in response to Zn, the vacuolar pHs of the wild-type and *Δpma2* strains were not changed, whereas those of the *Δvma2* and *Δvma3* mutants were significantly dropped to approximately 6.0 (Fig. 6). Since the *vma* mutants lacking V-ATPase activity are unable to pump excessive protons into vacuole, we hypothesized that the cytosolic pHs of these mutant would be more acidic than that of the wild-type strain, causing a sensitivity to heavy metals. Surprisingly, our results revealed that, after a shift to fresh YPD media for 12 hours, the cytosolic pHs of the *Δvma2* and *Δvma3* strains (pH 5.9) were more alkaline than those of the wild-type and *Δpma2* strains containing functional V-ATPase (pH 5.6) (Fig. 7). Although the cytosolic pHs of all strains were dropped after exposure to

Cd, Co, and Ni, the cytosolic pHs of the *Δvma2* and *Δvma3* mutants were more alkaline than those of the wild-type and *Δpma2* strains (Fig. 7). On the other hand, Zn seemed not to have any effects on alteration of cytosolic pH (Fig. 7). Based on these results, we concluded that the cytosolic pH does not account for the sensitivity to heavy metals in the *vma* mutants. The changes of media pH of these strains were also examined. We found that the media pHs of all strains were slightly increased after exposure to Cd, Co, Ni, and Zn. Moreover, the media pHs of the *vma* mutants were higher than those of the wild-type and *Δpma2* strains at all conditions (Fig. 8).

The effect of heavy metals on induction of oxidative stress in the *vma* mutants

Heavy metal has been reported to induce oxidative stress in the yeast cell. Furthermore, it has been shown that a loss of vacuolar ATPase activity also causes a chronic oxidative stress (Milgrom *et al*., 2007). Therefore, the sensitivity to heavy metals of the *vma* mutant may be due to the increased endogenous oxidative stress caused by heavy metals. To test this hypothesis, we examined the growth of the wild-type, *Δvma2, Δvma3,* and *Δpma2* strains in the presence of heavy metals under aerobic and anaerobic conditions. The oxidative stress-sensitive *Δsod1* mutant lacking Cu, Zn-superoxide dismutase and oxidative stress-inducing agent H_2O_2 were used as control. We found that the wild-type and *Δpma2* strains were able to grow in the YPD media supplemented with 30 μM $CdCl_2$, 2 mM $CoCl_2$, 2 mM $NiCl_2$, and 3 mM $ZnCl_2$ under both aerobic and anaerobic conditions (Fig. 9). On the other hand, the sensitivity to Cd, Co, Ni, and H_2O_2, of the *vma* and *Δsod1* mutants under aerobic condition was suppressed under anaerobic condition (Fig. 9). These results suggested that the sensitivity to heavy metals of the *vma* mutants may be due to increased oxidative stress induced by heavy metals.

To further investigate the effect of heavy metals on induction of oxidative stress, we measured the level of reactive oxygen species (ROS) in the yeast cells after exposure to heavy metals by using oxidant-sensitive probe 2′, 7′-dichlorofluorescin diacetate (DCFDA). This probe is trapped inside the cells after cleavage of the diacetate by an intracellular esterase (Royall & Ischiropoulos, 1993), and

then oxidized by radical species (mainly H_2O_2) to be a more fluorescent compound (Tsuchiya *et al.*, 1994). To determine ROS level of yeast cells in response to heavy metal and oxidative stresses, the wild-type, *Δvma2*, *Δvma3*, and *Δpma2* strains were incubated with 30 µM $CdCl_2$, 2 mM $CoCl_2$, 2 mM $NiCl_2$, and 3 mM $ZnCl_2$, and 1 mM H_2O_2 for 12 hours prior to the measurement of ROS levels. We found that the relative fluorescence units (RFU) of ROS generation in the wild-type and *Δpma2* strains under physiological condition were similar, whereas those of the *Δvma2* and *Δvma3* mutants were approximately 2.5-fold higher than that of the wild-type strain (Fig. 10). Our results revealed that the ROS level of all strains seemed to be increased after exposure to Cd, Co, Ni, Zn, and H_2O_2. Furthermore, the ROS levels of the *vma* mutants were significantly higher than those of the wild-type strain only after exposure to Cd, and H_2O_2, whereas, after exposure to Co, Ni, and Zn, the ROS accumulations in the *vma* mutant were at high levels similar to those in the wild-type strains (Fig. 10).

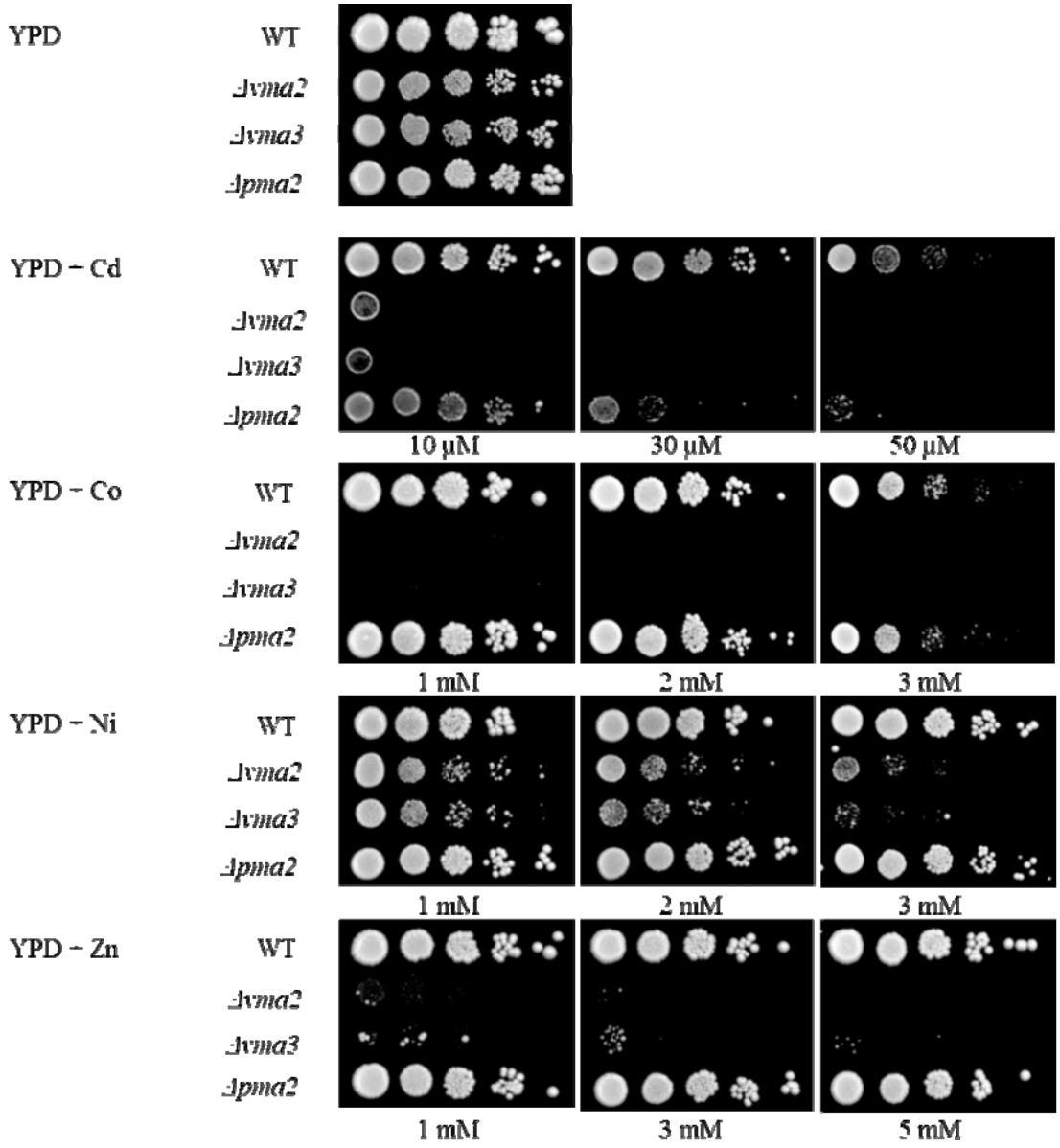

Figure 1. Growth of the wild-type, *Δvma2*, *Δvma3*, and *Δpma2* strains in YPD media containing various concentrations of heavy metals. Cells grown to log phase in YPD broth were serially diluted ten-fold from an initial OD_{600} of 0.1. Aliquots (3 µl) were spotted on the YPD agar plates and YPD agar plate containing 10, 30, and 50 µM $CdCl_2$; 1, 2, and 3 mM $CoCl_2$ and $NiCl_2$; 1, 3, and 5 mM $ZnCl_2$, and incubated at 30 °C for 3 days.

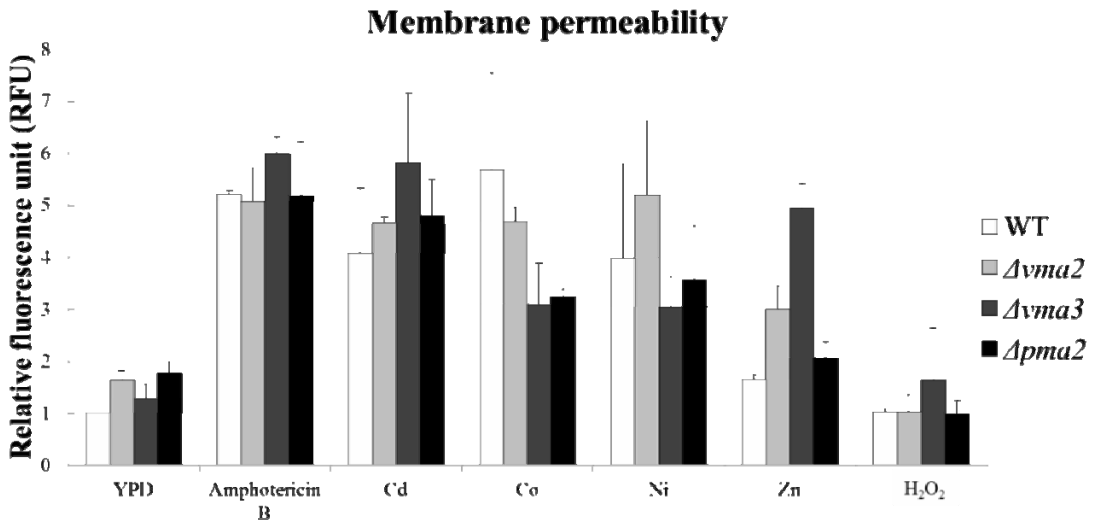

Figure 2. SYTOX green uptakes of the yeast strains grown in the presence of heavy metals and amphotericin B. The wild-type BY 4742 (□), *Δvma2* (▒), *Δvma3* (■), and *Δpma2* (■) strains were grown in the YPD medium and YPD media containing 0.5 μM amphotericin B, 30 μM $CdCl_2$, 2 mM $CoCl_2$, 2 mM $NiCl_2$, or 3 mM $ZnCl_2$ at 30 °C for 12 hours. The cells of an OD_{600} = 1 were loaded with SYTOX green, and immediately used for the measurement of the fluorescence intensity at the excitation wavelength of 544 nm and the emission wavelength of 488 nm. The relative fluorescent unit (RFU) of the treated cells was normalized by fluorescent intensity of the wild-type strain grown under physiological condition.

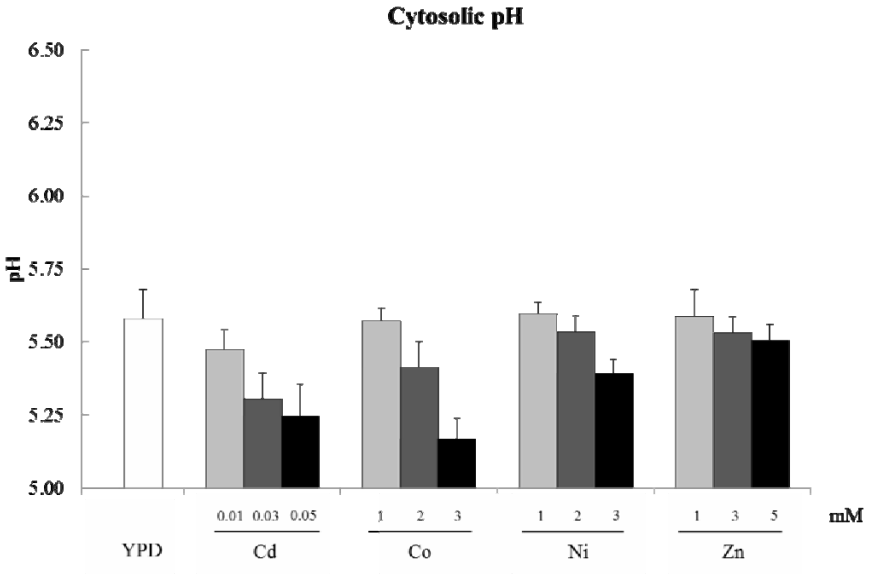

Figure 3. The cytosolic pH of the wild-type strain under heavy metals stress conditions. The wild-type (BY 4742) strain cultivated in YPD medium and YPD media containing 10, 30, and 50 µM $CdCl_2$; 1, 2, and 3 mM $CoCl_2$ and $NiCl_2$; 1, 3, and 5 mM $ZnCl_2$ for 12 hours, was used for the measurement of cytosolic pH.

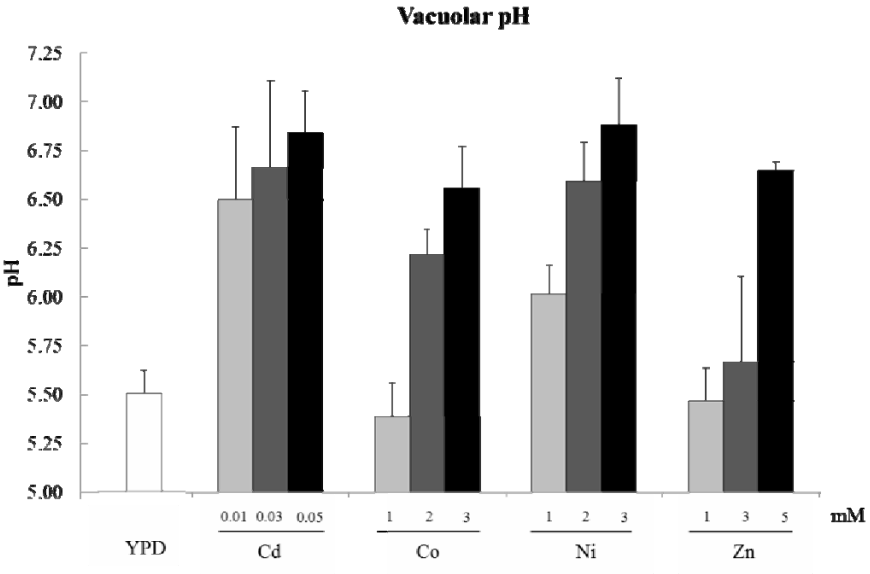

Figure 4. The vacuolar pH of the wild-type strain under heavy metals stress conditions. The wild-type (BY 4742) strain cultivated in YPD medium and YPD media containing 10, 30, and 50 µM $CdCl_2$; 1, 2, and 3 mM $CoCl_2$ and $NiCl_2$; 1, 3, and 5 mM $ZnCl_2$ for 12 hours, was used for the

measurement of vacuolar pH.

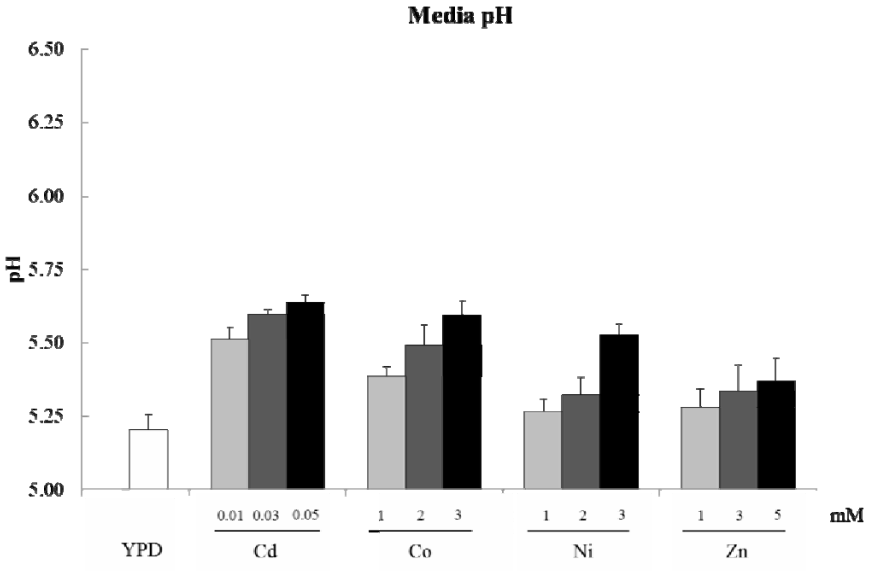

Figure 5. The media pH of the wild-type strain under heavy metals stress conditions. The wild-type (BY 4742) strain cultivated in YPD medium and YPD media containing 10, 30, and 50 μM $CdCl_2$; 1, 2, and 3 mM $CoCl_2$ and $NiCl_2$; 1, 3, and 5 mM $ZnCl_2$ for 12 hours, was used for the measurement of media pH.

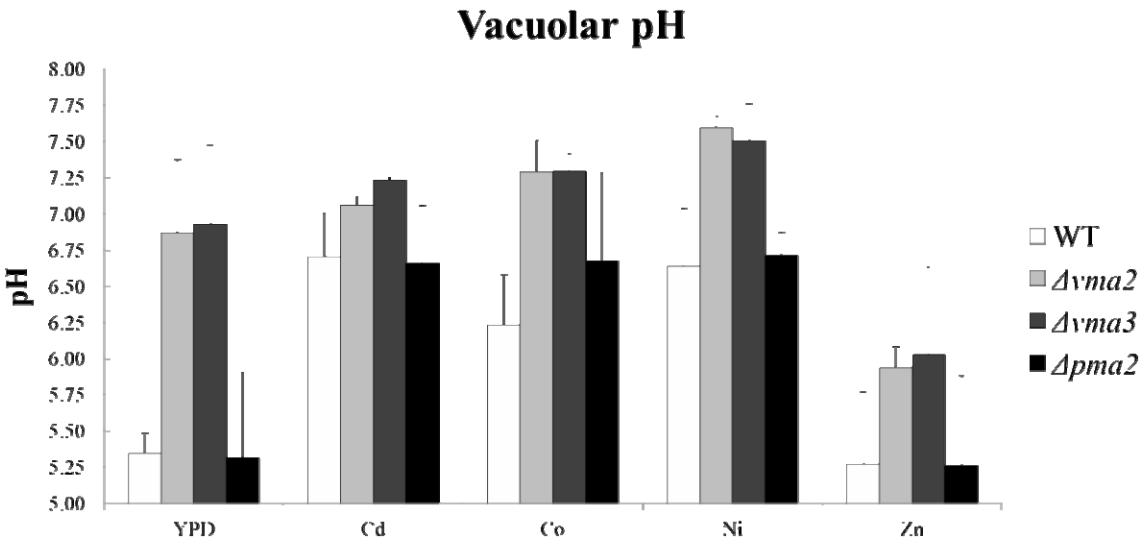

Figure 6. The vacuolar pHs of the mutants lacking V-ATPase and P-ATPase after exposure to heavy metals. The wild-type (BY 4742) (□), *Δvma2* (▒), *Δvma3* (■), and *Δpma2* (■) strains cultivated in YPD medium and YPD media containing 30 μM $CdCl_2$, 2 mM $CoCl_2$, 2 mM $NiCl_2$, and

3 mM $ZnCl_2$ for 12 hours, was used for the measurement of vacuolar pH.

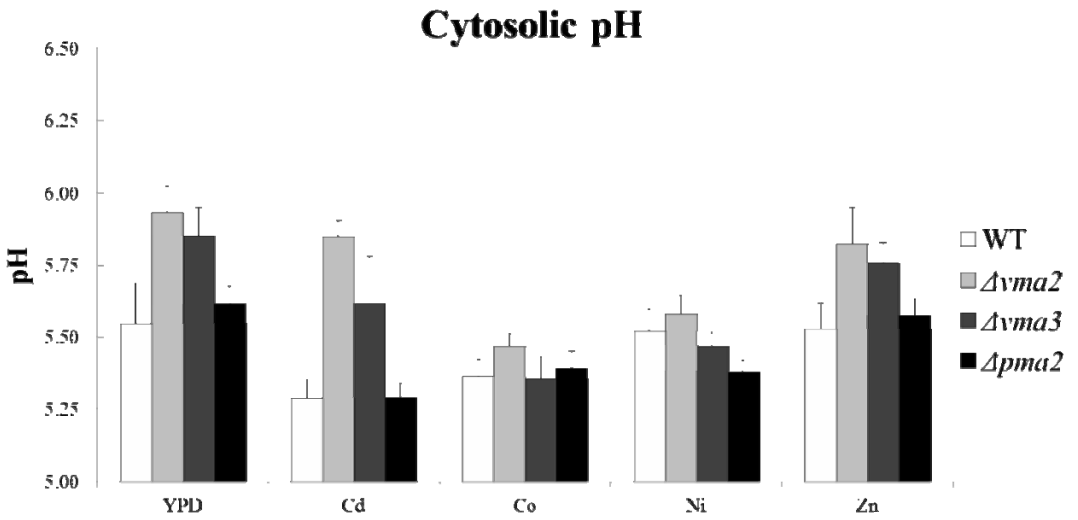

Figure 7. The cytosolic pHs of the mutants lacking V-ATPase and P-ATPase after exposure to heavy metals after exposure to heavy metals. The wild-type (BY 4742) (☐), *Δvma2* (▒), *Δvma3*(■), and *Δpma2* (■) strains cultivated in YPD medium and YPD media containing 30 µM $CdCl_2$, 2 mM $CoCl_2$, 2 mM $NiCl_2$, and 3 mM $ZnCl_2$ for 12 hours, was used for the measurement of cytosolic pH.

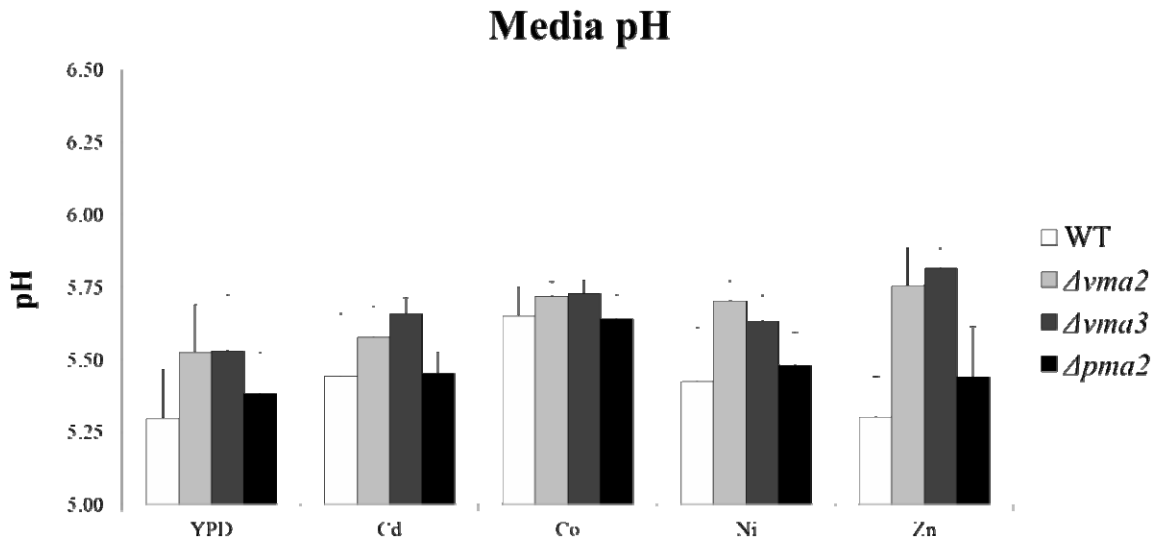

Figure 8. The media pHs of the mutants lacking V-ATPase and P-ATPase after exposure to heavy metals after exposure to heavy metals. The wild-type (BY 4742) (☐), *Δvma2* (▒), *Δvma3* (■), and *Δpma2* (■) strains cultivated in YPD medium and YPD media containing 30 µM $CdCl_2$, 2 mM $CoCl_2$, 2 mM $NiCl_2$, and 3 mM $ZnCl_2$ for 12 hours, was used for the measurement of media pH.

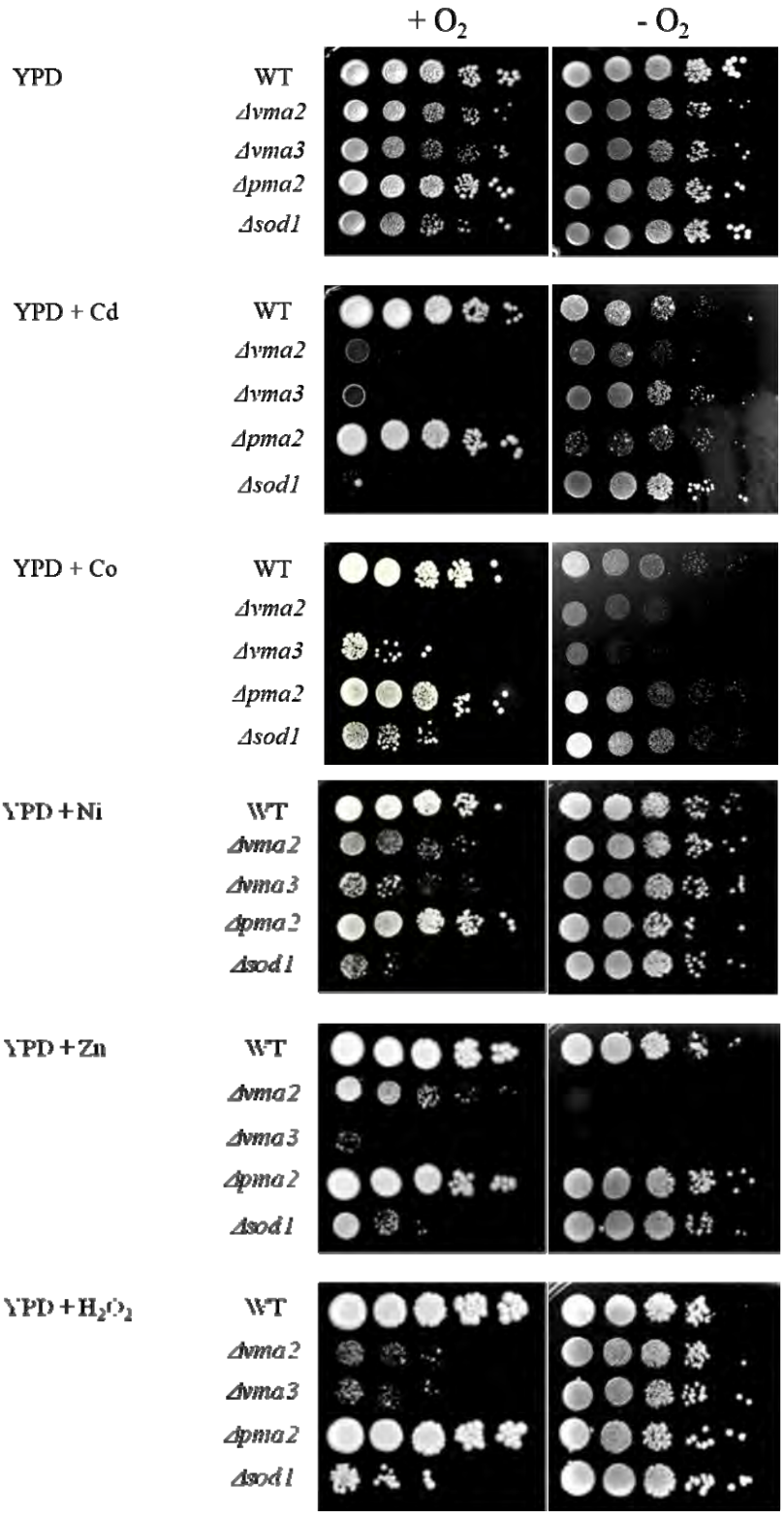

Figure 9. Growth of yeast strains under anaerobic conditions. Cells grown to log phase in YPD broth were serially diluted ten-fold and spotted on the YPD agar plates and YPD agar plate containing the concentrations of 30 μM $CdCl_2$, 2 mM $CoCl_2$, 2 mM $NiCl_2$, 3 mM $ZnCl_2$, and 1 mM H_2O_2, and incubated at 30 °C under aerobic and anaerobic conditions for 3 days.

DISCUSSION

Effect of heavy metal on membrane permeability and pH alteration.

Our previous study on genomic screening for genes required for cadmium tolerance in yeast demonstrated that genes involved in vacuolar functions are essential for Cd tolerance. In addition, it has been reported that the yeast mutants lacking genes and/or proteins responsible for vacuolar functions often displayed a reduced growth in the presence of heavy metals such as Co, Ni, and Zn (Ramsay & Gadd, 1997). Consistent with the previous reports, we found that the *Δvma2* and *Δvma3* mutants were sensitive to Cd, Co, Ni, and Zn (Fig. 1). On the other hand, the *Δpma2* mutant lacking minor plasma membrane H^+-ATPase did not show sensitivity to heavy metals (Fig. 1). This may be due to the fact that the *Δpma2* mutant contains the functional major plasma membrane H^+-ATPase Pma1p. Since *PMA1* is an essential gene whose protein product functions in creating the electrochemical gradient of protons across the plasma membrane (Portillo *et al.*, 1989; Serrano, 1984). Therefore, it is impossible to use a deletion mutant of this gene to examine the role of this protein in tolerance to heavy metals. Moreover, *PMA2* is weakly expressed under non-stress conditions and not essential for viability and growth of yeast cells (Viegas *et al.*, 1994). Based on our results, it is likely that Pma2p does not play an important role in heavy metal tolerance.

Most heavy metal ions have high affinity for polar molecules, such as phosphates and side chains of proteins, which are all abundant in microbial cells (Gardarin *et al.*, 2010). It has been reported that cadmium induced plasma membrane permeabilization (Howlett & Avery, 1997), and many metals including Zn, Cd, Cr, Cu, Fe, and Pb increased membrane fluidity Garcia (Garcia *et al.*, 2005). Thus, the toxicity of heavy metal might be associated with activities of cellular ions and small metabolites including proton (Madeira *et al.*, 2010). Supporting this hypothesis, Cd, Co, Ni and Zn evidently induced an increase of the SYTOX green uptake similar to the effect of Amphotericin B (Fig. 2), indicating that the membrane permeability of the yeast wild-type cell is increased after exposure to these metals. These membrane permeabilization induced by heavy metals seem to cause decreases of cytosolic pH and increase of media pH in dose-dependent manner (Fig. 3 and 5),

possibly due to proton influx to cytoplasm.

The loss of V-ATPase activity did not cause cytosolic acidification.

V-ATPases and P-ATPase are responsible for pumping H^+ to maintain the optimal cytosolic pH, which are essential for most cellular functions (Martinez-Munoz & Kane, 2008). Therefore, both H^+-ATPase functions seem to be required for the regulation of the cytosolic pH in response to proton influx caused by heavy metals. However, the *Δpma2* mutant lacking minor plasma membrane H^+-ATPase was not sensitive to heavy metals (Fig. 1) and the pH alteration pattern of this mutant in response to metals was similar to that of the wild-type strain (Fig. 5-7). These results indicated that Pma2p does not play an important role in maintaining cytosolic pH during heavy metal stresses.

A loss of V-ATPase activity was supposed to promote vacuolar alkalization due to an in ability to pump proton into vacuole and cytoplasmic acidification due to an accumulation of excessive protons in cytoplasm. As expected, the vacuolar pHs of the *Δvma2* and *Δvma3* mutants were more alkaline than that of the wild-type strain, due to a loss of ability to pump protons into vacuole. However, even though the cytosolic pHs of the *Δvma2 and Δvma3* mutants were decreased after exposure to heavy metals, their cytosolic pHs were more alkaline than that of the wild-type strain (Fig. 6). Based on this observation, it is unlikely that the cytosolic pH is contributed to sensitivity to heavy metals in the *vma* mutants. The high cytosolic pH in the *vma* mutants after exposure to heavy metals may be due to the activation of major plasma membrane H^+-ATPase Pma1p during cytosolic acidification to compensate for the loss of V-ATPase (Eraso & Gancedo, 1987; Orij *et al.*, 2011; Yenush *et al.*, 2005). However, it has been reported that, in the *vma* mutants, Pma1p is mislocalized to the vacuole (Martinez-Munoz & Kane, 2008), raising the possibility that some other proteins may be involved in controlling cytosolic pH. Although the alteration of cytosolic pH did not account for increased sensitivity to heavy metal in the *vma* mutants, vacuole is still important for heavy metal detoxification through compartmentalization. For instance, cobalt is transported into the vacuole by the vacuolar transporter Cot1p (Conklin *et al.*, 1992), whereas nickel and zinc are transported into

the vacuole via active transport driven by proton gradient generated by V-ATPase (Joho *et al.*, 1995; MacDiarmid *et al.*, 2002; Nishimura *et al.*, 1998).

The role of V-ATPase in inhibiting ROS generation during heavy metal stress.

Heavy metals is known to induce ROS production and oxidative stress (Valko *et al.*, 2005), while the *vma* mutants have been shown to be sensitive to H_2O_2 and various oxidants (Milgrom *et al.*, 2007; Thorpe *et al.*, 2004), possibly due to the inability to neutralize ROS, which will lead to increased protein damage even in the absence of exogenous oxidant (Kane, 2007). We therefore hypothesized that the sensitivity of the *vma* mutant to heavy metals may be a consequence of a lack of ability to eliminate intracellular ROS. Although, under anaerobic condition without oxygen to induce ROS generation, the sensitivity to Cd, Ni, and Co of the *vma* mutants was suppressed (Fig. 8), the ROS levels in the *vma* mutants, after exposure to Co and Ni, but not Cd, were similar to that of wild-type strain (Fig. 9). These result suggested that increase of oxidative stress may not be a major cause of heavy metal sensitivity in the *vma* mutants. In case of zinc, although the *vma* mutants were sensitive to zinc under aerobic condition, this growth defect was not restored under anaerobic condition (Fig. 8). Given that zinc is an essential metal having antioxidant activity, exposure to zinc may cause interference with the redox activities of some heavy metals, such as Fe and Cu (Pagani *et al.*, 2007). In addition, at the metabolic level, excessive zinc has been shown to disturb iron homeostasis through interfering with the assembly and/or function of iron-sulphur containing proteins (Pagani, *et al.*, 2007). The inconsistency in our results suggested that the sensitivity to heavy metals in the *vma* mutants does not solely arise from an increase in the ROS level (Fig. 8 and 5). What is a major cause of heavy metal sensitivity in the *vma* mutant? It has been reported that a loss of proton motive force energized by V-ATPase causes a defect in vesicle trafficking from late endosome to the vacuole, resulting in an excessive ion accumulation at toxic levels in cytosol (Brett *et al.*, 2005; Cagnac *et al.*, 2010). Moreover, since heavy metal induced an increase in membrane

permeability (Fig. 2), it is possible that the loss of V-ATPase affects influx and efflux rates of monovalent cations such as Na^+ or K^+, leading to an ionic imbalance in cytosol and inhibition of cellular metabolisms (Bleackley & MacGillivray, 2011; Howlett & Avery, 1997).

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 Jindarungruang S, Techo T, and Auesukaree C. The Roles of Vacuolar H^+-ATPase in Response to Heavy Metal Stress in *Saccharomyces cerevisiae*. (paper in preparation)

2. การนำเสนอผลงานในที่ประชุมวิชาการ

2.1 Techo T, Jindarungruang S, and Auesukaree C. The Roles of Vacuolar H+-ATPase in Response to Heavy Metal Stress in Saccharomyces cerevisiae. การประชุมวิชาการวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ครั้งที่ 37 (วทท 37) โรงแรมเซ็นทาราแกรนด์ กรุงเทพ 10-12 ตุลาคม 2554

2.2 Jindarungruang S, Bhubhanil S, and Auesukaree C. The Roles of Vacuolar H^+-ATPase in Response to Heavy Metals in *Saccharomyces cerevisiae*. International Conference The 3rd Biochemistry and Molecular Biology (BMB) Conference "From Basic to Translational Research for a Better Life" Chiang Mai, Thailand; April 6-8, 2011.