Vacuolar H\textsuperscript{+}-ATPase subunit Vma1p functions as the molecular ligand in the vacuole-targeting fungicidal activity of polymyxin B

Maki Iida,\textsuperscript{1} Keiichi Yamada,\textsuperscript{2} Yoshiya Nango,\textsuperscript{1} Yoshihiro Yamaguchi,\textsuperscript{1} Akira Ogita,\textsuperscript{1,3} Ken-ichi Fujita\textsuperscript{1} and Toshio Tanaka\textsuperscript{1,\*}

Abstract

Polymyxin B (PMB) is a cationic cyclic peptide that can selectively inhibit the growth of Gram-negative bacteria by disrupting the outer membrane permeability barrier through binding to lipopolysaccharide (LPS). Here, a fluorescent PMB derivative (PMB-Ds) was applied to visually confirm the vacuole as a direct lethal target of PMB against fungal cells, which lack LPS. PMB-Ds could be visualized in the normal rounded vacuolar membrane of Saccharomyces cerevisiae cells, suggesting the presence of a molecular ligand assisting the vacuole-targeting mobilization of the peptide in the organism. Vma1p, a cytoplasmic subunit constituent of the yeast vacuolar-type ATPase, was identified as one of the PMB-binding proteins by means of mass spectrometry. Mutant cells carrying a deletion of Vma1p but not those with deletions in two separate PMB-binding proteins were shown to be resistant to the vacuolar membrane disruptive action of PMB. Furthermore, the mutant cells were resistant to PMB lethality even when treated with PMB in combination with allicin, an allyl sulfur compound, which can selectively enhance the vacuole-targeting fungicidal activity of the peptide. In contrast, the parent cells were not made resistant to the vacuolar membrane disruptive action of PMB even if cells were pre-treated with bafilomycin A1, a specific inhibitor of the yeast vacuolar-type H\textsuperscript{+}-ATPase. However, the parent cells were rendered more resistant to PMB consequent to Vma1p-GFP localization in the cytoplasm. These findings suggested a role for Vma1p in the vacuole-targeting fungicidal activity of PMB comparable to that of LPS in the outer membrane of Gram-negative bacteria.

INTRODUCTION

Polymyxin B (PMB) (Fig. 1a) is a cationic cyclic peptide that can selectively inhibit the growth of Gram-negative bacteria. Notably, in spite of its nephro- and neurotoxic side effects, its re-introduction into clinical use has been considered because of the emergence of multidrug-resistant bacterial strains [1, 2]. PMB initially disrupts a permeability barrier of the bacterial outer membrane by binding to lipopolysaccharide (LPS) as a PMB-specific molecular ligand, with subsequent incorporation across the outer membrane into the inner membrane [3]. This peptide antibiotic can ultimately alter the permeability of the acidic phospholipids that constitute the bacterial plasma membrane to intracellular molecules [4, 5]. PMB can also inhibit the growth of yeasts and fungi, but to lesser extents, which is consistent with the absence of LPS on the cell surfaces of microbes other than Gram-negative bacteria [6–8]. The binding ability of PMB to LPS has been clearly demonstrated [9]; thus, it is routinely applied clinically for the removal of LPS as an endotoxin from human blood with the aid of PMB-immobilized fibres [10].

Plasma membrane permeability change has been accepted as a cause of PMB fungicidal activity as well as for its antibacterial effects, as reflected by the synergistic effect between PMB and an azole-type antifungal agent [7, 8, 11]. In our previous study, PMB was found to enhance K\textsuperscript{+} ion efflux from the cytoplasm of Saccharomyces cerevisiae cells; however, the level of PMB-induced K\textsuperscript{+} ion leakage was only slightly elevated in response to the marked enhancement of PMB lethality in a medium supplemented with allicin, an allyl sulfur compound from garlic (Fig. 1c) [12]. This suggested the involvement of an unknown mechanism in the fungicidal activity of PMB, resulting in the discovery of a lethality dependent upon vacuolar membrane disruptive action. Subsequently, the vacuole-targeting fungicidal activity of PMB was confirmed by its selective enhancement using an ionophore...
compound such as salinomycin as well as zwiebelane A, a sulfur-containing compound from onions [13, 14]. However, it remains unclear whether PMB mobilization into the fungal vacuole is dependent upon a specific transport system, such as the role of autophagy in amphotericin B trafficking to the luminary space of an organelle [15]. Without such direction, the water-soluble PMB peptide might instead diffuse uniformly throughout the fungal cytoplasm, allowing its potential binding with a target molecule to effect its fungicidal action, similar to the binding to LPS in its bactericidal activity against Gram-negative bacteria. Therefore, in this study we assumed the presence of a protein that could facilitate the binding of PMB to the vacuolar membrane. To verify this premise, candidate PMB-binding proteins were isolated by means of affinity chromatography using PMB as a molecular ligand and identified by mass spectrometry, and potential relevance in PMB fungicidal activity was assessed by functional analysis following candidate gene deletion.

**METHODS**

**Total synthesis of dansyl-labelled PMB (PMB-Ds)**

Total synthesis of PMB-Ds (Fig. 1b) was carried out according to the method described previously for the total synthesis of PMB using PMB$_3$ instead of PMB$_1$ [16]. PMB-Ds (1.6 mg) was obtained as a colourless solid with a 5% yield after HPLC-dependent purification. The electrospray ionization low resolution mass spectrometry (LR-MS) spectrum value of m/z 681.3 ([M+2H]$^{2+}$) detected with PMB-Ds coincided with the half of calculated value of m/z 1362.76 for C$_{60}$H$_{106}$N$_{18}$O$_{15}$S. The scheme of PMB-Ds synthesis is shown in Fig. S1 (available in the online Supplementary Material); the details of the synthetic procedure will be reported elsewhere.

**Strains and media**

*S. cerevisiae* strain BY4741 and its gene deletion mutants Δvma1, Δrach1, Δats1 and Δap1k2 were obtained from the Yeast Knockout Strain Collection (Thermo Scientific Open Biosystems, Waltham, MA, USA). *S. cerevisiae* strains ATCC 201388 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and its genetically manipulated strain for production of Vma1p–GFP fusion protein in the vacuolar membrane was from the Yeast GFP Clone Collection of Thermo Scientific Open Biosystems (Waltham, MA, USA). This strain was designated *S. cerevisiae* ATCC 201388 (Vma1p–GFP). Unless otherwise stated, yeast cells were grown in nutrient medium (pH 7.0) consisting of 1% yeast extract (Difco Laboratories, Detroit, MI, USA), 2% bacto-peptone (Difco Laboratories) and 2% D-glucose (YPD) at 30°C with vigorous shaking for the measurement of cell growth. The pH of the YPD medium was increased to 7.5 in the experiment using bafilomycin A1 in

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**Fig. 1.** Structures of PMB (a), PMB-Ds (b), and allicin (c).
order to fully achieve its inhibition of the vacuolar-type H+-ATPase. SD (–uracil) medium was used for the colony isolation of plasmid-transformed cells and their overnight cultivation at 30°C [17].

**Plasmid construction and transformation**

The VMA1 gene was amplified by PCR using *S. cerevisiae* dTC063 genomic DNA as a template with the gene-specific primers VMA1 forward: GGG GAG CTC AAA AAA AAA TGG CTG GTG CAA TTG AAA AC, and VMA1 reverse: GGG GAG CTC TTA ATC GGT AGA TTC AGC AAA TCT TTC, and cloned into the SacI site of the plasmid pAD4 [18]. The resulting pAD4 plasmid carrying VMA1 was transformed into cells of *S. cerevisiae* ATCC 201388 (Vma1p-GFP) via the lithium acetate method [19]. Cells of *S. cerevisiae* ATCC 201388 (Vma1p-GFP) were also transformed with the empty vector pAD4 and the corresponding transformed cells were used as the control cells, in which Vma1p–GFP normally localizes in the vacuolar membrane rather than in the cytoplasm. In comparison, the cytoplasmic localization of Vma1p-GFP in *S. cerevisiae* ATCC 201388 (Vma1p-GFP) cells could be achieved as a result of the plasmid-dependent overproduction of Vma1p and its formation into a complex with the other subunits of vacuolar-type ATPase in the vacuolar membrane as a substitute for Vma1p-GFP. *S. cerevisiae* ATCC 201388 (Vma1p-GFP) cells transformed with either an empty vector or the vector carrying VMA1 were selected on the basis of their colony-forming ability after incubation on SD (–uracil) agar medium overnight at 30°C.

**Affinity chromatography and SDS-PAGE**

PMB-binding proteins were purified using a column (Detoxi-Gel Endotoxin Removing Columns, Thermo Fisher Scientific), in which PMB was used as a molecular ligand for binding with LPS. The column was equilibrated with 50 mM Tris-HCl buffer (pH 7.4) containing 1% sodium deoxycholate. Parent cells of *S. cerevisiae* BY4741 were grown to mid-logarithmic phase in 200 ml YPD medium and were collected by centrifugation and were washed several times with 50 mM Tris-HCl buffer (pH 7.4). The obtained cells were suspended in a small volume of the buffer and disrupted by repeated vortexing with glass beads in an ice bath. The supernatant (0.8 ml) of the thus-obtained cell lysate was applied to the column and then the column was extensively washed with the buffer alone. The column was then washed with 50 mM Tris-HCl buffer (pH 7.4) containing PMB at 1.0 mM. Fractions of 0.3 ml were collected; those containing the eluted proteins were combined and concentrated using a centrifugal filter with a 10 kDa membrane (Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-30 membrane, Darmstadt, Germany). To confirm the removal of unbound proteins from the column prior to elution with PMB-containing buffer, the final fraction (0.5 ml) eluted from the column with 50 mM Tris-HCl buffer (pH 7.4) was similarly concentrated and analysed for its protein content. These two samples were subjected to SDS-PAGE using a 15% polyacrylamide gel and an ATTO Mini-Slab Electrophoresis System (Tokyo, Japan) at a constant current of 10 mA. The gel was stained with 0.25% Coomassie brilliant blue for 35 min and de-stained with a mixture of 7% acetic acid and 5% methanol in distilled water.

**Identification of PMB-binding proteins by matrix-assisted laser desorption ionization/time-of-flight mass spectroscopy (MALDI-TOF-MS)**

The major protein bands were excised from the gel and digested with trypsin [20]. Mass spectra were collected by MALDI-TOF-MS using a Brucker Daltonics autoflex speed TOF/TOF system (Billerica, MA, USA) for protein identification. Peptide mass fingerprinting was carried out using the MASCOT program for protein identification (www.matrixscience.com/).

**Measurement of cell growth**

Cells grown overnight in YPD medium were diluted into freshly prepared YPD medium at 1×10⁷ cells ml⁻¹. The cell suspensions were incubated at 30°C with vigorous shaking in YPD medium in which PMB and allicin were added at various concentrations for the measurement of viable cell numbers as colony-forming units [12]. The minimum growth inhibitory concentrations (MICs) of PMB, PMB-Ds and bafilomycin A1 were determined in YPD medium as described in our previous paper [12].

**Vacuole staining**

Vacuoles were stained with the fluorescent probe FM4-64 according to published methods with some modifications [21, 22]. Briefly, overnight cultures were incubated for another 1 h at 30°C in YPD medium with 5 µM FM4-64. Cells were then collected by centrifugation and suspended in freshly prepared YPD medium at 1×10⁷ cells ml⁻¹. Cell suspensions were then incubated with vigorous shaking at 30°C for 2 h to investigate the effects of PMB and PMB-Ds on vacuole morphology.

**Fluorescent microscopy**

After treatment with PMB or PMB-Ds, FM4-64-stained cells (1×10⁷ cells ml⁻¹) were collected by centrifugation, washed, resuspended in 100 µl PBS and imaged by bright-field and fluorescence microscopy. Cells labelled with FM4-64 were imaged following excitation at 520–550 nm and emission at 580 nm. PMB-Ds was visualized with excitation at 480 nm and emission at 530 nm.

**Statistical methods**

Statistical evaluation was performed using Student’s *t*-test, in which *P*<0.05 was considered statistically significant.

**Chemicals**

PMB and allicin were purchased from Wako Pure Chemical Industries (Osaka, Japan) and LKT Laboratories (St Paul MN, USA), respectively. Bafilomycin A1 was a product of Sigma-Aldrich (St Louis, MO, USA). FM4-64 was purchased from Molecular Probes (Eugene, OR, USA). The
other chemicals used for PMB-Ds synthesis were of analytical reagent grade.

RESULTS

Cellular localization of PMB-Ds

In our prior study, we were able to confirm the cellular uptake of PMB across the plasma membrane of *S. cerevisiae* cells by means of HPLC analysis of the cell-free extract from PMB-treated cells [12]. However, it remains unknown whether PMB is mobilized to the vacuole and penetrates into the vacuolar membrane to exert a direct disruptive action. Therefore, in the current study we visually confirmed the cellular localization of PMB with the aid of PMB-Ds. Notably, in spite of the presence of the dansyl moiety, PMB-Ds was shown to be lethal to *S. cerevisiae* cells at the MIC value of 100 µM (data not shown), a value that represented only twice the MIC value of PMB itself in YPD medium. As shown in Fig. 2, PMB-Ds was mostly visible in the vacuolar membrane after 1 h incubation, categorizing the vacuole as a direct lethal target of PMB. This finding also suggested the presence of a molecule to facilitate the specific binding of PMB to the vacuolar membrane. During an additional 1 h of incubation, PMB-Ds was observed to be scattered throughout the cytoplasm in association with the fragmented vacuolar membrane dots, reflecting a vacuolar membrane disruptive action of PMB-Ds identical to that observed in PMB-treated cells.

Isolation and identification of PMB-binding proteins

Based on the results from the current study, we postulated the presence of a protein that could assist PMB binding to the fungal vacuolar membrane, as in the case of the effective achievement of PMB bactericidal plasma membrane disruptive action through a specific binding to LPS. As shown in Fig. 3(a), more than 10 protein bands were identified as representing PMB-binding proteins by affinity chromatography followed by SDS-PAGE, suggesting that these proteins were mostly bound to PMB in a non-specific fashion, possibly owing to the cationic property of the peptide or to the matrix component of the column. We considered that if the PMB-binding protein relevant to the current study could specifically bind to PMB as a ligand of the column, this protein might then also be included in the fraction eluted from the column by using the PMB-containing buffer.

As summarized in Fig. 3(b), we were able to identify three of the proteins detected by SDS-PAGE as Vma1p (also see Fig. 3c), Rack1p and Tsa1p, on the basis of their mass spectral data. Rack1p is a translation inhibitor core component of the 40S ribosomal unit, whereas Tsa1p is a kind of thiorodoxin peroxidase. These two proteins, therefore, appear to have no relation to the molecular components of the fungal vacuole. In contrast, Vma1p exhibits a likely direct relationship to the vacuole, as reflected by its function as a subunit of vacuolar-type H^+-ATPase in *S. cerevisiae*, wherein it is involved in the H^+ translocation function of the enzyme [23, 24].

Vacuole-targeting fungicidal activity of PMB against Δrack1, Δtsa1 and Δvma1

We examined the effects of PMB on the viability of Δvma1 using Δrack1 and Δtsa1 as references for deletions of PMB-binding proteins other than Vma1p. PMB was apparently lethal to cells with Δrack1 and Δtsa1 at 50 µM, as was observed for the parent cells, whereas this peptide was only weakly effective in reducing the viability of Δvma1 cells (Fig. 4). The lethality of PMB was increased in a dose- and time-dependent fashion against cells of PMB-sensitive strains, whereas only a marginal increase was observed against cells of Δvma1.

The effects of PMB on vacuole morphology were also examined among cells of PMB-sensitive parent strains, Δrack1 and Δtsa1, in addition to cells of the PMB-resistant strain Δvma1, in order to confirm the relationship between the PMB lethality and its vacuole-disruptive action. As shown in Fig. 5, PMB was able to cause vacuolar membrane disruptive damage against cells of PMB-sensitive strains at the lethal concentration of 50 µM; however, this phenomenon was observed in cells of the PMB-resistant Δvma1 strain to a significantly lower extent. Δvma1 cells were also characterized by their lower growth rate even in unmodified YPD medium (Fig. 4d), which was consistent with the partial

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**Fig. 2.** Microscopic observation of cells of the *S. cerevisiae* BY4741 parent strain exposed to PMB-Ds. Cells (1×10⁷ cells ml⁻¹) were preincubated with FM4-64, followed by incubation at 30 °C in YPD medium with 100 µM PMB-Ds for the indicated times and microscope imaging using bright-field (top) or fluorescence with FM4-64 (middle) or PMB-Ds-specific excitation (bottom). The most representative images are shown. Bars represent 5 µm. Cells with non-disrupted vacuole images were observed at the ratio of 92±4 % at 1 h incubation and at 34±12 % at 2 h incubation. The data represent the means±SD of the ratio of cells with non-disrupted vacuoles upon observation of 10 cells from each of 10 different stages.
impairment of the H⁺-translocation function of vacuolar H⁺-ATPase.

**Effects of allicin on the vacuole-targeting fungicidal activity of PMB**

Allicin acts to stimulate the fungicidal activity of PMB by selectively enhancing the vacuole disruptive activity of the peptide, but not by enhancing PMB-induced plasma membrane permeability change [12]. Therefore, inclusion of allicin was considered likely to more precisely address the question of whether the PMB-resistant phenotype of Δvma1 was dependent on the lack of Vma1p in the vacuolar membrane of the mutant cells. In accordance with the previous finding [12], allicin was weakly inhibitory to the growth of the parent cells at 50 µM, whereas the presence of allicin at this concentration rendered the parent cells highly susceptible to the lethal action of PMB even at 25 µM (Fig. 6a, b). In contrast, Δvma1 cells were markedly resistant to the lethal action of PMB at 25 µM even if the mutant cells were treated with this peptide in combination with 100 µM allicin (Fig. 6c, d). In accordance with this result, the enhancement effect of allicin on PMB lethality accompanied the disruption of the vacuoles in the parent cells, in which Vma1p normally exists as a cytoplasmic subunit component of the vacuolar membrane H⁺-ATPase. However, PMB was ineffective in its vacuolar membrane-disruptive action against PMB-resistant Δvma1 cells regardless of whether allicin was added simultaneously (Fig. 7a, b).

**Effect of a vacuolar H⁺-ATPase inhibitor on the vacuolar membrane disruptive action of PMB**

It was considered possible that the PMB-resistant phenotype of Δvma1 might alternatively originate from the impairment of vacuolar H⁺-ATPase activity owing to a genetic defect of Vma1p rather than from the absence of Vma1p as a molecular ligand on the surface of the vacuolar membrane.

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**Fig. 3.** SDS-PAGE of PMB-binding proteins (a), summary of identified PMB-binding proteins (b), and Vma1p peptide mass fingerprinting in MALDI-TOF-MS (c). In (a), after applying the protein sample from cells of the *S. cerevisiae* BY4741 parent strain and washing the column with 5 ml 50 mM Tris-HCl buffer (pH 7.4), an aliquot (40 µl) of the final eluted fraction (1 ml) was assessed by electrophoresis to confirm the removal of contaminating proteins (A). The fraction (300 µl) eluted from the column with 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM PMB was concentrated to 40 µl and was subjected to electrophoresis (B). SDS-PAGE standard markers (Bio-Rad) were also included (M).

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membrane. Therefore, we examined whether the vacuolar membrane disruptive action of PMB could be protected when the parent cells were treated with bafilomycin A1, a well-known specific inhibitor of the yeast vacuolar H^+\text{-ATPase} [25]. The parent cells were pre-incubated in YPD medium at 30 °C for 2 h with and without bafilomycin A1 at a concentration of 0.375 µM, which is suitable for selective inhibition of the vacuolar H^+\text{-ATPase} activity [26, 27]. After pre-incubation, the parent cells were further incubated with the addition of 50 µM PMB at 30 °C for an additional 2 h. Cells with non-disrupted vacuoles appeared at ratios of 56±5 % in cells pre-treated with bafilomycin A1 and 50 ±11 % in untreated cells, respectively. These values are slightly higher than the values obtained from PMB-treated cells without pre-incubation (see Fig. 5a, e), but are not statistically different from each other. These results supported the idea that PMB lethality consequent to vacuolar membrane disruptive action depends on the presence of Vma1p on the surface of the yeast vacuolar membrane.

**Protection of PMB lethality owing to cytoplasmic localization of Vma1p-GFP**

We next examined whether Vma1p could protect against PMB lethality if this protein was localized in the cytoplasm of *S. cerevisiae* cells. Vacular membrane localization of Vma1p could be visually confirmed with the aid of GFP-derived fluorescence in cells of *S. cerevisiae* strain ATCC 201388 (Vma1p-GFP). Subsequent transformation of the yeast cells with a vector carrying the *VMA1* gene to induce overproduction of Vma1p resulted in out-competition of Vma1p-GFP at the vacuolar membrane and its consequent localization in the cytoplasm of transformed cells (Fig. 8a). As shown in Fig. 8b, the transformed cells were more resistant to the lethal action of PMB than control cells similarly transformed with the empty vector. These findings supported the concept that Vma1p in the cytoplasm prevented PMB from being bound to the Vma1p subunit constituting the vacuolar membrane H^+\text{-ATPase} complex, thereby protecting the yeast vacuole against its disruptive action.

**DISCUSSION**

Vacuolar membrane disruptive damage has been evaluated as a cause of the fungicidal action of the polyol macrolide niphimycin and the polyene macrolide AmB [28]. However, the value of AmB as an antifungal agent might be enhanced if the serious side-effects consequent to its complexation with cholesterol embedded in the plasma membrane of mammalian cells could be reduced. As expected from the re-evaluation of PMB as a potent bactericidal agent against multi-drug resistant bacteria [1, 2], this peptide antibiotic might be similarly classified as a novel type of fungicidal agent if its vacuole-targeting fungicidal activity could be selectively enhanced. It is therefore important to confirm the mechanism enabling its vacuole-targeting mobilization. In this study, we first attempted, with the aid of the fluorescent probe PMB-Ds, to confirm whether PMB, a water-soluble cationic peptide, could be transported to the vacuole after penetration across the fungal plasma membrane (Fig. 2). The assembly of this probe into the vacuolar membrane prior to the stage of disruption suggested the presence of a molecule that facilitated the specific binding of the peptide to the organelle, as was the case for the specific binding of PMB to LPS in bacteria.

Various protein bands were detected via SDS-PAGE following affinity chromatography for the identification of potential PMB-binding yeast proteins (Fig. 3a), which were likely to have resulted from the occurrence of non-specific ionic interactions.
interactions between PMB and acidic amino acid residues spatially localized on the surface of the yeast proteins. Furthermore, as deduced from the inhibitory effect of PMB on protein kinase C [29], a protein other than those involved in fungal vacuolar membrane function or architecture may exhibit a specific binding ability to this cyclic peptide. Consistent with this indication, Rack1p, a translation inhibitor core component of the 40S ribosomal unit, was isolated as one of the PMB-binding proteins in this study (Fig. 3b). Notably, PMB exhibited a marked lethal effect against S. cerevisiae cells in a synergistic relationship with hygromycin B, an inhibitor of the ribosomal translation reaction in both prokaryotic and eukaryotic cells [30]. The molecular interaction between PMB and Rack1p thus may have a certain implication in the combined lethal actions between PMB and hygromycin B, although Δrack1 cells were not rendered more sensitive to PMB lethality than the parent cells, precluding its likely relevance to the vacuolar disruption effect of PMB.

Vma1p, another candidate PMB-binding protein isolated in the current study, comprises one of the cytoplasmic subunits of the vacuolar-type H⁺-ATPase complex and is thereby responsible for H⁺-translocation across the vacuolar membrane [23]. Vma1p deletion mutant (Δvma1) cells are viable; however, their growth rate was lower than that of the parent cells in YPD medium possibly because of the lower H⁺-translocation ability of the vacuolar H⁺-ATPase. In contrast, Δvma1 cells were rendered extremely resistant to the vacuolar membrane disruptive action of PMB even when this peptide was added at a concentration higher than the MIC value for the parent strain (Fig. 5d). However, allicin, which has been shown to selectively enhance the vacuolar membrane disruptive action of PMB and also to inhibit the mechanism of yeast vacuolar membrane integrity maintenance [12, 31], nevertheless had no impact on the PMB-resistant phenotype of Δvma1 cells. This finding indicated the essential role of Vma1p on the surface of the vacuolar membrane as a molecular ligand for PMB binding to the organelle. Furthermore, PMB could similarly cause vacuolar membrane disruption in cells of the parent strain treated with or without bafilomycin A1, a well-known inhibitor of vacuolar type H⁺-ATPase. On the other
hand, the mutant with reduced plasma membrane H\textsuperscript{+}-ATPase activity is tolerant to hygromycin B because of the failure in cellular uptake of this cationic molecule [32, 33]. This fact means that the PMB-resistant phenotype of \(Dvma1\) cells can be also elucidated by the failure in cellular uptake of this cationic peptide because of a decrease in the plasma membrane Pma1p level as a result of impaired function of vacuolar membrane H\textsuperscript{+}-ATPase [34]. In order to consider this possibility, we examined PMB lethality together with its vacuolar membrane disruptive action against \(Dptk2\) cells, which are deficient in a protein kinase Ptk2p, essential for activation of the yeast plasma membrane H\textsuperscript{+}-ATPase [32, 33]. Unlike this postulation, PMB was able to cause lethal action along with the vacuolar membrane disruption against the parent cells and \(Dptk2\) cells to similar extents, reflecting its successful uptake across the plasma membrane of \(Dvma1\) cells (see Fig. S2). The cellular uptake of PMB in \(Dptk2\) cells and \(Dvma1\) cells may be achieved concomitantly with plasma membrane permeability increase in PMB-treated cells [35]. Thus, the PMB-resistant phenotype of \(Dvma1\) could be attributed to the loss of Vma1p as a molecular ligand for PMB binding.
to the vacuole rather than to a failure of efficient H⁺-translocation across the vacuolar membrane or the loss of plasma membrane potential in the mutant cells.

However, despite these suggestive findings regarding the functionality of Vma1p, the detection of Vma1p by SDS-PAGE indicated the possibility that this protein was able to bind to PMB via a non-specific ionic linkage in a manner distinct from a specific molecular interaction. Future x-ray crystallographic analysis might provide more reliable information related to the molecular interaction between Vma1p and PMB as a trigger for PMB binding to the vacuolar membrane. In the current study, to obtain preliminary evidence with respect to this molecular interaction, we attempted to examine the protective effects of Vma1p on PMB lethality resulting from the vacuolar membrane disruptive action by engineering the cytoplasmic localization of Vma1p (Vma1p-GFP) in the parent cells. This process rendered the yeast cells highly resistant to the vacuolar membrane disruptive action of PMB, allowing their growth even in a medium containing PMB at the lethal concentration of 50 µM. However, the means by which PMB could be mobilized from LPS to the outer membrane and ultimately to the inner membrane after its administration to Gram-negative bacterial cells remains to be determined. Similarly, the movement of PMB once bound to Vma1p on the vacuolar membrane is an ongoing area of study.

Vma1p of S. cerevisiae and the corresponding subunit (ATP6V1A) of human V-ATPase show 66% identity in their amino acid sequences (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov/genbank/). This value may be insufficient to refer to the possibility of PMB binding with V-ATPase of human lysosome, and the resulting disruptive action on this organelle membrane. However, such a possibility should be carefully considered as a cause of its nephro- and neurotoxic side effects if PMB is re-introduced into clinical use against pathogenic fungal strains in addition to multidrug-resistant bacterial strains [1, 2].

Fig. 8. Microscopic observation of Vma1p localization in cells of the S. cerevisiae ATCC 201388 (Vma1p-GFP) strain transformed with the vector alone and with the vector carrying the VMA1 gene (a), and the effects of PMB on the viability of the ATCC 201388 parent strain transformed with the vector alone and with the vector carrying the VMA1 gene (b). For (a), cells (1×10⁷ cells ml⁻¹) were pre-treated with FM4-64, followed by incubation at 30°C for 2 h in YPD medium and imaged by bright-field (top) or fluorescence microscopy with excitation for FM4-64 (middle) or GFP (bottom). Bars represent 5 µm. For (b), cells (1×10⁷ cells ml⁻¹) were incubated for 2 h at 30°C in YPD medium containing 50 µM PMB. Data represent the means±sd of triplicate experiments. *P<0.05.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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