NOTE

The Aspergillus fumigatus P-Type Golgi Apparatus Ca\(^{2+}\)/Mn\(^{2+}\) ATPase PmrA Is Involved in Cation Homeostasis and Cell Wall Integrity but Is Not Essential for Pathogenesis

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The Aspergillus fumigatus \(\Delta \text{pmrA}\) (Golgi apparatus Ca\(^{2+}\)/Mn\(^{2+}\) P-type ATPase) strain has osmotically suppressible basal growth defects and cationic tolerance associated with increased expression of calcineurin pathway genes. Despite increased \(\beta\)-glucan and chitin content, it is hypersensitive to cell wall inhibitors but remains virulent, suggesting a role for PmrA in cation homeostasis and cell wall integrity.

Calcineurin, a conserved serine/threonine-specific phosphatase, mediates environmental stress adaptation in fungi through the transcription factor Crz1/Prz1/CrzA (4, 6, 9, 10, 12, 16, 22). The P-type ATPases, encoded by \(\text{PMR1}, \text{PMC1}\), and \(\text{ENA1}\), have also been characterized as targets of calcineurin in Saccharomyces cerevisiae (3, 5, 11, 20), but their filamentous fungal counterparts have not been completely investigated. \(\text{PMR1}\), encoding the Ca\(^{2+}\)/Mn\(^{2+}\) P-type ATPase, localizes to the Golgi apparatus, where it regulates Ca\(^{2+}\) homeostasis and supplies Mn\(^{2+}\) required for protein glycosylation (7). The \(\text{S. cerevisiae PMR1}\) mutant secretes misfolded proteins and exhibits defective growth under low-Ca\(^{2+}\) conditions and in the presence of Mn\(^{2+}\) (1, 13, 18, 20). In Candida albicans, \(\text{PMR1}\) deletion results in loss of virulence (2). In Aspergillus niger, the \(\text{\Delta pmrA}\) strain exhibits growth retardation on EGTA-containing medium, suggesting the involvement of \(\text{pmrA}\) in Ca\(^{2+}\) homeostasis in Aspergillus species (27). Ectopic expression of an Aspergillus fumigatus PMR1-like ATPase in \(\text{S. cerevisiae}\) demonstrated its requirement for Ca\(^{2+}\) and Mn\(^{2+}\) homeostasis (23), but no functional characterization in \(\text{A. fumigatus}\) was reported. BLAST search using the \(\text{S. cerevisiae PMR1}\) sequence revealed 3 open reading frames (ORFs) (AFUA_2G05860, AFUA_G01320, and AFUA_6G06740) with homology to the \(\text{Pmr1}\) protein (65%, 30%, and 37% identities, respectively). We constructed the \(\text{pmrA}\) mutant by deleting the ORF (AFUA_2G05860) with the highest similarity to \(\text{S. cerevisiae PMR1}\) (E value, 5.6e−220) and examined its growth, cation homeostasis, cell wall integrity, and pathogenicity.

The \(\text{A. fumigatus \Delta pmrA}\) strain was generated by replacing the \(\text{pmrA}\) ORF with the Aspergillus parasiticus \(\text{pyrG}\) gene and confirmed by Southern analysis (Fig. 1A). Successful reconstitution of \(\text{pmrA}\), employing a construct containing the entire \(\text{pmrA}\) coding region, including the 2.5-kb upstream sequence and 500-bp downstream flanking sequence, was confirmed by Southern analysis (data not shown). Growth of the \(\text{\Delta pmrA}\) strain was reduced to ~60% of the wild type (WT) under standard conditions (Fig. 1B), and complementation (not shown) restored radial growth. In the yeasts \(\text{S. cerevisiae and Schizosaccharomyces pombe}\), \(\text{PMR1}\) functions in Ca\(^{2+}\) sequestration and Ca\(^{2+}\) and Mn\(^{2+}\) tolerance (3, 5); therefore, we tested growth of the \(\text{\Delta pmrA}\) strain in the presence of cations (Fig. 1C). While the WT was sensitive to 200 mM CaCl\(_2\), 0.8 M NaCl, and 10 mM MnCl\(_2\), the \(\text{\Delta pmrA}\) strain was more tolerant (Fig. 1C). Treatment with 25 mM EGTA revealed greater sensitivity with the \(\text{\Delta pmrA}\) strain (~60% inhibition) than with the WT (~50% growth inhibition) (Fig. 1C). Each strain’s growth recovered with the addition of 200 mM CaCl\(_2\) (data not shown). Interestingly, culture on osmotically stabilized medium (1 M sorbitol) improved growth of the \(\text{\Delta pmrA}\) strain by ~40%, indicating cell wall instability (Fig. 1C). Growth on sorbitol rescues yeast cell wall integrity mutations by providing an isotonic environment (3). Since the cation sequestration process can indirectly affect intracellular pH, the strains were grown under acidic and alkaline pH conditions. While the \(\text{\Delta pmrA}\) strain showed tolerance at pH 4.0, the WT showed 60% inhibition (Fig. 1C). Each strain’s growth recovered with the addition of 200 mM CaCl\(_2\) (data not shown). Notably, the role of \(\text{pmrA}\) in growth under alkaline or acidic pH conditions remains to be established.

In contrast to the \(\text{S. cerevisiae PMR1}\) mutant, which exhibits growth sensitivity to Ca\(^{2+}\) and Mn\(^{2+}\) (1, 13, 18, 20), our data revealed cation tolerance in the \(\text{\Delta pmrA}\) strain. This discrepancy prompted us to investigate the expression levels of the major genes of the calcineurin pathway, including \(\text{cnaA}, \text{crzA}\), and...
FIG. 1. Construction of the ΔpmrA strain and phenotypic analysis. (A) Schematic representation of the genomic locus of the WT and ΔpmrA strains. The entire coding sequence of the A. fumigatus pmrA gene was replaced with that of the A. parasiticus pyrG gene by homologous recombination. Southern analysis with SacI-digested genomic DNA and the pmrA left flank probe shows the replacement of pmrA with pyrG as a 7.1-kb fragment in the ΔpmrA strain. (B) Culture morphology of the WT and ΔpmrA strains on glucose minimal medium agar (GMM) after 5 days of growth at 37°C. (C) Radial growth of the ΔpmrA strain under different stress conditions, compared to that of the WT strain. A total of 1 × 10⁴ conidia were inoculated onto GMM plates containing different salt concentrations. In addition, growth analysis was also performed on alkaline (pH 9.0) and acidic (pH 4.0) media. Three independent experiments were performed, and the results shown are the means and standard deviations obtained after 4 days of growth at 37°C. (D) RT-PCR expression analysis in response to salt stress. The expression levels of calcineurin catalytic subunit A (cnaA), a calcipressin (cbpA), a Na⁺ ATPase (enaA), and two zinc finger transcription factors (crzA and sltA) were analyzed after 20 min of exposure to 0.8 M NaCl. Three independent experiments were performed, and the results shown are the means and standard deviations.

FIG. 2. (A) Effect of cell wall inhibitors on the WT and ΔpmrA strains. A total of 10⁴ conidia/ml of the WT and ΔpmrA strains were inoculated onto coverslips immersed in GMM medium and grown in the presence or absence of the indicated concentrations of nikkomycin Z (NZ) or caspofungin (CA) for 16 to 20 h at 37°C. Coverslips were removed and observed by microscopy. Scale bar, 20 μm. (B) β-1,3-Glucan content was quantified using the aniline blue assay as previously described (8, 25, 26), using curdlan, a β-1,3-glucan analog, as the standard. Values are expressed as relative fluorescence units (RFU) per mg of mycelial tissue. (C) Quantification of chitin was performed based on a modified protocol (8) and reported as glucosamine equivalents (μg/ml).
and cbpA. For real-time reverse transcription (RT)-PCR analysis, RNA extracted from 15-h cultures incubated for 20 min with 0.8 M NaCl was subjected to first-strand cDNA synthesis. Triplicate RT-PCR assays were performed using 1× iQ SYBR green master mix with two biological replicates, and expression levels were normalized to those for β-tubulin. The 2−ΔΔCT method was used to determine fold changes of expression.

Following 0.8 M NaCl treatment, the expression levels of cnaA and its downstream effector crzA increased 2.1- and 1.7-fold in the ΔpmrA strain, respectively (Fig. 1D). The ΔpmrA strain was tolerant to FK506 treatment (data not shown), which correlates with the increased expression of cnaA. The expression of the endogenous calcineurin inhibitor CbpA (19) was not significantly altered. As the S. cerevisiae Na+/Li+ ATPase encoded by ENA1 and PMR2 is regulated by calcineurin and CRZ1 during alkaline pH, salt, and osmotic stress adaptation (11, 14–16, 18, 21), we analyzed the expression of the A. fumigatus homolog of ENA1 (AFUA_6G03690; enaA) in the ΔpmrA strain after exposure to NaCl. Furthermore, considering the role of the Aspergillus nidulans sltA gene, encoding a C2H2 zinc finger transcription factor, in alleviating sensitivity to salt stress (17), we also studied the expression of the homologous gene (AFUA_1G13050) in the ΔpmrA strain. Increased expression of both enaA (2.3-fold) and sltA (1.7-fold) genes was noted (Fig. 1D), suggesting a role for PmrA in Na+ homeostasis and salt stress adaptation of A. fumigatus. Recently, sltA, which has no identifiable homolog in S. cerevisiae but, like crzA, is involved in cation adaptation and homeostasis, has been shown to positively regulate the transcription of enaA (24). Interestingly, both the sltA and enaA genes contain po-
tential calcineurin-dependent response elements (CDRE) in their promoters (data not shown), implicating the involvement of calcineurin in Na⁺ homeostasis. Although increased cytosolic Ca²⁺ and the subsequent activation of calcineurin may be helpful during salt stress, perturbed Ca²⁺ homeostasis and prolonged calcineurin activity may be harmful. Loss of Ca²⁺ ATPase activity in the ΔpmrA strain probably results in prolonged Ca²⁺ signaling, which in turn affects radial growth. Therefore, PmrA likely acts as the major Ca²⁺ ATPase during salt stress.

Since deletion of pmrA induced a growth defect which was overcome by addition of sorbitol, indicating a cell wall defect, we examined the susceptibility of the ΔpmrA strain to the cell wall-destabilizing antifungals nikkomycin Z (NZ) and caspofungin (CA). Broth microdilution analysis revealed minimal effective concentrations (MECs) of NZ and CA to be 0.03125 μg/ml and 0.5 μg/ml, respectively, for the ΔpmrA strain. As shown in Fig. 2A, the ΔpmrA strain was more susceptible to nikkomycin Z than the WT, showing drastically swollen hyphae and weakened cell wall architecture. Caspofungin treatment (0.5 to 1 μg/ml) also caused distorted hyphae in the ΔpmrA strain (Fig. 2A). As the enhanced sensitivity of the ΔpmrA strain to cell wall antifungals suggests a role for pmrA in cell wall integrity and possibly biosynthesis of cell wall components, we assayed for total cell wall β-glucan and chitin content. Interestingly, the β-glucan and chitin content increased by 12% and 25%, respectively, in the ΔpmrA strain (Fig. 2B and C). Despite decreased 1,3-β-D-glucan synthase activity, an increase in cell wall α- and β-glucans has been reported for S. pombe (3). A similar increase of glucans was noted in the C. albicans pmrA strain as a reciprocal response to decreased mannans (2).

Calcofluor white staining was performed to verify any variations in cell wall morphology and showed aggregation of cell wall polysaccharides in the conidial vesicles and thickened septa in the ΔpmrA strain (Fig. 3). Uniform accumulation of cell wall material was seen at the hyphal tips of the WT strain but not in the ΔpmrA strain, indicating that perturbed cell wall assembly leads to chitin and glucan accumulation at the septum in the ΔpmrA strain.

Next, the influence of pmrA deletion on virulence was investigated in an inhalational murine model of invasive pulmonary aspergillosis, as previously described (4, 19, 25). Briefly, 6-week-old CD1 male mice were immunosuppressed with cyclophosphamide and triamcinolone. In a Hinners inhalational chamber, three groups of 20 unanesthetized mice were each exposed by inhalation to 40 ml of an aerosolized suspension of 1 x 10⁶ conidia/ml of the WT and ΔpmrA strains as well as a control diluent. Survival was assessed by Kaplan-Meier analysis and pairwise comparison using the log rank test. Statistical significance was defined as a two-tailed test with a P value of <0.05. Unlike the Candida albicans Δpmr1 strain, which was significantly attenuated in virulence (2), there was no difference in virulence between the WT and ΔpmrA strains (Fig. 4A). Histopathological analysis of lungs harvested from mice euthanized at defined time points and stained with Gomori’s methenamine silver and hematoxylin-eosin revealed similar hyphal invasion patterns and extensive inflammation in both the WT and ΔpmrA strains (Fig. 4B). Despite the basal growth defect, different cation sensitivities, increased susceptibility to cell wall antifungals, and increased cell wall chitin and β-glucan, deletion of pmrA did not affect overall pathogenesis. It is possible that a hyphal growth threshold exists between being pathogenic and nonpathogenic. Further studies are required to understand the persistent virulence of the ΔpmrA strain in spite of these aberrancies. In conclusion, we demonstrate that PmrA is involved in hyphal growth, cation homeostasis, and cell wall integrity and likely acts as the major Ca²⁺ ATPase during salt stress in A. fumigatus.

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