

NOTE

The *Aspergillus fumigatus* P-Type Golgi Apparatus Ca²⁺/Mn²⁺ ATPase PmrA Is Involved in Cation Homeostasis and Cell Wall Integrity but Is Not Essential for Pathogenesis[∇]

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The *Aspergillus fumigatus* $\Delta pmrA$ (Golgi apparatus Ca²⁺/Mn²⁺ P-type ATPase) strain has osmotically suppressible basal growth defects and cationic tolerance associated with increased expression of calcineurin pathway genes. Despite increased β -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 *PMR1*, *PMCL1*, and *ENAI*, 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. *PMR1*, encoding the Ca²⁺/Mn²⁺ P-type ATPase, localizes to the Golgi apparatus, where it mediates Ca²⁺ homeostasis and supplies Mn²⁺ required for protein glycosylation (7). The *S. cerevisiae* *PMR1* mutant secretes misfolded proteins and exhibits defective growth under low-Ca²⁺ conditions and in the presence of Mn²⁺ (1, 13, 18, 20). In *Candida albicans*, *PMR1* deletion results in loss of virulence (2). In *Aspergillus niger*, the $\Delta pmrA$ strain exhibits growth retardation on EGTA-containing medium, suggesting the involvement of *pmrA* in Ca²⁺ homeostasis in *Aspergillus* species (27). Ectopic expression of an *Aspergillus fumigatus* *PMR1*-like ATPase in *S. cerevisiae* demonstrated its requirement for Ca²⁺ and Mn²⁺ homeostasis (23), but no functional characterization in *A. fumigatus* was reported. BLAST search using the *S. cerevisiae* *PMR1* sequence revealed 3 open reading frames (ORFs) (AFUA_2G05860, AFUA_G01320, and AFUA_6G06740) with homology to the Pmr1 protein (65%, 30%, and 37% identities, respectively). We constructed the *pmrA* mutant by deleting the ORF (AFUA_2G05860) with the highest similarity to *S. cerevisiae* *PMR1* (E value, 5.6e–220) and examined its growth, cation homeostasis, cell wall integrity, and pathogenicity.

The *A. fumigatus* $\Delta pmrA$ strain was generated by replacing

the *pmrA* ORF with the *Aspergillus parasiticus* *pyrG* gene and confirmed by Southern analysis (Fig. 1A). Successful reconstitution of *pmrA*, employing a construct containing the entire *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 $\Delta pmrA$ 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 *S. cerevisiae* and *Schizosaccharomyces pombe*, *PMR1* functions in Ca²⁺ sequestration and Ca²⁺ and Mn²⁺ tolerance (3, 5); therefore, we tested growth of the $\Delta pmrA$ strain in the presence of cations (Fig. 1C). While the WT was sensitive to 200 mM CaCl₂, 0.8 M NaCl, and 10 mM MnCl₂, the $\Delta pmrA$ strain was more tolerant (Fig. 1C). Treatment with 25 mM EGTA revealed greater sensitivity with the $\Delta pmrA$ strain (~60% inhibition) than with the WT (~50% growth inhibition) (Fig. 1C). Each strain's growth recovered with the addition of 200 mM CaCl₂ (data not shown). Interestingly, culture on osmotically stabilized medium (1 M sorbitol) improved growth of the $\Delta pmrA$ strain by ~40%, indicating cell wall instability (Fig. 1C). Growth on sorbitol rescues yeast cell wall integrity mutations by providing an isosmotic environment (3). Since the cation sequestration process can indirectly affect intracellular pH, the strains were grown under acidic and alkaline pH conditions. While the $\Delta pmrA$ strain showed tolerance at pH 4.0, the WT showed reduced growth at alkaline pH 9.0. However, the exact role of *pmrA* in growth under alkaline or acidic pH conditions remains to be established.

In contrast to the *S. cerevisiae* *PMR1* mutant, which exhibits growth sensitivity to Ca²⁺ and Mn²⁺ (1, 13, 18, 20), our data revealed cation tolerance in the $\Delta pmrA$ strain. This discrepancy prompted us to investigate the expression levels of the major genes of the calcineurin pathway, including *cnaA*, *crzA*,

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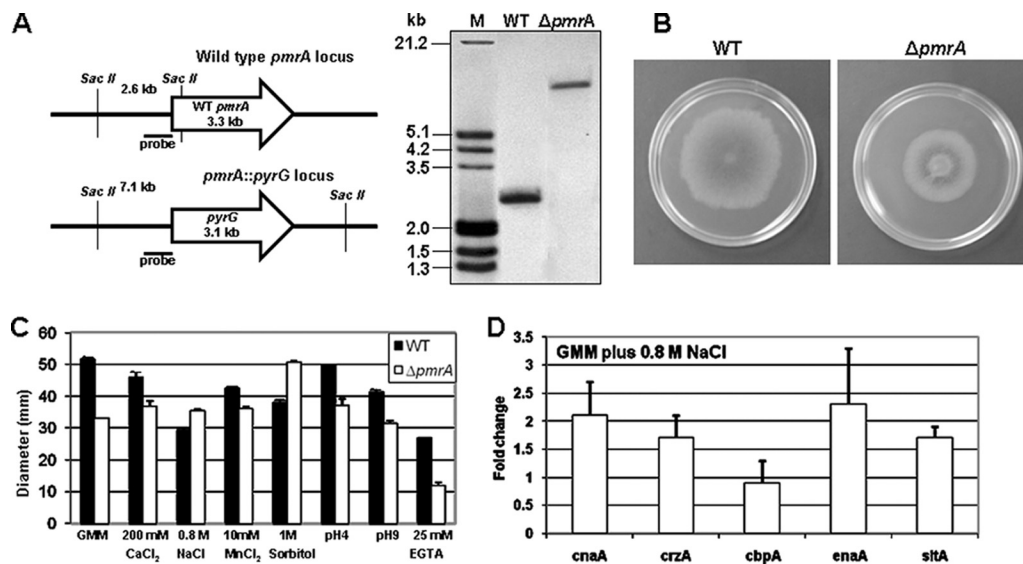


FIG. 1. Construction of the $\Delta pmrA$ strain and phenotypic analysis. (A) Schematic representation of the genomic locus of the WT and $\Delta pmrA$ strains. The entire coding sequence of the *A. fumigatus pmrA* gene was replaced with that of the *A. parasiticus pyrG* gene by homologous recombination. Southern analysis with *Sac*II-digested genomic DNA and the *pmrA* left flank probe shows the replacement of *pmrA* with *pyrG* as a 7.1-kb fragment in the $\Delta pmrA$ strain. (B) Culture morphology of the WT and $\Delta pmrA$ strains on glucose minimal medium agar (GMM) after 5 days of growth at 37°C. (C) Radial growth of the $\Delta pmrA$ strain under different stress conditions, compared to that of the WT strain. A total of 1×10^4 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.

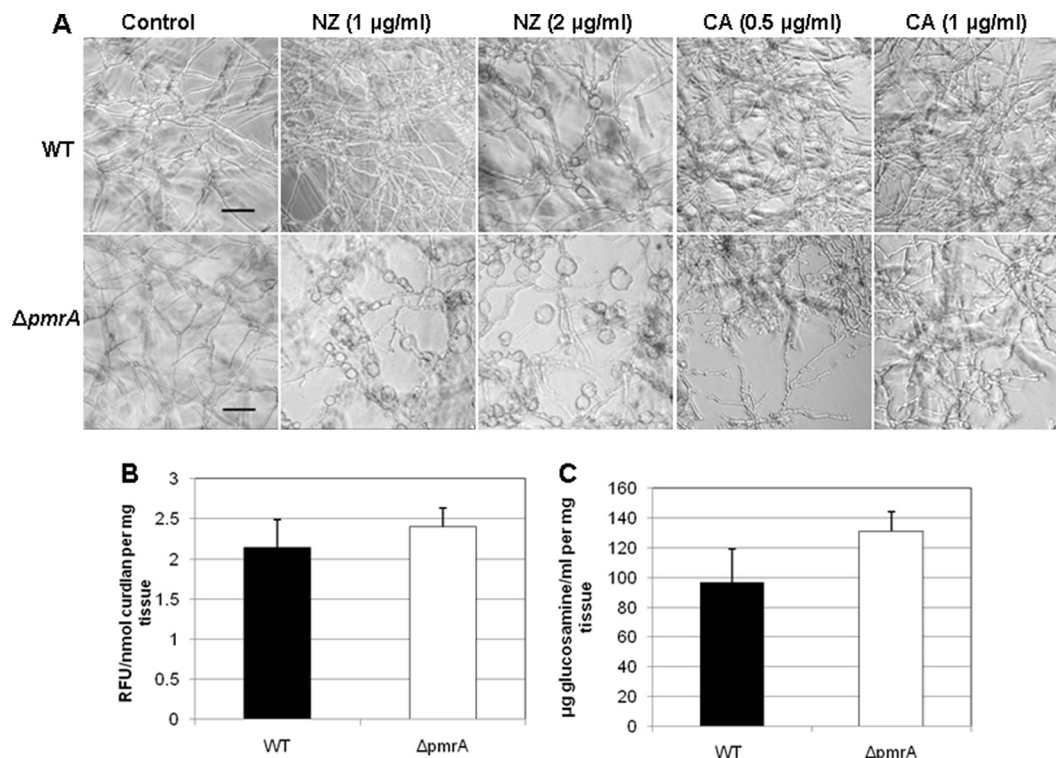


FIG. 2. (A) Effect of cell wall inhibitors on the WT and $\Delta pmrA$ strains. A total of 10^3 conidia/ml of the WT and $\Delta pmrA$ strains were inoculated onto coverslips immersed in GMM medium and grown in the presence or absence of the indicated concentrations of nikkomycin Z (NZ) or caspofungin (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).

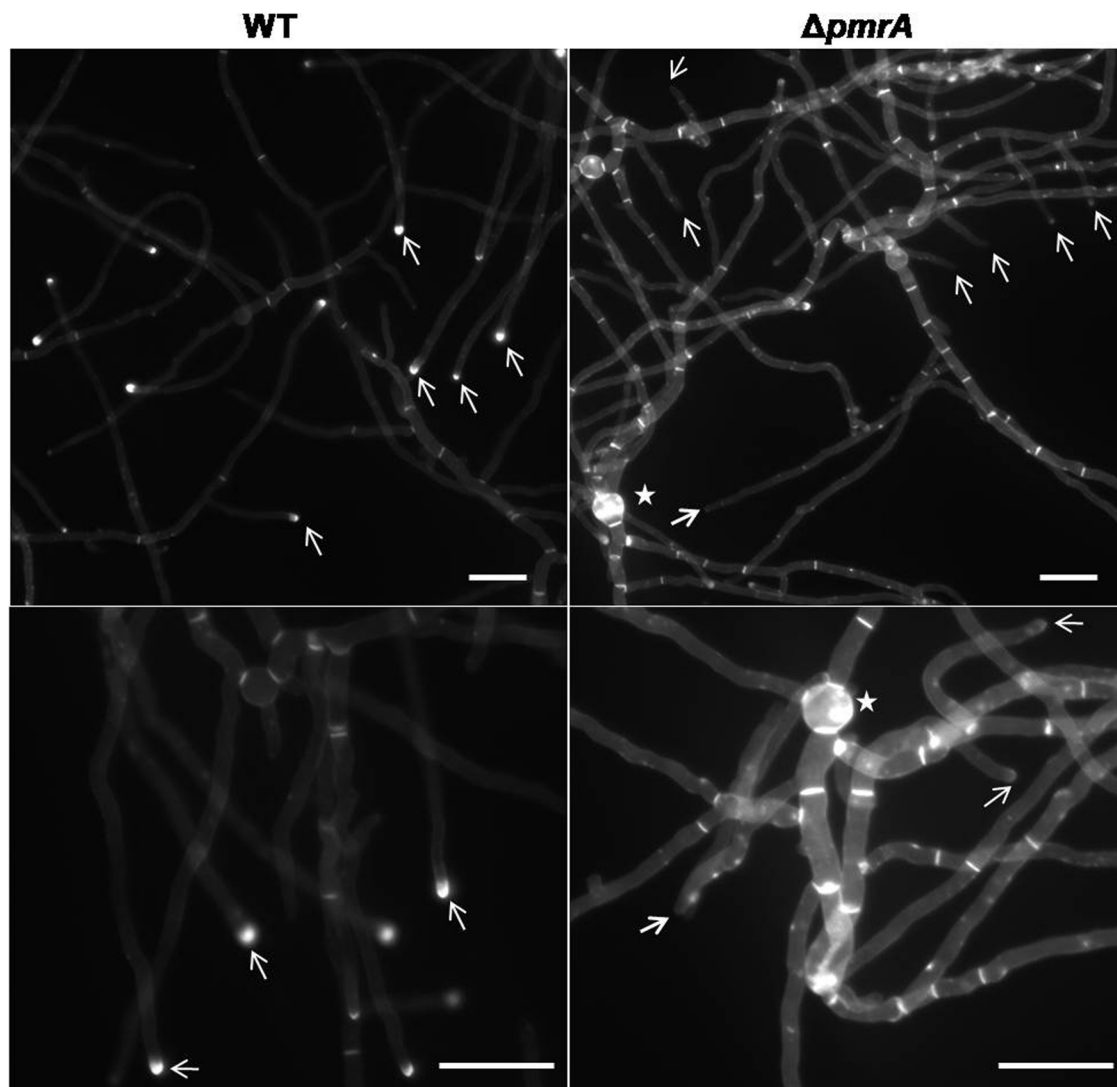


FIG. 3. Calcofluor white staining of the WT and $\Delta pmrA$ strains. A total of 10^3 conidia/ml of the WT and $\Delta pmrA$ strains were inoculated onto coverslips immersed in GMM medium and grown for 24 h at 37°C. The coverslips were rinsed in sterile water and inverted onto a 200- μ l drop of calcofluor white staining solution for 5 min at room temperature. Coverslips were then rinsed twice for 10 min in sterile water and mounted in Cytoseal 60 mounting medium. To ensure that the results of the calcofluor white staining of different samples were appropriately compared, the ultraviolet light exposure time for each sample was set at 25 ms for the WT untreated control, and the strains were exposed for the same lengths of time. Scale bar, 50 μ m.

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 $1 \times$ iQ SYBR green master mix with two biological replicates, and expression levels were normalized to those for β -tubulin. The $2^{-\Delta\Delta C_T}$ 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 $\Delta pmrA$ strain, respectively (Fig. 1D). The $\Delta pmrA$ 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 cal-

cinurin 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 $\Delta pmrA$ strain after exposure to NaCl. Furthermore, considering the role of the *Aspergillus nidulans* *sltA* gene, encoding a C_2H_2 zinc finger transcription factor, in alleviating sensitivity to salt stress (17), we also studied the expression of the homologous gene (AFUA_1G13050) in the $\Delta pmrA$ 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 Ca^{2+} and the subsequent activation of calcineurin may be helpful during salt stress, perturbed Ca^{2+} homeostasis and prolonged calcineurin activity may be harmful. Loss of Ca^{2+} ATPase activity in the $\Delta pmrA$ strain probably results in prolonged Ca^{2+} signaling, which in turn affects radial growth. Therefore, PmrA likely acts as the major Ca^{2+} 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 $\Delta pmrA$ 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 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$, respectively, for the $\Delta pmrA$ strain. As shown in Fig. 2A, the $\Delta pmrA$ 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 $\mu\text{g/ml}$) also caused distorted hyphae in the $\Delta pmrA$ strain (Fig. 2A). As the enhanced sensitivity of the $\Delta pmrA$ 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 $\Delta pmrA$ 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 pmr1* Δ 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 $\Delta pmrA$ strain (Fig. 3). Uniform accumulation of cell wall material was seen at the hyphal tips of the WT strain but not in the $\Delta pmrA$ strain, indicating that perturbed cell wall assembly leads to chitin and glucan accumulation at the septum in the $\Delta pmrA$ 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 1×10^9 conidia/ml of the WT and $\Delta pmrA$ 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* $\Delta pmr1$ strain, which was significantly attenuated in virulence (2), there was no difference in virulence between the WT and $\Delta pmrA$ 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 $\Delta pmrA$ strains (Fig. 4B). Despite the basal growth defect, different cation sensitivities, increased susceptibility to cell wall antifungals, and increased cell wall chitin and β -glu-

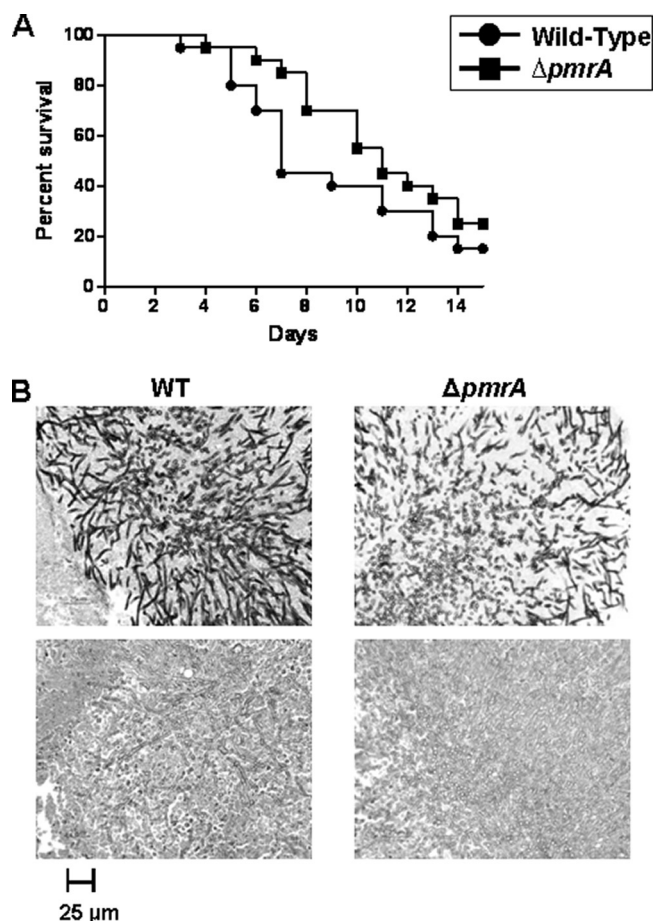


FIG. 4. Virulence analysis in a murine inhalational model of invasive aspergillosis. (A) Kaplan-Meier survival curve. At day 14 postinfection, mice infected with the $\Delta pmrA$ strain displayed about 70% mortality, which is comparable to that of mice infected with the WT strain ($P = 0.1774$). (B) Lung histopathology at day 7 postinfection at $\times 400$ magnification. (Top) Gomori's methenamine silver staining demonstrated comparable extensive hyphal proliferation patterns in the lung tissue of mice infected with the WT and $\Delta pmrA$ strains. (Bottom) Hematoxylin and eosin staining shows necrosis and inflammation in the tested strains.

can, 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 $\Delta pmrA$ 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 Ca^{2+} ATPase during salt stress in *A. fumigatus*.

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