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Cloning of the *Aspergillus niger pmrA* gene, a homologue of yeast *PMR1*, and characterization of a *pmrA* null mutant

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Abstract

The *pmrA* gene, a yeast *PMR1* homologue, was isolated from *Aspergillus niger*. Sequence analysis of the *pmrA* cDNA and the genomic DNA revealed that two introns exist in the coding region, and that an open reading frame in the cDNA encodes a polypeptide of 1056 amino acids containing all the conserved regions present in P-type Ca^{2+}-ATPases. The predicted pmrA protein exhibited a high degree of sequence similarity to the Pmr1 proteins from yeasts and mammalians (50–59% identity). The expression of the *pmrA* cDNA partially restored the growth defect of *Yarrowia lipolytica pmr1* null mutant on EGTA-containing medium. This indicates that the *A. niger pmrA* gene encodes a functional homologue of the yeast P-type Ca^{2+}-ATPase involved in the secretory pathway. An *A. niger pmrA* null mutant exhibited growth retardation on EGTA-containing medium and the growth defect was overcome by adding Ca^{2+} or Mn^{2+} into the medium. This suggests an involvement of the pmrA protein in Ca^{2+} and Mn^{2+} homeostasis in *A. niger*.

Keywords: Secretory pathway Ca^{2+}-ATPase; *pmrA*-disrupted mutant; Ca^{2+} and Mn^{2+} homeostasis; *Aspergillus niger*

1. Introduction

A novel P-type Ca^{2+}-ATPase, Pmr1p, which becomes localized to the Golgi apparatus and displays features distinct from sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and plasma membrane Ca^{2+}-ATPase (PMCA), was identified in *Saccharomyces cerevisiae* a decade ago [1]. As considerable evidence indicates that *S. cerevisiae* Pmr1p plays an important role in the secretory pathway, P-type Ca^{2+}-ATPases with homologies to Pmr1p have been grouped into a third class of P-type Ca^{2+}-ATPases, secretory pathway Ca^{2+}-ATPases (SPCA) [1–5]. Several secretory pathway Ca^{2+}-ATPases have been cloned from yeasts (*S. cerevisiae*, *Yarrowia lipolytica*, *Hansenula polymorpha*, and *Kluyveromyces lactis*) [1,6–8], rat [9], and human [10]. Among them, the *PMR1* gene encoding the secretory pathway Ca^{2+}-ATPase of *S. cerevisiae* has been extensively studied and provided most of the information concerning the in vivo function of SPCA [2–4,11,12]. Mutations in the *PMR1* gene caused Ca^{2+}- or Mn^{2+}-dependent growth retardation. Moreover, strains lacking a functional *PMR1* gene showed defects in many cellular events that need Ca^{2+} or Mn^{2+} for normal functioning: protein glycosylation, ER-associated protein degradation, protein processing and sorting, suppression of various *sec* mutants blocked in ER and/or Golgi and post-Golgi transport, and suppression of oxidative damage in superoxide dismutase-deficient cells [2,3,13]. However, one of the most interesting phenotypes of the *pmr1* mutant is the supersecretion of several heterologous proteins [14]. *pmr1* null mutants of yeast strains secreted much more bovine prothymosin, bovine growth hormone, and a nonglycosylated variant of human urinary plasminogen activator than its isogenic wild-type strains although secretion levels of some other heterologous proteins did not increase at all [14–16].

In this study, we report the cloning of the *Aspergillus*

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niger pmrA gene, a yeast *PMR1* homologue. We also show that the *A. niger pmrA* gene product could functionally complement the defective growth phenotype of a *Y. lipolytica pmr1*-disrupted mutant on EGTA-containing medium. Furthermore, we describe an *A. niger pmrA*-disrupted mutant that displayed growth retardation on EGTA-containing medium, a phenotype found in *pmr1*-disrupted mutants of yeasts.

2. Materials and methods

2.1. Strains and growth conditions

The *A. niger* FGSC A888 (*cspA1 argB13 nicA1*) strain was used to clone the *pmrA* gene and construct a *pmrA*-disrupted mutant. A *pmr1* null mutant of *Y. lipolytica* (*MatA ade1 ura3 xpr2 pmr1::ADE1*) was used for complementation study [6]. *Escherichia coli DH5a* [17] was used for plasmid propagation.

A. niger strains were grown at 30°C on complete medium (CM), or minimal medium (MM) [18] supplemented with arginine and nicotinamide. *Y. lipolytica* was grown in YPD medium (1% yeast extract, 1% proteose peptone, 2% glucose) and synthetic complete (SC) medium containing 0.67% yeast nitrogen base w/o amino acid, 1% glucose and amino acids or bases required.

2.2. Recombinant DNA technology and transformation

Isolation of chromosomal DNA and plasmid DNA, Southern blotting, and other recombinant DNA techniques were essentially carried out as described by Sambrook et al. [17]. PCR was performed to generate a probe specific for the secretory pathway Ca^{2+}-ATPase with a set of degenerate primers that had been used to clone the *PMR1* genes of *Y. lipolytica* and *H. polymorpha* [6,7]. A single 2 kb PCR product was obtained, cloned into pCR2.1 (Invitrogen, USA), and subsequently used as the probe to clone the full-length *pmrA* gene.

The PCR amplified 2 kb *pmrA* gene fragment in pCR2.1 was cut with *Eco*RI and subcloned into pGEM-11Zf(+) (Promega, USA), resulting in pG11Z-PD. The 0.5 kb *Pst*I-*Spe*I fragment in the 2 kb *pmrA* gene fragment of pG11Z-PD was replaced with the 2 kb *Aspergillus nidulans argB* gene which was amplified with two primers designed to have a *Pst*I or *Spe*I site, generating a *pmrA* disruption vector, pG11Z-PD-I. Plasmid pIMR53-AnpmrA, which was used for the complementation study, was constructed by inserting the *pmrA* cDNA into pIMR53-AUX, a low-copy replicative *Y. lipolytica* plasmid that has the *URA3* gene as a selection marker. The *pmrA* cDNA was inserted between the *XPR2* promoter and terminator such that the expression of the *pmrA* cDNA could be controlled by the *XPR2* promoter which is turned on at pH 6.8 by proteose peptone [19].

Reverse transcription was performed for 1 h at 48°C using an RT-PCR kit (Stratagene, USA) according to the manufacturer's instructions. The primers used for the reverse transcription were the random primers supplied by the manufacturer and the synthesized oligonucleotide corresponding to the 3′-UTR of *pmrA* (PMRCT, 5′-CTATACATATAGCCTATAACC-3′).

For the transformation of *A. niger*, approximately 2×10^6 spores, collected from the cells grown on CM plates for 2 days, were inoculated into 100 ml liquid CM in a 500 ml flask and cultured for 16–18 h at 30°C. Protoplasts were prepared by treating mycelia with 5 mg ml^{-1} Novozyme-234. The protoplasts were transformed according to the method of Punt and van den Hondel [20] with a slight modification. *Y. lipolytica* transformation was carried out by the lithium acetate method [21].

2.3. DNA sequencing and analysis

Sequencing was carried out using an ABI Model 373A automated DNA sequencer (Applied Biosystems, USA). The DNA sequence data of the *pmrA* gene presented in this paper were submitted to GenBank with the accession number AF232827. Sequence comparisons were made using the GCG program.

3. Results and discussion

3.1. Cloning of the A. niger pmrA gene and primary structure of its deduced protein

To prepare a probe that could be used to clone a yeast *PMR1* homologue from *A. niger*, PCR was performed using the degenerate primers which had been used successfully to amplify the *PMR1* gene fragments of *Y. lipolytica* and *H. polymorpha* [6,7]. We obtained one PCR product approximately 2 kb in length and in which the deduced amino acid sequence was homologous to those of the yeasts' *PMR1* genes. The PCR fragment was used as a probe to clone the entire gene in *A. niger*. We digested *A. niger* chromosomal DNA completely with several restriction enzymes and analyzed the Southern hybridization patterns. Among the bands exhibiting a hybridization signal, 6 kb fragments (*Bam*HI-*Hin*dIII) were chosen to prepare a partial genomic library. Plasmids were obtained from the positive clones isolated by colony hybridization of the partial library and the sequences encompassing the *PMR1* homologous gene were determined from both strands (Fig. 1).

To investigate if introns were present in the gene, the sequence of the cDNA obtained by RT-PCR was compared with that of the genomic DNA clone. Alignment of the cDNA and genomic DNA sequences showed that two introns interrupted the open reading frame (ORF) responsible for encoding a 1056 amino acid polypeptide

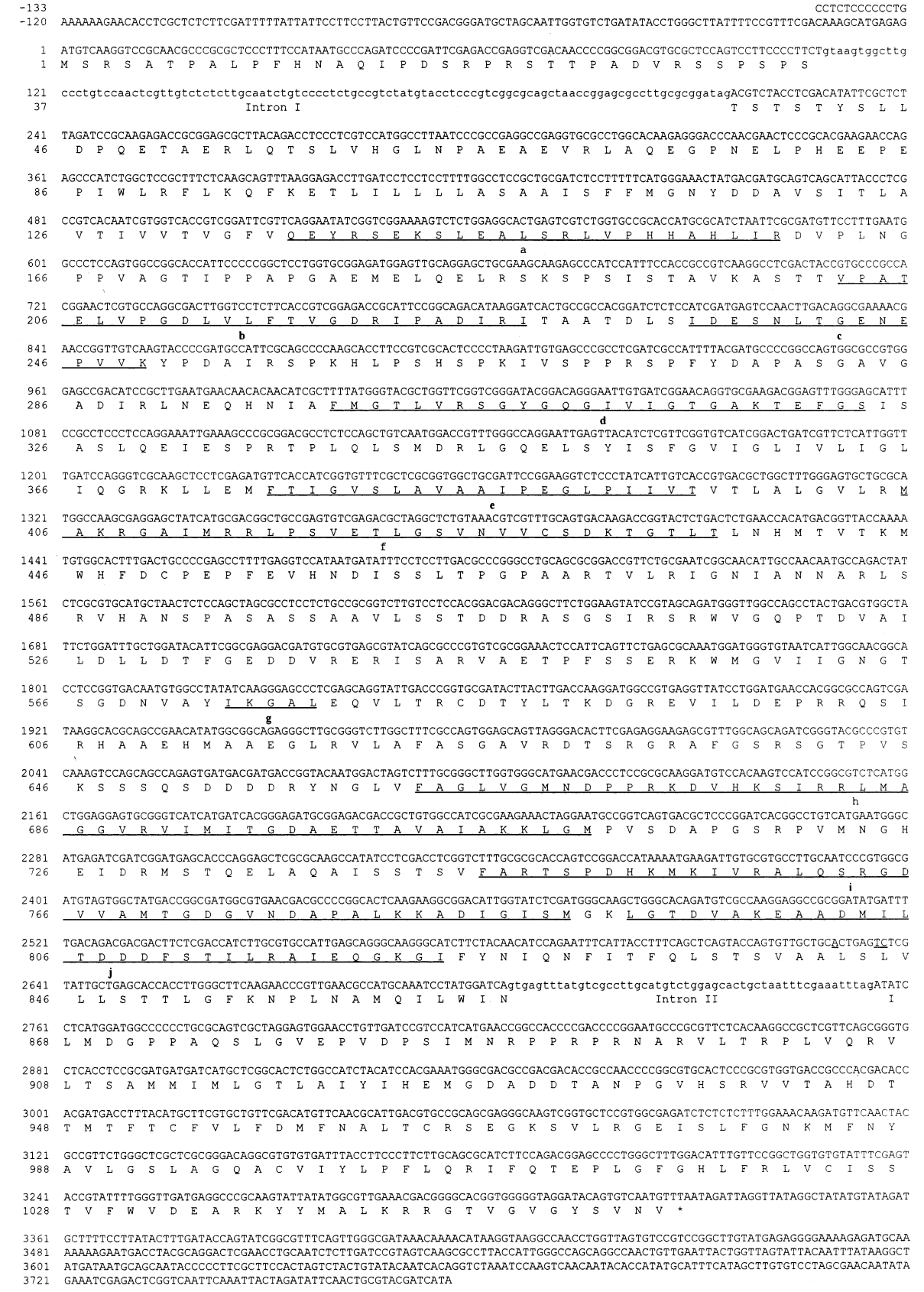

Fig. 1. Nucleotide sequence of the *A. niger pmrA* gene (GenBank AF232827) and its predicted amino acid sequences in single-letter code. The positions of the introns were deduced by alignment with the cDNA sequence. The introns are given in lowercase. The amino acid sequences corresponding to the regions (a–j) conserved in all P-type ATPases are underlined.

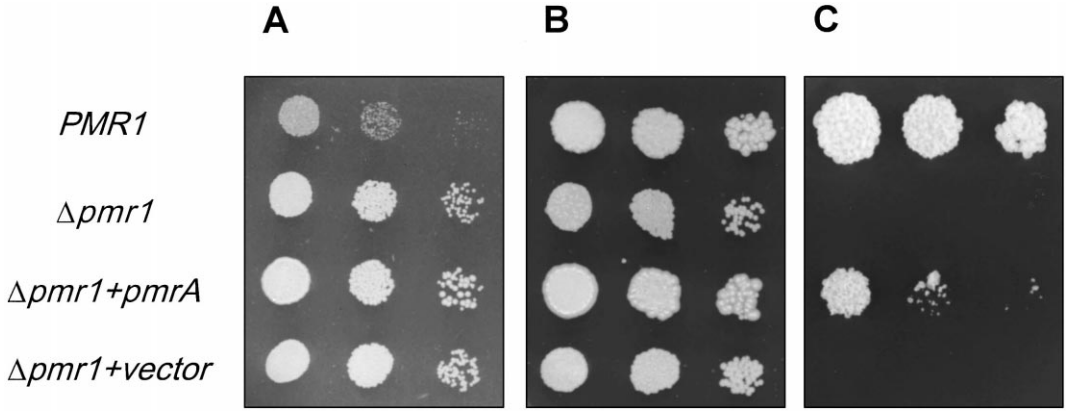

Fig. 2. Functional complementation of *Y. lipolytica pmr1* mutation by *A. niger pmrA* cDNA. The *pmr1* null mutant strain of *Y. lipolytica* was transformed with the control plasmid pIMR53-AUX and the plasmid pIMR53-AnpmrA containing the *A. niger pmrA* cDNA. The strains were suspended in water and serially diluted. The suspensions were spotted in parallel on SC medium (pH 6.8) (A), SC medium (pH 6.8) containing 2% proteose peptone (B), and SC medium (pH 6.8) containing 5 mM EGTA and 2% proteose peptone (C). The plates were incubated at 28°C for 2 days.

with a calculated molecular mass of 113 877 Da and a theoretical p*I* of 7.1 (Fig. 1). The two introns have the following consensus sequences that are required for intron excision in *A. niger*: a 5′-splice site (5′-GTPuNGT3-′), an internal lariat site (5′-PuCTPuAC3-′), and a 3′-splice site (5′-PyAG3-′) [22].

The ORF of the cDNA has all 10 conserved regions (a–j) of the P-type ATPase family (Fig. 1). Comparison of the amino acid sequences showed that the ORF has high sequence identities with other secretory pathway Ca^{2+}-ATPases (58.7%, 57.1%, 56.1%, 55.8%, 51.7%, and 50.4% with *H. polymorpha*, *S. cerevisiae*, *Y. lipolytica*, *K. lactis*, rat, and human, respectively). In contrast, relatively low sequence similarities were observed in comparisons with the sarco/endoplasmic reticulum Ca^{2+}-ATPases and plasma membrane Ca^{2+}-ATPases. These results suggested that the cloned gene product was a member of the secretory pathway Ca^{2+}-ATPase family. The cloned gene was therefore designated as the *pmrA* gene of *A. niger*.

3.2. Complementation of *Y. lipolytica pmr1* mutation by *A. niger pmrA*

In order to investigate whether the *A. niger pmrA* gene is a functional homologue of the yeast *PMR1* gene, we examined the effect of *pmrA* expression on the growth phenotype of the *Y. lipolytica pmr1* mutant in EGTA-containing medium. We introduced the pIMR53-AnpmrA plasmid carrying the *pmrA* cDNA (see Section 2) into a *Y. lipolytica pmr1* null mutant. Four *Y. lipolytica* strains, *PMR1* wild-type, *pmr1* mutant, *pmr1* mutants carrying pIMR53-AnpmrA, or a control plasmid pIMR53-AUX, were inoculated onto synthetic complete media with or without 5 mM EGTA. Some of the media were also supplemented with 2% proteose peptone to induce the expression of the *pmrA* cDNA from the *XPR2* promoter [19]. The *pmr1* mutant strain bearing pIMR53-AnpmrA partially overcame the defective growth when grown in the EGTA-containing medium supplemented with proteose peptone (Fig. 2), while the other two *pmr1* mutant strains exhibited growth characteristics associated with the defective growth phenotype. This result suggests that the expression of the pmrA protein could, at least in part, complement the defective growth phenotype of *Y. lipolytica* caused by *PMR1* gene disruption. Along with the high degree of sequence identity with other secretory pathway Ca^{2+}-ATPases, the functional complementation of the *Y. lipolytica pmr1* mutation by the *A. niger pmrA* gene clearly demonstrates that the *A. niger pmrA* gene product indeed belongs to the family of the secretory pathway P-type Ca^{2+}-ATPases.

3.3. Characterization of a *pmrA*-disrupted mutant strain

To study the in vivo role of the pmrA protein in *A. niger*, we attempted to construct a *pmrA* null mutant of *A. niger* in which the wild-type *pmrA* gene was replaced with a disrupted *pmrA* allele. *A. niger* was transformed directly with the 3.5 kb DNA fragment, the *argB* gene flanked by the *pmrA* gene fragments, which was amplified by PCR using pG11Z-PD-I as the template. After selecting $argB^+$ transformants, we performed PCR to identify homologous recombinants among the transformants. One transformant out of 25 candidates was found to give rise to the PCR products (3.5 kb and 2 kb in size) expected for the *pmrA* disruptant, which suggested that the transformant had the disrupted *pmrA* gene. Southern blotting result confirmed that a homologous recombination event only occurred at the *pmrA* gene (data not shown).

Since the primary characteristic of the yeast *pmr1* mutant is defective growth in the Ca^{2+}-deficient medium, we tested if the *pmrA*-disrupted mutant of *A. niger* had the similar phenotypic characteristics. The wild-type strain ($argB^-$), the *pmrA*-disrupted mutant ($argB^+$), and two transformants ($argB^+$) that did not carry the *pmrA*-disrupted allele, were grown on the medium containing 0,

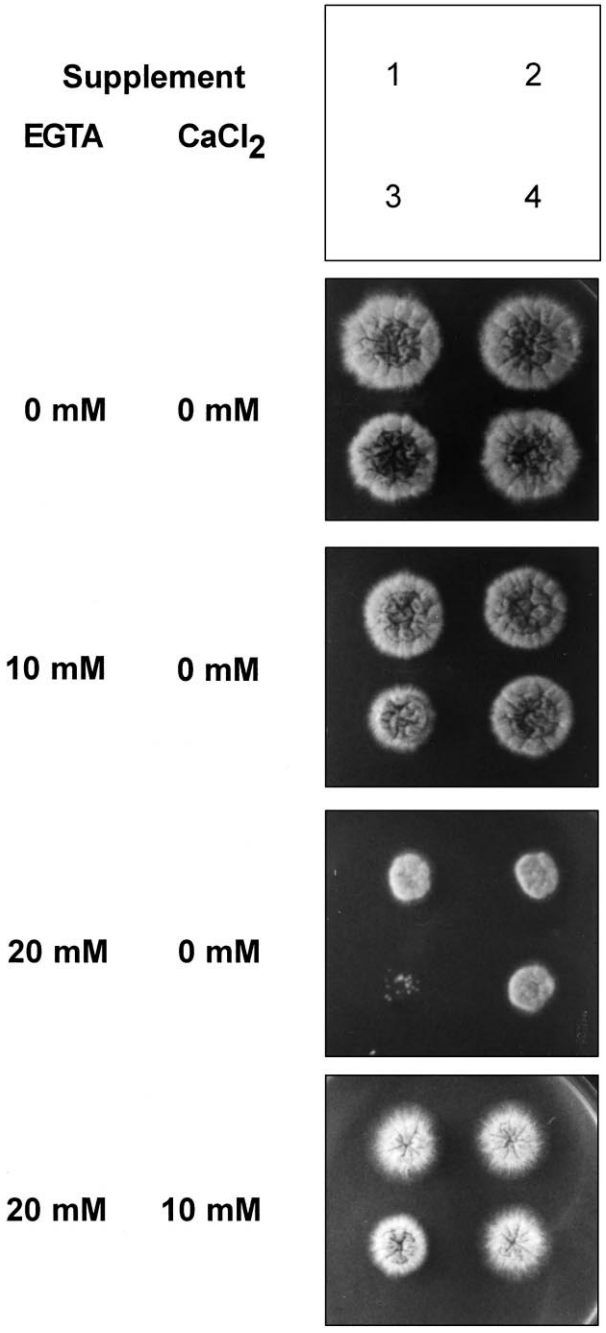

Fig. 3. Growth properties of the *pmrA*-disrupted mutant of *A. niger* on EGTA-containing medium. 1, *pmrA* wild-type strain (argB$^-$); 3, *pmrA*-disrupted mutant strain (argB$^+$); 2 and 4, *pmrA* wild-type strain (argB$^+$). The strains were grown in complete medium containing 0, 10, or 20 mM EGTA and in complete medium containing 20 mM EGTA and 10 mM Ca^{2+}. The plates were incubated at 30°C for 2 days.

10, or 20 mM EGTA. As shown in Fig. 3, the *pmrA*-disrupted mutant could not grow on the medium containing 20 mM EGTA whereas the *pmrA* wild-type strains (argB$^-$ and argB$^+$) formed colonies on the same medium. However, the inhibitory effect of EGTA on the growth of the *pmrA* mutant was overcome by adding 10 mM Ca^{2+} into the 20 mM EGTA medium (Fig. 3). Similar results were observed when 10 mM Mn^{2+} was added to the medium (data not shown). It therefore seems likely that the *pmrA* gene plays an important role in maintaining intracellular Ca^{2+} and Mn^{2+} ion concentrations as the *PMR1* gene does in yeast [1,3,4,6].

In the *pmr1* mutant of yeasts, abnormally high cytoplasmic or low Golgi divalent cation (Ca^{2+} or Mn^{2+}) concentrations impaired glycosylation, proteolytic processing and sorting of some secretory proteins. Whether the *pmrA* mutant of *A. niger* exhibits the same defects in post-translational modification as the yeasts' *pmr1* mutants was not tested in this work but remains to be elucidated. Although it is not clearly known which of the divalent cations, Ca^{2+} or Mn^{2+}, is mainly related to the protein supersecretion phenotype of the *pmr1* mutant, the disruption of the *PMR1* gene resulted in supersecretion of some homologous and heterologous proteins in yeasts [1,3,16]. It would therefore be interesting to investigate if the *pmrA* mutant of *A. niger* is able to supersecrete some heterologous proteins, particularly therapeutically valuable ones.

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