February 2007

Identification and characterization of calcium and manganese transporting ATPase (PMR1) gene of Pichia pastoris

Michael P. Dux

Mehmet Inan

Identification and characterization of calcium and manganese transporting ATPase (*PMR1*)geneof *Pichia pastoris*

Abstract

A gene homologous to *Saccharomyces cerevisiae* *PMR*1 has been cloned in the methylotrophic yeast *Pichia pastoris*.The entire *P*. *pastoris* *PMR*1 gene (*PpPMR*1) codes a protein of 924 amino acids. Sequence analysis of the *PpPMR*1 cDNA and the genomic DNA revealed that there is no intron in the coding region. The putative gene product contains all of the conserved regions observed in P-type ATPases and exhibits 66.2%, 60.3% and 50.6% identity to *Pichia angusta (Hansenula polymorpha)*, *Saccharomyces cerevisiae* *PMR*1 and human *ATP2C*1 gene products, respectively. A *pmr*1 null mutant strain of *P*. *pastoris* exhibited growth defects in media with the addition of EGTA, but with supplementation of Ca^{2+} to a calcium-deficient media reversed the growth defects of the mutant strain. Manganese reversed the growth defects of the mutant strain; however, the cell growth was not as profound as the Ca^{2+}-supplemented media. The results demonstrated that the *P*. *pastoris* gene encodes the functional homologue of the *S*. *cerevisiae* *PMR*1 gene product, a P-type Ca^{2+}/Mn^{2+}-ATPase. The DNA sequence of the *P*. *pastoris* *PMR*1 gene has been submitted to GenBank under Accession No. DQ239958.

Keywords: Pichia pastoris; PpPMR1 ; ATPase; calcium; manganese

Introduction

A number of ion-motive ATPases have been identified in a broad variety of organisms, from yeast to human. They have been group into four major classes, the F-, V-, P-and ABC-type ATPases (Pederson, 2002). The P-type Ca^{2+} ATPases, having very distinct biochemical characterization from the other ATPases, includes secretory pathway Ca^{2+} ATPases (SPCA), sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA), and plasma-membrane Ca^{2+} pumps (PMCA) (Ton *et al*., 2002). The secretory pathway Ca^{2+} ATPase (SPCA) or their yeast counterparts (*PMR*1 pumps) have attracted attention recently when Hailey–Hailey disease was linked to a mutation in the human *ATP2C*1 gene (Hu *et al*., 2000; Sudbrak *et al*., 2000).

The yeast *PMR*1 gene product was localized only in later compartments of the secretory pathway (Antebi and Fink, 1992; Sorin *et al*., 1997) whereas SERCA pumps are expressed both in the ER and in the Golgi complex (Wuytack *et al*., 2003). The yeast *pmr*1 mutants fail to concentrate Ca^{2+} and Mn^{2+} within the Golgi secretory pathway, leading to defective growth in media containing BAPTA or EGTA (Rudolph *et al*., 1989; Wei *et al*., 2000). Addition of extracellular Ca^{2+} reversed these defects in *pmr*1 mutant yeast strains (Antebi and Fink, 1992). Given the central importance of Ca^{2+} as an intracellular messenger, it should not be surprising that complex mechanisms exist in cells to manage and control Ca^{2+}. Much of the Ca^{2+} accumulates in the endoplasmic reticulum and calciosomes. Ca^{2+} is released when messenger signals are generated. These signals are translated into desired intracellular responses by calcium-binding proteins, which in turn regulate many cellular processes, including secretion of proteins. Recently, it has been shown that, in addition to its role as Ca^{2+} and Mn^{2+} pumps, the *PMR*1 gene product plays a central role with cellular functions, such as glycosylation, sorting (Rudolph *et al*., 1989), endoplasmic reticulum-associated degradation (ERAD) (Durr *et al*., 1998; Ramos-Castaneda *et al*., 2005), salt tolerance (Park *et al*., 2001), cell shape (Cortes *et al*., 2004) and virulence (Bates *et al*., 2005). The *pmr*1 mutant strains were also evaluated for improved secretion of heterologous recombinant protein productions (Ko *et al*., 2002; Sohn *et al*., 1998).

*P. pastori*s has become an organism of interest for heterologous protein expression for industrial applications. This organism is also suitable for studying cellular processes such as peroxisome biogenesis and protein secretion (Johnson *et al*., 1999; Mogelsvang *et al*., 2003; Payne *et al*., 2000). In this study, we report identification of the *Pichia pastoris PMR*1 gene (*PpPMR*1), which codes for a *S. cerevisiae Pmr1*p homologue and phenotypic characterization of the *PpPMR1*-disrupted mutant strain.

Materials and methods

Strains, media and standard methods

The *P. pastoris* strain used in this study was GS115 (*his*4). *Escherichia coli TOP1*0 cells and the plasmid pAO815 were purchased from Invitrogen (Carlsbad, CA). *S. cerevisiae* AAY247 (a gift from Audrey Atkin, University of Nebraska, Lincoln, NE) was used as a positive control for PCR. The medium used for cell growth was YSD (yeast extract 10 g/l, Soytone 10 g/l, dextrose 20 g/l) or minimal dextrose (MD: yeast nitrogen base with ammonium sulphate without amino acids 1.34 g/l, dextrose 10 g/l, biotin 0.0004 g/l). MDH medium consisted of MD with the addition of histidine (0.0004 g/l). Solid media were prepared with a 15 g/l agar addition. Synthetic Ca^{2+} deficient medium was prepared as MD medium with the omission of CaCl2, as previously

described in Rudolph *et al*. (1989). The only calcium ions in this medium were from calcium salt of panthothenate (0.84 μM). The cultures were grown at 30 °C in a stationary incubator on plates or with shaking at 225 rpm in a reciprocal shaker.

All DNA manipulations were performed using methods described by Sambrook *et al*. (1989). The DNA probes were labelled with digoxigenin, using a High Prime DNA Labeling and Detection Starter Kit from Roche Molecular Systems Inc. (Alameda, CA). Restriction enzymes and the Quick ligation kit were from New England Biolabs (Ipswich, MA). The SMART™ RACE cDNA Amplification Kit was from BD Clontech (Palo Alto, CA). The primers were purchased from Eurogentec North America Inc. (San Diego, CA).

Cloning of P. pastoris PMR1 gene

To identify the *P. pastoris PMR*1 gene, forward (5'GGTTGCTGCTATTCCAGAAGG-3') and reverse (5'-CCAACGGCGTAGTTGAACAT-3') primers were designed, based on the consensus sequence of the *PMR*1 DNA sequence from *S. cerevisiae* (P13586), which were highly homologous to *P. angusta* (AAC68831) and *Yarrowia lipolytica* (O43108). The genomic DNA of *P. pastoris* GS115 strain was used as a template, along with the above primers to amplify the *PpPMR*1 gene fragment. As a positive control, another PCR reaction was run using *S. cerevisiae* genomic DNA with the same primers. The expected ~1.6kb PCR product, based on the *S. cerevisiae PMR*1 gene, was gel-purified from the PCR product of *P. pastoris* genomic DNA and subcloned into a pCRII-TOPO vector. The fragment was sequenced at the University of Nebraska Lincoln Genomics Core Research Facility (GCRF) by the dideoxy method.

Total RNA of *P. pastoris* GS115 was prepared from YSD-grown cells. The cells were broken with
0.5 mm Zirconia/silica beads in a Bead Beater (BioSpec Products Inc., Bartlesville, OK) with 1 min breaking time followed by a 5 min resting interval. Total RNA was extracted with TRI reagent (Molecular Research Center Inc., Cincinnati, OH). mRNA was purified from total RNA with Oligotex Spin Column Purification Kit (Qiagen Inc., Valencia, CA).

The entire DNA sequence of PpPMR1 gene was obtained by the rapid amplification of cDNA ends (RACE) PCR method. The 5' and 3' RACE Ready cDNA was prepared following the instruction manual from the SMART RACE cDNA amplification kit (BD Biosciences, San Jose, CA). This cDNA was used as a template for 5' and 3' race PCR reactions, using a 5' RACE primer (CTGCATGGCATTCAATGGATTTGGCAGGTTG) and a 3' RACE primer (GCTGAAGGTATCGCCACTCCACTCACAG), respectively, in separate reactions. The PCR products were gel-purified and subcloned into a pGEM-T vector (Promega, Madison, WI). Insert fragments were sequenced and then assembled into one sequence. The entire *PMR*1 gene sequence was amplified from 5' Race Ready cDNA and GS115 total genomic DNA with forward (GGACAACTGTTATTTGCTTCTTTCCTGG) and reverse (GCACATGAAACTATATCTAATG) primers. The PCR products were then subcloned into the pCRIITOPO vector and the insert fragments were sequenced, with 12 primers covering the entire sequence twice.

Disruption of the PpPMR1 gene

The *PpMR*1 gene was disrupted with a DNA fragment encoding the functional *HIS*4 gene, which was excised from the plasmid pAO815 (Invitrogen) with *Bam*HI and *Bg*l II. The gel-purified fragment was inserted into the *Bgl*II site of the *PpPMR*1 gene in the pCRIIPp*PMR*1 plasmid.

Then the plasmid was digested with *Afl*II and *Sac*I restriction enzymes. The 6.1 kb linear *pmr*1 disruption fragment (Figure 3B) was gel-purified and transformed into *P. pastoris* GS115 electrocompetent cells prepared according to the method described by Wu and Letchworth (2004). The transformed cells were plated on MD + 10 mM CaCl2 plates.

The single colonies grown on MD + Ca^{2+} plates were inoculated in YSD broth and grown overnight at 30 °C. Chromosomal DNA from the yeast cells was prepared from the YSD culture using a Yeast Pure Chromosomal DNA kit (Epicentre, Madison, WI). Chromosomal DNA (3 µg) from the chosen clones was cut with *Eco*RI enzyme and then separated on a 0.8% agarose gel. The DNA fragments were transferred to a positively charged nylon membrane (BioRad, Hercules, CA). The 600 bp of *PMR*1 gene (*Eco*RI and *Bgl*II) fragment was used as a probe for Southern blot analysis by following the instructions of a High Prime DNA Labeling and Detection Starter Kit from Roche Molecular Systems, Inc. (Alameda, CA).

Results and discussion

Cloning and sequence analysis of the *P. pastoris PMR*1 gene

The PMR1 genes of several yeasts, including S. *cerevisiae* (Rudolph *et al*., 1989), *Y. lipolytica* (Park *et al*., 1998), *H. polymorpha* (Kang *et al*., 1998), *Neurospora crassa* (Benito *et al*., 2000), *Aspergillus niger* (Yang *et al*., 2001), *Schizosaccharomyces pombe* (Cortes *et al*., 2004), *Candida albicans* (Bates *et al*., 2005) and *Aspergillus fumigatus* (Soriani *et al*., 2005) have been identified and characterized. The *pmr*1 null mutants of these yeasts showed growth defects in media containing EGTA or BAPTA. The addition of Ca^{2+} reversed the growth defects of these mutants.

In order to clone the *PpPMR*1 gene, a PCR experiment was carried out with two primers which were specific to the *S. cerevisiae PMR*1 gene. The *S. cerevisiae PMR*1 gene-specific primers were used rather than degenerate primers, due to high homology of the DNA sequences of conserved regions from *S. cerevisiae*, *P. angusta* and *Y. lipolytica* pmr1 proteins. A 1.6 kb PCR product was obtained both from *S. cerevisiae* AAY247 and *P. pastoris* GS115 genomic DNA with the same primers. The sequence of the DNA fragment of *P. pastoris* revealed 65% homology to the *S. cerevisiae PMR*1 gene. This fragment was likely a product of the *PpPMR*1 gene. Specific RACE PCR primers were designed, based on the DNA sequence of the 1.6 kb fragment. The entire *PMR*1 gene was obtained by RACE PCR. The 5′ and 3′ RACE PCR products were sequenced and assembled to one sequence. Finally, a full-length sequence encoding the entire *PpPMR*1 gene was amplified, using first-strand cDNA and genomic DNA as templates with two specific primers. The sequences of the cDNA and genomic DNA products were identical, suggesting that the *PpPMR*1 gene does not contain any intron.

The DNA sequence of *PpPMR*1 and deduced amino acid sequence revealed that the coding region was 2772 bp, encoding 924 amino acids with a calculated molecular mass of 101 000 Da (Figure 1). The pmr1 protein contained all of the 10 highly conserved regions (a–j) of P-type ATPases (Serrano *et al*., 1986) (Figure 2). Comparison of amino acid sequences with secretory pathway ATPases revealed 66.2% and 60.3% identity with

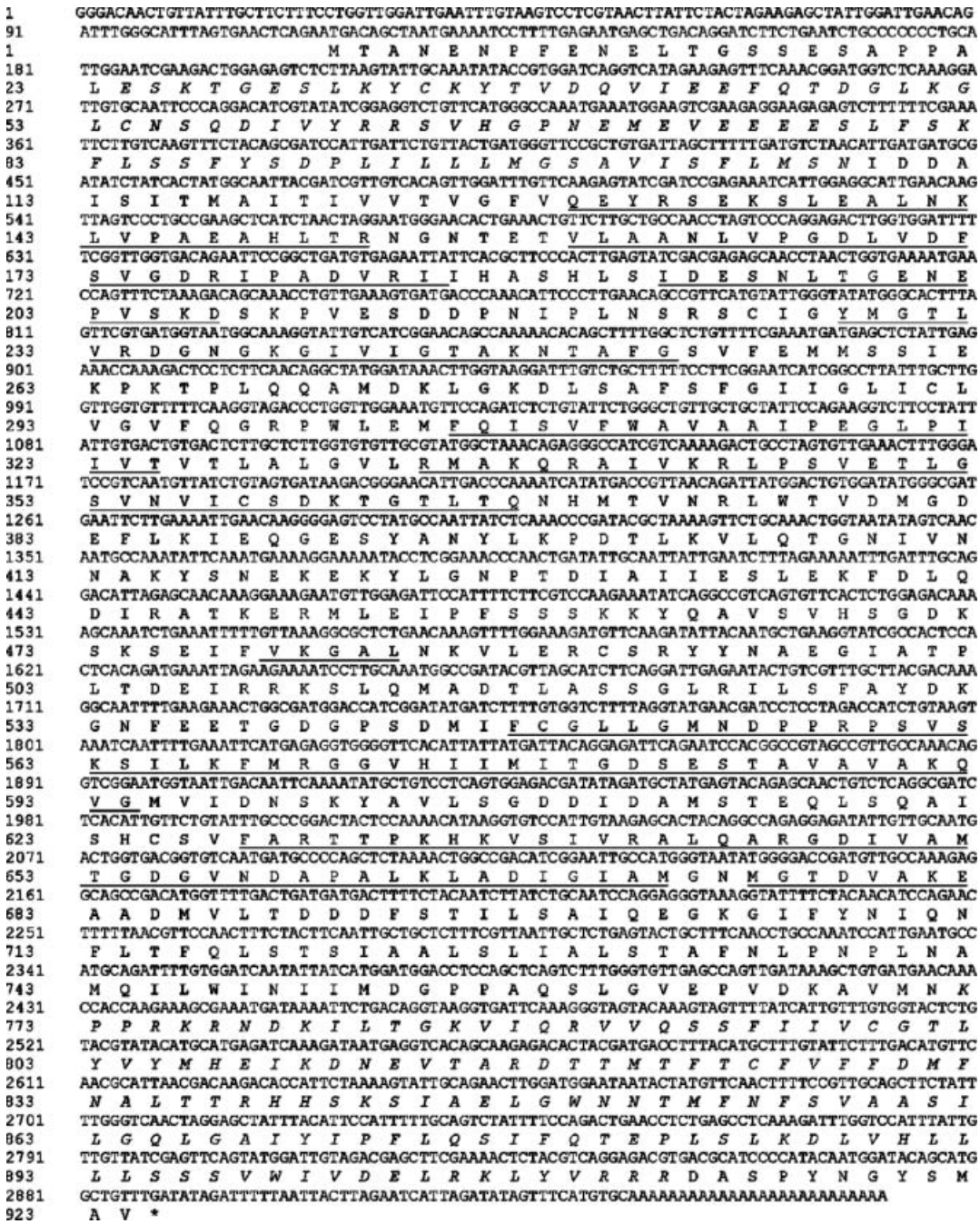

Figure **1**. Nucleotide sequence and predicted amino acid sequence of *P*. *pastori*s *PMR*1 gene (GeneBank Accession No. DQ239958). The amino acid sequences corresponding to regions a–j of ATPases are underlined (Serrano, 1988)

those of *P*. *angust*a and *S*. *cerevisi*e pmr1, respec-(regions a–j), the following putative functional tively. Relatively high sequence identity was found regions were detected: residues E318,N749 and with the human *ATP2C*1 (50.6%), human *ATP2C*2 D753 are predicted to be involved in binding of (47.2%) and mouse *ATP2C*1 (51.2%) gene prod-calcium ions, whereas Q_{758} is predicted to be ucts. Other than those highly conserved domains involved in manganese ion binding (Wei *e*t *al*.,

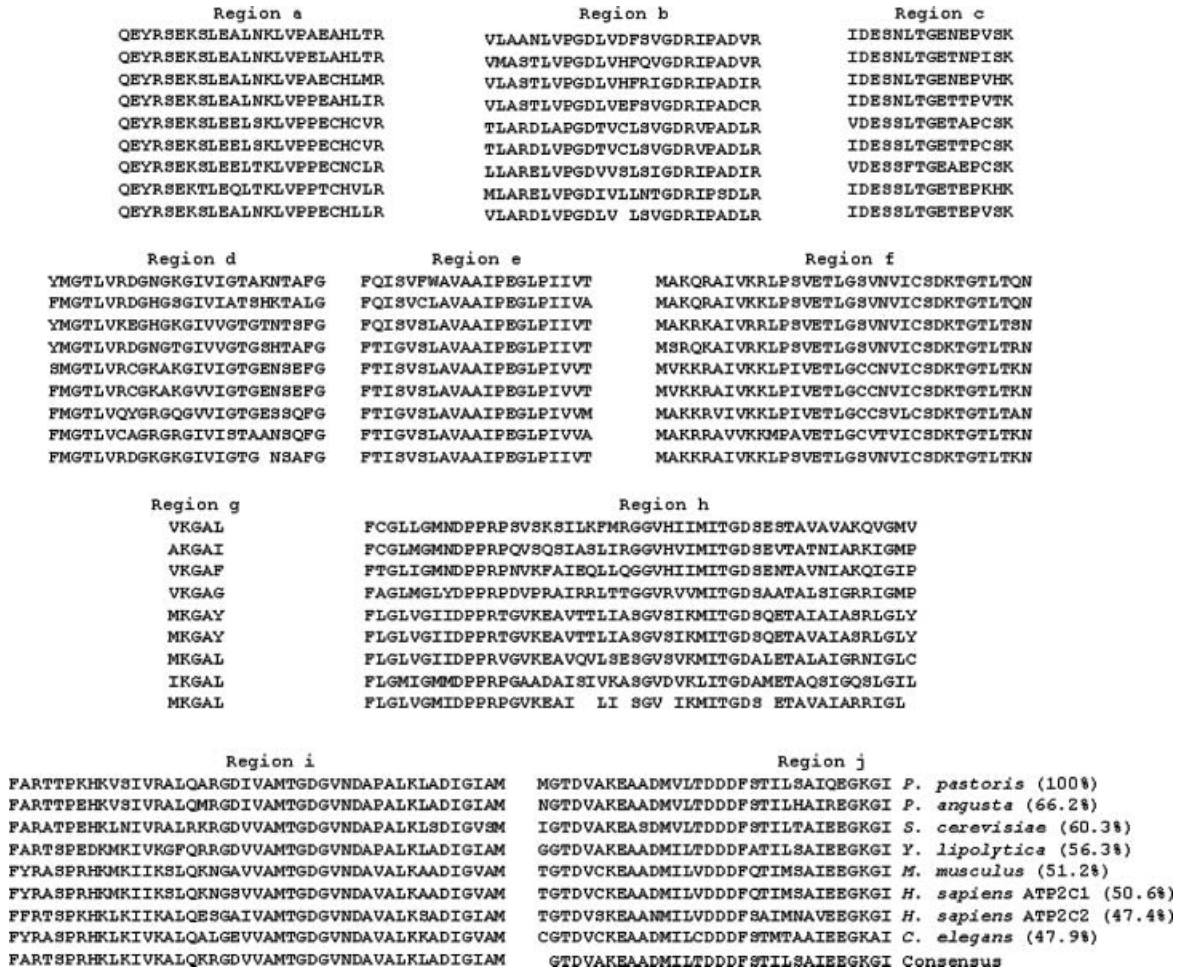

Figure **2**. Amino acid sequence alignment of with conserved domains (regions a–j) of homologous *PMR*1 genes of *P. pastori*s (ABB70815), *P. angust*a (AAC68831), *S. cerevisia*e (P13586), *Y. lipolytic*a (O43108), *M. musculu*s (CAD82864), *C. elegan*s (CAC19896), *H. sapien*s ATP2C1 (AAH28139) and *H. sapien*s ATP2C2 (NP 055676). GeneBank protein Accession Nos are given in parentheses following each organism name

2000; Mandal *et al*., 2000). These analysis results demonstrate that the *PMR*1 gene product is probably a Ca^{2+}/Mn^{2+} ATPase pump.

Disruption of PpPMR1 and gene characterization of a null mutant

To disrupt the *PpPMR*1 gene,a4 kb *Bgl*II/*Bam*HI fragment harbouring the functional *HIS*4 gene of
*P. pastori*s from the pAO815 plasmid was inserted at the internal *Bgl*II site of the *PpPMR*1 gene in the plasmid pCRII-PMR1 (Figure 3A, B). The 6.1 kb *Afl*II–*Sca*I fragment was gel-purified and transformed into the GS115 (*his*4) strain. The colonies were screened for growth on MD plates with 10 mM CaCl2. Four colonies were further characterized by Southern blot analysis, using the DIG-labelled E*co*RI/*Bgl*II (400 bp) fragment as a probe (Figure 3C). As expected, the mutant strains had a 4.5 kb band, while the host strain, GS115, had a 617 bp band with an expected intact *PMR*1 gene. This result proves that the 6.1 kb fragment disrupted the *PpPMR*1 gene, since the genomic DNA digested with *Eco*RI enzyme in Figure 3C shows the different band patterns.

After confirming that the *PpPMR*1 gene was disrupted, clone #2 was chosen for further characterization experiments. To show that mutation in the *PMR*1 gene of *P. pastori*s was

consistent with the previous *PMR*1 mutations in different yeasts,

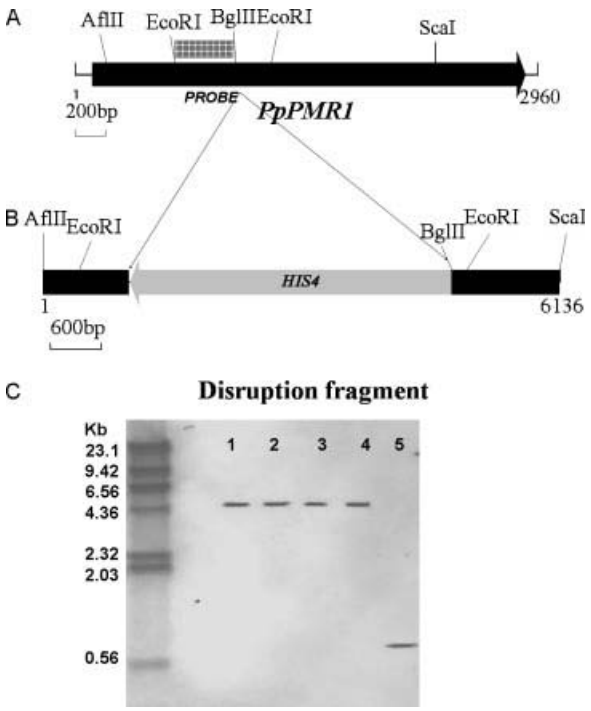

Figure **3**. Disruption strategy of the *PpPMR*1 gene. (A) Diagram of the coding region of the *PpPMR*1 gene. The 400 bp *Eco*RI–*Bgl*II fragment of *PpPMR*1 was DIG-labelled and used as a probe for Southern blot analysis. Only relevant restriction enzymes are shown. (B) *PMR*1 disruption fragment. A 4 kb *Bam*HI–*Bgl*II fragment encoding the *HIS*4 gene is inserted at the *Bgl*II site of the *PpPMR*1 gene. The 6.1 kb *Afl*II–*Sca*I fragment was transformed into *P. pastori*s GS115 strain. (C) Southern blot analysis of *P. pastori*s chromosomal DNA. Lanes 1–4, *Pppmr*1 mutant strains; lane 5, GS115. The molecular size of the DIG-labelled marker is also shown

the GS115 wild-type strain and the mutant *Pppmr*1 strain were plated on MDH plates, and MDH plates with the addition of either 40 mM EGTA or 30 mM CaCl2. As shown in Figure 4, the *pmr*1 mutant did not grow on minimal media containing EGTA, and the growth was reduced on MDH plates which had low Ca^{2+} concentration, but with the addition of 30 mM CaCl2 the *Pppmr*1 strain growth recovered and was identical as the GS115 wild-type strain. The addition of 40 mM EGTA and 30 mM CaCl2 did not affect the growth of the GS115 strain on the plates.

The mutant strains' growth characteristics were evaluated further in liquid media. Figure 5A shows growth curves of GS115 in MDH and *Pppmr*1 mutant in MD. It is clear from Figure 5A that the mutant had a longer lag time than GS115. The addition of 10 mM calcium to the medium slightly shortened the lag time by about 5 h. alternatively, the addition of EGTA (10 mM) prolonged the lag time and 40 mM EGTA stopped growth completely. These observations were in agreement with the report for other *pmr*1 mutant yeast strains (Kang *et al*., 1998; Park *et al*., 1998; Rudolph *et al*., 1989). Similar results were observed with Ca^{2+}-deficient media. The only calcium source in this medium was from the calcium salt of panthothenate, which was not enough to support the growth of the *Pppmr*1 mutant, while GS115 growth was not affected (Figure 5B). The mutant strain did not grow during the 25 h of analysis on the Ca^{2+}-deficient media. Again, the

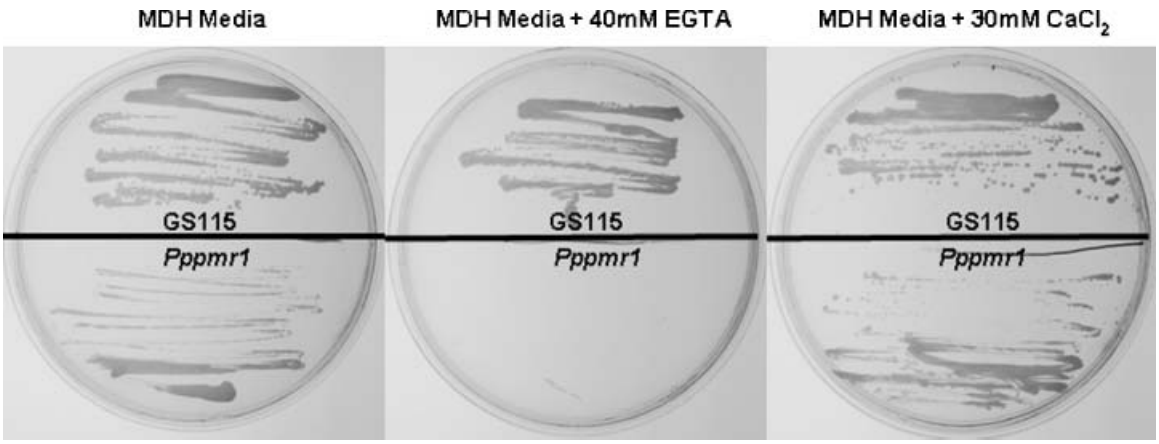

Figure **4**. Growth characteristics of *P. pastoris Pppmr*1 and GS115 on solid media. The indicated strains were grown for 2 days in YSD medium, then streaked on MDH, MDH + 40 mM EGTA, and MDH + 30 mM CaCl2 plates. The plates were incubated for 3 days at 30 °C

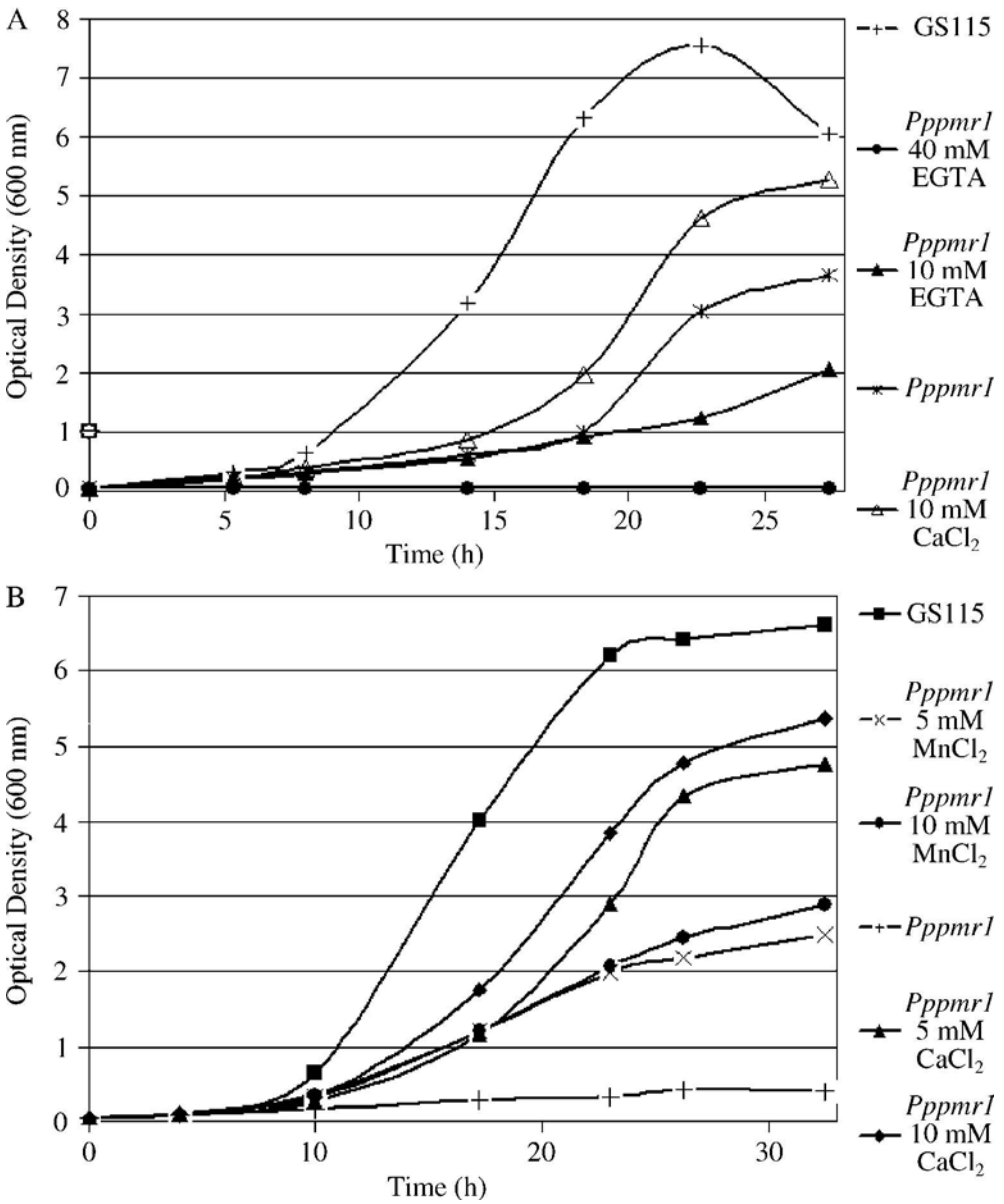

Figure **5**. Growth characteristics of *P*. *pastori*s *Pppmr*1 and GS115 on defined media. (A) MD media. Growth of GS115 wild-type in MDH and the *Pppmr*1 mutant in MD, MD + 10 mM CaCl2,MD + 10 mM EGTA, and MD + 40 mM EGTA. The results are an average of two separate experiments run in duplicate. (B) Calcium-deficient media. Growth (A600)ofGS115 wild-type in calcium-deficient medium + histidine and the *Pppmr*1 mutant in calcium-deficient medium, calcium-deficient medium + 5 and 10 mM CaCl2, and calcium-deficient medium + 5 and 10 mM MnCl2. The results are an average of two separate experiments run in duplicate

addition of 5 and 10 mM Ca^{2+} did partially recover pathway Ca^{2+}/Mn^{2+}-ATPase, which acts as a growth. The addition of 5 and 10 mM MnCl2 was Ca^{2+}/Mn^{2+} pump. ableto promote the growth of the *pmr*1 mutant, We have tested the *Pppmr*1 mutant for man-but was not as significantly as Ca^{2+}-containing ganese sensitivity, as reported in *S*. *cerevisia*e media. These results demonstrate that the *Pppmr*1 by Wei *e*t *al*. (2000). Interestingly, the *P*. *pas*-mutant strain is defective in a P-type secretion *tori*s *Pppmr*1 mutant did not show sensitivity to

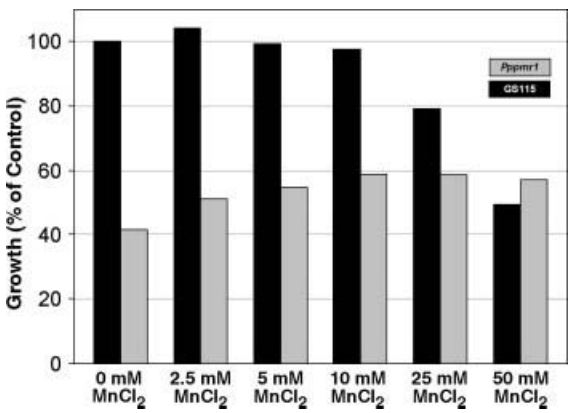

Figure **6**. Effect of MnCl2 on growth of GS115 and *Pppmr1*. Growth of GS115 wild-type in MDH and the *Pppmr*1 mutant in MD medium with varying concentrations of MnCl2 (2.5–50 mM MnCl2), was measured after 48 h and is plotted as a percentage of growth of the control GS115 culture. The results are an average of two separate experiments run in duplicate

manganese concentrations up to 50 mM in MD medium (Figure 6). Regardless of the amount of manganese in MD medium containing about 1 mM CaCl2, *Pppmr*1 showed 40–60% of growth of the GS115 strain, which was grown in MD medium without manganese supplementation. The GS115 strain showed about a 50% reduction in growth at the 50 mM MnCl2 concentration, but not *Pppmr1*. This was contrary to *S*. *cerevisia*e *pmr*1 mutants, which were shown to be hypersensitive to higher concentrations of manganese (>2mM) due to a loss of Mn^{2+} transport outside the cell through the secretory pathway. (Lapinskas *e*t *al*., 1995; Wei *e*t *al*., 2000). However, this is not surprising, since manganese toxicity was also not observed in the *C. albican*s *pmr*1 mutant (Bates *e*t *al*., 2005). Further investigation is needed to truly understand the differences between the *Pppmr*1 mutant and the mutants shown by Lapinskas *e*t *al*. (1995) and Wei *e*t *al*. (2000).

Future research will also be focused on the effect of *Pppmr*1 mutations on the secretion and processing of recombinant proteins. Mutations in the *PMR*1 gene in different types of yeast have been shown to reduce outer chain glycosylation of secreted proteins (Ko *e*t *al*., 2002; Sohn *e*t *al*.,

1998). *P. pastoris* has shown to be a very useful host system and this mutant strain should increase the usefulness in future of recombinant protein production.

Acknowledgements

We would like to thank the University of Nebraska Lincoln Biological Process Development Facility's Molecular Biology Group, Mehtap Yilmaz, Tarlan Mamedov, Sarah Plautz, Vijay Parasmal Jain and Aaron Stubbendieck, for support with this project.

References

Antebi A, Fink GR. 1992. The yeast Ca(2+)-ATPase homologue, *PMR1*, is required for normal Golgi function and localizes in a novel Golgi-like distribution. *MolBiolCel*l **3**: 633–654.

Benito B, Garciadeblas B, Rrodriguez-Navarro A. 2000. Molecular cloning of the calcium and sodium ATPases in *Neurospora crassa*. *Mo*l *Microbio*l **35**: 1079–1088.

Bates S, MacCallum DM, Bertram G, *et al*. 2005. *Candida albicans* Pmr1p, a secretory pathway P-type Ca^{2+}/Mn^{2+} ATPase, is required for glycosylation and virulence. *JBio*l *Che*m **280:** 23408–23415.

Cortes JC, Katoh-Fukui R, Moto K, Ribas JC, Ishiguro J. 2004. *Schizosaccharomyces pombe* Pmr1p is essential for cell wall integrity and is required for polarized cell growth and cytokinesis. *Eukaryot Cel*l **3**: 1124–1135.

Durr G, Strayle J, Plemper R, *et al*. 1998. The medial-Golgi ion pump Pmr1 supplies the yeast secretory pathway with Ca^{2+} and Mn^{2+} required for glycosylation, sorting, and endoplasmic reticulum-associated protein degradation. *MolBiolCel*l **9**: 1149–1162.

Hu Z, Bonifas JM, Beech J, *et al*. 2000. Mutations in *ATP2C1*, encoding a calcium pump, cause Hailey–Hailey disease. *Nat Gene*t **24**: 61–65.

Johnson MA, Waterham HR, Ksheminska GP, *et al*. 1999. Positive selection of novel peroxisome biogenesis-defective mutants of the yeast *Pichia pastoris*. *Genetic*s **151**: 1379–1391.

Kang HA, Kim JY, Ko SM, *et al*. 1998. Cloning and characterization of the *Hansenula polymorpha* homologue of the *Saccharomyces cerevisiae PMR*1 gene. *Yeas*t **14**: 1233–1240.

Ko JH, Hahm MS, Kang HA, Nam SW, Chung BH. 2002. Secretory expression and purification of *Aspergillus niger* glucose oxidase in *Saccharomyces cerevisiae* mutant deficient in *PMR*1 gene. *Protein Expr Purif* **25**: 488–493.

Lapinskas PJ, Cunnungham KW, Liu XF, Fink GR, Culotta VC. 1995. Mutations in *PMR*1 suppress oxidative damage in yeast cells lacking superoxide dismutase. *Mo*l *Cel*l *Bio*l **15**: 1382–1388.

Mandal D, Woolf TB, Rao R. 2000. Manganese selectivity of Pmr1, the yeast secretory pathway ion pump, is defined by residue Gnl783 in transmembrane segment 6; residue Asp778 is essential for cation transport. *JBio*l *Che*m **275**: 23933–23938.

Mogelsvang S, Gomez-Ospina N, Soderholm J, Glick BS, Staehelin LA. 2003. Tomographic evidence for continuous turnover of Golgi cisternae in *Pichia pastoris*. *Mo*l *Bio*l *Cel*l **14**: 2277–2291.

Park CS, Kim JY, Crispino C, Chang CC, Ryu DD. 1998. Molecular cloning of *YlPMR1*,a *S. cerevisiae PMR*1 homologue encoding a novel P-type secretory pathway Ca^{2+}-ATPase, in the yeast *Yarrowia lipolytica*. *Gene* **206**: 107–116.

Park SY, Seo SB, Lee SJ, Na JG, Kim YJ. 2001. Mutation in PMR1, a Ca(2+)-ATPase in Golgi, confers salt tolerance in *Saccharomyces cerevisiae* by inducing expression of *PMR2*, an Na(+)-

ATPase in plasma membrane. *JBiol Chem* **276**: 28 694–28 699.

Payne WE, Kaiser CA, Bevis BJ, *et al*. 2000. Isolation of *Pichia pastoris* genes involved in ER-to-Golgi transport. *Yeast* **16**: 979–993.

Pederson PL. 2002. Transport ATPases in biological systems and relationship to human disease: a brief overview. J *Bioenerg Biomemb* **34**: 327–332.

Ramos-Castaneda J, Park YN, Liu M, *et al*. 2005. Deficiency of *ATP2C1*, a Golgi ion pump, induces secretory pathway defects in endoplasmic reticulum (ER)-associated degradation and sensitivity to ER stress. *JBiolChem* **280**: 9467–9473.

Rudolph HK, Antebi A, Fink GR, *et al*. 1989. The yeast secretory pathway is perturbed by mutations in *PMR1*, a member of a Ca^{2+} ATPase family. *Cell* **58**: 133–145.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular Cloning*: A *Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Serrano R. 1988. Structure of proton translocating ATPase in plasma membranes of plants and fungi. *Biochim Biophys Acta* **947:** 1–28.

Sohn YS, Park CS, Lee SB, Ryu DD. 1998. Disruption of *PMR1*, encoding a Ca^{2+}-ATPase homolog in *Yarrowia lipolytica*, affects secretion and processing of homologous and heterologous proteins. J *Bacteriol* **180**: 6736–6742.

Soriani FM, Martins VP, Magnani T, *et al*. 2005. A PMR1like calcium ATPase of *Aspergillus fumigatus*: cloning, identification and functional expression in *S. cerevisiae*. *Yeast* **22:** 813–824.

Sorin A, Rosas G, Rao R. 1997. PMR1, a Ca^{2+}-ATPase in yeast Golgi, has properties distinct from sarco/endoplasmic reticulum and plasma membrane calcium pumps. *JBiolChem* **272**: 9895–9901.

Sudbrak R, Brown J, Dobson-Stone C, *et al*. 2000. Hailey–Hailey disease is caused by mutations in *ATP2C*1 encoding a novel Ca(2+) pump. *Hum Mol Genet* **9**: 1131–1140.

Ton VK, Mandal D, Vahadji C, Rao R. 2002. Functional expression in yeast of the human secretory pathway Ca(2+), Mn(2+)-ATPase defective in Hailey-Hailey disease. *JBiol Chem* **277**: 6422–6427.

Wei Y,Chen J,Rosas G, *et al*. 2000. Phenotypic screening of mutations in Pmr1, the yeast secretory pathway Ca^{2+}/Mn^{2+} ATPase, reveals residues critical for ion selectivity and transport. *JBiol Chem* **275**: 23927–23932.

Wu S, Letchworth GJ. 2004. High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol. *Biotechniques* **36**: 152–154.

Wuytack F, Raeymaekers L, Missiaen L. 2003. PMR1/SPCA Ca^{2+} pumps and the role of the Golgi apparatus as a Ca^{2+} store. *Pflugers Arch* **446**: 148–153.

Yang J, Kang HA, Ko SM, *et al*. 2001. Cloning of the *Aspergillus niger PMR*A gene, a homologue of yeast *PMR1*,and characterization of a *pmr*A null mutant. *FEMS Microbiol Lett* **199:** 97–102.