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

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Abstract

A gene homologous to Saccharomyces cerevisiae PMR1 has been cloned in the methylotrophic yeast Pichia pastoris. The entire P. pastoris PMR1 gene (PpPMR1) codes a protein of 924 amino acids. Sequence analysis of the PpPMR1 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 PMR1 and human ATP2C1 gene products, respectively. A pmr1 null mutant strain of P. pastoris exhibited growth defects in media with the addition of EGTA, but with supplementation of Ca²⁺ 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 Ca²⁺-supplemented media. The results demonstrated that the P. pastoris gene encodes the functional homologue of the S. cerevisiae PMR1 gene product, a P-type Ca²⁺/Mn²⁺-ATPase. The DNA sequence of the P. pastoris PMR1 gene has been submitted to GenBank under Accession No. DQ239958.

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

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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 Ca²⁺ ATPases, having very distinct biochemical characterization from the other ATPases, includes secretory pathway Ca²⁺ ATPases (SPCA), sarco/endoplasmic reticulum Ca²⁺ ATPases (SERCA), and plasma-membrane Ca²⁺ pumps (PMCA) (Ton *et al.*, 2002). The secretory pathway Ca²⁺ 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 pmr1 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 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 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 pmr1 mutant strains were also evaluated for improved secretion of heterologous recombinant protein productions (Ko et al., 2002; Sohn et al., 1998).

P. pastoris 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 (*hi4*). *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 Ca²⁺ 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 μ M). 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. cerevisia*e (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. cerevisia*e genomic DNA with the same primers. The expected 1.6kb PCR product, based on the *S. cerevisia*e PMR1 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 3 RACE а 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 PMR1 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 PpMR1 gene was disrupted with a DNA fragment encoding the functional *HIS4* gene, which was excised from the plasmid pAO815 (Invitrogen) with *Bam*HI and *Bg*1 II. The gel-purified fragment was inserted into the *Bg1*II site of the *PpPMR1* gene in the pCRIIPpPMR1 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 + Ca²⁺ 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 μ g) 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 PMR1 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), Schizosaccharomycess pombe (Cortes *et al.*, 2004), Candida albicans (Bates *et al.*, 2005) and Aspergillus fumigatus (Soriani *et al.*, 2005) have been identified and characterized. The pmr1 null mutants of these yeasts showed growth defects in media containing EGTA or BAPTA. The addition of Ca²⁺ 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* PMR1 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

GGGACAACTGTTATTTGCTTCTTTCCTGGTTGGATTGAATTTGTAAGTCCTCGTAACTTATTCTACTAGAAGAGCTATTGGATTGAACAG 1 91 ATTTGGGCATTTAGTGAACTCAGAATGACAGCTAATGAAAATCCTTTTGAGAATGAGCTGACAGGATCTTCTGAATCTGCCCCCCTGCA M T A N E N P F E N E L T G S S E S A P P A 181 TTGGAATCGAAGACTGGAGAGTCTCTTAAGTATTGCAAATATACCGTGGATCAGGGTCATAGAAGAGTTTCAAACGGATGGTCTCAAAGGA L E S K T G E S L K Y C K Y T V D Q V I E E F Q T D G L K G 23 TTGTGCAATTCCCAGGACATCGTATATCGGAGGTCTGTTCATGGGCCAAATGAAATGGAAGTCGAAGAGGAAGAGAGTCTTTTTTCGAAA 271 L C N S Q D I V Y R R S V H G P N E M E V E E E E S L F 53 SK 361 TTCTTGTCAAGTTTCTACAGCGATCCATTGATTCTGTTACTGATGGGTTCCGCTGTGATTAGCTTTTTGATGTCTAACATTGATGATGATGCG 83 F L S S F Y S D P L I L L M G S A V I S F L M S N I D D A 451 ATATCTATCACTATGGCAATTACGATCGTTGTCACAGTTGGATTTGTTCAAGAGTATCGATCCGAGAAAATCATTGGAGGCATTGAACAAG 113 I S I T M A I T I V V T V G F V <u>Q E Y R S E K S L E A L N K</u> TTAGTCCCTGCCGAAGCTCATCTAACTAGGAATGGGAACACTGAAACTGTTCTTGCTGCCAACCTAGTCCCAGGAGACTTGGTGGATTTT 541 143 L V P A E A H L T R N G N T E T V L A A N L V P G D L V D TCGGTTGGTGACAGAATTCCCGGCTGATGTGAGAATTATTCACGCTTCCCACTTGAGTATCGACGAGAGCAACCTAACTGGTGAAAATGAA 631 173 <u>S V G D R I P A D V R I </u>I H A S H L S <u>I D E S N L T G E N</u> CCAGTTTCTAAAGACAGCAAACCTGTTGAAAGTGATGACCCAAACATTCCCTTGAACAGCCGTTCATGTATTGGGTATATGGGCACTTTA 721 203 P V S K D S K P V E S D D P N I P L N S R S C I G Y M G GTTCGTGATGGTAATGGCAAAGGTATTGTCATCGGAACAGCCAAAAACACAGCTTTTGGCTCTGTTTTCGAAATGATGAGCTCTATTGAG 811 <u>G</u>SVFEMMSSIE V R D G N G K G I V I G T A K N T A F G S V F E M M S S I E AMACCAAAGACTCCTCTCCAACAGGCTATGGGATAAACTTGGGTAAGGATTTGCTGCTTTTTCCTTCGGAATCATCGGCCTTATTTGCTTG 233 901 263 K P K T P L Q Q A M D K L G K D L S A F S F G I I G L I C L GTTGGTGTTTTTCAAGGTAGACCCTGGTTGGAAATGTTCCAGATCTCTGTATTCTGGGCTGTTGCTGCTATTCCAGAAGGTCTTCCTATT 991 293 V G V F Q G R P W L E M F Q I S V F W A V A A I P E G L P I 1081 ATTGTGACTGTGACTCTTGCTCTTGGTGTGTGTGCGTATGGCTAAACAGAGGGCCCATCGTCAAAAGACTGCCTAGTGTTGAAACTTTGGGA 323 <u>I V T V T L A L G V L R M A K Q R A I V K R L P S V E T L G</u> TCCGTCAATGTTATCTGTAGTGATAAGACGGGAACATTGACCCAAAATCATATGACCGTTAACAGATTATGGACTGTGGATATGGGCGGAT 1171 <u>S V N V I C S D K T G T L T Q</u> N H M T V N R L W T V D M G D 353 1261 GAATTCTTGAAAAATTGAACAAGGGGAGTCCTATGCCAATTATCTCAAACCCGATACGCTAAAAGTTCTGCAAACTGGTAATATAGTCAAC 383 E F L K I E Q G E S Y A N Y L K P D T L K V L Q T G N I V N 1351 AATGCCAAATATTCAAATGAAAAGGAAAAATACCTCGGAAACCCAACTGATATTGCAATTTTGAATCTTTAGAAAAATTTGATTTGCAG N A K Y S N E K E K Y L G N P T D I A I I E S L E K F D L Q 413 1441 GACATTAGAGCAACAAAGGAAAGAATGTTGGAGATTCCATTTTCTTCGTCCAAGAAATATCAGGCCGTCAGTGTTCACTCTGGAGACAAA 443 1531 D I R A T K E R M L E I P F S S S K K Y O A V S V H S G D K AGCAAATCTGAAATTTTTGTTAAAGGCGCTCTGAACAAAGTTTTGGAAAGATGTTCAAGATATTACAATGCTGAAGGTATCGCCACTCCA 473 SKSEIF<u>VKGAL</u>NKVLERCSRYYNAEGIATP CTCACAGATGAAATTAGAAGAAAATCCTTGCAAATGGCCGATACGTTAGCATCTTCAGGATTGAGAATACTGTCGTTTGCTTACGACAAA 1621 503 L T D E I R R K S L Q M A D T L A S S G L R I L S F A Y D K 1711 GGCAATTTTGAAGAAACTGGCGATGGACCATCGGATATGATCTTTTGTGGTCTTTTAGGTATGAACGATCCTCCTAGACCATCTGTAAGT 533 GNFEETGDGPSDMIFCGLLGMNDPPRPSVS AAATCAATTTTGAAATTCATGAGAGGTGGGGGTTCACATTATTATGATTACAGGAGATTCAGAATCCACGGCCGTAGCCGTTGCCAAACAG K S I L K F M R G G V H I I M I T G D S E S T A V A V A K Q 1801 563 1891 GTCGGAATGGTAATTGACAATTCAAAATATGCTGTCCTCAGTGGAGACGATATAGATGCTATGAGTACAGAGCAACTGTCTCAGGCGATC 593 M V I D N S K Y A V L S G D D I D A M S T E Q L S Q A 1981 TCACATTGTTCTGTATTTGCCCGGACTACTCCAAAACATAAGGTGTCCATTGTAAGAGCACTACAGGCCAGAGGAGATATTGTTGCAATG S H C S V F A R T T P K H K V S I V R A L Q A R G D I V A M ACTGGTGACGGTGTCAATGATGCCCCAGCTCTAAAACTGGCCGACATCGGAATGCCATGGGTAATATGGGGACCGATGTTGCCAAAGAG 623 2071 T G D G V N D A P A L K L A D I G I A M G N M G T D V A K E 653 2161 GCAGCCGACATGGTTTTGACTGATGATGATGACTTTTCTACAATCTTATCTGCAATCCAGGAGGGTAAAGGTATTTTCTACAACATCCAGAAC 683 A A D M V L T D D D F S T I L S A I Q E G K G I F Y N I Q N 2251 TTTTTAACGTTCCAACTTTCAACTTCCAATTGCTGCTCTTTCGTTAATTGCTCTGAGTACTGCTTTCAACCTGCCAAATCCATTGAATGCC F L T F Q L S T S I A A L S L I A L S T A F N L P N P L N A 713 2341 ATGCAGATTTTGTGGATCAATATTATCATGGATGGACCTCCAGCTCAGTCTTTGGGTGTTGAGCCAGTTGATAAAGCTGTGATGAACAAA MQILWINIIMDGPPAQSLGVEPVDKAVMNK 743 2431 773 2521 Y V Y M H E I K D N E V T A R D T T M T F T C F V F F 803 DMF 2611 N A L T T R H H S K S I A E L G W N N T M F N F S V A A S I TTGGGTCAACTAGGAGCTATTTACATTCCATTTTTGCAGTCTATTTTCCAGACTGAACCTCTGAGCCTCAAAGATTTGGTCCATTTATTG 833 2701 863 L G Q L G A I Y I P F L Q S I F Q T E P L S L K D L V H L L TTGTTATCGAGTTCAGTATGGATTGTAGACGAGCTTCGAAAACTCTACGTCAGGAGACGTGACGCATCCCCATACAATGGATACAGCATG 2791 893 L L S S S V W I V D E L R K L Y V R R R D A S P Y N G YSM 2881 A V . 923

Figure 1. Nucleotide sequence and predicted amino acid sequence of *P. pastoris 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 *ATP2C1* (50.6%), human *ATP2C2* D753 are predicted to be involved in binding of (47.2%) and mouse *ATP2C1* (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 *et al.*,

Region a		Region	b	Region c
OEYRSEKSLEALNKLVPARAHLTR				IDESNLTGENERVSK
OFVD SEKSLEAT.NKLUDELAHLTD		LAANLVPGDLVDFSVGDRIPADVR		TDESNITGETNETSK
OFVDSRKSLEALNKLUDAECHIMD		MASTLVPGDLVHFQVGDRIPADVR		IDESNLTGENERVHK
OFYRSEKSLEALNKLUPPEAHLTR		LASTIVEGDIVNERIGDRIERDIR		IDESNLTGETTPVTK
OFVRSEKSLEELSKLUPPECHCUR		ABDIADCONUCIAUCODUDADID		VDESSLTGETAPCSK
OFVESEKSLEELSKLUPPECHCUB		ARDIAPGDTVCLOVGDRVPADIR		TDESST.TGETTECSK
OFVESEKSLEELTKLUPPECNCLE				VDESSFTGEAEPCSK
OFYRSEKTLEOLTKLUPPTCHULB		LARELUGOTULINTGDETOOLE		TDESSLTGETERKHK
OFYRSEKSLEALNKLUPPECHLLB		ARELVEGDIVLENTGDRIPSDLK		TDESSLTGETERVSK
go i nobilitati di ti toni	VLAR	DTAEGDTA T:	JVGDRIFADIR	199009109191 (Mt
Region d	Region e		F	Region f
YMGTLVRDGNGKGIVIGTAKNTAFG	FQISVFWAVAAIPEGLPIIVT		MAKQRAIVKRLPSVETLGSVNVICSDKTGTLTQN	
FMGTLVRDGHGSGIVIATSHKTALG	FQISVCLAVAAIPEGLPIIVA		MAKQRAIVKKLPSVETLGSVNVICSDKTGTLTQN	
YMGTLVKEGHGKGIVVGTGTNTSFG	FQISVSLAVAAIPEGLPIIVT		MAKRKAIVRRLPSVETLGSVNVICSDKTGTLTSN	
YMGTLVRDGNGTGIVVGTGSHTAFG	FTIGVSLAVAAIPEGLPIIVT		MSRQKAIVRKLPSV	ETLGSVNVICSDKTGTLTRN
SMGTLVRCGKAKGIVIGTGENSEFG	FTISVSLAVAAIPEGLPIVVT		MVKKRAIVKKLPIV	ETLGCCNVICSDKTGTLTKN
FMGTLVRCGKAKGVVIGTGENSEFG	FTISVSLAVAAIPEGLPIVVT		MVKKRAIVKKLPIVETLGCCNVICSDKTGTLTKN	
FMGTLVQYGRGQGVVIGTGESSQFG	FTIGVSLAVAAIPEGLPIVVM		MAKKRVIVKKLPIV	ETLGCCSVLCSDKTGTLTAN
FMGTLVCAGRGRGIVISTAANSQFG	FTIGVSLAVAAIPEGLPIVVA		MAKRRAVVKKMPAVETLGCVTVICSDKTGTLTKN	
FMGTLVRDGKGKGIVIGTG NSAFG	FTISVSLAVAAIPEG	LPIIVT	MAKKRAIVKKLPSV	ETLGSVNVICSDRTGTLTKN
Region g		Re	gion h	
VKGAL	FCGLLGMNDPPRPSVSKSILKFMRGGVHIIMITGDSESTAVAVAKQVGMV			
AKGAI	FCGLMGMNDPPRPQVSQSIASLIRGGVHVIMITGDSEVTATNIARKIGMP			
VKGAF	FTGLIGMNDPPRPNVKFAIEQLLQGGVHIIMITGDSENTAVNIAKQIGIP			
VKGAG	FAGLMGLYDPPRPDVPRAIRRLTTGGVRVVMITGDSAATALSIGRRIGMP			
MRGAY	FLGLVGIIDPPRTGVKEAVTTLIASGVSIKMITGDSQETAIAIASRLGLY			
MKGAY	FLGLVGIIDPPRTGVKEAVTTLIASGVSIKMITGDSQETAVAIASRLGLY			
MKGAL	FLGLVGIIDPPRVGVKEAVQVLSESGVSVKMITGDALETALAIGRNIGLC			
IKGAL	FLGMIGMMDPPRPGAADAISIVKASGVDVKLITGDAMETAQSIGQSLGIL			
MKGAL	FLGLVGMIDPPRPGVKEAI LI SGV IKMITGDS ETAVAIARRIGL			
Perion i			Per	ion i
REGION I RADMMDENEUGIUDALOADCOTUAMMCDCUNDADALELADICIAM		MCEDUAREAADMUIEDDDESETISATOECHCI P. pastoric (100%)		
FARTTPEHKUSTURALOMRGDTUAMTGDGUNDAPALKLADIGTAM		MGTDUAKRAADMULTDDDFSTIDSAIQBGKGI P. pastoris (1000)		
PARAMERINTUDAT.BYDCDUUAMMCDCUNDADALYL.GDTCUM		TGTDUAKEASDMULTDDDESTILTATEEGKGT S cerevisias (60.28)		
PARATEBICINI VIABARAGO VIANGO GU	CONDUARDA	DMTTEDDDF STIDIA	TERGEGI V lipolation (56 28)	
FYRASPEHKMKTTKSLOKNGAUUAMTGDGUI	TGTDUCKEAADMILUDDDFOTTMSATERGKGT M. musculus (51 24)			
FVDASDDHKMKTTKSLOKNGSUUAMTGDGU	TGTDUCKEAADMILUDDDFOTTMSATEEGKGI H. sanjens ATP2C1 (50 6%)			
FERTSPEHELKITKALOESGATUAMTGDGU	TOTOVSKEA	TOTOTOTOTOTOTOTOTOTOTOTOTOTOTOTOTOTOTO		
FYRASPRHKLKTUKALOALGEUUAMTGDGU	CGTDVCKEAA	CGTDUCKEAADMILCDDDFSTMTAAIEEGKAI C. elegans (47.9%)		
FARTSPRHKLKTVKALOKRGDVVAMTGDGV	GTDUAKEAA	DMTLTDDDF9TT.9A	TERGEGT Consensus	
	A CONTRACTOR OFFICE	JIDVANDA	WITHING BITTON	

Figure 2. Amino acid sequence alignment of with conserved domains (regions a–j) of homologous *PMR*1 genes of *P. pastoris* (ABB70815), *P. angusta* (AAC68831), *S. cerevisiae* (P13586), *Y. lipolytica* (O43108), *M. musculus* (CAD82864), *C. elegans* (CAC19896), *H. sapiens* ATP2C1 (AAH28139) and *H. sapiens* 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 Ca²⁺/Mn²⁺ 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. pastoris 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 EcoRI/*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 PpPMR1 gene was disrupted, clone #2 was chosen for further characterization experiments. To show that mutation in the PMR1 gene of P. pastoris was

consistent with the previous PMR1 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 *AfIII–ScaI* fragment was transformed into *P. pastoris* GS115 strain. (C) Southern blot analysis of *P. pastoris* 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 Ca²⁺ 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 Ca²⁺ -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 Ca²⁺-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 $^{\circ}$ C



Figure 5. Growth characteristics of *P. pastoris 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 CaCl2. The results are an average of two separate experiments run in duplicate

addition of 5 and 10 mM Ca²⁺ did partially recover pathway Ca²⁺/Mn²⁺ ATPase, which acts as a growth. The addition of 5 and 10 mM MnCl2 was Ca²⁺/Mn² 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 Ca²⁺ containing ganese sensitivity, as reported in *S. cerevisia*e media. These results demonstrate that the *Pppmr*1 by Wei *et al.* (2000). Interestingly, the *P. pas*-mutant strain is defective in a P-type secretion *toris 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 *Pppmr1* 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, *Pppmr1* 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. cerevisiae pmr1* mutants, which were shown to be hypersensitive to higher concentrations of manganese (>2mM) due to a loss of Mn²⁺ transport outside the cell through the secretory pathway. (Lapinskas *et al.*, 1995; Wei *et al.*, 2000). However, this is not surprising, since manganese toxicity was also not observed in the *C. albicans pmr1* mutant (Bates *et al.*, 2005). Further investigation is needed to truly understand the differences between the *Pnpmr1* how the *Pnpmr1* how the pnpmr1 how the pnpmr1

the differences between the *Pppmr*1 mutant and the mutants shown by Lapinskas *et al.* (1995) and Wei *et 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 *et al.*, 2002; Sohn *et 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.

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