RESEARCH ARTICLE

Inactivation of the *Hansenula polymorpha PMR1* gene affects cell viability and functioning of the secretory pathway

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Abstract

In yeast, functions of the endoplasmic reticulum (ER) depend on the Golgi apparatus Ca^{2+} pool, which is replenished by the medial-Golgi ion pump Pmr1p. Here, to dissect the role of the Golgi Ca^{2+} pool in protein folding and elimination of unfolded proteins in the ER, the manifestations of the *pmr1* mutation in yeast *Hansenula polymorpha* were studied. The *PMR1* gene was disrupted in a *H. polymorpha* diploid strain. Haploid segregants of this diploid bearing the disruption allele were viable, though they showed a severe growth defect on synthetic medium and rapidly died during storage at low temperature. Disruption of *H. polymorpha PMR1* led to defects of the Golgi-hosted protein glycosylation and vacuolar protein sorting. This mutation increased the survival rate of *H. polymorpha* cells upon treatment with the proapoptotic drug amiodarone. Unlike *Saccharomyces cerevisiae*, the *H. polymorpha pmr1* mutant was not hypersensitive to chemicals that induce the accumulation of unfolded proteins in the ER, indicating that the elimination of unfolded proteins from the ER was not essentially affected. At the same time, the *pmr1* mutation improved the secretion of human urokinase and decreased its intracellular aggregation, indicating an influence of the mutation on the protein folding in the ER.

Introduction

Most of the processes taking place in the eukaryotic secretory pathway depend on Ca^{2+}. In *Saccharomyces cerevisiae*, Ca^{2+} is sequestered into the secretory organelles mainly by means of the medial Golgi P-type ATPase, Pmr1p. The loss of this protein blocks the Golgi-hosted processes, e.g. modification of oligosaccharide chains of secretory proteins and sorting of certain proteins to the vacuole (Rudolph *et al*., 1989; Durr *et al*., 1998). Unlike higher eukaryotes, yeast cells do not possess the sarco/endoplasmic reticulum Ca^{2+}-ATPase. The requirement of the endoplasmic reticulum (ER) for Ca^{2+} is partially satisfied by Pmr1p, as in the ER of the *pmr1* mutant, Ca^{2+} concentration reaches *c.* 50% of the wild-type level (Strayle *et al*., 1999). As a result, the ER functions in the *pmr1* mutant are also affected. For example, the *S. cerevisiae pmr1* mutant is hypersensitive to drugs, which disturb protein-folding in the ER, and is defective in the degradation of misfolded proteins in this compartment (Durr *et al*., 1998). However, the mechanisms of Ca^{2+} delivery to the yeast ER are still unclear. The recent study of the *Hansenula polymorpha ret1* mutant by the authors indicates that Ca^{2+} can be transported to the ER via the COPI-coated vesicles furnishing the Golgi-to-ER retrograde transport (Chechenova *et al*., 2004). Indeed, C-terminal truncation of the α subunit of the COPI complex in *H. polymorpha* led to a sensitivity to Ca^{2+} shortage and to a disturbance of the ER-hosted processes, while the Golgi functions remained unaffected. This differs *H. polymorpha* from *S. cerevisiae*, as the C-terminal domain of α-COP in *S.*

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cerevisiae is life essential (Eugster *et al.*, 2000). However, the importance of *PMR1* for Ca^{2+} homeostasis in *H. polymorpha* had not been studied yet. In particular, although *HpPMR1* was shown to complement the *S. cerevisiae pmr1* mutation (Kang *et al.*, 1998), it remained unclear whether this gene is crucial for maintenance of Ca^{2+} pools in the secretory organelles of *H. polymorpha* cells. To study this, the *H. polymorpha PMR1* gene was disrupted and the different effects of this mutation were analyzed.

Materials and methods

Strains

Standard nomenclature was used for designation of yeast genes. When necessary, *H. polymorpha* genes were marked with '*Hp*' and *PMR1* disruption alleles were designated as *pmr1*-Δ. To disrupt the *PMR1* gene, *H. polymorpha* strains were transformed with a *LEU2*-equipped *PMR1*-disruption cassette (the pBB plasmid digested with EcoRI).

The diploid MA77 used to disrupt the *PMR1* gene was obtained from the cross of two derivatives of the *H. polymorpha* strain CBS4732 (ATCC34438): 1BQ (*leu2 ade2 mox:: uPA-Q*^{302}) bearing episomal plasmid pA331 with the *HpLEU2* marker (Bogdanova *et al.*, 1998) and 8 V (*leu2*) (Agaphonov *et al.*, 1994). Prior to the transformation with the disruption cassette, the plasmid pA331 was lost from the MA77 diploid strain. The haploid strain 1MA77/12 (*leu2 ade2 mox::uPA-Q*^{302} *pmr1::LEU2*) was selected among meiotic segregants of the MA77/12 diploid transformant disrupted for the *PMR1* gene. To restore the *leu2* auxotrophic marker in the 1MA77/12 strain, the *LEU2* marker of the genomic *pmr1::LEU2* allele was replaced with the G418 resistance marker ($G418^R$). To perform this, the strain 1MA77/12 was transformed with the $G418^R$-equipped *PMR1*-disruption cassette (pAF14 digested with EcoRI). The strain with the replacement was then transformed with the plasmid pDLMOX-GOD (Kim *et al.*, 2004), bearing the *LEU2* marker and the *Aspergillus niger* glucose oxidase (GOD) expression cassette. One of the transformants expressing GOD was designated as 1MA77/12G-GOD.

Plasmids

The plasmid pE1 possessing the EcoRI–EcoRI DNA fragment of the *H. polymorpha* DL-1 *PMR1* locus has been described earlier (Kang *et al.*, 1998). The plasmid pBB deriving from pE1 was constructed by the replacement of the BamHI–BstEII fragment inside the *PMR1* ORF with the SnaBI–BamHI fragment of pCHLX carrying the *LEU2* marker (Sohn *et al.*, 1996). The plasmid pAF14 was constructed by the replacement of the StuI–NheI fragment in the pE1 plasmid with the HincII–NheI fragment of the pGAG418-43 plasmid carrying $G418^R$ (Sohn *et al.*, 1999). The plasmid pCAT1 was constructed by the insertion of the SmaI–Psp124BI fragment of the pE1 plasmid carrying the *HpPMR1* gene between the HpaI and Psp124BI sites of the p2CHA6 plasmid possessing the *HpADE2* marker (M. Agaphonov, unpublished). Prior to yeast transformation, the pCAT1 and p2CHA6 plasmids were digested with EcoRI to induce their integration into the genome.

Analyses of uPA, GOD and chitinase

A qualitative test for the ability of yeast transformants to secrete a urokinase-type plasminogen activator (uPA) was performed by the examination of their capability to form haloes during growth on fibrin-containing medium (Agaphonov *et al.*, 1995). The production of uPA in *H. polymorpha* cells was induced by cultivation in the induction medium (1% yeast extract, 3% peptone, 0.1 mM NaCl, 0.8% methanol, 0.2% glycerol), which was supplemented in some cases with ammonium phosphate buffer [25 mM $NH_4H_2PO_4$, 25 mM $(NH_4)_2HPO_4$]. Samples were incubated at 37 °C for ∼70 h if extracellular uPA was analyzed or for 40 h in the case of analysis of cell lysates. Proteins from the culture supernatants were concentrated 20-fold by precipitation with trichloroacetic acid and analyzed by Western blotting with antibody against uPA. Expression of GOD was induced by cultivation in the induction medium lacking ammonium phosphate (see above) for 70 h at 30 °C. The amounts of samples loaded on the gel were normalized to the total cellular protein. Native chitinase from supernatants of saturated YPD cultures was isolated by binding to chitin and was analyzed by polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate (SDS-PAGE) as described elsewhere (Kuranda & Robbins, 1991).

Electrophoresis, antibodies, Western blotting and *in vitro* DNA manipulations

Proteins were resolved by the SDS-PAGE in 6% polyacrylamide gel for the analyses of chitinase and GOD, or in 10% gel for uPA-Q^{302} analysis as described by (Laemmli, 1970). The immunoblots were developed using chemiluminescent detection (Amersham Pharmacia Biotech). Antiserum against carboxypeptidase Y (CPY), antibody against GOD and monoclonal antibody specific to the uPA protease domain have been described elsewhere (Kratasyuk *et al.*, 1989; Kim *et al.*, 2004; Agaphonov *et al.*, 2005a). *In vitro* DNA manipulations were carried out according to the standard procedures (Sambrook *et al.*, 1989). Yeast DNA was isolated as described by Sherman *et al.* (1986) with minor modifications: to ensure precipitation of the low molecular weight fraction, DNA was precipitated by 2 volumes of isopropanol in the presence of 200 mM NaCl.

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Genetic methods and culture conditions

Hansenula polymorpha cells were cultivated at 37 °C in complex medium YPD (1% Yeast extract, 2% Peptone, and 2% glucose) or in synthetic complete media SC-D [0.67% Yeast Nitrogen Base (Difco), supplemented with 2% glucose]. Preparation of Ca^{2+}-deficient medium, supplemented with EGTA, was described elsewhere (Rudolph *et al.*, 1989). Strains of *H. polymorpha* were crossed, and hybrids were sporulated on maltose-containing medium. *H. polymorpha* was transformed according to the modified lithium acetate method (Bogdanova *et al.*, 1995). The number of dead cells in the cell suspensions was estimated by staining with Methylene blue as described by Kucsera *et al.* (2000). The sensitivity of cells to amiodarone was studied according to the method described earlier (Pozniakovsky *et al.*, 2005) with minor modifications, as follows. Cells grown overnight on the YPD plate were inoculated into liquid YPD and cultivated for 4 h with vigorous agitation. Cell suspensions were diluted with fresh YPD to $OD_{600\,nm} = 0.2$ and amiodarone was added to the final concentration of 80 μM. Cell suspensions were incubated at room temperature for 1.5 h and serially diluted aliquots were spread on to YPD plates along with the suspension of untreated cells. The survival rate was determined as a ratio of the number of colonies produced by the treated and untreated cell suspensions.

Results

Disruption of the *PMR1* gene

It was shown that in *S. cerevisiae* the *pmr1* mutation caused an improvement in the secretion of human uPA (Melnick *et al.*, 1990), allowing the suggestion that disruption of *PMR1* in *H. polymorpha* should have the same effect. To enable the monitoring of uPA secretion upon *PMR1* inactivation, the diploid strain MA77, used for *PMR1* disruption, carried a cassette expressing the unglycosylated variant of human uPA, uPA-Q^{302}. It is expected that the decrease of the *PMR1* dosage due to the disruption of its one copy in the diploid strain would lead to some increase in uPA-Q^{302} secretion. Indeed, some of the transformants obtained with the *PMR1* disruption cassette appeared to secrete uPA-Q^{302} more efficiently. PCR analysis of these clones revealed that some of them carried the *pmr1::LEU2* disruption allele (data not shown). One of these clones, designated MA77/12, was transferred to the sporulation medium and haploid segregants were obtained. Among them, the Leu^+ clones were identified, indicating that *HpPMR1* is dispensable. The disruption of *PMR1* in several Leu^+ segregants was confirmed by Southern analysis (data not shown). One of these segregants, which carried the *ade2* auxotrophic mutation, was designated 1MA77/12 and used for further analysis.

Table 1. Influence of the *pmr1*-Δ mutation on survival of cells upon amiodorone treatment and incubation at low temperature

	Survival upon amiodarone treatment (%)	Survival at 5 °C (%)	
		3 days	6 days
PMR1	0.15	97	110
pmr1-Δ	3	37	0.53

The 1MA77/12 strain carried the plasmid with wild-type *PMR1* (*PMR1*) or the empty vector (*pmr1*-Δ). The percentage of survived cells was calculated as a ratio of the number of colonies produced by cell suspensions after and before treatment.

Viability of the *pmr1*-Δ mutant upon different physiological conditions

To obtain the pair of isogenic strains, which differed only by the *PMR1* status, the strain 1MA77/12 (*ade2 leu2 mox::uPA-*Q^{302} *pmr1::LEU2*) was transformed with the pCAT1 plasmid, bearing the *PMR1* wild-type allele, or with the p2CHA6 empty vector. Transformants bearing wild-type *PMR1* grew much faster than transformants with the empty vector, especially on synthetic medium (data not shown). This difference was at least in part due to a decreased viability of the *pmr1*-Δ mutant. Indeed, the number of dead cells accumulating methylene blue stain was four to fivefold higher in the overnight culture of the *pmr1*-Δ mutant than in the culture of the control strain (~30% vs. 6–7%). Cell suspension of the mutant strain grown in YPD produced approximately threefold fewer colonies on SC-D medium than on YPD, though the number of cells accumulating methylene blue in cultures grown in YPD and SC-D was similar (data not shown). The density of the saturated YPD culture of the mutant strain was normally approximately threefold lower than that of the strain bearing the *PMR1* wild-type allele. Mutant cells rapidly died in the saturated YPD culture upon storage at +5 °C, while the number of viable cells in the culture of the control strain was not noticeably changed (Table 1). No decrease in the number of viable cells in the same culture of the *pmr1*-Δ cells stored at room temperature for 6 days was observed (data not shown).

It was reported earlier that the *S. cerevisiae pmr1* mutation leads to an increased sensitivity to amiodarone (Gupta *et al.*, 2003), the drug inducing apoptotic cell death via disruption of calcium homeostasis (Pozniakovsky *et al.*, 2005). Surprisingly, the disruption of *PMR1* increased the survival rate of *H. polymorpha* cells upon treatment with amiodarone (Table 1).

Like the corresponding *S. cerevisiae* mutant, the *H. polymorpha pmr1*-Δ strain could not grow in a shortage of Ca^{2+} (Fig. 1). However, unlike *S. cerevisiae* (Durr *et al.*, 1998), inactivation of *PMR1* in *H. polymorpha* did not

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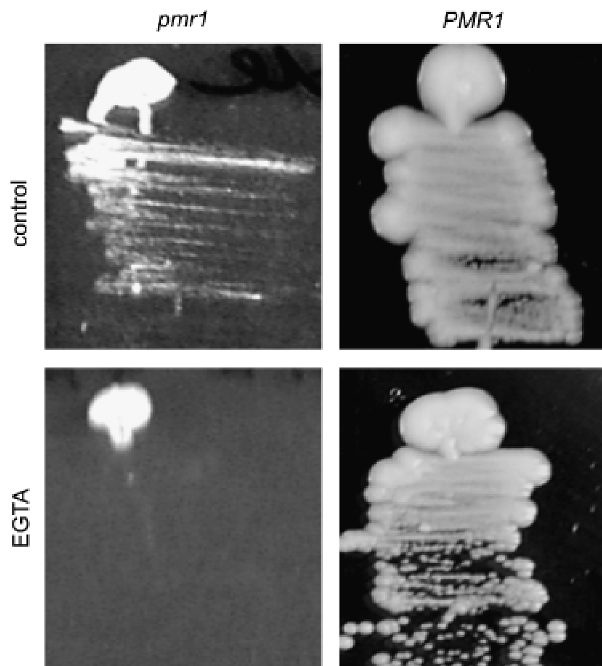

Fig. 1. The growth of the *pmr1*-Δ mutant is inhibited by a shortage of Ca^{2+} in the culture medium. Cells of the 1MA77/12 strain transformed with the plasmid pCAT1 carrying the wild-type *PMR1* (*PMR1*) or the empty vector p2CHA6 (*pmr1*) were streaked on the medium supplemented (EGTA) or not supplemented (control) with 25 mM EGTA (for details see Materials and methods).

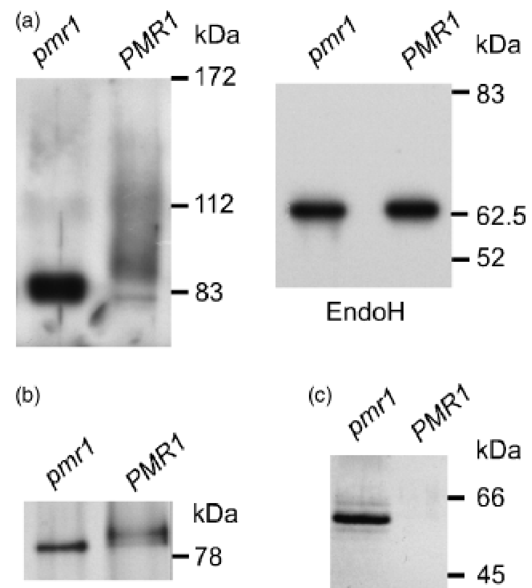

Fig. 2. Analysis of GOD, chitinase and CPY in culture supernatants of the *H. polymorpha pmr1*-Δ transformants bearing either the pCAT1 plasmid with wild-type *PMR1* (*PMR1*) or the empty vector p2CHA6 (*pmr1*). (a) Western blotting of GOD secreted by transformants of the 1MA/12G-GOD strain. Panel 'EndoH' represents samples treated with endoglycosidase H. (b) SDS-PAGE of extracellular chitinase from culture supernatants of transformants of the 1MA77/12 strain (for details see Materials and methods). (c) Western blotting of CPY in culture supernatants of transformants of the 1MA77/12 strain.

change the sensitivity of cells to dithiothreitol (DTT) and tunicamycin, the drugs that disturb protein-folding in the ER (data not shown).

Hansenula polymorpha pmr1-Δ mutation affects protein glycosylation

To analyze the dependence of protein *N*-glycosylation on Pmr1p, the *A. niger* glucose oxidase expression cassette was introduced into the genome of the *pmr1*-Δ mutant. Plasmid pCAT1, containing *PMR1*, was integrated into the genome of this strain to obtain the control strain. GOD secreted by the *H. polymorpha* cells bearing wild-type *PMR1* migrated in SDS-PAGE as a smear. This was due to an irregular size of the *N*-glycoside chains, because after their removal by endoglycosidase H the protein migrated as a distinct 63-kDa band (Fig. 2a). GOD secreted by the *pmr1*-Δ mutant migrated as a distinct band of ~80 kDa, while the endoglycosidase H treatment made the mobility of GOD from the *pmr1*-Δ mutant and control strain indistinguishable (Fig. 2a). This indicated that GOD secreted by the *pmr1*-Δ mutant possessed core *N*-glycoside chains, which had not received the irregular extensions in the Golgi apparatus.

Extracellular chitinase is an exclusively O-glycosylated protein (Kuranda & Robbins, 1991; Agaphonov *et al*., 2005b), which normally migrates upon SDS-PAGE as a dispersed band due to the presence of a multitude of O-glycoside chains of irregular length. Such a pattern in the strain bearing the *PMR1* wild-type gene was observed, while the protein from the culture supernatant of the *pmr1*-Δ mutant migrated as a distinct band of a lower molecular weight (Fig. 2b). This indicated that the *pmr1*-Δ mutation affects the O-glycosylation of secretory proteins.

Hansenula polymorpha pmr1-Δ mutation affects sorting of CPY

In *S. cerevisiae*, CPY, like some other vacuolar proteins, is sorted to the vacuole from the late Golgi compartments by means of the Vps10p receptor in a Ca^{2+}-dependent manner. Disruption of this process by inactivation of the sorting receptor (Marcusson *et al*., 1994) or by decrease of Ca^{2+} concentration in the Golgi apparatus (Durr *et al*., 1998) leads to the secretion of this protein into the culture medium. In *H. polymorpha*, the vacuolar sorting of CPY also depends on Vps10p (Agaphonov *et al*., 2003). Disruption of *PMR1* caused the defect of vacuolar protein sorting in *H. polymorpha*, as the *pmr1*-Δ mutant secreted CPY in the culture medium while its transformant bearing the *PMR1* wild-type allele did not (Fig. 2c).

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pmr1-Δ mutation increases efficiency of uPA secretion in *H. polymorpha*

uPA is a serine protease, which induces lysis of fibrin clots. This protein is synthesized as the 48-kDa zymogen, which is converted into the active form by the cleavage of the K^{158}-I^{159} peptide bond producing the 30-kDa polypeptide chain [for review, see Lijnen & Collen (1991)]. Thus the size of haloes formed by yeast colonies secreting uPA on fibrin plates depends both on the secretion levels of this enzyme and the efficiency of its proteolytic activation (Agaphonov *et al.*, 2005a). Inactivation of *H. polymorpha PMR1* in the strain expressing unglycosylated uPA variant, uPA-Q^{302}, greatly increased the halo size on fibrin-containing medium (Fig. 3a). Because the *pmr1*-Δ mutation caused a defect of vacuolar protein sorting (see above), which could lead to an increased proteolytic activation of uPA (Agaphonov *et al.*, 2005a), uPA-Q^{302} secreted by the *pmr*-Δ mutant and wild-type control strain was analyzed by Western blotting. It was previously observed that buffering of the culture medium with ammonium phosphate decreased the proteolysis of uPA (Tsujikawa *et al.*, 1996; Agaphonov *et al.*, 2005a). Indeed, the major band of uPA-Q^{302} from the culture supernatants migrated in SDS-PAGE as a ∼45-kDa protein (the calculated molecular weight of uPA-Q^{302} is 46 kDa). However, the intensity of the band in the case of the *pmr1*-Δ mutant was not increased (data not shown). At the same time, an obvious increase of uPA-Q^{302} secretion was observed when cells were incubated in the induction medium lacking ammonium phosphate buffer (Fig. 3b). Though the culture supernatants were ∼20-fold concentrated prior to the electrophoresis, uPA-Q^{302} could not be detected by Western blotting in the culture medium of the strain bearing the *PMR1* wild-type allele (Fig. 3b). uPA-Q^{302} secreted by the *pmr1*-Δ mutant migrated as a ∼45-kDa protein, even though the cells were incubated in an induction medium lacking ammonium phosphate buffer (Fig. 3b). This indicated that, unlike some other mutations affecting vacuolar protein sorting (Agaphonov *et al.*, 2005a), the *pmr1*-Δ mutation did not increase, but rather inhibited uPA-Q^{302} cleavage.

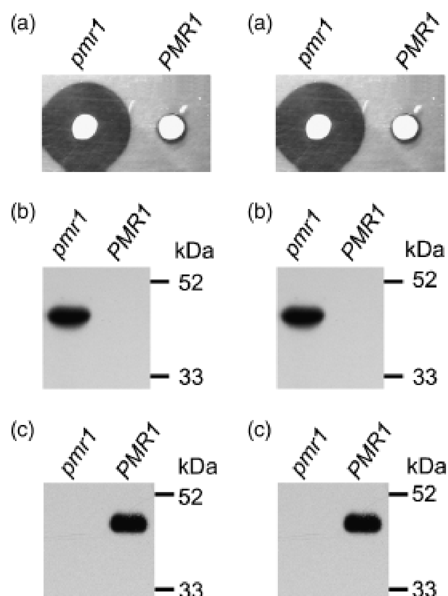

Fig. 3. Analysis of uPA-Q^{302} production in the transformants of the 1MA77/12 strain bearing either the pCAT1 plasmid with wild-type PMR*1* (*PMR1*) or the empty vector p2CHA6 (*pmr1*). (a) Haloes on fibrin containing medium formed by the transformants. (b) Western blotting of uPA in culture supernatants. (c) Western blotting of uPA in cell lysates.

uPA was shown to be poorly secreted by yeast cells due to inefficient folding in the ER that causes its intracellular accumulation in a form of high molecular weight aggregates. Absence of the *N*-glycosylation site in uPA-Q^{302} decreased the secretion efficiency and increased the intracellular accumulation of this protein (Agaphonov *et al.*, 2002). The *pmr1*-Δ mutation led to a decrease in the intracellular amount of uPA-Q^{302} (Fig. 3c), indicating that the higher rate of secretion was due to an improved folding in the ER.

Because in the experiments described above the *pmr1*-Δ mutation noticeably affected cell viability, it was suggested that the presence of ammonium phosphate buffer in the induction medium might cause cell death decreasing the uPA-Q^{302} secretion levels. Indeed, no colonies was obtained on YPD plates from the suspension of the *pmr1*-Δ cells incubated for 48 h in the induction medium containing ammonium phosphate buffer, while the same aliquots of cell suspension cultured without ammonium phosphate produced more than 1000 colonies. To test whether the cell death was due to apoptosis, DNA from cells cultured in the induction medium in the absence and in the presence of ammonium phosphate buffer was isolated and analyzed by electrophoresis in 2% agarose gel. In contrast to DNA isolated from the strain possessing the *PMR1* wild-type gene, DNA from the mutant contained a low molecular weight fraction, which migrated as a smear and was more pronounced if cells were cultured in presence of ammonium phosphate (Fig. 4). Similar patterns, indicating fragmentation of chromosomal DNA upon apoptosis, were observed in *S. cerevisiae* after treatment with proapoptotic agents (Wissing *et al.*, 2004; Pozniakovsky *et al.*, 2005). These data suggest that the death of the *pmr1*-Δ cells incubated in the induction medium was apoptotic. It is noteworthy that such a fraction of the lower molecular weight DNA was not observed in the *pmr1*-Δ cells grown in regular YPD medium (data not shown).

Discussion

In this work the *PMRI* gene was disrupted in the diploid strain obtained in the cross between derivatives of

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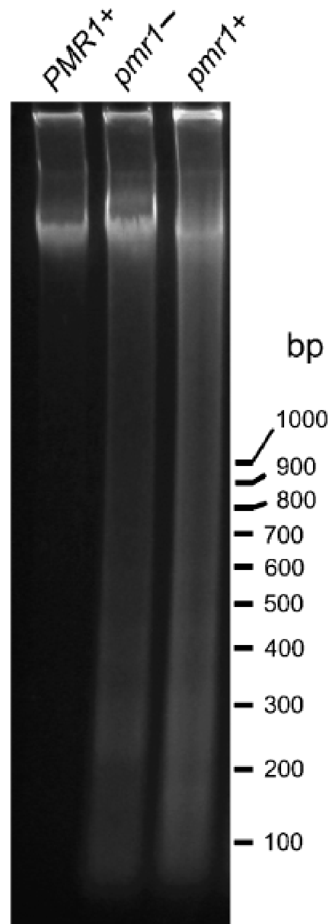

Fig. 4. Electrophoresis in 2% agarose gel of DNA isolated from the transformants of the 1MA77/12 strain bearing either the pCAT1 plasmid with wild-type PMR*1* (*PMR1*) or the empty vector p2CHA6 (*pmr1*). Transformants were incubated for 24 h at 37 °C in the induction medium with (+) or without (−) ammonium phosphate buffer.

H. polymorpha CBS4732. Analysis of haploid segregants of this diploid has shown that *PMRI* is dispensable. In spite of this, *PMR1* in the haploid *H. polymorpha* DL-1 strain could not be disrupted (data not shown), which was surprising, as the frequency of integration of transforming DNA into genome via homologous recombination in DL-1 is several-fold higher than in CBS4732 (unpublished). This allowed us to assume that the lack of *PMR1* decreased an ability of transformed haploid cells to survive and produce colonies. This agrees with the observation that the *pmr1*-Δ mutation strongly decreases *H. polymorpha* viability in some physiological conditions.

Like in *S. cerevisiae*, inactivation of the *H. polymorpha PMR1* gene affected both the Golgi steps of protein glycosylation and the vacuolar protein sorting. This indicated that *HpPMR1* is crucial for the maintenance of the Ca^{2+} pool in the Golgi compartments. At the same time, the effects of the *pmr1* mutation on cell viability appeared to be much more pronounced in *H. polymorpha* than in *S. cerevisiae*. It seems to be unlikely that such Ca^{2+}-dependent Golgi-hosted processes, such as protein glycosylation or vacuolar protein sorting, are less important for the viability of *S. cerevisiae* than of *H. polymorpha* cells. Therefore, it is suggested that more pronounced effects of the *pmr1*-Δ mutation in *H. polymorpha* was due to its effects on some other cellular processes.

The inactivation of *PMR1* in *S. cerevisiae* affects the degradation of misfolded proteins in the ER (Durr *et al.*, 1998) and increases sensitivity to amiodarone (Gupta *et al.*, 2003). The antiarrhythmic drug amiodarone was shown to disrupt Ca^{2+} homeostasis in yeast (Gupta *et al.*, 2003) and to induce programmed cell death (Pozniakovsky *et al.*, 2005). On the one hand, mutations causing a rise of cytosolic Ca^{2+} lead to higher sensitivity of yeast cells to amiodarone (Gupta *et al.*, 2003). On the other hand, the accumulation of unfolded proteins within the ER can also induce programmed cell death due to the generation of the reactive oxygen species (Haynes *et al.*, 2004). Thus, hypersensitivity of the *S. cerevisiae pmr1* mutant to amiodarone can be mediated by either mechanism. The *H. polymorpha pmr1*-Δ mutant was not hypersensitive to DTT or tunicamycin, indicating that the elimination of misfolded proteins in the ER was not affected. This might explain why the *pmr1* mutation did not increase the sensitivity of *H. polymorpha* to amiodarone, but hardly explains why this mutation significantly increased cell survival upon the amiodarone treatment. At the same time, it was observed that during cultivation in the medium for induction of uPA-Q^{302} expression, chromosomal DNA of the *pmr1*-Δ strain underwent the fragmentation that is a hallmark of apoptosis. It is speculated that the influence of the *pmr1*-Δ mutation on the switch to the apoptotic program is similar to the action of amiodarone. Thus, the *pmr1*-Δ mutation may increase the resistance of cells to amiodarone due to the 'preconditioning' effect of the disturbance of Ca^{2+} homeostasis.

The data obtained indicate that, unlike in *S. cerevisiae*, in *H. polymorpha* the elimination of unfolded proteins was not essentially altered by the *pmr1*-Δ mutation. However, this does not mean that other functions of the ER were unaffected. Indeed, this mutation improved the secretion of uPA-Q^{302} and decreased its intracellular accumulation, indicating an improvement of the folding of this protein in the ER. A similar effect of the *pmr1* mutation on uPA secretion was observed in *S. cerevisiae* (Melnick *et al.*, 1990). Thus, the influence of the *pmr1* mutation on protein-folding in the ER is similar in two yeast species, while the influence on the elimination of unfolded proteins is different. This discrepancy can be explained in two ways. First, it is possible to suggest that in *H. polymorpha* Pmr1p does not essentially contribute to the maintenance of the Ca^{2+} pool within the

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ER, and its influence on protein-folding in the ER is indirect, e.g. through the Golgi steps of protein glycosylation. The latter is indirectly supported by the observation that in *S. cerevisiae* some genes involved in the Golgi steps of protein glycosylation are regulated by the unfolded protein response signalling pathway (Travers *et al*., 2000), indicating that they may influence the ER functions. The second explanation is based on the assumption that efficient elimination of misfolded proteins in the ER requires a lower concentration of Ca^{2+} than in *S. cerevisiae*, or is not sensitive to Ca^{2+} at all. Indeed, the major mechanism of the unfolded protein elimination can be different in *H. polymorpha* and *S. cerevisiae*, as the expression of well-known targets of the unfolded protein response in *S. cerevisiae*, genes *KAR2* and *PDI1*, are not induced in *H. polymorpha* cells cultured in the presence of DTT or tunicamycin (Agaphonov *et al*., 2005b).

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