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Pleiotropic effect of anionic phospholipids absence on mitochondrial morphology and cell wall integrity in strictly aerobic *Kluyveromyces lactis* yeasts

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Abstract Cardiolipin and phosphatidylglycerol are anionic phospholipids localized to the inner mitochondrial membrane. In this study, it is demonstrated by fluorescence and transmission electron microscopy that *atp2.1pgs1*Δ mutant mitochondria lacking anionic phospholipids contain fragmented and swollen mitochondria with a completely disorganized inner membrane. In the second part of this study, it was shown that the temperature sensitivity of the *atp2.1pgs1*Δ mutant was not suppressed by the osmotic stabilizer glucitol but by glucosamine, a precursor of chitin synthesis. The *atp2.1pgs1*Δ mutant was hypersensitive to Calcofluor White and caffeine, resistant to Zymolyase, but its sensitivity to caspofungin was the same as the strains with the standard *PGS1* gene. The distribution of chitin in the mutant cell wall was impaired. The glucan level in the cell wall of the *atp2.1pgs1*Δ mutant was reduced by 4–8 %, but the level of chitin was almost double that in the wild-type strain. The cell wall of the *atp2.1pgs1*Δ mutant was about 20 % thinner than the wild type, but its morphology was not significantly altered.

Introduction

Cardiolipin (CL) and its precursor phosphatidylglycerol (PG) are anionic phospholipids whose synthesis is exclusively localized to mitochondria. Most knowledge about the function of these phospholipids in eukaryotic cells was obtained using the model organism *Saccharomyces cerevisiae*. It was found that disruption of *PGS1*, the gene encoding phosphatidylglycerolphosphate synthase, results in the complete loss of both phospholipids, but the physiology of this *petite*-positive yeast is not dramatically affected (reduced growth due to damage of the respiratory activity) (Subik 1974; Janitor and Subik 1993; Chang et al. 1998; Koshkin and Greenberg 2000). The simultaneous loss of both phospholipids and mitochondrial DNA (mtDNA) converts this petite-positive yeast into a petite-negative one and is lethal (Janitor et al. 1996).

Over the past two decades, *Kluyveromyces lactis* has developed into a second eukaryotic model as an alternative to the baker's yeast. Unlike the yeast *S. cerevisiae*, *K. lactis* is a strictly aerobic, petite-negative species, for which the loss of both anionic phospholipids PG and CL, caused by deletion of the *PGS1* gene, is lethal (Tyciakova et al. 2004). It was later shown that a specific mutation in the *ATP2* gene (*atp2.1* mutation) suppresses the lethality of the *pgs1* mutation in the *K. lactis* strain (Obernauerova and Palovicova 2009; Patrasova et al. 2010) by increasing the hydrolysis of ATP resulting in the maintenance of sufficient membrane potential required for the biogenesis of mutant mitochondria and survival of cells (Palovicova et al. 2012). The resulting *atp2.1pgs1*Δ *K. lactis* cells, lacking the oxidative phosphorylation reactions, exhibit a reduced growth, but they were able to form colonies even after the induction of mtDNA deletions (Palovicova et al. 2012).

CL and its precursor PG are essential for several functions in cell physiology. CL is specifically required for the organization and optimal activity of oxidative phosphorylation complexes in mitochondria. It plays a structural and functional role in the yeast *bc*1 complex (complex III) (Lange et al. 2001), cytochrome *c* oxidase (complex IV) (Sedlak et al. 2006) and ATP/ADP carrier (Nury et al. 2005) and enhances ATP

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synthase activity (Bogdanov et al. 2008). Likewise, the association of these individual complexes into a functional higher-order "respirasome" is dependent on CL levels (Pfeiffer et al. 2003). Respiratory chain supercomplexes and complex V (ATP synthase) are significantly enriched in mitochondrial cristae. Reduced levels of CL/alteration of its composition or its absence in the mutant cells is the factors resulting in the aberrant morphology of the mitochondrial cristae membrane (Pfeiffer et al. 2003; Mileykovskaya and Dowhan 2010).

The CL biosynthetic pathway is important not only for mitochondrial bioenergetics, but also for cell wall biogenesis. A connection between the *pgs1* phenotype and cell wall biogenesis was presented by Lussier et al. (1997). It has been shown that the absence of anionic phospholipids in the *S. cerevisiae* mutant leads to hypersensitivity to cell wall-perturbing agents such as Calcofluor White, caspofungin, caffeine or Zymolyase. In another study, it was found that the *S. cerevisiae pgs1*Δ mutant has a reduced level of β-1,3-glucan, a major component of the cell wall (Zhong et al. 2005).

The cell wall of yeast determines its cell shape and integrity during growth and cell division. Three main groups of polysaccharides form the cell wall: polymers of mannose (ca. 40 % of the cell wall dry mass); polymers of glucose (β-glucan, ca. 60 % of the cell wall dry mass) and polymers of *N*-acetylglucosamine (chitin, ca. 2 % of the cell wall dry mass). The cell wall is a dynamic structure that responds to physiological, morphological, genetic and environmental stimuli through cell wall remodeling mechanisms. One of the major outcomes of these mechanisms is changes in the cell wall composition (Dallies et al. 1998; Klis et al. 2002).

The aim of this study was to analyze how the dynamic organization of the mitochondrial membrane and integrity of the cell wall will be affected in strictly aerobic *K. lactis* yeasts deficient in the biosynthesis of anionic phospholipids.

Materials and methods

Strains, media and cell growth conditions

The following strains of *K. lactis* were used in this study: CW75-1D (*MAT***a**, *ade1*, *lys1*, *ura3.1*, *atp2.1*), CW75-1D-1a (*MAT***a**, *ade1*, *lys1*, *ura3.1*, *atp2.1*, *pgs1::*KanMX), CW75-1D-pATP2 (*MAT***a**, *ade1*, *lys1*, *atp2.1*, pRS306K-*ATP2*) (strains were prepared according to Palovicova et al. 2012) and JBD 100 (*MAT***a**, *trp1*, *ura3.1*, *lac 4-1*) (University of Leiden, the Netherlands). Cells were grown aerobically at 30 °C in an incubator in minimal yeast nitrogen base (YNB) medium containing 6.7 g/L YNB without amino acids and 20 g/L glucose and supplemented with the auxotrophic requirements (40 μg/mL). Solid media were prepared from 20 g/L Difco agar.

Transmission electron microscopy (Kopecka et al. 2000)

Exponential phase cells were fixed for 3 h with glutaraldehyde (30 g/L) in 200 mmol/L cacodylate buffer (pH 7.4), postfixed with 20 g/L osmium tetroxide for 2 h and dehydrated in an alcohol series for 2 h. Finally, the cells were embedded in the durcupan and allowed to polymerise for 3 days at 60, 70 and 80 °C. Ultrathin sections were contrasted 30 min with 25 g/L uranyl acetate and lead citrate for 6 min. The sections were viewed in a Morgagni 268D transmission electron microscope (USA) at 70 kV and photographed in Veleta 2kx2k TEM CCD camera, Olympus (GmbH).

Fluorescence microscopy (Marchi 2009)

Mid-exponential phase cells were resuspended in 10 mmol/L HEPES buffer containing 50 g/L glucose and incubated with 1 μmol/L Rhodamine B solution in total darkness at room temperature for 30 min. Mitochondria were examined directly under fluorescence microscope connected with digital camera Axioskop 2 FS plus, Zeiss, and digital camera DP72, Olympus, and viewed with ×100 oil immersion objective. Ultraviolet illumination with the following excitation filters was used for fluorescence microscopy: filter TBP 400 nm + 495 nm + 570 nm, beam splitter TFT 410 nm + 505 nm + 585 nm and emission filter TBP 460 nm + 530 nm + 610 nm. Images show at least 200 observed cells.

Temperature sensitivity testing

Cells in exponential phase grown at 30 °C in liquid YNB medium with glucose were diluted to a concentration of 10^7 cells per millilitre in sterile water. Ten microliters of cell suspension and 10-fold serial dilutions of cells were spotted on YNB and YNB + 1 mol/L glucitol media. Cells were incubated for 5 days at 30 °C or 37 °C.

Drug susceptibility testing

The strains were grown overnight at 30 °C in liquid YNB medium containing glucose. Cells were diluted to a concentration of 10^7 cells per millilitre in sterile water. Ten microliters of cell suspension in 10-fold serial dilutions was spotted onto minimal solid media supplemented with glucose containing various drug concentrations of caspofungin 1093.31 kDa (g/mol) (Sigma, USA), caffeine 194.1906 kDa (g/mol) (Sigma, USA) and Calcofluor White 916.98176 kDa (g/mol) (Sigma, USA), followed by 5-day incubation at 30 °C.

The growth of cells in the presence of glucosamine

Cells of the tested strains were grown in YNB medium in the presence or absence of 10 mmol/L glucosamine at 37 °C. The

growth was monitored by cell counting during incubation in a rotary shaker at the indicated temperature.

Zymolyase assay (Uccelletti et al. 2000)

Cells of tested strains were grown into exponential phase, and 5×10^8 cells were resuspended in 4 mL of buffered glucitol medium (20 mmol/L TRIS-HCL, pH 7.2; 1.2 mol/L glucitol; 10 mmol/L $MgCl_2$ with 30 g/L β-mercaptoethanol). After 10-min incubation, 1 mL Zymolyase 20T (62.5 U) was added and the cells were incubated for 30 °C. Cell lysis in samples was determined by measurements of A_{660} after dilution 1:10 in water taken in 10-min intervals. The data represent the mean value of three independent experiments.

Cell wall isolation

Stationary phase cells were washed twice with cold water and disintegrated with glass beads (0.5-mm diameter) in a rotary disintegrator immersed in an ice bath (4 °C). The suspension was filtered through a Miracloth nylon filter, and the cell walls were collected by centrifugation of the filtrate at 4000*g* for 10 min. The sediment was washed out consecutively 10 times with 1 mol/L NaCl and finally with water until no absorbance at 280 nm could be detected in the washings. The washed cell walls were lyophilized and stored in a desiccator until further analysis. Three independent cultivations were done.

Determination of glucans

For determination of glucans, 10 mg of lyophilized cell walls was wetted at 0 °C with 0.2 mL 720 g/L (*w*/*v*) H_2SO_4, and after 12-h incubation at 0 °C, the acid was diluted with 2 mL water. The tubes were sealed, and hydrolysis was carried out at 105 °C for 8 h. The hydrolysate was neutralized with 5 mol/L NaOH and 10 μL phenolphthalein. At the end, the volume was adjusted with water to 5 mL. From this solution, samples were taken for determination of glucose with glucose oxidase-peroxidase test (Bio-La-Test, Erba Lachema, Czech Republic). The data reported are the means of three independent experiments.

Determination of chitin

For determination of chitin, 10 mg lyophilized cell walls were extracted in 1 mol/L NaOH at room temperature overnight. The insoluble residues were washed out once with 1 mol/L acetic acid and several times with water until neutral pH. Afterwards, they were resuspended in 500 μL of 0.1 mol/L phosphate buffer (pH 6.5) containing 10 mg/mL lyophilized dialyzed snail gut juice and 2 mg/mL of chitinase from *Serratia marcescens* (Sigma, USA). Finally, 0.2 g/L sodium azide was added to prevent contamination. The hydrolysis was carried out at 37 °C overnight. The enzymatic hydrolysate was used for determination of *N*-acetylglucosamine according to Reissig et al. (1955). The data reported are the means of three independent experiments.

Results

Mitochondrial morphology and ultrastructure in *K. lactis atp2.1pgs1*Δ mutant cells

To highlight the relationship between the respiratory activity of *K. lactis* cells influenced by the absence of anionic phospholipids and mitochondrial morphogenesis, the cells in the mid-exponential phase of growth on glucose medium were stained with the mitochondria-specific dye Rhodamine 123 and then observed by fluorescence microscopy. Cells with the standard *PGS1* gene (*atp2.1*pATP2, *atp2.1* strains) contained an adequate number of mitochondria corresponding to the growth conditions and metabolic activity, and their mitochondria exhibited standard tubular morphology (Fig. 1a, b). In contrast, *atp2.1pgs1*Δ mutant cells under the same growth conditions contained a reduced number of mitochondria, and the mitochondria were fragmented, forming the so-called mitochondrial patches (Fig. 1c). The morphological aspect of the absence of anionic phospholipids was also investigated by electron microscopy. As Fig. 2a shows, mitochondria from the wild type exhibited an extended "standard" mitochondrial shape, with numerous cristae. The presence of the *atp2.1* mutation had no significant effect on the mitochondrial shape and inner membrane organization (Fig. 2b). In contrast, *atp2.1pgs1*Δ mutant mitochondria were swollen and their inner membrane was completely disintegrated without cristae (Fig. 2c).

*K. lactis atp2.1pgs1*Δ mutant displays cell wall stress phenotype

Analysis of the behaviour of *K. lactis* strains under conditions of physiological stress (the absence of anionic phospholipids) and environmental stress revealed that none of the tested strains grew at 37 °C, and in addition, the stabilizing effect of glucitol was only reflected in strains with the standard *PGS1* gene (Fig. 3). The spot-test sensitivity of *K. lactis atp2.1pgs1*Δ cells to cell wall-perturbing agents such as Calcofluor White (CFW), caffeine (CAF) and caspofungin (CAS) showed that the double mutant exhibited an increased susceptibility to CFW and caffeine compared to the wild type, but its susceptibility to caspofungin compared to strains with the standard *PGS1* gene was not altered over the range of concentrations tested (Fig. 4). Relationship between CFW sensitivity and chitin distribution in the cell walls of tested strains was investigated using fluorescence microscopy. As

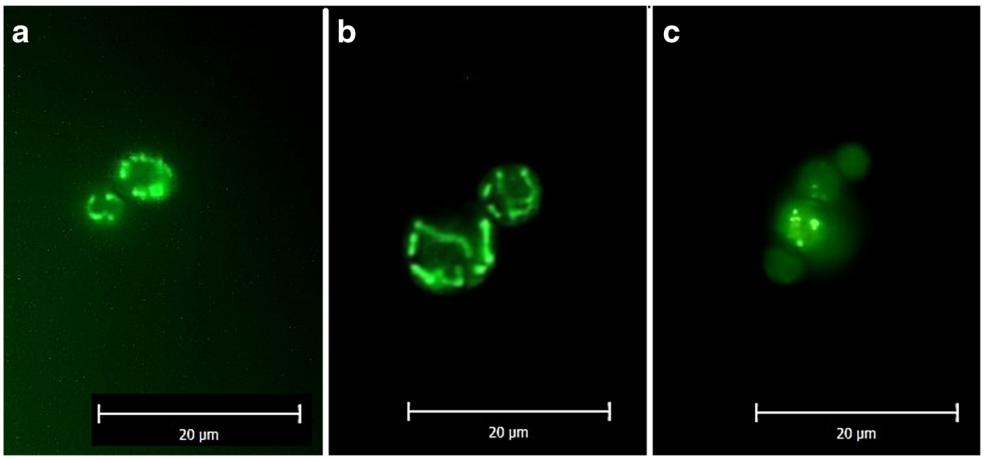

Fig. 1 Mitochondrial morphology of the *K. lactis* strains. The cells were grown up to the mid-exponential phase aerobically at 30 °C in synthetic liquid medium containing 2 % glucose, stained with mitochondria-specific dye Rhodamine B and examined by fluorescent microscopy. **a** Wild-type *atp2.1*pATP2, **b** *atp2.1* and **c** *atp2.1pgs1*Δ cell mitochondria

Fig. 5a shows, the wild-type cells exhibit a typical localization of chitin in the neck between mother cells and emerging buds as well as in the rings of bud scars. The pattern of chitin distribution in the cell wall of the *atp2.1* mutant was very similar to the wild-type cells (Fig. 5b). Maldistribution of chitin can be seen in *atp2.1pgs1*Δ cells (Fig. 5c). The mutant cells had a bright fluorescence staining pattern distributed not only in the region of the cell wall, but also throughout the whole of the cells. These observations indicate that the localization and distribution of chitin in the *atp2.1pgs1*Δ mutant are impaired.

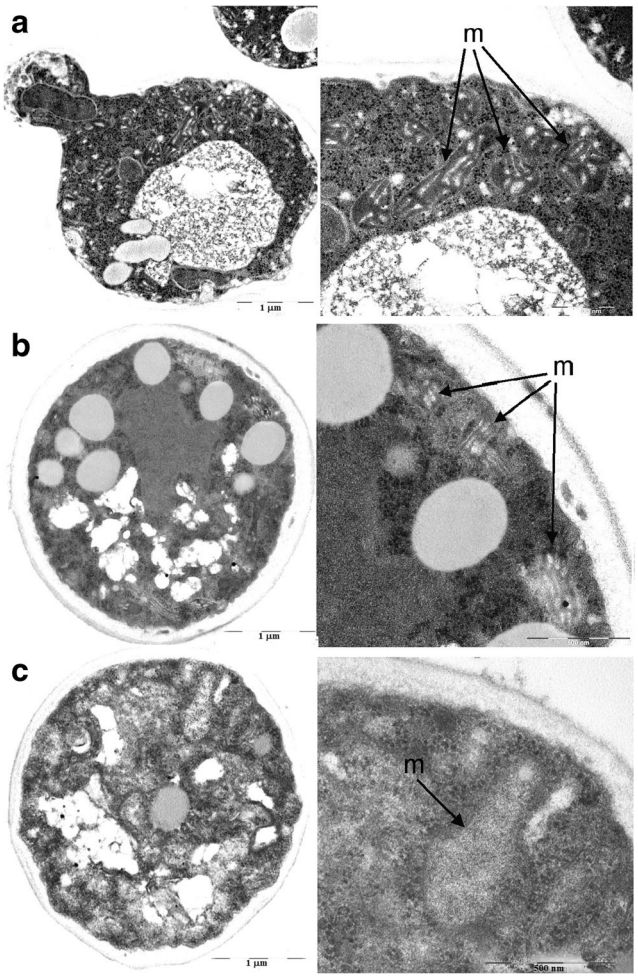

Fig. 2 Mitochondrial ultrastructure of the *K. lactis* strains. Transmission electron microscopy of mutant and standard cells grown aerobically at 30 °C in minimal medium containing 2 % glucose into the mid-exponential phase. **a** Wild-type *atp2.1*pATP2 cell, **b** *atp2.1* cell and **c** *atp2.1pgs1*Δ cell. *m* mitochondria

Alterations of chitin and glucan content in cell wall of *atp2.1pgs1*Δmutant

Based on phenotypic tests and the chitin staining pattern of the analyzed cells, a quantitative analysis of the glucan and chitin content in the cell walls of the tested strains was performed. The results reported in Table 1 show that the glucan content in the cell walls of wild-type (*atp2.1*pATP2), *atp2.1* and *atp2.1pgs1*Δ mutants did not vary significantly. The level of glucan in the *atp2.1pgs1*Δ mutant was only reduced by 4–8 %

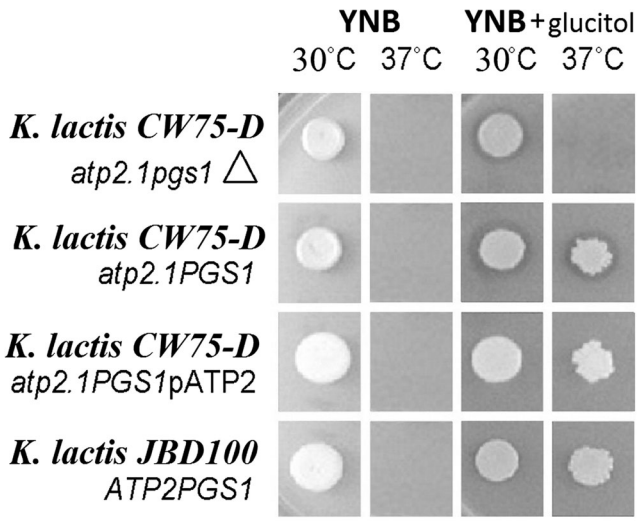

Fig. 3 Effect of elevated temperature on *K. lactis* growth. Cells were serially diluted, spotted on YNB and YNB+ glucitol plates and incubated at 30 and 37 °C for 5 days

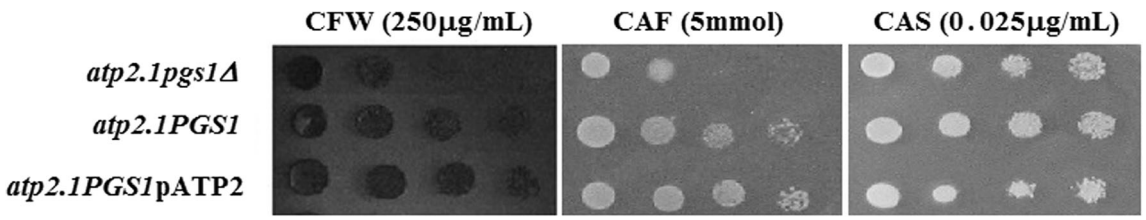

Fig. 4 Effect of cell wall-modifying agents on growth of the *K. lactis* strains. Spotting assays were performed with 10-fold dilution of overnight cultures in minimal glucose medium containing cell wall inhibitors in the concentration ranges: *CFW* Calcofluor White 0–700 µg/mL, *CAF* caffeine 0–10 mmol/L, *CAS* caspofungin 0–0.5 µg/mL. The plates were incubated for 5 days at 30 °C

compared to the strains with the standard *PGS1* gene. In contrast, the level of chitin in the cell walls of the *atp2.1pgs1*Δ mutant was about 89 % higher than in the wild-type cells and nearly 32 % higher than in the *atp2.1* mutant cells. Based on the fact that glucosamine is a precursor of chitin, a substance that helps maintain the integrity of the cell wall, we tried to analyze whether glucosamine may affect the growth and viability of the *atp2.1pgs1*Δ mutant at 37 °C. As Fig. 6c shows, the presence of glucosamine supported the growth of *atp2.1pgs1*Δ cells. Despite the mutant growth at 37 °C being delayed and the growth yield being three times lower than under the standard conditions (30 °C), cells were still viable after 52 h of cultivation. In contrast, the presence of glucosamine did not affect the growth rate and yield of strains with the standard *PGS1* gene (Fig. 6a, b). Thus, supplementing with glucosamine, unlike glucitol, was able to support the growth and maintain the viability of the *atp2.1pgs1*Δ mutant at the elevated temperature.

Zymolyase is another of the substances that is used to detect in vivo alterations in yeast cell wall structure. The susceptibility of the tested strains to Zymolyase digestion is shown in Fig. 7. The *atp2.1pgs1*Δ mutant was significantly more resistant to the lytic action of the enzyme than strains with the standard *PGS1* gene. These results indicate that, most likely, the change in glucans/chitin ratio (increased chitin level) is responsible for the observed resistance of *atp2.1pgs1*Δ cells to the action of the lytic enzyme.

In order to identify the morphological aspects induced by changes in the content of components of the cell wall, electron microscopy of conventional ultra-thin sections of more than 50 analyzed cells from each strain was performed. As is apparent from Fig. 8a, b, the cell wall of the wild-type and *atp2.1* mutant was slightly thicker (an average of 160 ±48 µm) than that of the *atp2.1pgs1*Δ mutant (an average of 130±32 µm), but aberrations in cell wall morphology were not observed in the *atp2.1pgs1*Δ mutant.

Discussion

Mitochondria are key organelles in intermediate cellular metabolism, in energy conversion, in controlling apoptosis, in cell wall integrity and in several other processes associated with cell viability. Energy conversion occurs at the inner mitochondrial membrane that consists of two subcompartments: the inner boundary membrane and the cristae membrane. The cristae membrane is significantly enriched in respiratory chain complexes and the F_1F_0-ATP synthase complex. Previous studies have shown that the association of individual respiratory chain complexes into a functional higher-order respirasome is dependent on CL, whose biosynthesis takes place in the inner mitochondrial membrane (Schafer et al. 2007; Zick et al. 2009; Schlame and Ren 2009; Mileykovskaya and Dowhan 2010).

Mitochondria are organelles that exhibit an extremely large variability in their ultra-structures, depending on the physiological state or developmental state. As was indicated in our earlier study, the *atp2.1pgs1*Δ mutant lacking anionic phospholipids was not able to utilize a non-fermentative carbon source, and its growth on glucose medium was reduced more than 40 % compared to isogenic strains with a standard *PGS1* gene. The growth defect was caused by significantly reduced

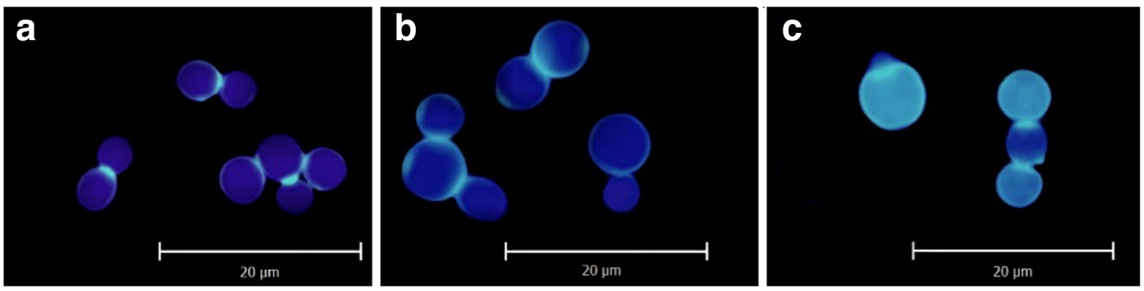

Fig. 5 Analysis of chitin localization in the cell wall of the *K. lactis* strains. Cells were grown into a late exponential phase in minimal glucose medium and stained by Calcofluor White. **a** Wild-type *atp2.1*pATP2, **b** *atp2.1* and **c** *atp2.1pgs1*Δ

Table 1 The composition of cell wall glucan and chitin in the *K. lactis* cells

Strains	Glucan (μg/mg)	Chitin (μg/mg)
*atp2.1pgs1*Δ	368 ± 24.18	24.76 ± 2.66
atp2.1PGS1	382 ± 4.76	18.76 ± 1.38
*atp2.1PGS1*pATP2	400 ± 27.43	13.09 ± 0.54

Cells were grown in minimal glucose medium at 30 °C and harvested at the later exponential phase. Glucan and chitin levels were determined as described in "Materials and methods" section. Data represent three independent experiments. The values are calculated in units of micrograms per 1 mg of the dry matter of the cell walls

respiration activity due to the absence of cytochrome *b* and reduced content of cytochrome *a* and cytochrome *c* (Palovicova et al. 2012). We have shown in this study that this defect was reflected in the morphology and topology of the mutant mitochondria. Fluorescently stained mutant cells contained a reduced number of mitochondria, and moreover, mutant mitochondria were fragmented and form aggregates (Fig. 1c). Transmission electron micrographs of *atp2.1pgs1*Δ cells showed that mutant mitochondria are swollen with a completely disorganized inner membrane topology (Fig. 2c). The *atp2.1* mutation (a specific mutation of the *β*-subunit in the F_1 part of the F_1F_0-ATP synthase) had no effect on the mitochondrial morphology and topology of the inner mitochondrial membrane (Clark-Walker and Chen 1966) (Fig. 2b). This is consistent with the observations which show that the altered morphology of the inner mitochondrial membrane in yeast is associated with the absence of subunits *e* and *g* of this ATP synthase complex (Paumard et al. 2002).

Alterations to mitochondrial structures are a well-known cause of numerous diseases in humans. A direct link between CL content in mitochondrial membranes, mitochondrial functions and mitochondrial ultrastructure was demonstrated in the mitochondria of patients suffering from the Barth syndrome (Schlame and Ren 2006). The Barth syndrome is a mitochondrial disorder caused by mutations in tafazzin, which is involved in the biosynthesis of this mitochondrial phospholipid (Bione et al. 1996). Electron microscopy of the Δ *taz1* mutant mitochondria also identified alterations in their morphology, such as elongated cristae and onion-like structures (Mileykovskaya and Dowhan 2010), but these changes were not as destructive as was observed in the *atp2.1pgs1*Δ *K. lactis* mitochondria. In recent years, several other examples of human diseases related to alterations in mitochondrial functions and mitochondrial morphology have been reported such as Parkinson's and Alzheimer's diseases (Trimmer et al. 2000), Wolf-Hirschhorn syndrome (Dimmer et al. 2008) and others. All of the above examples demonstrate a direct relationship between mitochondrial biogenesis and mitochondrial morphology, despite the fact that damage to mitochondria can have various origins, and the consequences of damage on the viability of the cells can be different, depending on the

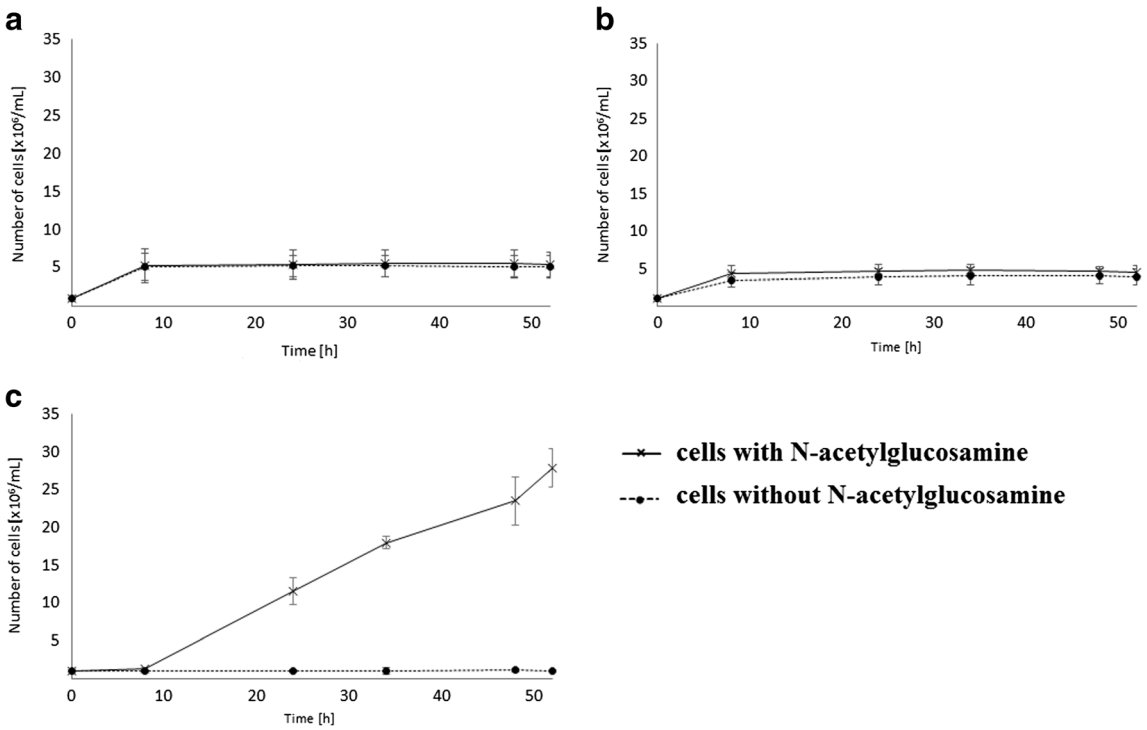

Fig. 6 The effect of glucosamine on growth of the *K. lactis* strains in glucose medium at 37 °C. Cells were grown in YNB medium in the presence or absence of 10 mmol/L glucosamine. The growth was monitored by cell counting during cultivation in a rotary shaker at the indicated temperature. Values shown are averages of two independent experiments

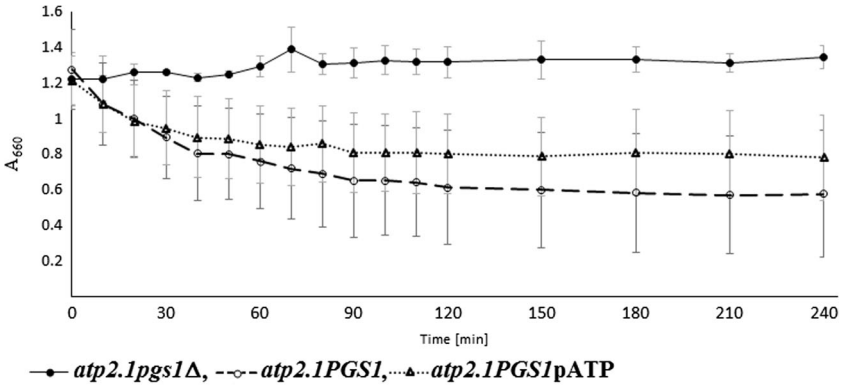

Fig. 7 The effect of Zymolyase on stability of *K. lactis* cells. Cells were incubated in buffered glucitol medium containing 3 % β-mercaptoethanol in the presence of Zymolyase 20T. Cell lysis was determined in 10-min intervals for 4 h (A_{600})

physiology of the cells. In this study, we have shown that in the case of strictly aerobic yeasts *atp2.1pgs1*Δ *K. lactis*, the absence of anionic phospholipids has a destabilizing effect on components of the respiratory chain, which significantly affects the inner membrane topology of this mutant. Our observations are in good correlation with other in vitro and in vivo studies showing the direct relationship between mitochondria-dependent cell growth, the functionality and stability of respiratory chain complexes and the topology of the inner cristae membrane (Cogliati et al. 2013).

The yeast cell wall is essential for the maintenance of cell shape, prevention of lysis and adverse environmental stress factors. Despite its apparent rigidity, the yeast cell wall is a dynamic structure that can be modulated in response to various physiological and morphological changes (Orlean 1998; de Nobel et al. 2000). A link between mitochondrial dysfunction and cell wall biogenesis has been implicated in the study by Zhong et al. (2005), where it was shown that disruption of the *PGS1* gene (causing the loss of both anionic phospholipids) in the yeast *S. cerevisiae* results in several cell wall defects including thermosensitivity, hypersensitivity to cell wall-perturbing agents and a marked reduction in β-1,3-glucan. The phenotype of *pgs1*Δ mutant cells was suppressed by the *kre5*^{w1166X} mutation, suggesting a defect in its cell wall integrity.

In this study, we demonstrated the impact of the absence of anionic phospholipids on the cell wall integrity of a *K. lactis* yeast. We have shown (Fig. 3) that the *atp2.1pgs1*Δ *K. lactis* mutant, which lacks anionic phospholipids CL and PG, as well as *K. lactis* strains with the standard *PGS1* gene, did not grow on minimal medium with a fermentable carbon source at 37 °C. Given that no other genetically different *K. lactis* strain (JBD100) grew at elevated temperature, we can assume that thermosensitivity is a natural characteristic of wild-type strains of this species. However, in contrast to *K. lactis* strains with the standard *PGS1* gene, as in the *S. cerevisiae pgs1*Δ mutant (Zhong et al. 2005), the thermosensitivity of the *atp2.1pgs1*Δ mutant was not reduced by glucitol, an osmotic stabilizer, in the medium (Fig. 3).

Another characteristic feature of mutants defective in cell wall biogenesis is their hypersensitivity to cell wall-perturbing agents (Lussier et al. 1997). Analysis of the sensitivity of the tested *K. lactis* strains showed that the *atp2.1pgs1*Δ mutant is hypersensitive to CFW (Fig. 4). Hypersensitivity to CFW is associated with the delocalization of chitin all along the cell wall (Molano et al. 1980). This fluorescence staining pattern uniformly distributed throughout the cells was observed in the *atp2.1pgs1*Δ mutant (Fig. 5c), in contrast to the *atp2.1* mutant and wild-type strain which exhibited a typical localization of

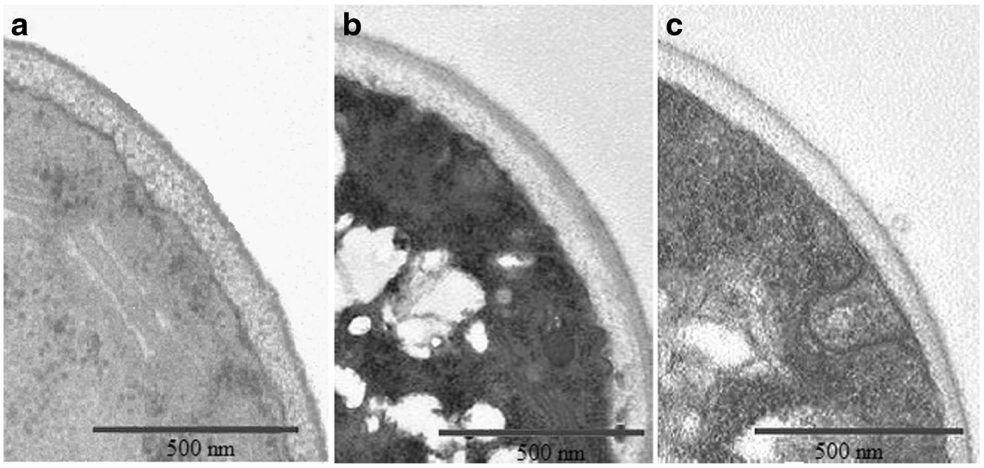

Fig. 8 Transmission electron microscopy of *K. lactis* cell wall. Transmission electron microscopy of mutant and standard cells grown aerobically at 30 °C in minimal medium containing 2 % glucose into the mid-exponential growth phase. **a** Wild-type *atp2.1*pATP2, **b** *atp2.1* and **c** *atp2.1pgs1*Δ. The *horizontal scale bar* represents 500 nm

chitin only at the sites of active growth and in the rings of bud scars (Fig. 5a, b). On the basis of these findings, we assume that deficiency in mitochondrial anionic phospholipids results in the cell wall defects (a content/distribution of chitin) of the *atp2.1pgs1*Δ mutant.

The basic structural components of the yeast cell wall are glucan and chitin. Glucan is responsible for the elasticity and chitin for the mechanical strength of the cell wall (Smits et al. 1999). In addition, several studies have demonstrated that the level of glucan in the cell wall usually correlates with the sensitivity of cells to the lytic action of Zymolyase (Uccelletti et al. 2000; Aguilar-Uscanga and Francois 2003). In our study, we have shown that the *atp2.1pgs1*Δ mutant exhibits a slightly lower glucan level (Table 1), but its resistance to the Zymolyase was significantly higher than the control strains (Fig. 7). In several studies, it was indicated that one of the cell wall compensatory mechanisms activated to protect the cells against lysis is an increase in cell wall chitin (Popolo et al. 1997; Lagorce et al. 2002). The chitin level in the *atp2.1pgs1*Δ mutant was almost twice as high as that of wild type (Table 1). The hyperaccumulation of chitin is one of the mechanisms that can help maintain the integrity of the cell wall in conditions affecting cell viability (Klis et al. 2002). Glucosamine, the precursor of chitin, stimulated the growth and increased the viability of the *atp2.1pgs1*Δ mutant at 37 °C (Fig. 6c). A positive effect of glucosamine on the growth of *atp2.1* and wild-type cells at this temperature was not observed (Fig. 6a, b). It follows that chitin stabilizes the growth of *atp2.1pgs1*Δ mutant cells in which the synthesis of anionic phospholipids is impaired. A defect in the cell wall structure of the *atp2.1pgs1*Δ mutant resulted in a reduction in cell wall thickness (about 20 %), but the cell wall morphology was not significantly affected (Fig. 8).

In summary, we have demonstrated, by fluorescent and transmission electron microscopy, the destructive impact of the absence of anionic phospholipids on the morphology and topology of the inner mitochondrial membrane of the *atp2.1pgs1*Δ *K. lactis* mutant.

Another interesting result of this study is that the strength of the cell wall structure of *K. lactis* yeasts unable to synthesize anionic phospholipids, as judged by the lytic action of Zymolyase on whole cells and stimulation of mutant viability by glucosamine under temperature stress, is not directly dependent on the absolute level of glucan but more linked to that of chitin. This polymer of *N*-acetylglucosamine is implicated in covalent linkage with glucans, and these cross-linkages may contribute to the modular structure of the *K. lactis* cell wall. These findings confirm the important role of anionic phospholipids in mitochondrial morphology as well as in the modulation of the structure of the *K. lactis* cell wall under conditions of environmental stress.

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