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A novel cholesterol-producing *Pichia pastoris* strain is an ideal host for functional expression of human Na,K-ATPase α3β1 isoform

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Abstract The heterologous expression of mammalian membrane proteins in lower eukaryotes is often hampered by aberrant protein localization, structure, and function, leading to enhanced degradation and, thus, low expression levels. Substantial quantities of functional membrane proteins are necessary to elucidate their structure–function relationships. Na,K-ATPases are integral, human membrane proteins that specifically interact with cholesterol and phospholipids, ensuring protein stability and enhancing ion transport activity. In this study, we present a *Pichia pastoris* strain which was engineered in its sterol pathway towards the synthesis of cholesterol instead of ergosterol to foster the functional expression of human membrane proteins. Western blot analyses revealed that cholesterol-producing yeast formed enhanced and stable levels of human Na,K-ATPase α3β1 isoform. ATPase activity assays suggested that this Na,K-ATPase isoform was functionally expressed in the plasma membrane. Moreover, [^{3}H]-ouabain cell surface-binding studies underscored that the Na,K-ATPase was present in high numbers at the cell surface, surpassing reported expression strains severalfold. This provides evidence that the humanized sterol composition positively influenced Na,K-ATPase α3β1 stability, activity, and localization to the yeast plasma membrane. Prospectively, cholesterol-producing yeast will have high potential for functional expression of many mammalian membrane proteins.

Keywords *Pichia pastoris* · Membrane protein · Cholesterol · Na,K-ATPase · Protein–lipid interaction · Lipid engineering

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Introduction

Human membrane proteins are prime drug targets, and therefore, a lot of effort is put into the investigation of their structure and function (Freigassner et al. 2009). Biochemical studies are often hindered by low amounts of membrane proteins that can be extracted directly from mammalian tissue. Consequently, attempts have been made to produce sufficient amounts of membrane proteins by heterologous expression in different microbial host systems, including yeasts, for biochemical characterization and crystallization studies.

However, fungi—including yeasts—contain ergosterol, while animal cells contain cholesterol as major sterol, which may be a bottleneck for the heterologous expression of mammalian membrane proteins in fungi (Fig. 1). Despite their very similar structure, these sterols have distinct functions in biological systems as well as in artificial membranes (Xu et al. 2001). The first steps in sterol biosynthesis are the same in animals, plants, and fungi (Nes 2011). In order to synthesize ergosterol, fungi add an additional methyl group at C-24, which is accomplished by sterol C-24 methyl transferase (Erg6p). Furthermore, a double bond is introduced by sterol C-22 desaturase (Erg5p). In mammals, by contrast, sterols are saturated at positions C-7 and C-24 by dehydrocholesterol reductase 7 (*DHCR7*) and 24 (*DHCR24*), respectively. Despite the different membrane sterols, yeast offers advantages as recombinant expression host for mammalian membrane proteins as it is much easier to handle than mammalian or insect cells (Bill 2001; Gatto et al. 2001). The methylotrophic yeast *Pichia pastoris* is especially advantageous for expression of membrane proteins as it can grow to

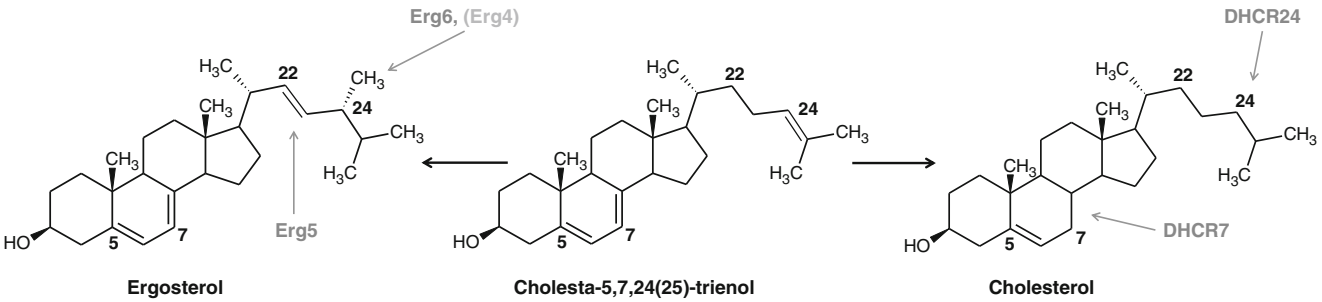


Fig. 1 Structures of ergosterol and cholesterol. The major yeast sterol, ergosterol, differs from the mammalian cholesterol lacking two double bonds at positions C-7 and C-22 and one methyl group at position C-24. The enzymes involved in ergosterol synthesis are the sterol C-22 desaturase encoded by *ERG5* and the sterol C-24 methyl transferase encoded by *ERG6*. For cholesterol synthesis, two dehydrocholesterol reductases, *DHCR7* and *DHCR24*, are required to saturate specifically the double bonds at positions C-7 and C-24. Cholesta-5,7,24(25)-trienol is shown as a theoretical, common biosynthetic intermediate of ergosterol and cholesterol biosynthesis. However, cholesta-5,7,24(25)-trienol is hardly detectable in ergosterol-producing yeast strains due to Erg6p action

high cell density, potentially enhancing the yield of recombinant protein. Many membrane proteins, including Na,K-ATPases, have already been expressed successfully in *P. pastoris* (Asada et al. 2011; Chloupková et al. 2007; Katz et al. 2010; Krettler et al. 2013; Lundstrom et al. 2006; Mao et al. 2004; Reina et al. 2007; Strugatsky et al. 2003; Zeder-Lutz et al. 2006). Furthermore, heterologous protein expression can be tightly regulated by using the methanol-inducible alcohol oxidase 1 (*AOX1*) promoter as reviewed in Bill (2001) and Freigassner et al. (2009).

Since the Na,K-ATPase is an important mammalian membrane protein (Skou 1957), its biochemical and structural properties have been studied extensively (Kaplan 2002). It belongs to the P-Type ATPase family of cation transporters and fulfils several essential functions in human cell physiology. The main function is to maintain the Na^+ and K^+ gradients across the plasma membrane, which is necessary for the contractility of heart and muscle cells as well as for neuronal excitability in the nervous tissue (Geering 2006). Moreover, the ion pump is an important target for the binding of cardiac glycosides such as ouabain and digitalis, which have been used for centuries in the treatment of heart failure (Aperia 2007). The catalytic α subunit is mainly responsible for ATP hydrolysis and ion transport across the membrane, whereas the β subunit supports correct and stable assembly into the plasma membrane (Beguin et al. 1998; Geering 2001; Hasler et al. 1998). Biochemical experiments have shown that cholesterol and also phospholipids have a notable influence on the stability and activity of Na,K-ATPases (Cohen et al. 2005; Cornelius et al. 2003; Cornelius 2001; Haviv et al. 2007; Lifshitz et al. 2007). Different isoforms of this enzyme family have been expressed heterologously in *Xenopus* oocytes (Crambert et al. 2000), *Saccharomyces cerevisiae* (Horowitz et al. 1990; Müller-Ehmsen et al. 2001; Pedersen et al. 1996), *P. pastoris* (Cohen et al. 2005; Reina et al. 2007), and insect cells (Blanco 2005; Koenderink et al. 2000; Liu and Guidotti 1997), respectively. Recently, cholesterol was identified in the crystal structure of Na,K-ATPase from shark, hence confirming the structural importance of this sterol (Toyoshima et al. 2011). Moreover, it was described that not only in the β2-adrenergic receptor, but also in the Na,K-ATPase protein family amino acid residues forming proposed cholesterol-binding sites are strongly conserved (Adamian et al. 2011). A cholesterol-binding consensus motif had been proposed earlier for G-protein-coupled receptors (GPCRs) (Hanson et al. 2008). New insight on lipid stabilization of membrane proteins has been derived quite recently (Goddard and Watts 2012; Jafurulla and Chattopadhyay 2013; Oates et al. 2012; Oates and Watts 2011; Zheng et al. 2012).

The utility of *P. pastoris* in membrane protein expression combined with the cholesterol dependence of many mammalian membrane proteins triggered our interest in creating a *P. pastoris* strain capable of producing cholesterol. Here, we describe the construction of a *P. pastoris* strain forming cholesterol as main sterol. We followed a similar strategy that was lately shown to work for *S. cerevisiae* (Morioka et al. 2013; Souza et al. 2011). Furthermore, we provide evidence that cholesterol-producing *P. pastoris* is capable of expressing the human Na,K-ATPase α3β1 isoform more efficiently in terms of stability, activity, and localization than other expression strains available so far. We propose that our cholesterol-producing strain will be a favorable tool for the expression of many other membrane proteins requiring specific interaction with cholesterol.

Materials and methods

Strains and culture conditions

Escherichia coli TOP10F′ cells (Life Technologies, Carlsbad, CA) were used for cloning experiments and propagation of expression vectors. *P. pastoris* strains used and generated in this study are listed in Table 1. All strains were derived from *P.*

Table 1 Description of *P. pastoris* strains used in this study

Name	Description	Source
WT	CBS7435 Δ*his4*Δ*ku70*	Näätsaari et al. (2012)
SMD1168	*SMD1168* Δ*his4*Δ*pep4*	Life Technologies, Carlsbad, CA
Δ*erg5*::*DHCR7*	CBS7435 Δ*his4*Δ*ku70* Δ*erg5*::*pPpGAP-Zeocin*^{TM}-[*DHCR7*]	This work
Cholesterol strain	CBS7435 Δ*his4*Δ*ku70* Δ*erg5*::*pPpGAP-Zeocin*^{TM}-[*DHCR7*] Δ*erg6*::*pGAP-G418*[*DHCR24*]	This work
WT+ATPase	*CBS7435* Δ*his4*Δ*ku70* Δ*aox1*::*pAO815*[*5′-AOX1-α3-TT-5′-AOX1-β1-TT-HIS4*]	This work
SMD1168+ATPase	*SMD1168* Δ*his4*Δ*pep4* Δ*aox1*::*pAO815*[*5′-AOX1-α3-TT-5′-AOX1-β1-TT-HIS4*]	This work
S-α3β1 (+ ATPase)	*SMD1168* Δ*his4*Δ*pep4* Δ*aox1*::*pAO815*[*5′-AOX1-α3-TT-5′-AOX1-β1-TT-HIS4*]	Reina et al. (2007)
Cholesterol strain+ATPase	CBS7435 Δ*his4*Δ*ku70* Δ*erg5*::*pPpGAP- Zeocin*^{TM}-[*DHCR7*] Δ*erg6*::*pGAP-G418*[*DHCR24*]Δ*aox1*::*pAO815*-[*5′-AOX1-α3-TT-5′-AOX1-β1-TT-HIS4*]	This work

pastoris CBS7435 Δ*his4*Δ*ku70* (Näätsaari et al. 2012) or from protease-deficient *P. pastoris* SMD1168 (Life Technologies, Carlsbad, CA), respectively. The control strain *P. pastoris* S-α3β1, already containing the genes for both Na,K-ATPase subunits integrated in the genome, was kindly provided by Laura Popolo (Reina et al. 2007). Knockout strains of *P. pastoris* were selected on YPD with antibiotics (1 % yeast extract, 2 % peptone, 2 % glucose, 2 % agar, 300 mg/l geneticin sulfate, or 100 mg/l Zeocin^{TM}). Minimal dextrose (MD) plates (1.34 % yeast nitrogen base (YNB), 4×10^{-5} % biotin, 2 % dextrose, and 1.5 % agar) were used to screen for His^{+} transformants containing the Na,K-ATPase expression cassette. In expression studies, *P. pastoris* cells were pregrown at 28 °C in BMGY (1 % yeast extract, 2 % peptone, 0.1 M phosphate buffer, pH 6, 1.34 % YNB, 4×10^{-5} % biotin, 1 % glycerol) for 48 h, followed by induction with BMMY medium containing 1 % methanol instead of glycerol at the same temperature. Protein expression was carried out for up to 72 h on 50 or 200 ml scale in baffled 300 ml and 2 l flasks, respectively.

Construction of a cholesterol-producing *P. pastoris* strain

The *ERG5* and *ERG6* coding sequences were sequentially disrupted and replaced by knock-in constructs for constitutively expressing dehydrocholesterol reductases specific for positions C-7 (*DHCR7*) and C-24 (*DHCR24*) in the sterol molecule, respectively (Fig. 2). Codon-optimized sequences for *DHCR7* and *DHCR24* from zebrafish (*Danio rerio*) were kindly provided by Howard Riezman (Souza et al. 2011) and were amplified with primers 1–4 (Supplemental Table S1). The genes originating from zebrafish had been codon-optimized for expression in *S. cerevisiae*. As the mean difference in codon usage between *S. cerevisiae* and *P. pastoris* is <5 %, according to Graphical Codon Usage Analyzer (GCUA) Software (Fuhrmann et al. 2004), no *P. pastoris*-specific codon optimization of the reductase genes was performed. The *DHCR7* coding sequence was cloned into pGAPZ A (Life Technologies, Carlsbad, CA), whereas the *DHCR24* coding sequence was cloned into pPpKan_S (GenBank Accession: JQ519694.1) using *Eco*RI and *Not*I restriction sites in both cases. To achieve constitutive expression of *DHCR24*, the *AOX1* promoter of pPpKan_S was replaced by the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter obtained from the pGAPZ A vector by *Eco*RI and *Bgl*II restriction and cloning. Expression cassettes with 5′ and 3′ stretches homologous to *ERG5* and *ERG6* flanking sequences, respectively, were created to achieve gene replacement by homologous recombination in the desired locus of the host strain (Fig. 2). DNA stretches of 500 bp flanking *ERG5* and *ERG6* coding sequences on the 5′ and 3′ sides, respectively, were amplified from *P. pastoris* CBS7435 genomic DNA using primers 5–12 (Supplemental Table S1). The 5′-flanking regions of *ERG5* and *ERG6* coding sequences were inserted in front of the *DHCR7* and *DHCR24* expression cassettes using *Bgl*II restriction sites. The 3′-flanking regions of *ERG5* and *ERG6* coding sequences, respectively, were blunt-end-cloned into pJET1.2/blunt vector (Thermo Scientific, Waltham, MA). *DHCR7* and *DHCR24* expression constructs were amplified using primers 13–16 (Supplemental Table S1) and were *Xho*I-cloned into pJET1.2/blunt vectors containing the respective 3′-flanking regions. Final expression/knock-in constructs were verified by sequencing. To obtain linear DNA fragments at suitable amounts for transformation of *P. pastoris*, the *DHCR7* and *DHCR24* knock-in cassettes were amplified using primers 17–20 (Supplemental Table S1). *P. pastoris* CBS7435 Δ*his4*Δ*ku70* was transformed sequentially with the *5′ERG5-GAP-DHCR7-zeocin*^{R}*-ERG5-3′* and the *5′ERG6-GAP-DHCR24-G418*^{R}*-ERG6-3′* cassettes (Fig. 2) as described (Lin-Cereghino et al. 2005). The *ERG5* gene was replaced by *DHCR7* using the knock-in cassette shown in Fig. 2a and yielded in *P. pastoris* Δ*erg5*::*DHCR7-z*eocin^{R} strain producing mainly campesterol (ergosta-5-enol, data not shown). This strain was transformed with the second knock-in cassette

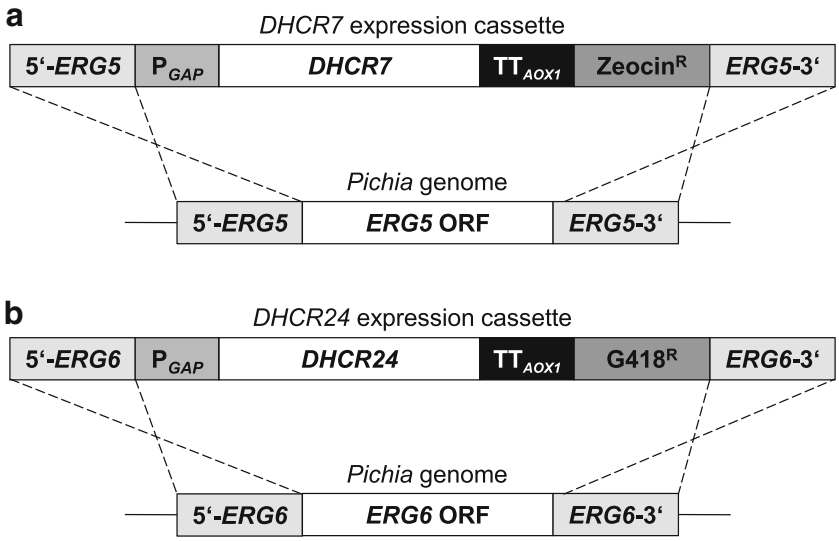


Fig. 2 Expression cassettes used for the generation of a cholesterol-producing *P. pastoris* strain. **a** The *DHCR7* expression cassette contains regions homologous to the 5′- and 3′-flanking sequences of the *ERG5* locus. Transformants were selected for Zeocin™ resistance. **b** The *DHCR24* expression cassette is flanked by 5′- and 3′-regions homologous to the sequences upstream and downstream of the *ERG6* coding sequence to assure homologous recombination in the *ERG6* locus. Transformants were screened for geneticin sulfate (G418) resistance. *GAP* promoter and *AOX1* terminator were used for both expression cassettes. The two cassettes were transformed sequentially into *P. pastoris* WT to obtain a cholesterol-producing strain

containing the *DHCR24* gene to generate the cholesterol-producing *P. pastoris* strain resistant to Zeocin™ and geneticin sulfate (Fig. 2b). Colony PCR using primers 21–24 (Supplemental Table S2) confirmed the correct integration of the expression cassettes.

Gas chromatography–mass spectrometry (GC-MS) analysis of yeast sterols

Total sterols were extracted from 15 OD_{600} units of cells cultivated under protein expression conditions, i.e., methanol induction for 72 h. Sterol extraction was performed essentially according to Quail and Kelly (1996). Briefly, cells were resuspended in 0.6 ml of methanol, 0.4 ml of 0.5 % pyrogallol in methanol, and 0.4 ml of 60 % KOH. Ten micrograms of cholesterol (Sigma-Aldrich, St. Louis, MO) dissolved in ethanol was added as internal standard to all samples except for the strains that were expected to produce cholesterol. Samples were heated at 90 °C for 2 h and saponified lipids were extracted three times with 1 ml *n*-heptane. The extracted sterols were dissolved in 10 μl of pyridine and derivatized with 10 μl of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (Sigma-Aldrich, St. Louis, MO). Derivatized samples were dissolved in 50 μl of ethyl acetate and sterols were analyzed by GC-MS as described previously (Ott et al. 2005). Compounds were identified based on their mass fragmentation pattern and their retention time relative to cholesterol using MSD ChemStation Software (Agilent Technologies, Santa Clara, CA).

Expression of Na,K-ATPase α3β1 isoform

The plasmid pAO815-α3/β1 encoding both α3 and β1 subunits of Na,K-ATPase under the control of the *AOX1* promoter was kindly provided by Cristina Reina (Reina et al. 2007). The vector was linearized with *Bgl*II and transformed into electrocompetent *P. pastoris* cells as described (Lin-Cereghino et al. 2005). Transformants were checked for integration of the expression cassette at the *AOX1* locus via colony PCR using primer numbers 25–28 (Supplemental Table S2). Positive clones were inoculated in 25 or 100 ml of BMGY in 300 ml or 2 l baffled Erlenmeyer flasks, respectively, for cultivation at 28 °C and 120 rpm for 48 h. Na,K-ATPase expression was induced by the addition of 25 or 100 ml BMMY to obtain a final methanol concentration of 1 %. Methanol was added every 12 h to a final concentration of 1 % for up to 72 h of induction.

Cell disruption and membrane fraction preparation

To prepare total cell lysates, yeast culture aliquots of 1 ml were spun for 5 min at 3,000×*g* at 4 °C, and cell pellets were resuspended in 200 μl of ice-cold breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5 % glycerol). Phenylmethylsulfonyl fluoride (PMSF) was freshly added from a 1 M stock in dimethyl sulfoxide (DMSO) to a final concentration of 1 mM. An equal volume of glass beads (0.25–0.5 mm diameter, Carl Roth GmbH, Karlsruhe, Germany) was added, and cells were disrupted by vortexing for 30 s followed by

cooling for 30 s on ice. Disruption and cooling cycles were repeated eight times. After centrifugation at 3,000×*g* and 4 °C for 5 min, the supernatant containing the total cell lysate was harvested and stored at −20 °C until use.

Membrane fractions were prepared according to the following procedure: 200 ml of the cell culture was harvested at 3,000×*g* and 4 °C for 5 min. The cells were washed with ice-cold water and the pellet was resuspended in 1 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4) and 2 μl of 1 M PMSF in DMSO per gram of cell wet weight. Disruption was performed with a Merckenschlager homogenizer (Sartorius, Goettingen, Germany) under CO_2 cooling for 3 min with 30 s cooling intervals. Unbroken cells, cell debris, and glass beads were spun out at 3,000×*g* for 10 min. The total cell lysate was centrifuged at 12,000×*g* for 15 min to obtain supernatant S12 and pellet P12 fraction. Supernatant S12 was spun at 20,000×*g* for 15 min to receive supernatant S20 and pellet P20. Ultracentrifugation of supernatant S20 at 100,000×*g* for 45 min yielded the fractions S100 and P100. The pellets were resuspended in 10 mM Tris–HCl buffer, pH 7.4, and all aliquots were frozen at −80 °C until use.

SDS-PAGE and western blot analysis

Proteins were precipitated by adding 0.25 volumes of 50 % trichloroacetic acid and solubilized in 0.1 % sodium dodecyl sulfate (SDS) dissolved in 0.1 M sodium hydroxide (NaOH). Protein concentrations were quantified by the method of Lowry using bovine serum albumin as standard (Lowry et al. 1951). Twenty micrograms of protein was separated on 12.5 % SDS-PAGE gels following standard procedures (Laemmli 1970). Western blot analysis was performed as described (Haid and Suissa 1983). Rabbit anti-KETYY and anti-GERK antisera recognizing Na,K-ATPase α subunit and β subunit, respectively, were kindly donated by Steven J. D. Karlish (Weizmann Institute of Sciences, Rehovot, Israel). An antibody against yeast plasma membrane H^+-ATPase (Pma1p) produced in rabbit was provided by Guenther Daum (Institute of Biochemistry, Graz University of Technology) and was used as marker for plasma membrane localization. Goat anti-rabbit IgG-peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) was used as secondary antibody. Visualization of immunoreactive bands was accomplished with the SuperSignal® West Pico Chemiluminescent substrate (Thermo Scientific, Waltham, MA) using the G:Box HR16 BioImaging system (Syngene, Cambridge, UK).

Na,K-ATPase activity assay

Na,K-ATPase activity was determined as previously described with minor modifications (Kapri-Pardes et al. 2011). Aliquots of the crude membrane fractions containing 1–3 μg of protein were added to 400 μl reaction medium containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, and 25 mM histidine, pH 7.4, in the presence or absence of 10 mM ouabain (Merck KGaA, Darmstadt, Germany). To start the reaction, ATP was added freshly to 0.1 mM and the mixture was incubated at 37 °C and 350 rpm for 15 min. The released P_i was detected with "P_iColorLock Gold" (Innova Biosciences, Cambridge, UK), and the absorbance of the green malachite dye complex was measured at 635 nm. Specific Na,K-ATPase activity was defined as ATPase activity susceptible to inhibition by ouabain and was calculated as the difference in ATP hydrolysis without and with 10 mM ouabain in the assay.

[^{3}H]-ouabain binding assay

Saturation binding of [^{3}H]-ouabain (13 Ci/mmol; PerkinElmer, Waltham, MA) was performed for 90 min as previously described (Pedersen et al. 1996; Reina et al. 2007). Cell surface-binding capacity of 10^9 cells per strain and time point was estimated upon cell harvest and incubation with 500 nM [^{3}H]-ouabain. To estimate nonspecific binding, equivalent samples were incubated with 500 nM [^{3}H]-ouabain together with 1 mM cold ouabain. Subsequent to incubations, cells were pelleted at 1,000×*g* and 4 °C for 5 min and washed twice with ice-cold water. Bound [^{3}H]-ouabain was measured with a Packard Tri-Carb2900TR Liquid Scintillation Analyzer (PerkinElmer, Waltham, MA), and counts per minute (c.p.m.) values for nonspecific binding to each strain were subtracted.

Results

Characterization of a cholesterol-producing *P. pastoris* strain

Growth tests in baffled shake flasks with BMGY medium showed a reduced specific growth rate of the cholesterol-producing *P. pastoris* strain (0.11 h^{-1}) compared to the corresponding wild-type strain (0.25 h^{-1}). The cholesterol-producing *Pichia* strain is still capable of growing to high cell densities, as it reached a final OD_{600} of 42–61 after 48–60 h of growth on BMGY medium in shake flasks, while the wild-type cells grew to a final OD_{600} of ~75 under the same conditions. Upon methanol induction for 72 h, the final OD_{600} was 70–75 for wild-type strains, while cholesterol-producing strains only reached an OD_{600} of 45. Expression of Na,K-ATPases did not significantly alter the growth behavior and final OD_{600} in these two strain backgrounds.

GC-MS analyses of total sterol patterns showed that under standard protein expression conditions, i.e., 72 h of methanol induction, the *P. pastoris* WT strain (Fig. 3a) contained 88 % ergosterol and some ergosterol precursors, whereas the cholesterol-producing *P. pastoris* strain (Fig. 3b) formed approximately 89 % of cholesterol besides several cholesterol precursors. The mass fragment spectrum of the yeast-derived

cholesterol peak (Fig. 3b) was identical to the spectrum of the cholesterol reference standard (Fig. 3a), confirming cholesterol biosynthesis in the novel *P. pastoris* strain. The overall sterol patterns of wild-type and cholesterol-producing *P. pastoris* strains and the relative retention times of the identified sterols are listed in Table 2. To our knowledge, this is the first

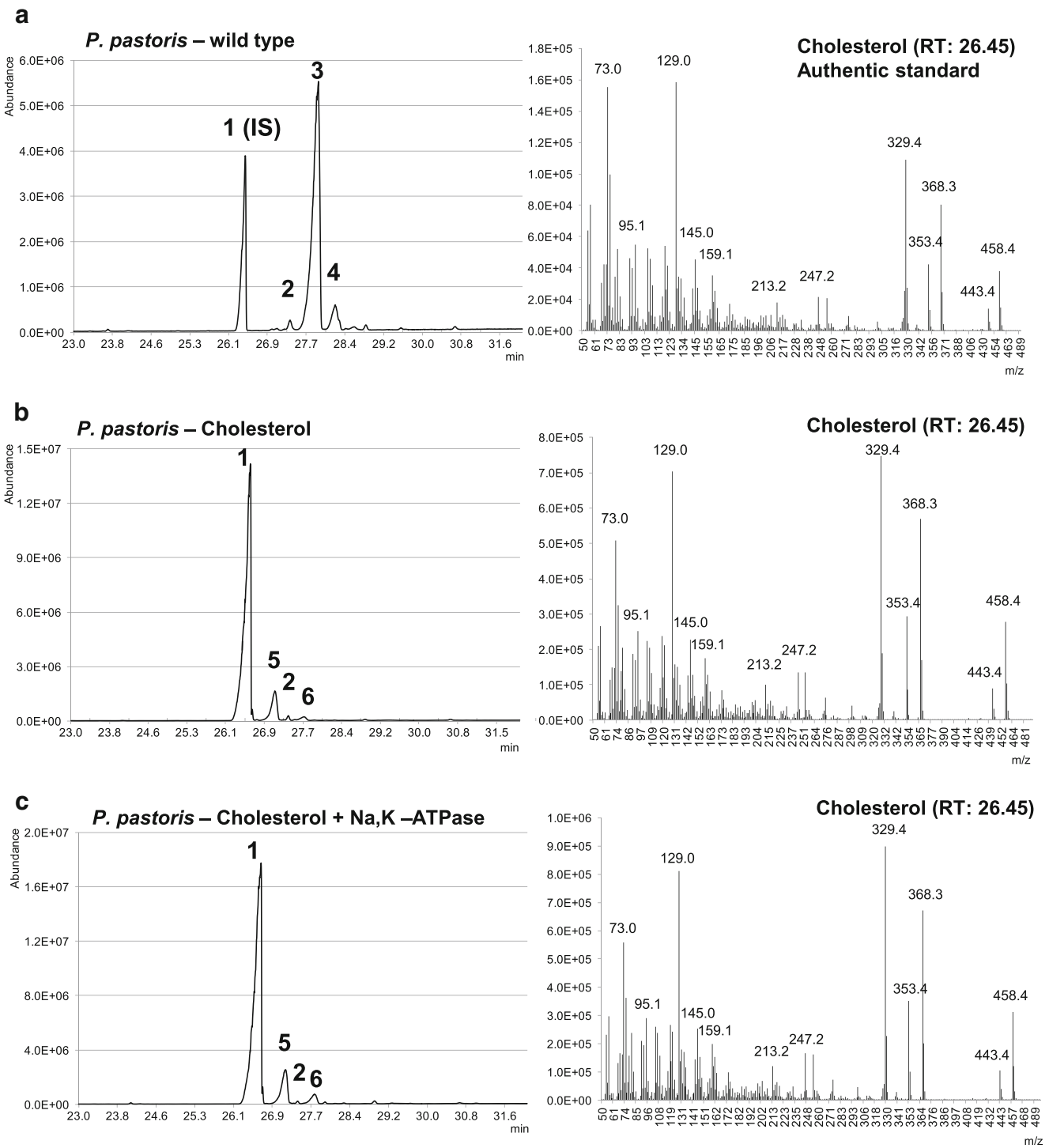


Fig. 3 GC-MS analysis of sterol extracts from *P. pastoris*. Representative chromatograms of sterols isolated from wild-type (**a**), cholesterol-producing (**b**), and cholesterol-producing as well as Na,K-ATPase expressing (**c**) *P. pastoris* strains induced in BMMY medium for 72 h are shown. The analyses were performed in triplicate and quantifications are shown in Table 2. Authentic standards, relative retention times, and MS fragmentation patterns allowed identification of the following compounds: cholesterol (*1*), zymosterol (*2*), ergosterol (*3*), ergosta-5,7,22,24(28)-tetraenol (*4*), 7-dehydrocholesterol (*5*), and cholesta-5,7,24(25)-trienol (*6*). MS fragmentation patterns of authentic cholesterol standard (internal standard, IS) and cholesterol produced in *P. pastoris* were identical

Table 2 GC-MS analysis of sterols isolated from *P. pastoris* strains upon 72 h of methanol induction

Sterol	Relative amount (%)[a]	Relative retention time
P. pastoris WT		
Cholesterol (internal standard)	–	1
Zymosterol (cholesta-8,24-dienol)	1.6±0.1	1.034
Ergosterol	88.2±0.2	1.054
Ergosta-5,7,22,24(28)-tetraenol	10.2±0.1	1.068
P. pastoris cholesterol strain		
Cholesterol (cholesta-5-enol)	89.2±3.0	1
7-Dehydrocholesterol	8.9±2.1	1.018
Zymosterol (cholesta-8,24-dienol)	0.6±0.2	1.029
Cholesta-5,7,24(25)-trienol	1.3±0.1	1.041
P. pastoris cholesterol strain+ATPase		
Cholesterol (cholesta-5-enol)	85.8±0.4	1
7-Dehydrocholesterol	10.8±0.3	1.019
Zymosterol (cholesta-8,24-dienol)	0.4±0.1	1.028
Cholesta-5,7,24(25)-trienol	3.0±0.2	1.041

[a] Mean ± standard deviations of three biological replicates are given

documentation of cholesterol formation in an engineered *P. pastoris* strain. As the sterol patterns for both the cholesterol-producing and the wild-type *P. pastoris* strain showed that roughly 90 % of their total sterols are the respective terminal sterols, this situation was considered ideal to analyze the sterol dependence of Na,K-ATPase α3β1 expression and function in *P. pastoris*.

Expression of Na,K-ATPase α3β1 in *P. pastoris*

P. pastoris wild-type, SMD1168, and cholesterol strains were transformed with the *Bgl*II linearized pAO815-α3β1 plasmid for co-expression of both Na,K-ATPase subunits (Table 1). *P. pastoris* S-α3β1 containing the same expression plasmid served as control for our experiments (Reina et al. 2007). Induction time dependence of α3 subunit expression was explored by taking 1 ml aliquots after 0, 8, 24, 48, and 72 h of methanol induction from 50 ml cultures grown at 28 °C in 300 ml baffled flasks. After cell harvest and disruption, the α3 subunit was detected in lysates as 110 kDa band on western blots using an antibody specifically recognizing the KETYY amino acid sequence (Fig. 4). Expression level of the α3 subunit reached its maximum at 8 h of methanol induction for *P. pastoris* wild-type, S-α3β1, and SMD1168 strains, before levels decreased significantly with progressing induction time. Strikingly, the amount of expressed α3 subunit increased in the cholesterol-producing strain with prolonged induction period, which was the first indication that recombinant Na,K-ATPase α3β1 showed an enhanced protein half-life in the sterol-engineered strain.

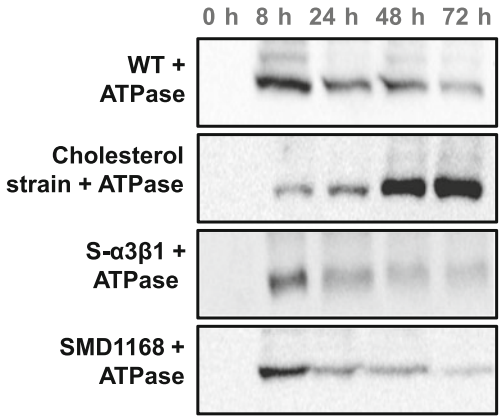


Fig. 4 Western blot detection of Na,K-ATPase subunit α3 in total cell lysates of expression strains. Expression of α3 subunit (110 kDa) was determined after 0, 8, 24, 48, and 72 h of methanol induction in cell lysates. Samples of *P. pastoris* wild-type (WT), cholesterol-producing, S-α3β1, and SMD1168 strains all expressing Na,K-ATPase α3β1 isoform were harvested by centrifugation for 5 min at 3,000×*g* and 4°C. Twenty micrograms of total cell extract protein was loaded onto a 12.5 % SDS-PAGE gel, separated by electrophoresis, and probed by anti-KETYY antibody

In their native hosts, Na,K-ATPases are localized to the plasma membranes. Thus, it was of particular interest to determine whether recombinant Na,K-ATPase α3β1 is transported to the plasma membrane in *P. pastoris* and whether this process was influenced by the available sterol structures. To characterize in more detail the membrane localization of the α3 and β1 subunits, cells were grown in baffled 2 l flasks to obtain sufficient cell material for membrane preparation after 8 and 72 h of methanol induction (Fig. 5). Several centrifugation steps yielded subfractions comprising the total cell lysate or homogenate (H) at 3,000×*g*, the supernatant and pellet at 12,000×*g* (S12, P12), the supernatant and pellet at 20,000×*g* (S20, P20), and the supernatant and pellet after ultracentrifugation at 100,000×*g* (S100, P100). An antibody against yeast plasma membrane ATPase (Pma1p, 100 kDa) was used as plasma membrane marker for the particular fractions. Colocalization of α3 and β1 with Pma1p was taken as an indicator for plasma membrane localization of Na,K-ATPase. Pma1p was found in different amounts in every fraction except S100, which should contain mainly cytosolic proteins but no membranes (Zinser and Daum 1995). In *P. pastoris* strains containing ergosterol as major sterol, Pma1p was observed mainly in fractions P12 and P20 and only to a lesser extent in the P100 fraction. In cholesterol-producing strains, Pma1p was equally prominent in P20 and P100 fractions and was also found in P12 fractions. These trends were independent of the expression of Na,K-ATPase. Specificity of the employed anti-α3 (anti-KETYY) and anti-β1 (anti-GERK) antisera was underscored by the lack of signal in the empty wild-type and cholesterol-producing strains (Fig. 5).

After 8 h of induction, the α3 subunit was detected in all expression strains and almost perfectly colocalized with the

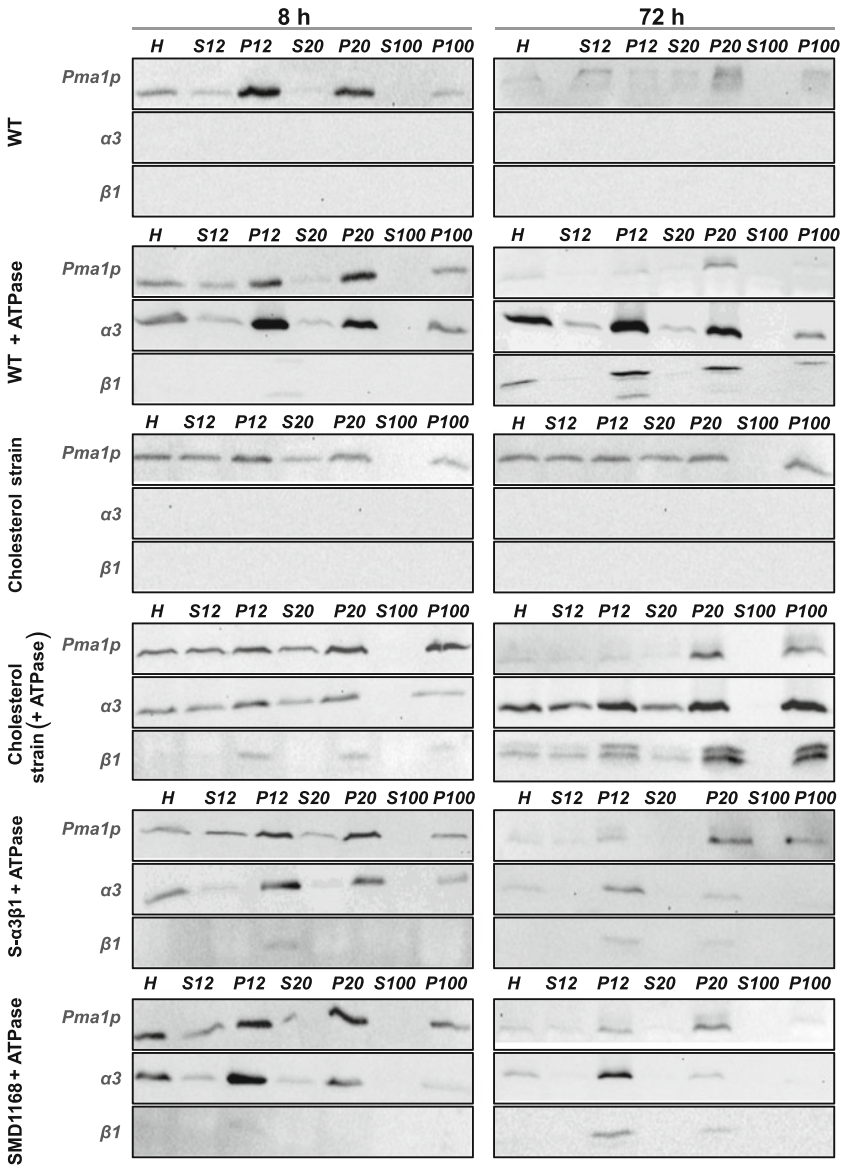


Fig. 5 Western blot detection of Na,K-ATPase subunits α3 and β1 and plasma membrane marker Pma1p in subcellular fractions. *P. pastoris* WT+ATPase, *P. pastoris* cholesterol strain+ATPase, *P. pastoris* S-α3β1(+ATPase), and *P. pastoris* SMD1168+ATPase were induced for 8 and 72 h, respectively. *P. pastoris* WT and *P. pastoris* cholesterol-producing strain without expression plasmid were treated the same way serving as negative control. After membrane fractionation, 20 μg of total protein samples were separated on a 12.5 % SDS-PAGE gel and incubated with antibodies against subunits α3 (anti-KETYY) and β1 (anti-GERK) and Pma1p. Different fractions after centrifugation are indicated in the lines as cell homogenate (H), supernatant and pellet at 12,000×*g* (S12, P12), supernatant and pellet at 20,000×*g* (S20, P20), and supernatant and pellet at 100,000×*g* (S100, P100)

plasma membrane marker in each of the strains. The major signals for α3 and Pma1p were observed in the P12 and P20 fractions. The β1 subunit was not very well expressed at 8 h in any of the strains and was visible as a very faint band in the P12 fraction in the wild-type, SMD1168, and S-α3β1 strains. A small amount was also detectable in the P20 and P100 fractions in the cholesterol-producing strain, which correlated nicely with the Pma1p signal in this background. Interestingly, after 72 h of induction, the α3 subunit hardly colocalized with Pma1p in all strains with wild-type sterol background. Whereas Pma1p peaked in the P20 fraction in these strains, the strongest signals for α3 and β1 subunits were obtained in P12 fractions trailed by P20 fractions. The signals for the β1 subunit were weaker than for the α3 subunit, but usually colocalized with the latter. Occasionally, an additional, smaller band of 35 kDa was observed, particularly in the P12 fraction. This indicates that β1 is not fully glycosylated in ergosterol-containing strains as has already been described (Reina et al. 2007). In contrast, the expression of α3 and β1 subunits in the cholesterol-producing strain was relatively strong after 72 h showing absolute colocalization with Pma1p in the fractions P20 and P100 and, on a lower level, also in P12 fraction. Remarkably, the β1 subunit was much better expressed than in the ergosterol-producing strains and, additionally, showed a much more advanced glycosylation pattern with apparent sizes of 44 and 40 kDa in the western blot. Improved expression and enhanced glycosylation of the β1 subunit in the cholesterol-producing *P. pastoris* strain indicated an enhanced overall stability of the heterodimer when colocalizing with the plasma membrane marker Pma1p. Recombinant expression of Na,K-ATPase α3β1 in the cholesterol-producing *Pichia* strain did not significantly alter the sterol pattern of this strain (Fig. 3c and Table 2).

Determination of Na,K-ATPase α3β1 activity in membrane fractions

The same membrane fractions that had been subjected to western blot analyses were assayed for ATPase activity using "P_iColorLock Gold" reagent to detect inorganic phosphate released by ATP hydrolysis at 37 °C. We refrained from adding SDS for the particular reason that the membrane environment of the ion pump in the intact, cholesterol- or ergosterol-containing lipid bilayer should not be altered. Specific activities were calculated based on the differences in absorbance at 635 nm without and with the addition of 10 mM ouabain. The assay originally is supposed to detect all kinds of cellular ATPase activity, not only the activity of recombinant Na,K-ATPase. Thus, the ouabain-sensitive part of ATPase activity was determined to exclude all intrinsic *Pichia* ATPase activities in these assays and detect specifically Na,K-ATPase function that is known to be inhibited by ouabain (Reina et al. 2007). We observed a certain background of ouabain-sensitive activity in all strains tested independent of Na,K-ATPase expression (Fig. 6). After 8 h of methanol induction, no difference in Na,K-ATPase activity could be detected between the strains and the membrane fractions assayed (data not shown).

Notably, a significant ouabain-sensitive ATPase activity was detected only for the membranes of cholesterol-producing Na,K-ATPase expression strain but not for all the other strains after 72 h of induction (Fig. 6). Consistent with the western blot analysis (Fig. 5), high Na,K-ATPase activities were derived for the P20, P100, and P12 fractions of the cholesterol-producing Na,K-ATPase expression strain, but also for the total cell lysate. It appears that the activity of recombinant Na,K-ATPase was too low to be detectable in the crude membrane fractions of conventional *Pichia* expression hosts with the available method. On the other hand, ouabain-sensitive ATPase activity made up ~40 % of total ATPase activity in the membranes of the cholesterol-producing expression strain. In these experiments, the highest activities were found for the P20 fraction (Fig. 6c), which also harbors the highest amounts of plasma membrane marker Pma1p (Fig. 5).

[^{3}H]-ouabain binding to Na,K-ATPase on the cell surface of intact *P. pastoris* cells

Assaying ouabain-sensitive ATPase activity had turned out to be of very limited reliability in characterizing the abundance of Na,K-ATPase in membrane preparations of different expression strain backgrounds. Thus, we measured [^{3}H]-ouabain binding to intact cells and used the number of binding sites as an indicator for functional Na,K-ATPase expression on the cell surface (Pedersen et al. 1996; Reina et al. 2007). After 8 h of methanol induction, minor amounts of cell-associated [^{3}H]-ouabain were detected for some of the tested strains (Fig. 7). Only the wild-type, cholesterol-producing, and protease-deficient SMD1168 strains expressing Na,K-ATPase α3β1 showed radioligand binding above the background signal. After 72 h of induction, in contrast, significant and specific binding of [^{3}H]-ouabain was detected for every ATPase expression strain as the negative controls did not show any binding capacity. Calculation of the average number of ouabain-binding sites per cell (B_{max}/cell) yielded values in the order of magnitude described for Na,K-ATPase α3β1 expression in *P. pastoris* (Reina et al. 2007). The SMD1168 (B_{max}/cell 127) and S-α3β1 (B_{max}/cell 116) protease-deficient Na,K-ATPase expression strains showed similar binding capacity, which is consistent as both have the same strain background (Reina et al. 2007). About 60 % more [^{3}H]-ouabain binding was observed for the wild-type-based expression strain (B_{max}/cell 200). Remarkably, the cholesterol-producing expression strain (B_{max}/cell 478) had about 2.5- and 4-fold more radioligand-binding sites on the cell surface than the wild-type and protease-deficient expression strains, respectively.

Discussion

The overexpression of membrane proteins from higher eukaryotes in yeasts is highly desired for elucidation of protein structures as well as for studying membrane protein function in vitro and in vivo (Freigassner et al. 2009). Moreover, the number of membrane proteins regulated in their stability, localization, and function by molecular interaction with other membrane components is increasing rapidly (Haviv et al. 2013; Lifshitz et al. 2006). There is considerable interest in studying sterol-dependent membrane protein function (Heese-Peck et al. 2002; Kato and Wickner 2001; Morioka et al. 2013; Munn et al. 1999; Souza et al. 2011; Umebayashi and Nakano 2003; Wriessnegger and Pichler 2013), and there is already some interest in applying sterol-engineered yeast cells for membrane protein expression (Kitson et al. 2011). Basically, all of the yeast sterol-engineering studies to date have been conducted in *S. cerevisiae*. In this work, we have focused on *P. pastoris* as the preferred host for membrane protein expression and present for the first time a *P. pastoris* strain that does form cholesterol as its main sterol instead of the yeast-specific ergosterol. Furthermore, we show that our cholesterol-producing *Pichia* strain is perfectly suited for functional expression of Na,K-ATPase α3β1 isoform, which exerts its function in a cholesterol-dependent manner (Haviv et al. 2007).

Following a similar approach as described for *S. cerevisiae* (Souza et al. 2011), we obtained a *P. pastoris* strain that produces cholesterol with almost the same efficiency as the corresponding baker's yeast strain, i.e., approximately 90 % of total sterols is cholesterol (Fig. 3b and Table 2). In both yeasts, the constitutive expression of *DHCR7* and *DHCR24* integrated into the genome was the key element in generating a stable, cholesterol-producing cell line. It should be noted, however,

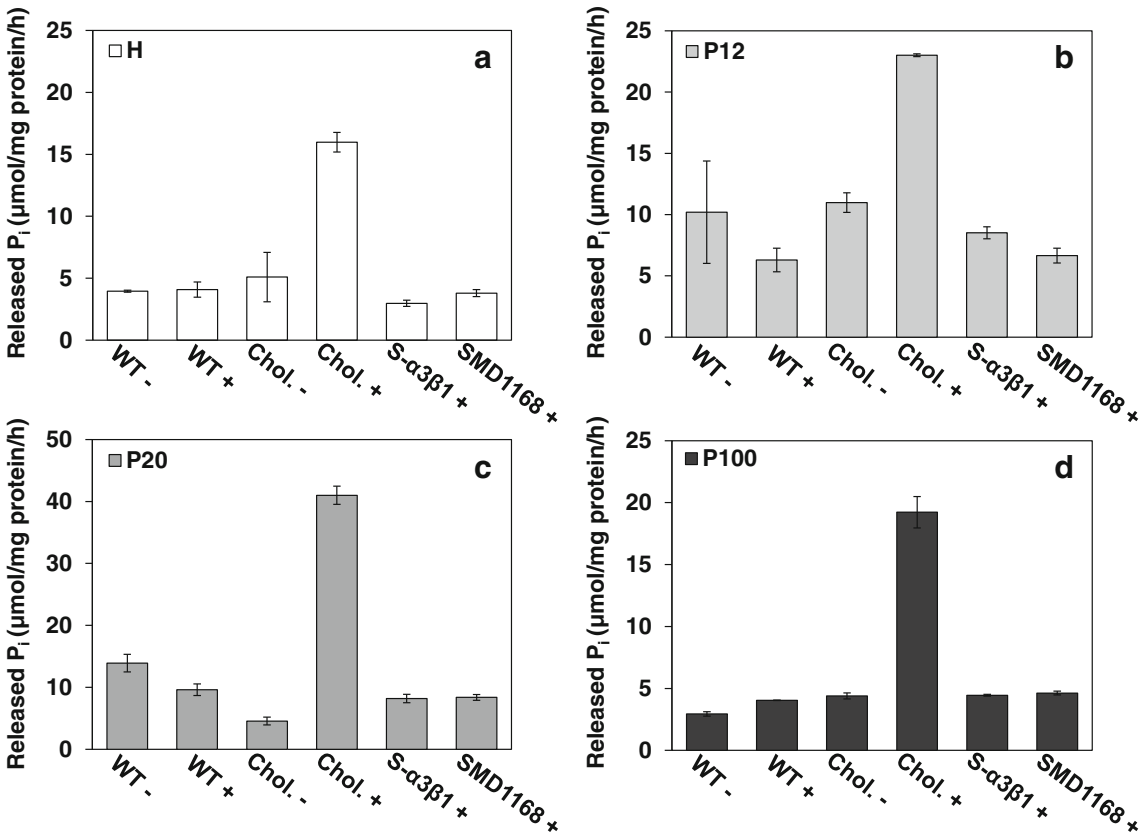
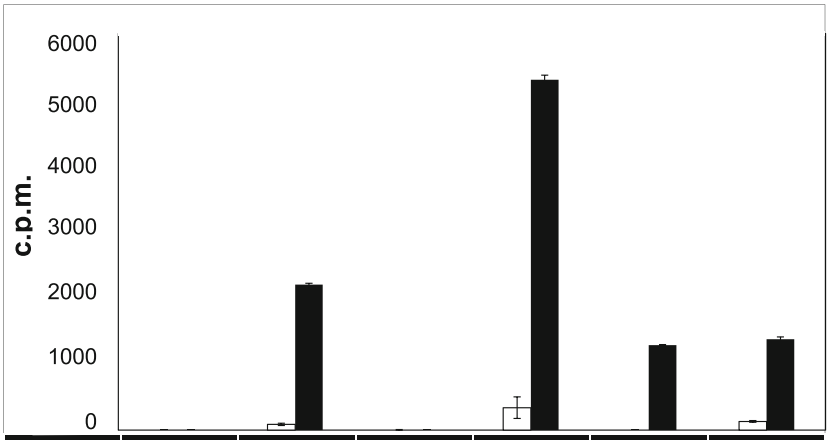


Fig. 6 Determination of ouabain-sensitive Na,K-ATPase activity. Membrane fractions H, homogenate of total cell extract, (**a**), P12 (**b**), P20 (**c**), and P100 (**d**) were isolated from cells pregrown on BMGY and induced on BMMY medium for 72 h as described in the "Materials and methods" section. One to 3 μg of total protein from each membrane fraction was incubated with the reaction mixture containing 0.1 mM ATP in the absence or presence of 10 mM ouabain at 37°C. Specific ouabain-sensitive ATPase activity was calculated from the difference in absorbance at 635 nm as micromole of liberated P_i per hour and milligram protein. Membrane fractions of Na,K-ATPase α3β1 isoform expressing strains (+) in wild type (WT), cholesterol-producing (Chol.), published control (S-α3β1), and protease-deficient (SMD1168) strain background and of empty WT as well as cholesterol-producing strains (−) were compared. The *bars* show the mean value and range of two independent experiments

that the high rate of cholesterol formation in *P. pastoris* was observed under the conditions of Na,K-ATPase expression by methanol induction while *DHCR7* and *DHCR24* expression was driven by supposedly constitutive glyceraldehyde-3-phosphate dehydrogenase promoters (P_{GAP}, Fig. 2). When cholesterol-producing *Pichia* was grown on glucose or glycerol media, it became apparent that cholesterol formation by DHCR7 and DHCR24 protein action was incomplete as sterol analysis yielded about 50 % of cholesterol and 50 % of cholesterol precursors cholesta-7,24(25)-dienol, cholesta-5,7,24(25)-trienol, and 7-dehydrocholesterol under these conditions (data not shown). Thus, it may be speculated that under methanol induction conditions, the reduced proliferation rate of *Pichia* as well as a potentially lower transcription rate of dehydrocholesterol reductase genes is beneficial for cholesterol formation in the methylotrophic yeast. Too high transcriptional activity from P_{GAP} on glucose or glycerol medium might be detrimental to folding of the recombinant DHCR proteins. Most important for recombinant membrane protein expression studies, methanol induction conditions yielded similarly efficient cholesterol and ergosterol production in the engineered and wild-type strains, respectively, providing a fair chance to assess sterol-dependent effects (Fig. 3). The cholesterol-producing *P. pastoris* strain had a lower specific growth rate compared to the wild-type strain, which very much resembles the situation in *S. cerevisiae* (Souza et al. 2011). Apparently, yeasts are restricted in their growth behavior by the production of a nonnatural sterol emphasizing the importance of specific sterol structures for the cell physiology of eukaryotic organisms. Despite the reduced maximum growth rate, cholesterol-producing *P. pastoris* is capable of reaching high cell densities during standard protein expression protocols.

When expressing Na,K-ATPase α3β1 isoform in diverse strain backgrounds, western blot experiments were performed on total cell lysates (Fig. 4) and on different membrane fractions (Fig. 5). The results showed that cholesterol-containing membranes afford a good environment for stability of the α3 subunit, whereas it is less stable in ergosterol-containing strains, which has already been documented in the past (Reina et al. 2007). Furthermore, the β1 subunit was strongly expressed in the cholesterol-producing *P. pastoris* strain. Both



Strain	Wild type	Wild type	Chol. producing	Chol. producing	SMD1168 (S-α3β1)	SMD1168
ATPase	-	+	-	+	+	+
□ 8 h	0	87 ± 17	0	340 ± 163	0	132 ± 13
■ 72 h	0	2201 ± 22	0	5302 ± 68	1287 ± 8	1376 ± 35

Fig. 7 Quantification of [^{3}H]-ouabain binding capacity of Na,K-ATPase expression strains. Strains were pregrown in BMGY and induced in BMMY medium for 8 (*open bars*) and 72 h (*filled bars*) as described in the "Materials and methods" section. Radioligand binding was determined for 10^9 cells per strain and experiment by liquid scintillation counting, and values for unspecific binding were subtracted for strains expressing (+), or not expressing (−), Na,K-ATPase α3β1 isoform in wild-type, cholesterol-producing, and protease-deficient strains (S-α3β1 and SMD1168). The counts per minute (c.p.m.) values are given as mean ± standard deviation of representative single cultivations analysed in triplicate

ATPase subunits were detected in the same fractions as plasma membrane marker Pma1p. Earlier, it had been demonstrated that expression of the α subunit without the β subunit leads to its ER retention and degradation (Beggah et al. 1996; Gatto et al. 2001; Reina et al. 2007). This correlates with our observations that when β1 is badly expressed, also α3 is susceptible for degradation. In the cholesterol-producing strain, in contrast, the β1 subunit was very well expressed and could therefore stabilize the α3 subunit, promoting transport and correct integration into the plasma membrane. Cholesterol was recently found to be associated with Tyr^{40} of the β subunit in the crystal structure of Na,K-ATPase (Toyoshima et al. 2011). This amino acid forms a hydrogen bond with Gln^{856} on transmembrane domain 7 of the α subunit. This highly conserved tyrosine residue was also previously described to interact with the α subunit (Hasler et al. 2001). Consequently, the cholesterol in the membranes of our engineered *P. pastoris* strain is likely to interact with the β1 subunit, hence improving the assembly of the recombinant α3β1 dimer. The β subunit is a 35 kDa protein with three glycosylation sites (Ovchinnikov et al. 1986), which—upon full glycosylation—lead to an apparent size of 55 kDa in mammalian cells, but to only 44 kDa in *P. pastoris* due to different glycosylation patterns. Our observations showed that in the cholesterol-producing strain an additional 40 kDa protein is produced. In the ergosterol-producing strain, in contrast, a protein with an apparent size of 35 kDa can be detected besides the 44 kDa band. Similar findings have also been described earlier (Katz et al. 2010; Reina et al. 2007). This leads to the assumption that glycosylation is performed differently in our novel *Pichia* strain, probably also contributing to the subunit assembly. It is described, though, that only the complete lack of glycosylated β subunits truly influences the assembly and activity of Na,K-ATPase (Beggah et al. 1997).

Na,K-ATPase activity was detected in crude membrane fractions of cholesterol-producing yeast without further purification steps or the addition of stabilizing lipids, which were so far deemed essential to document protein function (Haviv et al. 2007; Lifshitz et al. 2007). In accordance with the western blot results, the highest activity was measured in the P20 fraction (41 ± 1.5 μmol P_i/mg protein/h). Also the measured activity in the P100 fraction (19 ± 1.3 μmol P_i/mg protein/h) is remarkable for our cholesterol-producing yeast (Fig. 5). Published data indicates that the Na,K-ATPase αβ complex is assembled in the endoplasmic reticulum (ER), where the protein already exerts its function (Gatto et al. 2001). This could be a reason for the detection of specific Na,K-ATPase activity in fractions containing membrane parts other than the plasma membrane. Unexpectedly, no significant Na,K-ATPase activity beyond the background level was detected in wild-type and protease-deficient expression strains. This may be due to inferior expression of the β1 subunit leading to impaired stability of the heterodimeric protein in the ergosterol-containing membranes. Furthermore, SDS had been used for purification, solubilization, and unmasking of Na,K-ATPases that are enclosed in sealed vesicles, and therefore, accessibility by either ouabain or ATP is reduced (Ivanov et al. 2004). Preceding incubation of membranes with SDS

had been shown to inhibit yeast endogenous H^+-ATPases and, furthermore, had increased Na,K-ATPase activity by 20 % due to improved accessibility of Na,K-ATPases in closed vesicles (Pedersen et al. 1996). To preserve the natural membrane environment, our assay setup did not include SDS treatment of the membranes, which has to be taken into account when interpreting the results (Fig. 6). At first sight, it seemed puzzling that no significant amount of ouabain-sensitive ATPase activity was detectable in the membranes of ergosterol-containing Na,K-ATPase expression strains. However, in these strains, total ATPase activity was roughly one order of magnitude higher than ouabain-sensitive activity limiting the accuracy of the applied procedure. Furthermore, due to the omission of SDS, a certain part of Na,K-ATPases may have been sealed in outside-in vesicles and may therefore not have been accessible for the inhibitor ouabain. Following the same lines of argumentation, the minor levels of apparently ouabain-sensitive ATP hydrolysis observed for the membranes of nonexpressing strains can only be explained by the inaccuracies in determining ouabain-sensitive from total ATPase activity levels. Nonetheless, Na,K-ATPase activity in the membrane fractions of the cholesterol-producing *P. pastoris* strain clearly surpassed the measured activities of membrane fractions from all other strains used in this study.

Initial evidence for recombinant Na,K-ATPase localization had been derived from western blot analyses and ATPase assays. Additionally, we examined how much of the protein is effectively transported to the cell surface of the cell by [^{3}H]-ouabain-binding studies with intact cells. This ligand binds specifically to the Na,K-ATPase α subunit at the outer leaflet of the membrane and, therefore, can be used to trace the sodium pump in the plasma membrane (Reina et al. 2007). The cell surface [^{3}H]-ouabain binding capacity measured for the *P. pastoris* S-α3β1 strain which was used for control experiments correlated well with the published data. Strikingly, the cholesterol-producing strain showed about four times more surface-binding sites for ouabain proving that properly folded Na,K-ATPase α3β1 is located on the cell surface to a higher extent than in all of the ergosterol-containing strains. Low Na,K-ATPase activities and inefficient transport to the plasma membrane as described in Reina et al. (2007) were therefore significantly enhanced by producing cholesterol in the *P. pastoris* expression host. In Chinese hamster ovary cells, cholesterol positively influences membrane protein exit from the ER (Ridsdale et al. 2006). Similar processes may be stimulated in cholesterol-producing yeasts. Although attempts have been made earlier to create a *S. cerevisiae* cholesterol strain for enhanced membrane protein production (Kitson et al. 2011), our work provides the first evidence that expression of a human membrane protein is improved in a yeast strain capable of producing cholesterol instead of ergosterol. To conclude, our results show that changing the lipid environment of a heterologous host system such as *P. pastoris* can contribute to the improvement of recombinant expression and stability of a human membrane protein.

Ongoing and future work in our laboratory will be focusing on three particularly urgent issues. First, what is the physiological response of *P. pastoris* to the production of the nonnative sterol compound and which compensatory reactions might be taking place. Secondly, it has been shown that expression of the Na,K-ATPase α3β1 is limited in shaking flask cultures, but could be improved by cultivating cells in a bioreactor (Reina et al. 2007). How will cholesterol-producing *P. pastoris* behave in bioreactors? The equivalent *S. cerevisiae* strain (Souza et al. 2011) performed very well in this situation (Howard Riezman, personal communication). Last, but not least, it will be interesting to learn which further mammalian membrane proteins will be expressed to higher levels or enhanced stability and/or activity in cholesterol-producing *P. pastoris*.

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