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Tuning of *Pichia pastoris* for the Expression of Membrane Proteins and Small Peptides

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Abstract

Pichia pastoris (aka Komagataella phaffii) is one of the most popular organisms for biotechnological production of pharmaceuticals and industrially important enzymes. Meanwhile, this yeast is also used in basic research for a better understanding of mechanisms like protein secretion, peroxisome biogenesis or methanol metabolism. This work addresses the optimized expression of proteins, which are targeted to the plasma membrane or the cell exterior via the classical secretory pathway. On the one hand, the secretion of small proteins, so-called antimicrobial peptides, was influenced by the co-expression of *S. cerevisiae* pro-peptide processing proteases. On the other hand, this thesis deals with the expression of plasma membrane proteins in sterol-modified *P. pastoris* strains. Our strategy proved that the exchange of yeast ergosterol for cholesterol can have a positive effect on recombinant production of mammalian membrane proteins. Furthermore, these sterol-engineered strains were characterized thoroughly for their physiological and genetic properties via spot-tests, electron microscopy, RNA sequencing, and random mutagenesis leading to synthetic phenotypes or complementation. Thereby, we found connections between yeast sterol metabolism and cell wall biogenesis. These findings represent the basis for future research for a better understanding of cellular interactions between sterols (and possibly other lipids) and cell wall biosynthesis, including stress-induced regulation mechanisms in *P. pastoris*.

Zusammenfassung

Pichia pastoris ist seit einigen Jahren einer der beliebtesten Hefe-Organismen für die biotechnologische Herstellung von Pharmazeutika und industriell eingesetzten Enzymen. Auch in der Grundlagenforschung wird diese Hefe mittlerweile genutzt, um Mechanismen der Proteinsekretion, Peroxisomen-Biogenese oder des Methanol-Stoffwechsels besser zu verstehen. Diese Arbeit beschäftigt sich intensiv mit der Optimierung der Expression von Proteinen, die über den sekretorischen Weg in (Plasma-)Membranen eingebaut, oder aus der Zelle ausgeschleust werden sollen. Einerseits handelt es sich dabei um kleine antimikrobielle Peptide, deren Sekretion durch die Co-expression von Proteasen aus der Bäckerhefe *Saccharomyces cerevisiae* beeinflusst wird. Zum anderen beschäftigt sich diese Arbeit mit der Herstellung von Membranproteinen in *P. pastoris* Stämmen mit modifizierter Plasmamembran-Zusammensetzung. Diese Strategie stellte unter Beweis, dass der Austausch von Hefe-eigenem Ergosterol zu Cholesterol einen positiven Einfluss auf die rekombinante Herstellung humaner Membranproteine haben kann. Des Weiteren wurden diese Sterol-modifizierten Stämme mittels Spot-tests, Elektronenmikroskopie, RNA-Sequenzierung und Zufallsmutagenese/Komplementationsanalyse eingehend hinsichtlich ihrer physiologischen und genetischen Eigenschaften studiert. Dabei wurden Zusammenhänge zwischen dem Sterolmetabolismus und der Zellwandsynthese in Hefe entdeckt. Diese Erkenntnisse sind die Grundlage für zukünftige Forschungsarbeiten zum besseren Verständnis der zellulären Interaktionen zwischen Sterolen (und ggf. auch anderen Lipiden) und der Zellwandbiosynthese einschließlich Stress-induzierter Regulationsmechanismen in *P. pastoris*.

1. Introduction

1.1. Aims of this Thesis

In contemporary Biotechnology, *P. pastoris* has become one of the most important and most widely used hosts for production of heterologous proteins for the pharmaceutical industry or industrial applications. Heterologous expression in *P. pastoris* can be achieved via secretion, intracellular expression or by targeting proteins to the plasma membranes. This thesis aims to approach several strategies to better understand and improve the *P. pastoris* expression system:

Chapter 1 gives a detailed introduction to the generals of the *P. pastoris* expression system. Both, the *Pichia* Protocols book chapter and the Mini-Review focus on introducing and describing the majority of different strains and vectors available. Furthermore, methods for heterologous protein expression are described, including cloning, selection of desired expression strains via Mut^+/Mut^S screening, integration into defined loci and promoting multicopy integration events.

Chapter 2 describes novel strategies to improve the secretion of antimicrobial peptides in *P. pastoris*. These small proteins are emerging as potential novel antibiotic drug candidates and are, therefore, highly interesting targets for overexpression in *P. pastoris*. We designed gene expression cassettes as four tandem repeats and co-expressed two enzymes of the *S. cerevisiae* secretory pathway, Kex1p and Kex2p, to improve the proteolytic processing of the pre-pro α-mating factor signal peptide. For plectasin, we could show a beneficial effect of *KEX2* and *KEX1* co-expression by agar-diffusion tests, wherein the secreted peptide inhibited growth of the Gram-positive bacterium *Bacillus subtilis*.

In chapter 3, we elaborate on the heterologous expression of mammalian membrane proteins in *P. pastoris*. The review "Overexpression of Membrane Proteins from Higher Eukaryotes in Yeasts" gives a thorough introduction about recent advances in the field. Subsequently, we present a strategy to foster the expression of a mammalian membrane protein in *P. pastoris*: By engineering the sterol pathway towards the production of cholesterol instead of ergosterol, mammalian Na,K-ATPase α3β1 could be expressed in a more stable and active way as compared to respective control strains. This opens the possibility to study further membrane proteins known for their interaction with sterols, such as G-protein coupled receptors.

The slow growth phenotype of the sterol-modified *P. pastoris* strains prompted us to investigate their physiology in more detail. In chapter 4, we focus on the phenotypic characterization of these strains. Initial growth tests revealed substantial differences in the ability to deal with stressors such as calcofluor white, SDS, NaCl or low temperatures. By a genetic knockout screening, putative effector genes could be identified, which need to be further studied. We aimed to find connections between the structural organization of the cell wall, induction of the cell wall integrity pathway and the sterol metabolism by various methods including electron microscopy, RNA sequencing and HPLC analysis of cell wall

sugars. These experiments revealed that the sterol composition has a major impact on cell physiology. We give first insights into a complex regulatory network, which interconnects changes in the sterol composition to an altered cell wall structure and the regulatory response on the transcriptional level. Future studies should aim at finding links in this regulatory network in *P. pastoris* to better understand the physiology and stress response of this important yeast.

Finally, the results of this thesis are summarized in the conclusion in chapter 5. Last but not least, the appendix in chapter 6 contains methods and results of additional experiments performed, which did not fit into the scope of the other chapters.

1.2. Introduction: *Pichia* Protocols Book Chapter

Strains and Vectors for Protein Expression in *P. pastoris*

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My contribution (90%):

- Writing of the chapters "Introduction", "Materials", and "Methods" (80%)
- Preparation of Tables 1-3 (100%)

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Abstract

Many industrially and pharmaceutically important proteins can be successfully expressed in *P. pastoris*, but the overall yields depend on several factors. One major factor is the selection of proper host strain/vector combinations that are suitable for heterologous protein expression. In this chapter, we summarize available host strains and describe basic and novel vector systems. These host strains are optimized for protein expression and reduction of proteolytic activity, for example by providing different auxotrophies and optimized N-glycosylation or by lacking endogenous proteases. The described vectors are optimized for intracellular and secretory expression by varying promoters, secretion signals or integration sites. In the last part, we discuss diverse methods comprising cloning strategies, selection of desired expression strains via Mut^+/Mut^S screening, integration into defined loci and promoting multi-copy integration events. These straight-forward approaches can strongly influence the outcome of a project.

Key Words

P. pastoris host strains, protease-deficient strains, expression vectors, multi-copy integration

1. Introduction

At the beginning of each expression experiment in *P. pastoris*, several questions arise. In fact, it will have a substantial impact on the success of heterologous protein expression in *P. pastoris*, if the right choices concerning host strains and vectors are made from the start. Which promoter should be chosen? Which selection marker is suitable for the experiment, and does it allow for screening of multi-copy integration events? Which host strain is desirable for the successful outcome of the project? In this chapter, these questions are addressed by discussing major advantages and disadvantages of using specific host strains and vectors. We provide tables listing standard strains and vectors together with recently developed alternatives. These new developments include i) clean, marker-free auxotrophic and protease-deficient strains, ii), glyco-engineered platform strains and vectors for a human-like N-glycan structures, iii) generally applied strain engineering strategies for improved protein production and iv) novel vector systems, offering the choice for different selection markers, secretion signals and promoters.

Additionally, we discuss cloning issues, possible influences of methanol utilization (Mut) phenotypes on protein expression, and we describe general methods to generate strains having single or multiple copies of the desired gene integrated.

2. Materials

2.1 Strains

Since *P. pastoris* is a work horse in biotechnology, a diverse set of strains has been developed for various purposes, for example to reduce proteolytic activity (see section 2.1.2) or to alter the glycosylation pattern towards human-like N-glycan structures (see section 2.1.3). Most of these strains are derived from *P. pastoris* NRRL Y-11430 (=CBS7435) or NRRL Y-48124 (Invitrogen expression kit strain, Carlsbad CA, USA). In 2009, these strains were re-classified and now belong to the genus *Komagataella phaffii* ***(1)***. The strains were deposited at the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL, and the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. The strain NRRL Y-11430, however, is not available anymore from Centraalbureau voor Schimmelcultures, but can be obtained from ATCC (http://www.lgcstandards-atcc.org) or from NRRL upon request.

Nowadays, full genome sequences are available, which greatly facilitated host strain development and can be accessed via NCBI (http://www.ncbi.nlm.nih.gov) or through *Pichia* genome browsers (http://www.pichiagenome.org and http://bioinformatics.psb.ugent.be/orcae/overview/Picpa) ***(2)***. The type strain of *Pichia pastoris* NRRL Y-1603 (=CBS704), belonging to the species *Komagataella pastoris*, was sequenced by Mattanovich et al. ***(3)***. Although there is no full genome sequence available yet for the *Komagataella phaffii* type strain NRRL Y-7556 (=CBS2612), the derivatives NRRL Y-11430/CBS7435 and GS115 were sequenced independently ***(4, 5)***. To improve readability, all strains are further on termed "*P. pastoris*" in this chapter. The sequence of the CBS7435 strain was recently further refined by Sturmberger et al. ***(6)***, closing twelve major sequence gaps and manually curating over 5000 open reading frames, which were then confirmed by RNA sequencing.

Various sets of strains are commercially available from companies such as Life technologies^{TM} and BioGrammatics. For industrial use, however, license fees have to be paid, which may constitute a limitation. Such limitations can be overcome by using the *P. pastoris* NRRL Y-11430 (=CBS7435) strain, initially patented by Philips Petroleum Company for single cell protein production, and its derivatives. For this lineage of strains there is freedom of use for the scientific community as well as in industrial settings. An overview of tailored platform strains can be found in Table 1.

Table 1. *P. pastoris* Host Strains.

Wildtype Strains			
Strain	**Species**	**Phenotype**	**Source**
NRRL Y-1603/ CBS704/ DSMZ 70382	*K. pastoris (type strain)*	WT	Agriculture Research Service culture collection, Peoria IL, USA
NRRL Y-7556/ CBS2612	*K. phaffii (type strain)*	WT	Agriculture Research Service culture collection, Peoria IL, USA
NRRL Y-11430/ CBS7435	*K. phaffii*	WT	Agriculture Research Service culture collection, Peoria IL, USA
X-33	*K. phaffii* GS115 *HIS4*	WT	Life Technologies™
BG10	*K. phaffii* NRRLY-11430	WT, killer plasmid free	BioGrammatics Inc.
Auxotrophic Strains	**Genotype**	**Phenotype**	**Source**
GS115	*his4*	His^-	Life Technologies™
PichiaPink™ 1	*ade2*	Ade^-	Life Technologies™
KM71	*aox1::ARG4, arg4, his4*	His^-, Mut^S	Life Technologies™
KM71H	*aox1::ARG4, arg4*	Mut^S	Life Technologies™
BG09	*arg4::nourseo^R Δlys2::hyg^R*	Lys^-, Arg^-, $Nourseothricin^R$, $Hygromycin^R$	BioGrammatics Inc.
BG12	*his4*	His^-	BioGrammatics Inc.
GS190	*arg4*	Arg^-	***(7)***
GS200	*arg4, his4*	His^-, Arg^-	(Waterham et al. 1996)
JC220	*ade1*	Ade^-	***(7)***
JC254	*ura3*	Ura^-	***(7)***
JC227	*ade1 arg4*	Ade^- Arg^-	***(8)***
JC300-JC308	combinations of *ade1, arg4, his4, ura3*	Combinations of Ade^-, Arg^-, His^-, Ura^-	***(8)***
YJN165	*ura5*	Ura^-	***(9)***
CBS7435 *his4*	*his4*	His^-	TU Graz *Pichia* Pool 1 ***(10)***
CBS7435 mutS *his4*	*aox1, his4*	Mut^S, His^-	TU Graz *Pichia* Pool 1 ***(10)***
CBS7435 mutS *arg4*	*aox1, arg4*	Mut^S, Arg^-	***(10)***
CBS7435 *pha2*	*pha2*	Phe^-	TU Graz *Pichia* Pool 2 ([a]*Pp*7029)
CBS7435 *met2*	*met2*	Met^-	TU Graz *Pichia* Pool 2 ([a]*Pp*7030)
CBS7435 *met2 arg4*	*met2 arg4*	Met^- Arg^-	TU Graz *Pichia* Pool 2 ([a]*Pp*7031)
CBS7435 *met2 his4*	*met2 his4*	Met^- His^-	TU Graz *Pichia* Pool 2 ([a]*Pp*7032)
CBS7435 *lys2*	*lys2*	Lys^-	TU Graz *Pichia* Pool 2 ([a]*Pp*7033)
CBS7435 *lys2 arg4*	*lys2 arg4*	Lys^- Arg^-	TU Graz *Pichia* Pool 2 ([a]*Pp*7034)
CBS7435 *lys2 his4*	*lys2 his4*	Lys^- His^-	TU Graz *Pichia* Pool 2 ([a]*Pp*7035)
CBS7435 *pro3*	*pro3*	Pro^-	TU Graz *Pichia* Pool 2 ([a]*Pp*7036)

CBS7435 *tyr1*	*tyr1*	Tyr$^-$	TU Graz *Pichia* Pool 2 ([a]*Pp*7037)
Protease-deficient Strains			
SMD1163	*his4 pep4 prb1*	His$^-$	***(11)***
SMD1165	*his4 prb1*	His$^-$	***(11)***
SMD1168	*his4 pep4::URA3 ura3*	His$^-$	Life Technologies^{TM}
SMD1168H	*pep4*		Life Technologies^{TM}
SMD1168 *kex1*::*SUC2*	*pep4::URA3 kex1::SUC2 his4 ura3*	His$^-$	***(12)***
PichiaPink^{TM} 2-4	Combinations of *prb1/pep4*	Ade$^-$	Life Technologies^{TM}
BG20	*pep4*		BioGrammatics Inc.
BG21	*sub2*		BioGrammatics Inc.
CBS7435 *prc1*	*prc1*		([a]*Pp*6676)
CBS7435 *sub2*	*sub2*		([a]*Pp*6668)
CBS7435 *sub2*	*his4 pep4*	His$^-$	([a]*Pp*6911)
CBS7435 *prb1*	*prb1*		([a]*Pp*6912)
CBS7435 *his4 pep4 prb1*	*his4 pep4 prb1*	His$^-$	([a]*Pp*7013)
Glyco-engineered Strains			
SuperMan$_5$	*his4 och1::pGAPTrα1,2-mannosidase*	His$^-$, Blasticidin^R	BioGrammatics Inc.
	och1::pGAPTrα1,2-mannosidase	Blasticidin^R	BioGrammatics Inc.
	pep4 och1::pGAPTrα1,2-mannosidase	Blasticidin^R	BioGrammatics Inc.
***Pp*FWK3**	*aox1 och1*	Mut^S, defective for mannosyltransferase	***(13)***
Other Strains			
BG11	*aox1*	Mut^S	BioGrammatics Inc.
GS241	*fld1*	Growth defect on methanol as sole C-source or methylamine as sole N-source	***(14)***
MS105	*his4 fld1*	See GS241; His$^-$	***(14)***
MC100-3	*his4 arg4 aox1::ScARG4 aox2::PpHIS4*	Mut$^-$	***(15)***
CBS7435 mutS	*aox1*	Mut^S	TU Graz *Pichia* Pool 1 ***(10)***
CBS7435 *ku70*/ CBS12694	*ku70*	WT	TU Graz *Pichia* Pool 1 ***(10)***, Centraalbureau voor Schimmelcultures
CBS7435 *ku70 his4*	*ku70 his4*	His$^-$	TU Graz *Pichia* Pool 1 ***(10)***
CBS7435 *ku70 gut1*	*ku70 gut1*	Growth defect on glycerol; Zeocin^R	***(10)***
CBS7435 *ku70 ade1*	*ku70 ade1*	Ade$^-$, Zeocin^R	***(10)***
CBS7435 cholesterol strain	*ku70 his4 erg5::DHCR7*^{Zeo} *erg6::DHCR24*^{G418}	His$^-$, Zeocin^R, Geneticin^R	***(16)***

[a] TU Graz culture collection number

2.1.1 Auxotrophic Strains

Many auxotrophic strains have become available, which can be conveniently used for DNA transformation and selection ***(8, 17)***. Strains with a deleted or mutated histidinol dehydrogenase gene *his4* are still most widely used for selection due to the broad variety of vectors available harboring the intact *HIS4* gene for complementation. It should be mentioned, that the *his4* auxotrophic *P. pastoris* GS115 strain was created by nitrosoguanidine mutagenesis ***(18)*** and, therefore, it is possible that the strain spontaneously reverts the mutation in the *HIS4* gene without having the expression cassette integrated, resulting in false-positive clones. So-called "clean" knockouts, generated by completely disrupting the coding sequence with a knockout-cassette via homologous recombination and subsequent marker recycling using the Flp-FRT recombinase system are more stable and thus favorable ***(10)***. Clean knock-out strains auxotrophic for *his4, arg4*, *met2, lys2, pro3, pha2* and *tyr1* are available from the TU Graz *Pichia* Pool (see Table 1). Additional auxotrophic strains, which were constructed by mutagenesis, are available at the Keck Graduate Institute together with vectors containing the complementing biosynthetic gene ***(19)***.

Transformation experiments using auxotrophic markers are usually highly efficient with hardly any background colonies showing up, in contrast to experiments employing antibiotic selection markers. It is, however, not possible to directly screen for multi-copy strains by using standard auxotrophic markers. Vectors need to have an additional antibiotic selection marker conferring resistance to, for example, geneticin disulphate (G418). The PichiaPink™ system has overcome this limitation by exploiting the feature that the *ade2* defect makes strains appear pink due to accumulation of products of the adenine biosynthetic pathway. By using high-copy plasmids, which have a truncated and, thus, weaker *ADE2* promoter, colonies can be directly screened on plate for multi-copy integration events (see section 3.4.2). The colonies which have more copies of the vector integrated appear white on the plate ***(20)***.

A more detailed overview of vectors with auxotrophic markers is provided in section 2.2.3 and in Tables 3 and 4.

2.1.2 Protease-deficient Strains

In some cases, one might run into the problem that the desired protein is not stable under secretory expression conditions in *P. pastoris*. Proteolytic activity, arising from vacuolar, secreted or intracellular proteases after cell lysis, can be problematic and strongly exacerbate downstream processing from culture supernatants, leading to a loss of final product yield. Especially during high cell density cultivation of *P. pastoris*, cell lysis might liberate proteases into the culture medium. There are several options to overcome this problem, such as media optimization and supplementation with casamino acids or soytone ***(21, 22)***. Additionally, strains can be used for expression, which are deficient for the major vacuolar proteases Pep4p and Prb1p. These strains called SMD1168 (Δ*his4* Δ*pep4*), SMD1165 (Δ*his4* Δ*prb1*) and SMD1163 (Δ*his4* Δ*pep4* Δ*prb1*) are available from Life technologies™. The *PEP4* gene

product, an aspartyl protease, is responsible for activating itself and other proteases such as proteinase B (*PRB1*) and carboxypeptidase Y (*PRC1*). Strains deficient in *pep4* and *prb1* have, therefore, a strongly reduced proteolytic activity. Several studies report that the use of these strains led to increased expression of intact protein, as in the cases of Human Insulin-Like Growth Factor I (IGF-I) ***(23)***, mouse 5-HT5A serotonin receptor ***(24)*** or mouse epidermal growth factor ***(21)***. However, there are some studies reporting no beneficial effect of using a protease-deficient strain as compared to wild type strains ***(25–27)***.

The construction of additional strains deficient for certain proteases, such as Yps1p ***(28–30)***, Kex1p ***(31, 32)*** and Kex2p ***(30)***, for improved protein expression are also reported with variable success. Knockout of the serine carboxypeptidase Kex1p, which is specific for basic amino acid residues, can be beneficial for proteins that are prone to C-terminal degradation. Typically, the beneficial effect of using protease-deficient strains is strongly dependent on the protein of interest. One cannot generally advise the use of a certain protease-deficient strain from the start, because it is reported that these strains are not as robust as wild type strains and frequently several different proteases are involved in degradation of the end product. Therefore, the knockout of single proteases might be insufficient. However, if there are problems with proteolytic degradation, it is definitely a viable option to use protease-deficient strains for expression. Table 1 provides an overview of protease-deficient strains available.

2.1.3 Glyco-engineered Strains

The early steps of protein N-glycosylation in the endoplasmic reticulum (ER) leading to the core glycan structure $(Man)_8(GlcNac)_2$ are highly conserved in higher eukaryotic species and yeasts. In mammals, however, final N-glycan structures are more diverse and complex as they contain besides N-acetyl glucosamine and mannose also galactose, fucose and terminal sialic acid. Yeasts, such as *P. pastoris,* tend to attach high-mannose glycan structures to proteins that enter the secretory pathway, although hyper-mannosylation is not as pronounced as in *S. cerevisiae*. Still, this can have severe impacts on the properties of the recombinant proteins, especially in the case of therapeutic proteins due to possible immunogenic reactions and decreased serum half-life ***(33)***. Several successful attempts have been made to change the glycosylation patterns towards a more human-like N-glycan structure ***(34–36)***. BioGrammatics now offers commercially available GlycoSwitch strains, which are derived from GS115 and are also available as *HIS4* prototrophic, protease-deficient or Mut^S variants. The major engineering steps in these strains were the knockout of Golgi-resident Och1p, an α-1,6-mannosyl-transferase located in the Golgi apparatus, preventing the extensive addition of mannose residues, and the introduction of α-1,2-mannosidase from *Trichoderma reesei*. These modifications trim the typical yeast high-mannose structure to a more homogenous glycan structure. Additional plasmids are available for overexpression of different glycosyltransferases to further modify glycan structures. A comprehensive guide to produce complex human-like N-glycan structures in *P. pastoris* strains using the GlycoSwitch technology is provided by Jacobs et al. ***(37)***. Krainer et al. described furthermore the construction of a clean Och1p

knockout strain in the *P. pastoris* CBS7435 Mut^S background, which has been proven to be a versatile host for secretory expression of a more uniformly mannosylated horseradish peroxidase, despite the observed growth defects ***(13)***.

2.1.4 General Strain Engineering Strategies

Besides using protease-deficient strains or glyco-engineered strains, there are several other strategies to generate efficient, high-yielding *P. pastoris* production strains. To improve folding capacity during protein secretion, co-overexpression of folding helpers such as the ER resident chaperone protein-disulfide isomerase (PDI) from either *S. cerevisiae* or *P. pastoris* turned out to be of advantage in several reported cases *e.g.* for secretion of human parathyroid hormone ***(27)***, *Necator americanus* secretory protein (Na-ASP1) ***(38)***, *Rhizopus chinensis* lipase ***(39)***, or *Plasmodium falciparum* transmission-blocking vaccine candidate Pfs25 ***(40)***. Although it was favorable in these cases, it seems not to be a generally applicable strategy, as there are also studies reporting no or adverse effects on protein production, *e.g.* for A33 single chain antibody fragment secretion ***(41)***. The same study describes, however, the positive effect of overexpressing immunoglobulin binding protein (BiP), an Hsp70 class heat shock protein, on protein secretion. There are two *P. pastoris* CBS7435 Mut^S platform strains available from VTU and TU Graz, having one or more copies of PDI integrated into the genome. As it was already shown in several cases to be of advantage, overexpression of folding helpers is an engineering strategy worth trying.

Co-expression of the spliced, active transcription factor HAC1p, which regulates transcription of unfolded protein response (UPR) target genes, can also improve protein secretion or expression of membrane proteins. This was demonstrated, for example, for the secretion of *Citrobacter amalonaticus* phytase ***(42)*** and for several other secreted, surface displayed and membrane proteins ***(41, 43–46)***.

Another potential bottleneck for the expression of more complex proteins, such as mammalian membrane proteins, is the sterol composition of yeast membranes. Yeast contains ergosterol as major sterol, whereas mammalian cells contain cholesterol, offering an engineering target for improved expression of membrane proteins. A study published at our Institute targeted the engineering of the ergosterol pathway towards production of cholesterol in *P. pastoris* ***(16)***. By overexpressing two enzymes of the cholesterol biosynthesis pathway, total sterols consisted of roughly 90% cholesterol. Hence, expression of the mammalian Na,K-ATPase $\alpha 3\beta 1$ was improved in terms of protein stability and activity. This approach might be applicable for other membrane proteins such as GPCRs as well, which require a special lipid environment to be fully functional and stable.

2.2 Vectors

The selection of a suitable vector is one crucial factor that largely influences heterologous protein expression levels in *P. pastoris*. Genomic integration of expression constructs is the preferred method

in *P. pastoris* due to instability issues of autonomously replicating (ARS) plasmids. There might be progress in the development of more stable ARS plasmids in the near future, though. Currently, most of the available expression vectors are applied in a two-step procedure; the cloning and amplification of an expression vector is carried out in *E. coli*, followed by linearization and transformation of the expression cassette to generate *P. pastoris* expression strains. For this purpose, vectors are equipped with an origin of replication and a marker cassette for plasmid maintenance and selection in *E. coli*. In addition, each vector contains a marker cassette for selection in *P. pastoris* and an expression cassette, which is composed of a promoter (in most cases P_{AOX1} or P_{GAP}), a multiple cloning site (MCS) and a terminator sequence. The gene of interest (GOI) can be cloned by using any of the available restriction sites. However, it is recommended to use restriction sites that introduce the least number of interfering nucleotides between the promoter sequence and the start codon of the heterologous gene. Some vectors also contain an additional 3' untranslated region of the *AOX1* gene (3'UTR) for targeting the expression cassette via homologous recombination to the *AOX1* locus to generate strains with a slow methanol utilization phenotype (Mut^S). To allow secretion of heterologous proteins, different secretion signals are employed, *e.g.* from the *S. cerevisiae* alpha-mating factor or from the *P. pastoris* endogenous acid phosphatase *PHO1*. The respective coding sequences are fused in frame upstream of the GOI.

This section will further give an overview of important vector parts, *i.e.* standard and novel promoters, biosynthetic or antibiotic markers as well as commonly used secretion signals. Additionally, a comparative list of available vectors is provided in Table 2.

2.2.1 Promoters

Alcohol oxidase 1 promoter (P_{AOX1}) is by far the most studied and most commonly used promoter to drive protein expression in *P. pastoris*. P_{AOX1} is a tightly regulated promoter which is repressed in the presence of glucose and can be induced up to 1000-fold by growing cells on methanol as a sole carbon source. The high degree of process control renders this promoter ideal for heterologous protein expression by uncoupling the growth phase from production phase, particularly in case of toxic proteins. Other strong methanol-inducible promoters have also been used to express proteins in *P. pastoris,* such as the formaldehyde dehydrogenase (*FLD1*) and the dihydroxy-acetone synthase (*DAS1/2*) promoters ***(14, 47)***. In addition to methanol, the P_{FLD1} promoter can be induced by methylamine as a single nitrogen source in the presence of glucose as a carbon source ***(14)***. The *PEX8* promoter, controlling expression of the peroxisomal matrix protein, has infrequently been used to this end. P_{PEX8} can drive gene transcription on glucose at low but considerable levels and is induced up to 3-5 times with methanol or oleate ***(48)***.

In some cases, the use of a strong promoter may lead to undesirable results, especially in secretory expression mode, as there appears to be insufficient time for proper protein folding and processing of recombinant proteins through the secretory pathway ***(48, 49)***. Promoters having a similar regulatory

profile as P_{AOX1} but moderate expression levels such as the alcohol oxidase 2 promoter (P_{AOX2}) can be used for these particular applications. In addition, several variants of P_{AOX1} are available for fine-tuned expression of heterologous genes. These promoter variants have been shown to possess a range of 6% to 160% of the wild type promoter activity ***(50)***. Some variants are furthermore de-repressed under glycerol feeding conditions and were employed recently to secrete 18 g/L of TcHB1 in small scale fermenter cultivations ***(50–52)***. These promoter variants and respective expression vectors are available from VTU technology (www.vtu-technology.com).

A recently published study by Vogl et al. describes the characterization of novel promoters derived from the methanol utilization pathway, which show different strengths and regulatory profiles. These diverse promoters naturally differ in their DNA sequence, thereby facilitating fine-tuned co-expression of multiple genes for pathways by increasing the genetic stability ***(53)***.

Methanol is, however, an extremely toxic and flammable substance and its use in large scale cultivations requires specialized and costly handling procedures. In addition, being a derivative of petroleum, its use is not appropriate in food industry. During high density cultivations, excess of methanol can promote cell death phenomena, releasing intracellular proteins into the culture broth, which not only complicates the downstream processing and purification, but may also result in undesirable proteolysis of secreted recombinant proteins ***(54)***. Therefore, several constitutive promoters such as P_{GAP}, P_{TEF1}, and P_{YPT1} have been used to express proteins ***(55–57)***. P_{GAP} is the most commonly used alternative promoter in place of P_{AOX1} and has been shown to govern expression of heterologous proteins to similar levels like P_{AOX1} ***(55)***. A major advantage of using constitutive promoters is that the need for switching carbon sources is eliminated, thereby reducing production time, effort and increasing overall productivity. However, constitutive promoters can only be used to express proteins that are not toxic to the host cell. An extensive review of available *P. pastoris* promoters and their regulatory properties has been published by Vogl et al. ***(58)***.

2.2.2 Selectable Markers

Genetic modifications and amplification of *P. pastoris* vectors are usually carried out in *E. coli*. Vectors may still contain the *bla* gene encoding ampicillin resistance for selection in *E. coli*. However, many vectors are available either from Life Technologies™ or TU Graz that contain a single resistance marker gene, *Sh ble* from *Streptoalloteichus hindustanus*, which confers resistance to Zeocin™ in both organisms. The two most frequently used selection markers are *HIS4* and Zeocin™. In addition, several other auxotrophic and dominant selection markers are available, as discussed below.

Auxotrophic Selection Markers

Auxotrophic selection markers have major benefits due to their ease of handling, cost effectiveness and superior genetic stability of expression clones. However, they can only be used with corresponding auxotrophic strains. Secondly, due to the independent marker cassettes for selection in bacteria and yeast, auxotrophic marker-based expression vectors are usually larger in size, which might complicate the cloning and transformation processes. A number of auxotrophic selection markers, readily cloned in expression vectors, are available for genetic manipulation of *P. pastoris: HIS4* (histidinol dehydrogenase) ***(59)***, *ARG4* (argininosuccinate lyase), *ADE1* (phosphoribosyl-aminoimidazole succinocarboxamide synthase), *URA3* (orotidine 5′-phosphate decarboxylase) ***(8)***, *ADE2* (phosphoribosyl aminoimidazole carboxylase) ***(20)***, *URA5* (orotate-phosphoribosyl transferase) ***(9)***, *MET2* (homoserine-O-transacetylase) ***(17)***, and *GUT1* (glycerol kinase 1) ***(10).*** In addition, a set of expression vectors containing *ARG1*, *ARG2*, *ARG3* and *HIS1*, *HIS2* and *HIS5* as auxotrophic markers was constructed by Nett et al. ***(60)***. These vectors can be used to disrupt arginine pathway genes with histidine pathway genes and *vice versa* in a sequential manner. During the process, multiple heterologous genes can be integrated at defined loci into the genome of *P. pastoris*. The main disadvantage of this strategy is the time-intensive screening of transformants to identify auxotrophic strains with targeted integration of the expression cassette, which can then be used for further transformations.

Dominant Selection Markers

There are several dominant selection markers available conferring resistance to zeocin^{TM} (*She ble*) ***(61)***, geneticin/G418 (*Tn903kan*^{r}) ***(62, 63)***, blasticidin S (*BSD*) ***(64)***, formaldehyde (*FLD1) **(65)***, hygromycin (*HPH*) ***(66)*** and nourseothricin (*NAT1*) ***(67)***. From this listing, zeocin^{TM} is the most commonly used substance for selection, even though it is also the most expensive. One of the major advantages of these markers is that they are not limited to a complementary genetic host and can, therefore, be used for genetic modifications of wild type or industrial production strains. Additionally, some of the dominant markers, such as zeocin^{TM} and hygromycin resistance, can be used for selection in both, *E. coli* and *P. pastoris,* which significantly reduces the size of the expression vectors. The use of dominant selection markers also enables the screening for multi-copy clones, which is discussed in section 3.4 in more detail. One major drawback is, however, that the industrial use of strains harboring antibiotic resistance genes may be undesired and/or problematic.

2.2.3 Secretion Signals

One of the most outstanding features of *P. pastoris* is its ability to secrete properly processed and active recombinant proteins into the culture medium. *P. pastoris* secretes only low levels of endogenous proteins and, as a result, the secreted protein is often the most prominent protein in the culture supernatant. This greatly reduces the downstream processing efforts, otherwise needed for the

purification of intracellular proteins ***(61)***. The decision to target a recombinant protein to the secretory pathway depends upon the native situation of the protein in its natural host. Thus, protein secretion in *P. pastoris* is worth trying if the protein is naturally secreted. Intracellular proteins are usually more problematic and, therefore, intracellular expression may be more promising. However, there are rare examples in the literature describing secretion of originally intracellular protein ***(69, 70)***. Another important point to consider is the choice of the secretion signal. A commonly used strategy is to design and compare several expression constructs with different secretion signals, including the native secretion signal as well as the *S. cerevisiae* α-mating factor secretion signal ***(71–73)***.

Other secretion signals have also been used to direct proteins to the secretory pathway in *P. pastoris* with varying success. These include leader peptides from *PHO1* (*P. pastoris* acid phosphatase) ***(74, 75)***, *SUC2* (invertase) ***(73, 76)***, *PHA-E* (*Phaseolus vulgaris* agglutinin-E form) ***(77)*** and Killer Toxin ***(69, 78)***. However, these secretion signals have not been used extensively. Either available data is very limited or results have been variable for a broad range of proteins.

Life Technologies™ and DNA2.0 offer vectors harboring different secretion signals to easily test and screen for the best solution. The most commonly, and by far the most successfully, used secretion signal is the α-mating factor pre-pro signal peptide, which is readily available in most of the expression vectors available from Life Technologies™, TU Graz, Biogrammatics, DNA2.0 and the Keck Graduate Institute. In order to have authentic N-terminal amino acids of the secreted recombinant protein, *Xho*I or compatible *Sal*I sites can be used to clone the heterologous gene in frame, but the Kex2 cleavage site needs to be restored through the cloning process.

There are two problems commonly reported for the MF-α secretion signal approach. The first one is the occurrence of incomplete signal peptide processing at the Kex2 cleavage site (Lys-Arg) due to inefficient protease activity. This might be overcome by including Glu-Ala repeats between the Kex2 cleavage site and the amino acid sequence of the protein of interest or by optimizing the Kex2 recognition site ***(79, 80)***. For certain proteins, it can be beneficial to co-express Kex2 for improved signal peptide processing (own unpublished results), although these strategies are not generally applicable and should be tested on a case-to-case basis. The second problem can be incomplete processing of Glu-Ala residues by Ste13 protease in the Golgi complex, resulting in heterogeneous N-termini of the recombinant protein. A reported strategy is to use only the pre-region of MF-α, which in some cases not only improved the proper processing but also increased the overall secretion of the recombinant protein ***(81)***. Four different variants of MF-α signal sequences along with seven other alternative secretion signals are readily available from DNA 2.0 (www.dna20.com).

Table 2. Vectors for Intracellular Expression

Name	Selection in *P. pastoris*	Promoter	General Features	Reference
pHIL-D2	*HIS4*	P_{AOX1}	*NotI* linearization site for *AOX1* replacement, *EcoRI* for cloning, *f1* Origin of replication, *Sal*I or *Stu*I linearization for *his4* insertion	Life Technologies™
pAO815	*HIS4*	P_{AOX1}	*EcoRI* for cloning, *BglII* and *BamHI* sites for *in vitro* multimerization, *Bgl*II linearization for *AOX1* replacement, *Sal*I or *Stu*I linearization for *his4* insertion	Life Technologies™
pPIC3.5K	*HIS4*/G418^r	P_{AOX1}	MCS, *Bgl*II linearization for *AOX1* replacement, *Sac*I linearization for *AOX1* insertion, *Sal*I linearization for *his4* insertion, G418 selection for multi-copy strains	Life Technologies™
pPICZ (A, B, C)	Zeo^r	P_{AOX1}	different MCS (A, B and C), C-terminal 6XHis-tag, c- *myc* epitope, *BglII* and *BamHI* sites for *in vitro* multimerization, zeocin™ selection for multi-copy strains	Life Technologies™
pPIC6 (A, B, C)	*Bsd^r*	P_{AOX1}	Similar to pPICZ, except for blasticidin selection for multi-copy strains	Life Technologies™
pGAPZ (A, B, C)	Zeo^r	P_{GAP}	different MCS (A, B and C), C-terminal 6XHis-tag, c- *myc* epitope, zeocin™ selection for multi-copy strains	Life Technologies™
pFLD	Zeo^r	P_{FLD}	MCS, C-terminal 6XHis-tag, V5 epitope, targets integration into *FLD1* locus, induction with methanol or methylamine	Life Technologies™
PichiaPink™ (pPINK-HC, pPINK-LC)	*ADE2*	P_{AOX1}	Colour-based selection of strains, high-copy and low-copy plasmids, MCS, truncated promoter for marker gene, integration into *trp2* or *AOX1* locus possible	Life Technologies™ ***(20)***
pJL-IX	*FLD1*	P_{AOX1}	*NotI* linearization site for *AOX1* replacement, *EcoRI* for cloning, formaldehyde selection for multi-copy expression strains, transformed strain must be *FLD1* deficient	***(65)***
pBLHIS-IX	*HIS4*	P_{AOX1}	Different combinations of MCS/auxotrophic selection markers available, different restrictions sites for *in vitro* multimerization, linearization site located in the marker gene	Keck Graduate Insitute ***(8, 17)***
pBLARG-IX	*ARG4*			
pBLADE-IX	*ADE1*			
pBLURA-IX	*URA3*			
pBLMET-IX	*MET2*			
pKAN B	*Tn903kan^r*	P_{AOX1}	MCS, resistance marker under control of P_{GAP} for direct selection of transformants using kanamycin in *E. coli* and G418 in *P. pastoris*	***(63)***
pJAN/pJAZ/ pJAG	*NAT1*/Zeo^r /G418^r	P_{AOX1}	Seamless cloning based on Type IIS restriction enzymes	Biogrammatics
pD902, pD905	Zeo^r	P_{AOX1}/P_{GAP}	Integrated vectors, IP-Free	DNA 2.0
pRSFC plasmid family (18 variants)	Zeo^r/*HIS4*	P_{AOX1}/P_{GAP}	Seamless cloning of a PCR product using Type IIS restriction enzymes, multiple combinations of N- or C-terminal tags (6xHis, FLAG, *myc*, Strep, MBP and eGFP)[a], blunt end ligation, IP-Free	TU Graz ***(82)***

pXYZ plasmid family	*HIS4*/*ARG4*/ Zeo^r/G418^r	P_{AOX1}/P_{GAP}	*Bgl*II/*Sph*I/*Swa*I linearization sites for *AOX1* gene replacement, *ARG4* promoter drives expression of marker gene, restriction sites for easy marker exchange, IP-Free	TU Graz *Pichia* Pool 2 ***(71)***
pPpT4/B1 family	Zeo^r /G418^r	P_{AOX1}/P_{GAP}	MCS, low copy (T4) and high copy (B1) plasmids, depending on promoter strength in front of resistance marker, *Bgl*II or *Swa*I for linearization	TU Graz *Pichia* Pool 1 ***(10)***
pAHYB/ pGHYB	*Hygromycin* ^{r}	P_{AOX1}/P_{GAP}	MCS, 6XHis-tag, c- *myc* epitope, *Bgl*II site for linearization	***(66)***

[a] His-, MBP- and Strep-tag fusion plasmids are also available with a TEV-protease cleavage site.

Table 3. Vectors for Secretory Expression

Name	Selection in *P. pastoris*	Promoter	General Features	Reference
pHIL-S1	*HIS4*	P_{AOX1}	*PHO1* secretion signal, MCS for in-frame fusion of the GOI, *Bgl*II linearization site for *AOX1* replacement, *Sal*I or *Stu*I linearization for *his4* insertion	Life Technologies^{TM}
pPIC9K	*HIS4*/G418^r	P_{AOX1}	α-mating factor secretion signal, MCS for in-frame fusion of the GOI, *Bgl*II linearization for *AOX1* replacement, *Sal*I linearization for *his4* insertion, G418 selection for multi-copy strains	Life Technologies^{TM}
pPICZα (A, B, C)	Zeo^r	P_{AOX1}	α-mating factor secretion signal, different MCS (A, B and C), C-terminal 6XHis-tag, c-*myc* epitope, *BglII* and *BamHI* sites for *in vitro* multimerization, zeocin^{TM} selection for multi-copy strains	Life Technologies^{TM}
pPIC6α (A, B, C)	*Bsd^r*	P_{AOX1}	Similar to pPICZα except for blasticidin selection for multi-copy strains	Life Technologies^{TM}
pGAPZα (A, B, C)	Zeo^r	P_{GAP}	α-mating factor secretion signal, different MCS (A, B and C), C-terminal 6XHis-tag, c-*myc* epitope, zeocin^{TM} selection for multi-copy strains	Life Technologies^{TM}
pFLDα	Zeo^r	P_{FLD}	α-mating factor secretion signal, MCS, C-terminal 6XHis-tag, V5 epitope, targets integration into *FLD1* locus, induction with methanol or methylamine	Life Technologies^{TM}
PichiaPink^{TM} (pPINKα-HC)	*ADE2*	P_{AOX1}	α-mating factor secretion signal, colour-based selection of strains, truncated promoter for marker gene, low-copy and high-copy plasmids, 7 other secretion signals are available and can be cloned via three-way ligation	Life Technologies^{TM} ***(20)***
pJL1-IX	*FLD1*	P_{AOX1}	α-mating factor secretion signal, MCS, *NotI* linearization site for *AOX1* replacement, formaldehyde selection for multi-copy expression strains, transformed strain must be *fld1* deficient	***(65)***
pBLHIS-SX	*HIS4*	P_{AOX1}	α-mating factor secretion signal, different combinations of MCS/auxotrophic selection markers available, different restrictions sites for *in vitro* multimerization, linearization site located in the marker gene	KGI ***(8, 17)***
pBLARG-SX	*ARG4*			
pBLADE-SX	*ADE1*			
pBLURA-SX	*URA3*			
pBLMET-SX	*MET2*			
pKANα B	*Tn903kan^r*	P_{AOX1}	α-mating factor secretion signal, MCS, resistance marker under control of P_{GAP} for direct selection of transformants using Kanamycin in *E. coli* and G418 in *P. pastoris*	***(63)***
pRSFC plasmid family (22 variants)	Zeo^r /*HIS4*	P_{AOX1}/P_{GAP}	Seamless cloning of a PCR product using Type IIS restriction enzymes, multiple combinations of N- or C-terminal tags (6xHIS, FLAG, MYC, Strep, MBP and	TU Graz ***(82)***

			eGFP)[b] and signal sequences, blunt end ligation, IP-Free	
pJAN-s1/ pJAZ-s1/ pJAG-s1	*NAT1*/Zeo^r/ G418^r	P_{AOX1}	α-mating factor secretion signal, seamless cloning based on Type IIS restriction enzymes	BioGrammatics
pXYZ plasmid family	*HIS4*/*ARG4* /Zeo^r/G418^r	P_{AOX1}/P_{GAP}	α-mating factor secretion signal, *Bgl*II/*Sph*I/*Swa*I linearization sites for *AOX1* gene replacement, *ARG4* promoter drives expression of marker gene, IP-Free	TU Graz *Pichia* Pool 2 ***(71)***
pPpT4/B1 family	Zeo^r/G418^r	P_{AOX1}/P_{GAP}	MCS, low copy (T4) and high copy (B1) plasmids, depending on promoter strength in front of resistance marker, *Swa*I for linearization	TU Graz *Pichia* Pool 1 ***(10)***
pD912/pD915	*Zeo^r*	P_{AOX1}/P_{GAP}	10 different secretion signals available[a], IP-Free	DNA 2.0

[a] The MF-α secretion signal is provided once with Kex2 (KR) and Ste13 cleavage sites (EAEA), once lacking EA repeats, and once as truncated version (pre-region only).
[b] His-, MBP- and Strep-tag fusion plasmids are also available with a TEV-protease cleavage site.

3. Methods

3.1 Cloning Strategies

Usually, knowledge of standard cloning procedures is sufficient for the successful generation of *P. pastoris* expression vectors. There are, however, some points to consider, which are discussed in the section below.

Most of the conventional vector systems are equipped with a multiple cloning site (MCS) for cloning of the GOI based on type II restriction enzymes, which often leaves a cloning scar resulting in a non-optimal 5' untranslated region (5' UTR). Mutations in the 5' UTR have been shown to negatively affect the translation efficiency of heterologous gene expression ***(83, 84)***. Therefore, several new vector systems have been developed to clone the GOI seamlessly with flanking regulatory sequences, *e.g.* promoter, terminator, secretion signal and fusion tag. A set of expression vectors termed "*Pichia* pool 2 plasmid family" is available from TU Graz. Cloning is performed via a single *Eco*RI site introduced into the promoter region. Thereby, the immediate 5' region upstream of ATG is identical to the natural *AOX1* gene. The Kozak consensus sequence (TTCGAAACG) between *Eco*RI and the start ATG has to be added to the GOI sequence when vectors for intracellular expression are used ***(71)***. There are also vector systems available from BioGrammatics (www.biogrammatics.com), DNA 2.0 (www.dna20.com), and lately from TU Graz based on type II S restriction enzymes, which cleave outside of their recognition sequence. This cloning strategy enables the fusion of the GOI seamlessly with upstream or downstream sequences to circumvent any potential problems arising from intervening nucleotides. A set of 40 expression vectors was developed based on this cloning strategy termed restriction site free cloning (RSFC), employing the type IIS endonuclease *Mly*I. A single PCR product can be cloned in frame with multiple promoters, secretion signals and N- and C-terminal tags to screen for optimal protein expression and purification ***(82)***. It has to be considered, however, that the proper orientation of the cloned GOI needs to be confirmed before further experiments are performed.

If secretory expression vectors encoding the MF-α signal sequence are used, there is mainly one aspect that needs to be considered. It is of great importance to maintain the Kex2 cleavage site for proper signal peptide processing. If restriction enzymes are chosen that eliminate the bases encoding the Lys-Arg residues from the vector backbone, the respective bases need to be inserted through the GOI fragment to ensure the proper processing of the secreted protein.

3.2 Mut^+/Mut^S Screening

P. pastoris contains two alcohol oxidase genes, *AOX1* and *AOX2*, which are necessary for the cells to grow on methanol as sole carbon source ***(15, 85)***. The two peroxisomal enzymes catalyze the first step in the methanol assimilation pathway by oxidizing methanol to hydrogen peroxide and formaldehyde. Despite the high similarity of Aox proteins, the *AOX1* gene product is synthesized to a much higher

extent due to the very strong *AOX1* promoter, resulting in Aox1p constituting approximately 30% of total cellular protein on methanol ***(86)***. After a heterologous gene has been integrated into the *AOX1* locus via double cross-over, the cells become defective for *aox1* and have to rely solely on *AOX2* for methanol utilization, which results in a slow growth phenotype on methanol (Mut^S, methanol utilization slow). If the *AOX1* gene remains intact, cells grow very well on methanol (Mut$^+$, methanol utilization plus).

There are several reports that the use of a Mut^S over a Mut$^+$ strain is of advantage, for example in the case of horseradish peroxidase ***(87)*** or the antibody single chain variable fragment scFvA33 ***(88)***. On the contrary, there are also studies showing high expression levels using Mut$^+$ strains, *e.g.* for *Coprinus cinereus* peroxidase ***(89)***, or studies where the Mut phenotype seemed not to be of particular importance for product yield, like in the case of tetanus toxin fragment C ***(90)***. In the case of *Rhizopus oryzae* lipase, the maximum lipase activity and the specific activity were higher using a Mut^S strain, but overall productivity was higher for the Mut$^+$ strain, *i.e.* more enzyme was produced in shorter cultivation periods with the same amount of biomass. The same study describes, furthermore, a different behavior for multi-copy strains. The Mut$^+$ strains seemed to be more robust when expressing lipase from multiple gene copies ***(91)***. A clear advantage of using a Mut^S strain is, however, that less methanol is required and the cultivation process can be controlled more easily. Schwarzhans et al. reported that Mut^S clones show less genetic variance and are, therefore, more robust and more comparable to each other ***(92)***.

It should be evaluated for each protein of interest, which Mut phenotype performs best during cultivation. After transformation of the vector, clones should be investigated for growth behavior on minimal media containing either glucose or methanol to determine their Mut phenotype. This can be easily done by streaking clones on minimal methanol (1%) and minimal dextrose (2%). If it is already known for a protein of interest, that Mut^S is the preferred phenotype, it is possible to use *P. pastoris* Mut^S strains from the start, which are available from Life technologies™ (KM71) or from the TU Graz *Pichia* Pool.

3.3 Single Copy Integration – Targeting a Defined Locus

For some experiments, it is necessary to integrate one single copy of a heterologous gene into a specific locus. This can be the case if Mut^S strains are desired for heterologous expression, if overexpression of other helper-proteins such as PDI has positive effects on expression, or if it is already known that multiple copies do not enhance protein expression efficiency.

The problems with generating multi-copy strains might be genetic instability and, as the integration loci are frequently unknown, pleiotropic effects that arise from multiple integrations. It will be difficult to compare *P. pastoris* strains regarding the positive effects of gene overexpression as long as the exact genotypes of the strains are not known. For that reason, it may be desired to target a specific integration locus to be able to obtain comparable results. This can be achieved by homologous recombination at

defined loci via targeting of the expression cassette with 5' and 3' homologous flanking regions. This strategy has been mainly used for integrating expression cassettes into the *AOX1* locus followed by screening for Mut^S mutants (see chapter 3.2). Additionally, expression cassettes can be targeted to the *HIS4* or *ARG4* locus, followed by screening for histidine or arginine auxotrophic mutants, respectively. Vectors for integration into these loci are available from the TU Graz *Pichia* Pool.

The generation of auxotrophic strains by integration into *HIS4* or *ARG4* loci has the advantage that additional markers become available for further rounds of transformation. To ensure, that there are no further copies integrated randomly into the genome, copy numbers can also be determined, *e.g.* by quantitative PCR ***(93)***. In general, the majority of obtained clones after transformation have one single copy integrated, especially if auxotrophic markers instead of antibiotic resistance genes are used for selection. This was also nicely demonstrated in a recent study by Schwarzhans et al. by gene copy number determination of GFP-expressing strains ***(92)***. Therein, 2-3 µg of a *HIS4* containing pAHBgl-GFP expression vector was transformed, which resulted in 95% of single copy strains. All of the analyzed strains showed integration into the *AOX1* locus via single or double crossover recombination events.

3.4 Multi-copy Integration

Integration of linear expression cassettes into the genome of *P. pastoris* is mostly preferred over ARS plasmids due to superior genetic stability of final expression strains. One of the key strategies to achieve maximal protein expression in *P. pastoris*, beside minimizing the negative effects of non-optimal 5' untranslated region, mRNA secondary structure, protein stability and locus of integration, is by increasing the copy number of heterologous genes. In case of intracellular expression, there seems to be a direct correlation of copy number and expression levels. However, this may not hold true for secretory expression due to a possible overload of the secretory pathway. Several well-established protocols are available to generate multi-copy expression strains and have been reviewed in detail elsewhere ***(94, 95)***. Briefly, one of these methods includes screening for spontaneously occurring multi-copy strains based on protein expression levels using sodium dodecyl sulphate-polyacylamide gel electrophoresis (SDS-PAGE), immunoblotting, direct enzymatic assays, or by DNA hybridization techniques at genomic level.

Another strategy is based on *in vitro* multimerization of expression cassettes using different expression plasmids provided with specific restriction sites[3]. Plasmids for this purpose are available from either Life technologies™ (*i.e.* pAO815) or from Keck Graduate Institute ***(8, 17)*** (see Tables 2 and 3). The main disadvantage of this strategy is the time and effort required to perform increasingly difficult cloning steps and problems associated with transformation to generate expression strains due to the very large size of the resulting plasmid. However, this strategy is ideal for situations when absolutely defined

genetic conditions are required. Moreover, the study of Schwarzhans et al. describes a positive correlation of GFP expression and head-to-tail orientation of the integration cassettes ***(92)***.

This could also be combined with *in vivo* multimerization using post-translational vector amplification (PTVA) ***(96)*** or integration into the rDNA locus to generate extremely high copy number clones ***(97)***.

The fastest and, thereby, most commonly applied method to generate multi-copy clones is still the screening of transformants for increased resistance on high antibiotic concentrations using geneticin/G418 ***(63, 90)***, zeocin™ ***(61)***, or hygromycin ***(66)***. It is also possible to screen clones for enhanced resistance to other substances such as formaldehyde ***(65)*** or 3-amino-1,2,4 triazol (3-AT) ***(98)***.

3.4.1 Generation of Multi-copy Strains using Dominant Markers (zeocin™, G418, hygromycin and blasticidin)

The most widely used method to screen for multi-copy transformants is based on selection of transformants on increasing concentrations of the antibiotic zeocin™. Resistance to zeocin™ is conferred by the *Sh ble* gene product, which sequesters the zeocin™ glycopeptide by stoichiometrically binding it instead of catalyzing its degradation ***(99)***. Therefore, increased resistance of transformants to the drug would most probably result from increased expression of the *Sh ble* gene product. In most of the *P. pastoris* expression plasmids, expression of the *Sh ble* gene is controlled by a strong constitutive promoter such as P_{TEF1} or P_{ILV5} ***(10, 61)***, which may be a heavy metabolic burden to the cells. This could explain the existence of low-copy transformants even on high zeocin™ concentrations, *e.g.* 2000 µg/ml ***(94, 100, 101)***. With the aim to render selection conditions more stringent, we have newly constructed expression vectors (*Pichia* Pool 2, TU Graz), using the weaker *P. pastoris ARG4* promoter to drive expression of the zeocin™ resistance gene. The basal expression levels from this promoter ensure that transformants bearing single to multiple copies can be selected in a range of 25 – 400 µg/mL of zeocin™. Only a few colonies are formed on the higher antibiotic concentrations. These colonies have an increased chance of being multi-copy clones, making the screening process more efficient.

To generate multi-copy expression strains based on increasing resistance to zeocin™, G418/geneticin[2], hygromycin or blasticidin, the linearized expression vector should be transformed into electrocompetent or spheroplasted cells. Immediately after electroporation, the cells are re-suspended in 1 ml of 1 M sorbitol and YPD (1:1) and are regenerated at 28°C, 200 rpm for 2 h[1]. Selection can then be performed on BYPD plates containing different concentrations of antibiotic (for Zeocin™ 100-2000 µg/ml, for G418/geneticin 500-1000 µg/ml, for hygromycin 100-300 µg/ml and for blasticidin 50-500 µg/ml).

It is described, and we can confirm based on own observations, that chances of generating multiple integrations are increased, if the expression vector is linearized with *SacI* for insertions at the *AOX1* locus, followed by transformation into Mut^S strains like KM71 or CBS7435 Mut^S ***(102)***.

3.4.2 Generation of Multi-copy Strains using Pichia Pink

A color based method was developed by Du et al. employing an attenuated *ADE2* gene and its complementary expression using native truncated promoters to compensate adenine auxotrophy ***(20)***. The gene product of *ADE2*, phosphoribosyl aminoimidazole carboxylase, is required to catalyze the sixth step in the formation of purine nucleotides. Inefficient expression of the marker gene from a truncated promoter results in a build-up of purine precursors inside the cells, giving them a reddish color. Based on this principle, two vectors named pPink-LC and pPink-HC are available from Life technologies^{TM} for selection of single copy and high copy clones, respectively. In pPink-HC, the expression of *ADE2* is controlled by a truncated and thereby weaker promoter. Hence, only clones having multiple copies integrated are able to produce sufficient protein to complement adenine auxotrophy. Consequently, transformants having multiple integrations of expression cassettes can be readily identified based on the white color of the colonies formed.

Notes

[1] In order to have more stringent conditions for selection of multi-copy transformants and to suppress generation of single copy transformants on plates with higher zeocin^{TM} concentrations, regeneration time after transformation should be short, and only big colonies appearing on plates after three days of incubation should be selected.

[2] Selection on G418/geneticin is sensitive to high cell density.

[3] Cloning of multiple expression cassettes into a single vector may lead to rearrangements in *E. coli*.

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1.3 Introduction: Mini-Review

Protein Expression in *Pichia pastoris*: Recent Achievements and Perspectives for Heterologous Protein Production

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MINI-REVIEW

Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production

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Abstract *Pichia pastoris* is an established protein expression host mainly applied for the production of biopharmaceuticals and industrial enzymes. This methylotrophic yeast is a distinguished production system for its growth to very high cell densities, for the available strong and tightly regulated promoters, and for the options to produce gram amounts of recombinant protein per litre of culture both intracellularly and in secretory fashion. However, not every protein of interest is produced in or secreted by *P. pastoris* to such high titres. Frequently, protein yields are clearly lower, particularly if complex proteins are expressed that are hetero-oligomers, membrane-attached or prone to proteolytic degradation. The last few years have been particularly fruitful because of numerous activities in improving the expression of such complex proteins with a focus on either protein engineering or on engineering the protein expression host *P. pastoris*. This review refers to established tools in protein expression in *P. pastoris* and highlights novel developments in the areas of expression vector design, host strain engineering and screening for high-level expression strains. Breakthroughs in membrane protein expression are discussed alongside numerous commercial applications of *P. pastoris* derived proteins.

Keywords Yeast · *Pichia pastoris* · Protein expression · Protein secretion · Protease-deficient strains · Chaperone

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Introduction

The methylotrophic yeast *Pichia pastoris*, currently reclassified as *Komagataella pastoris*, has become a substantial workhorse for biotechnology, especially for heterologous protein production (Kurtzman 2009). It was introduced more than 40 years ago by Phillips Petroleum for commercial production of single cell protein (SCP) as animal feed additive based on a high cell density fermentation process utilizing methanol as carbon source. However, the oil crisis in 1973 increased the price for methanol drastically and made SCP production uneconomical. In the 1980s, *P. pastoris* was developed as a heterologous protein expression system using the strong and tightly regulated *AOX1* promoter (Cregg et al. 1985). In combination with the already developed fermentation process for SCP production, the *AOX1* promoter provided exceptionally high levels of heterologous proteins. One of the first large-scale industrial production processes established in the 1990s was the production of the plant-derived enzyme hydroxynitrile lyase at >20 g of recombinant protein per litre of culture volume (Hasslacher et al. 1997). This enzyme is used as biocatalyst for the production of enantiopure *m*-phenoxybenzaldehyde cyanohydrin — a building block of synthetic pyrethroids — on the multi-ton scale.

Through a far-sighted decision this expression system, initially patented by Phillips Petroleum, was made available to the scientific community for research purposes. A major breakthrough was the publication of detailed genome sequences of the original SCP production strain CBS7435 (Küberl et al. 2011), the first host strain developed for heterologous protein expression GS115 (De Schutter et al. 2009), as well as of the related *P. pastoris* DSMZ 70382 strain (Mattanovich et al. 2009b). Equally important breakthroughs for the commercial application of the *P. pastoris* cell factory were the Food and Drug Administration (FDA) GRAS (generally recognized as safe) status for a protein used in animal

feed, phospholipase C (Ciofalo et al. 2006), and the FDA approval of a recombinant biopharmaceutical product, Kalbitor®, a kallikrein inhibitor (Thompson 2010).

The classical *P. pastoris* expression system has been extensively reviewed over the years (Cereghino and Cregg 2000; Daly and Hearn 2005; Gasser et al. 2013; Jin et al. 2006; Macauley-Patrick et al. 2005). In this review, we focus on recent developments for heterologous protein production and describe examples for the commercial use of this expression system. In the first chapter, we refer to the established basic vector systems and elaborate on developments thereof with an emphasis on newly developed promoter systems. Herein, also some aspects of secretion will be summarized. The second part is devoted to the most recent developments regarding host strain development. As a specific novelty, a new platform based on the CBS7435 strain is described, for which patent protection has ceased and no specific material rights are pending. In the third chapter, we describe specific strategies for obtaining high-level expression strains and summarize important applications of *P. pastoris* for production of biopharmaceuticals, membrane proteins and industrial proteins. The last section provides an outlook on future perspectives covering recent progress in molecular and cell biology of *P. pastoris* and possibilities for implementing new strategies in expression strain development.

Basic systems for cloning and expression in *P. pastoris*

When devising strategies for cloning and expression of heterologous proteins in *P. pastoris* some points need to be considered from the start, that is, the choice of promoter–terminator combinations, suitable selection markers and application of vector systems for either intracellular or secreted expression including selection of proper secretion signals (Fig. 1). The choice of the proper expression vector and complementary host strain are a most important prerequisite for successful recombinant protein expression.

Promoters

The use of tightly regulated promoters such as the alcohol oxidase (*AOX1*) promoter holds advantages for overexpression of proteins. By uncoupling the growth from the production phase, biomass is accumulated prior to protein expression. Therefore, cells are not stressed by the accumulation of recombinant protein during growth phase, and even the production of proteins that are toxic to *P. pastoris* is possible. Furthermore, it may be desirable to co-express helper proteins like chaperones at defined time points, for example, before the actual target protein is formed. On the other hand, use of constitutive promoters may ease process handling. Constitutive promoters are usually also applied to express selection markers. Metabolic pathway engineering strategies might further take advantage of fine-tuned constitutive promoters to ensure a controlled flux of metabolites. An extensive summary of promoters used for heterologous expression in *P. pastoris* has recently been published by Vogl and Glieder (2013). An overview of broadly used and extensively studied as well as recently examined promoters is given in Table 1.

Inducible promoters

The tightly regulated *AOX1* promoter (P_{AOX1}), which was first employed for heterologous gene expression by Tschopp et al. (1987a), is still the most commonly used promoter (Lünsdorf et al. 2011; Sigoillot et al. 2012; Yu et al. 2013). P_{AOX1} is strongly repressed when *P. pastoris* is grown on glucose, glycerol or ethanol (Inan and Meagher 2001). Upon depletion of these carbon sources, the promoter is de-repressed, but is fully induced only upon addition of methanol. Several studies have identified multiple regulatory elements in the P_{AOX1} sequence (Hartner et al. 2008; Kranthi et al. 2006, 2009; Ohi et al. 1994; Parua et al. 2012; Staley et al. 2012; Xuan et al. 2009). Positively and negatively acting elements have been described (Kumar and Rangarajan 2012; Lin-Cereghino et al. 2006; Polupanov et al. 2012), but the molecular details of P_{AOX1} regulation are still not completely elucidated.

Methanol is a highly flammable and hazardous substance and, therefore, undesirable for large-scale fermentations. Alternative inducible promoters or P_{AOX1} variants, which can be induced without methanol but still reach high expression levels, are desired. A recently published patent application describes such a method, wherein expression is controlled by methanol-inducible promoters, such as *AOX1*, methanol oxidase (*MOX*) or formate dehydrogenase (*FMDH*), without the addition of methanol (Takagi et al. 2008). This was achieved by constitutively co-expressing the positively acting transcription factor Prm1p from either of the *GAP*, *TEF* or *PGK* promoters. The relative activity of a phytase reporter protein was 3-fold increased without addition of methanol as compared to a control strain with *PRM1* under its native promoter. However, phytase expression levels were not compared for standard methanol induction and constitutive Prm1p expression conditions. Hartner et al. have constructed a synthetic *AOX1* promoter library by deleting or duplicating transcription factor binding sites for fine-tuned expression in *P. pastoris* (Hartner et al. 2008). Using EGFP as reporter, some promoter variants were found to confer even higher expression levels than the native P_{AOX1} spanning a range between 6 % and 160 % of the native promoter activity. These P_{AOX1} variants have also proven to behave similarly when industrially relevant enzymes such as horseradish peroxidase and hydroxynitrile lyases were expressed.

Numerous further controllable promoters are currently being investigated for their ability to promote high-level

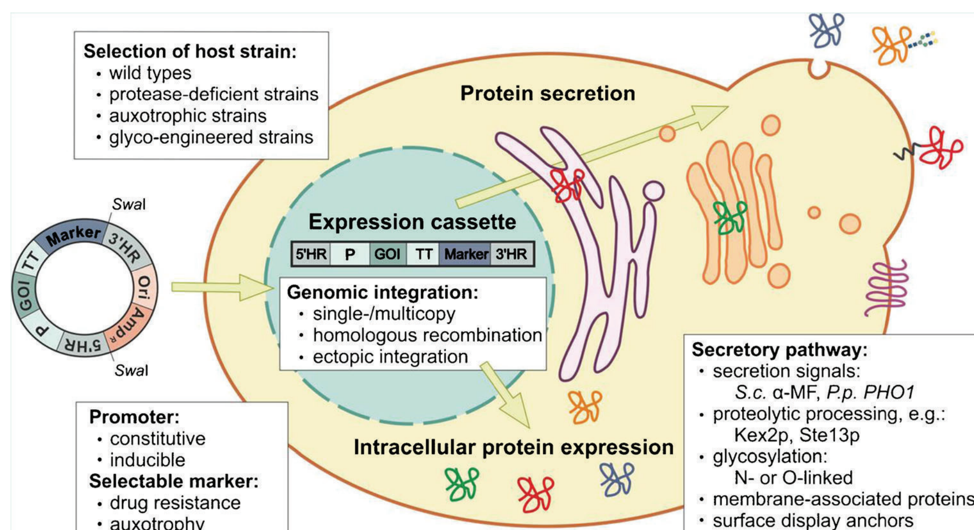

Fig. 1 General considerations for heterologous gene expression in *P. pastoris*. Expression plasmids harbouring the gene(s) of interest (*GOI*) are linearized prior to transformation. Selectable markers (e.g., Amp^R) and origin of replication (*Ori*) are required for plasmid propagation in *E. coli*. The expression level of the protein of interest may depend on (i) the chromosomal integration locus, which is targeted by the 5′ and 3′ homologous regions (5′HR and 3′HR), and (ii) on the gene copy number. A representative promoter (*P*) and transcription terminator (*TT*) pair are shown. Proper signal sequences will guide recombinant protein for intracellular or secretory expression, and will govern membrane integration or membrane anchoring

expression (Table 1). For example, a recently published patent application describes the use of three novel inducible promoters from *P. pastoris*, *ADH1* (alcohol dehydrogenase), *GUT1* (glycerol kinase) and *ENO1* (enolase), showing interesting regulatory features (Cregg and Tolstorukov 2012). However, due to a lack of absolute expression values the performance of these novel promoters cannot be compared to the widely used *AOX1* and *GAP* promoters.

Table 1 The most prominently used and very recently established promoters for heterologous expression in *P. pastoris*

Inducible	Corresponding gene	Regulation	Reference
AOX1	Alcohol oxidase 1	Inducible with MeOH	(Tschopp et al. 1987a)
DAS	Dihydroxyacetone synthase	Inducible with MeOH	(Ellis et al. 1985; Tschopp et al. 1987a)
FLD1	Formaldehyde dehydrogenase 1	Inducible with MeOH or methylamine	(Shen et al. 1998)
ICL1	Isocitrate lyase	Repressed by glucose, induction in absence of glucose/by addition of ethanol	(Menendez et al. 2003)
PHO89	Putative Na$^+$/phosphate symporter	Induction upon phosphate starvation	(Ahn et al. 2009)
THI11	Thiamine biosynthesis gene	Repressed by thiamin	(Stadlmayr et al. 2010)
ADH1	Alcohol dehydrogenase	Repressed on glucose and methanol, induced on glycerol and ethanol	(Cregg and Tolstorukov 2012)
ENO1	Enolase	Repressed on glucose, methanol and ethanol, induced on glycerol	(Cregg and Tolstorukov 2012)
GUT1	Glycerol kinase	Repressed on methanol, induced on glucose, glycerol and ethanol	(Cregg and Tolstorukov 2012)
Constitutive	**Corresponding gene**	**Regulation**	**Reference**
GAP	Glyceraldehyde-3-P dehydrogenase	Constitutive expression on glucose, to a lesser extent on glycerol and methanol	(Waterham et al. 1997)
TEF1	Translation elongation factor 1	Constitutive expression on glycerol and glucose	(Ahn et al. 2007)
PGK1	3-Phosphoglycerate kinase	Constitutive expression on glucose, to a lesser extent on glycerol and methanol	(de Almeida et al. 2005)
GCW14	Potential glycosyl phosphatidyl inositol (GPI)-anchored protein	Constitutive expression on glycerol, glucose and methanol	(Liang et al. 2013b)
G1	High affinity glucose transporter	Repressed on glycerol, induced upon glucose limitation	(Prielhofer et al. 2013)
G6	Putative aldehyde dehydrogenase	Repressed on glycerol, induced upon glucose limitation	(Prielhofer et al. 2013)

Constitutive promoters

Constitutive expression eases process handling, omits the use of potentially hazardous inducers and provides continuous transcription of the gene of interest. For this purpose, the glyceraldehyde-3-phosphate promoter (P_{GAP}) is commonly used, which — on glucose — reaches almost the same expression levels as methanol-induced P_{AOX1} (Waterham et al. 1997). Expression levels from P_{GAP} drop to about one half on glycerol and to one third when cells are grown on methanol (Cereghino and Cregg 2000). Alternative constitutive promoters and promoter variants have been described recently (Table 1). The constitutive P_{GCW14} promoter, for example, was described to be a stronger promoter than the *GAP* and *TEF1* promoters, which was assessed by secretory expression of EGFP (Liang et al. 2013b). It was found that EGFP expression from P_{GCW14} yielded in a 10-fold increase compared to P_{GAP} driven expression when cells were cultivated on glycerol or methanol, and a 5-fold increase on glucose.

A recent DNA microarray study identified novel promoters that are repressed on glycerol, but are being induced upon shift to glucose-limited media (Prielhofer et al. 2013). Supposedly, the most interesting promoters discovered by this approach control expression of a high-affinity glucose transporter, *HGT1*, and of a putative aldehyde dehydrogenase. The former promoter was reported to drive EGFP expression to even higher levels than could be reached with P_{GAP}. In glycerol fed-batch fermenter cultures, human serum album was expressed from the novel promoter to a 230 % increase in specific product yield as compared to P_{GAP} driven expression.

In some cases, it is desired that expression levels can be fine-tuned in order to (1) co-express accessory proteins facilitating recombinant protein expression and secretion or (2) provide protein post-translational modifications as well as to (3) engineer whole metabolic pathways consisting of a cascade of different enzymatic steps. For such applications, a library of *GAP* promoter variants with relative strengths ranging from 0.6 % to 16.9-fold of the wild type promoter activity was developed and tested using three different reporter proteins, yEGFP, β-galactosidase and methionine acetyltransferase (Qin et al. 2011).

Vectors

The standard setup of vectors is a bi-functional system enabling replication in *E. coli* and maintenance in *P. pastoris* using as selection markers either auxotrophy markers (e.g., *HIS4*, *MET2*, *ADE1*, *ARG4*, *URA3*, *URA5*, *GUT1*) or genes conferring resistance to drugs such as Zeocin™, geneticin (G418) and blasticidin S. Although there are some reports of using episomal plasmids for heterologous protein expression or for the screening of mutant libraries in *P. pastoris* (Lee et al. 2005; Uchima and Arioka 2012), stable integration into the host genome is the most preferred method. Unlike in *Saccharomyces cerevisiae*, where homologous recombination (HR) predominates, non-homologous end-joining (NHEJ) is a frequent process in *P. pastoris.* The ratio of NHEJ and HR can be shifted towards HR by elongating the length of the homologous regions flanking the actual expression cassettes and by suppressing NHEJ efficiency (Näätsaari et al. 2012).

The standard vector systems for intracellular and secretory expression provided by Life Technologies (Carlsbad, CA, USA) include constitutive (P_{GAP}) and inducible promoters triggered by methanol or methylamine (P_{AOX1}, P_{FLD}). The recently introduced PichiaPink™ expression kit for intracellular or secreted expression enables easy selection of multicopy integration clones by differences in colour formation based on *ade2* knockout strains and truncated *ADE2* promoters of varying strengths in front of the *ADE2* marker gene (Du et al. 2012; Nett 2010).

Additionally, BioGrammatics (Carlsbad, CA, USA) holds licences for selling standard *P. pastoris* expression vectors and strains and also provides GlycoSwitch® vectors for humanized glycosylation of target proteins (Table 2). Several vectors for disruption of *OCH1* and expression of different glycosidases or glycosyltransferases are available to achieve mammalian-type N-glycan structures in *P. pastoris*. These vectors harbour, for example, the human GlcNAc transferase I, the mannosidase II from rat, or the human galactosyl transferase I. A detailed protocol for humanizing the glycosylation pattern using the GlycoSwitch® vectors is provided (Jacobs et al. 2009).

James Cregg's laboratory at the Keck Graduate Institute, Claremont, CA, USA, has developed a set of plasmids for protein secretion and intracellular expression in *P. pastoris* containing the strong *AOX1* promoter. These vectors are based on different auxotrophy markers, such as *ARG4*, *ADE1*, *URA3* and *HIS4*, for selection necessitating the use of the appropriate host strains (see section "Host strain development"). The vectors contain restriction sites for linearization within the marker genes to target the expression cassettes to the desired locus as well as for multicopy integration (Lin-Cereghino et al. 2001). Moreover, a set of integration vectors for sequential disruption of *ARG1*, *ARG2*, *ARG3*, *HIS1*, *HIS2*, *HIS5* and *HIS6* in *P. pastoris* was applied to provide the host strains for engineering the protein glycosylation pathway (Nett et al. 2005).

The Institute of Molecular Biotechnology, Graz University of Technology, Austria, provides vectors and strains to the *P. pastoris* community through the so-called '*Pichia* Pool'. The p*Pp* plasmids described by Näätsaari et al. (2012) comprise vectors containing the *GAP* or *AOX1* promoters and, for secretory expression, the *S. cerevisiae* α-mating factor (α-MF) secretion signal. The antibiotic selection marker cassettes were placed under the control of *ADH1* or *ILV5* promoters in the p*Pp*B1 and p*Pp*T4 vectors, respectively. It is

Table 2 Commercial vector systems

Supplier	Promoter	Signal sequences	Selection in yeast	Selection in bacteria	Comments
Life Technologies™	*AOX1, FLD1, GAP*	*S. cerevisiae* α-MF; *P. pastoris PHO1*	Blasticidin, G418, Zeocin™, *HIS4*	Zeocin™, Ampicillin, Blasticidin	c-myc epitope, V5 epitope, C-terminal 6× His-tag available for detection/purification
Life Technologies –PichiaPink™	*AOX1*	α-MF; set of eight different signal sequences – not ready to use[a]	*ADE2*	Ampicillin	Low- and high-copy vectors available, *TRP2* sequence for targeting
BioGrammatics	*AOX1*	α-MF	Zeocin™, G418, Nourseothricin	Ampicillin	Intracellular or secreted expression
BioGrammatics – GlycoSwitch®	*GAP*	–	Zeocin™, G418, Hygromycin, *HIS4*, Nourseothricin	Zeocin™, Ampicillin, Kanamycin, Nurseothricin	Human GlcNAc transferase I, rat Mannosidase II, human Gal transferase I
DNA2.0	*AOX1*	Ten different signal sequences – ready to use[b]	Zeocin™, G418	Zeocin™, Ampicillin	Intracellular or secreted

[a] The different secretion signals have to be cloned into the vector by a three-way ligation step

[b] The α-MF secretion signal is provided once with Kex2p (KR) and Ste13p cleavage sites (EAEA), once lacking EA repeats, and once as truncated version (pre-region only)

described that the p*Pp*T4-based vectors usually lead to lower gene copies in the cell as compared to the p*Pp*B1-based vectors.

Further vectors based on either the *GAP* or the *AOX1* promoter and a series of strains have recently been added to this pool, both for intracellular and secretory protein expression (M. Ahmad, unpublished results). For intracellular expression, cloning of the target genes is accomplished by using *Eco*RI and *Not*I, whereby the Kozak consensus sequence has to be restored for efficient translation initiation (Fig. 2a). A special characteristic of these vectors is that the *Eco*RI site has been introduced by a single point mutation directly into the *AOX1* promoter sequence without changing the promoter activity. Thereby, the gene of interest may be fused to the promoter without having additional nucleotides between the promoter and the start codon. Another advantage is the use of the short *ARG4* promoter for the expression of the selection markers. The weaker *ARG4* promoter used for selection marker cassettes enables selection at lower concentrations of Zeocin™ (i.e., 25 instead of 100 μg/ml) without obtaining false-positive clones. For secretory expression governed by the *S. cerevisiae* α-MF signal sequence, *Xho*I and/or *Not*I sites are used for cloning the genes of interest (Fig. 2b).

Aspects of secretory expression

One of the main advantages of using *P. pastoris* as a protein production host is its ability to secrete high titres of properly folded, post-translationally processed and active recombinant proteins into the culture media. As a rule of thumb, proteins secreted in their native hosts will also be secreted in *P. pastoris*. However, there are also some reports of successful secretion of typically intracellular proteins such as GFP or human catalase (Eiden-Plach et al. 2004; Shi et al. 2007). The most commonly employed secretion signals in *P. pastoris* are derived from *S. cerevisiae* α-MF, *S. cerevisiae* invertase (*SUC2*) and the *P. pastoris* endogenous acid phosphatase (*PHO1*) (Daly and Hearn 2005). As listed in Table 2, commercial kits also provide vectors with different secretion signals, which allows for screening of the best-suited signal sequence.

The α-MF signal sequence is composed of a pre- and pro-region and has proven to be most effective in directing protein through the secretory pathway in *P. pastoris*. The pre-region is responsible for directing the nascent protein post-translationally into the endoplasmic reticulum (ER) and is cleaved off subsequently by signal peptidase (Waters et al. 1988). The pro-region is thought to play a role in transferring the protein from ER to Golgi compartment and is finally cleaved at the dibasic KR site by the endo-protease Kex2p (Julius et al. 1984). The two EA repeats are subsequently trimmed by the *STE13* gene product (Brake et al. 1984). One of the common problems encountered while using the α-MF secretion signal is non-homogeneity of the N-termini of the recombinant proteins due to incomplete *STE13* processing. Constructs without the EA repeats may enhance homogeneity at the N termini of recombinant proteins. However, the removal of these sequences may affect protein yield. While no reports on enhanced co-expression of *STE13* are available, co-overexpression of *HAC1*, a transcription factor in the unfolded protein response (UPR) pathway, with the membrane protein adenosine A2 receptor had a positive effect on proper processing of the α-MF signal sequence (Guerfal

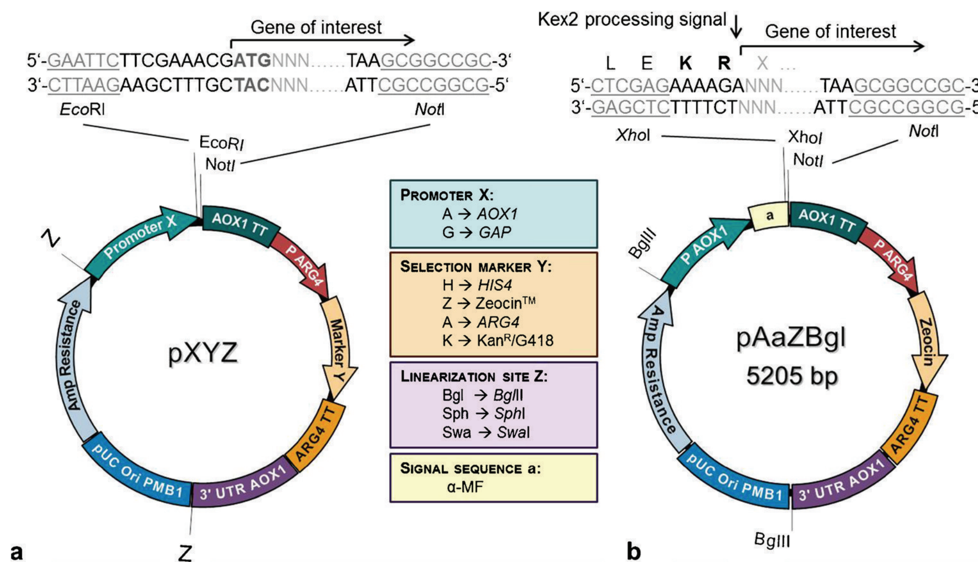

Fig. 2 Novel '*Pichia* Pool' plasmid sets for intracellular and secretory expression. **a** General features of pXYZ vector for intracellular expression. Letters refer to the choice of promoters (*X*), selection markers (*Y*), and restriction enzymes (*Z*) for linearization. Available elements are shown in boxes. The vector backbone harbours an ampicillin resistance marker and origin of replication for maintenance of the plasmid in *E. coli*. The GOI is *Eco*RI–*Not*I cloned directly after the promoter of choice. The Kozak consensus sequence for yeast (i.e., CGAAACG), should be restored between the *Eco*RI cloning site and the start codon of the GOI in order to achieve optimal translation. In addition, sequence variation within this region will allow fine-tuning translation initiation efficiency. Expression in *P. pastoris* is driven either by the methanol inducible *AOX1* or the constitutive *GAP* promoter. Positive clones can be selected for by antibiotic resistance (i.e., to Zeocin™ or geneticin sulphate) or by selection for His or Arg prototrophy. Selection marker expression is uniformly driven by the *ARG4* promoter–terminator pair. **b** Plasmid pAaZBgl from '*Pichia* Pool' is shown as an example of a vector made for secretory expression encoding *S. cerevisiae* α-MF signal sequence in front of the GOI cloning site. The Kex2 processing site AAAAGA should be restored between the *Xho*I cloning site and the fusion point of the GOI

et al. 2010). Recently, Yang et al. (2013) reported enhanced secretory protein production by optimizing the amino acid residues at the Kex2 P1′ site.

Multiple strategies have been followed to enhance the secretory potential of the α-MF signal sequence including codon optimization (Kjeldsen et al. 1998), directed evolution (Rakestraw et al. 2009), insertion of spacers and deletion mutagenesis (Lin-Cereghino et al. 2013). Directed evolution of the α-MF signal sequence in *S. cerevisiae* resulted in up to 16-fold enhanced full-length IgG_1 secretion as compared to the wild type. Furthermore, when this improved leader sequence was combined with strain engineering strategies comprising *PDI* overexpression and elimination of proteins involved in vacuolar targeting, up to 180-fold enhanced secretion of the reporter protein was observed (Rakestraw et al. 2009). Deletion mutagenesis based on a predicted structure model of α-MF signal peptide resulted in 50 % increased secretion of horseradish peroxidase and *C. antarctica* lipase B (CALB) in *P. pastoris* (Lin-Cereghino et al. 2013). It appears that decreasing the hydrophobicity of the leader sequence by deleting hydrophobic residues or substituting them with more polar or charged residues increased the flexibility of the α-MF signal sequence structure, which enhanced the overall secretory capacity of the pro-region. Alternative signal sequences used to direct protein secretion and their features and applications are summarized in Table 3.

Beyond the choice of the secretion signals there are several other factors that govern efficient protein secretion. The newly synthesized proteins are translocated co- or post-translationally into the ER lumen through the Sec61p translocon. Then, proteins may undergo one or several post-translational modifications, folding into the native state, disulphide-bond formation, glycosylation and membrane-anchoring. When the recombinant protein fails to fold into its native state or protein expression exceeds the folding capacity of the ER (Sha et al. 2013), unfolded proteins may start to aggregate, triggering the UPR pathway. UPR is responsible for induction of genes that are involved in protein folding. In parallel to UPR pathway, ER-associated degradation (ERAD) by the proteasome may relieve blocks in protein secretion (recently reviewed by Idiris et al. 2010 and Damasceno et al. 2012). Inappropriate mRNA structure and gene copy numbers, limits in transcription, translation and protein translocation into the ER, incomplete protein folding and inefficient protein targeting to the exterior of the cell are major bottlenecks encountered in secretory expression of heterologous proteins. Commonly used strategies to overcome such secretory bottlenecks comprise the overexpression of folding helper proteins like BiP/Kar2p, DnaJ, PDI, PPIs and Ero1p or, alternatively, overexpression of *HAC1*, a transcriptional regulator of the UPR pathway genes. Unlike in *S. cerevisiae*, Guerfal et al. (2010) reported that *HAC1* is

Table 3 Signal sequences used to secrete the protein into the extracellular space

Secretion signal	Source	Target protein(s)	Length	Reference
α-MF	*S.c.* α-mating factor	Most commonly used secretion signal in *P. pastoris*	85 aa, with or without EA repeats	(Brake et al. 1984)
PHO1	*P.p.* acid phosphatase	Mouse 5-HT5A, porcine pepsinogen,	15 aa	(Payne et al. 1995; Weiss et al. 1995; Yoshimasu et al. 2002)
SUC2	*S.c.* Invertase	Human interferon, α-amylase, α-1-antitrypsin	19 aa	(Moir and Dumais 1987; Paifer et al. 1994; Tschopp et al. 1987b)
PHA-E	Phytohemagglutinin	GNA, GFP and native protein	21 aa	(Raemaekers et al. 1999)
KILM1	Kl toxin	CM cellulase	44 aa	(Skipper et al. 1985)
pGKL	pGKL killer protein	Mouse α-amylase	20 aa	(Kato et al. 2001)
CLY and CLY-L8	C-lysozyme and syn. leucin-rich peptide	Human lysozyme	18 and 16 aa	(Oka et al. 1999)
K28 pre-pro-toxin	K28 virus toxin	Green fluorescent protein	36 aa	(Eiden-Plach et al. 2004)
Scw, Dse and Exg	*P.p.* Endogenous signal peptides	CALB and EGFP	19, 20 and 23 aa	(Liang et al. 2013a)
Pp Pir1	*P.p.* Pir1p	EGFP and Human α1-antitrypsin	61 aa	(Khasa et al. 2011)
HBFI and HBFII	Hydrophobins of *Trichoderma reesei*	EGFP	16 and 15 aa	(Kottmeier et al. 2011)

constitutively expressed and spliced in *P. pastoris* under normal growth conditions, which may explain the higher titers of secreted proteins obtainable with this organism. A contradictory observation was reported by Whyteside et al. (2011). Unspliced *HAC1* mRNA was detected under normal growth conditions and splicing of *HAC1* mRNA was only detected when cells were grown in presence of dithiothreitol (DTT) to activate the UPR. It should be mentioned, though, that sometimes overexpression of folding helpers actually reduced protein secretion or did not have any effect (van der Heide et al. 2002).

Host strain development

Elucidation of full genome sequences and gene annotation were great steps toward rational strain engineering, identifying new promoters and progressing in the (systems) biology of *P. pastoris* (Küberl et al. 2011; Mattanovich et al. 2009a; De Schutter et al. 2009). Two online databases (http://bioinformatics.psb.ugent.be/orcae/overview/Picpa and http://www.pichiagenome.org) provide convenient access to genome sequences and annotations. Frequently used commercially available strains are the *his4* strain GS115, the reconstituted prototrophic strain X-33, the *aox1* knockout strains KM71 and KM71H as well as protease-deficient strains SMD1168 and SMD1168H and the *ade2* auxotrophic PichiaPink™ strain. Use of these strains for commercial applications, however, is restricted by patent protection and/or materials ownership policy. Strains derived from *P. pastoris* CBS7435, in contrast, are not covered by patent protection and, therefore represent an alternative for production purposes. Furthermore, the CBS7435 Mut^S strain provided by the Graz *Pichia* Pool has the advantage of being marker-free as it was constructed using the Flp/FRT recombinase system for marker removal (Näätsaari et al. 2012). Using the same strategy, *ade1* and *his4* knockout strains were created along with the CBS7435 *ku70* strain (CBS 12694), which is impaired in the NHEJ mechanism, thereby enhancing the efficiency of HR. A selection of most relevant strains is compiled in Table 4.

Auxotrophic strains

Several auxotrophic strains (e.g., *ade1*, *arg4*, *his4*, *ura3*, *met2*), and combinations thereof are available together with vectors harbouring the respective genes as selectable markers (Lin-Cereghino et al. 2001; Thor et al. 2005, Graz *Pichia* Pool). Auxotrophic strains have been useful for in vivo labelling of proteins, for example in the global fluorination of *Candida antarctica* lipase B (CALB) in a *P. pastoris* X-33 *aro1* strain deficient in tryptophan, tyrosine, and phenylalanine biosynthesis (Budisa et al. 2010). Fluorinated analogues of these amino acids were supplemented and incorporated into the heterologous protein, thereby, for example, prolonging CALB shelf-life but lowering its lipase activity. The proteolytic pattern of CALB was retained, though. Another example is the use of a *lys2 arg4* double knockout strain for stable isotope labelling by amino acids in cell culture (SILAC) (Austin et al. 2011).

Protease-deficient strains

Undesired proteolysis of heterologous proteins expressed in *P. pastoris* does not only lower the product yield or biological

Table 4 *P. pastoris* host strains

Strain	Genotype	Phenotype	Source
Wild-type strains			
CBS7435 (NRRL Y-11430)	WT	WT	Centraalbureau voor Schimmelcultures, the Netherlands
CBS704 (DSMZ 70382)	WT	WT	Centraalbureau voor Schimmelcultures, the Netherlands
X-33	WT	WT	Life Technologies™
Auxotrophic strains			
GS115	*his4*	His⁻	Life Technologies™
PichiaPink™ 1	*ade2*	Ade⁻	Life Technologies™
KM71	*his4, aox1::ARG4, arg4*	His⁻, Mut^S	Life Technologies™
KM71H	*aox1::ARG4, arg4*	Mut^S	Life Technologies™
BG09	*arg4::nourseo^R Δlys2::hyg^R*	Lys⁻, Arg⁻, Nourseothricin^R, Hygromycin^R	BioGrammatics
GS190	*arg4*	Arg⁻	(Cregg et al. 1998)
GS200	*arg4 his4*	His⁻, Arg⁻	(Waterham et al. 1996)
JC220	*ade1*	Ade⁻	(Cregg et al. 1998)
JC254	*ura3*	Ura⁻	(Cregg et al. 1998)
JC227	*ade1 arg4*	Ade⁻ Arg⁻	(Lin-Cereghino et al. 2001)
JC300-JC308	Combinations of *ade1 arg4 his4 ura3*	Combinations of Ade⁻, Arg⁻, His⁻, Ura⁻	(Lin-Cereghino et al. 2001)
YJN165	*ura5*	Ura⁻	(Nett and Gerngross 2003)
CBS7435 *his4*[a]	*his4*	His⁻	(Näätsaari et al. 2012)
CBS7435 Mut^S *his4*[a]	*aox1, his4*	Mut^S, His⁻	(Näätsaari et al. 2012)
CBS7435 Mut^S *arg4*[a]	*aox1, arg4*	Mut^S, Arg⁻	(Näätsaari et al. 2012)
CBS7435 *met2*[a]	*met2*	Met⁻	(*Pp*7030)[b]
CBS7435 *met2 arg4*[a]	*met2 arg4*	Met⁻ Arg⁻	(*Pp*7031)[b]
CBS7435 *met2 his4*[a]	*met2 his4*	Met⁻ His⁻	(*Pp*7032)[b]
CBS7435 *lys2*[a]	*lys2*	Lys⁻	(*Pp*7033)[b]
CBS7435 *lys2 arg4*[a]	*lys2 arg4*	Lys⁻ Arg⁻	(*Pp*7034)[b]
CBS7435 *lys2 his4*[a]	*lys2 his4*	Lys⁻ His⁻	(*Pp*7035)[b]
CBS7435 *pro3*[a]	*pro3*	Pro⁻	(*Pp*7036)[b]
CBS7435 *tyr1*[a]	*tyr1*	Tyr⁻	(*Pp*7037)[b]
Protease-deficient strains			
SMD1163	*his4 pep4 prb1*	His⁻	(Gleeson et al. 1998)
SMD1165	*his4 prb1*	His⁻	(Gleeson et al. 1998)
SMD1168	*his4 pep4::URA3 ura3*	His⁻	Life Technologies™
SMD1168H	*pep4*		Life Technologies™
SMD1168 kex1::SUC2	*pep4::URA3 kex1::SUC2 his4 ura3*	His⁻	(Boehm et al. 1999)
PichiaPink 2-4	Combinations of *prb1/pep4*	Ade⁻	Life Technologies™
BG21	*sub2*		BioGrammatics
CBS7435 *prc1*[a]	*prc1*		(*Pp*6676)[b]
CBS7435 *sub2*[a]	*sub2*		(*Pp*6668)[b]
CBS7435 *sub2*[a]	*his4 pep4*	His⁻	(*Pp*6911)[b]
CBS7435 *prb1*[a]	*prb1*		(*Pp*6912)[b]
CBS7435 *his4 pep4 prb1*	*his4 pep4 prb1*	His⁻	(*Pp*7013)[b]
Glyco-engineered strains			
SuperMan$_5$	*his4 och1::pGAPTrα1,2-mannosidase*	His⁻, Blasticidin^R	BioGrammatics
	och1::pGAPTrα1,2-mannosidase	Blasticidin^R	BioGrammatics
	pep4 och1::pGAPTrα1,2-mannosidase	Blasticidin^R	BioGrammatics

Table 4 (continued)

Strain	Genotype	Phenotype	Source
Other strains			
GS241	*fld1*	Growth defect on methanol as sole C-source or methylamine as sole N-source	(Shen et al. 1998)
MS105	*his4 fld1*	See GS241; His$^-$	(Shen et al. 1998)
MC100-3	*his4 arg4 aox1::ScARG4 aox2::PpHIS4*	Mut$^-$	(Cregg et al. 1989)
CBS7435 *ku70* [a]	*ku70*	WT	(Näätsaari et al. 2012)
CBS7435 *ku70 his4* [a]	*ku70, his4*	His$^-$	(Näätsaari et al. 2012)
CBS7435 *ku70 gut1*	*ku70, gut1*	Growth defect on glycerol; Zeocin^R	(Näätsaari et al. 2012)
CBS7435 *ku70 ade1*	*ku70, ade1*	Ade$^-$, Zeocin^R	(Näätsaari et al. 2012)

[a] These *P. pastoris* CBS7435 derived strains are marker-free knockouts

[b] Strains from '*Pichia* Pool' of TU Graz (M. Ahmad, unpublished results)

activity, but also complicates downstream processing of the intact product as the degradation products will have similar physicochemical and affinity properties. Proteolysis may occur either during vesicular transport of recombinant protein by secretory pathway-resident proteases (Werten and de Wolf 2005; Ni et al. 2008) or in the extracellular space by proteases being secreted, cell wall-associated (Kang et al. 2000) or released into the culture medium as a result of cell disruption during high cell density cultivation (Sinha et al. 2005). Different strategies have been employed to address the proteolysis problem, namely, modifying fermentation parameters (pH, temperature and specific growth rate), changing the media composition (rich medium, addition of casamino acids or peptone as competing substrates), lowering the salt concentration and addition of soytone (Zhao et al. 2008), applying protein engineering strategies (Gustavsson et al. 2001) and engineering of the expression host to obtain protease-deficient strains (reviewed by Idiris et al. 2010 and Macauley-Patrick et al. 2005). However, in some cases, optimization of the fermentation media and protein engineering strategies failed to alleviate the proteolysis problem and tuning the expression host itself was the only viable option (Li et al. 2010). The use of protease-deficient strains such as SMD1163 (*Δhis4 Δpep4 Δprb1*), SMD1165 (*Δhis4 Δprb1*) and SMD1168 (*Δhis4 Δpep4*) has been well documented for the expression of protease-sensitive proteins (Gleeson et al. 1998). *PEP4* encodes a major vacuolar aspartyl protease which is able to activate itself as well as further proteases such as carboxypeptidase Y (*PRC1*) and proteinase B (*PRB1*). The use of protease-deficient strains other than the above mentioned (e.g., *yps1*, *kex1*, *kex2*) was reported with variable success (Ni et al. 2008; Werten and de Wolf 2005; Wu et al. 2013; Yao et al. 2009). A general conclusion from these studies is that in many cases several proteases are involved in degradation events and, therefore, it is not an easy task to optimize protein expression by knocking out just a single one. However, the *pep4* and *prb1* knockout strains are still the most effective ones in preventing recombinant protein degradation, and, hence, also the most widely applied. Although it has been reported that protease-deficient strains show typically slower growth rates, lower transformation efficiencies and reduced viability (Lin-Cereghino and Lin-Cereghino 2007), experiments in our laboratory showed robust growth behaviour of 28 protease-deficient strains that were recently created (M. Ahmad, unpublished results).

Glyco-engineered strains

When yeasts such as *P. pastoris* are chosen for production of therapeutic proteins, N- and O-linked glycosylation are of tremendous relevance. Although the assembly of the core glycans, that is, $(Man)_8$-$(GlcNAc)_2$, in the ER is highly conserved in mammals and yeasts, mammals provide a much higher diversity in the ultimate glycan structure assembled in the Golgi cisternae. Yeasts, in contrast, produce high mannose glycan structures, which may lead to decreased serum half-life and may trigger allergic reactions in the human body (Ballou 1990). While in *P. pastoris* the hyper-mannosylation is not as prominent as in *S. cerevisiae*, it is still a problem that needs to be tackled, and is therefore a target for intensive strain engineering. A very detailed summary of the glycosylation machinery and the targets for glyco-engineering in different yeast species, including *P. pastoris*, has been given recently (De Pourcq et al. 2010). To sum up briefly, engineering strategies included the introduction of a *Trichoderma reesei* α-1,2-mannosidase (Callewaert et al. 2001), the knockout of the highly conserved yeast Golgi protein α-1,6-mannosyltransferase encoded by *OCH1*, which is responsible for hyperglycosylation (Choi et al. 2003; Vervecken et al. 2004), as well as co-overexpression of several glycosyltransferases and glycosidases carrying proper targeting signals (Hamilton et al. 2003). Terminally sialylated glycoproteins

produced for the first in *P. pastoris* were obtained by introducing a complex sialic acid pathway (Hamilton et al. 2006). Key to success was the correct localization of the heterologous glycosyltransferases and glycosidases in the ER and Golgi networks. Combinatorial genetic libraries and high throughput screening methods were successfully applied to find the best targeting signal/enzyme combinations for N-linked glycoengineering (Nett et al. 2011). Furthermore, a useful guide to glyco-engineering in *P. pastoris* by using the GlycoSwitch® technology was described by Jacobs et al. (2009). These strategies, altogether, enable the production of valuable biopharmaceuticals with a more homogeneous, 'humanized' N-glycosylation pattern.

However, as yeasts also carry out O-glycosylation that differs structurally from the mammalian type (Strahl-Bolsinger et al. 1999), O-glycosylation has also been an interesting target for engineering. In *P. pastoris*, O-linked glycosylation is initiated with a mannose monosaccharide, which is further elongated by α-1,2-mannose residues and finally capped with β- or phospho-mannose residues. Until lately, the engineering strategies were limited to the use of an inhibitor of the major ER located protein-O-mannosyltransferases (PMTs) as the deletion of these genes did not yield robust and viable strains. The characterization of the *P. pastoris* PMT gene family was an important step forward in O-glycosylation engineering (Nett et al. 2013). In this study, the knockout of PMTs as well as the use of PMT inhibitors led to a reduced number of O-mannosylation events and, furthermore, to reduced chain lengths of the O-glycans. A follow-up study described the production of a TNFR2:Fc[1] fusion protein carrying sialylated O-linked glycans in *P. pastoris* (Hamilton et al. 2013). Therein, an α-1,2-mannosidase as well as a protein-O-linked-mannose β-1,2-N-acetylglucosaminyl-transferase 1 (PomGnT1) were co-expressed in a *P. pastoris* strain, that was already engineered in its N-glycosylation pathway. Hence, the mannose residues were first trimmed to single O-linked mannose residues, which were then capped with N-acetylglucosamine. This structure was extended with sialic acid residues to achieve human-like O-glycan residues similar to the α-dystroglycan-type. However, there is still room for improvement, for example by engineering *P. pastoris* towards human mucin-type O-glycosylation.

Expression strategies and industrial applications

Screening for high level expression

Subsequent to the choice of suitable expression vectors and proper host strains, and transformation of the expression cassettes, it is important to select for transformants which show high expression levels of the desired protein. Single copy transformants can be easily generated by targeting the linear expression cassettes to the *AOX1* locus resulting in gene replacement events. Ectopic integrations may simultaneously occur, however. Transformants resulting from gene replacement at the *AOX1* locus have methanol utilization slow phenotype (Mut^S) and can be easily identified by replica-plating on minimal methanol plates. The most commonly applied strategy to screen for high-yielding *P. pastoris* transformants focusses on screening for clones having multicopy integrations of the expression cassette. A recent detailed review describes the methods applied to obtain strains containing multiple expression cassettes and provides a summary of published data showing correlations between copy number and expression levels of intracellular as well as secreted proteins. It also highlights the problem of genetic instability of the integration cassettes that might be encountered when cultivating multicopy strains. Due to the highly recombinogenic nature of *P. pastoris*, expression cassettes might be excised through loop-out recombination. This effect seems to be more pronounced the more copies are integrated (Aw and Polizzi 2013).

Regarding the correlation between copy number and expression level, a number of recent studies have shown a direct correlation especially for intracellular expression (Marx et al. 2009; Vassileva et al. 2001). The direct correlation of expression level and gene copy number is, however, not necessarily valid when the protein is directed to the secretory pathway. The most commonly employed method of generating multicopy expression strains in *P. pastoris* is based on plating the transformation mixture directly on selection plates containing increasing concentrations of antibiotics (e.g., 100 to 2,000 μg/ml of Zeocin™). The majority of transformants will have a single copy of the expression vector integrated into the genome, and numerous clones will have to be screened to find high-copy transformants (Lin-Cereghino and Lin-Cereghino 2007). Therefore, several high-throughput methods have been established to screen a large number of clones based on small-scale cultivation in deep well plates (Mellitzer et al. 2012; Weinhandl et al. 2012; Weis et al. 2004). The selected clones, however, might not perform as well in fermenter cultivations due to different cultivation conditions. A further pronounced problem of resistance marker based screening is a high prevalence of false-positive colonies. This so-called high transformation background is supposedly caused by cell stress and cell rupture. Depending on the mechanism of antibiotic resistance conferred by the resistance marker, un-transformed cells may survive in the vicinity of ruptured transformants. This problem was addressed by constructing expression vectors based on marker gene expression driven by the weak *ARG4* promoter (*Pichia* Pool, Fig. 2). This ensures basal levels of expression, thereby allowing handlers to select single copy to

[1] Ectodomain of tumor necrosis factor 2 with crystallizable fragment of IgG1 (Fc)

multicopy strains by plating the transformants directly on low concentrations of Zeocin™ (i.e., 25 μg/ml for single copy and up to 400 μg/ml for multi-copy transformants). Thus, transformants having 1 to 20 (±5) copies can be selected. To reduce the chances of having single copy transformants, regeneration time should be kept short and transformants should be plated directly on increased concentrations of antibiotic. By employing this method, only few transformants survive on high concentrations of antibiotic, but will most likely contain multiple copies, which can be determined by quantitative (qPCR) or Southern blot analysis (M. Ahmad, unpublished results). Performance can then be tested directly under production conditions in bioreactor cultivations instead of small-scale cultivations in deep well plates or shake flasks.

Membrane protein expression

P. pastoris has been shown to produce 15+ g of soluble recombinant protein per litre of culture intracellularly (Hasslacher et al. 1997) or in secretory mode (Werten et al. 1999). Key to such high titres is the ability of *P. pastoris* to grow to very high cell densities reaching up to 150 g cell dry weight per litre of fermentation broth in fed-batch bioreactor cultivations (Jahic et al. 2006). At very high cell densities, even proteins that are present in limited entities per single cell can be produced with reasonable volumetric yields in *P. pastoris*. Typical examples of non-abundant proteins with high scientific and commercial relevance are integral membrane proteins. Being the targets of >50 % of drugs applied on humans (Arinaminpathy et al. 2009), only very few membrane proteins have been characterized on the molecular level regarding structure–function relationships. The simple reason is that it is difficult to obtain sufficient purified membrane protein for structural and biochemical studies, unless affinity-tagged membrane proteins are obtained at reasonable yield. Actually, *P. pastoris* has been applied routinely to produce affinity-tagged membrane proteins for protein purification and subsequent biochemical studies (Cohen et al. 2005; Haviv et al. 2007; Lifshitz et al. 2007). Furthermore, *P. pastoris* has been the expression host of choice for elucidating the crystal structures of membrane proteins from diverse origins, even from higher eukaryotes (Brohawn et al. 2012; Hino et al. 2012; Ho et al. 2009).

Evolutionary proximity of a heterologous expression host and the origin of an expressed membrane protein are beneficial for successful recombinant expression (Grisshammer and Tateu 2009). In addition to the intramolecular forces and bonds, ions, cofactors and interacting proteins that stabilize soluble proteins, membrane proteins are usually interacting with and are partially also stabilized by the lipids of the surrounding bilayers (Adamian et al. 2011). As *P. pastoris* and other yeast expression hosts do significantly differ in their membrane compositions from bacterial, plant or animal cells (Wriessnegger et al. 2007, 2009; Zinser and Daum 1995), heterologous membrane proteins may face stability issues upon expression in distantly related hosts. Thus, multiple approaches have been undertaken to improve *P. pastoris* host strains and expression conditions for membrane protein production. Applying similar tools as for the optimisation of soluble protein expression — that is, manipulation of expression conditions, addition of chemical chaperones, co-expression of chaperones or of proteins activating UPR, use of protease deficient strains, etc. — has been showing some, however often target-specific success in membrane protein expression. A novel approach is the engineering of *P. pastoris* cellular membranes for improved accommodation of heterologous membrane proteins. In the first reported example, a cholesterol-producing *P. pastoris* strain was shown to stably express an enhanced level of ligand-binding human Na,K-ATPase moieties on the cell surface (Hirz et al. 2013).

Products on — or on the way to — the market

The *P.* pastoris expression system has gained importance for industrial application as highlighted by the number of patents published on heterologous expression in and cell engineering of *P. pastoris* (Bollok et al. 2009). Products obtained by heterologous expression in *P. pastoris* have already found their way to the market, as FDA approved biopharmaceuticals or industrial enzymes have shown. The www.pichia.com web page provides a list of proteins produced in *P. pastoris* with the commercial expression system licensed by Research Corporation Technologies (RCT) and their applications: Phytase (Phytex, Sheridan, IN, USA) is applied as animal feed additive to cleave plant derived phytate, thereby providing a source of phosphate. Trypsin (Roche Applied Science, Germany) is used, for example, as protease in proteomics research to obtain peptide patterns for MS analysis. Further examples listed are nitrate reductase (The Nitrate Elimination Co., Lake Linden, MI, USA), used for water testing and treatment, phospholipase C (Verenium, San Diego, CA, USA/DSM, The Netherlands), used for degumming of vegetable oils, and Collagen (Fibrogen, San Francisco, CA, USA), used in medical research and as dermal filler. Thermo Scientific (Waltham, MA, USA) sells recombinant *Tritirachium album* Proteinase K produced in *P. pastoris*. Concerning biopharmaceuticals, a famous example is Kalbitor® (ecallantide), produced in *P. pastoris* by Dyax (Cambridge, MA, USA). Kalbitor® is a plasma kallikrein inhibitor indicated against hereditary angioedema. This product was the first biopharmaceutical to be approved by the FDA for market release in 2009 (Walsh 2010). As can be found on the web page of RCT (www.rctech.com), *Pichia*-manufactured Jetrea®, a drug used for treatment of symptomatic vitreomacular adhesion, was recently approved by the FDA and the European Commission. Other *Pichia*-

derived products provided by the Indian company Biocon are recombinant human insulin and analogues thereof (Insulin, Glargine). Products under development, such as Elastase inhibitor against Cystic fibrosis or Nanobody® ALX antibody fragments developed by Ablynx (Belgium), are also listed by Gerngross (2004) and on www.pichia.com. In 2008, Novozymes (Denmark), which found a highly active antimicrobial agent, the plectasin peptide derivative NZ2114 (Andes et al. 2009; Mygind et al. 2005), granted Sanofi-Aventis (France) an exclusive licence for the production and commercialisation of this compound in *P. pastoris.* This might be the first antimicrobial peptide approved for the market in the future.

Although not yet approved for medical use, many products can be found on the market for research purposes. GenScript (Piscataway, NJ, USA) provides recombinant cytokines and growth factors, such as human HSA-IFN-Alpha 2b, human Stem Cell Factor SCF, murine TNF-α and ovine IFN-τ, to name just a few examples. Recombinant human angiostatin can be found for instance in the reagents offered by Sigma-Aldrich (St. Louis, MO, USA).

Future perspectives — outlook

Successful expression of many industrial enzymes as well as pharmaceutically relevant proteins has rendered the methylotrophic yeast *P. pastoris* one of the most suitable and powerful protein production host systems. It is also an emerging host for the expression of membrane proteins (Hirz et al. 2013) and of small bioactive and antimicrobial peptides, which could be a forthcoming alternative to chemical synthesis (Zhang et al. 2014). Although many basic elements of this expression system are now well developed and one can make use of a broad variety of vectors and host strains, there is still space for further optimization of protein expression and secretion, which, in many cases, will be highly dependent on the desired product. One general interest is to find effective alternatives for induction to replace methanol for industrial scale fermentations (Delic et al. 2013; Prielhofer et al. 2013; Stadlmayr et al. 2010).

Improving protein secretion performance is one of the first and foremost goals for engineering *P. pastoris*. There is still potential to increase yields, for example, by employing different secretion signals (Vadhana et al. 2013) or mutating *S. cerevisiae* α-MF (Lin-Cereghino et al. 2013). In contrast to the well-studied secretory pathway of *S. cerevisiae*, *P. pastoris* still is a black box regarding factors influencing secretion efficiency. Current studies try to identify these factors by mutagenesis approaches and screening for enhanced secretion of reporter proteins (Larsen et al. 2013; C. Winkler and H. Pichler, unpublished results). The well-developed tools for strain engineering, including marker-free integration and deletion of desired genes, will provide a powerful set of engineered designer host strains in the near future. These will provide optimized cell factories by fine-tuned co-expression of important homologous or heterologous protein functions needed for efficient and accurate functional expression, secretion and post-translational modification of proteins. Moreover, knockout or knockdown of undesired functions such as proteolytic decay will increase product quality and process performance. Considering the scope of this review on heterologous protein expression, it was not feasible to address all possible applications for *P. pastoris* as production organism, such as metabolic engineering for production of small molecules and metabolites, or for whole-cell biocatalysis. However, developments in these fields may also be relevant for constructing improved host strains dedicated for protein production. There are several recent reviews and research articles describing advances in these fields in detail (Abad et al. 2010; Araya-Garay et al. 2012; Wriessnegger and Pichler 2013).

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Chapter 2: Engineering of Antimicrobial Peptide Secretion in *P. pastoris*

Secretion of Antimicrobial Peptides Plectasin and Protegrin in *Pichia pastoris:* Effects of co-expressing *Saccharomyces cerevisiae KEX2* and *KEX1* on Pro-peptide Processing

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Analyzed the data: MH and CO

Wrote the manuscript: MH

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Abstract

Pichia pastoris is one of the most important hosts for heterologous protein production and secretion, which is highly relevant due to facilitating downstream processing. Still, there are multiple bottlenecks in the secretory transport of recombinant proteins which may be alleviated by host cell engineering to improve yields. This study elaborates on the co-expression of processing enzymes of the protein secretory pathway to improve secretion of the antimicrobial peptides (AMP) plectasin and protegrin. For targeting these peptides to the secretory pathway, we used the commonly applied *Saccharomyces cerevisiae* α-factor signal sequence. In its natural host, *S. cerevisiae* mating factor α (MFα1) is organized in four tandem repeats and its processing is catalyzed by the proteases Kex2p, Kex1p and Ste13p. This setup is very efficient for the secretion of four identical copies of the mature α-factor from a single translation product. To imitate this natural setup, expression cassettes with four repeats of AMP separated by Kex2p cleavage sites KR were designed. Single AMP copy constructs were constructed as control, representing standard expression conditions. We tested functional expression and secretion of AMPs via agar diffusion assays of culture supernatants showing antimicrobial activity against *Bacillus subtilis*. Expression of 4xPlectasin was achieved after co-expression of *KEX2* and could be further enhanced by co-expression of *KEX1* by 20% on the average. Furthermore, *KEX2* activity also increased functional secretion of plectasin from the single copy constructs with an approximately 2-fold increase in total secreted plectasin. However, the same strategy was not functional for protegrin expression in any tested setup. The arrangement of four tandemly arrayed peptide sequences can therefore not be recommended as a universal strategy for high-level expression of small proteins and peptides. Functional co-expression of *S. cerevisiae KEX2* and *KEX1* proteases, however, was achieved in *P. pastoris* and promoted secretion of plectasin in two different genetic setups.

23 # Introduction

24 *P. pastoris* (syn. *Komagataella phaffii*) is an industrially important yeast that is used for secreting high
25 levels of heterologous proteins to the culture supernatant [1]. Protein titers therein can be up to 18 g/L,
26 as was shown for recombinant cellulase [2]. This feature renders the methylotrophic yeast extremely
27 interesting for basic and applied research. Many proteins are not secreted to such high titers, though.
28 Therefore, eliminating bottlenecks in the secretory pathway to enhance recombinant protein secretion is
29 a stated research goal. Engineering strategies comprise modifying the gene copy number [reviewed in
30 3], varying promoter strengths [2], overexpressing transcription factors [4] or folding helpers [5],
31 knocking out proteins related to cell wall assembly [6] or optimizing cultivation conditions [7]. One
32 potential bottleneck is the use of a proper signal peptide triggering protein secretion. In most of the
33 cases, the *S. cerevisiae* mating factor α pre-pro signal sequence is successfully used to target proteins
34 and peptides to the secretory pathway. Several studies report on optimization of this signal sequence,
35 for example by codon optimization [8], site-directed mutagenesis of the pre-pro region [9] or by
36 engineering of the Kex2 P1'site [10].

37 Here, we present two novel strategies to increase secretion yields of pharmaceutically relevant
38 antimicrobial peptides (AMP) in *P. pastoris*. First, we designed expression cassettes containing four
39 repeats encoding peptide sequences (4xAMP). Mimicking the natural gene setup of the *S. cerevisiae*
40 mating factor α, *MFα1,* the different peptide entities were to be separated by Kex2p cleavage at
41 intermittent basic residues (KR) [11] (Fig 1). The natural, pre-mature α-factor peptides are N-terminally
42 decorated by two or three Gln/Asn-Ala repeats, which are subsequently processed by dipeptidyl-
43 aminopeptidase A (*STE13*) [reviewed in 12]. Secondly, we co-expressed *S. cerevisiae* Kex2 and Kex1
44 peptidases to examine possible improvements of proteolytic processing of the AMP repeats to their
45 mature form. *P. pastoris* possesses an endogenous Kex2 protease. We speculated that the endogenous
46 activity might not be sufficient for trimming heterologous proteins and, thus, the proteolytic processing
47 capacity could be a potential bottleneck for recombinant peptide secretion. In *S. cerevisiae*, Kex2p
48 endopeptidase cleaves the α-factor pro-region at the dibasic processing sites KR or RR [11,13]. Kex1p
49 is a serine carboxypeptidase and cleaves off the C-terminal KR residues [14,15]. Additionally, Ste13p
50 is necessary to trim repetitive glutamic/aspartic acid and alanine residues, which are present in the
51 natural setup (Fig 1) [16]. This arrangement drives the formation of four identical peptide copies from
52 a single transcript. This system appears to be very efficient in its natural host and we intended to
53 investigate if this design can be exploited for heterologous peptide expression and secretion to improve
54 yields.

55 We aimed for expression of AMPs to test this strategy, because these peptides are of similar size as
56 mating factor α of *S. cerevisiae*. AMP activity can be easily detected via agar diffusion assays in culture
57 supernatants. The number of antibiotic-resistant bacteria is on the rise and alternatives to conventional

antibiotics are strongly desired. Generally, AMPs are amphipatic and cationic molecules present in many organisms as essential defense elements of the innate immune system. Most AMPs target bacterial cells by binding their negatively charged cytoplasmic membranes, leading to pore formation and rapid cell disruption. Some AMPs also have additional intracellular targets or different modes of action [reviewed in 17,18].

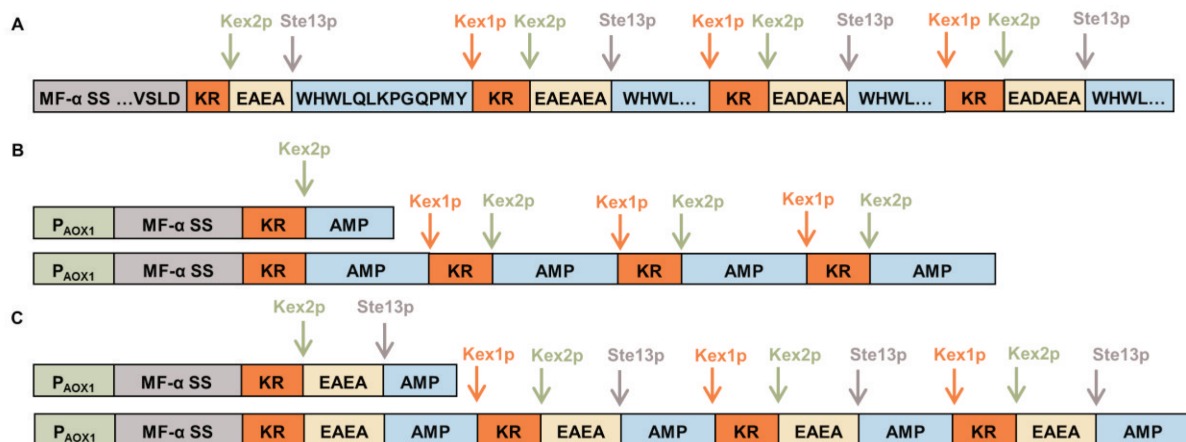


Fig 1. Strategy of AMP expression in tandem repeats. (A) The natural setup of *S. cerevisiae* mating factor α *MFα1* consists of 4 tandemly-arrayed α-factor peptide sequences, each separated by lysine and arginine (KR) residues as well as glutamic/aspartic acid and alanine (E/DA) residues. Kex1p, Kex2p, and Ste13p protease cleavage sites are indicated by arrows. (B) The AMP expression cassettes consist of the inducible *AOX1* promoter, the *S. cerevisiae* mating factor α secretion signal MF-α SS and one (1xAMP) or four copies (4xAMP) of plectasin or protegrin. The 4xAMP expression cassette contains four tandemly-arrayed sequences of plectasin or protegrin, separated by KR residues. (C) Arrangement of AMP expression cassettes with glutamic acid and alanine repeats (EA) between the Kex2p cleavage sites and the peptide sequences.

We chose two promising candidates for expression in *P. pastoris*: Plectasin, a fungal peptide discovered in 2005, shows strong antimicrobial activity against methicillin-resistant *Staphylococcus aureus*, rendering this peptide a promising therapeutic compound [19]. Five years after its discovery, a study was published describing that it acts by binding the bacterial cell wall precursor Lipid II rather than disrupting the membrane itself. Thereby, plectasin efficiently inhibits correct cell wall biosynthesis [20]. Protegrin-1 is found in porcine leukocytes and directly targets the bacterial membrane by pore formation, resulting in broad-spectrum antimicrobial activity [21–23]. In total, five protegrin peptides have been identified in *Sus scrofa*, which contain two disulfide bridges and show high sequence homology [24].

Plectasin and protegrin are both active against Gram-positive bacteria, allowing the use of *B. subtilis* as indicator strain in antimicrobial assays. We monitored AMP secretion and activity by agar diffusion assays against *B. subtilis* and this assay furthermore allowed for screening of positive or negative effects

of *KEX2/KEX1* co-expression. We checked for peptide levels in the culture supernatants using SDS-PAGE and confirmed *KEX2* co-expression via immunodetection of the C-terminal Flag-tag. We further quantified secreted plectasin by densitometry and by relative halo diameters in agar diffusion assays.

Our results showed that initially the 4xPlec setup did not result in any functional secretion, whereas the 1xPlec control showed good secretion levels. Protegrin secretion was achieved from the single copy construct (1xProt), but to a lesser extent. This observation indicated a limitation in the proteolytic processing capacity for the 4xAMP setup. Therefore, *KEX2* from *S. cerevisiae* was co-expressed in the plectasin and protegrin expression strains. The constitutive co-expression of *KEX2* led to an approximately 2-fold increased secretion of 1xPlec and even enabled secretion from the 4xPlec cassette. Co-expression of *KEX1* enhanced secretion from the 4xPlectasin construct by further 20%. We also tested plectasin constructs with EA repeats between the Kex2p cleavage sites, but we could not detect production of plectasin in this setup at all.

The secretion of protegrin-1 was abolished after introducing *KEX2* co-expression, obviously due to the arginine residues in protegrin, which can also be cleaved by Kex2p. Consequently, we mutated the Arg^{10} residue to proline to obtain the active isoform protegrin-5. Unfortunately, we also saw a loss of functional secretion after *KEX2* co-expression.

Collectively, our results show that the secretion of plectasin can be further improved by co-expression of secretory pathway processing enzymes Kex2 and Kex1 from *S. cerevisiae*. Nevertheless, this strategy did not apply for another AMP, protegrin. Improved AMP secretion was not achieved by the expression of tandemly arrayed peptide sequences, but by co-expression of two proteases of the secretory pathway, Kex2p and Kex1p, which significantly increased the functional secretion of plectasin in *P. pastoris*. This effect was, however, not observed for protegrin. Further peptide sequences need to be tested case by case.

108 # Materials and Methods

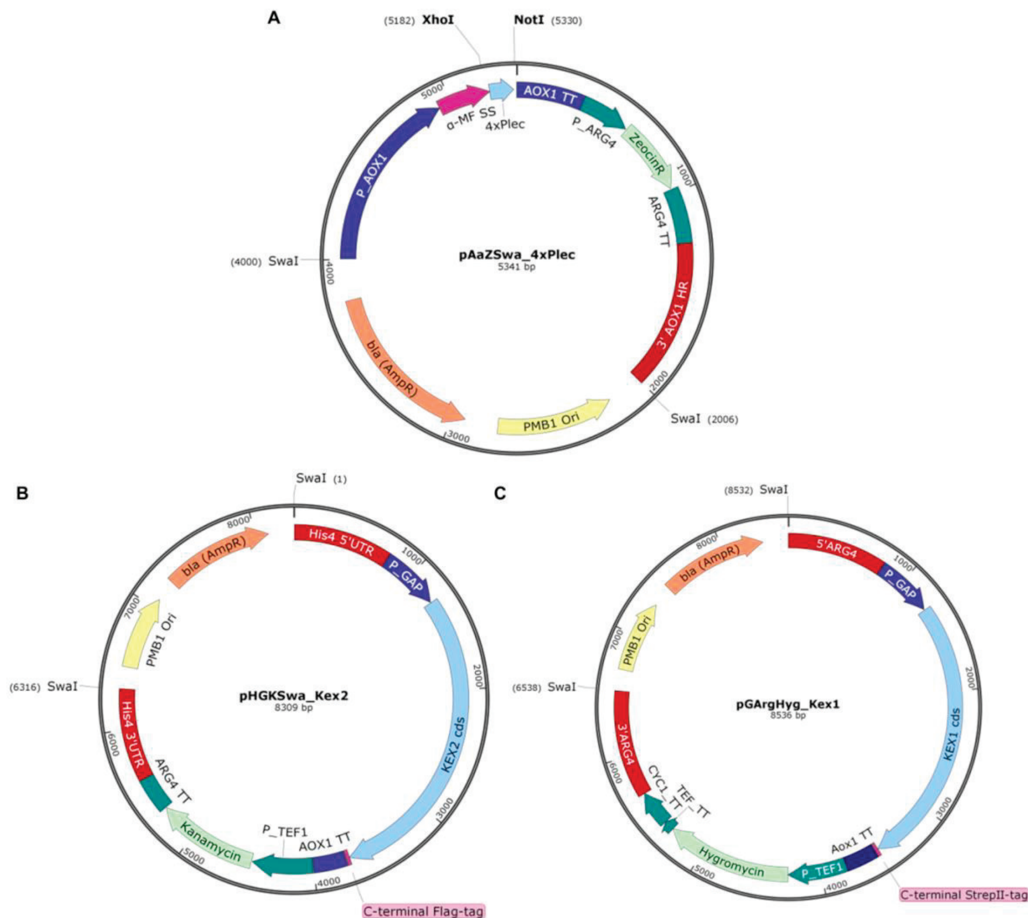
109 ## Media, Reagents and Cultivation Conditions

110 Unless otherwise stated, all chemicals and reagents were purchased from Carl Roth GmbH & Co. KG
111 (Karlsruhe, Germany) or Sigma-Aldrich (St. Louis, MO, USA). For propagation of plasmids in *E. coli*,
112 Luria Bertani (LB) solid media (1% peptone, 0.5% yeast extract, 1% NaCl, 2% agar) with 100 mg/l
113 ampicillin was used. *P. pastoris* transformants were selected on YPD with antibiotics (1% yeast extract,
114 2% peptone, 2% glucose, 2% agar, 300 mg/l geneticin sulfate, 300 mg/l hygromycin, or 100 mg/l
115 zeocin™). Hygromycin was purchased from Formedium (Norfolk, UK) and zeocin™ from Invivogen
116 (San Diego, CA, USA). Minimal dextrose (MD) plates (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 2%
117 dextrose, and 1.5% agar) and MD plates supplemented with 0.04% histidine or 0.01% arginine were
118 used to screen for *Δhis4*/*Δarg4* transformants containing *KEX2*/*KEX1* co-expression cassettes in the
119 desired loci, respectively. For AMP expression, *P. pastoris* cells were inoculated to an OD_{600} of 0.1 and
120 pre-grown at 28 °C in 25 ml buffered glycerol-complex medium (BMGY, 1% yeast extract, 2% peptone,
121 0.1 M phosphate buffer, pH 6, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol) for 36 h at 120 rpm. This
122 growth phase was then followed by induction with 25 ml of buffered methanol-complex medium
123 (BMMY) containing 1% of methanol instead of glycerol. Methanol was added twice a day to maintain
124 1% final concentration. Protein expression was carried out in 300 ml baffled shake flasks for up to 120
125 h at 28 °C. A slightly modified nutrient agar (0.5% peptone, 0.3% yeast extract, 0.8% agarose) was used
126 for testing the culture supernatants for antimicrobial activity.

127 ## Strain Construction and Cloning

128 ### Construction of AMP Expression Strains

129 The codon-optimized, synthetic genes for expression of plectasin
130 (GFGCNGPWDEDDMQCHNHCKSIKGYKGGYCAKGGFVCKCY) and protegrin-1
131 (RGGRLCYCRRRFCVCVGR) were designed in 4 tandem repeats as shown in Fig 1 and ordered from
132 Invitrogen GeneArt (Thermo Fisher Scientific GeneArt, Regensburg, Germany). *E. coli* TOP10F' was
133 used for cloning and propagation of plasmids (Life Technologies, Carlsbad, CA, USA). The sequences
134 containing four tandem repeats of plectasin or protegrin (4xPlec, 4xProt) were sub-cloned into the
135 previously constructed *P. pastoris* expression vector pAaZSwa [1] using *Xho*I/*Not*I (Fig 2A).



136

137 **Fig 2. Maps of plasmids constructed for AMP expression and Co-expression of *KEX2/KEX1*.** (A)
138 Plasmid pAaZSwa was used for cloning of 1xAMP and 4xAMP constructs, with and without EA repeats.
139 (B) Plasmid pHGKSwa was used for co-expression of *S. cerevisiae KEX2*. (C) Plasmid pGArgHyg was
140 used for co-expression of *S. cerevisiae KEX1*.

141 To obtain the constructs harboring a single AMP copy (1xPlec, 1xProt), sequences were PCR amplified
142 from pAaZSwa_4xPlec and pAaZSwa_4xProt using the primers 1-4 listed in Table S1. *Phusion* High-
143 Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., St. Leon-Rot, Germany) was used according
144 to the recommended protocol. For the plectasin constructs including EA repeats, a new codon-
145 optimized, synthetic gene 4xPlecEA was ordered from Invitrogen GeneArt (Thermo Fisher Scientific
146 GeneArt, Regensburg, Germany) and sub-cloned into pAaZSwa. To obtain the 1xPlecEA plasmid, the
147 respective sequence was PCR amplified using primers 5 and 6 listed in Table S1. All constructs were
148 confirmed by sequencing (LGC Genomics, Berlin, Germany).

149 *P. pastoris* CBS7435 was used for transformation of expression constructs. Transformation of *Swa*I-
150 linearized plasmids was carried out using a condensed protocol as described by Lin-Cereghino et al.
151 [25]. Transformants were selected on YPD + zeocin^{TM} (100 mg/l).

152

153 ## Mutagenesis of Protegrin R10P

154 The protegrin R10P variant was constructed by site-directed mutagenesis as described in the
155 QuikChange® Instruction Manual (Agilent Technologies, Santa Clara, CA). Q5® High-Fidelity DNA
156 Polymerase from New England Biolabs, Inc. (Ipswich, MA, USA) was used for amplification. The used
157 primer sequences 10 and 11 are listed in S1 Table. The PCR cycling conditions were: 98°C (30 s) –
158 [98°C (10 s) – 65°C (30 s) – 72°C (6 min)] x 35 – 72°C (2 min) – 4°C ∞. After 2 h of *Dpn*I digestion,
159 the plasmid was transformed into *E. coli* TOP10F' cells for amplification. Correct nucleotide exchange
160 was confirmed by sequencing. The *Swa*I linearized plasmid was transformed into *P. pastoris* CBS7435
161 and clones were selected on YPD + Zeocin™ (100 mg/l).

162 ## Cloning and Co-expression of *S. cerevisiae KEX2* and *KEX1*

163 Genomic DNA of *S. cerevisiae* CEN.PK2-1D (Euroscarf, Germany) was isolated [26] and used as
164 template for PCR amplification of sequences encoding Flag-tagged Kex2 and StrepII-tagged Kex1
165 sequences (primers 24, 25 and 42, 43 in Table S1). For *KEX2* co-expression, the vector pHGKSwa was
166 constructed (Fig 2B) via Gibson Assembly [27] using primers 12-15 (see Table S1). This vector is based
167 on the pHAKSwa and pGaHSwa vector series developed in our house [1] and carries a KanMX cassette,
168 conferring resistance to geneticin sulphate (G418). The *AOX1* promoter was replaced by the *GAP*
169 promoter. Furthermore, the *ARG4* promoter in front of the kanamycin resistance gene was exchanged
170 by the *TEF1* promoter from the plasmid pPpHyg [28] via *Bam*HI/*Nde*I cloning (see primers 16 and 17
171 in Table S1). This step optimized the screening for positive transformants, which were selected on YPD
172 + G418 (300 mg/l). Final assembly of the *KEX2* co-expression plasmid was done by Gibson Cloning
173 [27] using primers 18-23 (Table S1). To generate control strains with the same genetic *Δhis4*
174 background, the empty pHGKSwa plasmid, created using primers 26 and 27 (Table S1) was also
175 transformed. For cassette integration into the *HIS4* locus, the plasmid harbored 5'- and 3'-flanking
176 regions. This strategy was used to rule out any locus-specific effects. Transformants having the correct
177 integration locus were unable to grow on MD plates without histidine.

178 The plasmid pHGKSwa served as template for generation of the *KEX1* co-expression plasmid
179 pGArgHyg via Gibson Assembly [27]. The kanamycin resistance gene was replaced by the hygromycin
180 resistance marker from the plasmid pPpHyg [28] and the *HIS4* flanking sites were replaced by 5' and
181 3'*ARG4* homologous regions amplified from the *P. pastoris* CBS7435 genome using primer pairs 30,
182 31 and 38-41 listed in Table S1. This enabled us to screen for correct integration events by picking
183 clones unable to grow on MD plates without supplemented arginine. The empty pGArgHyg plasmid –
184 assembled using primers 26 and 27 (Table S1) – was transformed to generate control strains with the
185 same genetic *Δarg4* background. All strains constructed during this study are listed in Table 1.

186

187 **Table 1.** ***P. pastoris*** **strains used in this study.**

Name	**Description**	**Source**
WT	*P. pastoris* CBS 7435	CBS[a]
Plectasin expression strains		
1xPlec	WT *aox1*::1xPlec-Zeo^{R}	This work
1xPlec$^{[pHGK]}$	WT *Δhis4*::G418^{R} *aox1*::1xPlec-Zeo^{R}	This work
1xPlec$^{[pGArgHyg]}$	WT *Δarg4*::Hyg^{R} *aox1*::1xPlec-Zeo^{R}	This work
1xPlec$^{[pHGK, pGArgHyg]}$	WT *Δhis4*::G418^{R} *Δarg4*::Hyg^{R} *aox1*::1xPlec-Zeo^{R}	This work
1xPlec_Kex2	WT *Δhis4*::G418^{R} *aox1*::1xPlec-Zeo^{R} pHGKSwa_Kex2	This work
1xPlec_Kex2$^{[pGArgHyg]}$	WT *Δhis4*::G418^{R} *Δarg4*::Hyg^{R} *aox1*::1xPlec-Zeo^{R} pHGKSwa_Kex2	This work
1xPlec_Kex1	WT *Δarg4*::Hyg^{R} *aox1*::1xPlec-Zeo^{R} pGArgHygSwa_Kex1	This work
1xPlec_Kex1$^{[pHGK]}$	WT *Δhis4*::G418^{R} *Δarg4*::Hyg^{R} *aox1*::1xPlec-Zeo^{R} pGArgHygSwa_Kex1	This work
1xPlec_Kex1Kex2	WT *Δhis4*::G418^{R} *Δarg4*::Hyg^{R} *aox1*::1xPlec-Zeo^{R} pHGKSwa_Kex2 pGArgHygSwa_Kex1	This work
4xPlec	WT *aox1*::4xPlec-Zeo^{R}	This work
4xPlec$^{[pHGK]}$	WT *Δhis4*::G418^{R} *aox1*::4xPlec-Zeo^{R}	This work
4xPlec$^{[pGArgHyg]}$	WT *Δarg4*::Hyg^{R} *aox1*::4xPlec-Zeo^{R}	This work
4xPlec$^{[pHGK, pGArgHyg]}$	WT *Δhis4*::G418^{R} *Δarg4*::Hyg^{R} *aox1*::4xPlec-Zeo^{R}	This work
4xPlec_Kex2	WT *Δhis4*::G418^{R} *aox1*::4xPlec-Zeo^{R} pHGKSwa_Kex2	This work
4xPlec_Kex2$^{[pGArgHyg]}$	WT *Δhis4*::G418^{R} *Δarg4*::Hyg^{R} *aox1*::4xPlec-Zeo^{R} pHGKSwa_Kex2	This work
4xPlec_Kex1	WT *Δarg4*::Hyg^{R} *aox1*::4xPlec-Zeo^{R} pGArgHygSwa_Kex1	This work
4xPlec_Kex1$^{[pHGK]}$	WT *Δhis4*::G418^{R} *Δarg4*::Hyg^{R} *aox1*::4xPlec-Zeo^{R} pGArgHygSwa_Kex1	This work
4xPlec_Kex1Kex2	WT *Δhis4*::G418^{R} *Δarg4*::Hyg^{R} Zeo^{R} pHGKSwa_Kex2 pGArgHygSwa_Kex1	This work
1xPlecEA	WT *aox1*::1xPlecEA-Zeo^{R}	This work
1xPlecEA$^{[pHGK]}$	WT *Δhis4*::G418^{R} *aox1*::1xPlecEA-Zeo^{R}	This work
1xPlecEA_Kex2	WT *Δhis4*::G418^{R} *aox1*::1xPlecEA-Zeo^{R} pHGKSwa_Kex2	This work
4xPlecEA	WT *aox1*::4xPlecEA-Zeo^{R}	This work
4xPlecEA$^{[pHGK]}$	WT *Δhis4*::G418^{R} *aox1*::4xPlecEA-Zeo^{R}	This work
4xPlecEA_Kex2	WT *Δhis4*::G418^{R} *aox1*::4xPlecEA-Zeo^{R} pHGKSwa_Kex2	This work
Protegrin expression strains		
1xProt	WT *aox1*::1xProt-Zeo^{R}	This work
1xProt$^{[pHGK]}$	WT *Δhis4*::G418^{R} *aox1*::1xProt-Zeo^{R}	This work
1xProt_Kex2	WT *Δhis4*::G418^{R} *aox1*::1xProt-Zeo^{R} pHGKSwa_Kex2	This work
1xProt^{R10P}	WT *aox1*::1xProt^{R10P}-Zeo^{R}	This work
1xProt$^{R10P\ [pHGK]}$	WT *Δhis4*::G418^{R} *aox1*::1xProt^{R10P}-Zeo^{R}	This work
1xProt^{R10P}_Kex2	WT *Δhis4*::G418^{R} *aox1*::1xProt^{R10P}-Zeo^{R} pHGKSwa_Kex2	This work
4xProt	WT *aox1*::4xProt-Zeo^{R}	This work
4xProt$^{[pHGK]}$	WT *Δhis4*::G418^{R} 7435 *aox1*::4xProt- Zeo^{R}	This work
4xProt_Kex2	WT *Δhis4*::G418^{R} *aox1*::4xProt-Zeo^{R} pHGKSwa_Kex2	This work

188 [a]Centraalbureau voor Schimmelcultures

189

Expression of AMPs and Agar Diffusion Assay

Transformants were checked for correct integration of sequences by colony PCR (primers 7-9 in Table S1) and four clones for each construct were cultivated as described above. After 8, 24, 48, 72, 96 and 120 h, one ml aliquots were collected and cells were separated by centrifugation. Supernatants and cell pellets were frozen at -20°C until further use. Antimicrobial activity was tested via agar diffusion assays against *B. subtilis* DSM347 (DSMZ, Braunschweig, Germany). For this purpose, 200 µl of *B. subtilis* over-night cultures grown at 37°C (ranging from 5 to 5.7 x 10^8 cfu/ml) were added to 50 ml of nutrient agar at around 37°C to obtain a final cell density of approximately 2.5 x 10^6 cfu/ml. The agar containing *B. subtilis* was poured into sterile Nunc OmniTrays™ (Thermo Fisher Scientific Inc., St. Leon-Rot, Germany). After solidification, holes of 0.55 cm diameter were punched into the agar with sterile Pasteur pipettes. One hundred µl of culture supernatants were applied to the holes and plates were incubated at 37°C over night. Halos of growth inhibition were imaged the next day using G:Box HR16 BioImaging system (Syngene, Cambridge, UK). Halo diameters were measured and relative sizes to the reference strains within the same plate were calculated to eliminate plate-to-plate variations. Statistical analysis was performed using an unpaired, two-tailed Student's *t*-test. Strains showing antimicrobial activity in initial tests were further used for co-expression of *S. cerevisiae* Kex2 and Kex1.

SDS-PAGE and Immunoblotting

For the analysis of secreted peptides by SDS-PAGE, protein concentrations were determined with the Bio-Rad Protein Assay, based on the method of Bradford [29]. Thirty µg of protein were precipitated from culture supernatants with MeOH/$CHCl_3$ according to Wessel and Flügge [29] and resuspended in 1xLDS NuPAGE® sample buffer (Thermo Fisher Scientific Inc., St. Leon-Rot, Germany). After a denaturing step at 95°C for 10 min, samples were separated on a 4-12% Bis-Tris NuPAGE® Gel using MES buffer (50 mM MES, 50 mM Tris Base 0.1% SDS, 1 mM EDTA, pH 7.3). Gels were stained with Coomassie brilliant blue R-250. For densitometric evaluation of protein amounts, band intensities were determined using Gene Tools 4.0 Software from SynGene (Frederick, MD, USA). For detection of Flag-tagged Kex2p and StrepII-tagged Kex1p via Western blotting, cell pellets from 1 ml samples were collected and resuspended in 200 µl of ice-cold breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol, 1 mM PMSF). Acid-washed glass beads (0.25–0.5 mm diameter, Carl Roth, Karlsruhe, Germany) were added and cells were disrupted by eight subsequent 30 s vortexing and cooling steps on ice. Glass beads and cell debris were removed by centrifugation at 3,000 × g and 4°C for 5 min, and the total cell lysate was stored at -20 °C until further use. Protein concentration was determined by the Bio-Rad Protein Assay, based on method of Bradford [30]. After TCA precipitation, 20 µg of protein was dissolved in 1xLDS NuPAGE® sample buffer (Thermo Fisher Scientific Inc., St. Leon-Rot, Germany) and separated on 12.5% SDS-PAGE gels following standard procedures [31]. Western Blot analysis was performed according to Haid and Suissa [32]. Primary mouse antibodies

225 against Flag-tag (Sigma-Aldrich, St. Louis, MO, USA) and StrepII-tag (IBA, Göttingen, Germany) were
226 diluted 1:1000. Secondary peroxidase-conjugated rabbit anti-mouse IgG was purchased from Sigma-
227 Aldrich (St. Louis, MO) and diluted 1:10 000. Immunoreactive bands were visualized with the
228 SuperSignal® West Pico Chemiluminescent substrate (Thermo Fisher Scientific Inc., St. Leon-Rot,
229 Germany) using the G:Box HR16 BioImaging system (Syngene, Cambridge, UK).

Results

Plectasin and Protegrin are Secreted in *P. pastoris* under Standard Conditions

The heterologous expression of plectasin and protegrin was initially tested under standard conditions to set up and adapt the agar diffusion assay for clone screening. In our standard conditions, the expression cassette was constructed by fusing the single coding sequences to the *S. cerevisiae* mating factor α signal sequence without EA repeats. Peptide expression was driven from the methanol-inducible *AOX1* promoter. For monitoring of peptide secretion, we adapted a simple agar diffusion assay in which the growth of *B. subtilis* DSM347 is inhibited and halos are formed, if the peptide is secreted and active against the tester strain. This assay allowed us to test the *P. pastoris* culture supernatants for antimicrobial activity and gave direct information about peptide titers via the halo size. After 96-120 h of methanol induction, we observed moderate amounts of secreted protegrin and its mutated R10P variant with halo diameters ranging between 79-105 mm (Table 2, Fig S1 panel C) and high amounts of plectasin with halo diameters above 190 mm (Table 2, Fig 3A and B). In these shake flask cultivation experiments, cell growth was not impaired, meaning that expression of these two peptides was feasible in *P. pastoris* and did not exert (membrane) stress onto the cells.

Table 2. Summary of halo diameters determined in antimicrobial activity assays.

	Halo diameter range (mm)		Halo size relative to reference strain	
Strain	**min.**	**max.**	**Mean $\pm$ s. d.[c]**	**p-value[d]**
4xPlec_Kex2[a]	95	105	1	
4xPlec_Kex2Kex1	109	129	1.20 ± 0.06	1.34×10^{-6}
1xPlec[a]	>190[b]		1	
1xPlec_Kex1	>190[b]		0.97 ± 0.01	0.02
1xPlec_Kex2	>250[b]		1.18 ± 0.05	1.92×10^{-6}
1xProt^{R10P}[a]	79	105	1	
1xProt^{R10P}_Kex2	65	94	0.79 ± 0.14c	0.19

[a] Reference strain with a halo diameter set to 1.0

[b] Undiluted supernatants could not be measured more accurately due to assay limitations

[c] Mean values ± standard deviation were determined for 4xPlec_Kex2Kex1 (n = 11), 1xPlec_Kex1 (n = 5), 1xPlec_Kex2 (n = 10), and for 1xProt^{R10P}_Kex2 (n = 3), where n is the number of independent clones tested

[d] Statistical significance of the difference in halo diameters of co-expression strains compared to reference strains tested by an unpaired, two-tailed Student's *t*-test.

KEX1 and *KEX2* Co-expression assist Plectasin Processing and Secretion

We were successful in expressing the 1xAMP constructs, but for the 4xAMP constructs no inhibition zones were detectable in any tested setup (Fig 3E and Fig S1 panels B & C). One potential tailback is the proper post-translational processing of the nascent peptide chain. Therefore, we applied a strategy to foster proteolytic processing by the co-expression of *S. cerevisiae* Kex2 endopeptidase and Kex1 carboxypeptidase. 4xAMP strains were transformed with *KEX2* and *KEX1* co-expression plasmids (Fig 2) and 1xAMP expression strains transformed with the empty pHGK and pGArgHyg plasmids served as controls.

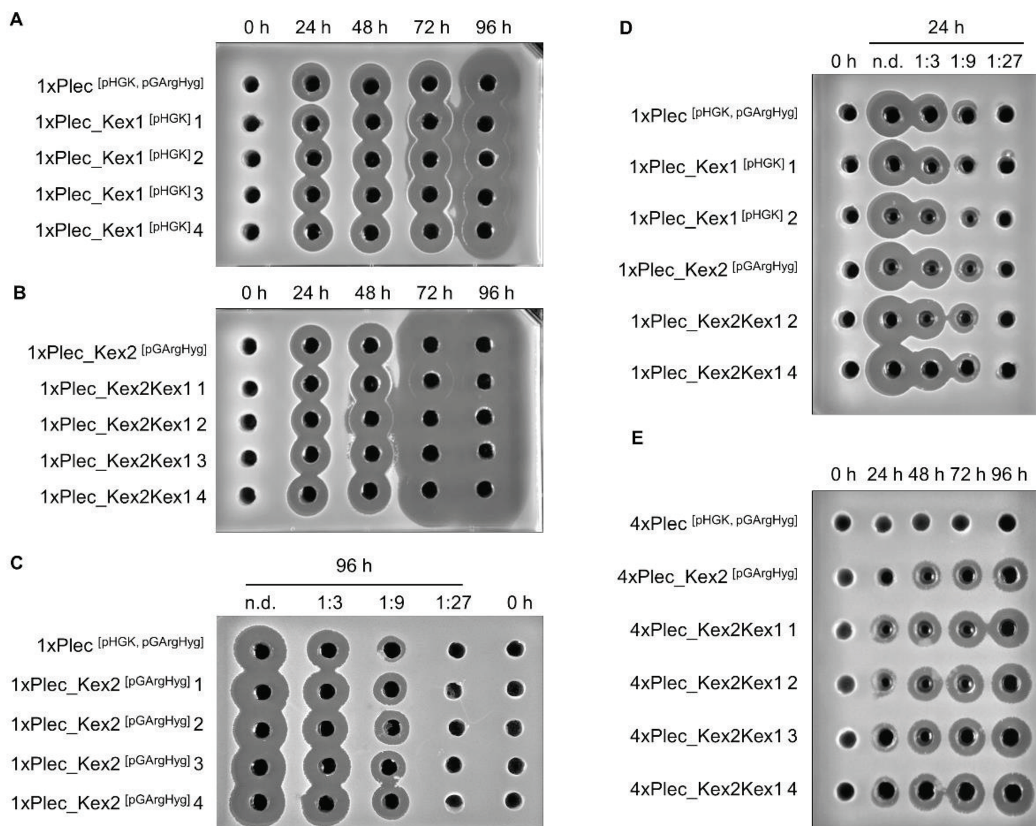


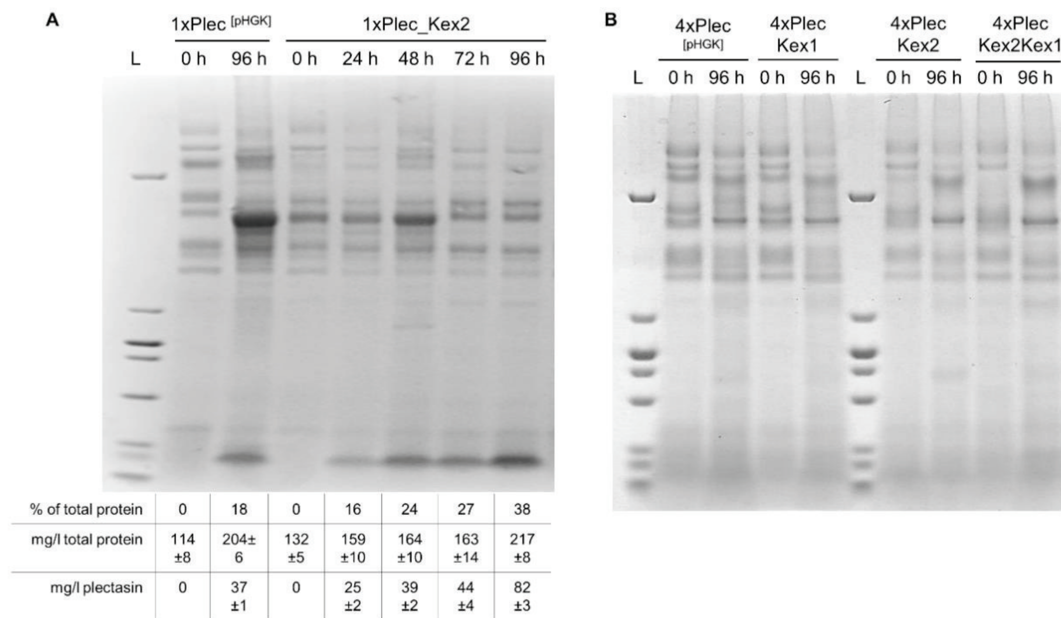
Fig 3. Antimicrobial activity assay of plectasin secreting strains. Plectasin secretion from 1xPlec strains over 96 h of methanol induction, (A) with and without *KEX1* co-expression, and (B) with both *KEX1* and *KEX2* co-expression. (C) 1xPlec strains with and without *KEX2* co-expression after 96 h of methanol induction. For better visibility of different halo diameters, supernatants were diluted as indicated and only 50 µl were applied. n. d. = not diluted. Four representative clones of each co-expression strain are shown. (D) Plectasin secretion with and without *KEX1* and *KEX2* co-expression after 24 h of methanol induction. For better visibility of different halo diameters, supernatants were

272 diluted and only 50 µl were applied. n. d. = not diluted. Two representative clones of each co-expression
273 strain are shown. (E) Plectasin secretion from 4xPlec strains with and without *KEX1* and *KEX2* co-
274 expression over 96 h of methanol induction. Samples from four independent clones are shown.
275 Antimicrobial activity was tested against *B. subtilis.*

276 Fig 3 demonstrates nicely that co-expression of *KEX2* in the 1xPlec strain background increased
277 antimicrobial activity, which was most likely due to increased levels of properly processed plectasin in
278 the culture supernatant. It can be seen clearly, that halo sizes were larger in all of the *KEX2* co-expression
279 strains (Fig 3B, C), as compared to the control strains without *KEX2* (Fig 3A). Samples after 24 h and
280 96 h of methanol induction were 3-fold serially diluted to be able to measure halo sizes properly. For a
281 better comparison between the co-expression conditions, samples from representative strains were
282 assayed again on the same agar plate (Fig 3D). Upon measurement of the halo sizes, we detected a
283 significant ($p < 0.05$) increase in halo sizes of averaged 18% (Table 2).

284 As expected, co-expression of heterologous *KEX1* did not have a strong impact on plectasin secretion
285 in the 1xPlec strains (Fig 3). This is in accordance to the ascribed function of cleaving carboxy-terminal
286 KR residues [15], which are not present in the 1xPlec setup. Measured halo sizes were on average 4%
287 smaller as compared to the empty control strains. The slightly decreased efficiency of peptide secretion
288 could be due to the additional burden on the cell caused by heterologous co-expression under the control
289 of the strong *GAP* promoter.

290 We quantified the amount of plectasin produced in our best 1xPlec expression strain by densitometric
291 analysis of the corresponding protein gel image after separation of 30 µg of total secretory proteins via
292 SDS-PAGE (Fig 4A). After 96 h of methanol induction, 38% of total secreted proteins consisted of
293 plectasin in the 1xPlec_Kex2 strain. This corresponded to roughly 82 mg/l plectasin produced, as
294 determined by the Bradford assay. In comparison, the control strain without *Sc*Kex2p only produced
295 37 mg/l plectasin, which corresponded to 18% of its total proteins secreted to the culture supernatant.
296 Hence, the amount of total secreted plectasin was increased around 2-fold in the *KEX2* co-expressing
297 strain.



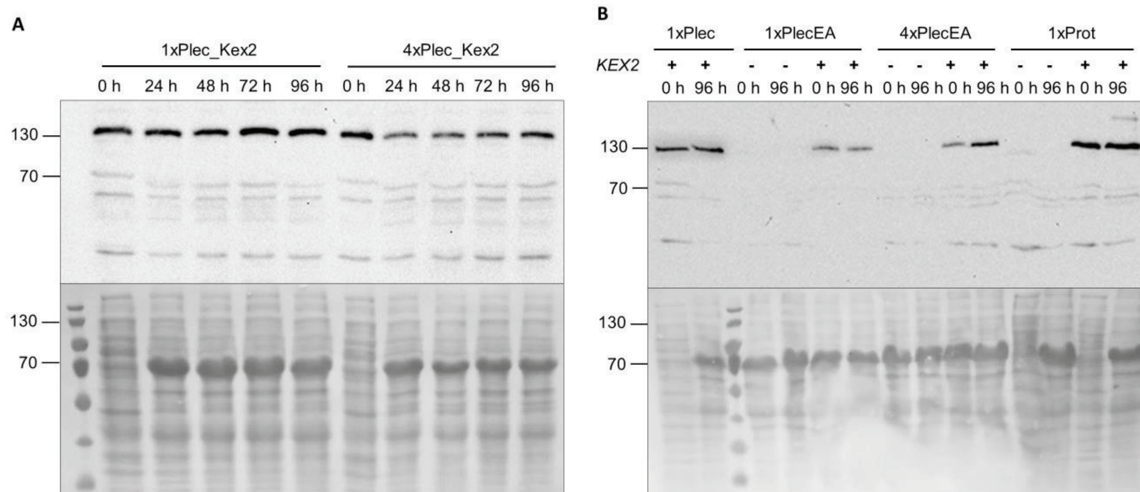
% of total protein	0	18	0	16	24	27	38
mg/l total protein	114 ±8	204± 6	132 ±5	159 ±10	164 ±10	163 ±14	217 ±8
mg/l plectasin	0	37 ±1	0	25 ±2	39 ±2	44 ±4	82 ±3

298

299 **Fig 4. SDS-PAGE of plectasin expressing strains.** (A) Thirty µg of proteins were precipitated from
300 culture supernatants of 1xPlec[pHGK] and 1xPlec_Kex2 strains and analyzed on a 4-12% Bis-Tris
301 NuPAGE® Gel using MES buffer. Percentage of total protein was quantified using Gene Tool Software
302 and total amounts were measured by the Bradford method. Mean values ± standard deviation are shown
303 for three technical replicates. (B) Thirty µg of proteins were precipitated from culture supernatants of
304 4xPlec strains with and without co-expressed *KEX2* and *KEX1* and were analyzed on a 4-12% Bis-Tris
305 NuPAGE® Gel using MES buffer. L = PageRuler^{TM} Low Range Unstained Protein Ladder (Thermo
306 Fisher Scientific Inc., St. Leon-Rot, Germany).

307 Strikingly, we not only achieved functional plectasin secretion in the 4xPlec strain by co-expression of
308 *S. cerevisiae KEX2*, but the secretion of antimicrobial activity was also further improved significantly
309 ($p < 0.05$) by 20% upon co-expression of *KEX1* (Fig 3E). Still, the overall amount of secreted plectasin
310 was too low in the 4xPlec setup to detect it via SDS-PAGE (Fig 4B). Therefore, absolute quantification
311 of protein amounts was not possible.

312 We also confirmed the presence of Flag-tagged Kex2p in the 1xPlec and 4xPlec expression strains via
313 Western Blot (Fig 5A). The predicted size of Kex2p is 90 kDa, which is smaller than the observed size
314 on the Western Blot, but its three potential N-glycosylation sites could be responsible for this apparent
315 size shift. Unfortunately, co-expression of Strep-tagged Kex1p protease could not be detected in total
316 cell lysates via Western Blot as only unspecific bands were observed (Fig S2). Since Kex1p is a Golgi-
317 resident, multispanning membrane protein, it is no surprise that detection from total cell lysates is
318 problematic. However, we did not isolate membranes for further Western blot analyses because we
319 consistently observed increasing halo sizes when *KEX1* was integrated in the 4xPlec expression strains.
320 This gives strong evidence, that the Kex1p protease is actively expressed in *P. pastoris*.



321

322 **Fig 5. Immunodetection of Kex2p in total cell lysates of plectasin and protegrin expressing strains.**
323 (A) Detection of intracellularly expressed, Flag-tagged Kex2p in 1xPlec and 4xPlec strains after 0-96 h
324 of methanol induction. (B) Detection of intracellularly expressed, Flag-tagged Kex2p in 1xPlec,
325 1xPlecEA, 4xPlecEA and 1xProt strains after 0 and 96 h of methanol induction. All strains without co-
326 expressed *KEX2* (-) harbour the pHGKSwa empty plasmid to provide an isogenic control strain.
327 L = PageRuler^{TM} Prestained Protein Ladder (Thermo Fisher Scientific Inc., St. Leon-Rot, Germany).

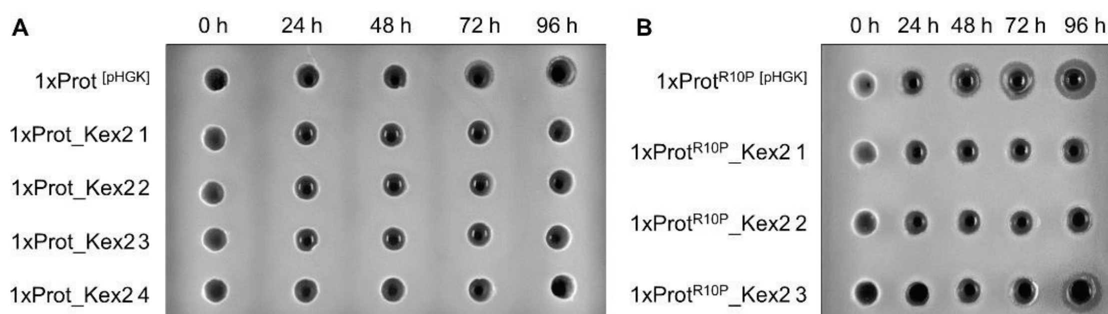
328 Additonally, the natural, untagged sequence variants of *KEX2* and *KEX1* were co-expressed to rule out
329 any negative effect of the Flag- and StrepII-tag. We could not detect any differences as all observed
330 effects on plectasin and protegrin secretion were reproducible (Fig S1). This confirmed that the tags did
331 not influence Kex2p and Kex1p activities in our strains. Also, presence of *Sc*Kex2p and *Sc*Kex1p did
332 not alter the growth behavior of the strains in shake flask cultivations. Thus, co-expression did not cause
333 major stress to the cells.

334 *KEX2* Co-expression is not beneficial for Protegrin Secretion

335 Initially, we worked with the protegrin-1 variant, which contains three subsequent arginine residues.
336 This variant was moderately secreted in the 1xProt strain (Fig 6A, uppermost lane). Co-expression of
337 *S. cerevisiae KEX2* abolished functional secretion of protegrin. Thereby, it was proven that Kex2 was
338 functionally expressed (Fig 6A), since Kex2p does not only cleave Lys-Arg (KR), but also Arg-Arg
339 (RR) residues. To test whether Kex2 co-expression can have a beneficial effect on protegrin secretion
340 as well, we constructed a variant without the putative RR cleavage sites. Therefore, the arginine 10
341 residue was exchanged for proline, thereby generating the Protegrin^{R10P} variant identical to the isoform
342 protegrin-5. This variant was also actively secreted in *P. pastoris* (Fig 6B, uppermost lane). However,
343 upon co-expression of *KEX2* in the 1xProt^{R10P} variant, we could not observe any beneficial effect on
344 secretion of protegrin to the culture supernatant. Halos from the tested strains were either smaller or
345 disappeared completely (Fig 6B). Upon measurement of the halo sizes of three independent clones, we

346 observed on average 21% smaller diameters (Table 2). The difference was, however, not significant, but
347 a positive effect of *KEX2* co-expression was ruled out.

348 To ensure that Kex2p was indeed co-expressed, we confirmed its presence by Western blotting (Fig 5B).
349 The same effect was also observed when the untagged variant of Kex2p was co-expressed (Fig S1 panel
350 C). Unfortunately, we cannot explain exactly why Kex2p co-expression was not beneficial in the case
351 of protegrin. We can only guess that the protegrin sequence itself is unfavorable for protease trimming
352 and can, possibly, be cleaved at additional positions by Kex2p. This also led us to the decision that it is
353 not worth testing any positive effects of *KEX2* and *KEX1* co-expression in the 4xProt strain.



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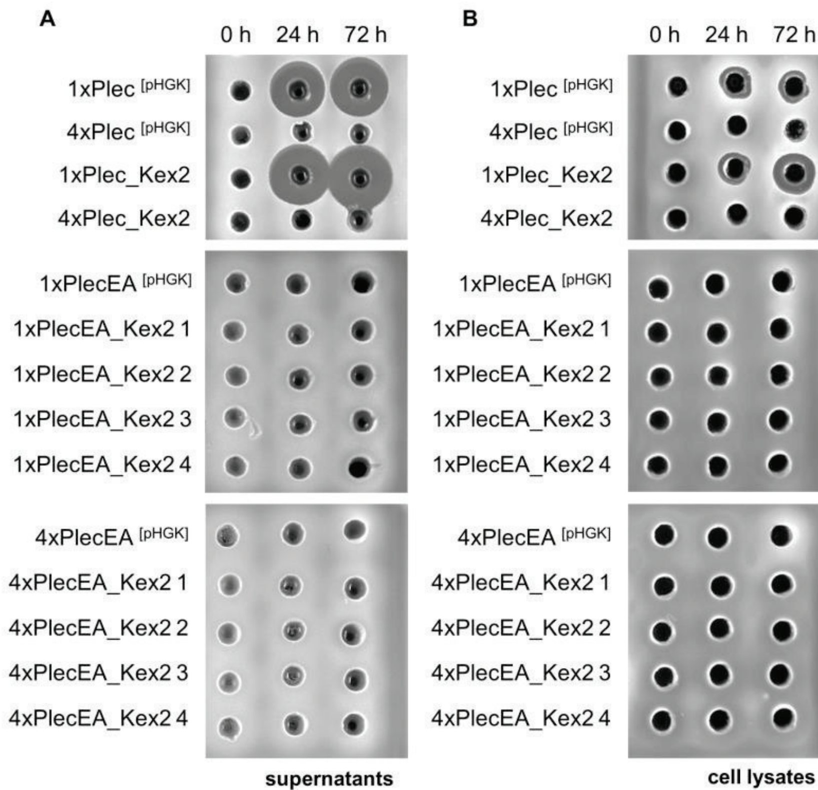
355 **Fig 6. Antimicrobial activity assay of protegrin secreting strains.** (A) Protegrin secretion from 1xProt
356 strains with and without *KEX2* co-expression over 96 h of methanol induction. Samples from four
357 independent clones are shown. (B) Protegrin secretion from 1xProt^{R10P} strains with and without *KEX2*
358 co-expression over 96 h of methanol induction. Samples from three independent clones are shown.
359 Antimicrobial activity was tested against *B. subtilis.*

360 Introduction of EA repeats is Detrimental for Plectasin Secretion

361 To test whether the presence or absence of glutamine-alanine repeats influences expression levels and/or
362 antimicrobial activity of plectasin, we ordered the synthetic, codon-optimized gene. This synthetic gene
363 consisted of 4 tandemly-arrayed plectasin sequences, connected by Lys-Arg-Glu-Ala-Glu-Ala
364 (KREAEA) sequences. Introduction of Glu-Ala repeats within the expression cassette completely
365 abolished the secretion of plectasin in the 1xPlecEA and 4xPlecEA strains, as neither activity nor protein
366 could be detected by inhibition assays or by SDS-PAGE, respectively (Fig S3). We assumed that the
367 proteolytic processing might cause problems in this setup. Consequently, we also tried co-expressing *S.*
368 *cerevisiae KEX2* in these strains, but we could neither achieve functional secretion (Fig 7A), nor see
369 any intracellularly retained activity (Fig 7B). Co-expression of *KEX2* was, however, confirmed by
370 immunodetection (Fig 5B). This result seemed puzzling, as the EA repeats are present in standard
371 commercial expression vectors. As we saw in our experiments, these amino acid repeats can be
372 detrimental for protein secretion, and it should be always worth trying both setups, with and without EA
373 repeats.



374

375 **Fig 7. Antimicrobial activity assays of plectasin secreting strains with EA repeats.** Plectasin activity
376 was monitored over 72 h of methanol induction from culture supernatants (A) or cell lysates (B) of
377 1xPlecEA and 4xPlecEA *KEX2* co-expression strains. Samples from four independent clones are shown.
378 Cell lysates were prepared as described for Western Blot sample preparation in the "Materials and
379 Methods" section. Control strains without *KEX2* and without EA repeats are shown in the respective
380 upper panels. Antimicrobial activity was tested against *B. subtilis*.

381

Discussion

In this study, we investigated novel strategies for improving the heterologous secretion of small peptides – in our case antimicrobial peptides – in *P. pastoris*. We chose AMPs because they are promising new alternatives to conventional antibiotics and are urgently needed due to fast-emerging antibiotic-resistant bacteria. In particular, we chose two model peptides, plectasin and protegrin, which are interesting candidates and were already successfully expressed in *P. pastoris*. Targeting these AMPs to the secretory pathway seemed promising, as they are also secreted in their natural hosts as defense peptides. For the design of the expression cassettes, we arranged four tandemly arrayed repeats of the AMP coding sequence after the *S. cerevisiae* mating factor α secretion signal. This 4xAMP setup resembled the arrangement of the natural *S. cerevisiae* mating factor α sequence (Fig 1). Heterologous expression was conducted under the control of the strong, inducible *AOX1* promoter. We also cloned the single sequences (1xAMP) in the same expression plasmids in order to have controls for expression levels.

In first expression experiments, we observed moderate secretion of protegrin and strong secretion of plectasin, but only in the 1xAMP setup. Neither the 4xProt, nor the 4xPlec construct allowed secretion initially. We figured that the proteolytic processing of the tandemly arrayed peptide structure might be a bottleneck cause a tailback in *P. pastoris*. Upon introduction of heterologous pro-peptide processing proteases Kex2p and Kex1p from *S. cerevisiae*, we observed several, yet, diverse effects. First of all, co-expression had no negative effect on cell growth (data not shown). This was beneficial since expression from a strong, constitutive promoter often causes problems and constitutes a burden for the cell. Secondly, positive effects could be proven for the expression of plectasin, a highly important fungal antimicrobial peptide. In both, the 1xPlec and 4xPlec setups, introduction of *Sc*Kex2p showed beneficial effects. In the 1xPlec strains, co-expression led to approximately 2-fold increased plectasin levels in the supernatants as detected by SDS-PAGE, and a significant increase in halo size of 18%, correlating the elevated antimicrobial activities to higher amounts of properly processed and secreted peptide. In the 4xPlec strain, we achieved functional peptide secretion upon co-expression of *KEX2*, which could be further enhanced significantly by 20% upon co-expression of *KEX1*. This is, to the best of our knowledge, the first report that co-expression of *S. cerevisiae* proteases leads to better processing and thereby increased secretion of this AMP.

Unfortunately, the secretion of protegrin could not be further improved by the co-expression of *S. cerevisiae* Kex2p. We did our experiments with two different protegrin isoforms, protegrin-1 having three arginine residues in its sequence, and protegrin-5, for which we mutated the arginine-10 residue of protegrin-1 to proline. For protegrin-1 we saw abolished secretion upon Kex2 co-expression, which can be explained by the ability of Kex2p to cleave RR residues as well. For protegrin-5 we expected an increase in secretion, but halo sizes also decreased. We cannot rule out additional detrimental effects, but the amino acid surrounding of the KR residues (P_3-$P_{3'}$) might play a crucial role for efficient Kex2p

417 cleavage. Manfredi et al. (2016) conducted a detailed study wherein they investigated the influence of
418 different amino acids in P_3-$P_{3'}$ positions on cleavage efficiency [33]. This work suggests that specific
419 cleavage of Kex2p is strongly dependent on the KR flanking amino acid residues. Therefore, the amino
420 acid surrounding should be closely investigated to increase chances of beneficial effects upon Kex2p
421 co-expression.

422 Additionally, the native protegrin is amidated at the Carboxy-terminus, which could outline another
423 bottleneck of heterologous expression in *P. pastoris*. There is very little data available on C-terminal α-
424 amidation of peptides expressed in yeast. However, one study shows that this post-translational
425 modification was not performed in *S. cerevisiae* when the human regulatory peptide cholecystokinin
426 was expressed [34]. Since the responsible enzyme, peptidylglycine α-amidating monooxygenase, is only
427 found in higher eukaryotes, it is not surprising that there was no homologue found in *S. cerevisiae*
428 capable of performing this amidation reaction. We speculate that also *P. pastoris* does not add a C-
429 terminal amide to secreted peptides, which can result in lowered activities [reviewed in 34]. Especially
430 for cationic peptides, neutralization of the negatively charged C-terminal carboxy group could be crucial
431 for antimicrobial activity.

432 Considering absolute yields of heterologously produced plectasin in *P. pastoris*, there is not a lot of data
433 available. One study reports about plectasin yields of around 540 mg/l obtained after 120 h of induction
434 in a 5-l fermenter [36]. However, these values cannot be directly compared to our yield of roughly
435 82 mg/l in 300 ml shake flasks after 120 h of induction due to the different cultivation conditions. It
436 would be highly interesting to test our best expression strains in high cell density bioreactor cultivations,
437 which should further increase plectasin yields.

438 Another interesting finding in our study was revealed when we investigated plectasin secretion from
439 strains harboring 1xPlec constructs with Glu/Asp-Ala (E/D-A) repeats. The native *S. cerevisiae* mating
440 factor α sequence contains at least two E/D-A repeats between the four α-factor copies, and the
441 proteolytic processing thereof works very well. Hence, it has been reported in several cases, that these
442 EA repeats facilitate, or at least do not hamper efficient secretion [37–39]. This is also why standard
443 expression vectors, such as the commercially available Invitrogen™ plasmids, still contain two to four
444 EA repeats. In our study, however, the introduction of EA repeats in the exactly same strain background
445 completely abolished secretion, which could not be restored by co-expression of Kex2p. This leads to
446 the assumption that the expression efficiency has a different bottleneck, probably on the transcriptional
447 level. It is a valuable advice to always evaluate both possibilities, with and without EA repeats, for
448 secretion of the protein of interest.

449 Of course, there are always many possibilities, where problems can arise in the secretion of heterologous
450 peptides or proteins, *e.g.* at the stages of transcription, translation or post-translational translocation into
451 the ER. Hence, it would be interesting to know more about transcript levels of the different expression

452 constructs. Obtaining deeper insights into transcriptional regulation, for example via qRT-PCR, would
453 be necessary to draw further conclusions.

454 As we have shown that *S. cerevisiae* Kex2p can be actively expressed in *P. pastoris*, its co-expression
455 might be beneficial for proteins where incomplete N-terminal cleavage is a known problem. Our strategy
456 could also be applied to other small bioactive proteins, like peptide hormones or cytokines, but a well-
457 established screening assay will be essential.

458 Conclusion

459 This study describes new concepts to improve peptide secretion in *P. pastoris.* We aimed for generally
460 applicable strategies, which were, however, not unveiled. Instead, we showed that co-expression of
461 Kex2p and Kex1p proteases from *S. cerevisiae* do influence the amount of actively secreted plectasin.
462 Unfortunately, this beneficial result could not be transferred to protegrin expression. We could still show
463 that there is an effect of Kex2p in the protegrin expressing strains, which is most likely on the level of
464 transcript processing of the amino acid chain during processing through the secretory pathway.
465 Furthermore, we showed that in our case the design of an expression construct with Glu-Ala repeats is
466 detrimental for expression. For a better understanding of maturation of protein or peptide precursors, it
467 will be necessary to study the processing enzymes in *P. pastoris* in more detail. Also, the observed
468 effects of Kex2 and Kex1 co-expression should be confirmed or disproved for additional peptides.

469

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473

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592

593 # Supporting Information

594 **Table S1**. **Primers used for this study.**

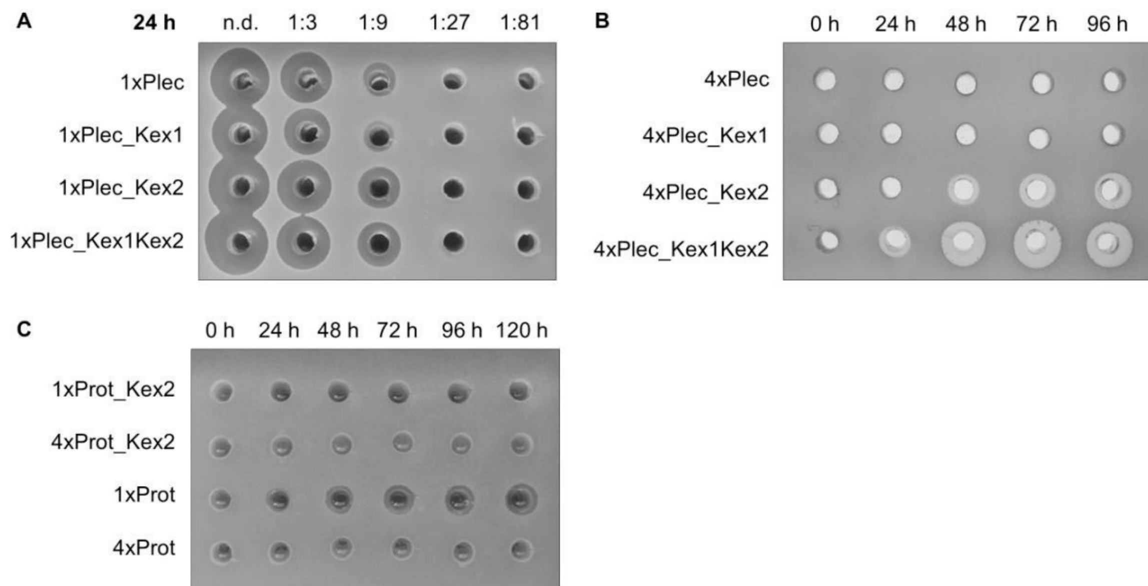
N°	Name	Sequence 5'-3'
	Primers for construction of single copy constructs	
1	Fw_1xPlecXhoI	GTAAACTCGAGAAGAGAGGTTTCGG
2	Rv_1xPlecNotI	GTTTGCGGCCGCTTAATAAC
3	Fw_1xProtXhoI	GTGTTCTCGAGAAGAGAAGAGGTGGTAGATTGTG
4	Rv_1xProtNotI	CGTTTGCGGCCGCTTATCT
5	Fw_1xPlecEAXhoI	TCTCTCGAGAAGAGAGAAGCTGAGG
6	Rv_1xPlecEANotI	TTTGCGGCCGCTTAGTAAC
	Primers for verification of *P. pastoris* transformants via colony PCR	
7	Fw_Seq	CAGTCTCTCTATCGCTTCTGAAC
8	Rv_Plect	TTAATAACATTTACAAACAAATCCAC
9	Rv_Prot	TTATCTTCCAACACAAACACAG
	Primers for site-directed mutagenesis of protegrin	
10	Fw_ProtR10P	GTAGACCAAGATTCTGTGTTTGTGTTGGAAGATAAGC
11	Rv_ProtR10P	GAATCTTGGTCTACAGTAACACAATCTACCACCTCTTC
	Primers for construction of *KEX2* co-expression plasmid	
12	Fw_pHAKSwa	ATGGCTATCCCCGAAGAG
13	Rv_pHAKSwa	TACATAGTTCGTACTCAAGTGATTGG
14	Fw_GAP	TTGAGTACGAACTATGTATTTTTGTAGAAATGTCTTGGTG
15	Rv_GAP	CTTCGGGGATAGCCATTGTGTTTTGATAGTTGTTGTTCAATT G
16	F_TEF1*Bam*HI	ATCGGGATCCCACACACCAT
17	R_TEF1*Nde*I	AATTCATATGGGTTTAGTTCCTCACCTTGTC
18	F_pHGK1	CCGGCTCCAGATTTATCAGC
19	R_pHGK1	TGTGTTTTGATAGTTGTTCAATTGATTG
20	F_pHGK2	TGAAAATACCCATCTTTCACAGAA
21	R_pHGK2	TACTTACTCTAGCTTCCCGGCA
22	F_pHGK3	TCAAGAGGATGTCAGAATGCC
23	R_pHGK3	GTTCGGTCTTCTGTTCGTCG
24	Fw_Kex2Flag	CACACTCGAGATGAAAGTGAGGAAATATATTACT
25	Rv_Kex2Flag	GAGAGCTCTTATTTATCGTCATCGTCTTTATAATCCGATCGT CCGGAAGA
26	Fw_pHGKTef1EVC3[a]	AAAACACACTCGAGGAGCTCTCAAGAGGATGTCAGAATG
27	Rv_pHGKTef1EVC1[a]	CTTGAGAGCTCCTCGAGTGTGTTTTGATAGTTGTTC
28	F_ScKex2	TTGAACAACTATCAAAACACAATGAAAGTGAGGAAATATAT TACT
29	R_ScKex2	CTGACATCCTCTTGATTACGATCGTCCGGAAG
	Primers for construction of *KEX1* co-expression plasmid	
30	Fw_HPH	ACGTTCGTTTGTGCCACACACCATAGCTTCAA
31	Rv_HPH	CACGTTCTTAAACTCTCCACCTAGGGTACCTTGCTCACAT
32	Fw_OriAmp	CATCTTTGTTGCGGTATTTAAATTCACTGACTCGC
33	Rv_OriAmp	TATCGGTCATCTTTCATTTAAATGACGAAAGGGC
34	Fw_GAP	AGCCAGGGGATTTTTGTAGAAATGTCTTGGT
35	Rv_GAP	TTGTAAAACATCGTTTCGTGTGTTTTGATAGTTGTTC

36	Fw_HYG	TGAGATGACTGATTTTTAATCAAGAGGATGTCAGAAT
37	Rv_HYG	TTAGAAGTTCCTCCGGCTTGCAAATTAAAGCC
38	Fw_3'ARG	TTAATTTGCAAGCCGGAGGAACTTCTAAGAC
39	Rv_3’ARG	CGAGTCAGTGAATTTAAATACCGCAACAAAGATGTTG
40	Fw_5'ARG4	CCCTTTCGTCATTTAAATGAAAGATGACCGATACTATTGGT
41	Rv_5'ARG4	CAAGACATTTCTACAAAAATCCCCTGGCTTCTCAACA
42	Fw_Kex1Strep	AGTCATCCTCAATTTGAAAAATAATAATCAAGAGGATGTCAGAATGCCATTTGC
43	Rv_Kex1Strep	TTATTTTTCAAATTGAGGATGACTCCAAAAATCAGTCATCTCAAAAGATTC
44	Fw_Kex1	TCAAAACACACGAAACGATGTTTTACAATAGGTGGCTC
45	Rv_Kex1	GCATTCTGACATCCTCTTGATTAAAAATCAGTCATCTCAAAAGAT

595 [a] was also used for construction of the pGArgHyg empty vector control

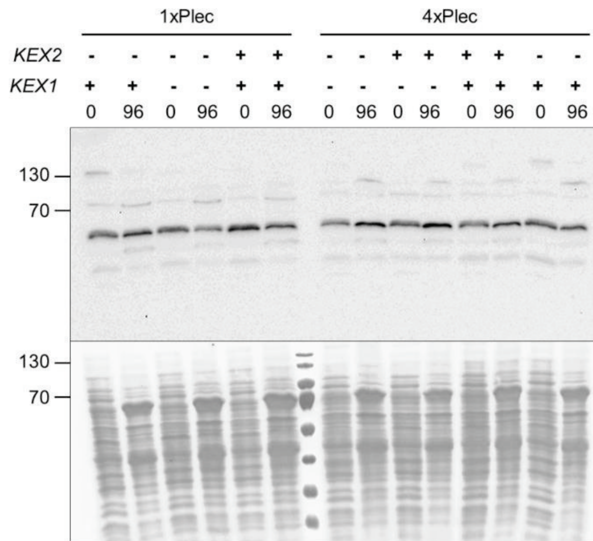
596

597 **Supplemental Figures**



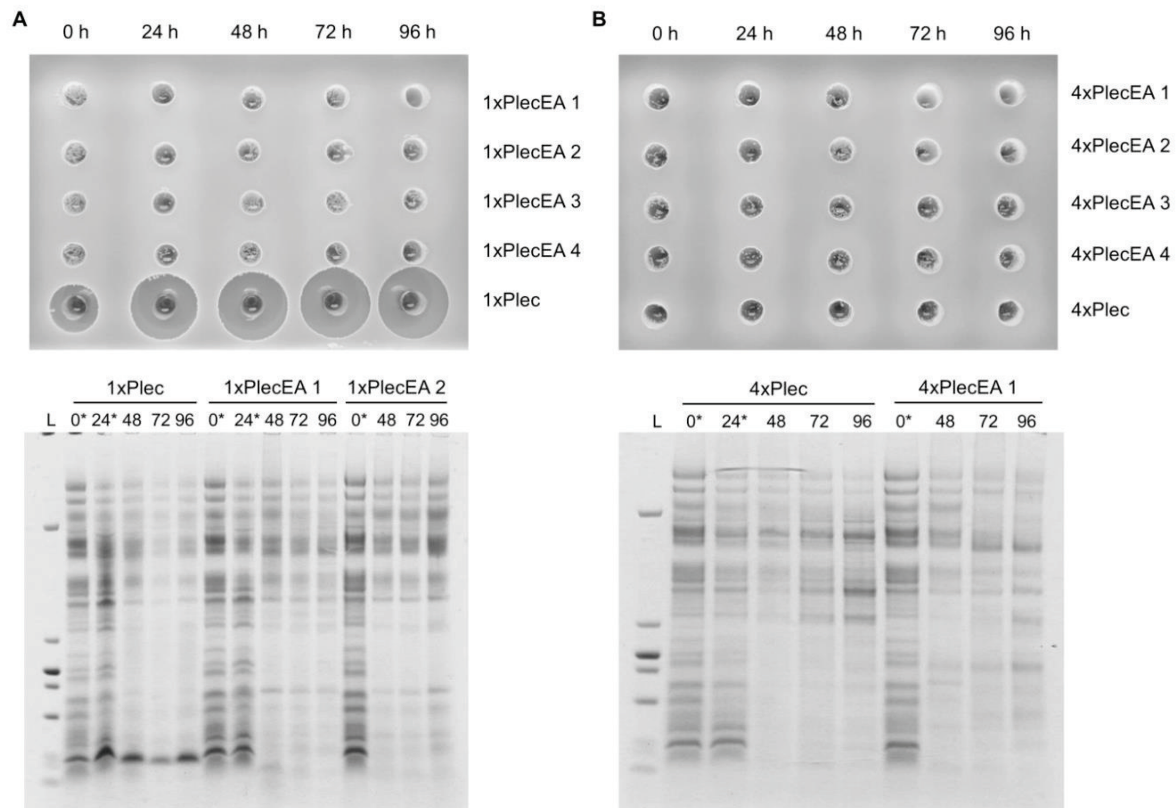
598

599 **Fig S1. Antimicrobial activity of secreted plectasin and protegrin-1.** Kex1p and Kex2p were co-
600 expressed in 1x/4xPlec and 1x/4xProt strains without tags. (A) Antimicrobial activity of plectasin was
601 tested for culture supernatants after 24 h of methanol induction. Samples were three-fold serially diluted
602 for better visualization of halo diameters. n. d = not diluted. (B) Plectasin activity in culture supernatants
603 was monitored over 96 h of methanol induction. (C) Secretion of active protegrin was detected in culture
604 supernatants after 0-120 h of methanol induction.



605

606 **Fig S2. Immunodetection of Kex1p in total cell lysates of plectasin expressing strains.** Detection of
607 intracellularly expressed StrepII-tagged Kex1 in 1xPlec and 4xPlec strains before and after 96 h of
608 methanol induction. L = PageRuler^{TM} Prestained Protein Ladder (Thermo Fisher Scientific Inc., St.
609 Leon-Rot, Germany). The predicted size of Kex1p is 83 kDa.



610

611 **Fig S3. Antimicrobial activity assays and SDS-PAGE of plectasin-secreting strains, with and**
612 **without N-terminal EA repeats on plectasin.** Secretion from 1xPlecEA strains (A) and 4xPlecEA
613 strains (B) was monitored over 96 h of methanol induction. Four independent clones were tested for
614 each construct. For protein visualization, 30 µg of proteins were precipitated from culture supernatants
615 and analyzed on a NuPAGE® Bis-Tris 4-12% gel. L = PageRuler^{TM} Low Range Unstained Protein
616 Ladder (Thermo Fisher Scientific Inc., St. Leon-Rot, Germany). The asterisk (*) indicates samples that
617 were frozen as cell broth before centrifugation, resulting in a partial release of intracellular proteins.
618 Antimicrobial activity was tested against *B. subtilis*.

3. Sterol-engineered *P. pastoris* for Membrane Protein Expression

3.1. Mini-Review

Overexpression of Membrane Proteins from Higher Eukaryotes in Yeasts

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My contribution (25%):

- Writing of the chapter "Membrane protein expression for interaction studies" (100%)
- Preparation of Fig. 1 (100%)
- Revision of chapters "Membrane protein expression for interaction studies", "Industrial applications of heterologous membrane proteins", and "References" (90%)

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MINI-REVIEW

Overexpression of membrane proteins from higher eukaryotes in yeasts

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Melanie Hirz · Harald Pichler

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Abstract Heterologous expression and characterisation of the membrane proteins of higher eukaryotes is of paramount interest in fundamental and applied research. Due to the rather simple and well-established methods for their genetic modification and cultivation, yeast cells are attractive host systems for recombinant protein production. This review provides an overview on the remarkable progress, and discusses pitfalls, in applying various yeast host strains for high-level expression of eukaryotic membrane proteins. In contrast to the cell lines of higher eukaryotes, yeasts permit efficient library screening methods. Modified yeasts are used as high-throughput screening tools for heterologous membrane protein functions or as benchmark for analysing drug–target relationships, e.g., by using yeasts as sensors. Furthermore, yeasts are powerful hosts for revealing interactions stabilising and/or activating membrane proteins. We also discuss the stress responses of yeasts upon heterologous expression of membrane proteins. Through co-expression of chaperones and/or optimising yeast cultivation and expression strategies, yield-optimised hosts have been created for membrane protein crystallography or efficient whole-cell production of fine chemicals.

Keywords Yeast · Heterologous expression · Membrane protein · Protein interactions · Protein structure

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Introduction

Approximately one third of all genes encode integral membrane proteins in each kingdom of life (Krogh et al. 2001). More than 50 % of current medication is targeting membrane proteins (Petschnigg et al. 2011). Therefore, it is evident that heterologous expression of membrane proteins for biochemical characterisation, structural analysis and industrial applications is attracting ever-increasing attention. Although dedicated research centres work on the elucidation of membrane protein structures, success rates are mediocre due to low protein yields, poor solubility in aqueous milieu and tedious, time-consuming purification methods employing detergents. Many eukaryotic membrane protein structures are still obtained either via direct purification from rich native sources (Kiser et al. 2009; Nyblom et al. 2013; Toyoshima et al. 2013), overexpression in mammalian cells (reviewed by Andréll and Tate 2013) or upon heterologous expression in *Escherichia coli* (Bernaudat et al. 2011). In contrast to the latter, yeasts provide endogenously many of the factors required for correct folding, posttranslational modification and intracellular transport of eukaryotic proteins (reviewed by Freigassner et al. 2009). Furthermore, yeasts offer low-cost screening and high-level production platforms meeting the demands of safety and authentically processed proteins (Gellissen et al. 2005b). Since the first groups published expression of human uncoupling proteins (Murdza-Inglis et al. 1991), human D_{2S} dopamine receptor (Sander et al. 1994) or rabbit SERCA1a (Centeno et al. 1994) in yeast, significant effort was put into recombinant membrane protein production in these hosts. In many cases, yeasts were employed to confirm the modes of action expected for selected membrane proteins of higher eukaryotes. Expression from high copy number plasmids and strong promoters provided sufficient material to demonstrate protein functionality as described for plant aquaporins (reviewed by Kaldenhoff

et al. 2007), G-protein coupled receptors (reviewed by Sarramegna et al. 2003) or uncoupling proteins (reviewed by Klingenberg 2001). Overall membrane protein expression levels are frequently not communicated or not even assessed. This might be due to inaccuracies in quantifying recombinant membrane proteins either in whole-cell extracts or upon challenging purification procedures. Though, not in every case strong expression yielded the best result. Based on progress in understanding cellular functions, fine-tuning of induction conditions increased the applicability of yeasts in membrane protein production (Bill 2014).

In this review, we point out the vast potential of yeasts in screening for diverse interaction processes, for example in drug–target protein relationships, protein–lipid and protein–protein interactions. As an update and extension of Freigassner et al. (2009), we provide a deeper insight into recent achievements focusing on yeast host engineering strategies, highlighting specific applications and listing membrane protein families successfully expressed for fundamental or applied research (Fig. 1).

Yeast systems for membrane protein expression

Saccharomyces cerevisiae is a good choice for straightforward heterologous protein expression. Baker's yeast exhibits incontestable advantages compared to other yeasts, i.e. several whole genome sequences of laboratory strains, well-characterised cell biology and metabolism, and many different strain collections, e.g. single-gene knockout (Entian et al. 1999; Giaever et al. 2002; Winzeler et al. 1999), GFP-tag (Huh et al. 2003) and GST-tag collections (Sopko et al. 2006; Zhu et al. 2001). Nevertheless, the use of *S. cerevisiae* in heterologous protein expression may hold some noteworthy drawbacks. *S. cerevisiae* has a tendency to hyperglycosylate proteins by attaching numerous mannose residues to N-linked carbohydrate chains (Conde et al. 2004). This can severely influence protein activity and translocation, and can also channel the recombinant protein to the endoplasmic reticulum (ER)-associated degradation (ERAD) pathway (Needham et al. 2011). The option of using episomal plasmids for protein expression allows high copy number expression and flexibility of transforming one and the same expression vector easily into multiple strains, but entails instability of recombinant strains due to vector loss or vector incompatibility (reviewed by Gellissen et al. 2005b). Recently, Debailleul et al. (2013) described the application of the *GAP1* promoter as particularly suitable for the production of membrane proteins.

The methylotrophic yeast *Pichia pastoris* is gaining popularity in heterologous protein expression (reviewed by Gonçalves et al. 2013; Ramón and Marín 2011; Hedfalk 2013). Comprising many advantages such as high cell density cultivation, strong and regulable promoter systems, and genetic manipulation techniques similar to *S. cerevisiae* make it a powerful expression host (Cereghino et al. 2002; Cregg et al. 2009; Macauley-Patrick et al. 2005). Although similarly organized in cell structure, *S. cerevisiae* and *P. pastoris* may yield in substantial differences in expression success (reviewed by Darby et al. 2012; Mattanovich et al. 2012). Many heterologously expressed membrane protein classes can be produced in both yeast hosts. For example, human monoamine oxidase was initially expressed in *S. cerevisiae*, which was quickly succeeded by *P. pastoris* as yields were much higher. Moreover, *P. pastoris* plays a prominent role in producing recombinant membrane protein among all yeast hosts in protein crystallography (Tables 1 and 2). Though, baker's yeast is favoured over *P. pastoris* when it comes to recombinant expression of uncoupling proteins or oleosins. A slight disadvantage in using the strong *AOX1* promoter for heterologous protein expression in *P. pastoris* still may be the need for methanol induction.

Although thoroughly promising, other non-conventional yeasts are only in the starting blocks for membrane protein expression. For example, *Yarrowia lipolytica* naturally offers interesting properties in conversion of hydrophobic substrates and, therefore, holds potential in expressing membrane-associated proteins especially for the metabolism of hydrophobic compounds (Thevenieau et al. 2009; Nicaud 2012). *Y. lipolytica* was applied in the production of several green notes used as aroma components and diverse substances such as gamma-decalactone for food chemistry (Fickers et al. 2005; Schrader et al. 2004). *Y. lipolytica* is powerful when it comes to studies on membrane-anchored cytochrome P450 enzymes (CYPs) like the human, hepatic CYP2D6 (Braun et al. 2012; Geier et al. 2012) and CYP1A1 (Nthangeni et al. 2004) or plant CYP53B1 (Shiningavamwe et al. 2006).

Schizosaccharomyces pombe, distantly related to *S. cerevisiae* (Sipiczki 2000), is called fission yeast because it reproduces technically similar to the proliferation of higher eukaryotic cells. By sharing many molecular, genetic and biochemical features with multicellular organisms, *S. pombe* is a particularly useful model for studying the function and regulation of genes from higher eukaryotes. Many cellular processes of more complex organisms, such as mRNA splicing, posttranslational modification and cell cycle control resemble those of *S. pombe* more closely than those of *S. cerevisiae* (Takegawa et al. 2009; Zhao and Lieberman 1995). Therefore, *S. pombe* was successfully used for expression of leukotriene LTC4 synthase (Ago et al. 2007), cytochrome P450 enzymes (Drăgan et al. 2005; Ewen et al. 2008; Hakki et al. 2008; Peters et al. 2009) and human D_{25} dopamine receptor (Sander et al. 1994). In general,

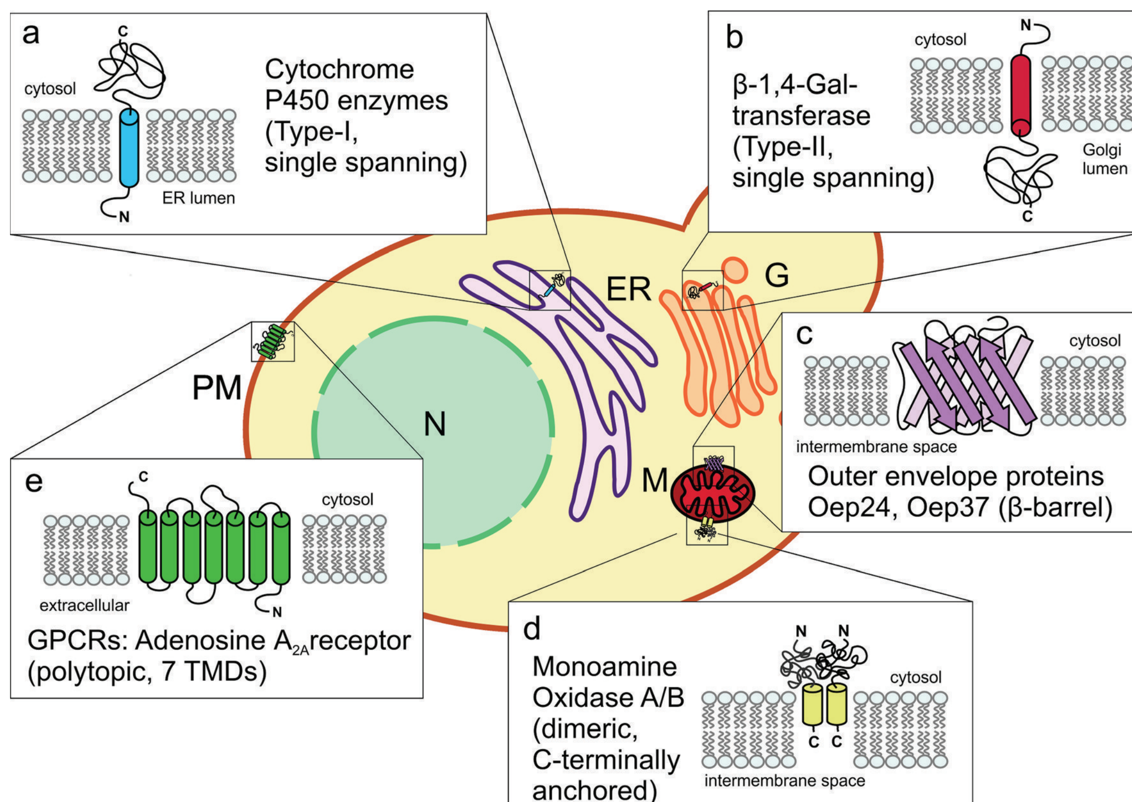

Fig. 1 Prominent membrane proteins expressed in yeast: topology and localization. This schematic drawing presents the basic topologies membrane proteins can adopt, highlighting examples that have been successfully expressed in yeast. Upon heterologous expression, membrane proteins can be, furthermore, localized to different compartments, which have to be taken into account for functional studies as well as for cell engineering purposes. Cytochrome P450 enzymes are typical type-I single spanning membrane proteins (*a*) localized to the endoplasmic reticulum (*ER*) with their largest, C-terminal part, facing the cytosolic side. They have been heterologously expressed in different yeasts, which are used as versatile whole-cell biocatalysts (Bernhardt 2006). Using a yeast membrane anchor, namely the N-terminal part of Golgi (*G*) localized Kre2p, a human β-1,4-Gal-transferase was functionally expressed in *S. cerevisiae*, facilitating localization of this type-II membrane protein (*b*) to the yeast Golgi apparatus (Schwientek et al. 1995). This strategy was further successfully employed to engineer the protein glycosylation pathway of *P. pastoris* (Vervecken et al. 2004). Beta-barrel membrane proteins (*c*) are typically found in the outer membranes of Gram-negative bacteria, mitochondria and chloroplasts. The plant chloroplast outer envelope membrane proteins Oep24 and Oep37 were exclusively localized to the mitochondria (*M*) when heterologously expressed in yeast, indicating a conserved membrane import mechanism. Monoamine oxidases A and B are α-helically anchored to mitochondrial outer membranes and form a dimeric complex in the active state (*d*). Polytopic membrane proteins (*e*) consisting of multiple membrane spanning alpha helices frequently localize to the plasma membrane of recombinant yeast, e.g. G-protein coupled receptors (GPCRs) such as the A_{2A} adenosine receptor comprising seven transmembrane domains (TMDs). *N*=Nucleus

S. pombe is well suited to examine receptors, especially G-protein coupled receptors (GPCRs), based on its G_α-subunit being more suitable than in *S. cerevisiae*, on a more highly developed intracellular membrane system and on better ligand accessibility (Ladds et al. 2003). Moreover, *S. pombe* is a very interesting host for analysis of olfactory receptors (Davey and Ladds 2011) and proteins comprising difficult glycosylation patterns can be produced in this yeast (De Pourcq et al. 2010).

Like *P. pastoris*, the thermotolerant, methylotrophic yeast *Hansenula polymorpha* grows to high cell densities. Growth at higher temperatures may be favourable for expression of human proteins (van Dijk et al. 2000; Gellissen et al. 2005a). Furthermore, *H. polymorpha* efficiently produces recombinant N-glycosylated proteins, which are much less hyper-mannosylated due to sophisticated glyco-engineering (Kim et al. 2006; Kim et al. 2004). This yeast was successfully used for expression of complex human β-1,2-*N*-acetylglucosaminyltransferase I (GnTI) (Cheon et al. 2012).

Overexpression of membrane proteins for fundamental research

Determination of high-resolution structures

Human monoamine oxidase B, a mitochondrial outer membrane protein, was the first higher eukaryotic membrane protein to be expressed in *P. pastoris* for crystallisation purposes (Binda et al. 2002). Table 1 gives an overview of all

Table 1 Membrane proteins of higher eukaryotes heterologously expressed in yeast for high-resolution structure determination (data largely obtained from http://blanco.biomol.uci.edu/mpstruc/)

Protein	Original host	Expression host	PDB coordinates	Resolution	Reference
Monotopic membrane proteins					
Oxidases					
Monoamine oxidase B, 1OJA: bound with Isatina	*H. sapiens*	*P. pastoris*	1GOS, 1OJA	3.0 Å/1.70 Å	Binda et al. (2003, 2002)
Monoamine oxidase A	*R. norvegicus*	*S. cerevisiae*	1O5W	3.20 Å	Ma et al. (2004)
Monoamine oxidase A with bound clorglycine	*H. sapiens*	*P. pastoris*	2BXR	3.00 Å	De Colibus et al. (2005)
Monoamine Oxidase A with bound harmine: 2Z5Y: G110A mutant with bound harmine	*H. sapiens*	*S. cerevisiae*	2Z5X, 2Z5Y	2.20 Å/2.17 Å	Son et al. (2008)
Transmembrane proteins: alpha helical					
GPCRs					
A_{2A} adenosine receptor in complex inverse-agonist antibody, 3VGA: bound with mouse Fab2838 in the presence of the antagonist ZM241385	*H. sapiens*	*P. pastoris*	3VG9, 3VGA	2.70 Å/3.10 Å	Hino et al. (2012)
Histamine H_1 receptor, complexed with doxepin	*H. sapiens*	*P. pastoris*	3RZE	3.10 Å	Shimamura et al. (2011)
Channels: potassium and sodium ion—selective					
Two-pore domain potassium channel K_{2P}1.1 (TWIK-1)	*H. sapiens*	*P. pastoris*	3UKM	3.40 Å	Miller and Long (2012)
Two-pore domain potassium channel K_{2P}4.1 (TRAAK)	*H. sapiens*	*P. pastoris*	3UM7	3.80 Å	Brohawn et al. (2012)
Two-pore domain potassium channel K_{2P}4.1 (TRAAK)	*H. sapiens*	*P. pastoris*	4I9W	2.75 Å	Brohawn et al. (2013)
Kv1.2 voltage-gated potassium channel, 3LUT: re-refinement of 2A79 using normal-mode X-ray crystallographic refinement	*R. norvegicus*	*P. pastoris*	2A79, 3LUT	2.9 Å/2.9 Å	Chen et al. (2010b); Long et al. (2005a, 2005b)
Kv1.2/Kv2.1 voltage-gated potassium channel chimera, 3LNM: F233W mutant	*R. norvegicus*	*P. pastoris*	2R9R, 3LNM	2.4 Å	Long et al. (2007); Tao et al. (2010)
Kir2.2 inward-rectifier potassium channel, 3SPI, 3SPC: in complex with dioctanoylglycerol pyrophosphate (DGPP), 3SPH: I223L mutant in complex with PIP_2, 3SPJ:I223L mutant, apo form, 3SPG: R186A mutant in complex with PIP_2	*G. gallus*	*P. pastoris*	3JYC, 3SPI, 3SPC, 3SPH, 3SPJ, 3SPG	3.1 Å/3.31 Å/ 2.45 Å/3.00 Å/ 3.31 Å/2.61 Å	Hansen et al. (2011); Tao et al. (2009)
GIRK2 (Kir3.2) G-protein-gated K^+ channel: 3SYA: wild-type protein+PIP_2, 3SYC: D228N mutant, 3SYP: R201A mutant, 3SYQ: R201A mutant+PIP_2, 4KFM*: the β and γ subunits of *H. sapiens* expressed in *S. frugiperda*	*M. musculus*	*P. pastoris*	3SYO, 3SYA, 3SYC, 3SYP, 3SYQ, 4KFM*	3.60 Å/3.00 Å/ 3.4 Å/3.1 Å/ 3.45 Å/3.45 Å	Whorton and MacKinnon (2013, 2011)
Channels: calcium ion selective					
Orai Calcium release-activated calcium (CRAC) channel, 4HKS: K163W mutant	*D. melanogaster*	*P. pastoris*	4HKR, 4HKS	3.35 Å/3.35 Å	Hou et al. (2012)
Channels: aquaporins and glyceroporins					
AQP4 aquaporin water channel	*H. sapiens*	*P. pastoris*	3GD8	1.8 Å	Ho et al. (2009)
AQP5 aquaporin water channel	*H. sapiens*	*P. pastoris*	3D9S	2.0 Å	Horsefield et al. (2008)
SoPIP2;1 plant aquaporin (closed conformation), 2B5F: open conformation, 3CLL: S115E mutant, 3CN5: S115E:S274E mutant, 3CN6: S274E mutant	*S. oleracea*	*P. pastoris*	1Z98,2B5F, 3CLL, 3CN5, 3CN6	2.10 Å/3.90 Å/ 2.30 Å/2.05 Å/ 2.95 Å	Nyblom et al. (2009); Törnroth-Horsefield et al. (2006)
Membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG)					
Leukotriene LTC_4 Synthase in complex with glutathione	*H. sapiens*	*S. pombe*	2PNO	3.3 Å	Ago et al. (2007)
Leukotriene LTC_4 Synthase in complex with glutathione, 2UUI: apo form	*H. sapiens*	*P. pastoris*	2UUH, 2UUI	2.15 Å/2.00 Å	Martinez Molina et al. (2007)

Table 1 (continued)

Protein	Original host	Expression host	PDB coordinates	Resolution	Reference
Leukotriene LTC_4 Synthase in complex with stable leukotriene analogue I, 4JC7: W116A mutant with analog I, 4JRZ: W116F mutant with analog I, 4J7T: wild-type with analog II, 4J7Y: wild-type with analog III	*H. sapiens*	*P. pastoris*	4JC7, 4JRZ, 4J7T, 4J7Y	2.70 Å/2.40 Å/ 3.20 Å/2.90 Å	Niegowski et al. (2014)
Major facilitator superfamily (MFS) transporters					
PiPT high-affinity phosphate transporter	*P. indica*	*S. cerevisiae*	4 J05	2.90 Å	Pedersen et al. (2013)
NRT1.1 nitrate transporter, apo form, member of the NPF (NRT1/PTR) family. 4CL5: in complex with nitrate	*A. thaliana*	*S. cerevisiae*	4CL4, 4CL5	3.70 Å/3.71 Å	Parker and Newstead (2014)
ATP binding cassette transporter					
P-Glycoprotein, 3G60: with bound QZ59-RRR, 3G61: with bound QZ59-SSS	*M. musculus*	*P. pastoris*	3G5U, 3G60, 3G61	3.8 Å/4.40 Å/ 4.35 Å	Aller et al. (2009)
P-Glycoprotein, refined structure, 4M2S: corrected structure of mouse P-glycoprotein bound to QZ59-RRR, 4M2T Corrected structure of mouse P-glycoprotein bound to QZ59-RRR	*M. musculus*	*P. pastoris*	4M1M, 4M2S, 4M2T	3.80 Å/4.40 Å/ 4.35 Å	Li et al. (2014)
P-Glycoprotein	*C. elegans*	*P. pastoris*	4F4C	3.40 Å	Jin et al. (2012)
Membrane integral pyrophosphatases (M-PPases)					
H^+-translocating M-PPase in complex with the non-hydrolysable substrate analog imidodiphosphate (IDP)	*V. radiata*	*S. cerevisiae*	4A01	2.35 Å	Lin et al. (2012)
Na^+-translocating M-PPase with metal ions in active site, 4AV6: in complex with phosphate and magnesium	*T. maritima*	*S. cerevisiae*	4AV3, 4AV6	2.60 Å/4.00 Å	Kellosalo et al. (2012)

membrane protein structures elucidated at high resolution, detailing the yeast host system used for overexpression. *P. pastoris* clearly outnumbers *S. cerevisiae* regarding successfully expressed heterologous membrane proteins applied for high-resolution structure determination. For instance, a noteworthy milestone was set by Long et al. (2007) crystallising a Kv channel expressed in *P. pastoris* in a defined lipid environment. Also with the help of *P. pastoris*, the first structure of a G-protein gated potassium-selective channel was clarified by Whorton and MacKinnon (2011). Largely, structures of membrane-bound oxidases were obtained upon expression in yeast as can be seen on http://blanco.biomol.uci.edu/mpstruc/ (Table 1). This webpage provides a detailed overview of all membrane protein structures elucidated so far regardless of protein sources. However, the protein structure with the highest number of transmembrane domains solved so far, a H^+-translocating pyrophosphatase from *Vigna radiata* displaying 16 TMDs, was achieved upon expression in *S. cerevisiae* (Lin et al. 2012). Beta-barrel transmembrane proteins were hardly expressed in yeasts for crystallisation attempts, which can be explained by the achievements in expressing this membrane protein group in *E. coli*.

Ion channels: guards of the gates

Analysis, screening and characterisation of ion channels from higher eukaryotes can elegantly be performed with yeast strains engineered to lack endogenous ion channels. For example, potassium channels were expressed in smartly constructed yeast host strains deficient for K^+ uptake. First, *trk1* and *trk2* were deleted in *S. cerevisiae* W303 yielding strain SGY1528. Then, heterologous Kir family channels that compensated the growth deficiency at low K^+ concentrations were identified and functionally characterized. Tang et al. (1995) expressed a K^+-channel isolated from pig heart in *S. cerevisiae* SGY1528 comparing the constitutive *PGK* and the inducible *GAL1* promoter. Expression from both promoters has proven to restore growth on low K^+ levels to the same extent. The tester strain SGY1528 was further used to identify K^+ channel activities in mammalian cDNA libraries expressed from the methionine repressible *MET25* promoter (Grishin et al. 2006). Moreover, the same strain and expression strategy was used to determine structural elements relevant for channel functionality as described by Yi et al. (2001). The W303-based *S. cerevisiae* B31 strain deficient in K^+ export, thus rendering it unable to grow at high K^+ levels, was applied to elucidate channel

Table 2 Some examples of recombinant protein levels of different families heterologously expressed in yeast. For each class of protein, obtained yields are listed chronologically

Protein	Origin	Host	Yield	Reference
GPCRs				
OR17 and OR17-40	*R. norvegicus*, *H. sapiens*	*S. cerevisiae*	327 pmol per mg of membrane protein, 1.44×10^5 receptor entities per cell	Minic et al. (2005a)
Adenosine (A_{2A}) receptor	*H. sapiens*	*S. cerevisiae*	4 mg active protein per L of yeast culture	Niebauer and Robinson (2006)
Neuromedin U type 1 and 2 receptor (NmU_1R/ NmU_2R)	*H. sapiens*	*P. pastoris*	6–9 pmol receptor per mg of total membrane protein	Shukla et al. (2007a)
12 different GPCRs (e.g. hFSHR, $hA_{2A}R$, CB_2R, NK_1R…)	*H. sapiens*	*S. cerevisiae*	Ranging from 0.7±0.1 mg to 10.0± 1.0 mg per L of yeast culture	O'Malley et al. (2009)
Muscarinic acetylcholine receptor subtype 2 (CHRM2)	*H. sapiens*	*P. pastoris*	51.2 pmol per mg of membrane protein; 1.9 mg per L of yeast culture	Yurugi-Kobayashi et al. (2009)
β2-adrenergic receptor	*H. sapiens*	*P. pastoris*	20 mg per L of yeast culture	Gerasimov et al. (2012)
Channels: aquaporins				
Aquaporin 1 (hAQP1)	*H. sapiens*	*S. cerevisiae*	0.5 mg purified protein per L of culture	Saparov et al. (2001)
Aquaporin PM28A	*S. oleracea*	*P. pastoris*	25 mg of purified protein per L of culture	Karlsson et al. (2003)
*Pv*TIP3;1	*P. vulgaris*	*P. pastoris*	~1 mg of purified protein from 50 g of wet cells	Daniels and Yeager (2005)
Aquaporin 1 (hAQP1)	*H. sapiens*	*P. pastoris*	90 mg purified hAQP1 per L of culture	Nyblom et al. (2007)
Aquaporin 4 (hAQP4)	*H. sapiens*	*P. pastoris*	~15 mg purified protein per L of culture	Ho et al. (2009)
Aquaporin 1	*H. sapiens*	*S. cerevisiae*	1,500 pmol per mg of total membrane protein; 8.5 % of total membrane protein	Bomholt et al. (2013)
Uncoupling proteins				
UCP	*R. norvegicus*	*S. cerevisiae*	70–100 pg per mg of mitochondrial protein	Murdza-Inglis et al. (1991)
UPC1	*M. musculus*	*S. cerevisiae*	11 μg per mg of mitochondrial protein	Stuart et al. (2001)
UCP1	*H. sapiens*	*S. cerevisiae*	~10 μg per mg of total mitochondrial protein	Douette et al. (2006)
Transporter				
ABC transporters BSEP and MDR3	*H. sapiens*	*P. pastoris*	~1 mg BSEP and ~6 mg MDR3 per 100 g of wet cell weight	Ellinger et al. (2013)
ATPases				
SERCA1a	*H. sapiens*	*S. cerevisiae*	100 pmol per mg of membrane protein	Jidenko et al. (2005)
SERCA1a	*H. sapiens*	*S. cerevisiae*	200–500 μg of a 50 % pure SERCA1 per L of yeast culture	Cardi et al. (2010a)
Membrane bound oxidases				
Monoamine oxidase A	*H. sapiens*	*S. cerevisiae*	15 mg per L of culture	Weyler et al. (1990)
Monoamine oxidase B	*H. sapiens*	*P. pastoris*	1,700 U or 200 mg of purified protein per 2 L of culture	Newton-Vinson et al. (2000)
Monoamine oxidase A	*H. sapiens*	*P. pastoris*	1,170 units or 660 mg of purified protein per 2 L of culture	Li et al. (2002)
Monoamine oxidase A	*R. norvegicus*	*S. cerevisiae*	10 mg highly pure protein per L of culture	Ma et al. (2004)
Monoamine oxidase A	*R. norvegicus*	*P. pastoris*	700 U or 200 mg of purified protein per L of culture	Wang and Edmondson (2010)
Monoamine oxidase A	*D. rerio*	*P. pastoris*	300 U or 200 mg of purified protein per L of culture	Arslan and Edmondson (2010)
Cytochrome b_{561}	*B. taurus*	*P. pastoris*	0.7 mg detergent-solubilized cyt b_{561} per L of culture	Liu et al. (2005)
Cytochrome b_{561}	*M. musculus*	*S. cerevisiae*	15 nmol per mg of total protein	Bérczi et al. (2005)
Cytochrome b_{561}	*A. thaliana*	*P. pastoris*	7.7 mg per 2 L of culture	Cenacchi et al. (2012)

functionality in the opposite direction (Kolacna et al. 2005). Overexpression of the K^+ export channel Kir2.1 rescued B31 on high K^+ concentrations. Kir2.1 was either expressed from copper inducible *CUP1* promoter on a multicopy plasmid or from the *PMA1* promoter integrated into the yeast genome. Expression from the multicopy plasmid resulted in higher

Kir2.1p activity. Strain B31 was further used for identification and structural investigation of K^+ channels by mutational analysis and growth complementation assays at high K^+ concentrations. Potassium channels were expressed from a *MET25* or *CUP1* promoter in the pYES2 or the pYEX-BX vector (Bernstein et al. 2013; Schwarzer et al. 2008). Following a very similar strategy, various cation/H^+ exchangers from *Arabidopsis thaliana* were expressed in a cation handling-deficient *S. cerevisiae* strain using multicopy vectors harbouring the *GAL1* or the *PMA1* promoter. Cell growth and protein transport were investigated at different alkaline pH values (Chanroj et al. 2011; Hernández et al. 2009; Maresova and Sychrova 2006).

Pump transporting or transporter pumping

An overview of different plant proton-pumping pyrophosphatases (H^+-PPases) and their characterisation in engineered yeast strains is given in Serrano et al. (2007). *S. cerevisiae* strain YPC3 expressing its endogenous pyrophosphatase *IPP1* only upon galactose induction was used for expressing H^+-PPases from the constitutive *PMA1* promoter on a 2 μ-based plasmid. Overexpression of H^+-PPases chimera led to altered intracellular localization in *S. cerevisiae* YPC3 (Drake et al. 2010), alleviated the phenotype of *S. cerevisiae* strains with vacuolar ATPase deficiency (Pérez-Castiñeira et al. 2011) and altered yeast resistance to high salinity and metal stressors (Yoon et al. 2013). Human bile salt export pump (BSEP) was analysed in *S. cerevisiae* using a new site-directed method called Directed Recombination-Assisted Mutagenesis (DREAM) (Stindt et al. 2013; Stindt et al. 2011). In parallel, *P. pastoris* was chosen as expression host for human liver BSEP (ABCB11) and MDR3 (ABCB4) (Ellinger et al. 2013). Recombinant transporters were cloned into a pPIC-derived vector and expressed under the control of the *AOX1* promoter. More than 100 detergents were tested for BSEP extraction and ATPase activity was assessed via ATP agarose binding and malachite green assays, respectively. Furthermore, soluble domains of human ABCA4 were expressed in *S. cerevisiae* to assess their structural properties (Tsybovsky and Palczewski 2014). Another human membrane protein, hepatic thiazide-sensitive NaCl-cotransporter (NCC, SLC12A3), containing 12 TMDs, was expressed in *S. cerevisiae* from the *GPD* promoter on the 2 μ plasmid pRS426 (Needham et al. 2011). Site-specific mutations causing Gitleman syndrome were shown to target the co-transporter for ERAD.

Metal deficiency is one of the most common nutritional disorders in plants. Thus, transporters causative for or capable of relieving metal deficiency are of highest interest. Iron transporters from *A. thaliana* (Korshunova et al. 1999; Vert et al. 2001), apple (Xiao et al. 2008; Zhang et al. 2013b), tomato (Bereczky et al. 2003; Eckhardt et al. 2001) and peas (Cohen et al. 2004) were expressed in iron uptake-deficient *S. cerevisiae* mutants. Most of these transporters were not only found to restore growth deficiencies at low Fe^{2+} concentrations but also to complement uptake of other metals such as Mn, Cu and Zn in the corresponding mutant strains. Moreover, zinc transporters from barley and rice (Ishimaru et al. 2005; Pedas et al. 2009) and a manganese transporter from barley (Pedas et al. 2008) were functionally characterised in zinc-, manganese-, iron- and copper-deficient *S. cerevisiae* strains BY4741 *Δzrt1/zrt2, Δsmf1, Δfet3/fet4 and Δctr1*, respectively. In these studies, plant genes were expressed from a variety of constitutive promoters, e.g. *PGK, ADH1 or PMA1* promoters, but also from P_{GAL1}. Ammonium is an important nutrient and nitrogen source. A *S. cerevisiae* strain defective for its endogenous ammonium transporter served as host for functional expression of plant NH_4^+ transporters (Bu et al. 2013; Gu et al. 2013; Neuhäuser and Ludewig 2014).

Uncoupling proteins: 'heating' up the cells

Altogether, five distinct isoforms of uncoupling proteins (UCP1–5) have been found in mammals based on sequence homologies (Jezek et al. 2004; Krauss et al. 2005). A considerable number of human and mammalian UCPs have been expressed in *S. cerevisiae* (reviewed by Klingenberg et al. 2001). It is not surprising that *S. cerevisiae* still is the model organism of choice for expression and analysis of uncoupling proteins because of the availability of detailed mitochondrial proteome maps providing comprehensive "gene-to-protein" datasets (Sickmann et al. 2003). Douette et al. (2006) heterologously expressed human UCP1 and showed that it has a dual influence in free radical generation. Furthermore, regulation of the mitochondrial proteome by hUCP1 was documented. After numerous achievements with vertebrate UCPs, the first UCP from an invertebrate, the amphioxus *Branchiostoma belcheri*, was successfully expressed in *S. cerevisiae* (Chen et al. 2010a). Furthermore, uncoupling protein from *Drosophila melanogaster* (Fridell et al. 2004) and skunk cabbage were functionally expressed in baker's yeast (Ito et al. 2006). Heterologous expression of UCPs almost exclusively was driven from galactose inducible promoters of 2 μ-based vectors such as pESC-URA, pRS426-*GAL1* and pYES2.

GPCRs: the receptor in general

Besides using yeasts as whole-cell sensors expressing GPCRs, fundamental research is pursuing biochemical characterisation of GPCRs (Table 2). Marsango et al. (2011) investigated dimerization of human prokineticin binding receptor PKR2 in *S. cerevisiae* by Western blot analyses. High-quantity

production and purification of A_{2A}-adenosin receptor in *S. cerevisiae* was realized on the milligram per litre scale (Niebauer and Robinson 2006; O'Malley et al. 2009). *P. pastoris* was used for production and characterisation of neuromedin U type 1 and 2 receptor (NmU_1R/ NmU_2R). The addition of 2 % DMSO improved protein yield from 1 to 5 pmol per mg of total membrane protein (Shukla et al. 2007a). André et al. (2006) compared expression levels of 20 GPCRs in *P. pastoris* under different cultivation conditions. The need for large amounts of purified membrane proteins for crystallisation experiments considerably pushed the efforts in obtaining high-yielding yeast expression strains (Table 2).

Yeast sensors and membrane protein expression for drug development

The analysis of membrane protein structure–function relationships is a major focus in fundamental and applied research. A popular strategy for correlating protein function and structure is to perform site-directed mutagenesis and to analyse—by simple assays—the effects on protein activity. Revealing and understanding drug–target relationships is of high relevance because it is crucial for the early-stages of drug discovery, toxicology studies and clinical trials. Focused on medical indications, several databases list drug–membrane protein relationships with clinical relevance (Hecker et al. 2012; Sun et al. 2012).

Receptors and olfactory signalling pathways: yeasts as sensors

The most extensively studied group of membrane proteins commonly targeted by drugs are by far GPCRs and their effectors. GPCR research in yeasts mainly deals with two approaches characterising either the specificity of ligand docking to a given receptor or screening 'orphan' GPCRs for their unknown effector ligand in recombinant sensor cells (reviewed by Ladds et al. 2005; Ladds et al. 2003; Minic et al. 2005b; Suga and Haga 2007). Yeasts constitute great recombinant hosts providing a low endogenous background for mammalian GPCRs and G-proteins. Therefore, they are interesting hosts in screening of (olfactory) receptors and their corresponding stimulants (reviewed in Pausch 1997). There are remarkable similarities between the signal transduction cascades of GPCRs in mammalian cells and the pheromone response pathway in yeast that can be exploited. Upon cellular engineering, an effector docking to a heterologous GPCR actuates a MAP kinase pathway leading to expression of the yeast Ste12p transcription factor. Ste12p in turn specifically activates the P_{FUS1} promoter cloned in front of β-galactosidase, fluorophores or auxotrophy markers. (Dowell and Brown 2009; Ladds et al. 2005). For example, human adenosine A_{2A} receptor function was characterised by a *S. cerevisiae* strain coupling receptor activation to cell growth (Bertheleme et al. 2013). Expression strategies for heterologous GPCRs are highly diverse and changed with time. During the early stages, GPCRs fused to the α-factor prepro leader sequence were rather expressed from high copy number plasmids with inducible promoters (King et al. 1990). Later, low copy number plasmids like pRS416-*GPD*, pRS413 or pRGP with moderately constitutive promoters were preferred to fine-tune intracellular expression levels of GPCRs (Crowe et al. 2000; Schmidt et al. 2003). Yet, only half of all heterologously expressed GPCRs couple to the pheromone signalling pathway of *S. cerevisiae*. Also termed the 'smelling yeast', *S. cerevisiae* strains expressing mammalian olfactory receptors were established as biosensors (Crowe et al. 2000; Dhanasekaran et al. 2009; Minic et al. 2005a). For example, a *S. cerevisiae* strain aiding in the detection of environmental toxins such as dinitrotoluene (DNT) was created (Radhika et al. 2007). Beside baker's yeast, *S. pombe* was successfully established as heterologous expression host for olfactory receptors (reviewed by Davey and Ladds 2011).

Mitochondrial flavin containing oxidoreductases

Monoamine oxidases (MAOs) are situated in the outer mitochondrial membrane of higher eukaryotic cells, mainly in neurons and the intestinal tract. They catabolize monoamines such as adrenalin and serotonin, which may accumulate due to endogenous signalling processes or uptake from foods. MAO dysfunction is linked to a number of psychiatric and neurological disorders. Thus, inhibitors of MAOs are applied as anti-depressive drugs (Meyer et al. 2006; reviewed in Tipton et al. 2004). Initially, human liver MAO-A expressed in *S. cerevisiae* from the constitutive *GPD* promoter on a 2μ plasmid yielded 15 mg of purified enzyme per litre of cell culture (Weyler et al. 1990). Unfortunately, these amounts were too low for protein crystallization. Significantly higher levels were obtained in *P. pastoris* at 329 mg of MAO-A per litre of cell culture later on (Li et al. 2002). Several further MAOs were successfully expressed to high yields in *P. pastoris* (Table 2), paving the way for drug screening and enzyme crystallisation attempts (Arslan and Edmondson 2010; Binda et al. 2002; Newton-Vinson et al. 2000; Wang and Edmondson 2010). High-level expression was achieved by cloning genes into the pPIC3.5 K vector harbouring the extraordinarily strong methanol-inducible *AOX1* promoter.

Transport proteins

Transport proteins have been investigated extensively as they may specifically enable drug delivery into cells as described

for peptidomimetic drugs such as β-lactam antibiotics, angiotensin-converting enzyme inhibitors, selected peptidase inhibitors and pro-drugs (Rubio-Aliaga and Daniel 2002). Targeted import of small peptides was studied by generating *P. pastoris* strains heterologously expressing mammalian peptide transporters PEPT1 and PEPT2 (Döring et al. 1998a; Döring et al. 1997; Foltz et al. 2004). PEPT1 and PEPT2 are highly expressed in epithelial cells of the human small intestine and kidney, which makes them a favoured target for drug delivery (reviewed in Brandsch 2009). Furthermore, delta-aminolevulinic acid (δ-ALA) was transported into *P. pastoris* cells by the same transporters, which explained δ-ALA accumulation in epithelial cells after oral administration (Döring et al. 1998b). The *S. cerevisiae* strain SM4 devoid of its endogenous vacuolar ABCC-type ATP binding cassette (ABC) transporters was engineered to produce phytochelatin. This strain was used as screening system for functionality of plant ABC-transporters abrogating growth deficiencies caused by heavy metals (Park et al. 2012; Song et al. 2010). Heterologous ABC transporters were expressed either from the pNEV or the pYES3 vector.

Investigating cellular ‘plumbing’ systems: aquaporins

Aquaporins are important in all kingdoms of life because accurately regulated water entry and exit is vital (King et al. 2004). Aquaporins play a role in the onset of many diseases such as dry skin, obesity and even cancer (reviewed in Verkman 2012). Heterologous expression of aquaporins in *P. pastoris* has been described by several groups using the strong *AOX1* promoter (Daniels and Yeager 2005; Karlsson et al. 2003; Nyblom et al. 2007). An assay was established to screen for potential effectors of aquaporin function based on isolating spheroplasts of recombinant *P. pastoris* strains followed by spectroscopic measurement of their swelling degree (Azad et al. 2009; Azad et al. 2008). Crystal structures of aquaporin water channels were resolved upon heterologous expression in *P. pastoris*. Taking advantage of protein structures (see Table 1) and homology modelling, site-directed mutational analysis of aquaporins was performed. Structure–function relationships were elucidated by measuring water channel activities of the spheroplasts of recombinant *P. pastoris* expression strains harbouring aquaporin muteins (Azad et al. 2012; Murata et al. 2000). Functional expression of plant aquaporins in *S. cerevisiae* has been described as well (Kaldenhoff et al. 2007; Murozuka et al. 2013; Otto et al. 2010).

Transmembrane ATPases: cell-membrane counter traders

Transmembrane ATPases actively transport small molecules across cellular membranes, as for example the most prominent P-type ATPase family (reviewed in Bublitz et al. 2010). A very effective purification method based on the biotin acceptor domain (BAD) fusion strategy was used to analyse altered ATPase activity of a number of muteins (Cardi et al. 2010a; Cardi et al. 2010b; Jidenko et al. 2006). For easy expression in *S. cerevisiae* W303.1b, genes of interest were cloned into the shuttle expression vector pYeDP60 and transcriptionally controlled by the strong hybrid promoter *GAL10*/*CYC1*. BAD is fused C-terminally to the protein of interest provoking the expression host to autonomously biotinylate the recombinant protein.

Membrane protein expression for interaction studies

Membrane proteins frequently associate in complexes and, therefore, the identification of subunits and interaction partners is of utmost importance for understanding membrane protein mode of action. Heterologous expression in yeast can be a valuable tool to study the interaction of membrane proteins with other proteins or lipids. Several methods have been applied to identify interaction partners of membrane proteins and characterise their interactions.

Protein–protein interaction

Yeast two-hybrid (Y2H) systems are standard strategies to investigate protein–protein interactions in vivo. However, Y2H is not applicable to membrane proteins due to the requirement for nuclear localization of the proteins to be tested. To overcome this shortcoming, a split-ubiquitin assay was established (Johnsson and Varshavsky 1994) and further optimised for the screening of membrane protein interaction partners (Stagljar et al. 1998). In the so-called membrane yeast two-hybrid (MYTH) assay, the carboxy-terminal part of ubiquitin (Cub) is fused to a membrane protein along with the artificial transcription factor protein A-LexA-VP16 (PLV). Upon interaction with another membrane protein, which is fused to the amino-terminal part of ubiquitin (NubG), the reconstituted ubiquitin is recognized by a specific protease liberating the PLV transcription factor. The transcription factor enters the nucleus and induces the transcription of reporter genes *lacZ* and *HIS3*, respectively. Recently, this method was used to identify ten novel interacting proteins of the μ-opioid receptor by screening human brain cDNA libraries (Petko et al. 2013). Whereas homo-oligomerisation of GPCRs has been proven important for GPCR activity (Wade et al. 2011), hetero-oligomerisation is believed to have a huge impact, but still is rather hard to analyse. A specific novelty was recently described by Kittanakom et al. (2014), wherein MYTH was coupled to DNA microarray analysis to investigate GPCR interactions upon drug treatment. MYTH has been a powerful method in screening for interaction partners (Nakamura et al.

2013), for example, in searching for novel ATP13A2 interactors (Usenovic et al. 2012). It has further been employed in identifying proteins interacting with TRPML3 during autophagy (Choi and Kim 2014), in investigating physical interaction of K^+-channel TASK-2 with human heterotrimeric G-protein subunits (Añazco et al. 2013) or in detecting membrane protein targets of human papilloma virus oncoproteins upon cell differentiation (Kotnik Halavaty et al. 2014). A modified version of MYTH is the mating-based split-ubiquitin assay (mbSUS) established by Obrdlik et al. (2004). mbSUS is optimised for systematic large-scale analyses of membrane interactions by employing in vivo cloning strategies. For example, this assay was used for interactome mapping of *Arabidopsis* membrane proteins (Chen et al. 2012). In a recent study, the mbSUS technique was used for the identification of plant glutamate-like receptors that show similar interaction characteristics as the homologous animal ionotropic glutamate receptors (Price and Okumoto 2013).

Another well-established method for the investigation of membrane protein interaction in yeast is the bimolecular fluorescence complementation assay, shortly BiFC (reviewed by Kerppola 2008). Therein, a fluorophore such as GFP is split and each half is fused to the supposedly interacting proteins. In the case of actual interaction, the respective fluorescence signal is detected. The major advantage of this method is that the interaction can be concomitantly localized through microscopy in living cells. The interactions of major intrinsic membrane proteins responsible for the transport of water and small neutral solutes in *A. thaliana* were studied lately by applying this technique in *S. cerevisiae* (Murozuka et al. 2013). The same assay had been used earlier to study the hetero-tetrameric assembly of tobacco aquaporins *Nt*AQP1 and *Nt*PIP2;1 (Otto et al. 2010). Further methods for detecting membrane protein interactions and their successful applications are reviewed by Petschnigg et al. (2011) describing, among others, the Ras recruitment systems, G-protein fusion technology or fluorescence (FRET) and bioluminescence (BRET) resonance energy transfer-based systems. BRET has been employed to analyse the protein interactions of GPCRs (Sanz and Pajot-Augy 2013). Library screening of randomly mutagenized GPCRs, for example human UDP-glucose receptor or muscarinic acetylcholine receptor, was preferentially performed in baker's yeast (Ault and Broach 2006; Stewart et al. 2010; and reviewed by Beukers and Ijzerman 2005; Celić et al. 2004). Recombinant membrane protein interactions should always be confirmed by in vitro experiments such as co-immunoprecipitation or pull-downs and would preferably be supported by experiments in their natural host organisms.

Protein–lipid interaction

The impact of certain lipid molecular species on the activity and stability of membrane proteins has been reviewed extensively (Adamian et al. 2011; Hunte 2005; Lee 2004; Opekarová and Tanner 2003). Membrane proteins may be surrounded by an annular lipid layer or undergo specific protein–lipid interactions. These interactions are of crucial importance for proper membrane targeting and protein folding as well as membrane protein stability and activity. When membrane proteins from higher eukaryotes are expressed in yeast, it might be that the different lipid composition negatively influences the yield of recombinant protein. Although membrane protein–lipid interaction studies are often performed using model membranes (reviewed by Zhao and Lappalainen 2012), heterologous expression in engineered yeast cells can also give insights into lipid requirements of these proteins. Lipid-engineered yeast strains can be a tool for expression of membrane proteins that require specific lipids that do not naturally occur in yeasts. Several studies underscore that cholesterol, for instance, is a strong interaction partner of certain mammalian membrane proteins as evidenced by defined cholesterol binding sites in crystal structures (Cherezov et al. 2007; Hanson et al. 2008; Liu et al. 2012; Manglik et al. 2012; Shinoda et al. 2009). Usually, yeasts contain ergosterol as major sterol compound and lack cholesterol. Through metabolic engineering, *S. cerevisiae* and *P. pastoris* cells have been re-programmed to produce cholesterol (Souza et al. 2011; Hirz et al. 2013). The effect of this change in the sterol composition was studied with regard to yeast growth characteristics as well as to homologous and heterologous membrane protein expression. A set of sterol-engineered strains of *S. cerevisiae* was produced in order to characterise sterol structure requirements of membrane proteins, in particular of the yeast ABC transporter Pdr12p. Pdr12p function required ergosterol and, thus, cells with modified sterol composition were less resistant to weak organic acids. Speculating that GPCRs require specific sterol interaction, sterol-engineered *S. cerevisiae* strains producing cholesterol-like sterols were employed for expression of β_3-adrenergic and μ-opioid receptors showing two- to threefold higher protein yield than wild-type strains (Kitson et al. 2011). Morioka et al. (2013), furthermore, investigated the influence of sterol composition on the activity of Ste2p, an endogenous yeast GPCR. Specifically, cholesterol exerted a negative effect on the signalling activity of Ste2p. A cholesterol-producing *P. pastoris* strain was superior in functional expression of human Na,K-ATPase $\alpha3\beta1$ isoform to the wild-type strain background. The modification in membrane sterol composition resulted in enhanced protein stability and proper plasma membrane localization of the human Na,K-ATPase $\alpha3\beta1$ isoform leading to a significantly higher number of receptor-ligand binding sites on the cell surface (Hirz et al. 2013). A detailed list of membrane proteins that require specific sterols for their activity was recently published in a comprehensive review about sterol-engineered yeast (Wriessnegger and Pichler 2013).

Bocer et al. (2012) demonstrated that membrane lipid composition, especially the availability of phosphatidylserine, influenced the activity of the murine A class ABC transporter (ABCA1), which was heterologously expressed in a protease-deficient *S. cerevisiae* strain. Moreover, phosphatidylserine stabilised the α2β1 isoform of human Na,K-ATPase (Kapri-Pardes et al. 2011). In a follow-up study, it was reported that neutral phospholipids such as phosphatidylcholine and phosphatidylethanolamine stimulated Na,K-ATPase α1β1 activity (Haviv et al. 2013).

Industrial applications of heterologous membrane proteins

Heterologous expression of membrane-associated enzymes, e.g. oxidoreductases, may play a key role in the production of hydrophobic fine chemicals, representing an environmentally friendly alternative to commonly used chemical synthesis routes. In particular, cytochrome P450 (CYP450) enzymes are industrially interesting catalysts as they functionalise hydrophobic substances by stereo- and regioselectively introducing hydroxyl groups. Hence, CYP450s provide access to highly demanded compounds (reviewed by Bernhardt 2006). CYP450 activity is irrevocably linked to a finely balanced system of cofactor recycling, oxygen supply, correct integration of iron into the active site and perfect teamwork with the corresponding reductase that delivers electrons (Gu et al. 2003; Henderson et al. 2003; Omura 2010). Szczebara et al. (2003) have developed a whole-cell system for hydrocortisone production in *S. cerevisiae*. A fully self-sufficient biosynthesis pathway was introduced by expressing mammalian CYP11A1, CYP11B1, CYP17A1 and CYP21A1 in combination with adrenodoxin (ADX) and adrenodoxin reductase (ADR). These modifications along with additional engineering steps to avoid unwanted side reactions enabled the production of hydrocortisone from simple carbon source. The fission yeast *S. pombe* has also been successfully used for bioconversion of 11-deoxycortisol to cortisol by expressing human CYP11B1 under the control of the *nmt1* promoter in a newly developed integration vector (Drăgan et al. 2005). Another prominent example for the application of CYP450 proteins is the production of the antimalarial drug artemisinin (reviewed by Arsenault et al. 2008; Brown 2010). Recently, a very efficient semi-synthetic approach was developed using *S. cerevisiae* for the production of up to 25 g L^{-1} of artemisinic acid, which can be further chemically converted into artemisinin. Complex metabolic engineering steps, including the balanced overexpression of cytochrome b_5 (CYB5), amorphadiene oxidase (CYP71AV1) and its associated reductase CPR1 from *Artemisia annua*, were required (Paddon et al. 2013). Various engineering strategies employing yeasts in producing high value terpenoids, e.g. carotenoids, steroidal hormones, aroma compounds or vitamin precursors, have been reviewed recently (Wriessnegger and Pichler 2013). The successful production of the sesquiterpenoid (+)-nootkatone in *P. pastoris* yielding up to 208 mg L^{-1} in bioreactor cultivations proved that *P. pastoris* also is a suitable host for terpenoid biosynthesis. Production of (+)-nootkatone from simple carbon sources was facilitated by genomic integration of plant-derived premnaspirodiene oxygenase, cytochrome P450 reductase (CPR) and valencene synthase (Wriessnegger et al. 2014).

Other industrially relevant compounds that can be produced or converted upon heterologous membrane protein expression in yeast include (poly-)unsaturated fatty acids (PUFAs), which are widely used as dietary supplements, as mammals are not able to synthesize omega-6 and omega-3 fatty acids. PUFAs are produced by expression of the corresponding membrane-bound fatty acid desaturases (FADs), which almost exclusively originate from plant cells (reviewed by Veen and Lang 2004). FAD2 (Δ12-) desaturases from different plants, amongst others soybean, olive or moss, were cloned and expressed in *S. cerevisiae* for the accumulation of fatty acids normally not present in baker's yeast such as linoleic acid (18:2) (Chodok et al. 2013; Hernández et al. 2005; Li et al. 2007). Plants differ from other higher eukaryotes by harbouring two different types of desaturases: ER-associated as well as plastidial enzymes (Ohlrogge and Browse 1995). Evolving from the same ancestor though, they display different substrate specificities arising from their distinct cellular localization (Sperling et al. 2003). Due to the lack of chloroplast membranes in yeast, expression of heterologous plastidial omega-3-desaturases led to unsatisfying quantities and activities in yeast (Domergue et al. 2003a, Domergue et al. 2003b). However, Venegas-Calerón et al. (2009) showed that co-expressing sunflower plastidial *Ha*FAD7 in *S. cerevisiae* with photosynthetic ferredoxin originating from the same species resulted in a tenfold increase in desaturase activity compared to the values obtained without the additional electron donor.

Oleosins, structural membrane proteins of plant oil bodies, have been studied for biotechnological applications such as fusion strategies for purification or as emulsifying agents (reviewed by Capuano et al. 2007; Bhatla et al. 2010). An important aspect of studies on oleosins included the intracellular translocation in *S. cerevisiae* (Beaudoin et al. 2000). C- and N-terminal truncations of wild-type oleosins did not seem to impair microsomal localization in yeast (Beaudoin and Napier 2002). Very recent studies have shown that overexpressed oleosins mainly accumulate in lipid droplets (LDs) and only accumulate in ER membranes if LD formation is impaired (Jacquier et al. 2013; Jamme et al. 2013; Vindigni et al. 2013). Interestingly, oleosins are not only structural proteins, but also comprise enzymatic activities as

monoacylglycerol acyltransferases and phospholipases (Parthibane et al. 2012). Oleosin-fusion proteins accumulating in oil bodies or being targeted to oleosomes can easily be separated via floatation. Subsequently, oleosins are removed by site-specific peptidases as shown for expression of mCherry and cohesin linked to sesame oleosins in *Y. lipolytica* (Han et al. 2013). This strategy may represent a smart and easy to handle protein purification approach.

Strategies for improving membrane protein expression in yeast

Despite many recent breakthroughs in the field of membrane protein expression, progress in structural analysis of membrane proteins lags behind studies of soluble proteins. Membrane protein structures are still dramatically under-represented in the structural databases, e.g. PDB (http://www.rcsb.org/pdb/home/home.do). Usually, membrane proteins perform poorly in overexpression systems and tend to be instable in detergent solutions required for the membrane extraction and purification steps. Consequently, the major bottleneck for obtaining membrane protein structures is obtaining milligram quantities of pure, stable and functional membrane protein. Newby et al. (2009) postulated that successful protein crystallization can actually be predicted by the behaviour of pure, homogeneous and stable protein solutions. Criteria for a high chance of successful crystallization should be >98 % pure, >95 % homogeneous and >95 % stable protein when stored in solution at 4 °C for at least 1 week. It was stated that an amount of 2 mg of protein meeting these criteria should be a useful starting point for crystallization screening, e.g. 200 μL of purified protein at a concentration of 10 mg mL^{-1}. To meet these criteria, large efforts have been undertaken in optimising target proteins, in developing versatile expression systems or in establishing purification strategies to yield sufficient membrane protein for structural determination (Bannwarth and Schulz 2003; Vinothkumar et al. 2013). Here, we describe strategies that have been shown to improve the production of heterologous membrane proteins in yeast (see Figs. 1 and 2). Developments and improvements in membrane protein structure determination have been recently reviewed (Bill et al. 2011; Lieberman et al. 2011; Sonoda et al. 2010).

Characteristics of membrane proteins overexpressed in yeast

Membrane proteins are very diverse in structure and physicochemical properties. Hence, they behave in an unpredictable way upon overexpression. Therefore, various constructs are often tested in diverse expression hosts. Frequently, an appropriate combination and optimisation of target gene, vector and expression host maximizes the amount and quality of protein produced (Bernaudat et al. 2011). Several studies using *E. coli* and/or *S. cerevisiae* investigated the correlation between protein expression level and protein characteristics. Overexpression of >300 homologous inner membrane proteins in *E. coli* did not provide any correlation between protein features and expression level (Daley et al. 2005). In contrast, White et al. (2007) correlated protein-specific parameters, i.e. size, number of transmembrane domains or hydrophobicity to the expression levels of 1,092 predicted membrane proteins of *S. cerevisiae*. Like for soluble proteins, the size of the membrane proteins was one decisive factor for high-level expression. More than 40 % of homologous membrane proteins smaller than 60 kDa were expressed well in *S. cerevisiae* compared to less than 20 % of proteins larger than 80 kDa. The inverse correlation between the number of transmembrane segments in a protein and level of expression had been reported previously (Gelperin et al. 2005) and was confirmed by White et al. (2007). Interestingly, high-level membrane protein expression was positively correlated with the hydrophobicity of predicted transmembrane segments. Hence, it was postulated that increasing the proportion of hydrophobic amino acids in transmembrane segments of membrane proteins or decreasing the overall content of aromatic residues could be favourable for membrane protein yields (White et al. 2007).

Improvements on the genetic level: optimising coding sequences and copy numbers

P. pastoris has become the most attractive host for high-level production of GPCRs and aquaporins (Krettler et al. 2013; Oberg et al. 2009; Singh et al. 2012a; Singh et al. 2012b; reviewed by Hedfalk 2013; Ramón and Marín 2011). Lately, major improvements in protein expression yields were implemented by optimising the nucleotide sequences encoding the protein of interest. Modulation of the translation initiation efficiency by optimisation of the surrounding of the start codon has been shown to be an important factor in expression of membrane proteins in *P. pastoris* (Oberg et al. 2009). Aquaporins with a G base in position +4 of the coding DNA sequence, representing a typical mammalian Kozak sequence, were better expressed than those using the yeast consensus sequence (ATGTCT). Thus, depending on the protein to be produced, changing the sequence to a host consensus sequence, e.g. of *P. pastoris*, may often not be advantageous for protein yields. The original codon usage and its compatibility to that of the host usually need to be considered. Krynetski et al. (1995) demonstrated that optimisation of the start codon context by insertion of adenosine residues upstream of the CYP2D6 gene improved the yields of the recombinant protein.

Furthermore, heterologous expression of aquaporins in *P. pastoris* strongly responded to an increase in recombinant

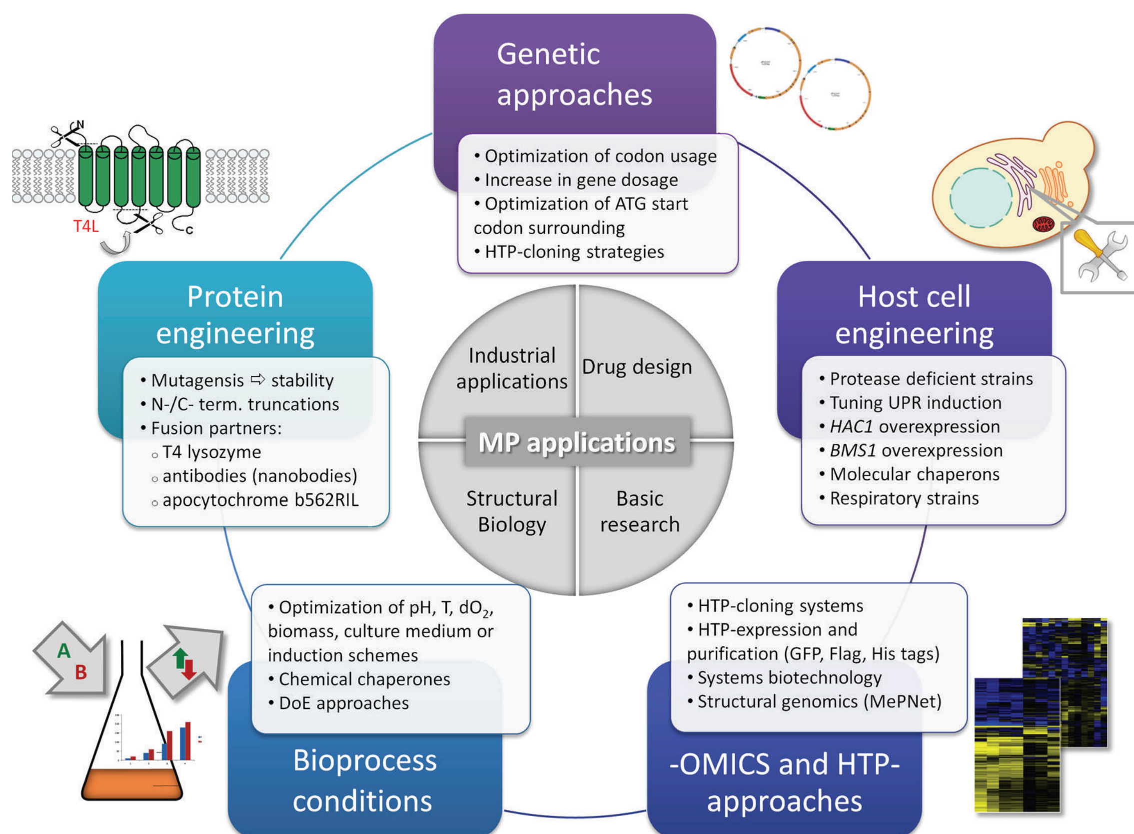

Fig. 2 Strategies for improving recombinant membrane protein expression in yeast

gene-dosage, whereupon protein folding and membrane localization seemed to be unaffected by increased expression levels (Nordén et al. 2011). Another expression study showed that a combination of codon optimisation, high gene-dosage and clone selection was important for production of aquaporins in *P. pastoris* (Öberg et al. 2011). Similarly, the human μ-opioid receptor was expressed at elevated levels in *P. pastoris* after optimising gene copy number, strain background, temperature, pH, and methanol induction (Sarramegna et al. 2002). For the HT_{5A} 5-hydroxytryptamine and the human β_2-adrenergic receptors, the yield of functional protein was increased up to twofold when the number of gene copies was increased from one to two or six (Weiss et al. 1998).

Protein engineering for enhanced protein stability and/or activity

Introduction of specific mutations may lead to improved stability and, therefore, higher yields of functional recombinant proteins as demonstrated for human aquaporin produced in *P. pastoris* (Oberg and Hedfalk 2013; Oberg et al. 2009) or for a eukaryotic nucleobase-ascorbate transporter expressed in *S. cerevisiae* (Leung et al. 2013). Mutagenesis techniques are also frequently and successfully applied to study the interaction of GPCRs with ligands, their mechanism of activation and their interaction with G-proteins (Beukers and Ijzerman 2005; Lundstrom et al. 2006). For several examples of GPCRs, protein engineering was required prior to crystallisation studies. Protein engineering measures involved truncation of N- and/or C-terminal domains (Shiroishi et al. 2011), implementation of point mutations stabilising the receptor in a specific conformation (Lebon et al. 2011; Serrano-Vega and Tate 2009; Serrano-Vega et al. 2008; Standfuss et al. 2007; Miller and Tate 2011) and insertion of T4-lysozyme (T4L) into the third intracellular loop critical for G-protein binding (Shiroishi et al. 2011; Mathew et al. 2013). The T4L fusion strategy was successful for the crystallisation of several receptors expressed in non-yeast host systems, e.g. insect cells (Ashok et al. 2013; Cherezov et al. 2007; Hanson et al. 2012; Jaakola et al. 2008; Rasmussen et al. 2011b; Wu et al. 2010; Xu et al. 2011; Zou et al. 2012). The expression of the human histamine H1 receptor is an example for the application of the T4L fusion strategy in combination with N-terminal truncations using *P. pastoris* as host system (Shiroishi et al. 2011).

GPCRs can be crystallized in different conformational states with the help of antibodies/nanobodies (Day et al. 2007; Rasmussen et al. 2011a; Rasmussen et al. 2007; Steyaert and Kobilka 2011). These functional antibodies can modulate the activity of a target protein, probably due

to stabilizing a particular conformation and/or preventing conformational changes of the protein (Hino et al. 2013). Previously, Hino et al. (2012) had crystallized the human A_{2A} adenosine receptor in complex with a Fab-fragment that prevented agonist but not antagonist binding to the extracellular ligand-binding pocket. The protein, expressed in *P. pastoris*, contained an intact intracellular loop 3, in contrast to many other GPCR structures, which had been fused to lysozyme.

Yeast host cell engineering strategies

Membrane proteins are expressed in various yeasts. However, the selection of the most appropriate host system may be essential for efficient membrane protein expression (Bernaudat et al. 2011; Bill 2014; Midgett and Madden 2007). Comparison of membrane protein yields in several expression hosts revealed that the best host usually was the evolutionarily most closely related one to the source of the target protein (Grisshammer and Tate 1995). Since then, major progress has been made in optimisation of strategies to improve the productivity of selected host systems and, especially, to understand the molecular mechanisms and bottlenecks for achieving high membrane protein yields (Ashe and Bill 2011; Bawa et al. 2011; Bill et al. 2011; Grisshammer 2006). Using the *P. pastoris* expression system, protease-deficient strains such as SMD1163 led to a two-to-eightfold improvement in expression levels of the mouse 5-HT_{5A} serotonin receptor (Weiss et al. 1995). The same group described a threefold enhancement in expression of the 5-HT_{5A} 5-hydroxytryptamine receptor in protease-deficient *P. pastoris* cells by fusing the gene to the *S. cerevisiae* α-factor leader sequence (Weiss et al. 1998). A clear correlation between Fps1 (eukaryotic glycerol facilitator) yield and an increase in *BMS1* transcript number was observed when analysing high-yielding host strains (Bonander et al. 2009). Engineering of host cells by tuning *BMS1* transcript levels in a doxycycline-dependent manner resulted in optimised yields of functional membrane proteins and soluble targets. Polysome profiling revealed that the reason for this high-yielding phenotype is a changed ribosomal subunit stoichiometry, hence, a change in translational efficiency. This is consistent with the role of the gene product of *BMS1* in ribosome biogenesis (Ashe and Bill 2011).

In a recent study, a respiratory *S. cerevisiae* strain TM6* showed at least a doubling in productivity over wild-type strains for three recombinant membrane proteins (Ferndahl et al. 2010). This strain mediates low sugar uptake rates and, thus, the strain does not produce ethanol even in the presence of high external sugar levels (Elbing et al. 2004). Hence, this *S. cerevisiae* strain represents a valuable host as its elevated biomass does lead to increased volumetric yields without the need for special cultivation conditions. The expression level of rat Na^+/H^+-antiporters in yeast plasma membrane was improved by using a strain harbouring an *npi1* mutation, which significantly lowered the amount of Rsp5 ubiquitin ligase in the cell leading to enhanced stability of the protein in the plasma membrane (Flegelova et al. 2006).

Several studies demonstrated that membrane proteins may have specific lipid requirements necessary for proper expression, activity, folding or stability (reviewed in Opekarová and Tanner 2003; Wriessnegger and Pichler 2013). Recently, the engineering of the *P. pastoris* sterol metabolism towards cholesterol production resulted in improved accommodation of active human Na,K-ATPase in cellular membranes (Hirz et al. 2013), suggesting to be a promising approach for successful expression of further human membrane proteins. Concurrently, Guerfal et al. (2013) engineered *Y. lipolytica* resulting in enhanced levels of eight different integral membrane proteins. Membrane hyperproliferation was achieved by deleting the phosphatidic acid phosphatase gene *PAH1*, which leads to a re-direction of fatty acids from storage lipids towards membrane lipids. Accumulation of intracellular membranes afforded enhanced expression levels and enhanced resistance to proteolytic degradation. Furthermore, co-induction of the unfolded protein response (UPR) pathway in such *Δpah1* strains improved the quality of the overproduced membrane proteins.

Membrane protein expression and yeast stress responses

Environmental stress response (ESR) of expression host cells may be triggered by the cultivation conditions. Genome-wide transcriptional changes after exposure of yeast cells to environmental stresses like heat shock, acidic pH, high osmolarity and different chemicals were intensively investigated (Causton et al. 2001; Gasch et al. 2000; Lelandais and Devaux 2010). However, little information is available on yeast stress response upon recombinant protein expression. ESR-related genes are up- or down-regulated transiently as a reaction to the stressful conditions and return to near-normal expression levels after adaptation to the new environmental conditions. During this transition phase, the cells reprogram their metabolism in order to reach a new homeostasis (Mattanovich et al. 2004). The ESR triggered by elevated temperature or heat shock has been investigated, but adaptation to cold shock or reduced temperatures is not well-described, although lowered temperatures are often applied for the production of heterologous proteins in yeasts. Several expression studies, mainly applying soluble target proteins, characterised the advantage of lowering the cultivation temperature (Jahic et al. 2003b) and/or pH (Curvers et al. 2001; Jahic et al. 2003a; Cregg et al. 2000) on protein production due to reduced cell lysis or lower host cell protease activity.

In contrast to soluble proteins, a major bottleneck in overexpression of membrane proteins appears to be the limitation in membrane capacity for accommodating additional proteins, due to intrinsic maintenance of an optimal ratio between lipids and membrane proteins (Drew et al. 2003; Wright et al. 1988). Enhanced synthesis and insertion of heterologous proteins into membranes affects membrane integrity and cell functionality, and therefore may lead to an activation of the cellular stress response pathway (Wagner et al. 2006). Overproduction of ER-resident membrane proteins in yeast led to enhanced proliferation of ER membranes as shown for HMG-CoA reductase and CYP52A3 (Menzel et al. 1997; Takewaka et al. 1999; Wiedmann et al. 1993; Wright et al. 1988). Activation of the UPR pathway has been documented as a consequence of the expression of soluble (Mori et al. 1992) and membrane-bound proteins (Griffith et al. 2003) in *S. cerevisiae*. The UPR senses the increase in unfolded protein within the ER and regulates the transcription of UPR target genes encoding chaperones, foldases and proteins involved in glycosylation or lipid metabolism. UPR in yeasts is activated by the non-conventional splicing of the *HAC1* mRNA (Drew and Kim 2012a; Patil and Walter 2001; Walter and Ron 2011). UPR has been suggested as a target for improvements in heterologous membrane protein yield (Griffith et al. 2003; Mattanovich et al. 2004). Overexpression of *P. pastoris HAC1* has been shown to improve the correct processing of the α-mating factor signal sequence in front of the adenosine A_{2A} receptor (Guerfal et al. 2010) leading to a more homogeneously dispersed receptor protein, but expression level of the receptor was not affected, as observed for soluble proteins. *HAC1* overexpression does not seem to be a generally applicable strategy and, therefore, needs to be evaluated on a case-to-case basis. Investigation of the expression levels of 12 GPCRs from the rhodopsin family of receptors in *S. cerevisiae* indicated that problems with GPCR folding and trafficking start at the point of translocation into the ER membrane, which led to the activation of downstream cellular stress responses (O'Malley et al. 2009). The expressed GPCRs associated with the ER-resident chaperone BiP/Kar2p, which is known to bind exposed hydrophobic regions of misfolded proteins. BiP/Kar2p was bound to most of the receptors except $hA_{2A}R$, which was properly folded and trafficked out of the ER. Another study on $hC_{B2}R$ had suggested that signal sequence processing may be critical for GPCR production in *P. pastoris (*Zhang et al. 2007*)*. Therefore, incomplete processing of the pre-pro leader sequence might have led to receptor misfolding and/or the adoption of an unexpected topology within the ER membrane (O'Malley et al. 2009).

Optimisation of yeast cultivation conditions

P. pastoris has been described to adapt easily to large-scale cultivation in bioreactors (Cereghino et al. 2002). Hence, Singh et al. (2008) developed a large-scale fermentation protocol for the production of significantly higher levels of functional $A_{2A}R$ compared to traditional shake flask cultures. In this work, the authors suggested that the protocol was also suitable for large-scale production of the human dopamine D2 and serotonin 5HT1D receptors (see also Singh et al. 2012b). A beneficial effect of increased pre-induction biomass accumulation on protein yield was described for several soluble proteins (Holmes et al. 2009). However, for membrane proteins, this correlation was not necessarily observed (Bonander et al. 2005). A recent study showed that an optimised induction scheme using non-selective rich medium yielded higher biomass and improved protein production by a factor >3 in *S. cerevisiae* (Drew and Kim 2012b). Growth in the simple Yeast nitrogen base (YNB)-based medium typically yielded low biomass and less recombinant membrane protein compared to Centralbureau voor Schimmelcultures (CBS) medium (reviewed in Bonander and Bill 2012). The latter is much more labour intensive to prepare, though. Medium optimisation studies showed that supplementing YNB-based medium with *myo*-inositol to levels similar to the CBS medium yielded improved growth rates and protein levels. The positive effect of *myo*-inositol was attributed to its essential role in the relief of cellular stresses during membrane protein expression (Gaspar et al. 2006). It has been shown previously that addition of soy peptides improves yeast tolerance to freeze–thaw stress or changes in lipid metabolism (Ikeda et al. 2011). Recently, the production of six of eight selected GPCRs in *S. cerevisiae* was enhanced ~2.3-fold when using soy peptide containing medium as compared to amino acid-based medium (Ito et al. 2012).

Improving membrane protein expression by proteinaceous and chemical chaperones

Prerequisite for the expression of functionally active and correctly targeted integral membrane proteins are proper folding, maturation and transport processes in the host cells. Typically, membrane proteins—excluding proteins destined for peroxisomal or mitochondrial membranes—enter the secretory pathway by translocating into the ER membrane where folding and maturation of the proteins take place (Alder and Johnson 2004; Hebert and Molinari 2007). Consequently, one strategy to improve the functional yield of membrane proteins has been co-expression of chaperones in analogy to successful approaches in the recombinant expression of soluble proteins. Butz et al. (2003) investigated whether the co-overexpression of ER-resident proteins like PDI, calnexin or BiP/Kar2p in yeast would improve total and/or active GPCR yields. They showed that expression of human A_{2A} adenosine receptor (hA_{2A}) and mouse substance P receptor (SPR) was not limited by any folding bottleneck. This conclusion was based on the facts that receptor yields were

unchanged upon co-expression of ER chaperones, that no ER-retention of GPCRs was observed by confocal microscopy, and that the trafficking dynamics of both receptors was insensitive to gene copy number. In contrast, the deletion of the *CNE1* gene, a yeast homologue of the mammalian chaperones calnexin and calreticulin, yielded higher levels of human transferrin receptor in *S. cerevisiae* (Prinz et al. 2003). Overall, modulation of chaperone levels has not been overly successful in boosting membrane protein expression in yeasts, probably because the knowledge on chaperones governing membrane protein folding is very limited.

Another strategy to improve GPCR expression for structural elucidation is the addition of ligands to the culture medium that may function as molecular chaperones by binding to the incompletely folded receptors and enhance their release from the ER-retention machinery (Bernier et al. 2004; Fraser 2006; Grünewald et al. 2004; Petäjä-Repo et al. 2002; Weiss et al. 1998). As part of a European membrane protein network (MePNet, see http://www.mepnet.org), with the aim to increase yields of functional GPCRs (Lundstrom et al. 2006), André and co-workers optimised the expression of 20 GPCRs in *P. pastoris* (André et al. 2006). Besides adjusting expression temperature and supplementing with chemical chaperones, the addition of GPCR-specific ligands to the *P. pastoris* culture media increased the yield of functional GPCR to more than eightfold over standard expression conditions. Supplementation of yeast culture media with chemical chaperones, such as DMSO, histidine or glycerol, has been beneficial for membrane protein yield (André et al. 2006; Drew and Kim 2012b; Figler et al. 2000; Fraser 2006; Shukla et al. 2007b; Weiss et al. 1998). Addition of DMSO to the growth medium dramatically altered the expression pattern of yeast (Zhang et al. 2003) and altered the membrane properties of several organisms by up-regulating genes involved in membrane lipid synthesis (Murata et al. 2003). Recent studies using genome-wide screens in *S. cerevisiae* identified major cellular processes that are sensitive to DMSO addition including ER/Golgi transport, chromatin remodelling, DNA repair and cell wall integrity (Gaytán et al. 2013; Zhang et al. 2013a). Furthermore, DMSO increased the permeability of membranes, thus enhancing the access of externally added ligands to membrane proteins (Yu and Quinn 1994). The addition of 2.5 % DMSO to *P. pastoris* cultivation medium resulted in sixfold increased protein production compared to standard conditions for 16 of 20 tested membrane receptors (André et al. 2006). Like for many chaperones, DMSO supplementation was not key to generally high heterologous membrane protein yields, as for several proteins no or a negative effect was observed (Shiroishi et al. 2012).

The addition of histidine to the culture medium was suggested to have a positive effect on the yield of membrane proteins. Twelve of 20 tested GPCRs showed enhanced yields upon supplementation of *P. pastoris* medium with histidine, but with a moderate improvement factor compared to other tested optimisation parameters (André et al. 2006). It is assumed that histidine, rather than other amino acids, can act as a physiological 'antioxidant' in yeast cells (Murakami et al. 1997), but actually there is no data available to support this assumption. Glycerol was used as chemical chaperone to obtain high yields of active human P-glycoprotein in *S. cerevisiae* (Figler et al. 2000). Cells cultured in media supplemented with 10 % glycerol showed a 3.3-fold increase in membrane-localized P-glycoprotein relative to controls. The positive effect was ascribed to glycerol stabilising the conformation of proteins, thus enhancing membrane protein folding and maturation.

High-throughput approaches for evaluation of membrane protein expression in yeast

A major challenge in optimising heterologous membrane protein expression is to quantify the obtained expression levels, ideally in a high-throughput approach. Especially when genomically integrating expression cassettes, it is required to carefully screen for the transformants that exhibit the best expression level. Multi-copy integrations and the actual integration loci might strongly influence expression yields (Grünewald et al. 2004; Macauley-Patrick et al. 2005). To facilitate the screening process for highly expressing 'jackpot' clones in *P. pastoris*, Brooks et al. (2013) developed a simple fluorescent plate assay using C-terminal GFP fusions for detection. Using this method, a large number of clones can obviously be screened in a simple and rapid way to search for highly expressing strains. Furthermore, the plate screen may obviate the need for further testing in liquid culture due to good correlation between plate fluorescence, liquid culture fluorescence and protein expression.

A high-throughput screening method based on fusion of GFP to membrane transporters has been developed by Newstead et al. (2007), and was recently further developed for large-scale membrane protein production for structural and functional studies (Drew and Kim 2012a). The advantage of GFP-fusion proteins is that the fluorescence resulting from overexpression can be measured in a fast, efficient and reliable way in liquid cultures, directly in standard SDS gels or in detergent-treated crude membrane preparations. The stability and monodispersity of proteins in detergent-solubilised membranes may be analysed by fluorescence size-exclusion chromatography (FSEC) for judging the quality of the recombinant material (Drew and Kim 2012c; Kawate and Gouaux 2006). This strategy allows for the selection of membrane protein-GFP fusions, which are well expressed and stable in detergent, and thus applicable for large-scale membrane protein production and purification (Drew and Kim 2012a). The successful GFP-based pipeline for rapid construction and evaluation of membrane protein yield, predominately shown

for transporters expressed in *S. cerevisiae*, was further developed for production of human GPCR variants for structural studies (Shiroishi et al. 2012). Recently, Scharff-Poulsen and Pedersen (2013) reported the production of eukaryotic nutrient transporters and transceptors at high quality in *S. cerevisiae* using a high-copy vector expression system (Pedersen et al. 1996) combined with the GFP-fusion methodology developed by Drew et al. (2008). The same strategy resulted in the production of human aquaporin-1 in *S. cerevisiae* at exceptionally high levels (Bomholt et al. 2013). Overall, the applicability of the GFP-based pipelines for screening and producing high amounts of functional membrane proteins was proven for a variety of eukaryotic membrane proteins.

Within the Membrane Protein Network initiative (MePNet), a versatile, high-throughput Dot-blot immunodetection methodology allowing for the rapid and easy quantification of expression levels of numerous GPCRs was developed. The method was applied for selecting GPCR expressing clones for further characterisation and optimisation (André et al. 2006). Furthermore, a medium-throughput pipeline to speed up the timelines and reduce the cost of identifying targets amenable to large-scale purification, crystallisation and functional characterisation was described (Li et al. 2009). By ligation independent cloning, 384 target genes were integrated into *S. cerevisiae* expression vectors and the expression levels of 272 targets were determined by semi-quantitative Western blotting. Initially, the approach had been developed for endogenous membrane proteins of *S. cerevisiae*, but its applicability to heterologously expressed membrane proteins was demonstrated by the expression of ten human integral membrane proteins from the solute carrier superfamily.

Summary and outlook

Yeasts are versatile and extremely powerful systems for the expression of membrane proteins of higher eukaryotes and the investigation of their functions. Many obstacles exacerbating the applicability of membrane proteins in biochemical and structural studies can be handled in yeasts. Membrane proteins are tricky to manipulate in solution, need correct intracellular targeting in eukaryotic hosts and require an appropriate membrane environment for optimal activity and/or structural function. Cell- and protein engineering strategies as well as the addition of chemical chaperones or ligands overcome most of these difficulties in yeast hosts. Moreover, fine-tuning of expression and cultivation conditions ultimately increases protein yields. Membrane proteins are highly diverse in structure, function and interaction with their environment; similarly diverse are their requirements in heterologous expression. Currently, there is no one-solution-fits-all approach in recombinant expression of membrane proteins from higher eukaryotes in yeast(s), and most probably there never will be. Consequently, it is difficult to provide general guidelines for which yeast host to choose and which additional measures to take if starting a novel membrane protein expression project in yeast. Based on the knowledge already derived and reviewed here, our advice is to compare the structural features of the to-be-expressed membrane protein, e.g. in terms of overall fold, number of TMDs, surface charges, known molecular interaction partners, etc., to the available literature. We would start with the yeast host and expression conditions of the closest fit. The approach will be different if considering application of the recombinant yeast harbouring heterologous membrane proteins as whole-cell biocatalyst for metabolite production. In this case, the availability of metabolic pathways and cellular metabolite transport routes should be the first issue to evaluate when selecting the yeast host and expression condition.

There is still a lot of room for engineering the membranes of yeasts towards improved heterologous membrane protein expression. Lipid-engineered yeast cells have not been extensively exploited, yet, and the involvement of (specific) chaperones in membrane protein folding is poorly understood. The potentials of *S. pombe*, *Y. lipolytica* and *H. polymorpha* in membrane protein expression shall be investigated more intensively. During the last 5 years, eukaryotic protein structures resolved with the help of yeast were increased almost threefold, suggesting yeast to be promising hosts for further crystallisation studies. Yeasts engineered to serve as screening tools for drug–target and other membrane protein interactions are one of the most promising fields of research, currently. Novel findings on the specific molecular interactions of membrane proteins will feed back into innovative cell-, protein- and cultivation engineering approaches that will improve heterologous membrane protein expression in yeasts in terms of protein quality and quantity.

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3.2. Research article

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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- Planning of experiments (50%)
- Performing the experiments (80%)
- Writing of the manuscript (95%)

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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

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

Melanie Hirz · Gerald Richter · Erich Leitner · Tamara Wriessnegger · Harald Pichler

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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

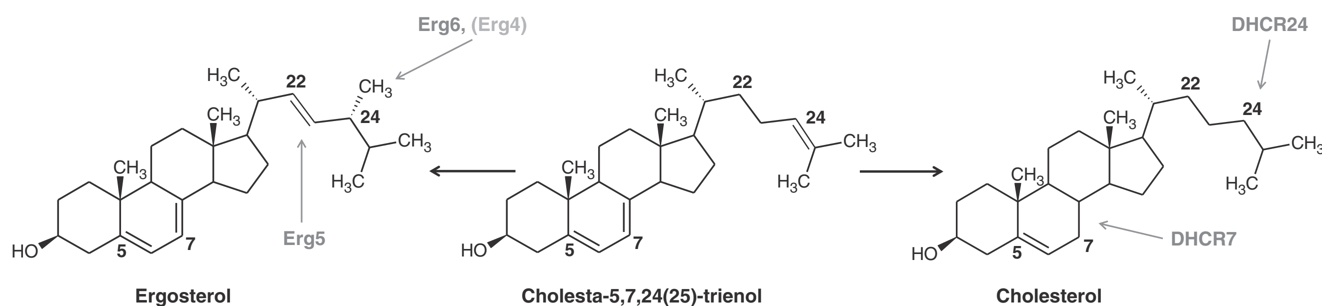


Fig. 1 Structures of ergosterol and cholesterol. The major yeast sterol, ergosterol, differs from the mammalian cholesterol lacking two double bonds at positions C-7 and C-22 and one methyl group at position C-24. The enzymes involved in ergosterol synthesis are the sterol C-22 desaturase encoded by *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 Δhis4Δpep4*	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 Δhis4Δku70 Δaox1*::*pAO815*[*5′-AOX1-α3-TT-5′-AOX1-β1-TT-HIS4*]	This work
SMD1168+ATPase	*SMD1168 Δhis4Δpep4 Δaox1*::*pAO815*[*5′-AOX1-α3-TT-5′-AOX1-β1-TT-HIS4*]	This work
S-α3β1 (+ ATPase)	*SMD1168 Δhis4Δpep4 Δaox1*::*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

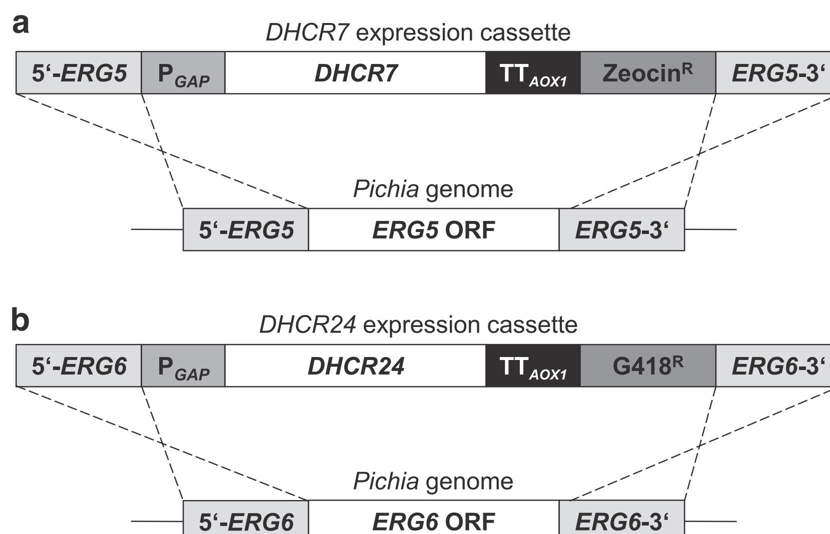


Fig. 2 Expression cassettes used for the generation of a cholesterol-producing *P. pastoris* strain. **a** The *DHCR7* expression cassette contains regions homologous to the 5′- and 3′-flanking sequences of the *ERG5* locus. Transformants were selected for Zeocin^{TM} resistance. **b** The *DHCR24* expression 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^{TM} 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

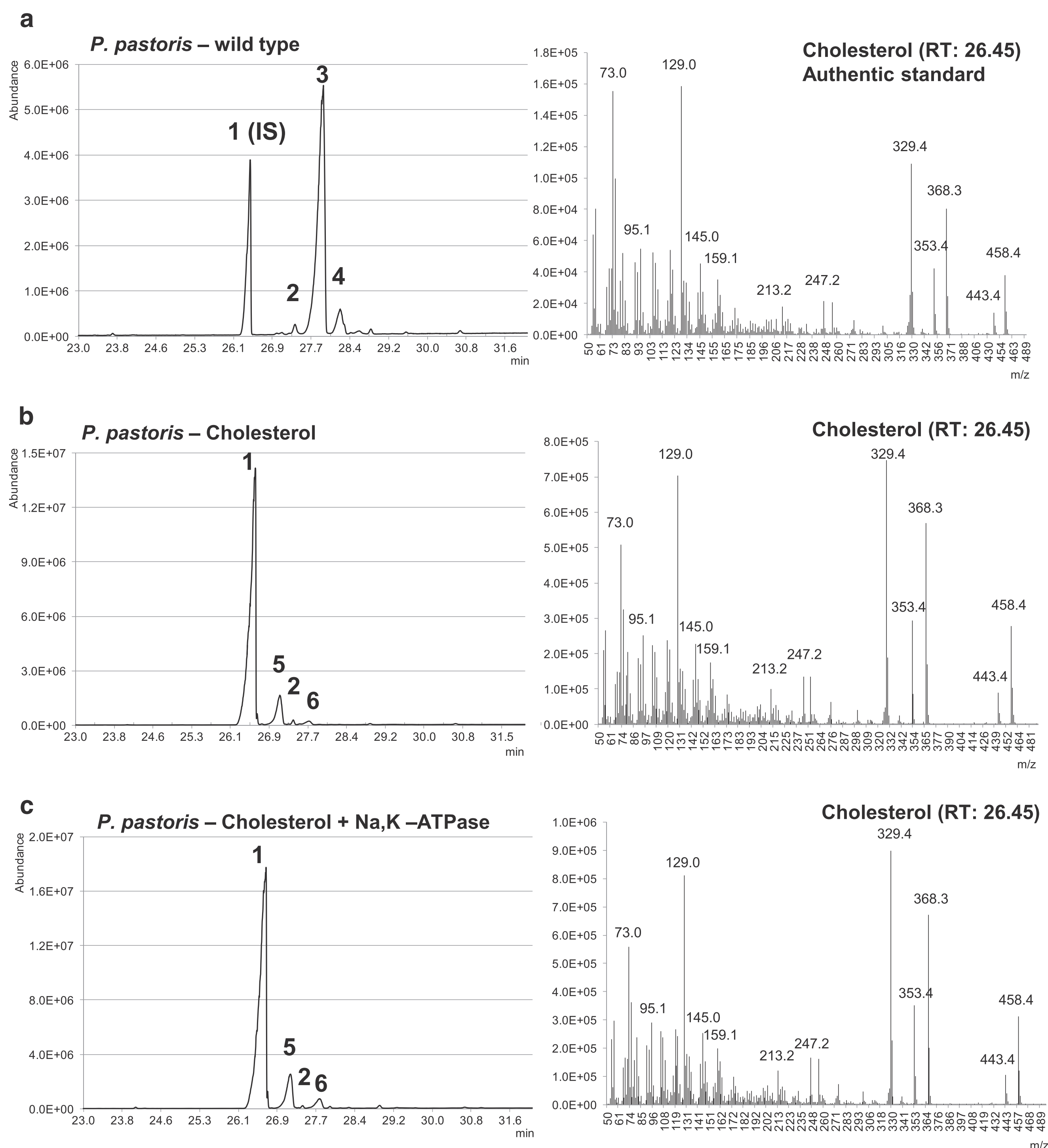


Fig. 3 GC-MS analysis of sterol extracts from *P. pastoris*. Representative chromatograms of sterols isolated from wild-type (**a**), cholesterol-producing (**b**), and cholesterol-producing as well as Na,K-ATPase expressing (**c**) *P. pastoris* strains induced in BMMY medium for 72 h are shown. The 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

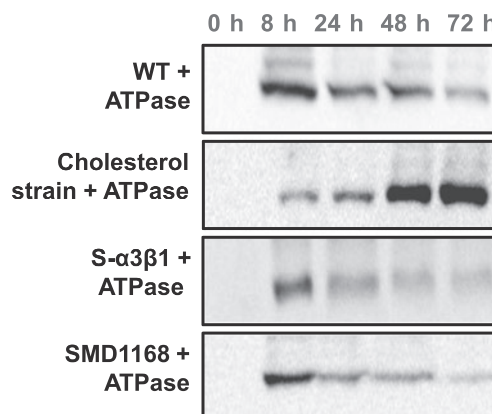


Fig. 4 Western blot detection of Na,K-ATPase subunit α3 in total cell lysates of expression strains. Expression of α3 subunit (110 kDa) was determined after 0, 8, 24, 48, and 72 h of methanol induction in cell lysates. Samples of *P. pastoris* wild-type (WT), cholesterol-producing, S-α3β1, and SMD1168 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

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.

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

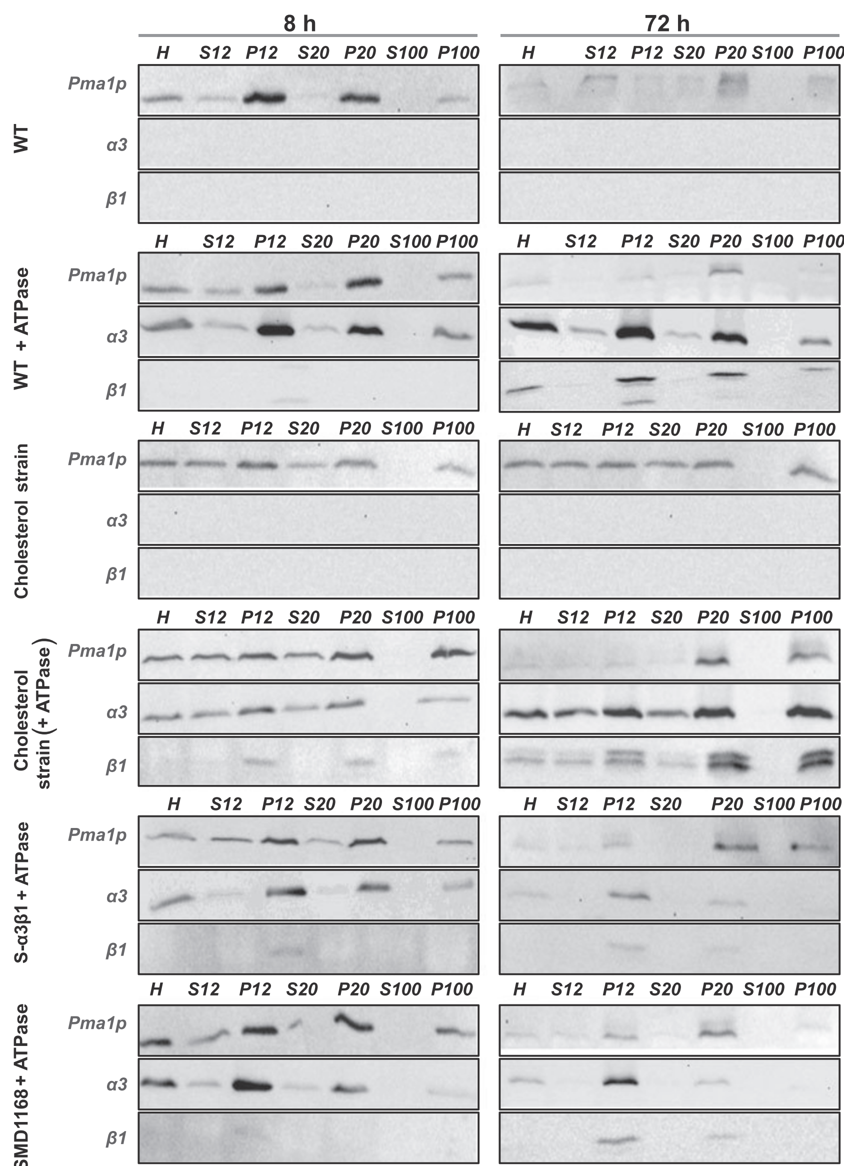


Fig. 5 Western blot detection of Na,K-ATPase subunits α3 and β1 and plasma membrane marker Pma1p in subcellular fractions. *P. pastoris* WT+ATPase, *P. pastoris* cholesterol strain+ATPase, *P. pastoris* S-α3β1(+ATPase), and *P. pastoris* SMD1168+ATPase were induced for 8 and 72 h, respectively. *P. pastoris* 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,

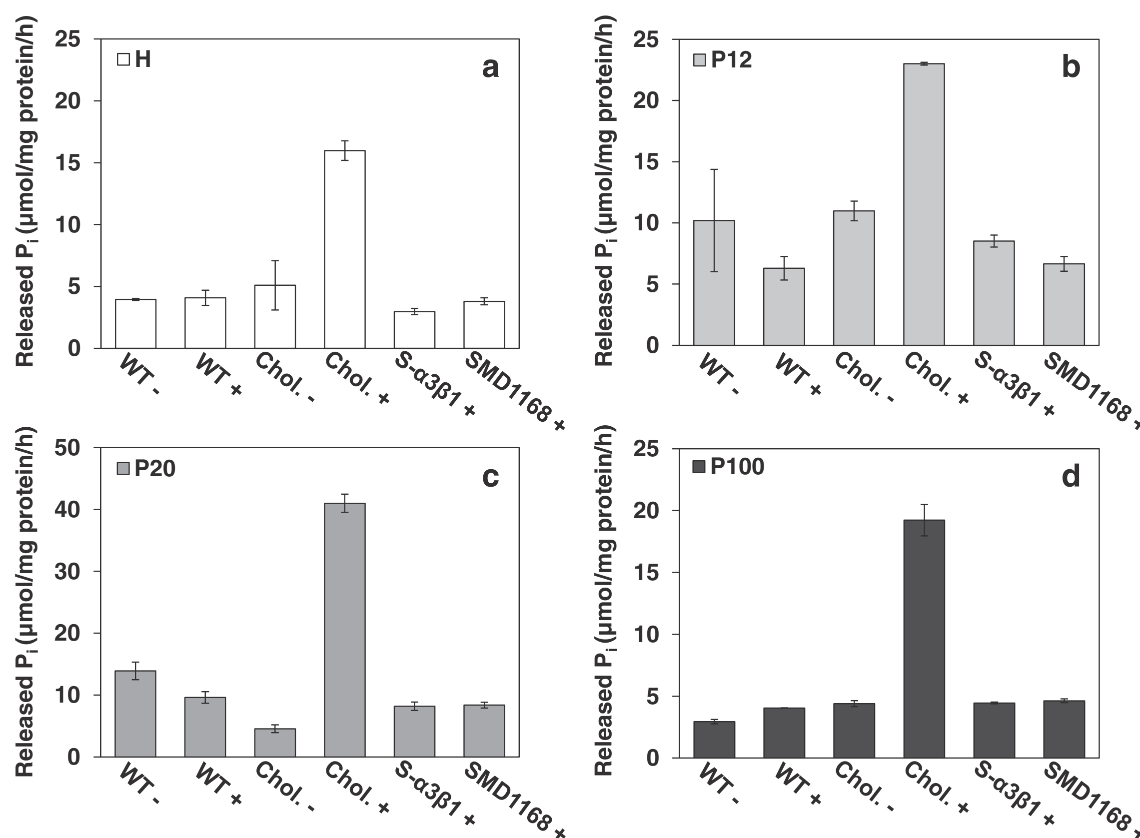
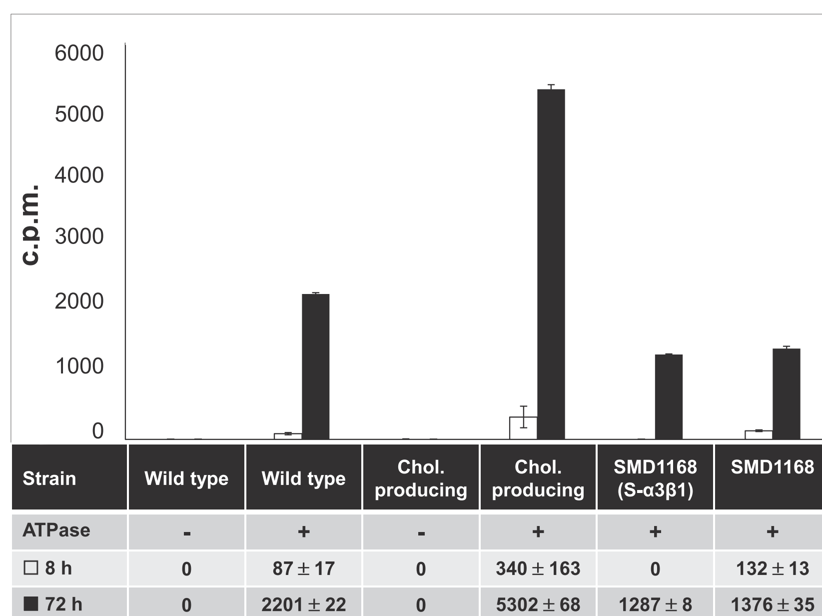


Fig. 6 Determination of ouabain-sensitive Na,K-ATPase activity. Membrane fractions H, homogenate of total cell extract, (**a**), P12 (**b**), P20 (**c**), and P100 (**d**) were isolated from cells pregrown on BMGY and induced on BMMY medium for 72 h as described in the "Materials and methods" section. 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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Chapter 4:

A Study of Sterol-engineered *P. pastoris*: Physiological, Morphological and Transcriptional Characterization

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HP and MH conceived and designed the experiments. MH performed the experiments on strain construction and characterization, spot-tests, as well as the knockout library construction and screening. MH and GZ planned and performed the electron microscopy experiments. MH, MS and JMF planned and performed experiments on cell wall isolation and characterization. MH, BD and RBG performed protein MS-analyses and MH and EL performed sterol analyses. MH analyzed the data and wrote the manuscript.

4.1. Abstract

We modified the sterol composition of the methylotrophic yeast *P. pastoris* to engineer this biotechnologically important organism for membrane protein production and to explore specific roles of sterols in cell physiology. We had demonstrated earlier, that especially the cholesterol-producing strain is a superior host for expression of the human membrane protein Na,K-ATPase α3β1. However, we also noticed that all strains with altered sterols showed slow growth phenotypes, which prompted us to examine the physiology of these sterol-engineered strains more closely.

Spot-tests revealed increased resistance of the cholesterol-producing strain towards cell wall stressors calcofluor white and congo red. Furthermore, the cholesterol-producing strain showed severe growth defects at low temperature, acidic pH, and elevated salt concentrations. We performed electron microscopy studies to take a closer look at the ultrastructure of the sterol-engineered strains under different cultivation conditions. In accordance with the observed resistance towards cell wall perturbing agents, we found extremely enlarged and highly unusual cell wall structures. Moreover, the cell wall protein Cwp1 was hyper-secreted to the culture medium and its transcription was upregulated. The cell wall polysaccharide composition, however, was not significantly altered, as determined by HPAEC-PAD following chemical hydrolysis. RNA sequencing furthermore revealed that mainly sterol-related biosynthesis genes, together with certain sphingolipid biosynthesis genes and genes of the secretory pathway were upregulated. We aimed to find molecular links between altered membrane sterols and cellular stress response mechanisms by a random gene knockout screening in the cholesterol-producing strain. By this screening, we identified several interesting genes – the TOR complex 2 subunit *TSC11,* the calmodulin dependent kinase *CMK2,* and the glycerophosphocholine acyltransferase *GPC1* – which are involved in the regulation of yeast stress response mechanisms and could be regulated by yet undefined sterol-dependent mechanisms.

Our studies show that, on the one hand, modification of sterols can be beneficial for heterologous expression of mammalian membrane proteins; on the other hand, strong effects on cell physiology, growth and stress response are inevitable consequences. Adequate sterol patterns play a tremendous role for cellular fitness. Prospectively, our sterol-modified strains can serve as interesting tool to study highly conserved eukaryotic regulatory mechanisms and stress response in *P. pastoris*.

4.2. Introduction

During the last two decades, *Pichia pastoris* (recently reclassified as *Komagataella* sp. [1]) has emerged as one of the most extensively used production hosts in biotechnology. There are plenty of well-established vector and host systems for constitutive or inducible expression systems, and also well-developed strategies to improve yields of proteins or metabolites of interest (reviewed in [2,3]). We recently added a novel strategy for heterologous expression of membrane proteins from higher eukaryotes by engineering the yeast membrane sterol content towards production of cholesterol instead of ergosterol. It has been reported for numerous membrane proteins, such as G-protein coupled receptors and ion channels, that their stability and activity strongly depend on proper sterols in their surrounding membranes [4–6]. Our strategy was shown to be beneficial for the expression of the human Na,K-ATPase α3β1 isoform [7].

Except for some research groups investigating lipid metabolism [8–10], peroxisome biogenesis [11–13], or transcriptional regulation of the *AOX1* promoter [14,15], there is little work focusing on basic research in *P. pastoris* compared to the vast knowledge available for *S. cerevisiae*. Especially when it comes to stress signaling and response mechanisms to cope with cell wall stress or environmental changes, there is very little information available for *P. pastoris*. Most of our knowledge so far is based on findings in *S. cerevisiae* or other yeasts such as *Candida albicans*. We figured that our sterol-engineered strains, which are retarded in growth and are sensitive towards different stress causing substances or conditions, may serve as well-suited models to investigate stress response in this methylotrophic yeast.

We created three different sterol-modified strains mainly producing ergosta-5-enol, 7-dehydrocholesterol and cholesterol via sequential knockout of the ergosterol biosynthesis genes *ERG5* and *ERG6*, and concomitant integration of the cholesterol biosynthesis genes *DHCR7* and *DHCR24* from *Danio rerio*. Figure 1 shows the chemical structures of ergosterol, cholesterol and their common, theoretical precursor cholesta-5,7,24(25)-trienol.

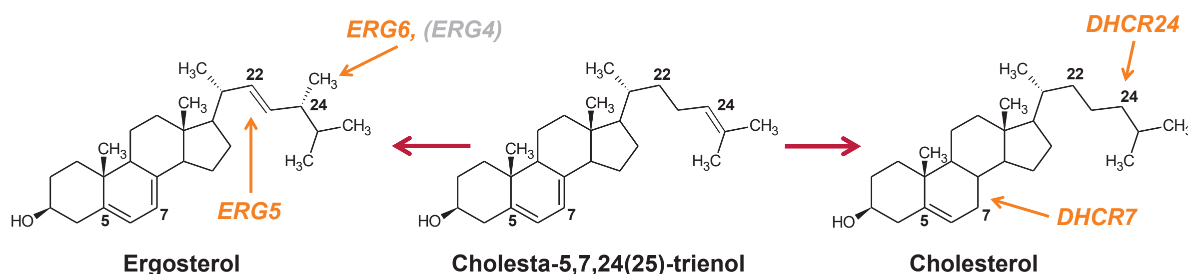

Fig. 1. Sterol structures and metabolism. To create cholesterol-producing *P. pastoris* strains, ergosterol synthesis genes *ERG5* and *ERG6* were knocked out and *Danio rerio* dehydrocholesterol reductases *DHCR7* and *DHCR24* were co-expressed. Orange arrows indicate the reaction site of the respective enzyme.

Yeast cells communicate with their environment and need to respond to external stress influences quickly. In our study, we put intrinsic stress on the cells by changing their sterol composition from ergosterol to the mammalian cholesterol. This could potentially influence the cellular interaction with other lipids, such as sphingolipids, or membrane proteins, which are often the first partners in complex cascades to transmit signals from the cell exterior to the inside. These signaling cascades result in transcriptional activation of certain genes to respond to unfavorable external conditions such as high salinity, extreme temperatures, or toxic compounds. By exposing the sterol-engineered strains to some of these stress conditions, we aimed to gain insight into potential connections between membrane sterol species and stress signaling pathways.

Yeasts have several mitogen-activated protein (MAP) kinase signaling pathways to handle changes in the environment. The best-studied pathways in *S. cerevisiae* are the response towards high osmolarity, cell wall stress, nutrient deprivation and the pheromone response. Figure 2 shows a general overview of transmembrane signaling pathways in *S. cerevisiae*. All of these processes need receptors, which are usually transmembrane proteins sensing and transmitting the exterior signal towards intracellular G-proteins or kinases. It is likely that many of these cascades are affected by an altered membrane sterol environment in our *P. pastoris* strains.

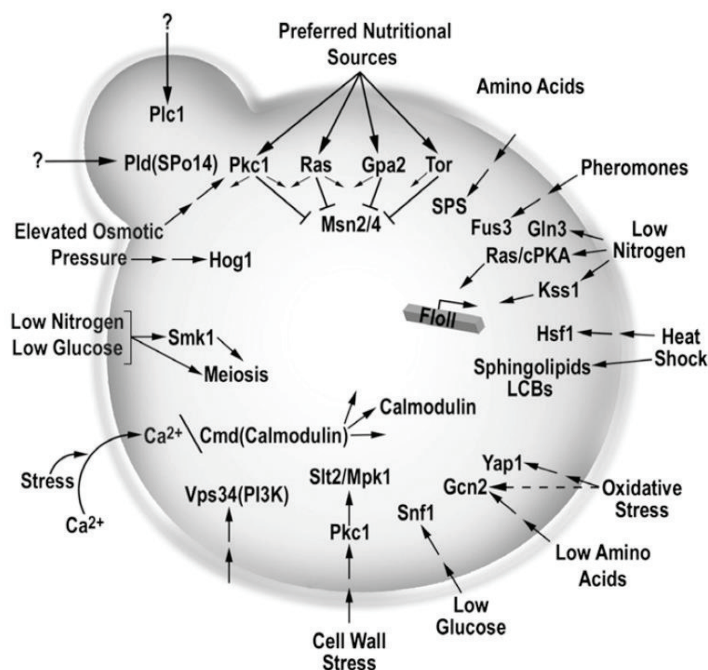

Fig. 2. Stress response mechanisms in *S. cerevisiae*. Image was taken from Engelberg et al. [16] with permission from Elsevier.

For our studies, the cell wall integrity (CWI) pathway, the high osmolarity glycerol (HOG) pathway as well as the regulation of cell growth and sphingolipid biosynthesis via target of rapamycin (TOR) complex 2 are of special interest. Therefore, these signaling pathways are shortly described.

Signaling via the CWI pathway is initiated by a group of sensor proteins located at the plasma membrane. These sensors encoded by *WSC1, WSC2, WSC3*, *MID2* and *MTL1* are coupled to Rho1p, a small G-protein, which in turn activates different effectors. This signaling cascade activates the transcription factors Rlm1p and Swi4/6p through phosphorylation mainly by the MAP kinase Slt2p/Mpk1p. Thereby, transcription of several processes is regulated, such as the synthesis of beta-glucan, cell wall biogenesis and the organization of the actin cytoskeleton (reviewed in [17]). In *P. pastoris*, a similar regulation has been described and the homologue for Slt2p, called Pim1p, was identified [18].

When cells are exposed to hyperosmotic stress, the HOG pathway is induced. This pathway prevents efflux of glycerol, thereby increasing intracellular glycerol levels to compensate for the high osmotic pressure. Mechanistically, the function of the glycerol efflux channel Fps1 is impaired by the action of the MAPK Hog1p [19]. Additionally, the active, open state of this channel is dependent on phosphorylation via TORC2-dependent Ypk1p kinase. Under hyperosmotic and other environmental stress conditions, this Fps1 phosphorylation is blocked and causes channel closure [20].

In *S. cerevisiae*, TOR1 and TOR2 are multiprotein complexes located at the intracellular membrane periphery and are mainly responsible for regulating cell growth. TOR complex 1 is activated upon nutrient starvation and induces translation initiation, ribosome biogenesis and cell cycle progression (reviewed in [21]). TOR complex 2 responds to different stress conditions, such as membrane-perturbing stress, sphingolipid depletion, heat shock and hypertonic as well as hypotonic conditions [22–25]. Interestingly, there are several studies describing that the HOG and TOR pathways also play a role in yeast cold response (reviewed in [26]).

Intracellular Ca^{2+} levels also rise upon hypertonic pressure via Mid1p and Cch1p channel influx, activating Calcineurin, a Ca^{2+}/Calmodulin dependent phosphatase, which in turn activates the transcription factor Crz1p for transcription of survival genes [27,28]. All these signaling pathways are usually interconnected, communicating with each other and ensuring cell survival in a highly fine-tuned fashion. These highly conserved pathways are most likely also affected by changing the sterol patterns in our *P. pastoris* cells.

In this study, we firstly aimed to characterize basic physiological responses of the sterol-modified strains. We conducted a thorough phenotypic characterization by spot tests to gain insights into which regulatory mechanisms are involved. Several hints pointed towards a dysregulation of the cell wall signaling cascade, such as the constitutive phosphorylation of Pim1p in the cholesterol-producing strain. Therefore, we further characterized the cell wall by electron microscopy and determined the cell wall sugar composition by high-performance anionic exchange chromatography. Based on initial atomic force microscopy experiments we tried to get a closer look at the nanomechanical properties of the

cholesterol-strain cell wall. Furthermore, we sequenced the total mRNA, which gave us deeper insight into the regulation of gene expression in *P. pastoris* strains when sterol metabolism is modified.

As we had identified growth phenotypes of the cholesterol-producing strain, we were able to set up a screening under very stringent conditions to find potential genes involved in complementation of sterol-dependent phenotypes. A knockout screening was performed as described in Christine Winkler's doctoral thesis [29], and unveiled three potential genes – the TOR complex 2 subunit *TSC11,* the calmodulin dependent kinase *CMK2,* and the glycerophosphocholine acyltransferase *GPC1* – involved in sterol-dependent stress response in *P. pastoris*. These genes, however, need to be investigated in more detail.

Gathering more information in this field is of special importance because many of these signaling cascades identified in yeasts have equivalent pathways in higher eukaryotes. To this day, there are yet unknown up- and downstream modulators in these signaling cascades. Yeasts still have a pioneering role of being model organisms for highly complex regulatory mechanisms in higher eukaryotes. By using another model organism than *S. cerevisiae*, it could be possible to identify yet unknown interaction partners in these pathways. As we have experienced that *S. cerevisiae* and *P. pastoris* are quite different regarding their sterol regulation, it will be highly interesting to use this methylotrophic yeast to add knowledge to the field.

4.3. Materials and Methods

4.3.1. Reagents and Media

Unless otherwise stated, all chemicals and reagents were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Sigma-Aldrich (St. Louis, MO, USA), or Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Enzymes were purchased from Thermo Fisher Scientific (St. Leon-Rot, Germany) or New England Biolabs (Ipswich, MA, USA). Oligonucleotides were obtained from Integrated DNA Technologies, BVBA (Leuven, Belgium). For propagation of plasmids in *E. coli*, cells were grown on LB solid media (1% peptone, 0.5% yeast extract, 1% NaCl, 2% agar) with 100 mg/l ampicillin. *P. pastoris* cells were routinely grown in 50 ml of YPD media (1% yeast extract, 2% peptone, 2% glucose, 2% agar) in baffled shake flasks at 28°C and 120 rpm. For testing different cultivation conditions, cells were pre-grown in 25 ml of buffered glycerol-complex medium (BMGY, 1% yeast extract, 2% peptone, 0.1 M phosphate buffer, pH 6, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol) or buffered minimal media (BMD, 0.1 M phosphate buffer, pH 6, 2% dextrose, 1.34% YNB, 4×10^{-5}% biotin, 0.04% histidine) for 48 h and 120 rpm. Then, cells were shifted to media containing methanol instead of glycerol or dextrose (BMMY or BMM, respectively). Induction was continued with 0.5% methanol for 48-72 h.

Transformation of linearized plasmids into *P. pastoris* was carried out according to the condensed protocol of Lin-Cereghino et al. [30]. Transformants were selected on YPD agar plates with antibiotics (1% yeast extract, 2% peptone, 2% glucose, 2% agar, 300 mg/l geneticin sulfate, 300 mg/l hygromycin, or 100 mg/l zeocin™). Hygromycin was purchased from Formedium (Norfolk, UK) and zeocin™ was from Invivogen (San Diego, CA, USA).

4.3.2. Strain Construction

P. pastoris CBS7435 *Δhis4*, in this study referred to as "WT", and *P. pastoris* CBS7435 *Δhis4 Δku70* henceforth referred to as "*Δku70*" were used for construction of sterol-modified strains. Construction of the cholesterol-producing strain in the *Δku70* background was described previously [7]. To eliminate possible effects arising from the *Δku70* deletion, we constructed a new cholesterol-producing strain based on *P. pastoris* CBS7435 *Δhis4* WT. For PCR amplification of the *ERG5* and *ERG6* knock-in cassettes, primers 1-4 were used (Table S1). We followed the same strategy as described previously [7] and confirmed presence of cholesterol via GC-MS analysis (see section 4.3.3). Table 1 lists all strains used and constructed during this study.

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

Name	Description	Source
Original strain (WT)	*P. pastoris* CBS7435	CBS[a]
WT	*P. pastoris* CBS7435 Δ*his4*	[31]
Δ*ku70*	*P. pastoris* CBS7435 Δ*his4* Δ*ku70*	[31]
Sterol-modified strains		
erg5DHCR7 Δ*ku70*	*P. pastoris* CBS7435 Δ*his4* Δ*ku70* Δ*erg5::DrDHCR7*-Zeo^{R}	[7]
erg6DHCR24 Δ*ku70*	*P. pastoris* CBS7435 Δ*his4* Δ*ku70* Δ*erg6::DrDHCR24*-G418^{R}	[7]
Cholesterol (Chol.) Δ*ku70*	*P. pastoris* CBS7435 Δ*his4* Δ*ku70* Δ*erg5::DrDHCR7*-Zeo^{R} Δ*erg6::DrDHCR24*-G418^{R}	[7]
erg5DHCR7	*P. pastoris* CBS7435 Δ*his4* Δ*erg5::DrDHCR7*-Zeo^{R}	This work
erg6DHCR24	*P. pastoris* CBS7435 Δ*his4* Δ*erg6::DrDHCR24*-G418^{R}	This work
Chololesterol (Chol.)	*P. pastoris* CBS7435 Δ*his4* Δ*erg5::DrDHCR7*-Zeo^{R} Δ*erg6::DrDHCR24*-G418^{R}	This work
Targeted knockout strains		
Chol. Δ*ku70* Δ*tsc11*[b]	*P. pastoris* CBS7435 Δ*his4* Δ*ku70* Δ*erg5::DrDHCR7*-Zeo^{R} Δ*erg6::DrDHCR24*-G418^{R} Δ*tsc11*	This work
Chol. Δ*ku70* Δ*gpc1*[c]	*P. pastoris* CBS7435 Δ*his4* Δ*ku70* Δ*erg5::DrDHCR7*-Zeo^{R} Δ*erg6::DrDHCR24*-G418^{R} Δ*gpc1*	This work
Chol. Δ*tsc11*[b]	*P. pastoris* CBS7435 Δ*his4* Δ*erg5::DrDHCR7*-Zeo^{R} Δ*erg6::DrDHCR24*-G418^{R} Δ*tsc11*	This work
Chol. Δ*gpc1*[c]	*P. pastoris* CBS7435 Δ*his4* Δ*erg5::DrDHCR7*-Zeo^{R} Δ*erg6::DrDHCR24*-G418^{R} Δ*gpc1*	This work

[a] Centraalbureau voor Schimmelcultures, Utrecht, NL
[b] *TSC11* was earlier annotated as *STE16* in the NCBI database
[c] *YGR149W* was recently named *GPC1* [32]

4.3.3. Sterol Analysis via Gas Chromatography-Mass Spectrometry (GC-MS)

Cells were grown over night in 10 ml of YPD at 28°C, 120 rpm and 15 OD_{600} units were harvested by centrifugation for 5 min at 1250 x g. Sterol extraction was basically performed as described by Quail and Kelly [33] and ourselves [7]. Briefly, pellets were resuspended in 600 µl of methanol, 400 µl of pyrogallol (0.5% in methanol) and 400 µl of 60% aqueous KOH. As internal standard, 5 µl of ergosterol or cholesterol (2 mg/ml in MeOH:$CHCl_3$, 2:1, v/v) were added to the cholesterol-producing strains or the WT control, respectively. Samples were heated for 2 h at 90 °C in a sand bath and saponified sterols were then extracted three times by adding 1 ml of n-heptane and centrifugation for 3 min at 450 x g. Combined sterol extracts were dried under a stream of nitrogen and dissolved in 10 µl of pyridine. For derivatization, 10 µl of N'O'-bis(trimethylsilyl)-trifluoroacetamide were added and incubated for 10 min. Samples were diluted with 50 µl of ethyl acetate analyzed via GC-MS as described previously [34]. Different sterols were identified based on their mass fragmentation pattern and their retention time relative to cholesterol using MSD ChemStation Software from Agilent Technologies (Santa Clara, CA, USA).

4.3.4. Phenotypic Characterization

Spot-tests

Single colonies were inoculated into 10 ml of YPD and were grown at 28°C and 120 rpm over night before 1.5 OD_{600} units were harvested by centrifugation. Cells were resuspended in 1 ml of sterile water and 10-fold serially diluted to 10^{-4}. Three µl of each suspension were spotted onto YPD agar plates supplemented with one of the following substances: NaCl (0.5-1.0 M), SDS (0.01%), calcofluor white (10 mg/l), congo red (8 mg/l), LiCl (50 mM), $NiCl_2$ (2 mM), caffeine (5 mM ± 0.5 M sorbitol), sorbitol (1 M), Na-orthovanadate (1 mM, 2 mM), or rapamycin (2 ng/ml, 5 ng/ml). For testing temperature sensitivity, cells were spotted onto YPD plates and incubated at 18°C, 28°C, and 37°C. For plates with pH 3, YPD media was adjusted to pH 3 with HCl and autoclaved separately from the agar before pouring the plates. Plates were incubated usually for 3 days, or until differences in growth were visible best. Photos were taken with a digital camera or with the G:Box HR16 BioImaging system (Syngene, Cambridge, UK).

Secretion of Endogenous Cell Wall Proteins

Cells were cultivated in BMGY or BMD media at 28°C and 120 rpm for 48 h. One ml samples were taken and cultures were then shifted to methanol-containing BMMY or BMM media. Induction was continued with methanol to maintain 0.5% final concentration for 72 h. For analysis of secreted proteins via SDS-PAGE, 200 µl of cell-free culture supernatants were precipitated with $MeOH/CHCl_3$ and deglycosylated with Endoglycosidase H following the instruction manual (New England Biolabs, Ipswich, MA, USA). After a denaturing step at 95°C for 10 min, 15 µl samples were separated on a 4-12% Bis-Tris NuPAGE® Gel using MES buffer (50 mM MES, 50 mM Tris base 0.1% SDS, 1 mM EDTA, pH 7.3). Gels were stained with Coomassie brilliant blue R-250.

Mass Spectrometry

The protein bands were excised from the SDS-PA gels, reduced, alkylated and digested with modified trypsin (Promega) according to the method of Shevchenko et al. [35]. Peptide extracts were dissolved in 0.1% formic acid/5% acetonitril and were separated by nano-HPLC (Dionex Ultimate 3000) equipped with a C18, 5 µm, 100 Å, 5 x 0.3 mm enrichment column and an Acclaim PepMap RSLC nanocolumn (C18, 2 µm, 100 Å, 500 x 0.075 mm) (all Thermo Fisher Scientific, Vienna, Austria). Samples were concentrated on the enrichment column for 2 min at a flow rate of 5 µl/min with 0.5% trifluoroacetic acid as isocratic solvent. Separation was carried out on the nanocolumn at a flow rate of 250 nl/min at 60°C using the following gradient, where solvent A was 0.1% formic acid in water and solvent B was acetonitrile containing 0.1% formic acid: 0-2 min: 4% B; 2-90 min: 4-25% B; 90-95 min: 25-95% B; 96-110 min: 95% B; 110-125 min: 4% B. The sample was ionized in the nanospray source equipped with stainless steel emitters (ES528, Thermo Fisher Scientific, Vienna, Austria) and was analyzed in a

Orbitrap velos pro mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) operated in positive ion mode, applying alternating full scan MS (m/z 400 to 2000) in the ion cyclotron and MS/MS by CID of the 20 most intense peaks with dynamic exclusion enabled. The LC-MS/MS data were analyzed by searching a database containing all *P. pastoris* sequences (downloaded 21.11.2016, 15223 sequences, 43946 queries) and all common contaminants with Mascot 2.3 (MatrixScience, London, UK). Detailed search criteria; enzyme: Trypsin, maximum missed cleavage sites: 2, N-terminus: hydrogen, C-terminus: free acid, Cys modification: carbamidomethylation, search mode: homology search, possible multiple oxidized methionine, maximum precursor charge 3; precursor mass tolerance 10 ppm, product mass tolerance ± 0.5 Da., 1% false discovery rate. Data was filtered according to stringent peptide acceptance criteria, including mass deviations of ± 10 ppm, minimum 2 peptides per protein, Mascot Ion Score of at least 17 and a position rank 1 in Mascot search.

Electron Microscopy

For investigation of phenotypes under methanol-induced conditions, cells were cultivated in 25 ml of BMGY in 300 ml baffled flask for 48 h at 28°C and 120 rpm. For methanol induction, 25 ml of 1% BMMY media was added to obtain 0.5% final concentration. Cells were fed twice a day with 2.5 ml of 10% BMMY for 48 h and were then harvested at 500 ×g for 5 min in an Eppendorf 5810R centrifuge. For investigation of glucose-fed phenotypes, cells were grown in YPD at 28°C and 120 rpm for 48 h and harvested as described above. Cell pellets were washed with distilled H_2O. Preparation of pellets for electron microscopy was essentially performed as described previously [36]. Briefly, cells were fixed for 5 min in 1% aqueous $KMnO_4$ at room temperature, washed with distilled H_2O, and fixed in 1% aqueous $KMnO_4$ for 20 min. Fixed cells were washed four times in distilled water and incubated in 0.5% aqueous uranyl acetate over night at 4°C. The samples were dehydrated for 20 min, each in a graded series of ethanol (50%, 70%, 90%, and 100%). Pure ethanol was then exchanged by propylene oxide, and specimen were gradually infiltrated with increasing concentrations (30%, 50%, 70% and 100%) of Agar 100 epoxy resin mixed with propylene oxide for a minimum of 3 h per step. Samples were embedded in pure, fresh Agar 100 epoxy resin and polymerized at 60°C for 48 h. Ultra-thin sections of 80 nm were stained for 3 min with lead citrate and viewed with a Philips CM 10 transmission electron microscope.

CWI Pathway Induction

For detection of phosphorylated Pim1p by Western blotting, cells were grown in 10 ml of YPD in 100 ml Erlenmeyer flasks at 28°C and 130 rpm for 15 h and cell wall integrity pathway was induced by addition of 90 µl calcofluor white (1 mg/ml). Samples were taken after 45 min, 2 and 4 h and cell pellets corresponding to three OD_{600} units were harvested by centrifugation for 10 min at 4°C. Cell disruption was performed according to the method of Riezman et al. [37], which preserved Pim1p in its phosphorylated state via rapid protein precipitation. Cell pellets were resuspended in 300 µl of 1.85 M

NaOH containing 7.5% β-mercaptoethanol. After 10 min of incubation on ice, 300 µl of 50% TCA was added and the suspension was again incubated on ice for at least 1 h. Precipitated proteins were collected by centrifugation at 13,000 rpm for 10 min and were washed with 500 µl of ice-cold water. Protein pellets were dissolved in 50 µl of 1xLDS NuPAGE® sample buffer (Thermo Fisher Scientific, St. Leon-Rot, Germany) and denatured for 20 min at 70°C. For SDS-PAGE, 15 µl of protein samples were applied and separated on a 12.5% polyacrylamide gel following standard procedures [38]. Western blot analysis was performed according to Haid and Suissa [39]. Primary rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody from New England Biolabs (Ipswich, MA, USA) was diluted 1:500. Secondary peroxidase-conjugated goat anti-rabbit IgG was purchased from Sigma-Aldrich (St. Louis, MO) and diluted 1:5000. Immunoreactive bands were visualized with the SuperSignal® West Pico Chemiluminescent substrate (Thermo Fisher Scientific, St. Leon-Rot, Germany) using the G:Box HR16 BioImaging system (Syngene, Cambridge, UK).

4.3.5. RNA sequencing for Transcriptome Analysis

Cultivation of strains was conducted under methanol-inducing conditions in BMGY/BMMY media for 48 h as described for the electron microscopy sample preparation (section 4.3.4). Samples were taken and cell pellets of 150-300 mg of cell wet weight were snap-frozen in liquid nitrogen. Pellets were sent on dry ice to BioGrammatics (Carlsbad, CA, USA) for RNA isolation and sequencing, which was performed on Illumina HiSeq NGS, single end read of 50-100 bases per cluster and a minimum of 20 million clusters per strain. Sequences were compared to the refined reference genome of *P. pastoris* CBS7435 recently published by Sturmberger et al. [40]. The results were evaluated using the CLC genomics workbench 9. Accepted reads were imported as .bam file from Illumina sequencing with unpaired read under default options. Read counts were normalized to total counts and interesting hits were chosen with a threshold of minimal fold change of three. The genes with the highest fold changes were manually curated. Additionally, interesting target genes of lipid metabolism and cell wall synthesis were examined. This list of genes was then analyzed by NCBI protein blast and the *S. cerevisiae* genome database [41] to obtain the description for the designated gene function.

4.3.6. Cell Wall Isolation and Characterization

Cells were cultivated in 200 ml of YPD in 1 l baffled shaking flasks at 30°C and 120 rpm for 48 h. Yeast cell walls were isolated as described previously [42]. Briefly, cells were harvested by centrifugation and pellets were washed with sterile water. Pellets corresponding to 10 OD_{600} units each were resuspended in 500 µl of cold Tris-HCl buffer (50 mM, pH 7.5) and placed in a 2 ml lysis tube containing 500 mg of 0.5 mm glass beads. Cells were broken using a Fastprep system (MP Biomedicals, Santa Ana, CA, USA) for 20 s with 1 min cooling intervals on ice. Disruption cycles were repeated until more than 95% of the cells were lysed as estimated by methylene blue staining according to Cot et al. [43]. Suspensions of broken cells were collected and glass beads were extensively washed with ice-cold deionized water. The

pooled supernatant and washings were centrifuged at 13,000 x g and 4°C for 15 min to pellet cell walls and membranes. The pellet was again washed two times with ice-cold deionized water and centrifuged at 3200 x g and 4°C for 5 min to remove membranes. Finally, yeast cell walls were frozen in liquid nitrogen and lyophilized until complete dryness.

Acid Hydrolysis and HPAEC-PAD

Acid hydrolysis of cell walls to release sugar monomers (*N*-acetylglucosamine from chitin, glucose from β-glucan, and mannose from mannans) was essentially performed as described previously [44]. Ten mg of dried cell wall were suspended in 75 µl of 72% H_2SO_4 and incubated at room temperature, vortexing every 30 min. After 3 h, samples were diluted with 905 µl of water to 2 N H_2SO_4. Hydrolysis was conducted in a heating block set to 100°C for 4 h with intermittent vortexing steps every hour. Sulfate ions were precipitated by drop-wise addition of saturated $Ba(OH)_2$ (40 g/l) until a pH 6-7 was reached. The pH was indicated by previously added 100 µl of 1% bromophenol blue and was also checked with pH papers. Samples were incubated over night at 4°C to allow precipitation of remaining sulfate ions. The volume was adjusted to 25 ml and the $BaSO_4$ precipitate was pelleted at 3000 x g for 15 min. Supernatants were then filtered through 0.2 µm syringe filters. Released monosaccharides were measured by high-performance anionic exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) on a Dionex™ ICS 3000 system (Thermo Fisher Scientific, Courtabœuf, France) as described [44]. Separation was performed on a CarboPac PA10 analytical column (250 x 4 mm) with a guard column CarboPac PA10 using an isocratic elution of 18 mM NaOH at 25°C and a flow rate of 1 ml/min. Sugar residues were detected on a pulsed amperometric system equipped with a gold electrode. A standard mix consisting of 100 µg/ml glucose, 100 µg/ml mannose and 10 µg/ml glucosamine was used for preparation of the calibration curve for quantification of released sugars.

Protein Isolation and Mass Spectrometry

Alkali soluble proteins were extracted from cell walls as follows: Ten µg of dry cell wall were resuspended in 1 ml of extraction buffer (20 mM Hepes, pH 7.1, 100 mM KCl, 1 mM EDTA, 1 mM DTT) and were mixed for 1 h on a VXR Vibrax® (IKA, Staufen, Germany). Then, NaOH was added to obtain a final concentration of 0.1 M, before vortexing and heating in a sand bath at 80°C for 1 h. After centrifugation for 10 min at 2000 rpm and 4°C, supernatants were transferred to fresh reaction tubes and protein content was determined with the Bio-Rad Protein Assay, based on the method of Bradford [45]. For MS analysis, 100 µg of proteins were precipitated with $MeOH/CHCl_3$ and 25 µg were loaded on a 4-12% Bis-Tris NuPAGE® Gel using MES buffer (50 mM MES, 50 mM Tris Base 0.1% SDS, 1 mM EDTA, pH 7.3). Electrophoresis was performed until total proteins migrated approximately 1 cm into the gel. Proteins in the gel were stained with Coomassie brilliant blue R-250 and the single band containing non-separated cell wall proteins was excised. Samples were further analyzed according to section 4.3.4, Mass Spectrometry.

Phospholipid Extraction and Thin-layer Chromatography

Phospholipids were extracted from 2-3 µg of yeast cell walls following the method of Folch [46] using $CHCl_3$/MeOH (2:1, v/v) as solvent. Individual phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel 60 plates (Merck, Darmstadt, Germany) using $CHCl_3$/MeOH/25% ammonia (65:35:5, per vol.) as first solvent, and $CHCl_3$/acetone/MeOH/acetic acid/water (50:20:10:10:5, per vol.) as second solvent. Phospholipid bands were visualized by staining with iodine vapor, scraped off the plate and quantified by the method of Broekhuyse [47].

Atomic Force Microscopy (AFM)

Strains were cultivated in 10 ml of YPD at 30°C and 200 rpm for 48 h. Cells were collected by centrifugation, washed once with 5 ml of acetate buffer (18 mM sodium acetate, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 5.2), and resuspended in 10 ml of the same buffer. Then they were immobilized in micro chambers made by polydimethylsiloxane (PDMS) stamps and prepared as described by Formosa et al. [48]. First, a glass/chromium mask presenting microstructured patterns was generated and these patterns were transferred onto a silicium wafer. The silicium wafer was generated by photolithography at LAAS-CNRS (Toulouse, France). The microstructured patterns of the silicon master were 1.5 µm to 6 µm wide squares, with a depth ranging from 1 µm to 4 µm and a pitch of 0.5 µm. Then, a solution of PDMS polymer was prepared containing a 10:1 mass ratio of PDMS oligomers and a reticular agent (Sylgard® kit 184, Sigma-Aldrich). The PDMS solution was degassed under vacuum and placed onto the silicium wafer. Bubbles were removed by degassing again under vacuum. Finally, the PDMS was cured for 1 h at 80°C and cooled down at room temperature. For immobilization of the cells, a microstructured PDMS motif was cut with a scalpel and removed. Then, the PDMS stamp was covered by 100 µL of the cell suspension and the cells were fixed in the microstructures of the stamp by convective/capillary assembly.

The atomic force microscope (Nanowizard III, JPK Instruments, Berlin, Germany) was coupled to an inverted microscope (Axio Vert Observer D1.m, Carl Zeiss, France) and a ProgRes® MFcool camera (Jenoptik, Jena, Germany). Before each experiment, the spring constant of the cantilevers k_{cant} (N/m) and the sensibility S (m/V) were measured. These calibration parameters were determined in acetate buffer on a glass slide by the thermal noise method [49,50].

AFM images were recorded in quantitative imaging (QI™) mode with MLCT AUWH silicon nitride cantilevers (Bruker, Camarillo, CA) with a nominal spring constant of 0.01 N/m. The measured spring constant values were between 0.01 and 0.02 N/m. The QI™ mode was recently developed by JPK instruments and is similar to the tapping mode, avoiding cell damage and lateral deflections [51]. To generate three-dimensional height images of the cells, they were scanned with a constant force of 1 nN and a speed of approach of 250 µm/s. The height image was obtained by analyzing the deflection of the oscillating cantilever. After imaging the yeast cell, a square of 1 µm x 1 µm was selected in the center

of the cell. On this area, the tip was pushed on the surface with a constant force of 0.5 nN on different locations (32 x 32 squares), resulting in 1024 recorded force-distance curves. The parameters in force spectroscopy mode were set to force = 0.5 nN, z-length = 1 µm, and speed = 2 µm/s.

Data were processed using JPK data processing software (JPK Instruments, Berlin, Germany). For calculating the elasticity of the cells, the Hertz model was applied to obtain Young's modulus *E* values according to equation *(1)*:

$$F = \frac{2E \tan \alpha}{\pi(1-\nu^2)} \delta^2 \qquad (1)$$

The force *F* is given as a function of the indentation δ (50 nm), the Young's modulus *E*, the tip opening angle α (35°) and the Poisson ratio ν (arbitrarily assumed to be 0.5). Thus, fitting each force curve of a batch, *e.g.* 1024 force curves, with the Hertz model generated a distribution of Young's modulus values. These values were adjusted to a Gaussian law using Origin 8 software (OriginLab, Northampton, MA, USA) in order to obtain the mean Young's modulus value and the standard deviation. A total of six viable cells per strain were analyzed in two independent experiments.

4.3.7. Construction and Screening of a Random Knockout Mutagenesis Library

Random Cassette Mutagenesis and Screening of Knockout Strains

The method for random integration of knockout cassettes and screening of mutant strains was essentially performed as described by Christine Winkler [29]. Instead of zeocin^{TM}, we used the hygromycin resistance gene as mutagenesis cassette, which was PCR amplified from pPpHyg [36] using primers 5 and 6 (Table S1). The *P. pastoris* Chol. Δ*ku70* strain was transformed with 1-2 µg of the cassette and primary clone screening was done on YPD with 300 mg/l of hygromycin. All clones positively selected on hygromycin (usually 700-800 cfu/µg DNA) were collected again by resuspending the cells from the plates in 10 ml of YPD media. After diluting 1:10 000 in YPD, 100 µl to 500 µl – depending on the number of obtained clones – were spread again and cultivated on selective conditions, *i.e.* YPD plates with 0.5 M NaCl or at 18°C incubation temperature. After 5 days of incubation at 28°C or 18°C, respectively, all clones that could grow were selected for re-screening. For the re-screening, clones were tested under the same selective conditions by spot-tests (see section 4.3.4, Spot-tests). Mutants were furthermore tested for presence of cholesterol as described in section 4.3.3 to rule out any mutations affecting cholesterol biosynthesis.

Genome Walking for Identification of Integration Loci

Genomic DNA was isolated from the mutant strains showing growth in the re-screening based on the method of Hoffman and Winston [52]. The genome walking protocol was adapted from Siebert et al. [53] and the GenomeWalker™ Universal Kit User Manual (Clontech Laboratories, Mountain View, CA, USA). Basically, genomic DNA was digested with *Bam*HI, *Bgl*II or *Hind*III and the respective adaptor was ligated. Adaptors (25 μM) were generated earlier by annealing oligonucleotides 7 and 8 (Table S1) for ligation to the *Bam*HI and *Bgl*II-digested fragments, and oligonucleotides 7 and 9 (Table S1) for *Hind*III digested fragments. For this purpose, equimolar amounts were mixed and incubated at 100°C for 2 min. Adaptors were slowly cooled to room temperature and were stored at -20°C. After adaptor ligation, 5' and 3' hygromycin resistance cassette flanking sequences were amplified in a first and second, nested PCR round using primers 10-12 and 13-15, respectively (Table S1). Amplified fragments were excised from agarose gels, purified and either directly sent for sequencing (LGC Genomics, Berlin, Germany) or cloned into pJET1.2/blunt (Thermo Fisher Scientific, St. Leon-Rot, Germany) prior to sequencing with primers 16 and 17 (Table S1). Sequencing results were compared to genome sequences of *P. pastoris* CBS7435 and GS115 using a BLAST Nucleotide Sequence Similarity Search [54,55]. The affected protein sequences were also compared to the *S. cerevisiae* proteome and other yeasts using the NCBI protein blast search and the *S. cerevisiae* genome database [41].

Construction of Targeted Gene Knockouts and Phenotype Verification

Knockout strains of the identified target genes were constructed according to a strategy described in the doctoral thesis of Mudassar Ahmad [56]. For this purpose, 5' and 3' flanking regions of *YGR149W (GPC1)* and *STE16* (*TSC11*) loci were PCR amplified using primers 18-21 and 22-25, respectively (Table S1). Flanking regions ranged from 980-1600 bp to ensure successful homologous recombination events. Fragments were then assembled via overlap expression PCR and finally cloned into knockout plasmids pPpKC3 via *Sfi*I. The pPpKC3_ste16 and pPpKC3_ygr149w plasmids harbor the *P. pastoris HIS4* gene for positive clone selection and the site-specific Flp recombinase for marker recycling. After transformation of *Swa*I linearized knockout cassettes, positive clones were selected on MD agar plates. Genomic DNA of positive clones was isolated and correct locus integration as well as gene deletion was confirmed by PCR using Primers 26-33 (Table S1) binding up- or downstream of the target locus. Phenotype characterization and verification was performed as described in section 4.3.4, Spot-tests. Growth of the knockout strains was investigated again under selective conditions, *i.e.* growth at 18°C and 0.5 M NaCl.

4.4. Results and Discussion

4.4.1. Sterol-engineered *P. pastoris* Show Strong Phenotypes Mainly Related to Cell Wall Synthesis

Spot-tests Reveal Unique Phenotypes in the Sterol-modified Strains

In order to characterize sterol-engineered *P. pastoris* strains in more detail, we began with a thorough phenotypic analysis conducted by spot-tests on YPD with different stress causing additives. We always investigated stationary phase cultures because of the observed differences in growth of the sterol-modified strains as compared to the WT and Δ*ku70* strain. By doing so, we could largely rule out these general growth differences and improve comparison of the results.

Starting with the cultivation temperature, we observed no major differences for the *erg5DHCR7* Δ*ku70* and *erg6DHCR24* Δ*ku70* strains compared to the WT and Δ*ku70* strain. The cholesterol strain was impaired in growth at 37°C, and growth was completely abolished at 18°C, though (Fig 3, A). When *S. cerevisiae* is cultivated above or below optimal growth temperatures, stress response is activated via various pathways (reviewed in [26,57]), which could be impaired in the Cholesterol Δ*ku70* strain.

For testing the response to osmotic stress, cells were spotted onto 0.5 M NaCl, 50 mM LiCl, and 1 M sorbitol plates [58]. For the latter, we observed generally a slower growth of all tested strains. The Chol. Δ*ku70* and *erg6DHCR24* Δ*ku70* strains were highly sensitive to 0.5 M NaCl. While the *erg6DHCR24* Δ*ku70* strain was still able to grow a little, a high salt concentration and thereby elevated osmotic stress was detrimental for the Chol. Δ*ku70* strain. Interestingly, the *erg5DHCR7* Δ*ku70* strain was even more resistant to NaCl than the WT, which became visible when salt concentrations were increased to 1 M (Fig. 3A). Growth of all three sterol-modified strains was abolished when cells were spotted onto YPD with 50 mM LiCl, potentially indicating a sterol-dependent function of membrane transporters regulating alkali metal efflux across the membrane. In *S. cerevisiae*, also calcineurin and HOG signaling pathways are known to contribute to the strict regulation of ion specific influx and efflux processes (reviewed in [59]).

Sterols could play an important role in proper function of the plasma membrane transporters involved in these signaling pathways. It is very interesting that despite the small differences in the sterol ring structures, huge differences occurred in the stress response of all three sterol-modified strains. The higher resistance towards 1 M NaCl was exclusive for the *erg5DHCR7* Δ*ku70* strain. This strain mainly contained ergosta-5-enol [7], which lacks the C7 and C22 double bonds. It could be that these double bonds or the C24 methyl group fulfill an important role for the membrane structure and thus for the interaction with membrane proteins.

The *erg6DHCR24* Δ*ku70* strain, which produces mainly 7-dehydrocholesterol [7], had more phenotypes in common with the cholesterol-producing strain. Interestingly, when both genetic modifications of the

sterol-modified strains were combined to yield the cholesterol-producing strain, the phenotypes added up and created novel, very specific phenotypes.

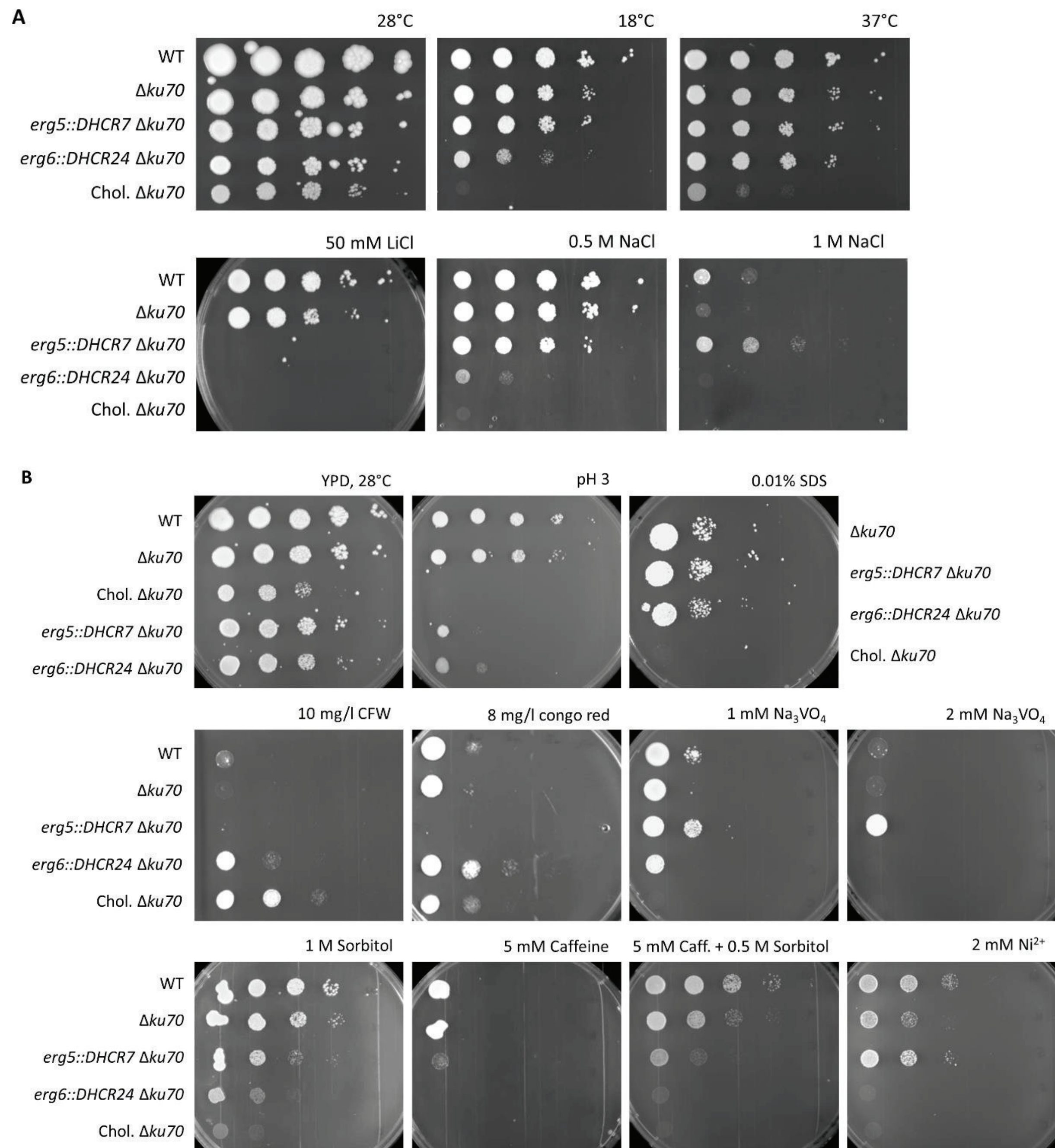

Fig. 3. Spot-tests of sterol-engineered *P. pastoris* Δ*ku70* strains. Strains were cultivated for 48 h on YPD. Cell pellets corresponding to 1.5 OD_{600} units were harvested, serially diluted to 10^{-4} and 3 µl were spotted onto YPD plates with the indicated additives. Plates were incubated at 28°C or 18°C and imaged after 3-4 days. **(A)** Growth of sterol-modified *P. pastoris* Δ*ku70* strains depending on incubation temperature and salt concentration. **(B)** Growth of sterol-modified *P. pastoris* Δ*ku70* strains in the presence of different cell wall integrity pathway and stress response inducing agents.

Another unique phenotype of the Chol. Δ*ku70* strain was the high sensitivity to pH 3 and SDS. Sensitivity to low pH could indicate vacuolar defects in this strain [58]. The detergent SDS generally affects plasma membrane stability and indirectly indicates cell wall defects due to a higher accessibility of SDS to the membrane [60]. Effects on the cell wall were more closely investigated by spotting the cells on calcofluor white and congo red, which are substances binding to chitin and disturbing the cell wall biogenesis [58,61]. The Chol. Δ*ku70* strain showed the highest resistance towards calcofluor white, indicating a defect in chitin synthesis and thus lower chitin levels. In contrast, the growth of *erg5DHCR7* Δ*ku70* strain was completely abolished. These findings suggest that the Chol. Δ*ku70* presumably bears the strongest changes in the cell wall structure among the three sterol-modified strains.

Caffeine has pleiotropic effects on the baker's cell, *e.g.* inhibition of the TOR complex 1 and induction of the cell integrity pathway by activation of the MAP kinase Mpk1p via Pkc1p [62,63]. Cells sensitive to caffeine due to defects in the CWI response are osmotically instable and, thus, can be rescued by the addition of sorbitol. In our case, the caffeine hypersensitive phenotype could not be reverted in *P. pastoris* by the osmotic stabilizer sorbitol for the Chol. Δ*ku70* and *erg6DHCR24* Δ*ku70* strains, pointing towards an additional influence in TORC1 signaling.

Strains resistant to Na-orthovanadate Na_3VO_4 are described to bear glycosylation defects in *S. cerevisiae* [58]. Interestingly, the *erg5DHCR7* Δ*ku70* strain showed a slight resistance, whereas the Chol. Δ*ku70* strain was more sensitive towards Na-orthovanadate. When cells were exposed to 2 mM nickel, both Chol. Δ*ku70* and *erg6DHCR24* Δ*ku70* were highly sensitive, but not the *erg5DHCR7* Δ*ku70* strain. This could indicate an impaired export mechanism of toxic metals via membrane ATPases and/or deficient vacuolar transport [58,64].

We also tested 200 mM sodium acetate and 200 mM $CaCl_2$ in YPD, pH 8, and growth on minimal media with 2% dextrose, but no specific effects besides the generally slower growth of the sterol-engineered strains was observed. The cholesterol strain did, furthermore, not grow on minimal media plates with 0.5% methanol (not shown). A slight difference was usually visible for the Δ*ku70* strain as compared to the wild type. To exclude any effects arising due to the Δ*ku70* deletion, new sterol-modified strains were constructed based on the WT background. We confirmed production of the expected sterols by GC-MS (Supplemental Fig. S1). The strains were tested again via spot-assays for the strongest phenotypes observed, such as growth at 18°C, 0.5 M NaCl, and 10 mg/l calcofluor white. Essentially, we could confirm the previously observed phenotypes also seen in the Δ*ku70* background strains (Fig. 4). The cold sensitive phenotype was the same and the new Chol. strain seemed to be even more susceptible to the membrane disturbing detergent SDS. Only the resistance against calcofluor white was not as pronounced as in the Chol. Δ*ku70* strain. In this series of spot-assays, cells were also tested for sensitivity towards 5 μg/l rapamycin. Interestingly, both cholesterol-producing strains were highly susceptible, whereas the growth of *erg5DHCR7* Δ*ku70* and *erg6DHCR24* Δ*ku70* strains was not affected. Although rapamycin selectively binds TOR complex 1, both TOR1 and TOR2 share a redundant, rapamycin-

sensitive regulation of signaling pathways. Sensitive yeast strains are therefore generally described to lack proper function of the shared, rapamycin-sensitive TOR signaling to control cell growth [65].

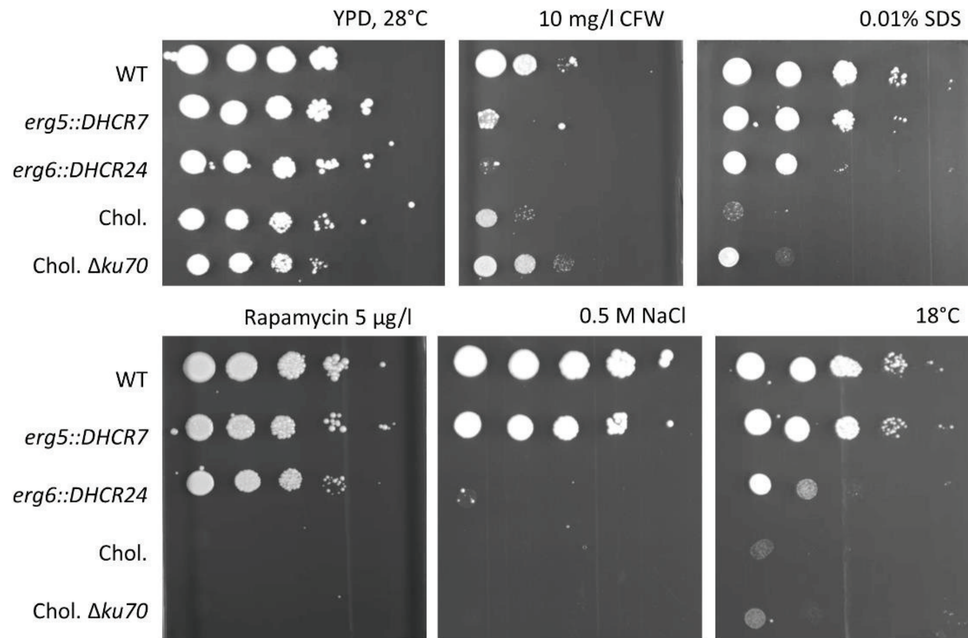

Fig. 4. Spot-tests of sterol-engineered *P. pastoris* strains. For verification of Δ*ku70*-independent phenotypes, newly constructed sterol-modified *P. pastoris* strains were cultivated for 48 h in YPD. Cell pellets corresponding to 1.5 OD_{600} units were harvested, serially diluted to 10^{-4} and 3 µl were spotted onto YPD plates with the indicated additives. Plates were incubated at 28°C or 18°C and imaged after 3-4 days.

Cwp1p and Flo5p are Hyper-secreted Cell Wall Proteins in the Chol. Δ*ku70* Strain

Upon cultivation on different carbon sources, we investigated the major secreted proteins of the sterol-modified strains compared to WT and Δ*ku70* control strains. In the culture supernatants of the Chol. Δ*ku70* strain, proteins of apparent sizes of 150 and 37 kDa occurred, which were neither observed in the other two sterol-engineered nor in the WT control strains (Fig. 5, A). These distinct bands occurred under each tested condition, independently of rich or minimal media, although bands were barely visible in BMDH media (Fig. 5, B). The intensity, and hence abundance, of the two major proteins was yet increased after methanol induction. After EndoH treatment, we observed that the 150 kDa protein was deglycosylated, as its size shifted to approximately 130 kDa. The 37 kDa protein, however, did not change in size. Therefore, it was likely not N-glycosylated. The protein bands excised from the gels for MS analysis are marked in Fig. 5 (1-4). Per analyzed sample, the fifteen hits with highest abundance, highest score and best coverage were listed in Table 2. However, some of the proteins from the list had rather low abundances, which is represented by the peptide spectrum matches (PSM) value. Therefore, we considered the 130 kDa protein most likely to be Flo5p and the 37 kDa protein to be Cwp1p.

Flo5p, a lectin-like cell wall protein, is described in *S. cerevisiae* to be involved in flocculation and the expression of the different *FLO* genes is dependent on several external factors such as oxygen, cations, pH and agitation, inducing several signaling pathways (reviewed in [66]). The *P. pastoris* Flo5p shares 31% sequence identity to the *S. cerevisiae* homologue. Bioinformatic analyses of the sequence using the CBS prediction servers (http://www.cbs.dtu.dk/services, 2.3.2017) and the Mendel GPI modification site prediction (http://mendel.imp.ac.at/gpi/gpi_server.html, 2.3.2017) revealed four potential N-glycosylation and several O-glycosylation sites, the presence of a signal peptide, but no predicted GPI anchor.

The earlier identified Cwp1p is meanwhile annotated in NCBI as hypothetical protein *Pp7435_Chr3-0879*. Upon protein blast search, we found that the protein was not well conserved, but shares 33% identity to *S. cerevisiae* Cwp1p. Therefore, we kept the name Cwp1p for this study. Bioinformatic analyses of the protein sequence revealed a predicted secretion signal with the cleavage site between amino acids 18 and 19, but no transmembrane domains, GPI-modification site, or any N- or O-glycosylation sites were found. In comparison, the secreted *S. cerevisiae* Cwp1p has a GPI anchor and one potential N-glycosylation site. Later, we also found by RNA sequencing and transcriptomics analysis that expression of several cell wall proteins is upregulated (see section 4.4.2). The transcription levels of *CWP1* were increased 24-fold compared to WT and 58-fold compared to Δ*ku70*. This difference between the WT controls is most likely due to an additional differential expression of *CWP1*, which was downregulated 2.5-fold in the Δ*ku70* strain compared to the WT. It seems that in the cholesterol-producing strain, this putative cell wall protein is upregulated and, thus, more strongly secreted to the culture supernatants. In *S. cerevisiae*, induction of Cwp1 expression is dependent on

Slt2p activity [67]. However, a physiological role of its potential homologue in *P. pastoris* is yet to be demonstrated.

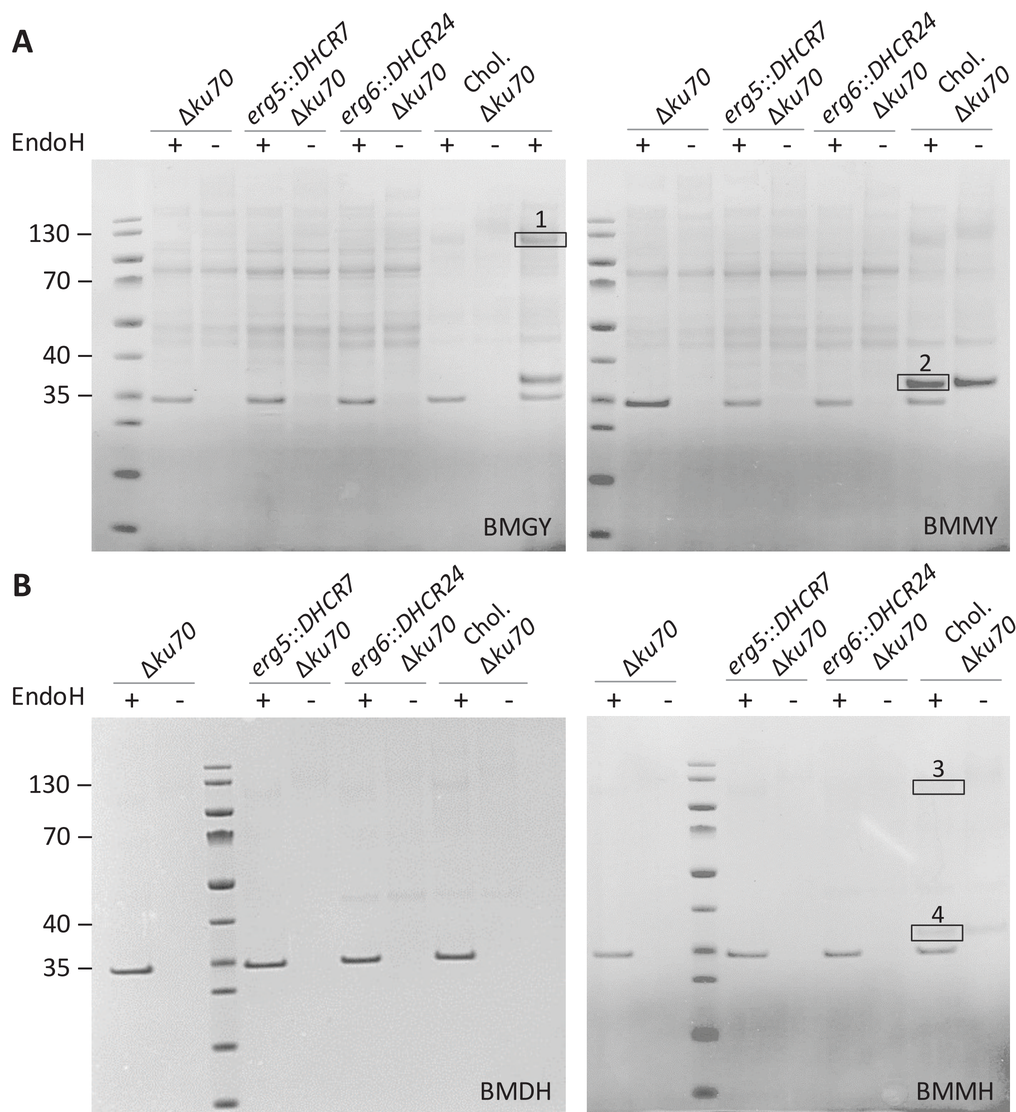

Fig. 5. SDS-PAGE analysis of culture supernatants. (A) Cells were cultivated in buffered complex BMGY media for 48 h (left image) and then induced for 48 h with methanol. (right image). **(B)** Cells were cultivated in buffered minimal BMDH media for 48 h (left image) and then induced with methanol for 48 h (right image). Numbers 1-4 indicate bands that were cut out and analyzed by mass spectrometry analysis.

Table 2. Mass spectrometry analysis of secreted proteins from the Chol. Δ*ku70* strain.

Accession	Description	Score	Coverage	#PSM[a]	MW [kDa]
130 kDa Protein					
Accession	**Description**	**Score**	**Coverage**	**#PSM[a]**	**MW [kDa]**
328351105	Putative lectin-like protein *FLO5*	6140,91	26,75	122	51,2
328351695	aminopeptidase 2[b]	4849,81	52,28	111	101,9
328351790	Protein with internal repeats *PpPIR1*	2008,53	28,83	43	29,5
328352275	Lysyl oxidase[b]	1822,82	21,50	37	90,0
238030806	hypothetical protein PAS_chr2-1_0812 [b]	1386,18	19,60	35	93,4
328352428	Cobalamin-independent methionine synthase	1012,10	19,92	26	85,8
328352741	Endochitinase	942,73	9,77	20	71,6
328353755	hypothetical protein	826,96	13,97	24	62,9
328352414	Non-essential glycogen phosphorylase [b]	797,88	18,83	21	97,6
328349991	1,3-beta-glucanosyltransferase *GAS1*	773,10	15,06	14	57,3
328352531	putative glucanase *SCW11* [b]	666,77	16,59	15	49,0
238033797	Putative chitin transglycosidase, cell wall protein	650,37	11,09	16	49,5
328351917	Alanine/arginine aminopeptidase [b]	589,69	12,09	20	99,7
328351366	Putative glucanase *SUN4*	527,83	16,47	12	45,0
238031855	Cell wall protein that contains a putative GPI-attachment site[c]	244,80	11,24	7	43,2
37 kDa Protein					
Accession	**Description**	**Score**	**Coverage**	**#PSM[a]**	**MW [kDa]**
328353433	Cell wall protein CWP1	8323,75	73,70	262	31,6
328351703	Mitochondrial porin	1737,66	65,02	41	29,6
328350946	Endo-beta-1,3-glucanase *BGL2*	1439,33	37,94	44	33,9
328350555	Glucose-6-phosphate 1-epimerase [b]	1196,01	53,69	36	33,9
238029970	G-protein beta subunit and guanine nucleotide dissociation inhibitor for Gpa2p[b]	1003,04	44,62	27	34,7
328352884	Intracellular esterase	791,56	42,81	23	33,3
328351807	Nascent polypeptide-associated complex subunit alpha[b]	629,70	30,69	14	21,7
328354450	Uncharacterized protein YMR244W[c]	628,57	17,62	15	38,8
328352001	Xylose and arabinose reductase [b]	623,54	22,06	17	32,1
328350992	40S ribosomal protein S0[b]	604,25	25,86	13	29,2
328353695	hypothetical protein PP7435_Chr3-1150[b]	553,09	26,52	14	28,3
238029542	Chitin deacetylase [b]	549,74	21,31	12	34,7
328353323	Hypothetical protein PP7435_CHR3-0767 [b]	528,44	16,72	10	33,7
328352141	glyceraldehyde 3-phosphate dehydrogenase[b]	525,77	26,13	12	35,6
328352125	Cell wall exo-1,3-beta-glucanase *EXG1* [c]	250,05	16,27	8	44,0

[a] Peptide spectrum matches

[b] only present upon cultivation in complex BMMY media

[c] only present upon cultivation in minimal BMMH media

4.4.2. Electron Microscopy Reveals Amorphous Cell Wall Structures

As the initial spot-tests made us speculate about defects in the cell wall morphology of the sterol-engineered *P. pastoris* strains, we investigated their cell wall ultrastructure grown on different carbon sources via transmission electron microscopy. These images revealed highly unusual, enlarged and porous cell wall structures especially in the Chol. and Chol. Δ*ku70* strains, but also in the precursor strains. Cells were either grown on glucose (Fig. 7, A) or on glycerol, following induction with 0.5% methanol (Fig. 7, B). The abnormal cell wall structures were largely independent of the added carbon source, although it seems that the effect was slightly more prominent upon methanol induction. This can be a result of the prolonged growth time or of additional stress that methanol could cause to the cells. We also confirmed that the phenotype occured independently of the Δ*ku70* deletion background.

An interesting observation was that the peripheral ER was much closer to the plasma membrane and cell wall in all sterol-modified strains. It has been described, that sterols synthesized in the ER are transferred via poorly understood, non-vesicular transport mechanisms to the plasma membrane [68,69]. An overproduction of sterols, which was also supported in our study by the strong upregulation of sterol biosynthesis genes, could stimulate the formation of contact points between the ER and the plasma membrane for incorporation of the sterols into the latter. In several images, we observed enlarged, net-like ER structures and vacuoles containing vesicular structures. These observations are very unusual but further investigations would be needed for a more detailed conclusion.

A closer look on the porous cell wall structure of the Chol. strain revealed that these holes enclose membraneous structures (Fig. 6). This might indicate a disturbed cell wall synthesis mechanism, where cell wall components are overproduced and membrane structures become enclosed within the beta-1,3-glucan, beta-1,6-glucan and chitin network.

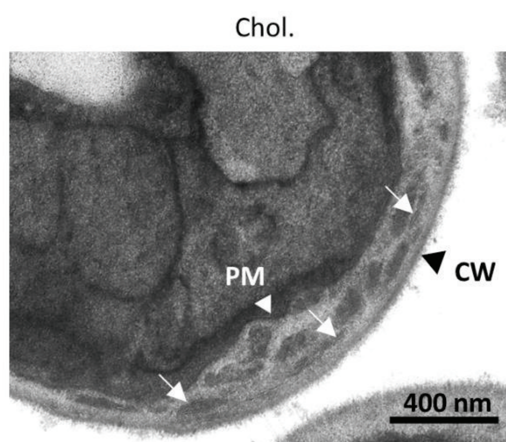

Fig. 6. Electron microscopy of the Chol. strain cell wall. Cells were cultivated on glycerol for 48 h and then induced with 0.5% methanol for 48 h. CW – cell wall; PM – plasma membrane. White arrows indicate membrane structures.

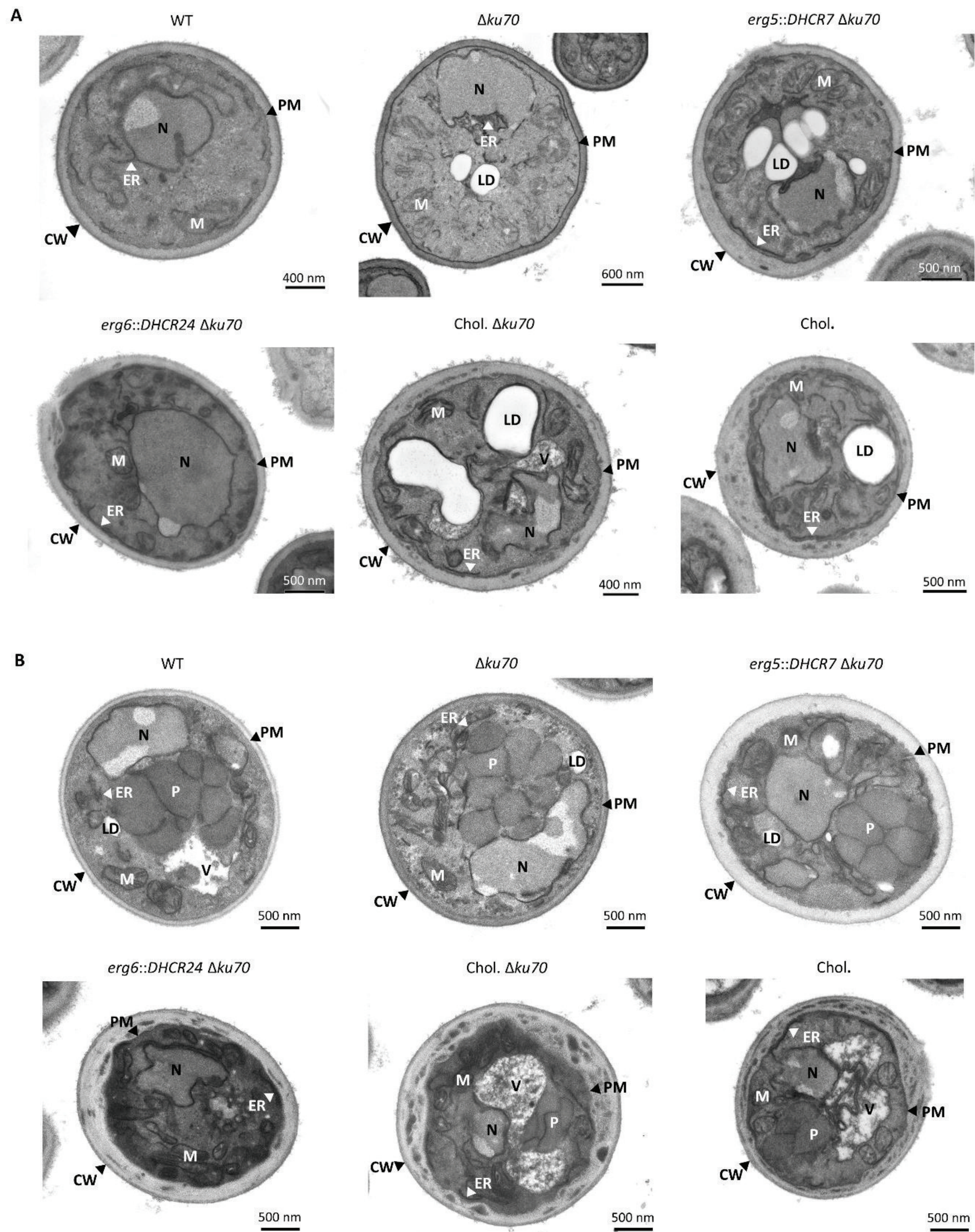

Fig. 7. Transmission electron microscopy of sterol-modified *P. pastoris*. (A) Cells were grown on YPD for 48 h. **(B)** Cells were grown for 48 h on glycerol following methanol induction for additional 48 h. P – peroxisomes. ER – endoplasmic reticulum; N – nucleus; PM – plasma membrane; LD – lipid droplet; M – mitochondria; CW – cell wall.

4.4.3. The Cell Wall Sugar Composition and Nanomechanical Properties Were Not Significantly Altered

The highly unusual morphology of Chol. and Chol. Δ*ku70* cell walls seen in the electron microscopic images prompted us to investigate the cell wall sugar composition in more detail. To do so, we isolated cell wall material, performed acid hydrolysis to release monosaccharides from glucans, mannans and chitin, and analyzed the monosaccharide composition via HPAEC-PAD. This analysis revealed that the major sugar composition was essentially identical in the WT and Chol. strain. Only when comparing strains with and without Δ*ku70* deletion, we spotted a slight decrease of glucose and mannose released after hydrolysis (Fig. 8, A). Our previous growth analysis on calcofluor white indicated that the chitin content should be decreased in the Chol. Δ*ku70* strain. Unfortunately, the chemical method used was not well-suited to hydrolyze and quantify chitin accurately. Therefore, enzymatic methods will be more suitable [42].

During acid hydrolysis, we observed large flocks forming, consisting of acid insoluble material. Moreover, the mass of released sugars per mg cell wall was very low (18-28%) when compared to the recovery yields of around 90% usually obtained for *S. cerevisiae* with this method [70]. Therefore, we speculated that we might have some contaminants in our cell wall preparations. We investigated the cell wall material for contaminants, such as large amounts of proteins or attached lipids. Upon phospholipid extraction, we detected between 15-25 µg of phospholipids per mg cell wall (Fig. 8, B). Differences between phospholipid species most likely arose because of the extraction and quantification procedure.

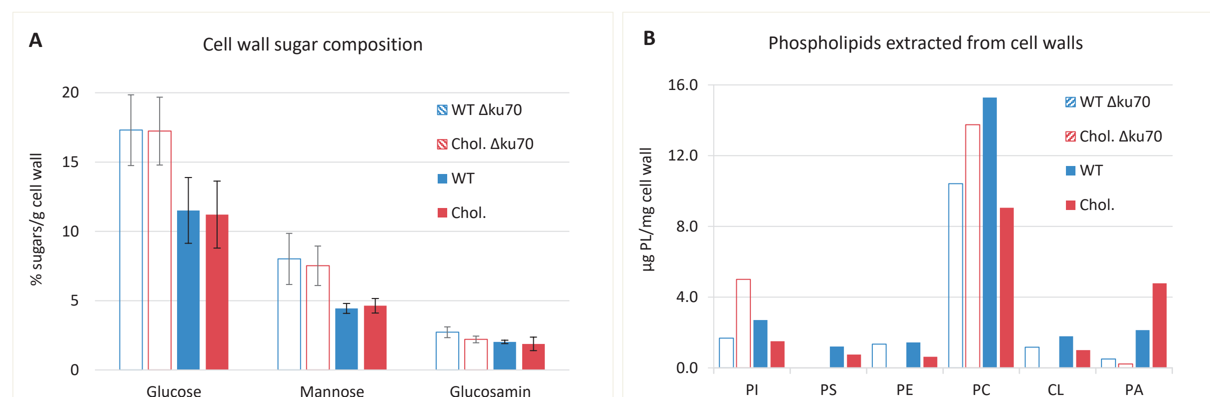

Fig. 8. Cell wall composition of *P. pastoris* WT, Δ*ku70*, Chol. and Chol. Δ*ku70* strains. (A) Sugar monomers of 10 mg cell wall were released by acid hydrolysis and analyzed by HPAEC-PAD. Error bars indicate standard deviation from three independent experiments (biological replicates). **(B)** Phospholipids were extracted from 2-3 mg of cell wall material from each strain and analyzed by 2-dimensional thin-layer chromatography followed by photometric phosphate determination. Phospholipids were quantified once.

Additionally, we found roughly 20% of cell wall mass to be protein in the WT and WT Δ*ku70* strains upon extraction under alkaline conditions and protein determination using the Bradford Assay. In the

Chol. and Chol. Δ*ku70* strains, protein content was less as compared to WT strains, comprising around 15-17% of total cell wall mass. It is possible that the large amount of proteins in the cell wall preparations inhibited acid hydrolysis. The proteins extracted from our cell wall preparations were also analyzed by mass spectrometry, and the most abundant species were listed in Table 3. It can be clearly seen that large amounts of intracellular proteins, such as ribosomal proteins and mitochondrial enzymes, were still present in the cell wall preparations. Therefore, the protocol for cell wall isolation will have to be optimized for *P. pastoris.*

Table 3. Mass spectrometry of cell wall proteins

Chol./Chol. Δ*ku70*				
Accession	**Description**	**Score**	**Coverage**	**#PSM[a]**
254573010	Major ADP/ATP carrier of the mitochondrial inner membrane	5207.42	27.96	234
254572796	Nuclear protein required for transcription of *MXR1*	3071.58	26.65	105
254568544	Mitochondrial alcohol dehydrogenase isozyme III	2807.45	14.00	83
254573014	Protein component of the small (40S) ribosomal subunit, nearly identical to Rps7Bp	1283.44	27.13	52
254565451	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl12Ap	986.37	23.64	35
254569780	One of two identical histone H4 proteins (see also HHF2)	904.83	40.78	37
254571763	Protein component of the small (40S) ribosomal subunit	865.85	10.41	26
254571387	Alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase	821.38	14.65	38
254567287	mitochondrial phosphate carrier protein	772.57	33.23	29
254569858	Beta subunit of the F1 sector of mitochondrial F1F0 ATP synthase	670.45	15.31	33
WT/WT Δ*ku70*				
Accession	**Description**	**Score**	**Coverage**	**#PSM[a]**
254573010	Major ADP/ATP carrier of the mitochondrial inner membrane	4460.14	29.93	202
254568544	Mitochondrial alcohol dehydrogenase isozyme III	2427.57	13.71	72
254572796	Nuclear protein required for transcription of *MXR*1	1873.24	24.94	71
254565451	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl12Ap	1136.10	23.64	44
254573014	Protein component of the small (40S) ribosomal subunit, nearly identical to Rps7Bp	852.54	27.13	34
254571387	Alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase	847.27	14.65	39
254570575	Major of three pyruvate decarboxylase isozymes	734.61	6.07	34
254569858	Beta subunit of the F1 sector of mitochondrial F1F0 ATP synthase	578.56	12.92	25
254573696	Ribosomal protein 10 (rp10) of the small (40S) subunit	522.99	13.67	20
254573092	Protein component of the small (40S) ribosomal subunit	514.66	10.99	22

To gain further insight into the nanomechanical properties of the cell wall, regardless of its composition, we performed atomic force microscopy experiments. We compared the WT Δ*ku70* and Chol. Δ*ku70* strains and imaged six cells from each strain in two independent experiments (Fig. 9). We imaged single cells from stationary phase cultures after 48 h of growth in YPD. Unfortunately, the long cultivation time resulted in analyzing many old cells having several bud scars, or even dead cells. The bud scars

hamper homogenous scanning of the surface elasticity, as the cells are more rigid close to bud necks due to higher chitin contents. In WT Δ*ku70* cells, the surface was additionally quite inhomogeneous resulting in a high deviation of the Young's modulus.

For some cells, we also generated adhesion images revealing that the cell surface of *P. pastoris* is generally not adhesive (not shown). Fig. 9C shows the zoom-in of a representative AFM stiffness image, which can be obtained upon force mapping with AFM. Each of the 1024 pixels were used to calculate the Young's Modulus values, which were then fitted to a Gaussian distribution to gain information about cell elasticity. Initial results might suggest a decreased stiffness of the Chol. Δ*ku70* strain, but many more cells need to be imaged to gain statistically significant data, and to solidify the assumption drawn from our preliminary experiments.

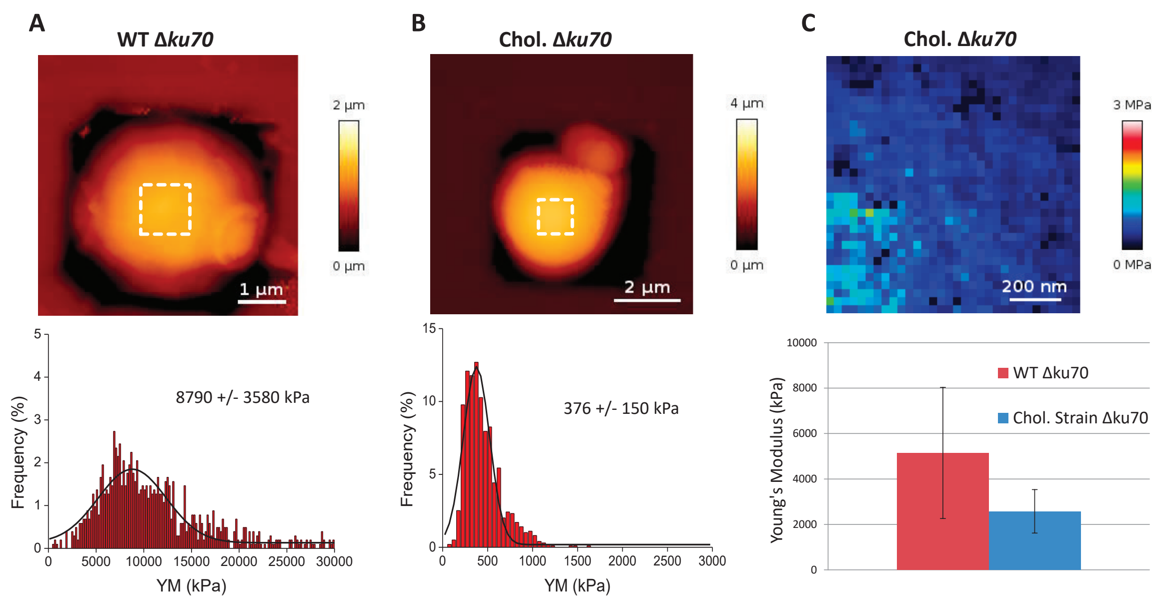

Fig. 9. Analysis of cell stiffness by atomic force microscopy. (A) Representative AFM height image of a WT Δ*ku70* cell **(A)** and a Chol. Δ*ku70* cell **(B)**. Young's Modulus values (kPa) were calculated from the area indicated by the white squares. **(C)** AFM stiffness image zoomed from the Chol. Δ*ku70* cell (white square, B). Mean values of Young's modulus (kPa) ± standard deviation from six imaged cells per strain are shown.

4.4.4. The CWI Pathway is Induced and Sterol Synthesis Genes are Strongly Upregulated

So far, our physiological characterization pointed towards strong impacts on the cell wall integrity pathway. To confirm the activation of CWI signaling via Pim1p phosphorylation, we monitored this important MAP kinase by Western blotting in the cholesterol-producing strain. Pim1p is the homologue of *S. cerevisiae* Slt2p, a major MAP kinase of the CWI pathway. Upon Slt2p phosphorylation, the expression of about 20 cell wall proteins is induced in *S. cerevisiae*, Cwp1p being among them [67].

Upon induction of the CWI pathway with calcofluor white, Pim1p phosphorylation was induced in all tested strains in *P. pastoris*. Astonishingly, we detected large amounts of phosphorylated Pim1p even before the addition of calcofluor white in the Chol. Δ*ku70* strain (Fig. 10). Although slight signals were also visible for the WT and Δ*ku70* strains at timepoint zero, the difference to the Chol. Δ*ku70* strain was remarkable. This indicated that the CWI pathway was constitutively activated in the cholesterol-producing strain. Upon RNA sequencing analysis, we saw that the transcript levels of *PIM1* were not significantly altered as compared to the WT and Δ*ku70* strains. Thus, there should be similar amounts of Pim1p in the Chol. Δ*ku70* strain, which are phosphorylated to a higher extent, even without adding any inducer of the CWI pathway. After 240 min, the signal of phosphorylated Pim1p decreased in all tested strains, which could be either due to instability of calcofluor white or dephosphorylation of Pim1p after signal transduction.

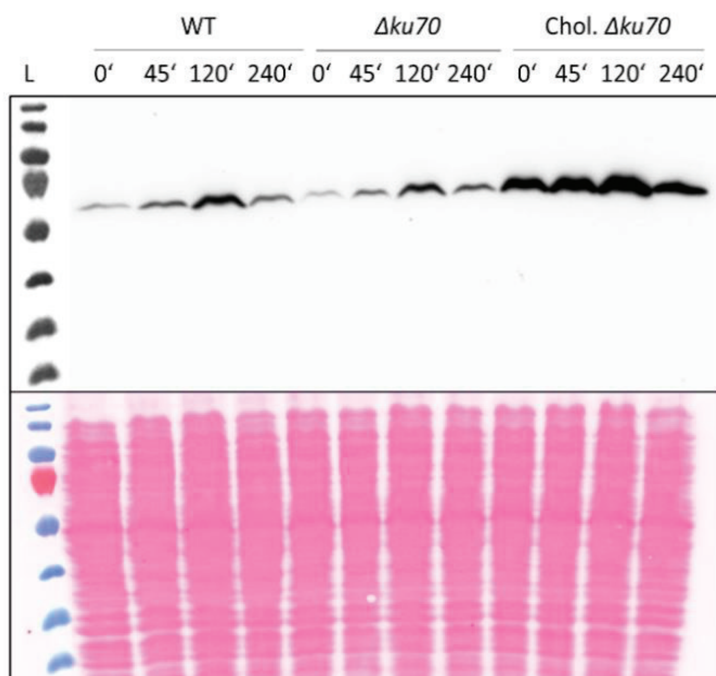

Fig. 10. Immunodetection of phosphorylated Pim1p. Cells were cultivated for 15 h in YPD media and the CWI pathway was induced by addition of calcofluor white (1 mg/ml). Three OD_{600} units were harvested after 0, 45, 120, and 240 min, and total proteins from cell lysates were separated by SDS-PAGE. Phosphorylated Pim1p was detected using anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody (upper panel). Total proteins were stained with Ponceau S (lower panel).

To identify genes that were up- or downregulated in the sterol-engineered strains, we sequenced total RNA upon induction with methanol. Table 4 shows the most interesting hits clustered by the annotated cellular function. Results of the *erg5*::*DHCR7* Δ*ku70* and *erg6*::*DHCR24* Δ*ku70* strains can be found in

supplemental Table S4. Furthermore, the description of interesting genes is summarized in supplemental Table S3. In total, we identified 773 genes that were 2-fold or higher upregulated and 1200 genes that were at least 2-fold downregulated in the Chol. Δ*ku70* strain. It would go beyond the scope of this work to analyze all genes in detail. Therefore, we chose the most interesting genes whose expression levels were changed 5-fold or higher. We found many genes of the ergosterol biosynthesis pathway to be strongly upregulated in the cholesterol-producing strain, with *ERG2* (240-fold), *ERG3* (92-fold), and *ERG11* (80-fold) being the most prominent. Also, RNA levels of the transcription factor *UPC2* regulating transcription of sterol related genes were 33-fold increased. Sphingolipid biosynthesis genes were also upregulated, although fewer genes were affected. Expression of the delta-4 sphingolipid desaturase *DES1* was 15-fold enhanced. Additionally, phospholipid biosynthesis was affected in the Chol. Δ*ku70* strain: *INO1* was upregulated, but other genes and transcription factors for phospholipid synthesis were downregulated. This indicates, that the cells responded to the changed sterols by remodeling the membrane lipid composition. The secreted cell wall protein *CWP1* gene was upregulated up to 58-fold. This protein was also found by mass spectrometry analysis to be highly abundant in the culture supernatants. Interestingly, three cell-wall glucanases *EXG1*, *RCE3* and *SUN4* were strongly upregulated in the Chol. Δ*ku70* strain. This could partially explain the abnormal cell wall structure, although many other important genes for cell wall synthesis were not differentially expressed. Higher activity of cell wall glucanases could also potentially explain the elevated secretion of Cwp1p.

Little is known about the *Pichia* homologue of secreted *PRY1/3*, but we observed that an altered membrane sterol composition also affected expression of this sterol binding protein involved in the export of acetylated sterols [71]. Surprisingly, also genes required for methanol utilization like *DAS1/2* and *FLD1,* as well as the peroxisomal *PMP20,* were more strongly expressed in the Chol. Δ*ku70* strain as compared to the WT and Δ*ku70* strains. The expression of *AOX1* and *AOX2* genes was, however, not strongly affected. We expected to find differentially expressed membrane proteins, which indeed held true. Some transporters such as the glucose transporter *HXT1* or the copper transporter *CTR1* were upregulated, but most of the affected proteins were strongly downregulated in the cholesterol-producing strain. This could be a protective mechanism when the cells are sensing that the membrane surrounding is not suitable for these proteins. Another reason could be that lower growth rates require less nutrient uptake and thus expression of transport proteins is diminished. To our surprise, we found many genes involved in amino acid metabolism, including transporters, to be downregulated in the Chol. Δ*ku70* strain. TORC1 is described to play a key role in nutrient sensing and responds to amino acid levels in *S. cerevisiae* [16]. One could speculate that amino acid levels in the Chol. Δ*ku70* strain are sufficient so that their catabolism is downregulated and thereby TORC1 is active. As a result, TORC1 could induce translation initiation, ribosome biogenesis and cytokinesis genes, as described in *S. cerevisiae* [16]. Furthermore, TORC1 inhibits the retrograde response transcription factor *RTG3*, which was indeed downregulated 34-fold in the Chol. Δ*ku70* strain.

Table 4. RNA sequencing results of WT, Δ*ku70* and Chol. Δ*ku70* strains.

Lipid biosynthesis				fold change		
	Name	NCBI Accession	Feature ID	Δ*ku70* vs. Chol.	WT vs. Chol.	Δ*ku70* vs. WT
Sterols	*ERG2*	CCA40964	ACIB2EUKG772851	235.7	184.9	1.3
	ERG3	CCA37283	ACIB2EUKG768951	91.7	53.5	1.7
	ERG11	CCA39186	ACIB2EUKG770976	79.1	65.5	1.4
	NCP1	CCA40319	ACIB2EUKG772172	13.7	18.5	1.4
	ERG24	CCA36873	ACIB2EUKG768514	55.7	38.3	1.5
	ERG25	CCA40131	ACIB2EUKG771974	45.5	18.2	2.5
	UPC2	CCA38869	ACIB2EUKG770635	33.3	22.3	1.5
	ERG26	CCA37528	ACIB2EUKG769204	28.7	20.4	1.4
	ERG4	CCA41104	ACIB2EUKG772999	27.3	25.8	1.1
	ERG1	CCA40055	ACIB2EUKG771896	15.7	19.4	1.2
	ERG13	SCV12104	ACIB2EUKG770547	15.6	19.3	1.2
	ERG27	CCA38340	ACIB2EUKG770066	15.4	21.1	1.37
	ERG20	CCA37230	ACIB2EUKG768894	13.8	34.2	2.5
	CYB5-1	CCA40773	ACIB2EUKG772644	10.6	16.9	1.6
	ERG7	CCA38589	ACIB2EUKG770328	11.0	12.7	1.2
	ERG10	CCA37220	ACIB2EUKG768884	11.0	11.7	1.1
	ERG9	CCA39850	ACIB2EUKG771673	6.5	8.4	1.3
	IDI1	CCA37348	ACIB2EUKG769019	5.1	8.6	1.7
Sphingolipids	*DES1*	CCA39205	ACIB2EUKG770996	14.6	10.1	1.4
	HET1	CCA37849	ACIB2EUKG769541	8.0	13.9	1.7
	SLD1	CCA36466	ACIB2EUKG768087	4.5	4.7	-1.0
	SCS7	CCA39626	ACIB2EUKG771439	2.3	1.5	1.5
	LAC1	CCA37899	ACIB2EUKG769594	1.7	1.7	1.0
	LAG1	CCA36821	ACIB2EUKG768456	1.5	1.4	1.0
	GCS	CCA39811	ACIB2EUKG771628	1.3	-1.0	1.3
	SUR1	CCA36384	ACIB2EUKG768002	-1.8	-1.9	-1.1
	SUR2	CCA39741	ACIB2EUKG771552	-1.1	-1.1	1.0
PL	*INO1*	CCA37816	ACIB2EUKG769506	15.0	63.7	4.23
	GPT2	CCA36245	ACIB2EUKG767856	-14.5	-17.5	-1.2
	PP7435_CHR3-0866	CCA39818	ACIB2EUKG771638	-9.4	-10.3	-1.1
	PLB3	CCA41015	ACIB2EUKG772901	-8.5	-9.2	-1.1
	OPI1	CCA36491	ACIB2EUKG768112	-6.5	-10.5	-1.6

Secretory pathway/Cell wall				fold change		
	Name	NCBI Accesion	Feature ID	Δ*ku70* vs. Chol.	WT vs. Chol.	Δ*ku70* vs. WT
Cell wall	*CWP1*	CCA39831	ACIB2EUKG771652	58.4	23.8	-2.5
	EXG1	CCA38524	ACIB2EUKG770258	55.5	74.6	1.3
	SUN4	CCA37765	ACIB2EUKG769450	14.7	20.2	1.4
	RCE3	CCA40496	ACIB2EUKG772352	11.2	11.3	1.0
	CHS2	SCV11969	ACIB2EUKG769486	3.1	6.9	2.2
	CHS1	CCA36855	ACIB2EUKG768494	3.2	2.2	-1.5
	CHS3	CCA38920	ACIB2EUKG770688	-1.5	-1.7	-1.1
	MNN9	CCA41064	ACIB2EUKG772955	2.0	2.3	1.2
	GAS1	CCA36391	ACIB2EUKG768009	2.0	1.7	-1.2
	FKS1	CCA38307	ACIB2EUKG770030	1.4	1.6	1.1
	FKS3	CCA36390	ACIB2EUKG768008	-1.2	1.5	1.8
	PIM1	CCA39249	ACIB2EUKG771047	1.3	-1.1	-1.4
	KRE6	CCA40635	ACIB2EUKG772494	1.1	1.1	-1.0
	SWI6	CCA40504	ACIB2EUKG772360	-1.8	-1.5	1.2
Secreted	*CWP1*	CCA39831	ACIB2EUKG771652	58.4	23.8	-2.5
	PRY1/PRY3	CAY70008	ACIB2EUKG771944	22.3	42.9	1.9
	FLO5	CCA37505	ACIB2EUKG769182	-1.2	1.9	3.2

Other differentially regulated genes				fold change		
	Name	**NCBI Accession**	**Feature ID**	**Δ*ku70* vs. Chol.**	**WT vs. Chol.**	**Δ*ku70* vs. WT**
MeOH utilization	*PMP20*	SCV11926	ACIB2EUKG769143	37.1	48.0	1.3
	DAS1	CCA39320	ACIB2EUKG771118	21.8	34.2	1.6
	DAS2	CCA39318	ACIB2EUKG771116	14.3	27.2	1.9
	FLD1	CCA39112	ACIB2EUKG770901	4.1	4.2	1.0
	AOX1	CCA40305	ACIB2EUKG772156	2.0	2.2	1.1
	AOX2	CCA41016	ACIB2EUKG772902	-2.7	-2.4	1.1
Membrane proteins/Transporter	*VMA21*	CCA36872	ACIB2EUKG768513	13.4	14.0	1.1
	AXL2	CCA38266	ACIB2EUKG769985	12.2	32.4	2.7
	PpHXT1	CCA37491	ACIB2EUKG769167	11.2	29.3	2.3
	CTR1	CCA40982	ACIB2EUKG772868	7.8	15.6	2.0
	SSO1	CCA37207	ACIB2EUKG768871	7.7	8.4	1.1
	KCH1	CCA38408	ACIB2EUKG770135	6.5	4.1	-1.6
	YCT1	CCA36184	ACIB2EUKG767791	-794.1	-930.1	-1.2
	XUT5	CCA36174	ACIB2EUKG767781	-656.1	-110.5	5.94
	FMO1-1	CCA36181	ACIB2EUKG767788	-651.8	-583.9	1.1
	GTH1	CCA36179	ACIB2EUKG767786	-204.5	-52.8	3.9
	ADY2	CCA36620	ACIB2EUKG768246	-102.9	-139.4	-1.4
	HGT2	CCA40159	ACIB2EUKG772004	-58.8	-87.2	-1.5
	MUP1-2	CCA40163	ACIB2EUKG772008	-48.5	-26.4	1.8
	EOS1	CCA41052	ACIB2EUKG772941	-15.4	-17.7	-1.15
	HSP12	CCA40512	ACIB2EUKG772368	-14.7	-13.4	1.1
	FMO1-4	CCA39292	ACIB2EUKG771090	-10.0	-3.6	2.8
	PP7435_Chr1-0971	CCA37105	ACIB2EUKG768765	-9.6	-7.5	1.3
	VHC1	CCA39516	ACIB2EUKG771325	-8.9	-9.1	-1.0
	ZRT1	CCA39646	ACIB2EUKG771459	-8.9	-5.3	1.7
	PHO89	CCA38747	ACIB2EUKG770500	-8.1	-9.4	-1.2
	ADY2-3	CCA36880	ACIB2EUKG768522	-6.1	-4.5	1.4
	PUT4-2	CCA38309	ACIB2EUKG770032	-6.0	-5.4	-1.12
	GDH2	CCA38671	ACIB2EUKG770416	-5.9	-6.0	-1.0
	DAL5-2	CCA36817	ACIB2EUKG768452	-5.7	-3.1	1.8
	SMF1	CCA39170	ACIB2EUKG770959	-5.7	-7.4	-1.3
	FPS1[a]	CCA40346	ACIB2EUKG772199	-5.1	-6.0	-1.17
Transcription factors	*PLM2*	SCV11828	ACIB2EUKG768437	10.5	58.8	5.6
	PP7435_Chr1-0006	CCA36178	ACIB2EUKG767785	-285.3	-289.9	-1.0
	PP7435_Chr1-0010	CCA36182	ACIB2EUKG767789	-79.6	-54.5	1.5
	PP7435_Chr1-0013	CCA36185	ACIB2EUKG767792	-69.0	-68.4	1.0
	RTG3	CCA40210	ACIB2EUKG772067	-34.3	-49.9	-1.5
	CAT8-2	CCA40601	ACIB2EUKG772458	-12.0	-15.1	-1.3
	CYC8	CCA38198	ACIB2EUKG769916	-10.7	-8.3	1.3
	PpAFT1	CCA37276	ACIB2EUKG768944	-10.5	-17.5	-1.7
	ZCF2	CCA36469	ACIB2EUKG768090	-10.5	-4.3	2.5
Amino acid metabolism	*PP7435_Chr1-0008*	CCA36180	ACIB2EUKG767787	-271.9	-173.0	1.6
	ARO9	CCA41021	ACIB2EUKG772908	-21.7	-16.0	1.4
	MXR1	CCA41165	ACIB2EUKG773061	-14.3	-17.7	-1.2
	SAM4	CCA40476	ACIB2EUKG772332	-10.9	-17.2	-1.6
	ARO10-1	CCA40086	ACIB2EUKG771927	-9.8	-14.5	-1.5
	MET1	CCA36242	ACIB2EUKG767853	-9.7	-2.2	4.4
	GDH3	CCA36566	ACIB2EUKG768193	-9.5	-12.3	-1.3
	PUT4-2	CCA38309	ACIB2EUKG770032	-6.0	-5.4	-1.12
	DIP5-1	CCA40867	ACIB2EUKG772748	-5.4	-4.0	1.4
Kinases	*KSS1*	CCA40612	ACIB2EUKG772469	6.5	10.0	1.5
	GUT1	CCA40348	ACIB2EUKG772200	-24.2	-26.6	-1.1
	KIN3	CCA37370	ACIB2EUKG769042	-5.6	-4.0	1.4
	YPK2	CCA40052	ACIB2EUKG771893	-5.1	-4.5	1.1

Other differentially regulated genes				fold change		
	Name	**NCBI Accession**	**Feature ID**	**Δ*ku70* vs. Chol.**	**WT vs. Chol.**	**Δ*ku70* vs. WT**
Cytokinesis	*CDC12*	CCA40863	ACIB2EUKG772742	9.7	12.6	1.3
	PCL2	CCA40206	ACIB2EUKG772071	9.7	18.8	1.9
	CDC10	CCA40281	ACIB2EUKG772131	5.7	8.7	1.5
	TEM1	SCV12368	ACIB2EUKG772478	5.5	7.4	1.3
	SHS1	CCA39164	ACIB2EUKG770953	5.1	7.4	1.5
	TUB4	CCA41020	ACIB2EUKG772907	-11.6	-8.5	1.4
Central carbon metabolism/Others	*PP7435_Chr1-0624*	CCA36773	ACIB2EUKG768404	55.7	114.9	1.35
	GEP3	CCA38771	ACIB2EUKG770524	44.2	88.4	2.0
	RKI1	SCV12413	ACIB2EUKG772836	38.3	51.8	1.4
	FBA1	CCA36787	ACIB2EUKG768418	28.1	47.4	1.7
	NUP60	CCA37527	ACIB2EUKG769203	16.0	23.7	1.5
	USP1	CCA39586	ACIB2EUKG771396	11.6	16.7	1.4
	RPS28B	CCA37146	ACIB2EUKG771711	10.2	15.6	1.5
	PP7435_Chr1-0005	CCA36177	ACIB2EUKG767784	-171.9	-197.3	-1.2
	PP7435_Chr1-0004	CCA36176	ACIB2EUKG767783	-64.2	-33.3	1.9
	PP7435_Chr1-0011	CCA36183	ACIB2EUKG767790	-57.2	-63.7	-1.1
	PP7435_Chr2-0422	CCA38112	ACIB2EUKG769823	-26.0	-71.3	-2.7
	DRE2	CCA37215	ACIB2EUKG768879	-25.4	-16.5	1.5
	PP7435_Chr1-0014	SCV11752	ACIB2EUKG767793	-25.0	-23.5	1.1
	PP7435_Chr2-0218	CCA37915	ACIB2EUKG769613	-14.8	-16.9	-1.1
	LRA2	CCA40813	ACIB2EUKG772689	-14.1	-21.5	-1.5
	PP7435_Chr2-1115	CCA38792	ACIB2EUKG770546	-10.5	-11.4	-1.1
	TMA17	CCA39072	ACIB2EUKG770860	-9.6	-12.3	-1.3

[a] annotated as putative channel-like protein *PP7435_Chr4-0171*. Only protein in *P. pastoris* with similarity to *S. cerevisiae* Fps1p.

Strong downregulation was also observed for a lot of hypothetical proteins with unknown function. Three of them (*PP7435_CHR1-0006*, *PP7435_CHR1-0010*, and *PP7435_CHR1-0013*) have similarity to transcription factors with conserved *GAL4*-like zinc cluster domains. Also, the potential fungal zinc cluster transcription factor *ZCF2* was found in the list of weakly downregulated genes. The gene *PP7435_CHR3-0866* is described to have low similarity to the transcriptional activator Ino2p, which forms a dimer with Ino4p in *S. cerevisiae*. These transcription factors are involved in phospholipid synthesis. It is astonishing that most of the transcription factors found by RNA sequencing are downregulated, with only one exception. The putative transcription factor *PLM2* was 10.5-fold upregulated in the Chol. Δ*ku70* strain and is described to play a role in response to DNA damage in *S. cerevisiae*.

We also sequenced total mRNA of the *erg5*::*DHCR7* Δ*ku70* and *erg6*::*DHCR24* Δ*ku70* strains and the most important results were listed in supplemental Table S4. In summary, we found 803 genes to be upregulated 2-fold or higher and 2226 genes downregulated 2-fold or higher in the *erg5*::*DHCR7* Δ*ku70* strain, whereas in the *erg6*::*DHCR24* Δ*ku70* strain only 122 genes were higher than 2-fold upregulated and 1882 were downregulated. Within the upregulated genes, we found certain genes exclusively in the *erg5*::*DHCR7* Δ*ku70* strain, such as *PDR17* (7-fold), encoding for a phosphatidylinositol transfer protein, *SEC62* (6-fold), a component of the Sec62/Sec63 complex involved in the posttranslational

translocation of proteins, and *SLD5* (5-fold), which is involved in assembly of the DNA replication machinery. It was noticeable that mitochondrial proteins were strongly regulated in either direction, which could indicate an adaptation of the energy metabolism in the *erg5*::*DHCR7* Δ*ku70* strain.

In the *erg6*::*DHCR24* Δ*ku70* strain, we also found a few exclusively regulated genes. For example, expression levels were increased for genes involved in RNA synthesis, processing or binding (SRB8, 12-fold; *MPT5*, 7-fold) and strongly decreased for the methionine-sulfoxide reductase *MXR2-1* (74-fold), which is involved in the response to oxidative stress and the regulation of life-span. Noticeably, many of the differentially expressed genes found in the *erg6*::*DHCR24* Δ*ku70* strain were of unknown function and encode for more or less conserved putative proteins.

Interestingly, all three sterol-modified strains showed a common upregulation of the ergosterol biosynthesis genes, although not all affected genes were identical. *UPC2*, the transcriptional activator of sterol biosynthesis and transport, was upregulated in the Chol. Δ*ku70* and *erg5*::*DHCR7* Δ*ku70* strains. Additionally, all three strains shared a strong downregulation in amino acid metabolism. Also, the expression of several membrane proteins was found to be differentially regulated, which was expected for the sterol-modified strains. It will be interesting to look at differentially expressed genes when the cells are grown on glucose and, consequently, confirm the most prominent hits by quantitative PCR.

4.4.5. A Knockout Library Screening Revealed Putative Roles for TORC2 Signaling, Ca^{2+}/Calmodulin Signaling and Phosphatidylcholine Metabolism in Stress Adaptation

Besides characterizing the cell wall morphology and composition, and identifying differentially expressed genes, we also wanted to find genes that are possibly involved in the regulation of osmotic and cold response signaling in the cholesterol-producing *P. pastoris* strain. For this purpose, we performed a random cassette mutagenesis of the whole genome of the Chol. Δ*ku70* strain. It has been shown previously, that fragments without any homology to the genome integrate randomly via non-homologous end-joining in *P. pastoris* (doctoral thesis Christine Winkler, [29]).

The strong growth phenotypes of the Chol. Δ*ku70* strain were well-suited to establish a stringent screening procedure to look for potential genes involved in sterol-dependent stress regulation. As the growth of the cholesterol-producing strain was completely abolished at 18°C and on 0.5 M NaCl, these conditions were suitable for a growth-based screening. First, we screened at 28°C for hygromycin resistant clones having the knockout cassette integrated, and then resuspended all obtained clones for the second phenotypic screening. High dilutions of the cell suspension were spread on YPD under selective conditions, *i.e.* containing 0.5 M NaCl or at 18°C. The benefit of this method was a very high clone throughput, which compensated for the elevated probability of screening multiple identical clones. In total, more than 4000 clones were screened for restored growth. In initial experiments, we also screened on 0.01% SDS, however, we never obtained any positive clones.

Twelve clones from each condition were re-screened and the six best clones were subjected to genome walking experiments. The re-screening was performed by spot-tests of serially diluted cultures. It revealed that the mutant strains indeed could grow under the selective conditions..WT-like growth was not reached though, still pointing towards multiple factors involved in sterol-related growth phenotypes (Fig. 11, A and B). To ensure that none of the cholesterol biosynthesis genes were affected upon random mutation, we also confirmed presence of cholesterol in all of the re-screened knockout strains via GC-MS (not shown).

The genome walking approach worked in principle, but it also had one major drawback in the Chol. Δ*ku70* strain: This strain already contained the zeocin and G418 resistance cassettes, which are controlled by the *TEF1* promoter, and thus provided additional binding sites for the mutagenesis cassette specific primer. Due to the additional, undesired amplicons, genome walking detected several fragments belonging to the previously integrated knock-in-cassettes such as *DHCR7*, *AOX1* terminator and zeocin resistance cassette sequences, and *ERG5* flanking regions. It is likely that the hygromycin cassette also integrated into these regions, and thus relieved sterol-engineering induced stress.

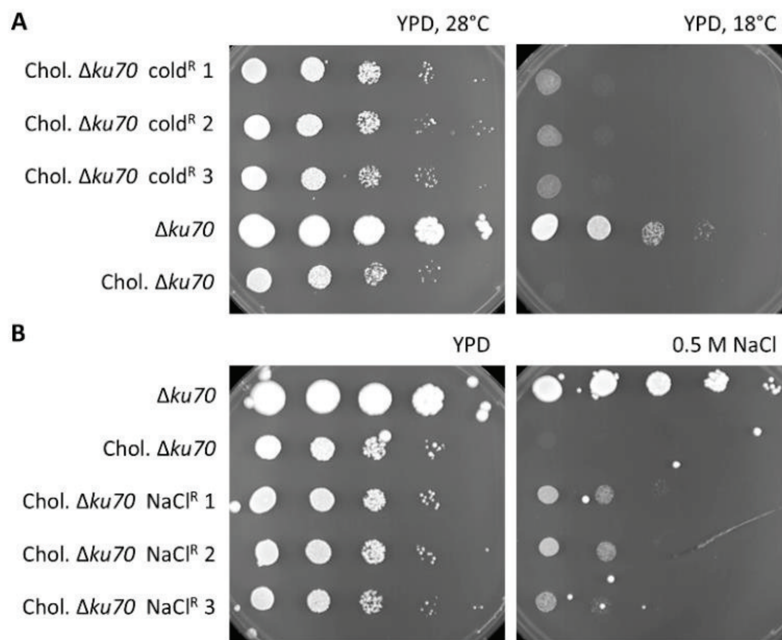

Fig. 11. Re-screening of *P. pastoris* Chol. Δ*ku70* mutants obtained through hygromycin cassette mutagenensis. Cells were grown for 48 h on YPD and 10-fold serially diluted to 10^{-4}. Plates were imaged after 3-4 days. **(A)** Three µl of each dilution were spotted onto YPD plates and incubated at 28°C or 18°C. Three representative cold-resistant mutant strains are shown. **(B)** Three µl of each dilution were spotted onto YPD plates with and without 0.5 M NaCl and were incubated at 28°C. Three representative NaCl-resistant mutant strains are shown.

Despite the difficulties of the genome walking approach, we were able to identify three loci wherein the hygromycin cassette was integrated. In two cases, the cassette integrated directly into the coding sequence of distinct genes, and in the third case it was located between two genes, possibly disturbing promoter or terminator sequences. The first hit was identified in the NaCl resistant mutants to be a putative membrane protein of unknown function (Table 5). Protein blast search revealed the *S. cerevisiae* homologue *YGR149W*, whose gene product was recently characterized to be a glycerophosphocholine (GPC) acyltransferase and was renamed *GPC1* [32,72]. The second hit was a gene initially identified as *STE16*, which was recently re-annotated to be the *P. pastoris* homologue of *TSC11*, a major subunit of the TOR complex 2. The hygromycin resistance cassette was directly integrated into the coding sequences of these two genes, thereby likely disrupting their function. A third integration locus was identified in between two genes. It was placed in front of the *CMK2* gene, possibly disrupting the promoter, and behind the coding sequence of a hypothetical protein, potentially disrupting the terminator sequence. This hypothetical protein shares 29% sequence identity with *S. cerevisiae ARH1*, an essential oxidoreductase of the mitochondrial inner membrane. In terms of their function, it seems that the *CMK2* target is of greater interest, as it might play a role in Ca^{2+}/calmodulin stress signaling. However, for the time being, we proceeded with analyzing only the two unambiguous genes that were targeted by the hygromycin knockout cassette.

Table 5. Genes targeted by cassette mutagenesis. Integration loci were identified by nucleotide blast search against *P. pastoris* CBS7435 and GS115.

NCBI Accession	Name	Remarks	*S. cerevisiae,* homologous gene
CCA37792	*YGR149W*	Found in NaCl resistant clone, 149 nt aligned; target sequence located within the cds; Glycerophosphocholine acyltransferase, predicted integral membrane protein	*GPC1* (57%)
CCA40770	*TSC11*	Found in cold-resistant clone, 1036 nt aligned; target sequence located within the cds; Subunit of TORC2	*TSC11*/*AVO3* (27%)
CCA37217	*CMK2*	Found in the same cold-resistant clone with *TSC11* insertion, 318 nt aligned; target sequence located within the promoter region; Ca^{2+}/calmodulin-dependent protein kinase 2	*CMK2* (49%)
SCV11891	Hypothetical protein	Found in the same cold-resistant clone with *TSC11* insertion, 318 nt aligned; target sequence located within the terminator region; Oxidoreductase of the mitochondrial inner membrane	*ARH1* (29%)

After having identified these two interesting genes, we wanted to confirm the observed complementation phenotypes for 0.5 M NaCl and cold sensitivity. Therefore, we created clean deletion strains following the strategy of Ahmad et al. (doctoral thesis, [73]) as described in the materials and methods section. We confirmed the deletion of *GPC1* and *TSC11* by PCR and investigated phenotypes via spot tests. It turned out that the *tsc11* deletion strain was also more resistant towards 0.5 M NaCl and the *GPC1* deletion also compensated the growth defect at 18°C, pointing towards more general mechanisms (Fig. 12, A). This might indicate that similar regulatory pathways are affected, which are beneficial for growth under these stress conditions. Interestingly, the *tsc11* knockout also reversed the Chol. Δ*ku70* strain's resistance towards calcofluor white, demonstrating a role in cell wall synthesis with respect to chitin contents.

Moreover, we intended to investigate a potential effect on rapamycin sensitivity by the *tsc11* deletion. It was described recently in *S. cerevisiae* that the Tsc11p/Avo3p subunit sterically blocks the rapamycin binding site and is therefore responsible for rapamycin insensitivity of TORC2 [74,75]. Spot tests showed that the parental Chol. Δ*ku70* and Chol. strains, as well as the *tsc11* deletion strains, were hyper-sensitive towards rapamycin. Therefore, we could not gain further information about the Tsc11p subunit in *Pichia*, but we can presume a general influence on proper TOR signaling to control cell growth in the parental Chol. Δ*ku70* and Chol. strains.

To rule out any Δ*ku70* deletion specific effect, we also created the same knockouts in the Chol. strain. We were able to reproduce and thereby confirm the complementation phenotypes also in this Chol. strain background (Fig. 12, B).

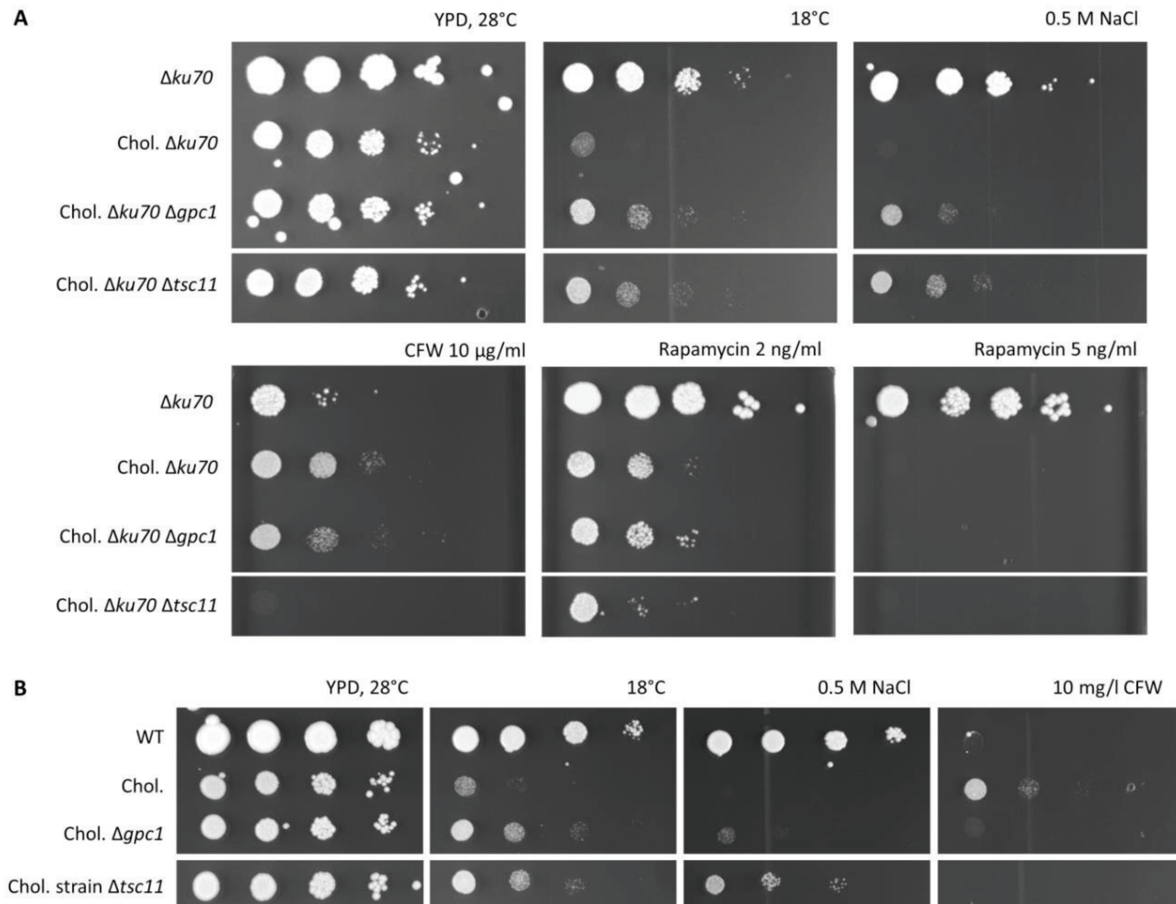

Fig. 12. Spot-tests of clean Δ*gpc1* and Δ*tsc11* knockout strains. Cell pellets corresponding to 1.5 OD_{600} units were harvested, serially diluted to 10^{-4} and 3 µl were spotted onto YPD plates with the indicated additives. Plates were incubated at 28°C or 18°C and imaged after 3 days. Knockout strains were generated in the Chol. Δ*ku70* background **(A)** and in the Chol. background **(B)**.

To learn more about the physiological roles of our identified targets, we compared them to the annotated gene functions in the *S. cerevisiae* literature and genome database. Our first hit was annotated as glycerophosphocholine acyltransferase *GPC1*. It has been reported that the turnover of phosphatidylcholine (PC) may contribute to osmotic adaptation of *S. cerevisiae* via biosynthesis of glycerophosphocholine, which acts as an osmoprotectant [76]. Upon knockout of the *GPC1* homologue in our cholesterol-producing strain, the reacylation of GPC could be hampered and, therefore, the osmoregulator GPC could accumulate. PC is mainly synthesized *de novo* from GPC via the CDP-choline pathway in *S. cerevisiae* [77]. However, a blocked reacylation of GPC seems to be effective in protecting the cells from high salt concentrations.

Our second hit, *STE16*, was found initially in the cold-resistant clones and was recently re-annotated in NCBI to be the homologue of *ScTSC11*. The *STE16* gene product has 27% sequence identity to *S. cerevisiae* Avo3/Tsc11p, an essential component of the target of rapamycin complex 2 (TORC2). TORC1 and TORC2 share important, common functions for regulating cell growth and cell wall

integrity in *S. cerevisiae.* TOR complex 2 is furthermore involved in the regulation of sphingolipid metabolism. Several growth-related phenotypes have been described for conditional *S. cerevisiae avo3* mutants, such as hypersensitivity towards caffeine, deficiency in actin cytoskeleton organization, or increased heat sensitivity – all of them pointing towards detrimental effects upon TORC2 depletion [78–80]. Thus, it is astounding that we did not encounter any problems in creating the *tsc11* knockout in *P. pastoris,* indicating that it is not an essential gene. It was also described recently that TORC2 is activated upon membrane stress and consequently regulates sphingolipid synthesis and the cell wall integrity pathway via Ypk2p and Pkc1p phosphorylation [33, 34]. In *S. cerevisiae,* temperature sensitive TORC2 mutants *avo3-30* furthermore showed reduced levels of ceramide [83]. Changes in sphingolipid contents could not be determined experimentally, but we hypothesize that additionally changing other lipid species in the cholesterol strain might have a strong impact on cell growth and stress response.

The exact mechanisms how the knockout of *P. pastoris tsc11* could have a positive impact on growth at 18°C and also on 0.5 M NaCl in the cholesterol-producing strain remains to be determined. One could hypothesize that lack of Tsc11p reduces TORC2 signaling, which could in turn promote calcineurin signaling by Slm1/2 dephosphorylation. This essential yeast stress signaling mechanism was lately described to co-regulate growth and stress response antagonistically with TORC2 [84]. By downregulating TORC2 activity, growth could be generally diminished, but due to the enhanced calcineurin stress signaling, the survival of the cells might be ensured.

Thanks to recently curated genome annotations for *P. pastoris*, we were able to identify an additional gene that was targeted by the knockout screening in the cold-resistant clones. It was annotated as *CMK2*, a calmodulin dependent protein kinase with a potential role in stress response. This target is also highly interesting, as – like mentioned above – the TORC2 signaling and the Ca^{2+}/Calcineurin signaling are interconnected antagonistically via Slm1/Slm2 phosphorylation and dephosphorylation in *S. cerevisiae*. Thus, knocking out one of the regulatory components could promote the other signaling pathway. Anyhow, these relationships are highly speculative and need further investigation.

The common motive of all our identified target genes was that they have designated roles in the regulation of cell wall stress, and the phospholipid or sphingolipid biosynthesis. This indicates that our knockout screening indeed worked and could be further used for the identification of yet unknown sterol stress related gene functions.

4.5. Conclusion and Outlook

Our descriptive study aimed to characterize sterol-modified *P. pastoris* strains in more detail regarding changes in morphology and stress response. Taking all our results together, we can draw several conclusions, which will serve as profound basis for further research to elucidate details in the cell wall integrity pathway of *P. pastoris.* We saw some very specific growth phenotypes of the Chol. Δ*ku70* strain, which were largely independent of the Δ*ku70* deletion. These characteristic phenotypes were partly also seen in the *erg6DHCR24 Δku70* strain. The *erg5DHCR7 Δku70* strain showed in some cases (NaCl, Calcofluor white, Congo red, and Na-orthovanadate) the opposite phenotype. This indicated a very specific role of the structure of membrane sterols. Despite the strong changes of the cell wall morphology observed under the microscope, we could not detect changes in the cell wall abundance of the oligosaccharides β-glucan, mannan and chitin. The resistance towards calcofluor white would actually point towards decreased chitin levels, which we could unfortunately not confirm by the acid hydrolysis with subsequent HPAEC analysis. There are enzymatic methods, which will supposedly be more suitable to determine the chitin content of the cells [42].

The isolation of *Pichia* cell wall material seems to be a challenging task. When we characterized the isolated material further, we found substantial amounts of proteins and phospholipids attached to the cell wall. It could be that the plasma membrane is more strongly attached to the cell wall in *P. pastoris* as compared to *S. cerevisiae*. Regarding the nanomechanical properties that we investigated by atomic force microscopy, we could unfortunately not obtain significant data. It will be interesting to examine more *P. pastoris* cells, ideally also in the logarithmic growth phase, to gain a better insight into changes in cell surface elasticity of sterol-modified *P. pastoris*.

We can conclude from our investigations that several signaling pathways are strongly affected in the sterol-modified strains, such as the CWI or the TOR signaling pathways, which are normally induced upon cell wall stress and nutrient depletion. We focused our study predominantly on investigating the cholesterol-producing strain, because it showed the strongest phenotypes and – in our hands - was the most relevant strain for mammalian membrane protein production. Although there was no external stress exerted, we observed strong responses and changes in the cell, such as a constitutively phosphorylated Pim1p kinase of the CWI pathway, a strongly altered cell wall structure, hyper-secretion of cell wall related proteins and a plethora of differentially regulated genes, especially of those involved in lipid metabolism and cell wall remodeling. Other regulation events at the post-translational level, such as protein phosphorylation, are likely to take place and will also influence cellular response mechanisms.

It was not surprising, that our sterol-engineered strains showed additionally strong phenotypes when external stress factors are applied. This can be used to study the regulatory mechanisms in *P. pastoris* in more detail, especially regarding the influence of membrane sterols. We started with this task by developing gene knockout and genomic library overexpression screenings. However, these procedures

are still under development and need further optimization. To date, there is a wealth of information about the regulatory pathways and signaling mechanisms in *S. cerevisiae,* but research is still in its infancy in *P. pastoris*. For the time being, we can only compare our findings to the already known regulatory pathways in *S. cerevisiae*, but it will be highly interesting to elaborate on the differences between the two yeasts.

In the future, it will be also interesting to study and apply the Chol. strain without the Δ*ku70* deletion in more detail. This strain has not been used yet for producing heterologous membrane proteins. Moreover, most of our experiments were conducted under standard "*Pichia*" protein expression conditions, meaning that cells are cultivated to high cell densities and further induced with methanol. These cultivation conditions inevitably result in a high proportion of stationary phase and partially dying cells. Presumably, more details will be revealed when our strains are studied in their early growth phases. Most certainly it will be possible to identify novel modifiers and genes involved in regulation of signaling pathways by thorough analyses of the affected signaling cascades, *e.g.* in our sterol-modified *P. pastoris* strains. Our cholesterol-producing strain can serve as tool for genetic screenings to identify genes and regulators involved in signaling pathways, which are induced by membrane and/or cell wall stress.

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4.7. References

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4.8. Supplemental Material

Table S1. Primers used in this study.

№	Name	Sequence 5'-3'
	Primers for construction of new cholesterol strain	
1	F_erg5dhcr7	GGTTGAAGAGAGAAGATGGTG
2	R_erg5dhcr7	TTGTGGTATCGTTTCTGGTTG
3	F_erg6dhcr24	GAGTAGAGCAGAGAGCAAGC
4	R_erg6dhcr24	ATTATTGGGCTTTCAGACGG
	Primers for amplification of hygromycin mutagenesis cassette	
5	TEFfw	CCCACACACCATAGCTTCAAAATG
6	CYC1rev	AGCTTGCAAATTAAAGCCTTCGAG
	Primers for genome walking	
7	F_Adaptor	GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT
8	R_CTAG	GATCACCAGCCCC*T*
9	R_TCGA	AGCTACCAGCCCC*T*
10	AP1	GTAATACGACTCACTATAGGGC
11	GSPTefa	TTCCAAACCTTTAGTACGGGTAATTAACGACAC
12	GSPCYC1a	GAGTTAGACAACCTGAAGTCTAGGTCCCTA
13	AP2	ACTATAGGGCACGCGTGGT
14	GSPTEFb	GCTGTGCTTGGGTGTTTTGAAGTGGT
15	GSPCYC1b	GTACAGACGCGTGTACGCATGTAACATTATAC
16	pJET.2 forward	CGACTCACTATAGGGAGAGCGGC
17	pJET1.2 reverse	AAGAACATCGATTTTCCATG GCAG
	Primers for cloning of pPpKC3 knockout plasmids[a]	
18	F_5'Ste16KC	CCAAGAAAAACAACACACCGGATTTAAATTTCACCTTCTCAGATCTCAG
19	R_5'Ste16KC	CTTCGGCCCTAGTGGCCCAATACAATTACGGGCTCAG
20	F_3'Ste16KC	CTTCGGCCGATCAGGCCCGTGAAGATTGAAATACAATGCC
21	R_3'Ste16KC	GATCTGAGAAGGTGAAATTTAAATCCGGTGTGTTGTTTTTCTTGG
22	F_5'YGRKC	CGCTCCTTCCCTAGATCATTTAAATTCGATCCTTCTAGATCAGTAAAAGCTTC
23	R_5'YGRKC	GAACTTCGGCCCTAGTGGCCAAATTACTCTGTACATTGTTTCTGGCG
24	F_3'YGRKC	CTTCGGCCGATCAGGCCTAGAGATCGTCATCCAGAA
25	R_3'YGRKC	GCTTTTACTGATCTAGAAGGATCGAATTTAAATGATCTAGGGAAGGAGC
	Primers for verification of *ygr149w* and *ste16* knockouts[a]	
26	F_Up5UTR_Ste16	AGTATTCCATTCGACCTCCTTAG
27	R_Down3UTR_Ste16	CAAGTTCTTCATGATGGTCGG
28	F_Ste16_cds	TGATAATGGGGGTGGAGTTCGATAT
29	R_Ste16_cds	ATGTCCAATTCCAAAAGTCTGGGC
30	F_Up5UTR_YGR	GGTGGACTTAGGGAGTTCAAAG
31	R_Down3UTR_YGR	TCTGAATACTGGTTGATGAGAGAGTAT
32	F_YGR_cds	ATCTTCTTTCTTTGCTTGCAATTGCGT
33	R_YGR_cds	ATGTCATCAGTAGGCTCTTTTGAAAGC

T = 3' inverted deoxythymidine, inhibitis unspecific extension by DNA polymerase [85]

[a] When knockouts had been created, *GPC1* was still called *YGR149W* and *TSC11* was still called *STE16*

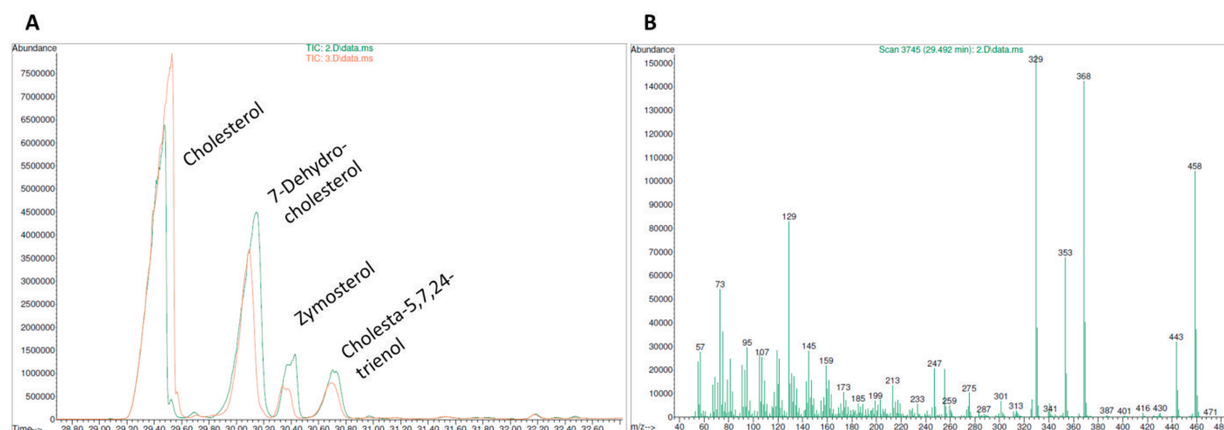

Fig S1. GC-MS analysis of newly constructed cholesterol-producing strain without Δ*ku70* background. Cells were cultivated in 10 ml of YPD at 28°C, 120 rpm for 48 h and sterols were extracted as described in the "Materials and methods" section. **(A)** Chromatogram overlay of Chol. (2, green) and Chol. Δ*ku70* (3, orange) strains. **(B)** Mass scan of the cholesterol peak with characteristic fragmentation pattern.

Table S2. GC-MS analysis of the new cholesterol-producing strain without Δ*ku70* background.

Chol. Δ*ku70* strain					
Peak #	**Compound**	**Ret. Time [min]**	**Rel. RT**	**Peak Area**	**% of total sterols**
1	Cholesterol	29.473	1	507209655	47,7
2	7-Dehydrocholesterol	30.145	1,0228	412902068	38,9
3	Zymosterol	30.428	1,0324	76966857	7,2
4	Cholesta-5,7,24-trienol	30.703	1,0417	65621194	6,2
Chol. strain					
Peak #	**Compound**	**Ret. Time [min]**	**Rel. RT**	**Peak Area**	**% of total sterols**
1	Cholesterol	29.528	1	756218410	69,1
2	7-Dehydrocholesterol	30.094	1,0192	251796557	23,0
3	Zymosterol	30.337	1,0274	34529016	3,2
4	Cholesta-5,7,24-trienol	30.694	1,0395	51579993	4,7

Table S3. Description of interesting genes found by RNA sequencing

Name	Description[a]
ADY2	Acetate transporter required for normal sporulation
ARO10-1	Phenylpyruvate decarboxylase, catalyzes decarboxylation of phenylpyruvate to phenylacetaldehyde, which is the first specific step in the Ehrlich pathway
ARO9	Aromatic aminotransferase II, catalyzes the first step of tryptophan, phenylalanine, and tyrosine catabolism
AXL2	Integral plasma membrane protein required for axial budding in haploid cells
CAT8-2	Zinc cluster transcriptional activator
CDC10/12	Component of the septin ring, required for cytokinesis
CTR1	High-affinity copper transporter of the plasma membrane; mediates nearly all copper uptake under low copper conditions, in *S. cerevisiae* the protein increases in abundance and relocalizes from nucleus to plasma membrane upon DNA replication stress
CYB5-1	Cytochrome b5, involved in the sterol and lipid biosynthesis pathways; acts as an electron donor to support sterol C5-6 desaturation
CYC8	General transcriptional co-repressor
DAL5-2	Allantoate permease
DES1	Delta-4-sphingolipid desaturase
DRE2	Component of the cytosolic Fe-S protein assembly (CIA) machinery
DIP5-1	Dicarboxylic amino acid permease; mediates high-affinity and high-capacity transport of glutamate and aspartate; also a transporter for Gln, Asn, Ser, Ala, and Gly; relocalizes from plasma membrane to vacuole upon DNA replication stress
ECM31	Ketopantoate-hydroxymethyl-transferase, required for pantothenic acid biosynthesis
EOS1	Protein involved in N-glycosylation
EXG1	Major exo-1,3-beta-glucanase of the cell wall, involved in cell wall beta-glucan assembly
FBA1	Fructose 1,6-bisphosphate aldolase
FKS3	1,3-beta-glucan synthase component
FMO1-1/4	Flavin-containing monooxygenase, localized in *S. cerevisiae* in the ER membrane
FPS1	Aquaglyceroporin, plasma membrane channel; involved in efflux of glycerol and xylitol; regulated by Rgc1p and Ask10p, which are regulated by Hog1p phosphorylation under osmotic stress; phosphorylation by Ypk1p required to maintain an open state
GAS1	Beta-1,3-glucanosyltransferase
GDH3	NADP(+)-dependent glutamate dehydrogenase
GEP3	Protein required for mitochondrial ribosome small subunit biogenesis
GPT2	Glycerol-3-phosphate/dihydroxyacetone phosphate acyltransferase located in *S. cerevisiae* in lipid particles and the ER; involved in the stepwise acylation of glycerol-3-phosphate and dihydroxyacetone in lipid biosynthesis
GTH1	High affinity glucose transporter
HET1	Putative sphingolipid transfer protein similar to *Candida* sp. *HET1*
HGT2	High-affinity glucose sensor or transporter similar to *C. albicans* HGT2, might be the analog of *S. cerevisiae* RGT2
HSP12	Plasma membrane protein involved in maintaining membrane organization in stress conditions; induced by heat shock, oxidative stress, osmostress, stationary phase, glucose depletion, oleate and alcohol; protein abundance in *S. cerevisiae* increased in response to DNA replication stress; regulated by the HOG and Ras-Pka pathways;
IDI1	IPP Isomerase, catalyzes activation step in isoprenoid biosynthetic pathway
IST2	Cortical ER protein involved in ER-plasma membrane tethering; one of 6 proteins (Ist2p, Scs2p, Scs22p, Tcb1p, Tcb2p, Tcb3p) that connect ER to the plasma membrane and regulate PM phosphatidylinositol-4-phosphate levels
KCH1	Potassium transporter
KIN3	Nonessential serine/threonine protein kinase; possible role in DNA damage response
KRE6	Type II integral membrane protein; required for beta-1,6 glucan biosynthesis
KSS1	Mitogen-activated protein kinase (MAPK); involved in signal transduction pathways that control filamentous growth and pheromone response; regulates septum assembly
MDM12	Mitochondrial outer membrane protein
MET1	S-adenosyl-L-methionine uroporphyrinogen III transmethylase, involved in the biosynthesis of siroheme, required for sulfate assimilation and methionine biosynthesis

MIP1	Mitochondrial DNA polymerase
MPT5	mRNA-binding protein of the PUF family; binds to the 3' UTR of specific mRNAs, including those involved in mating type switching, cell wall integrity, chronological lifespan, chromatin modification, and spindle pole body architecture
MRP17	Mitochondrial ribosomal protein of the small subunit
MRP49	Mitochondrial ribosomal protein of the large subunit
MUP1-2	High affinity methionine permease, integral membrane protein with 13 putative membrane-spanning regions; also involved in cysteine uptake
MXR1	Methionine-S-sulfoxide reductase, involved in the response to oxidative stress
NCP1	NADP-cytochrome P450 reductase; involved in ergosterol biosynthesis; associated and coordinately regulated with Erg11
NUP60	Nuclear pore complex FG-nucleoporin component
OPI1	Transcriptional regulator of a variety of genes; phosphorylation by protein kinase A stimulates Opi1p function in negative regulation of phospholipid biosynthetic genes; strongly correlated with overproduction of inositol;
PDR17	Phosphatidylinositol transfer protein; downregulates Plb1p-mediated turnover of phosphatidylcholine
PCL2	Cyclin, interacts with cyclin-dependent kinase Pho85p; involved in the regulation of polarized growth and morphogenesis and progression through the cell cycle
PHO89	Plasma membrane Na^+/P_i co-transporter
PLB3	Phospholipase B (lysophospholipase) involved in lipid metabolism
PLM2	Putative transcription factor; induced in *S. cerevisiae* in response to DNA damaging agents and deletion of telomerase
PMP20	Peroxiredoxin
PpHXT1	Low affinity glucose transporter of the major facilitator superfamily
PUT4-2	Proline permease, required for high-affinity transport of proline
RCE3	Endoglucanase
RKI1	Ribose-5-phosphate-ketol isomerase
RTG3	helix-loop-helix-leucine zipper transcription factor that forms a complex with Rtg1p to activate the retrograde (RTG) and TOR pathways; target of Hog1p
SAM4	S-adenosylmethionine-homocysteine methyltransferase
SHS1	Component of the septin ring, required for cytokinesis
SLD1	Delta-8-fatty acid sesaturase
SLD5	Subunit of the GINS complex, which id localized to DNA replication origins and implicated in assembly of the DNA replication machinery
SMF1	Divalent metal ion transporter
SRB8	Subunit of the RNA polymerase II mediator complex
SSO1	Plasma membrane t-SNARE; involved in fusion of secretory vesicles at the plasma membrane and in vesicle fusion during sporulation
SUN4	Cell wall protein related to glucanases, possibly involved in cell wall septation
SWI6	Activates endoglucanase
TEM1	GTP-binding protein of the Ras superfamily; involved in termination of M-phase; controls actomyosin and septin dynamics during cytokinesis
UPC2	Sterol regulatory element binding protein; induces transcription of sterol biosynthetic genes
USP1	Universal stress protein
VHC1	Vacuolar membrane cation-chloride cotransporter; likely mediates K^+ and Cl^- cotransport into the vacuole; has a role in potassium homeostasis and salt tolerance; localizes to sites of contact between the vacuole and mitochondria
XUT5	Putative xylose transporter
YCT1	High-affinity cysteine-specific transporter
YPK2	Protein kinase similar to serine/threonine protein kinase Ypk1p; functionally redundant with YPK1; participates in a signaling pathway required for optimal cell wall integrity; involved in the TORC-dependent phosphorylation of ribosomal proteins Rps6a/b, *P. pastoris* does not have the paralog *YPK1*

[a] Descriptions are according to *Saccharomyces* genome database SGD [41] and/or the National Center for Biotechnology Information NCBI (www.ncbi.nlm.nih.gov).

Table S4. RNA-Seq of *erg5*::*DHCR7* Δ*ku70* and *erg6*::*DHCR24* Δ*ku70* strains.

Lipid biosynthesis				fold change			
	Name	**NCBI Accession**	**Feature ID**	**Δ*ku70* vs. *erg5***	**WT vs. *erg5***	**Δ*ku70* vs. *erg6***	**WT vs. *erg6***
Sterols	*ERG3*	CCA37283	ACIB2EUKG768951	66.0	113.2	21.9	37.5
	ERG2	CCA40964	ACIB2EUKG772851	59.6	76.0	19.7	25.1
	ERG24	CCA36873	ACIB2EUKG768514	35.5	51.6	4.0	5.8
	ERG11	CCA39186	ACIB2EUKG770976	25.2	35.3	4.7	6.6
	ERG6	CCA39796	ACIB2EUKG771612	17.6	21.0	-	-
	ERG5	CCA39656	ACIB2EUKG771469	-	-	4.2	4.2
	ERG26	CCA37528	ACIB2EUKG769204	10.2	14.3	3.2	4.5
	CYB5-1	CCA40773	ACIB2EUKG772644	9.8	15.7	2.3	3.7
	ERG4	CCA41104	ACIB2EUKG772999	9.6	10.2	2.6	2.7
	ERG1	CCA40055	ACIB2EUKG771896	9.5	11.7	4.8	5.9
	ERG7	CCA38589	ACIB2EUKG770328	8.1	9.4	2.8	3.3
	UPC2	CCA38869	ACIB2EUKG770635	5.5	8.2	1.3	2.0
PL	*PDR17*	CCA39309	ACIB2EUKG771107	7.1	10.3	-1.4	1.0
	GPT2	CCA36245	ACIB2EUKG767856	-11.2	-13.5	-6.2	-7.5
	PLB3	CCA41015	ACIB2EUKG772901	-10.8	-11.7	-8.3	-8.9
	OPI1	CCA36491	ACIB2EUKG768112	-10.5	-17.0	-4.9	-7.9
	LPL1-2	CCA39362	ACIB2EUKG771160	-4.0	-3.9	-11.4	-11.0

Other differentially regulated genes				fold change			
	Name	**NCBI Accesion**	**Feature ID**	**Δ*ku70* vs. Chol.**	**WT vs. Chol.**	**Δ*ku70* vs. WT**	**WT vs. *erg6***
Cell wall	*CWP1*	CCA39831	ACIB2EUKG771652	5.6	2.3	1.7	-1.5
	GAS1	CCA36391	ACIB2EUKG768009	-8.3	-10.1	-6.5	-7.9
	PIM1	CCA39249	ACIB2EUKG771047	-4.4	-6.1	-1.4	-2.0
	KRE6	CCA40635	ACIB2EUKG772494	-4.9	-5.1	-3.5	-3.7
Membrane proteins/Transporter	*NUP60*	CCA37527	ACIB2EUKG769203	30.5	45.2	1.9	2.8
	VMA21	CCA36872	ACIB2EUKG768513	30.0	31.4	2.8	2.9
	SSO1	CCA37207	ACIB2EUKG768871	6.2	6.7	1.9	2.0
	SEC62	CCA39128	ACIB2EUKG770917	5.6	6.3	1.3	1.4
	ADY2-4	CCA36841	ACIB2EUKG768479	-1.1	-1.0	10.9	12.0
	PUT4-2	CCA38309	ACIB2EUKG770032	-19.8	-17.6	-10.7	-9.5
	SIT1-1	CCA39497	ACIB2EUKG771305	-18.4	-17.5	-4.3	-4.1
	FUR4	CCA36225	ACIB2EUKG767833	-17.2	-21.3	-4.1	-5.1
	ADY2-1	CCA36620	ACIB2EUKG768246	-16.6	-22.6	-7.4	-10.0
	IST2	SCV12063	ACIB2EUKG770208	-16.4	-13.8	-5.3	-4.5
	SLM1	SCV11910	ACIB2EUKG769007	-15.9	-9.4	-5.2	-3.1
	TPC1	CCA39399	ACIB2EUKG771201	-15.4	-17.9	-6.6	-7.7
	MUP1-2	CCA40163	ACIB2EUKG772008	-4.5	-2.5	-12.3	-6.7
	VHC1	CCA39516	ACIB2EUKG771325	-4.2	-4.3	-6.1	-6.2
	PHO89	CCA38747	ACIB2EUKG770500	-3.5	-4.0	-5.0	-5.8
	FPS1[a]	CCA40346	ACIB2EUKG772199	-8.2	-9.6	-2.0	-2.4
TF	*TEC1*	CCA40088	ACIB2EUKG771929	-21.7	-43.3	-14.5	-29.0
	ZCF2	CCA36469	ACIB2EUKG768090	-18.1	-7.3	-2.5	-1.0
	CAT8-2	CCA40601	ACIB2EUKG772458	-7.9	-9.9	-5.2	-6.5
AA metabolism	*GDH3*	CCA36566	ACIB2EUKG768193	-40.3	-52.5	-7.3	-9.6
	DIP5-1	CCA40867	ACIB2EUKG772748	-28.5	-21.1	-4.2	-3.1
	ASN2	CCA39482	ACIB2EUKG771290	-25.9	-22.4	-4.5	-3.9
	PUT4-2	CCA38309	ACIB2EUKG770032	-19.8	-17.6	-10.7	-9.5
	CAR1-1	CCA37997	ACIB2EUKG769700	-15.2	-14.5	-9.2	-8.9
	ARG5/6	CCA38818	ACIB2EUKG770578	-14.7	-12.3	-13.3	-11.1
	GLN1	CCA40345	ACIB2EUKG772198	-12.1	-9.5	-3.9	-3.1
	MXR2-1	CCA38968	ACIB2EUKG770741	1.3	1.2	-73.9	-82.5
	LEU2	CCA40147	ACIB2EUKG771992	-8.4	-5.1	-10.7	-6.5
	ARO10-1	CCA40086	ACIB2EUKG771927	-9.1	-13.5	-5.5	-8.2

Other differentially regulated genes				fold change			
	Name	NCBI Accesion	Feature ID	Δ*ku70* vs. Chol.	WT vs. Chol.	Δ*ku70* vs. WT	WT vs. *erg6*
Mitochondrial	*ECM31*	CCA39130	ACIB2EUKG770919	17.4	25.9	1.8	2.6
	MRP17	SCV12135	ACIB2EUKG770754	8.4	10.4	1.9	2.3
	MDM12	CCA39905	ACIB2EUKG771732	5.3	5.4	1.5	1.5
	MRP49	CCA39185	ACIB2EUKG770975	5.1	6.9	1.3	1.8
	MIP1	CCA39455	ACIB2EUKG771258	3.2	6.5	13.5	27.0
	FUM1	CCA39511	ACIB2EUKG771320	-24.2	-19.2	-2.8	-2.2
	PGI1	CCA39708	ACIB2EUKG771520	-20.2	-23.7	-3.9	-4.6
	ACS1	CCA38193	ACIB2EUKG769911	-19.1	-21.4	-7.2	-8.0
	TOM40	CCA40757	ACIB2EUKG772627	-17.8	-15.1	-3.0	-2.6
	CTA1	CCA37834	ACIB2EUKG769524	-17.4	-23.5	-2.4	-3.2
	DRE2	CCA37215	ACIB2EUKG768879	-15.5	-10.1	-8.0	-5.2
	GUT1	CCA40348	ACIB2EUKG772200	-12.9	-14.2	-5.7	-6.3
Central Carbon Metabolism/Others	*PP7435_Chr4-0590*	SCV12388	ACIB2EUKG772621	9.3	13.6	1.6	2.4
	PP7435_Chr1-0624	CCA36773	ACIB2EUKG768404	7.6	15.7	1.6	3.3
	NAT4	CCA38730	ACIB2EUKG770482	7.4	14.9	3.4	6.8
	USP1	CCA39586	ACIB2EUKG771396	5.8	8.3	1.1	1.5
	SLD5	CCA37363	ACIB2EUKG771192	5.3	8.3	-2.1	-1.3
	SRB8	CCA36665	ACIB2EUKG768294	-1.0	1.2	12.1	14.4
	MPT5	CCA39728	ACIB2EUKG771540	1.5	1.5	6.6	6.7
	TFC7	CCA40776	ACIB2EUKG772647	-1.5	1.4	5.9	12.0
	PDC1	CCA39987	ACIB2EUKG771819	-27.0	-25.1	-1.1	-1.1
	MDH3	CCA40311	ACIB2EUKG772162	-25.9	-25.3	-3.1	-3.1
	TAL1-1	CCA38051	ACIB2EUKG769757	-25.0	-22.0	-1.8	-1.6
	CUE5	CCA38003	ACIB2EUKG769707	-24.0	-23.3	-2.6	-2.5
	PP7435_Chr2-0990	CCA38670	ACIB2EUKG770414	-21.8	-12.5	-1.0	1.7
	RPS0B	CCA37392	ACIB2EUKG769064	-20.0	-19.0	-2.0	-1.9
	GLO3	CCA39604	ACIB2EUKG771415	-19.2	-18.6	-1.8	-1.8
	FDH1	CCA39210	ACIB2EUKG771003	-18.6	-25.7	-7.3	-10.0
	MIS1-2	CCA40925	ACIB2EUKG772807	-17.8	-21.8	-20.0	-24.5
	CCP1-1	CCA37830	ACIB2EUKG769520	-15.8	-18.4	-2.8	-3.3
	ISW2	CCA41039	ACIB2EUKG772928	-12.8	-10.4	-12.1	-9.8
	PP7435_Chr2-1115	CCA38792	ACIB2EUKG770546	-11.9	-12.9	-1.1	-1.2
	YPK2	CCA40052	ACIB2EUKG771893	-7.9	-7.0	-4.0	-3.5

5. Conclusion

This work aimed for the optimization and characterization of the biotechnologically important yeast *P. pastoris* for heterologous membrane protein expression and peptide secretion. These two rather different approaches are linked via the secretory pathway, as both, plasma membrane proteins and antimicrobial peptides, need to enter the endoplasmic reticulum and are routed via the Golgi compartment to the cell periphery. Antimicrobial peptides should be efficiently secreted to the culture medium for easily assessing their biological activity and facilitated downstream applications. Mammalian membrane proteins should be incorporated properly into the yeast plasma membrane to fulfil their receptor, transporter or signaling function even in the heterologous host system. Proper localization facilitates the biochemical investigation of membrane protein properties. To tackle these challenging tasks, we applied two distinct strategies. On the one hand, we engineered the secretory pathway of *P. pastoris* by co-expressing *S. cerevisiae* pro-peptide processing enzymes Kex2p and Kex1p to elevate the proteolytic processing capacity in the secretory pathway. On the other hand, we engineered the sterol composition in *P. pastoris* to produce cholesterol instead of ergosterol. Thus, a suitable surrounding for heterologous mammalian membrane proteins should be generated. Although both innovative strategies were successful for certain model proteins tested, we also encountered difficulties. Co-expression of Kex2p and Kex1p was so far only beneficial for plectasin secretion. The strategy did not improve secretion of protegrin, the second antimicrobial peptide we worked with. Unfortunately, the setup of four tandem repeats with Kex2 cleavage sites in-between was not the best suited strategy for secretion.

Considering the growth defects of sterol-modified *P. pastoris*, we aimed for a deeper characterization of these strains to uncover possible regulatory pathways affected by sterol homeostasis. Cholesterol in the yeast membranes successfully led to a more stable and active expression of human Na,K-ATPase α3β1 isoform as determined by Western Blot analyses, activity assays and radioligand binding studies. These positive effects, however, were accompanied by growth defects and the associated decreased cellular fitness. By exposing the sterol-modified strains to different conditions such as cell wall stress induced by calcofluor white or congo red, osmotic stress and decreased incubation temperatures, we could identify certain stress response mechanisms involved.

Especially in the cholesterol-producing strain, we observed a highly thickened and obviously porous cell wall. Also, the CWI pathway was constitutively activated via Slt2p phosphorylation. This indicated that cholesterol-producing cells are under cell wall stress and constantly build up new cell wall material. Upstream effectors are most likely membrane proteins which initiate MAPK signaling (*e.g.* Wsc11p in *S. cerevisiae*). Changes in the cell wall compositions could not be detected by analysis of the sugar composition as determined by HPAEC analysis of cell wall glucans, mannans and chitin content. The manifold up- and downregulated genes identified by RNA sequencing furthermore confirmed strong influences on transcriptional regulation of sterol metabolism and cell wall assembly. A genetic knockout screening hinted at, among other targets, the TOR2 complex that might be involved in cold and salt

response signaling. This complex additionally plays a role in cell wall maintenance, which was shown by a *tsc11* knockout in the cholesterol-strain reverting its insensitivity to calcofluor white.

Our results indicate that a plethora of adaptive events is happening within a sterol-modified *P. pastoris* cell, which most likely arise due to altered signaling events at or across the plasma membrane. We could, however, not yet pinpoint and investigate specific membrane proteins as being directly involved in the sterol-dependent response. This will be highly interesting, as it will pave the way for very specific analysis and applications of highly sterol-specific interactions of membrane proteins. We identified one pathway, namely the CWI pathway in *P. pastoris*, which is strongly affected by exchanging ergosterol for cholesterol. Also, several indications point towards altered TOR, CWI and HOG signaling in the sterol-engineered cells. However, there are still many more targets influencing cell signaling, which are triggered by the altered sterol content in the yeast, and which need to be analyzed. For instance, regulation events within sphingolipid biosynthesis may play an important role. *P. pastoris* is a very interesting host to study these effects, especially as there are plenty of differences found when compared to *S. cerevisiae*. Prospectively, better insights shall be gained to understand the role of sterol homeostasis and cell wall assembly within a simple eukaryotic cell.

6. Appendix: Additional Methods & Results

During this thesis, several additional methods were applied to characterize sterol-engineered *P. pastoris* strains. The methods and results are listed in this appendix, as they were not very conclusive or still need further experiments for confirmation. These include zymolyase sensitivity assays, calcofluor white microscopy, additional electron microscopy images of the Chol. Δ*ku70* Δ*tsc11* mutant, and preliminary results from a genomic overexpression library. In a short discussion, the drawbacks and problems encountered with each method are described.

6.1. Zymolyase Sensitivity Assays

Cells were grown in 25 ml YPD in 300 ml baffled flasks for 48 h at 28°C and 120 rpm. Per culture, 5 ml were harvested and washed twice with sterile deionized water. Pellets were resuspended in 10 mM potassium phosphate, pH 7.5, to 5 OD_{600} units/ml, and 14 µl of β-mercaptoethanol were added to a final volume of 10 ml. Before measuring the initial OD_{600}, 200 µl of the sample were mixed with 800 µl of 5% SDS. The reaction was started by addition of 500 µg zymolyase and was carried out in a water bath at 30°C and 200 rpm. OD_{600} was monitored every 10 min for 90 min. Decrease in optical density represented cell lysis due to zymolyase treatment (Fig. A1). Increased cell/spheroplast aggregation cannot be ruled out in these assays and may mask the expected cell lysis effects.

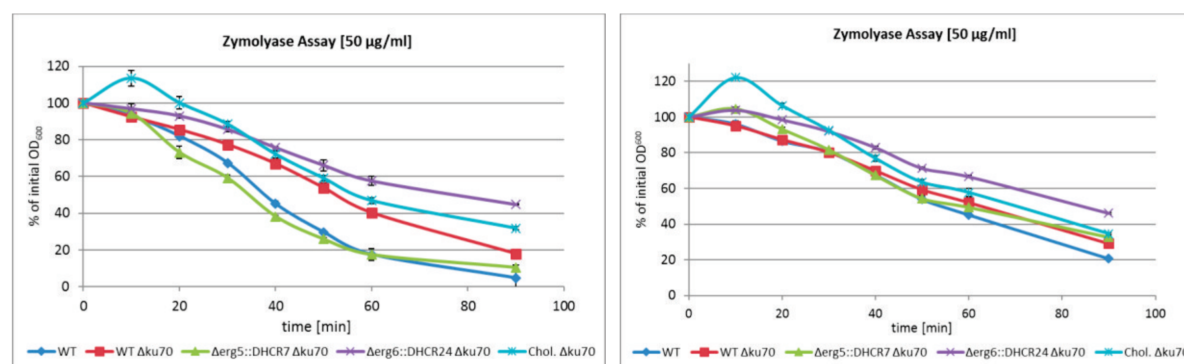

Fig. A1. Zymolyase sensitivity assay. Two independent experiments were performed. Sensitivity towards zymolyase was assayed by decrease in the OD_{600} values determined spectrophotometrically.

6.2. Calcofluor White Staining and Fluorescence Microscopy

In principle, calcofluor white staining was performed as described by de Groot et al. [1]. Pre-cultures were grown overnight in 10 ml of YPD media. For the main cultures, 2.5 ml of the ONC were added to 22.5 ml of YPD and were incubated for 5 h at 37°C in 300 ml baffled shake flasks to trigger cell wall related phenotypes. Then, 200 µl of the cultures were centrifuged for 1 min at 13,200 rpm. Pellets were resuspended in 50 µl of CFW (10 µg/ml, Fluorescent brightener 28). Microscopy was performed with a Leica DM LB2 microscope with an external LEJ EBQ 100 isolated Hg-lamp and a 100x immersion objective. The Leica filter cube A was used for visualization. Imaged cells are shown in Fig. A2.

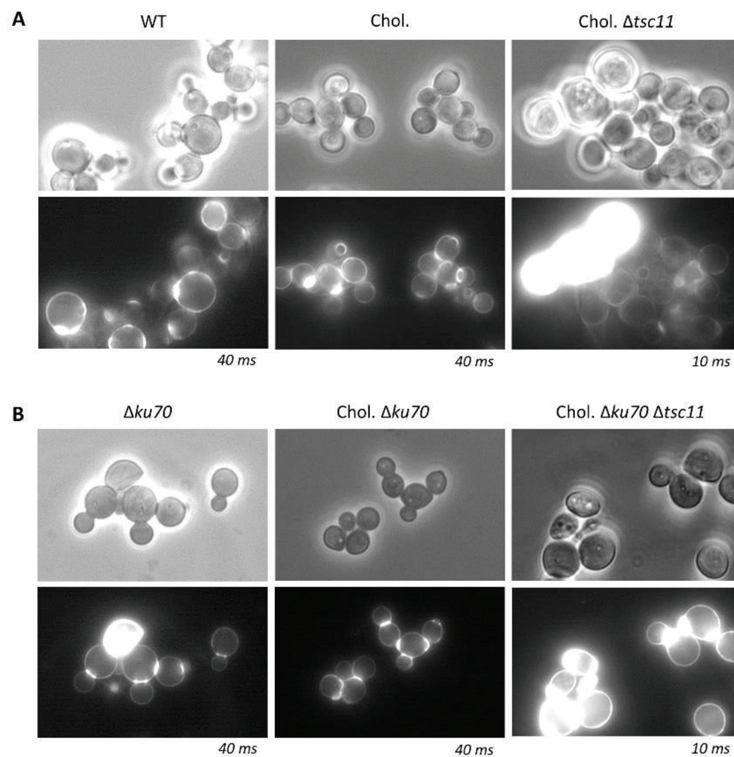

Fig. A2. Calcofluor white microscopy. Cells were imaged using a Leica DM LB2 microscope. Exposure time is indicated below the images.

6.3. Electron Microscopy of the Chol. Δ*ku70* Δ*tsc11* Knockout Strain

For methodological description, see Chapter 4 in the "Materials and Methods" section. The Chol. Δ*ku70* Δ*tsc11* strain was grown on YPD for 48 h. TEM images are shown in Fig. A3. This strain showed basically no difference compared to the parental Chol. Δ*ku70* strain. Also, the vesicular structures appeared several times in the other investigated strains.

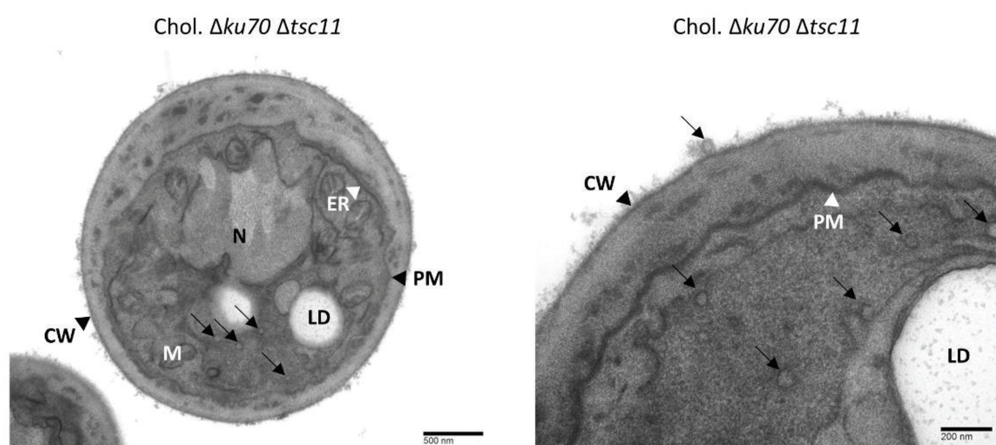

Fig. A3. Transmission electron microscopy of the *P. pastoris* Chol. Δ*ku70* Δ*tsc11* strain. ER – endplasmic reticulum; N – nucleus; PM – plasma membrane; LD – lipid droplet; M – mitochondria; CW – cell wall. Black arrows indicate vesicular structures.

6.4. Screening of a Genomic Overexpression Library in the Cholesterol-producing Strain

Genomic DNA was isolated from *P. pastoris* CBS7435 Δ*his4* and 10 µg were partially digested with 0.1 U of *Bsp*143I for 1 h at 37°C to obtain fragments in the size range of 1000-5000 bp. We constructed a plasmid for a genomic overexpression library based on pBSYA1Z (BISY e. U., Hofstätten an der Raab, Austria), which contains an ARS sequence for functional, extrachromosomal maintenance in *P. pastoris.* The zeocin resistance cassette was modified to a hygromycin resistance cassette based on pPpHyg [2] by Gibson Cloning [3] using primers 1-8 (Table A1) to obtain pBSYA1H (Fig. A4).

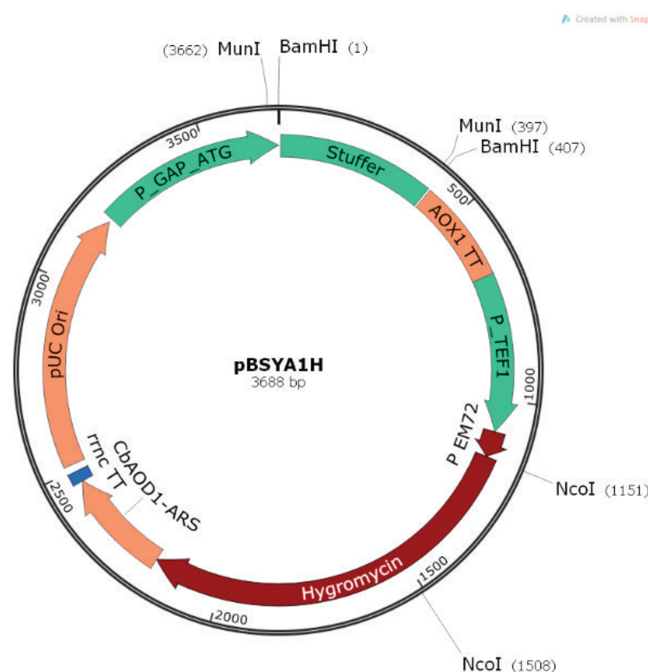

Fig. A4. Overexpression plasmid pBSYA1H used for library construction. Partially digested genomic DNA fragments from *P. pastoris* CBS7435 WT were *Bam*HI cloned into the plasmid. The constitutive GAP promoter should drive expression of fragments cloned without a native promoter sequence. Furthermore, the plasmid carries a hygromycin resistance gene for selection in *E. coli* and *P. pastoris*, as well as an autonomously replicating sequence (CbAOD1-ARS) for extrachromosomal maintenance in *P. pastoris*.

Fragments of the genomic library were *Bam*HI cloned into pBSYA1H and the whole ligation mixtures were transformed into *E. coli* TOP10F' to generate four independent libraries. *E. coli* clones were resuspended in resuspension solution and total plasmids were isolated from the genomic library population. Two-hundred ng of each library were transformed into the Chol. *Δku70* strain and clones were selected on YPD with hygromycin (100 µg/ml). The screening and re-screening procedures were the same as described for the knockout library in Chapter 04. In total, 2200 clones per library were obtained and after the first screening round 12 cold-resistant and 12 NaCl resistant clones were picked for re-screening. Plasmids were retrieved from interesting clones according to the protocol adapted from Singh et al. [4]. Briefly, *P. pastoris* cells were grown over night in 10 ml of YPD with hygromycin (50

μg/ml) at 28°C and 120 rpm, and were harvested by centrifugation. All reagents for plasmid isolation were used from the GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific, St. Leon-Rot, Germany). After resuspending the pellet in 200 μl of resuspension solution, 100 μl of zymolyase solution (0.5 M sorbitol, 50 mM KPi buffer, pH 7.4, 10 mg/ml zymolyase) was added. *Arthrobacter luteus* zymolyase 20T of 24,000 U/g activity was purchased from Nacalai tesque (Kyoto, Japan). The mixture was incubated at 37°C for 90 min, and then 300 μl Lysis solution was added. After incubation for 10 min at room temperature, 420 μl of Neutralization solution were added and samples were centrifuged for 10 min at 10,000 x g. Supernatants were loaded onto the purification column and further processed as described in the GeneJet Plasmid Miniprep Kit instruction manual. Isolated plasmids were re-transformed into *E. coli* TOP10F' and re-isolated to have sufficient DNA material for sequencing (LGC Genomics, Berlin, Germany) using primers 9 and 10 (Table A1). Sequencing results were compared to genome sequences of *P. pastoris* CBS7435 and GS115 using a BLAST Nucleotide Sequence Similarity Search [19,20]. Table A2 lists the hits found after nucleotide blast search.

Table A1. Primers used for construction of the overexpression library plasmid pBSYA1H.

№	Name	Sequence 5'-3'
1	F_ARS	GTCCGAGGGCAAAGGAATAAGGAGTATACGTAAATATATAATTAT
2	R_pGAPBamHI	CGATTTGGATCTTACCGGATCCCATTGTGTTTTGATAGTTG
3	F_BamHIStuffer	AACTATCAAAACACAATGGGATCCGGTAAGATCCAAATCGATG
4	R_StufferBamHI	ATTCTGACATCCTCTTGAGGATCCTGTTCAATTGAGGCTTGAAG
5	F_BamHIAOX1TT	CCTCAATTGAACAGGATCCTCAAGAGGATGTCAGAATG
6	R_AOX1TT	CATTTTGAAGCTATGGTGTGTGGCACAAACGAAGGTCTCA
7	F_Tef1P	GAGACCTTCGTTTGTGCCACACACCATAGCTTCAAAATGTTTCTAC
8	R_HYG	ATATATAATTATATATTTACGTATACTCCTTATTCCTTTGCCCTCGG ACGAG
9	F_GAP	ACCACCAGAATCGAATATAAAAGGC
10	R_Aox1TT	GATTTTCCCAAACCCCTACC

The re-screen of selected overexpression library mutants essentially showed similar results as compared to the knockout screening (Fig. A5). Growth of the cholesterol-producing strain on 0.5 M NaCl or at 18°C was partly restored, even though WT-like growth was not reached. Generally, the screening methods worked and the plasmid was obviously sustained in *P. pastoris*, under selective conditions. However, we encountered problems with the NaCl resistant clones afterwards. It was not possible to re-cultivate them in selective media, indicating a potential plasmid loss.

Upon plasmid isolation from the cold resistant clones, we saw that yields were very low and therefore insufficient for direct sequencing. Plasmids were either re-transformed into *E. coli* for amplification, or interesting regions were PCR amplified and sent for sequencing, yielding the same results. Sequencing results are presented it Table A2. The major problem with this method was the poor quality of the library, as we could only amplify very short fragments, which are actually unlikely to be responsible for the restored growth phenotypes.

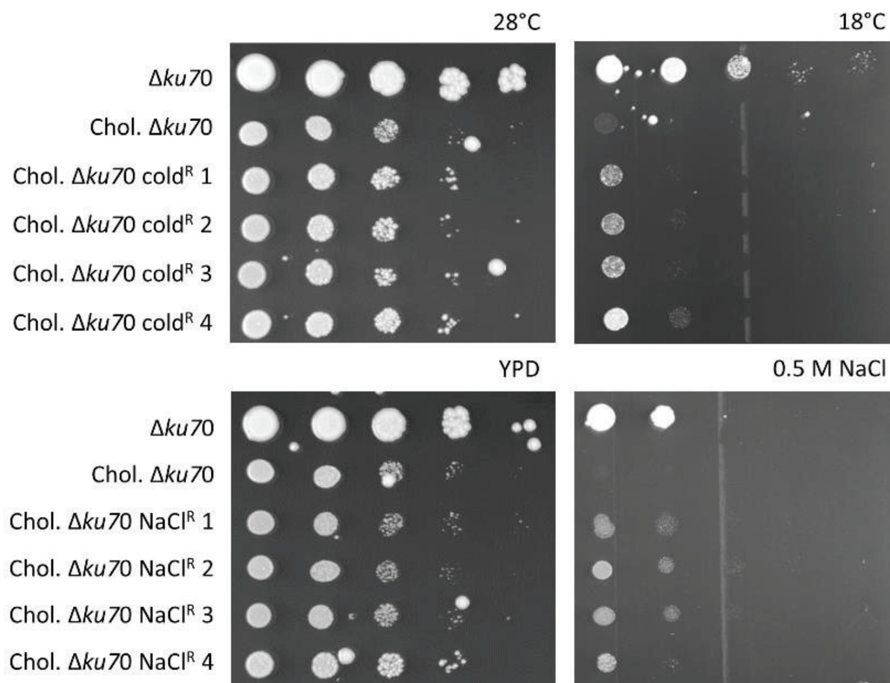

Fig. A5. Re-screen of Chol. Δ*ku70* overexpression library clones. Strains were cultivated for 48 h in YPD with hygromycin (100 µg/ml). Cell pellets corresponding to 1.5 OD_{600} units were harvested, serially diluted to 10^{-4} and 3 µl were spotted onto YPD plates with hygromycin (100 µg/ml). Plates were incubated at 28°C and imaged after 4 days. Here, four representative clones of each library re-screen are shown.

Table A2. Sequencing results of integrated genomic DNA fragments from Chol. Δ*ku70* cold^R isolated plasmids.

NCBI Accession	Name (aligned nt)	*S. cerevisiae* homologue
CCA36898	40S ribosomal protein S8-A (987 nt)	*RPS8B* (79%)
CCA39549	Protein of unknown function with similarity to phosphoserine phosphatases (279 nt)[a]	*YNL010W* (54%)
CCA41036	60S ribosomal protein L6-B (277 nt)	*RPL6B* (66%)
CCA39802	Hsp70 family ATPase (187 nt)	*SSC1*(82%)
CCA36901	Putative high mobility group proteins (166 nt)	*NHP10* (37%)
CCA37885	Cytoplasmic Ser/Thr protein kinase (139 nt)	*VHS1* (38%)
CCA37484	tRNA modification GTPase mnmE (mitochondrial) (102 nt)	*MSS1* (47%)
CCA38269	Hypothetical protein (91 nt)	no similarities found
CCA38627	E3 ubiquitin-protein ligase (72 nt)	*RSP5* (72%)
CCA38773	Vacuolar protein sorting-associated protein 30 (63 nt)	*VPS30* (32%)
CCA37034	Putative monocarboxylate permease; MFS superfamily (49 nt)	*MCH2* (46%)

[a] This target gene was found in two independent cold resistant clones

6.5. Discussion

We applied several additional methods to characterize the sterol-engineered strains, especially regarding the cell wall. However, some of these methods would need further optimization.

The zymolyase sensitivity assay was not very conclusive, as the normalization by OD_{600} was difficult for the sterol-engineered strains. It seemed that the SDS used in the assay influenced OD_{600} measurements, especially for the cholesterol-producing strain, as the values were increasing within the first ten min. The results were not well reproducible and might indicate cell clogging, particularly of sterol-modified strains. Unfortunately, no conclusion about sensitivity towards zymolyase could be drawn.

The calcofluor white staining did not show major differences between the WT and sterol-engineered strains. However, the *TSC11* knockout strain was very sensitive to calcofluor white as indicated by the spot-tests (see Chapter 4 for further details). When we investigated this strain under the microscope, we detected many cells that were strongly fluorescent due to binding of calcofluor white to chitin. It seems as if these strains have more chitin in the cell walls, but more experiments need to be performed to confirm this result.

We also tried to spot differences in the cell wall ultrastructure of the *tsc11* knockout strain via electron microscopy (Fig. A3). However, the abnormal cell wall structure was basically comparable to the parental Chol. Δ*ku70* strain. We also detected intra- and extracellular vesicular structures, which were also present in the other sterol-modified strains.

Besides the knockout library screening described in Chapter 4, we created a genomic overexpression library. For this purpose, we used the autonomously replicating pBSYA1 plasmid series and interchanged the zeocin selection marker for hygromycin. Cloning, screening and maintenance in *P. pastoris* generally worked well. Only for the NaCl resistant clones we observed potential plasmid loss, as we were not able to retrieve the plasmid from *Pichia* cells. Unfortunately, most of the sequencing results of the re-screened cold resistant clones were not very trustworthy, as the library analysis revealed that the cloned fragments were rather small. The smallest fragments were 100 bp and the largest fragments were 1800 bp, as determined by analyzing 28 random plasmid samples by restriction analysis. Also, the library contained 20-30% of re-ligated vector. Still, the identified targets are not completely irrational; we found genes encoding for a kinase and a phosphatase, for ribosomal subunits, for vacuolar sorting or for a membrane transporter. However, the restored phenotypes could also result from unspecified genomic integration events.

6.6. References Appendix

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