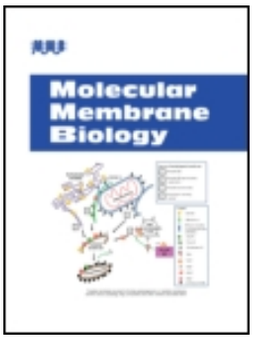

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Recombinant production of the human aquaporins in the yeast *Pichia pastoris* (Invited Review)

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Abstract
Aquaporins are water facilitating proteins embedded in the cellular membranes. Such channels have been identified in almost every living organism – including humans. These proteins are vital molecules and their malfunction can lead to several severe disorders and diseases. Hence, an increased understanding of their structure, function and regulation is of the utmost importance for developing current and future drugs. Heading towards this goal, the first problem to overcome is to acquire the proteins in sufficient amounts to enable functional and structural characterization. Using a suitable host organism, large amounts of target molecules can possibly be produced, but for membrane proteins limitations are frequently encountered. In the work described here, we have produced the 13 human aquaporins (hAQPs) in one of the most successful hosts for recombinant overproduction of eukaryotic proteins; the yeast *Pichia pastoris*, in order to explore the underlying bottleneck to a successful membrane protein production experiment. Here we present exceptional yield of hAQP1, whereas some other hAQPs were below the threshold needed for scaled up production. In the overproduction process, we have established methods for efficient production screening as well as for accurate determination of the initial production yield. Furthermore, we have optimized the yield of low producing targets, enabling studies of proteins previously out of reach, exemplified with hAQP4 as well as the homologue PfAQP. Taken together, our results. present insight into factors directing high production of eukaryotic membrane proteins together with suggestions on ways to optimize the recombinant production in the yeast *P. pastoris*.

Keywords: *Aquaporin, recombinant protein production, integral membrane proteins, Pichia pastoris*

Introduction

Membrane proteins serve crucial functions in the cell and they constitute the majority of all current drug targets (Lundstrom 2006). Thus, detailed understanding of the workings of this class of molecules is of great relevance for both academia and the pharmaceutical industry, encouraging biochemical, functional and structural analysis of this group of molecules. However, membrane proteins are generally poorly understood due to bottlenecks encountered all the way from production to characterization of the isolated protein and they are dramatically underrepresented in structural databases (White 2011). Hence, the first hurdle to encounter is the fact that the majority of membrane protein targets are present at very low concentrations in their native membranes (Mus-Veteau 2002), requesting novel innovative strategies for recombinant overproduction. Indeed, the main bottleneck for structural determination and characterization of a membrane protein today is the task of overproducing a stable and functional protein in sufficient amounts (Grisshammer and Tate 1995, Forstner et al. 2007). Membrane protein overproduction is often a matter of a trial-and-error exercise limiting the number of available targets to be studied (Grisshammer 2006). Interestingly, it has historically even been considered an art rather than science (Bonander and Bill 2009) giving the lack of knowledge and available methods to solve the problems associated with production of the specific protein of interest. Moreover, eukaryotic membrane proteins are known to be even more difficult to produce relative to their prokaryotic counterparts (Tate 2001, Grisshammer 2006). As a consequence, several ways of circumventing membrane protein overproduction exists, like extracting large quantities of protein from naturally abundant sources (Bill et al. 2011). While being quite successful, it limits the selection of targets, especially

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those of human origin. Consequently, for future progress on eukaryotic membrane proteins, this method has to be replaced by reliable recombinant overproduction, which takes development of effective strategies.

In general, there are reasons to avoid a prokaryotic host for production of eukaryotic membrane proteins; the translation rate, the translocon, and the lipid composition differ, which in combination could have a negative impact on the final yield (Tate 2001, Tate et al. 2003). The methylotrophic yeast *P. pastoris* is a commonly used eukaryotic host mainly due to its strong and tightly regulated alcohol oxidase 1 (AOX1) promoter used to drive recombinant protein production. In addition, the preferred respiratory growth of *P. pastoris* allows growth of high cell density cultures. Moreover, stable transformants from linearized vector DNA can easily be generated by homologous recombination resulting in stable host strains which can grow without selection pressure. Notably, the host *P. pastoris* has been a vital part of the pipeline leading up to structure determination of eukaryotic membrane proteins; *P. pastoris* is the most frequently used host-producing protein for structural characterization (Bill et al. 2011). Hence, as more researchers are attracted to use this production system in the future, increased understanding in the determinants of high membrane protein production levels is of vital importance.

In order to achieve an increased understanding of factors directing a successful membrane protein production experiment we have taken advantage of the family of human aquaporins (hAQPs); in total 13 homologues membrane proteins. The human aquaporins have a high sequence similarity; 63% of the protein sequence is identical between hAQP2 and hAQP5 (Figure 1). Aquaporins are commonly divided into two subgroups: the orthodox aquaporins (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8), mainly transporting water, and the aquaglyceroporins (AQP3, AQP7, AQP9, and AQP10), transporting water and glycerol. The two remaining aquaporins (AQP11 and AQP12) still have undetermined transport specificity and are usually placed in their own group called superaquaporins. In the work described here, we have produced the hAQPs in *P. pastoris* with the goal to identify patterns discriminating high and low producers, respectively. In addition, we have evaluated various optimization approaches in order to increase an initially low production level for a certain target. Altogether, reliable comparisons between the production levels of different targets, or modifications thereof, have been possible due to the establishment of a quantitative production screening protocol.

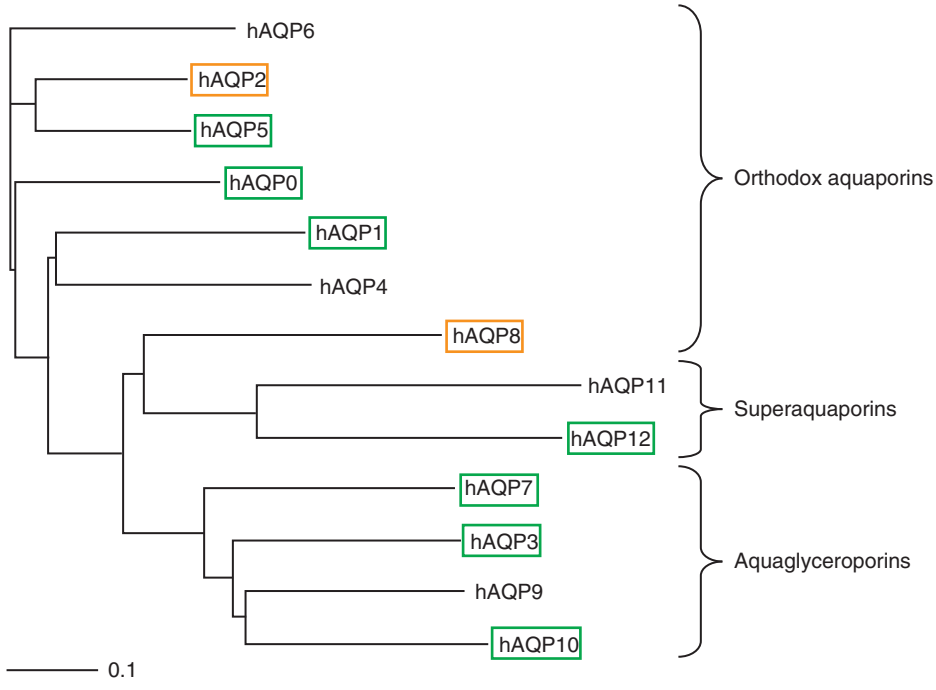


Figure 1. Phylogenetic tree of the 13 human aquaporins. Aquaporins giving a high protein yield are shown in black boxes, poor yield in grey boxes, and proteins with a yield below the detection limit in the quantitative production screen are shown without any boxes. This Figure is reproduced in colour in *Molecular Membrane Biology* online where high protein yield is shown in green boxes and poor yield in orange boxes, respectively.

Identifying high yielding clones by production screening

In order to conclude if the protein yield is high enough for further production and characterization or if optimization is needed, it is essential to have a fast and reliable method to determine the initial production level of a certain protein target. Thus, we established a protocol for small-scale production screening of novel targets produced in *P. pastoris* from 2 ml BMMY cultures using a 24-deep-well-block where samples were taken 6, 22 and 54 hours post induction (Fantoni et al. 2007). Even though the aeration and agitation are sub-optimal in these small culture vials, they are sufficient for identification of clones having production levels higher than a certain threshold level, as verified by the controls included in each growth experiment.

In theory, a higher gene dosage should, in principle, result in a higher level of protein product. While this, intuitively, is more likely to be the case for non trivial production targets like soluble proteins, it could be contra productive for membrane proteins possibly leading to intracellular traffic jam. To shed further light on this question, the correlation between the gene dosage and the aquaporin production level was evaluated providing an alternative small- scale production screen for integral membrane proteins in *P. pastoris* (Oberg et al. 2011a). In this screen we examined the relationship between the aquaporin yield and the ability of the recombinant *P. pastoris* cells to grow on high concentrations of Zeocin. Cells surviving on high Zeocin concentrations (2000 μg/ml) are likely to have multiple versions of the Zeocin resistance gene, thus, they will also contain multiple copies of the desired gene located on the same expression casette integrated into the *P. pastoris* genome. Indeed, for the human aquaporins there is a clear positive correlation between large colonies of the recombinant strain on high Zeocin concentrations and the production levels as analyzed by immunoblots (Figure 2A). Thus, increasing the gene dosage can be beneficial also for integral membrane proteins. Consequently, screening for improved growth on high Zeocin concentrations can circumvent the small scale production screening in 2 ml cultures having the additional advantage that more colonies could easily be screened in the search for high producing clones.

Following the observation that a high gene dosage is also beneficial for membrane protein production in *P. pastoris*, the influence of the transformation method used on the final aquaporin production level was evaluated. For generation of *P. pastoris* strains, the linearized expression plasmids have routinely been transformed by chemical transformation using the Lithium Chloride Method (Cregg and Barringer 1990). However, multiple insertion events occur at higher frequency when electroporation is used (Invitrogen 2010). Indeed, from our production trials of the human aquaporins in *P. pastoris* we found a significant improvement in yield when electroporation was used, which is most likely related to a higher frequency of multiple insertion events. Moreover, the impact by electroporation was mostly pronounced for

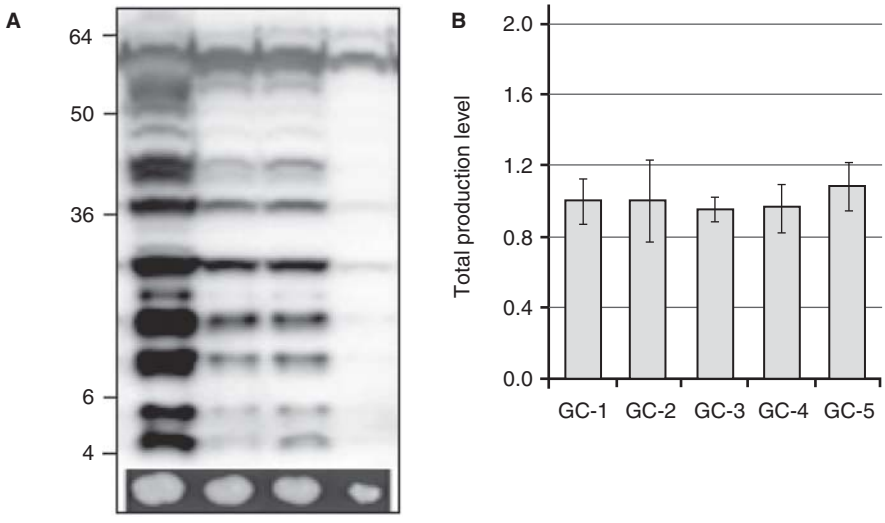


Figure 2. The screen on high Zeocin concentration. (A) Immunoblot showing the yields from the small scale production screen as compared to growth of the corresponding transformants on high Zeocin concentrations (2000 μg/mL), shown underneath. There is a clear correlation between large colonies grown on high Zeocin and the production yield analyzed by immunoblot. (B) Variation between the growth control (GC), SoPIP2;1, in five different experiments. The same control clone of SoPIP2;1 was cultured in five independent experiments ($n = 3$), over several years, and the production yield was estimated from independent immunoblots.

the low producing aquaporins, suggesting major benefits from an increased number of transcripts for these targets (Oberg et al. 2011a).

Independent of the choice of the initial production screen, 2 ml cultures or growth on high Zeocin concentrations, and the transformation method, it is important early on to be able to accurately estimate the relative production level for a certain target. Also, since aquaporins are integral membrane proteins, proper localization to the *P. pastoris* membrane could be a useful indication of properly folded and functional protein, something that has been confirmed by functional analysis of purified protein reconstituted into liposomes for four different aquaporin targets with varied production levels (see next section below). Hence, after the identification of a clone with a high enough production level from the small scale production screen, a quantitative production screen including a cell fractionation experiment is performed from triplicate 25 ml BMMY cultures in Erlenmeyer flasks 6 hours after induction. Growth in a shaker flask allows better aeration and is less sensitive to measuring errors due to the larger volume as compared to the growth in deep well blocks used in the small-scale production screen. After cell breakage, the crude extract (500 *g* supernatant) representing the total production and the membrane fraction (100,000 g pellet) are analyzed using immunoblots (Nyblom et al. 2007, Oberg et al. 2009). As a part of the quality assurance in each individual experiment, a growth control (SoPIP2;1) is always included in the 25 ml quantitative production screen. Its main purpose is to make sure the growth and production experiment progressed as expected, but it also serves as a reference in the estimation of the relative yield of a novel target.

Quantitative immunoblots are a particular challenge where a high variation in the signal strength is commonly observed if particular caution is not taken. Especially, the choice of detection system is critical to ensure that the read out of each signal is within the linear range; some of the commercially available detection kits indicate saturation of the signal while others enhance signals as much as possible on the cost of linearity (unpublished work: F. Öberg and K. Hedfalk). In addition, samples run on different gels at different time points have to be compared in a precise manner which requires that an internal standard is included in the experimental setup. For our study on aquaporin production in *P. pastoris*, we systematically used a defined amount of purified hAQP1* from one single batch as internal standard in all immunoblot experiments. By using this internal standard a certain signal could be related to a specific protein concentration and hence, we could remove the variation arising from the deviation in the total signal intensity from individual immunoblot experiments. Notably, the level of the growth control (SoPIP2;1) only showed minor variation after scaling to the internal standard (Figure 2B) providing an additional quality measurement for our established procedure used for quantitation of relative membrane protein production levels from individual immune blots.

Significant variation in recombinant production levels between homologous aquaporins

Even though the 13 human aquaporin homologues represent a family of highly-related proteins, there is a substantial variation in their yield when produced recombinantly in *P. pastoris* (Figure 3A). This is especially pronounced for the two closely-related proteins hAQP1 and hAQP4 where the former one is produced to high levels and the latter one is below the detection limit, as estimated from the quantitative production screen. Interestingly, the two aquaporins produced to the highest levels in *P. pastoris*, hAQP1 (Nyblom et al. 2007) and SoPIP2;1 from spinach leaves (Tornroth-Horsefield et al. 2006), also have a high natural abundance in their native plasma membranes. A possible interpretation could be that these proteins have natural intrinsic properties that allow them to be densely packed in the membrane and a concomitant high recombinant yield where aggregation and denaturation can be avoided.

Aquaporins have been localized to most organs in the human body. Some of the aquaporins have only been detected in intracellular vesicles whereas others are found in the plasma membrane. At least two of the human aquaporins are known to be trafficked to the plasma membrane (AQP2 and AQP8), thus they reside in intracellular vesicles when trafficking has not been triggered (Garcia et al. 2001, Nedvetsky et al. 2009). During protein synthesis, signals within the protein sequence itself or external recognition systems can determine the sub-cellular localization for a specific protein. To see whether the endogenous localization of a certain protein correlates with its recombinant yield in the *P. pastoris* production system, we analysed the estimated yield versus the native localization for each human aquaporin target (Table I). Although not statistically ensured, there is a clear tendency for proteins targeted to the plasma membrane to also have a high yield when recombinantly produced, as compared to the aquaporins found in intracellular vesicles. This observation suggests that yet unidentified signals or sequences within the protein itself are determinants for high or low recombinant yields, respectively.

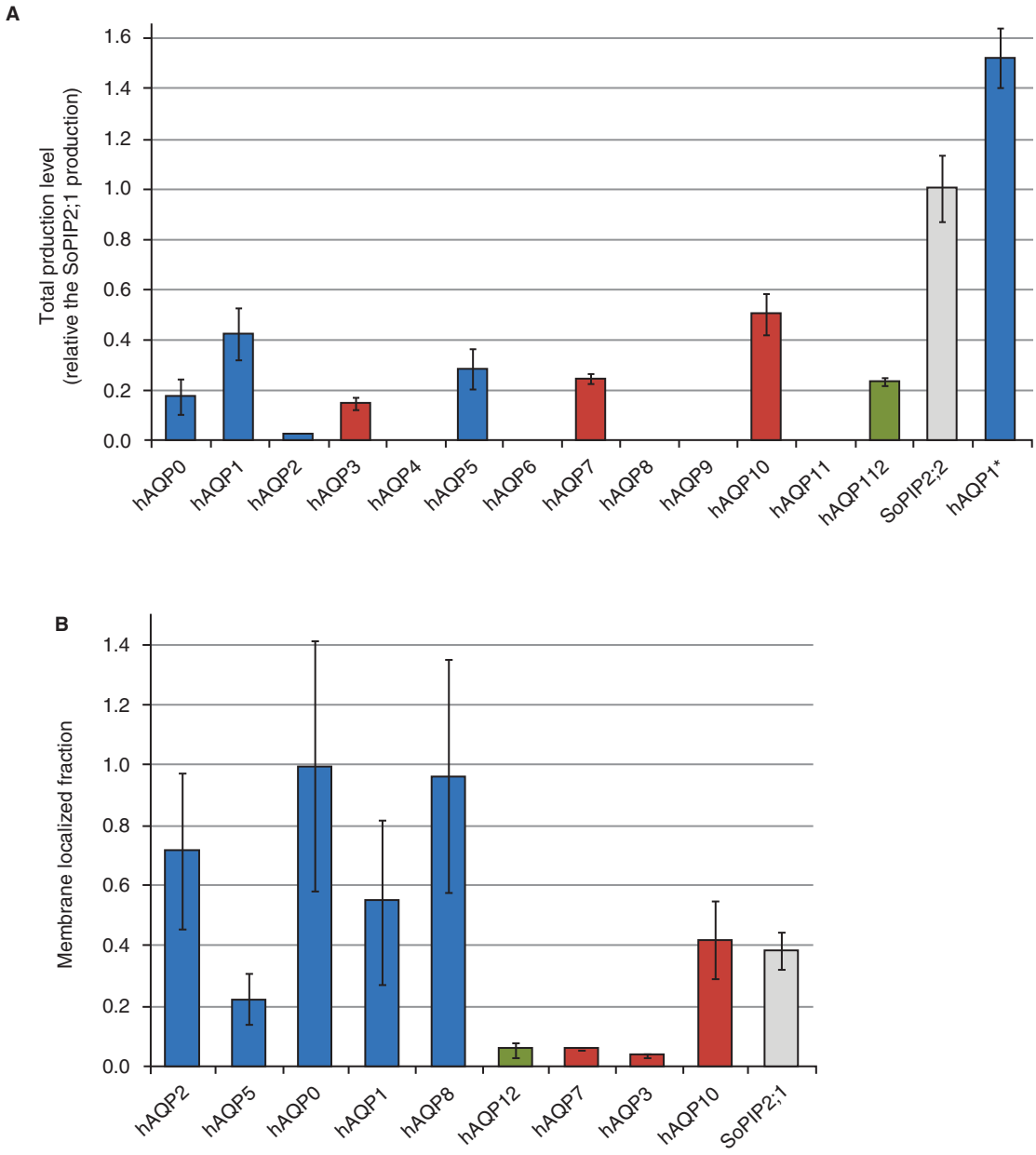


Figure 3. Production of the human aquaporin homologues in *P. pastoris*. (A) Bar chart showing the total production level of the human aquaporins homologues produced in the host *P. pastoris* relative the SoPIP2;1 production, for which the production is set to one (shown in grey). The y-axis represents the average from triplicate cultures and error bars show the standard deviation ($n = 3$). (B) Bar chart showing the membrane localized fraction for the hAQPs produced in the quantitative production screen. The typical membrane insertion is shown for the reference protein SoPIP2;1. The aquaporins are grouped by their position in the phylogenetic three (Figure 1). This Figure is reproduced in colour in *Molecular Membrane Biology* online where Orthodox aquaporins have blue bars, Aquaglyceroporins red bars and Superaquaporins green bars, respectively.

It is noteworthy that there is no apparent correlation between the recombinant yields (Figure 3A) and the location of a specific aquaporin in the phylogenetic tree (Figure 1). In contrast, such a correlation was apparent when analyzing the fraction of the protein localized to the membrane. To get the fraction of membrane localized material, the signal strength for the membrane fraction was divided by the total protein production for each specific target (Figure 3B). Due to error propagation arising from this exercise, the error bars are relatively wide. Nevertheless, our results showed a higher degree of membrane insertion for the orthodox aquaporins as compared to the superaquaporins and the aquaglyceroporins. A two- tailed Fisher's Exact Test comparing the orthodox aquaporins to the

Table I. Table showing the recombinant yield and sub-cellular localization in the native membrane for the different aquaporins found in mammalians. The localizations have been extracted from the shown references, but they are also stated, with only minor differences, in a review (King et al. 2004).

Protein	Recombinant yield	Native localization
AQP0	High	Plasma membrane (Chepelinsky, 2009)
AQP1	High	Plasma membrane (Nielsen et al. 1993)
AQP2	Poor	Intracellular vesicles (untrafficked) (Nedvetsky et al. 2009)
AQP3	High	Basolateral plasma membrane (Rai et al. 2006)
AQP4	No	Basolateral plasma membrane (Nielsen et al. 1997, Neely et al. 2001)
AQP5	High	Apical plasma membrane (Karabasil et al. 2009)
AQP6	No	Intracellular vesicles (Yasui et al. 1999)
AQP7	High	Apical plasma membrane (Skowronski et al. 2007)
AQP8	Poor	Intracellular vesicles (untrafficked) (Garcia et al. 2001)
AQP9	No	Plasma membrane (Elkjaer et al. 2000)
AQP10	High	Plasma membrane (Mobasheri et al. 2004)
AQP11	No	Intracellular (Morishita et al. 2005)
AQP12	High	Intracellular (Itoh et al. 2005)

non-orthodox gives a statistically significant association ($p < 0.05$) where the orthodox aquaporins are more prone to be membrane integrated. Thus, this suggests that the substrate specificity also gives rise to protein properties beneficial for proper folding and membrane insertion.

To evaluate the quality of an overproduction experiment, it would be appealing to directly measure the amount of correctly folded aquaporin in a membrane. We have conducted initial tests with GFP tagged AQPs and produced them in the *P. pastoris* cell. Our results support the production of correctly folded and properly inserted aquaporin for both a high (hAQP1) and low (hAQP8) yielding target in this particular host (Oberg et al. 2011a). However, their degree of membrane insertion appears to be different. Without staining, it is hard to distinguish between the different cellular compartments, but nevertheless, there seems to be a higher degree of insertion of the low producing hAQP8 (also see Figure 3B) as compared to the highly produced hAQP1 which has more pronounced intracellular GFP-signals (Oberg et al. 2011a). This implies the presence of aggregates due to saturation and overload of the cellular membrane protein secretion machinery for proteins being produced at exceptionally high yields, as has previously been suggested as a complication for related targets (Bonander et al. 2005).

To confirm our statement that proper localization to the *P. pastoris* membrane could be a useful indication of properly folded and functional protein, a selection of targets varying in production levels have been purified and reconstituted in liposomes. Notably, water transport was confirmed for all four targets, hAQP1 (Nyblom et al. 2007), hAQP4 (hAQP4^{m}-N185D) (Oberg et al. 2011a), hAQP5 (unpublished work: F. Öberg, J. Sjöhamn, and K. Hedfalk) and hAQP10 (Oberg et al. 2011b) (Table II). Moreover, the values for the osmotic water permeability (Pf) did not decrease as the protein yield was enhanced. For example, Pf was higher for hAQP1 than for hAQP5, indicating that the observed difference in functionality was due to the water transport capacity of the different aquaporin channels and not related to the overproduction yield as such. Hence, supported on the functional data for those aquaporins, our general conclusion is that human aquaporins recombinantly produced in the *P. pastoris* membrane are functional with the assumption that this is valid for the vast majority of the overproduced protein. Taken together, we can conclude that a high gene dosage in general also corresponds to a high total membrane protein production level for a certain target even though the yields of homologue proteins could vary. It is noteworthy that we have not seen any correlation between the production level and the function *per se*.

Table II. Table showing the osmotic water permeability (P_f) for hAQP4^{m}-N185D (Oberg et al. 2011a), hAQP5 (unpublished work: F. Öberg, J. Sjöhamn, and K. Hedfalk), hAQP10 (Oberg et al. 2011b and unpublished work: F. Öberg, J. Sjöhamn, and K. Hedfalk), and hAQP1 (Nyblom et al. 2007). The P_f for the control liposomes in each experiment is shown just before each protein sample.

Sample	P_f
Control	2.4 ± 0.01
hAQP4^{m}-N185D	7.5 ± 0.03
Control	2.4 ± 0.01
hAQP5	6.6 ± 0.01
hAQP10	7.3 ± 0.02
Control	3.1 ± 0.01
hAQP1	9.1 ± 0.4

Fermentor growth is essential to achieve high yields of stable aquaporins

When a high producing clone has been identified using the quantitative production screen described above, controlled growth and induction is vital to optimize the production and make use of the full capacity of the *P. pastoris* system. Ideally, the up-scaled growth takes place in a fermentor where the growth conditions are monitored and controllable. Essentially, the oxygen addition can be sufficient due to efficient aeration and agitation while the methanol addition can be maximized without any risk of oxygen limitation. This is especially important for *P. pastoris* since the protein being responsible for the first oxidation reaction in the methanol utilization pathway, alcohol oxidase 1, has a low affinity for oxygen. Hence, large amounts of oxygen are needed to allow higher methanol concentrations and thereby take the full advantage of the AOX1 promoter (Cregg et al. 2000). Furthermore, the controlled regimes accessible in a fermentor also allow fine tuning of the AOX1 promoter by mixed feeding protocol. Consequently, by cultivation in fermentors, we were able to achieve exceptional high yields of hAQP1; 90 mg of pure protein was extracted per litre of culture (Nyblom et al. 2007).

Moreover, by using the appropriate sensors, the amount of viable cells in the reactor can easily be monitored providing a sophisticated tool to control active growth as compared to the classical optical density measurements often used to analyze shake flask cultures where all cells are taken into account. Especially for *P. pastoris*, measuring the fraction of living cells is a useful tool in avoiding addition of excessive, and hence, toxic, amounts of methanol. Interestingly, we observed a difference in growth characteristics between aquaporins from the different sub-families. In general, cell-producing orthodox aquaporins continued to grow upon the switch from glycerol to methanol while clones overproducing aquaglyceroporins had a much slower growth rate on methanol, sometimes with a concomitant decay of living cells. A plausible explanation could be that the slightly wider channels provided by the aquaglyceroporins could allow transportation of the small methanol molecule into the cell where it would be toxic. To evaluate this possibility, three amino acids lining the pore entrance at the ar/R constriction region in hAQP5 were mutated to create a larger pore and thereby changing the pore specificity to not only be selective to water, as has previously been made for AQP1 (Beitz et al. 2006). Indeed, the growth on methanol was hampered for this AQP5 mutant indicating that methanol might be taken up by the broader channel (unpublished work: F. Öberg, J. Sjöhamn, and K. Hedfalk).

Following this notion, a mixed feed containing 60% sorbitol and 40% methanol was evaluated for hAQP10 with the intention to lower the concentration of the toxic methanol for aquaglyceroporins (described in Oberg et al. 2011b). Sorbitol is selected since it provides an additional carbon source that does not give rise to the gene repression associated with glycerol. In addition, growth on sorbitol has been shown to increase the protein yield by increasing biomass (Jungo et al. 2007a, Jungo et al. 2007b) as well as by weakening induction to better match the requirements of the metabolism of the cells (Holmes et al. 2009). For hAQP10, we observed no significant changes in the total protein yield from the mixed feed. However, a protein degradation product commonly seen in the pure methanol feed disappeared suggesting a reduced cellular stress response under these conditions. Hence, mixed feed could possibly provide a solution for stable production of aquaglyceroporins in general giving homogenous samples suitable for further characterization.

Finally, quantitation of the yield from the fermentor cultures made it possible for us to verify the quality, reliability and scale ability of the quantitative production screen performed in Erlenmeyer flasks. As mentioned above, we produced 90 mg hAQP1 per litre of fermentor culture (Nyblom et al. 2007). In comparison, a third of that amount was obtained for hAQP10; 30 mg hAQP10 per litre of fermentor culture (Oberg et al. 2011b). The relationship between the yields from the scaled-up cultures were fully consistent with the corresponding quantitated yields for those targets in 25 ml cultures where hAQP1 (denoted hAQP1*) gives exactly three times higher yield than hAQP10 (Figure 3A). In comparison, targets resulting in low yields in Figure 3A, such as hAQP2, hAQP6, and hAQP8, have been problematic to overproduce to sufficient yields for subsequent analysis, even by fermentor growth. Hence, to our satisfaction, the data from the quantitative production screen can be extrapolated to the large-scale production in the fermentor which equips us with a very useful tool early in the process to determine the suitability of a certain target for large scale production and characterization, a tool that saves time and energy as well as money.

Experimental details

The scaled up growth of *P. pastoris* was done in 3-litre fermentors (Infors) with an Initial Fermentation Volume (IFV) of 1.5 litres and a start OD_{600} of about

0.2 according to the Invitrogen Pichia Fermentation Process Guidelines (Invitrogen, 2002). During growth, the temperature was set to 30°C, pH adjusted to 5 by NH_3 addition, agitation and aeration were varied between 500–1500 rpm and 0.1–1 vvm, respectively, depending on the cell density, dissolved oxygen kept above 20% as verified by frequent oxygen spikes following carbon limitation and the density of living cells continuously monitored. The initial glycerol bath phase typically lasted for 20 hours consuming 60 g glycerol and giving an OD_{600} of about 25, followed by a glycerol fed-batch phase for 24 hours, just to acclimatize cells to growth during carbon-limited conditions. However, for targets with a limited growth on methanol, the length of the glycerol fed-batch phase could be doubled to allow the formation of more biomass before induction on methanol. The recombinant protein was produced during the methanol fed batch phase (200–400 ml MeOH) which lasted for 24–48 hours resulting in 400–600 g wet cells (OD_{600} about 200–400).

Table III. Table showing protein production yields for all human AQPs overproduced in *P. pastoris*. The hAQPs are listed based on their production level, starting with the hAQP giving the highest yield. The silent mutations introduced in the second codon are underlined. The original sequence is given in brackets. The two smallest nonpolar residues (alanine and glycine) in the second position are underlined.

Gene	Yield	Initiation sequence	2nd residue
hAQP1*	+++	C AAA ATG GCC	Ala
hAQP10	++	C AAA ATG GT**T**(C)	Val
hAQP1	++	C AAA ATG GC**T**(C)	Ala
hAQP5	++	C AAA ATG AAG	Lys
hAQP7	++	C AAA ATG GTT	Val
hAQP12	++	C AAA ATG GC**T**(A)	Ala
hAQP0	++	C AAA ATG TGG	Trp
hAQP3	++	C AAA ATG GGT	Gly
hAQP2	+	C AAA ATG TGG	Trp
hAQP8	+	T AAA ATG TCT	Ser
hAQP4 m1	-	C AAA ATG **TC**T(AG)	Ser
hAQP4 m23	-	C AAA ATG GT**T**(G)	Val
hAQP6	-	T AAA ATG GAT	Asp
hAQP9	-	C AAA ATG CAG	Gln
hAQP11	-	C AAA ATG TC**T**(G)	Ser

The construct design has a major impact on the final production level

The family of human aquaporins was used to evaluate several factors directing high production of eukaryotic membrane proteins including alterations around the initiation codon of the mRNA, fusion with Mistic or AQP, chimeric AQP constructs, as well as directing the topological maturation of the aquaporin monomer by directed mutagenesis.

The nucleotide sequence flanking ATG is of major importance

Translation is controlled by the rate of initiation, thus being affected by the 5′ sequence of the mRNA transcript (Romanos et al. 1992). For eukaryotic mRNA, the sequence flanking the initiator codon (underlined) was found to be (A/G)NNAUGG by Marilyn Kozak, henceforth called the Kozak sequence (Kozak 1981). In yeast, the consensus sequence is different; (A/Y)A (A/U)AAUGUCU (Cigan and Donahue 1987). For production of all 13 human aquaporins in *P. pastoris* (Oberg et al. 2009) we adapted the start sequence to the yeast consensus sequence A/YAA/TAATGTCT, as recommended in the EasySelect Pichia Expression Kit manual at the time (Invitrogen 2005). Hence, we consistently used Y (C/T) in the −4 position, AAA in the −1 to −3 positions and we aimed to mimic TCT at positions +4 to +6 by just allowing silent mutants in the second codon (Table III). When looking at the difference in production level and the sequence around the initiator ATG, we observed a clear preference of G at the +4 position among the highly produced aqupaorins (5/7 in Table III). The likely explanation to this is that a G in this position increases the probability for a small non-polar amino acid, like alanine and glycine, which are important for a successful cleavage of the initiator methionine from the nascent polypeptide (Xia 2007). To shed further light on the importance of G at the +4 position, we mutated a T to G in this position for an aquaporin produced to moderate yields, hAQP8, which resulted in a mutation of serine to glycine. Notably, the production yield was increased upon mutation from the wild type TCT sequence to GCT (Figure 4A). Another interesting observation was the significant decrease in production level for hAQP1 upon imitation of the yeast consensus sequence (Oberg et al. 2009) as compared to a construct with unaltered sequence for the second codon (Nyblom et al. 2007) (Figure 4B). A putative determinant for this observation could be the intrinsic cytosine in the +6 position, which upon change to a thymine reduced the yield. Most interestingly, the sequence flanking the initiator codon of the highly produced AOX1 in the wild type *P. pastoris* cell, (30% of the soluble protein content in methanol grown cells) was found to be ACGATGG (De Schutter et al. 2009), perfectly matching the Kozak sequence. This fact combined with our observation, that mimicking TCT for the second codon did not have any positive influence on the aquaporin yield, leads to the conclusion that the eukaryotic consensus sequence is superior to the yeast consensus sequence when overproducing eukaryotic membrane proteins in the yeast *P. pastoris*. It is worth mentioning that the

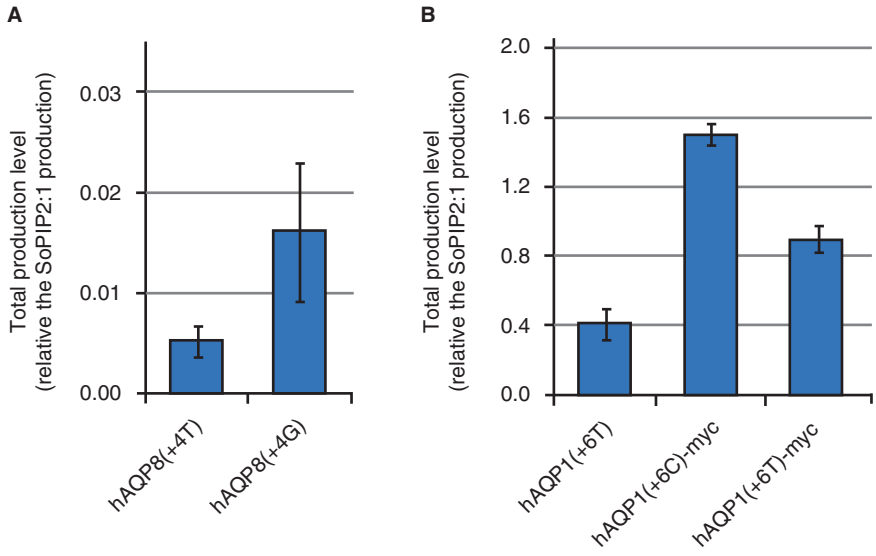


Figure 4. The influence of the second triplet on the production yield. Bar chart showing the total production yield for (A) two hAQP8 constructs with mutations in the +4 position, and (B) three hAQP1 constructs with mutations in the +6 position. Variations in the nucleotide sequence for the second codon are shown in brackets. All constructs have a C-terminal 6× histidine tag and some have the additional myc tag in the C-terminus, as shown in the figure. Production is relative to the SoPIP2;1 production, for which the production is set to one. The y-axis represents the average from triplicate cultures and error bars show the standard deviation ($n = 3$). This Figure is reproduced in colour in *Molecular Membrane Biology* online.

most recent EasySelect Pichia Expression Kit manual (Invitrogen 2010) recommends the (G/A)NNATGG consensus sequence with the notion that the yeast consensus sequence is a less strong alternative to the Kozak sequence showing a 2–3 fold effect in translation initiation efficiency. Interestingly, as compared to higher eukaryotes, translation in yeast is suggested to be more sensitive to secondary structures (Baim and Sherman 1988), supporting the importance of optimizing the initiation sequence.

Evaluation of fusion proteins in P. pastoris

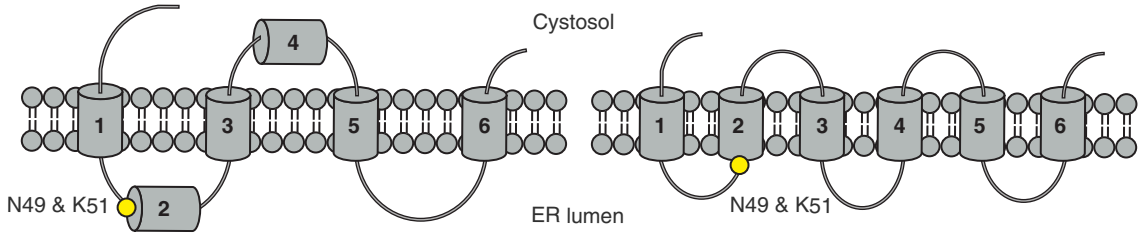
Apart from the flanking sequences of the initiator ATG codon, other aspects of the construct design were also evaluated for the human aquaporins produced in a simple eukaryotic host. The intention was to test whether the fusion to a stable and highly-produced protein, or peptide sequence, would enhance the production of the membrane protein of interest, analogous to similar approaches previously shown to be successful for soluble proteins in both *E. coli* and *S. cerevisiae* using maltose binding protein as well as other fusion partners (Wang et al. 2003, Hennig and Schafer 1998, Perez-Martin et al. 1997, Lian et al. 2009). In comparison, Mistic (acronym for 'membrane-integrating sequence for translation of integral membrane protein constructs'), a membrane anchored protein found in the bacteria *Bacillus subtilis*, has been applied as fusion partner in bacteria (Roosild et al. 2005) where it has been able to increase the production of G-protein coupled receptors among others (Petrovskaya et al. 2010). To evaluate the possibility of transferring this approach to a eukaryotic host, the Mistic sequence was codon optimized (Opt-Mistic) for *P. pastoris*. Notably, our results showed a remarkably stable and high level of production of Opt-Mistic alone, in the same range as hAQP1 (Oberg et al. 2011a). When fusing Opt-Mistic to either a high or a low yielding AQP, the Mistic-AQP fusions resulted in a lower yield than for the AQP alone. Hence, these data imply that the concept of Mistic fusions to increase eukaryotic membrane protein yields cannot be directly transferred from *E. coli* to a eukaryotic host like *P. pastoris*. Consequently, we evaluated the use of an aquaporin with an intrinsically high and stable yield in this particular host, hAQP1, as fusion partner. However, both full length hAQP1 and parts thereof failed to increase the moderate production level of hAQP8. A set of chimeric constructs, where either the amino-terminus, transmembrane domain 1 (TMD1), TMD1-2 or TMD1-3-of hAQP8 were substituted for the corresponding protein sequence of hAQP1, did not lead to any improvements in recombinant production of hAQP8 either, indirectly indicating an importance of the carboxyl terminal half of hAQP1 as determinant for high production. Indeed, increased production was also observed for SoPIP2;1, having a high and stable production in itself, having its C-terminus swapped for the one of hAQP1⋆ further supporting the importance of

the hAQP1 C-terminus for high recombinant production levels (Oberg et al. 2011a).

Application of protein engineering to influence folding and target stability

Yet another method of construct design was evaluated for the protein family of human aquaporins, this time based on detailed information available on the alternative topology maturation for AQP1 and AQP4, respectively. During folding of AQP1 in the ER, a four spanning intermediate is initially observed (Skach et al. 1994), which subsequently matures into the final six transmembrane fold (Lu et al. 2000) (Figure 5A). In contrast, AQP4 folds sequentially into the six spanning topology (Shi et al. 1995, Sadlish et al. 2005). Recent studies identified two amino acids near TMD2 as responsible for the difference in maturation between the two homologues aquaporins (Foster et al. 2000): Asn49 and Lys51 for AQP1 corresponding to Met48 and Leu50 in AQP4. Interestingly, engineering these specific amino acids from hAQP1 on hAQP4 (hAQP4^{m}) lead to a swap in folding pathway (Buck et al. 2007). Hence, it was tempting to test whether the settings for topology maturation could also have a positive influence on the aquaporin production level in *P. pastoris*. Indeed, a significant yield increase was observed for hAQP4^{m}, as compared to hAQP4, allowing scaled up production for this target (Oberg et al. 2009). When carefully comparing all the human aquaporin sequences in this specific region, a unique presence of positively charged amino acids is observed in hAQP1, something that is completely lacking in hAQP4 (Figure 5B). In correlation, the hydrophobicity of the amino acids in and in close

A



B CLUSTAL 2.0.12 multiple sequence alignment

hAQP1	(49)	**N**V**K**VSLAFGLSIATLAQSVGHISGAHLNPA	(78)
hAQP4 M23	(48)	**M**V**L**ISLCFGLSIATMVQCFGHISGGHINPA	(77)
hAQPO	(41)	**V**L**Q**VAMAFGLALATLVQSVGHISGAHVNPA	(70)
hAQP2	(41)	**V**L**Q**IAMAFGLGIGTLVQALGHISGAHINPA	(70)
hAQP3	(56)	**F**L**T**INLAFGFAVTLGILIAGQVSGAHLNPA	(85)
hAQP5	(42)	**I**L**Q**IALAFGLAIGTLAQALGPVSGGHINPA	(71)
hAQP6	(55)	**V**L**Q**IAITFNLVTAMAVQVTWKASGAHANPA	(84)
hAQP7	(67)	**Y**L**G**VNLGFGFGVTMGVHVAGRISGAHMNAA	(96)
hAQP8	(65)	**L**L**Q**PALAHGLALGLVIATLGNISGGHFNPA	(94)
hAQP9	(57)	**V**I**T**INVGFSMAVAMAIYVAGGVSGGHINPA	(86)
hAQP10	(55)	**F**F**T**MFLAGSLAVTIAIYVGGNVSGAHLNPA	(84)
hAQP11	(72)	**P**A**H**PTWTLTLVYFFSLVHGLTLVGTSSNPC	(101)
hAQP12	(66)	**D**F**G**PDLLLTLLFLLFLAHGVTLDGASANPT	(95)

Figure 5. The folding of human aquaporin 1. (A) Topology maturation for hAQP1 with a four spanning intermediate (left) and the mature fold with six TMD (right) (Lu et al. 2000). Asn49 and Lys51 in the N-terminal part of TMD2 (their position is marked with a black dot) are determinants for this fold (Foster et al. 2000). (B) Sequence alignment of all hAQPs around TMD2, with the amino acids corresponding to Asn49 and Lys51 in hAQP1 highlighted. For hAQP4 M23 the corresponding amino acids are Met48 and Leu50. Nonpolar amino acids (hydrophobic) are marked in bold and polar and electrically charged (hydrophilic) are marked in bold and underlined. This Figure is reproduced in colour in *Molecular Membrane Biology* online where specific positions in Figure 5A are marked with a yellow dot.

proximity of a given amino acid segment is a known determinant for the insertion of that TMD into the membrane (Hessa et al. 2005, Hessa et al. 2007). Hence, a plausible explanation for the observed difference in the aquaporin folding pathway could be the variation in hydrophobicity in the TMD2 segment, where hAQP1 is less hydrophobic due to the two positively charged residues before TMD2. Hence, the concept of increasing the production yield by redirecting the folding pathway is unlikely to be transferable to other family members since no other family members possess two hydrophobic amino acids in these specific positions (Figure 5B) and the folding pathway is yet to be established for those aquaporins. Nonetheless, the improvement of the hAQP4 yield by two point mutations exemplifies the great value of biochemical insight in the design of a successful overproduction experiment for a desired target.

Gene optimization to increases yield

An intuitive and attractive strategy to improve the production level of a certain target is to optimize its gene sequence to perfectly match the codon usage of the selected host system and the ease of creating synthetic genes has improved. Services offering gene optimization take various parameters into account: codon usage, GC content, splicing signals, stable mRNA secondary structures, DNA repeats and, where applicable, the most efficient signal peptide is identified for the gene of interest (GeneArt 2011, GenScript 2011). Among those parameters, the main focus has been the optimizing of the translation process based on the codon usage and host adaptation presented almost 25 years ago (Sharp and Li 1987). However, to produce high yields of functional proteins, the nascent polypeptide must also be correctly folded, properly translocated, and undergo any necessary posttranslational modifications.

The genetic code provides 64 possible ways of combining a triplet of nucleotides, codons, to code for either one of the 20 amino acids or the three stop codons giving that most amino acids can be encoded by several codons, methionine and tryptophan being the only exceptions having only one triplet coding for them. Moreover, the frequency of a given combination of a nucleotide codon and tRNA anticodon shows a great variation between different organisms (Table IV). Therefore, as a consequence of recombinant gene expression, the difference in the preferred codon usage in the native gene and the tRNA pools of the expression system may cause inefficient translation hampering the protein production. One solution is to increase the intracellular tRNA pool by overexpressing genes coding for the rare tRNAs. This strategy, commonly used for *E. coli*, was evaluated for the malaria parasite aquaporin (PfAQP), a putative drug target in the search for more efficient anti malarial treatment, without any successful outcome (Hedfalk et al. 2008). In comparison, moving this concept to higher organisms has been found to be impractical (Gustafsson et al. 2004). Hence, the alternative approach of changing the gene is left as a plausible solution. By codon optimization it is possible to change the codons for the encoded amino acids using silent mutations and thereby make use of the more favourable codons of the host. Codon Adaptation Index (CAI) is a measurement of how well the codon usage in a protein coding DNA sequence matches the bias of a certain host. CAI can also be used as a numerical estimator of gene expressivity where a high value would indicate a highly expressed gene (Wu et al. 2005). Table V shows CAI values for all hAQPs and PfAQP in their native host and in *P. pastoris*, respectively, using three different calculators; CAIcal (Puigbo et al. 2008), EMBOSS: cai (Bleasby 2001) and JCat (Grote et al. 2005), respectively. Two constructs optimized for production in *P. pastoris* have been included in Table IV and they are both named 'Opt-'. As seen here, these genes have exceptionally high CAI values in *P. pastoris* as compared to their native gene sequences and would thus be expected to give a higher protein yield. Indeed, the gene optimization of PfAQP is an illustrative example of production improvements in *P. pastoris* giving a functional and membrane localized protein product (Hedfalk et al. 2008). We anticipated that the explanation to this was mainly related to the unusually low GC content: only 31% in the PfAQP gene and 24% in the coding region of the whole genome (Tables IV and V). Whether the reason for an increased production of PfAQP in *P. pastoris* is found in the codon adaptation, in the alterations of the GC content or in the combination thereof is not obvious. It has been reported that the change in GC content is the major contributor to the increased translational efficiency in *P. pastoris* (Sinclair and Choy 2002) and considering the large deviation in GC content for *P. falciparum* it seems to be a probable explanation. Interestingly, the wild type PfAQP gene has a higher CAI in *P. pastoris* than all the human aquaporins suggesting it could be produced to high levels without any optimization. This, however, was not observed in our study. Consequently, the usage of CAI alone for prediction of membrane protein production levels is limited.

In comparison, the difference in GC content of the coding genome is not always as distinct as for *P. falciparum* and *P. pastoris*. In the case of human

Table IV. Table showing the codon usage for *P. pastoris* (Pp), *H. sapiens* (Hs) and *P. falciparum* (Pf), respectively. The genetic code includes 64 possible ways of making a triplet, of these, three are stop codons in most organisms and are here marked with a star (*). The codon usage is given as fractions where 1 equals 'always used' and the most commonly used codon is highlighted in bold. The GC content of the coded DNA is also given for each species. Gene frequencies are adapted from Codon Usage Database (Kazusa 2007) for *P. pastoris*, *H. sapiens* and *P. falciparum* where the species had the numbers 4922, 9606 and 36329 respectively.

Codon	AA	Pp	Hs	Pf
UAA	*	**0.51**	0.30	**0.69**
UGA	*	0.20	**0.47**	0.21
UAG	*	0.29	0.24	0.10
GCU	Ala	**0.45**	0.27	0.42
GCC	Ala	0.26	**0.40**	0.11
GCA	Ala	0.23	0.23	**0.43**
GCG	Ala	0.06	0.11	0.05
CGU	Arg	0.17	0.08	0.11
CGC	Arg	0.05	0.18	0.02
CGA	Arg	0.10	0.11	0.09
CGG	Arg	0.05	0.20	0.01
AGA	Arg	**0.48**	**0.21**	**0.60**
AGG	Arg	0.16	**0.21**	0.16
AAU	Asp	0.48	0.47	0.86
AAC	Asp	0.52	0.53	0.14
GAU	Asp	**0.58**	0.46	**0.87**
GAC	Asp	0.42	**0.54**	0.13
UGU	Cys	**0.64**	0.46	**0.87**
UGC	Cys	0.36	**0.54**	0.13
CAA	Gln	**0.61**	0.27	**0.87**
CAG	Gln	0.39	**0.73**	0.13
GAA	Glu	0.56	0.42	0.86
GAG	Glu	0.44	0.58	0.14
GGU	Gly	**0.44**	0.16	0.42
GGC	Gly	0.14	**0.34**	0.05
GGA	Gly	0.33	0.25	**0.44**
GGG	Gly	0.10	0.25	0.10
CAU	His	**0.57**	0.42	**0.86**
CAC	His	0.43	**0.58**	0.14
AUU	Ile	**0.50**	0.36	0.39
AUC	Ile	0.31	**0.47**	0.07
AUA	Ile	0.18	0.17	**0.54**
UUA	Leu	0.16	0.08	**0.63**
UUA	Leu	0.16	0.08	**0.63**
UUG	Leu	**0.33**	0.13	0.14
CUU	Leu	0.16	0.13	0.11
CUC	Leu	0.08	0.20	0.02
CUA	Leu	0.11	0.07	0.08
CUG	Leu	0.16	**0.40**	0.02
AAA	Lys	0.47	0.43	**0.82**
AAG	Lys	**0.53**	**0.57**	0.18
AUG	Met	**1.00**	**1.00**	**1.00**
UUU	Phe	**0.54**	0.46	**0.84**
UUC	Phe	0.46	**0.54**	0.16
CCU	Pro	0.35	0.29	0.40
CCC	Pro	0.15	**0.32**	0.10
CCA	Pro	**0.42**	0.28	**0.46**
CCG	Pro	0.09	0.11	0.05
UCU	Ser	**0.29**	0.19	0.23
UCC	Ser	0.20	0.22	0.08
UCA	Ser	0.18	0.15	0.26
UCG	Ser	0.09	0.05	0.05
AGU	Ser	0.15	0.15	**0.32**
AGC	Ser	0.09	**0.24**	0.06
ACU	Thr	**0.40**	0.25	0.26
ACC	Thr	0.26	**0.36**	0.12
ACA	Thr	0.24	0.28	**0.53**
ACG	Thr	0.11	0.11	0.09
UGG	Trp	**1.00**	**1.00**	**1.00**
UAU	Tyr	0.47	0.44	**0.89**
UAC	Tyr	**0.53**	**0.56**	0.11
GUU	Val	**0.42**	0.18	0.40
GUC	Val	0.23	0.24	0.06
GUA	Val	0.15	0.12	**0.41**
GUG	Val	0.19	**0.46**	0.12
Coding GC (%):		42.7	52.3	23.8

AQP4, the difference between *H. sapiens* and *P. pastoris* is significantly smaller and no obvious production problems can be predicted based on the GC content. The CAI values are also similar for hAQP4 in the native host and in *P. pastoris*, neither giving any indication of production problems related to differences in codon usage in the two hosts. Nevertheless, the wild type hAQP4 sequence was submitted to two large companies specialized in gene engineering: GenScript and GeneArt. As a comparison, a manual codon optimized gene was made using JCat (Grote et al. 2005). The result is presented in Table VI. The 'effective number of codons used', N_c, is a measurement of the extent of codon bias in a gene; its value range from 20, corresponding to one codon being exclusively used for an amino acid, to 61 where the probability is equal for all possible codons (Wright 1990). Independent of the method used, the major changes are seen in nucleotide no3 of each codon, as expected, since substitutions at this site more seldom change the amino acid. Interestingly, the variation between the optimizations performed by the companies is substantial. Moreover, it appears that human genes have a relatively high GC content, 52%, as compared to *P. pastoris*, 43% (Table IV). Commonly,

Table V. Table showing the codon adaptation index (CAI) for aquaporins in their native host and *P. pastoris*, respectively, where the optimized genes are highlighted in bold. CAI calculations using CAIcal (Puigbo et al. 2008) and EMBOSS:cai (Bleasby 2001) were based on the Codon Usage Database (Kazusa 2007) for *P. pastoris* (Pp), *H. sapiens* (Hs) and *P. falciparum* (Pf) where the species had the numbers 4922, 9606 and 36329 respectively. CAI calculations from JCat (Grote et al. 2005) use its own codon tables and since it does not contain data for Pp or Pf, these columns have been omitted. For clarity, the values obtained from the calculators are separated by a dotted line. The GC content and the N_c value is also given. N_c represents the extent of the gene's codon bias; 20: strong bias, 61: no bias.

			CAIcal			EMBOSS:cai			JCat
Gene	Nc	%GC	Pp	Hs	Pf	Pp	Hs	Pf	Hs
hAQP0	52.4	57.7	0.670	0.798		0.598	0.794		0.343
hAQP1	42.0	61.2	0.613	0.826		0.540	0.826		0.516
hAQP2	40.2	64.0	0.616	0.815		0.535	0.811		0.476
hAQP3	38.6	60.3	0.635	0.843		0.579	0.838		0.470
hAQP4	56.6	49.2	0.707	0.760		0.664	0.750		0.228
Opt-hAQP4	25.6	40.8	**0.907**	**0.750**		**0.909**	**0.693**		0.135
hAQP5	36.5	63.1	0.602	0.832		0.534	0.825		0.546
hAQP6	44.0	62.9	0.591	0.803		0.540	0.805		0.457
hAQP7	45.7	56.6	0.638	0.791		0.593	0.791		0.382
hAQP8	44.7	61.2	0.610	0.801		0.556	0.798		0.415
hAQP9	52.7	48.9	0.727	0.757		0.680	0.736		0.215
hAQP10	49.7	58.5	0.681	0.810		0.617	0.797		0.357
hAQP11	55.3	55.6	0.664	0.766		0.583	0.744		0.341
hAQP12	38.0	66.6	0.605	0.824		0.527	0.816		0.528
PfAQP	36.5	30.7	0.750		0.787	0.724		0.736	
Opt-PfAQP	32.6	44.0	**0.895**		**0.376**	**0.886**		**0.373**	

a decrease in GC content is observed for all the hAQP4 genes adapted to *P. pastoris* (TableVI) due to the more AT biased codon choice for this lower eukaryote. To further analyze the importance of the GC content, a plot was made for all the optimized genes in Table V and compared to the wild type hAQP4 (Figure 6A). From this GC-plot large variations in GC content are observed for the native protein, with some regions spiking over 67% and some dipping to 25%. In contrast, the general theme for the optimized genes is to stay within a much more narrow range of variation. To analyze the effect of the codon adaptation in practice, we used the GenScript optimized version of hAQP4 (Opt-hAQP4) for production in *P. pastoris* (Oberg et al. 2011a) which resulted in a significant increase in yield (Figure 6B). The precise explanation for this observation remains to be evaluated. Anyhow, we intuitively believe that the combinatorial effect of enhancing both the translation and translocation of a protein would result in the highest possible level of production, hence, we introduced the mutants directing the folding pathway of hAQP4 ($hAQP4^m$) in the codon optimized hAQP4, resulting in Opt-$hAQP4^m$. Certainly, the yield of hAQP4 was increased even further giving a yield comparable to the highly produced hAQP1 (Figure 6B). To summarize, the optimization strategies used for hAQP4 illustrates how detailed knowledge of the protein target provides the tools for designing a successful overproduction experiment. However, such knowledge is rarely available which emphasizes the importance of further studies of various aspects of novel protein targets. On the other hand, when information of this sort exists, combining strategies is likely to have a great impact on the final yield of functional membrane protein.

Conclusions

We have used the homologues proteins from the human aquaporin family in order to establish and validate quantitative screening protocols in the

Table VI. Table summarizing the different results obtained when optimizing hAQP4 using JCat, GenScript or GeneArt. Total GC content is shown as well as the GC content of the first, second and third position in the codon. The number of bases changed is also presented as a percentage of the 969 nucleotides in the gene. N_c is a value representing the extent of the codon bias; 20: strong bias, 61: no bias.

Name	%GC	%GC (1)	%GC (2)	%GC (3)	N_c	Changed
hAQP4 wt	49.2	53.3	43.3	51.1	55.9	—
JCat	43.6	45.5	43.3	41.8	20.0	25.3%
GenScript	40.8	45.5	43.3	33.4	32.7	24.0%
GeneArt	45.7	45.8	43.3	48.0	23.7	24.5%

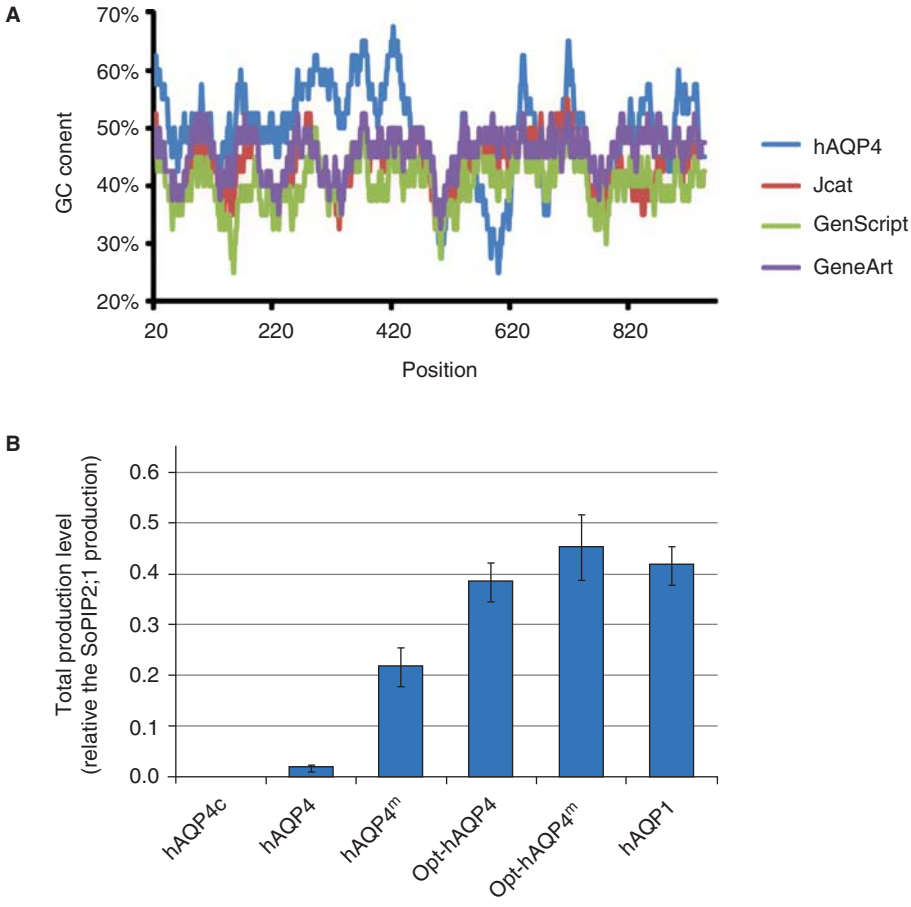


Figure 6. Various strategies to optimize the hAQP4 production in *P. pastoris*. (A) Plot showing the GC content in a 40 bp window centred at the indicated nucleotide position. Data were acquired using the EMBOSS:isochore (Rice 2011). (B) Bar chart showing the yield of four different hAQP4 versions. The genes have been transformed by electroporation except hAQP4c where chemical transformation has been used. Constructs having mutations causing an AQP1 like topology maturation have been indicated as hAQP4^{m}. The prefix Opt symbolizes constructs where the gene has been codon optimized to better match the *P. pastoris* host system. This Figure is reproduced in colour in *Molecular Membrane Biology* online.

methylotrophic yeast *P. Pastoris*, generic for eukaryotic membrane proteins. By systematically using these protocols we can early in the process estimate our final yield from scaled up fermentor cultures. The main gain is the possibility of evaluating whether the production level of a novel target will be sufficient for downstream purification and characterization or if optimization of its production needs to be undertaken. In brief, we recommend an initial small scale screen on high concentrations of Zeocin to identify high producing clones since increasing the gene dosage was found to be beneficial also for membrane protein production experiments. As a consequence, for the final protein yield, electroporation is superior to chemical transformation, something that was especially pronounced for targets produced at low to moderate initial levels.

For the 13 human aquaporins, we observed a significant variation in the total production level even between proteins having the highest degree of sequence identity. The underlying reason for this variation is not fully unravelled, but specific properties of the proteins are a plausible explanation. On the contrary, a relationship between the transport specificities and the degree of membrane localization was observed, indicating that the pure water channels are more likely to be densely packed in the membrane. In general, the highest yields are achieved when *P. pastoris* cells are grown in tightly controlled fermentor cultures, conditions allowing maximal use of the methanol-induced AOX1 promoter in high cell density cultures. This was particularly pronounced for one of our most highly produced human aquaporin homologues, hAQP1*, where fermentor growth resulted in nearly five times higher yields (90 mg of pure protein per litre) as compared to shaker flasks.

Further, using the recombinant hAQP clones we have evaluated the importance of the construct design for recovery of high membrane protein levels in *P. pastoris*. Obviously, the sequence flanking the

initiator methionine should mimic the Kozak sequence to improve the processing of the nascent polypeptide chain. Moreover, we found that the use of fusion partners is a non-trivial approach for eukaryotic membrane proteins in a eukaryotic host system. On the contrary, for the few cases where biochemical data is available for the target of interest, alteration of the topology maturation in the translation and translocation process might prove to be useful. A more generic approach is to apply gene optimization in order to increase the production level for membrane proteins. In this process, codons will be adapted to use the host tRNA pool in an efficient way, the GC content will be changed to suit the requirements of the host, and the mRNA stability will be enhanced. However, while shown to often be beneficial, the various algorithms used for codon adaptation and the precise reasons behind its actual effect remain elusive. PfAQP is an example where the production level was significantly improved when the GC content of the gene was closer to the GC content of the host's coding DNA. On the contrary, several of the wild type human aquaporins giving high yields, like hAQP1 and hAQP5, have a very high average GC content while some of the low producers, like hAQP4 and hAQP9, have a lower GC content resembling that of *P. pastoris*. Consequently, the reasons behind high production yields cannot simply be explained by the GC content of a certain gene. Rather, the explanation lies in the combination of different, possibly unknown, factors. However, when applicable, combining multiple strategies, such as alteration of the topology maturation and codon optimization, it is likely to be successful in the production experiment.

In conclusion, taking advantage of the 13 hAQP homologues, we have investigated and analyzed several factors of importance for a successful design of an overproduction experiment specifically for eukaryotic membrane proteins in the eukaryotic host *P. pastoris* where the transformation method, the effects of specific mutations, the protein stability, the sequence flanking the initiator methionine, the fusion partners, the GC content, and the codon adaptation have been taken into account. Our established screening protocol for production has made it possible to quantitate the membrane protein yield and carefully evaluate the impact of a certain modification on the final protein yield. Thus, we could efficiently identify the clone with the highest production level for each homologue, as well as quantitate the effect of any optimization experiment with great accuracy. Taken together, our results establish the possibility to take overproduction of recombinant eukaryotic membrane proteins to a quantitative level, which is an essential step towards revealing the complex factors influencing the final yield for this group of highly interesting and relevant protein targets.

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