Further advances in the production of membrane proteins in *Pichia pastoris*

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Membrane proteins have essential cellular functions and are therefore of high interest in both academia and industry. Many efforts have been made on producing those targets in yields allowing crystallization experiments aiming for high resolution structures and mechanistic understanding. The first step of production provides a crucial barrier to overcome, but what we now see, is great progress in membrane protein structural determination in a relatively short time. Achievements on recombinant protein production have been essential for this development and the yeast *Pichia pastoris* is the most commonly used host for eukaryotic membrane proteins. High-resolution structures nicely illustrate the successes in protein production, and this is the measure used by Ramón and Marin in their review "Advances in the production of membrane proteins in *Pichia pastoris*" from 2011. Here, additional advances on production and crystallization of eukaryotic membrane proteins are described and reflected on.

High-Resolution Structures: A Measure of Successful Overproduction

In June 2011, the review "Advances in the production of membrane proteins in *Pichia pastoris*" by Ana Ramón and Mónica Marin was published in the *Journal of Biotechnology*.[1] This article nicely describes the use of the *P. pastoris* host system for production of membrane proteins where the number of high resolution structures achieved

(11 structures representing four protein families) is used as a measure of success. Membrane proteins provide important cellular functions like transport, signaling, sensing, and energy generation. They constitute about 30% of any proteome and their crucial functions mirror their importance as drug targets.[2] High-resolution structures provide extremely valuable insight into the molecular mechanisms of proteins but take a lot of material to pursue. Since overproduction of membrane proteins is non-trivial, the first hurdle to overcome is achieving enough material for subsequent studies. In addition, membrane proteins are hydrophobic in their nature and therefore more difficult to handle as compared with soluble proteins. These facts taken together, explain why membrane protein structures constitute a minor fraction of the protein structures reported in PDB. Despite clear difficulties, great progress in membrane protein structure determination has been achieved in recent years which have been driven by a pronounced and determined interest in their structure and function. One key stone in this success is optimization of the recombinant production of eukaryotic membrane proteins where the host *P. pastoris* plays an important role.[1,3]

Eukaryotic Membrane Proteins Can be Produced in *P. pastoris*

By following the number of membrane protein structures reported per year, it is evident that large progress has been achieved in a short time. From this survey, we can also conclude that the

majority of the new structures are results of recombinant protein production. Hence, we are no longer limited to study membrane proteins that are endogenously produced to high levels in their native membranes. In this context, yeast has shown to be a promising host system for eukaryotic membrane proteins providing the most successful recombinant system for these targets.[3] Notably, *P. pastoris* is the most common yeast host among the 383 eukaryotic membrane proteins structures reported at 4 Å or lower (http://blanco.biomol.uci.edu/mpstruc/listAll/list). The collected knowledge on the production optimization in this yeast, clearly put together by Ramón and Marin in their review, can be divided into three approaches: (1) optimization of the nucleotide sequence of the gene to be expressed, (2) co-production of assisting proteins, and (3) optimization of the growth conditions.[1]

Generic Strategies to Improve Membrane Protein Production

The production of stable membrane proteins is often referred to as the main bottleneck for characterization of their structure and function.[4] Thus, it is worth reflecting on generic strategies for production optimization as learned from *P. pastoris*. To begin with, the correlation between gene dosage and final membrane protein yield is worth some consideration, an aspect that was not reflected on in the review by Ramón and Marin. When integrated in the *P. pastoris* genome, the expression cassette can end up as multimers giving increased template levels for the gene of interest. Intuitively, one could argue that more template would give more protein. For a membrane protein, this is not necessarily true since a high gene dosage could cause an overload of the cellular machinery with consequences for the protein maturation processes.[5] From production studies of the human aquaporins, however, it is clear that the presence of multimers and the concomitant improved growth on high Zeocin concentration is also beneficial for integral membrane proteins.[6] This would indicate that a high gene dosage is not in conflict with proper folding and translocation to the membrane. Similar effects have been reported for more challenging targets like GPCRs where 15 to 25 copies of the gene had no negative effect on the expression level of the human mu-opioid receptor.[7] Moreover, for the HT_{5A} 5-hydroxytryptamin and the human β_2-adrenergic receptor, the yield of functional protein was increased up to 2-fold when the number of gene copies was increased from one to two or six.[8] Taken together, a higher gene dosage is worth aiming for when planning a production experiment of a novel membrane protein target in *P. pastoris*. Two additional characteristics of the nucleotide sequence were pointed out in the review from 2011; the consensus sequence surrounding the start ATG and the optimization of codon usage for the selected production system. Both of these can have large impact on the final protein yield and should therefore be taken into consideration, also in those cases when the codon usage is apparently similar for the host and the origin of the gene.[9] In addition, Ramón and Marin highlighted gene fusions, using for example the α-factor from *Saccharomyces cerevisiae* and the FLAG-tag, as successful strategies. Worth adding to this notion, however, is that the fusion partner should be selected among those verified for the particular system of choice.[6] Moreover, a very efficient generic way to increase the protein production level, which was totally left out by Ramón and Marin, is the introduction of specific mutants that increase the stability of the protein and/or influences the folding pathway. This approach is substantially facilitated in cases where detailed knowledge is available for the target of interest, as exemplified by human AQP4; by introduction of two to three specific mutations, this protein went from no production to enough levels of functional target for subsequent purification and crystallization trials.[9] Another related example is the C116L mutant of the β-adrenergic receptor which gives a considerable increase in yield when produced in Sf9 cells without affecting the phenotype.[10]

An interesting feature brought up in the review from 2011 is the opposite effects observed for the co-production with proteins that activates the Unfolded Protein Response (URP). Activation of UPR can have a positive influence on the functionality of the recombinant GPCR adenosine A2A when co-produced with the transcription factor Hac1p from *S. cerevisie*. On the contrary, UPR activation can also have a negative influence in those cases when growth and production induce signals triggering cell death. As a consequence, one has to consider the fact that optimal growth conditions are not always ideal for protein production, thus the optimal conditions for a certain target has to be specifically addressed.[5] In this context, growth scale, temperature, pH and optimal time point for harvest should be considered in the optimization of a specific target where reduced temperature is a generic strategy applied to difficult targets like membrane proteins. Another crucial factor with respect to the growth of *P. pastoris* is the optimal methanol induction time. This is individual for each target and should therefore be carefully evaluated.[11,12] Finally, Ramón and Marin described how additives to the growth, like ligands, DMSO, or histidine, can lead to higher yields and/or a higher fraction of functional protein and could thus be tried in parallel with the actual growth parameters.

Eight New Membrane Protein Structures at High Resolution

Noteworthy, since the review by Ramón and Marin in June 2011, eight additional membrane protein structures have been published as results from recombinant production of eurkaryotic proteins in *P. pastoris* (**Table 1**). Especially, two additional families of proteins, G-protein coupled receptors and ion-selective calcium channels are now represented among *P. pastoris* produced proteins. When comparing these new structures, there are some common themes for the design of the DNA constructs where codon optimization, elimination of N-linked glycosylation, removal of disulfide bridges, truncations of the hydrophilic termini, and fusion with GFP are commonly applied (**Table 2**). Several of these protein engineering

Table 1. The 19 high resolution structures of eukaryotic integral membrane proteins produced recombinantly in *Pichia pastoris* (http://blanco.biomol.uci.edu/mpstruc/listAll/list)

Target	Origin	Ref
G protein-coupled receptors (GPCRs)		
A$_{2A}$ adenosine receptor in complex inverse-agonist antibody	*Homo sapiens*	18
Histamine H$_1$ receptor, complexed with doxepin	*Homo sapiens*	27
Channels: potassium and sodium ion-selective		
Two-pore domain potassium channel K$_{2P}$1.1 (TWIK-1)	*Homo sapiens*	26
Two-pore domain potassium channel K$_{2P}$4.1 (TRAAK)	*Homo sapiens*	14
Kv1.2 voltage-gated potassium channel (full length)	*Rattus norvegicus*	15
Kv1.2 voltage-gated potassium channel	*Rattus norvegicus*	23
Kv1.2/Kv2.1 voltage-gated potassium channel chimera	*Rattus norvegicus*	24
Kv1.2/Kv2.1 voltage-gated potassium channel chimera	*Rattus norvegicus*	29
Kir2.2 Inward-Rectifier Potassium Channel (Complete)	*Gallus gallus*	28
Kir2.2 Inward-rectifier potassium channel in complex with PtdIns(4,5)P_2	*Gallus gallus*	17
GIRK2 (Kir3.2) G-protein-gated K$^+$ channel	*Mus musculus*	31
Channels: calcium ion-selective		
Orai calcium release-activated calcium (CRAC) channel	*Drosophila melanogaster*	21
Channels: aquaporins and glyceroporins		
AQP4 aquaporin water channel	Human	19
AQP5 aquaporin water channel (HsAQP5)	Human	20
SoPIP2;1 plant aquaporin (closed conformation)	*Spinacia oleracea*	30
Aqy1 yeast aquaporin	*Pichia pastoris*	16
Membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG)		
Leukotriene LTC$_4$ Synthase in complex with glutathione	Human	25
ATP binding cassette (ABC) transporters		
P-glycoprotein	*Mus musculus*	13
P-glycoprotein	*Caenorhabditis elegans*	22

Eight new structures (underlined) have been published since the review by Ramón and Marin published in June 2011.[1] Among those, two new protein families are included; GPCRs and ion-selective calcium channels.

Table 2. Common themes in the construct design listed for the eight structures that have been published since the review by Ramón and Marin

Target	co	ge	sbr	trunc	GFP	T4L	Ref
Human A$_{2A}$ adenosine receptor	yes	yes	-	-	-	-	18
Human histamine H$_1$ receptor	yes	yes	yes	yes	yes	yes	27
Human potassium channel K$_{2P}$1.1 (TWIK-1)	-	yes	yes	yes	yes	-	26
Human potassium channel K$_{2P}$4.1 (TRAAK)	yes	yes	-	yes	yes	-	14
Chicken Kir2.2 potassium channel	-	-	-	-	yes	-	17
Mouse GIRK2 (Kir3.2) G-protein-gated K$^+$ channel	-	-	-	yes	yes	-	31
Worm P-glycoprotein	yes	-	-	-	yes	-	22
Drosophila Orai calcium channel (CRAC)	-	-	yes	yes	yes	-	21

The following shortenings are used; co (codon optimization), ge (N-glycosylation elimination by introducing asn to gln mutations), sbr (disulfide bridge removal), trunc (truncation of hydrophilic termini), GFP (fusion with GFP), and T4L (chimera with T4 lysozyme).

approaches were discussed by Ramón and Marin but some, worth noting, is extracted knowledge from production in other systems and successfully applied to *P. pastoris*, like the chimera with the 4 lysozyme (**Table 2**). All together, the main benefits with these modifications are to improve translation of the heterologous gene[6] and reduce flexible regions, as well as to increase the crystal contacts and hydrophilic surfaces.[3] Thus, all changes are done in order to improve the stability of the target of interest since this has a major impact on both the chance that the protein is produced as well as the likely hood that the protein will form well-ordered crystals.

Future Perspectives

Strikingly, all structurally determined membrane proteins recombinantly produced in *P. pastoris* are α-helical.[13-31] To date there is no example of a β-barrel protein, which should be added to the challenges for the future. The lack of eukaryotic β-barrel structures from proteins produced in *P. pastoris* could possibly relate to the localization of these targets to the mitochondrial membrane, rather than the plasma membrane, resulting in a lower production yield. Nevertheless, GPCRs constitute the largest family of drug targets,[32] and many efforts on production optimization are found in the literature. Hence, it is worth reflecting on what we can learn from the total experience in producing these targets. To date, there are high resolution structures for 15 members of this protein family whereof all but one is a result of recombinant production (http://blanco.biomol.uci.edu/mpstruc/listAll/list). The vast majority is produced in *Spodoptera frugiperda* and many of the receptors are engineered by T4 lysozyme increasing the hydrophilic surface to improve crystal formation. Another interesting strategy is to make the target more amendable for crystallization in short chain detergents which form micelles that expose larger area available for crystal contacts. However, to avoid denaturation of the protein in these detergents,

the target can be engineered to increase its stability by introduction of thermo stable mutants.[3] Intuitively, a more stable protein is also more likely to be produced to high levels, something that was indeed observed for human AQP4,[9] making this approach even more interesting. Thermo stable mutants have been introduced in the A_{2A} adenosine receptor and the $β_1$ adrenergic receptor, respectively,[33] using alanine scanning[34,35] resulting in high-resolution structures.[36,37] A related approach, using error prone PCR combined with directed evolution, has been applied to the rat neurotensin receptor which resulted in a substantial increase in functional production in *E. coli*, higher production levels in *P. pastoris*, and HEK293 cells, as well as enhanced stability during solubilization and purification.[38] Independent of the method used for creating the library of stable mutants, efficient screening protocols are necessary to assess the useful protein products. Such protocols have been presented for *S. cerevisiae*[39] as well as for *P. pastoris*,[40,41] which provides platforms for screening of membrane localized, and most likely also properly folded, proteins. If the efficient production screen can also be combined with assays on the protein function in the same system, the concept would be even more attractive. For the GPCR targets, the binding characteristics are helpful, but transport processes can also be assayed directly in yeast cells,[16]

making *P. pastoris* a useful system for both production and functional screening. However, as compared with *S. cerevisiae, P. pastoris* cannot be used for functional studies by complementation since libraries of deletion strains are not available for this yeast.[42] However, the genome of *P. pastoris* is known[43] providing the opportunity to create a similar library suitable for functional studies. Having such genetic tools at hand would create even more opportunities and wider use of the *P. pastoris* host system.

Conclusion

Importantly, *P. pastoris* has shown the ability to host high levels of demanding membrane protein targets in their functional form in its membrane. Tools for gene design, production screening and functional assessment are available for this host system. Thus, *P. pastoris* provides a complete system for high-through screening of all various steps from production to function. In conclusion, this yeast provides an attractive production host since it has an extraordinarily strong promoter that can be fine-tuned, it grows to high cell densities and it is robust to work with.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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