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Oral Presentation

Design of improved membrane protein production experiments in yeast: quantitation of the host response

Nicklas Bonander[1], Kristina Hedfalk[2], Christer Larsson[2], Petter Mostad[3], Celia Chang[4], Lena Gustafsson[2] and Roslyn M Bill*[5]

Address: [1]Department of Cell and Molecular Biology/Microbiology, Göteborg University, Box 462, 405 30 Göteborg, Sweden, [2]Department of Chemistry and Bioscience/Molecular Biotechnology, Chalmers University of Technology, Box 462, 405 30 Göteborg, Sweden, [3]Department of Mathematics, Chalmers University of Technology, 41296 Göteborg, Sweden, [4]The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104, USA and [5]School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, UK

* Corresponding author

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Background

Eukaryotic membrane proteins cannot be produced in a reliable manner for structural analysis. Consequently researchers still rely on trial-and-error approaches, which most often yield insufficient amounts. This means that membrane protein production is recognized by biologists as the primary bottleneck in contemporary structural genomics programs. Here we describe a study to examine the reasons for successes and failures in recombinant membrane protein production in yeast – a eukaryotic production organism – at the level of the host cell, by systematically quantifying cultures in high-performance bioreactors under tightly-defined growth regimes.

Results

In a first step to taking a systematic, quantitative approach to membrane protein production in yeast, we went back to first principles to collect a data set for our target protein, the glycerol channel Fps1p, in high-performance bioreactors under tightly-defined growth regimes. We chose to study expressing cultures at 20, 30 and 35°C, and pH 5 or 7, with the *FPS1* gene under the control of the *TPI1* promoter. Changes in temperature clearly affected the production time-course, which had a different profile under different conditions. Protein in both the total extract and the membrane-bound fraction was predominantly produced in the glucose phase. The data highlight major differences in production throughout the growth curve under a single condition, most pronounced at 35°C, pH 5 (7-fold in the total extract and 4-fold in the membrane fraction). Comparing all tested growth conditions, the overall difference in production was 10-fold in the membrane fraction (35°C pH7 *vs*. 20°C pH5 3). Importantly, it was clear that there is no correlation between the total production yield and the yield of membrane-localized protein, most pronounced at 35°C pH7. This result should be of particular interest to those setting up high-throughput platforms, since it is clear that a 'quick and dirty' analysis of total extracts can be very misleading since it is not representative of membrane-inserted protein yields.

In order to explain our membrane protein yield data, we first quantified the corresponding transcript levels. When we performed real time quantitative PCR (Q-PCR) on *TPI1* and the plasmid-borne *FPS1* genes, it was clear that the observed yields could not be explained by variations in the *TPI1* or *FPS1* transcripts. We therefore decided to identify genes (and their corresponding gene products) that are expressed or repressed in the host cell under specific culture conditions leading to a given yield of functional membrane protein, as this should allow for a better understanding of the critical parameters involved. To our knowledge, this approach has not been used to analyze any membrane protein production experiments: only a microarray analysis of soluble LuxA production in *E. coli*

has been reported [1]. We therefore performed an analysis using yeast miniarrays to rationalize the observations that changing the culture conditions from 30°C pH5 to either 35°C pH7 or 35°C pH5 gave good yields of total Fps1 protein, but dramatically reduced membrane-bound Fps1 protein yields. In essence, we sought to understand what was failing in the conversion of improved total Fps1 protein yields to membrane-inserted protein under these conditions. 84 genes changed their expression level on going from 30°C pH 5 to 35°C pH 5 and 111 on going from 30°C pH 5 to 35°C pH7, with 39 varying on going from 30°C pH 5 to 35°C pH 5 *and* 30°C pH 5 to 35°C pH7 ($p < 0.05$). Results for the genes, *MFα1* and *HOR7* provided a useful validation of our data set, as it is has already been reported that upregulation of *MFα1* results in down-regulation of *HOR7*, a gene of unknown function [2].

Both the increase in total Fps1 protein production at 35°C pH5 compared to 30°C pH5, and the similar total Fps1 protein yield at 30°C pH5 compared to 35°C pH7 were not reflected in membrane-bound yields. This was accompanied by down-regulation of three genes involved in ribosome biogenesis (*RPP1A*, *CGR1* and *BMS1*), a membrane component of the ER protein translocation apparatus, *SEC62*, and two genes involved in vacuolar trafficking, *APM3* and *VTC3*. Warner and coworkers have found that transcription of genes encoding both ribosomal proteins and rRNAs is repressed when the secretory pathway is defective [3], providing the cells with a mechanism for cellular stress adaptation [4]. This hypothesis is also consistent with our observed down-regulation of *SEC62*, since yeast *sec62* mutants are defective in the translocation of several secretory precursor proteins into the lumen of the endoplasmic reticulum, including α-factor precursors and certain membrane proteins [5]. Interestingly, upregulation of *MFα1* also indicates that the protein secretory pathway is compromised [6,7]. Indeed, the *S. cerevisiae* α-factor prepro-peptide leader sequence has been used to confer secretory competence to proteins such as insulin [8], and constructed leaders have been developed for efficient secretory expression in *Pichia pastoris*.

Other genes related to defects in the secretory pathway could also be correlated to poor membrane-bound yields of Fps1p, including *SRP102* which encodes the β-subunit of the signal recognition particle (SRP) receptor. Srp102p has been suggested to co-ordinate the release of the signal sequence from the SRP with the presence of the translocon [9], and appears to regulate this process through a 'switch cycle' of GTP/GDP binding [10]. The remaining genes have roles in key metabolic events, but not in protein quality control; specifically not in ubiquitination.

We compared our dataset of 39 genes to those published on the response of yeast cells to environmental changes [11,12], and specifically on moving cells from 27°C to 37°C [11]. Apart from the well-documented up-regulation of *HSP82* on raising culture temperature, we found no overall correlation between the datasets, lending further support to the fact that the genomic profile we observe is linked to failure in membrane protein production, rather than merely resulting from an increase in temperature.

Conclusion

Our data show that the most rapid growth conditions of those chosen are not the optimal production conditions. Furthermore, the growth phase at which the cells are harvested is critical: we show that it is crucial to grow cells under tightly-controlled conditions and to harvest them prior to glucose exhaustion; just before the diauxic shift. The differences in membrane protein yields that we observe under different culture conditions are not reflected in corresponding changes in mRNA levels of *FPS1*, but rather can be related to the differential expression of genes involved in membrane protein secretion and yeast cellular physiology.

In the search for generic membrane protein production systems, several solutions have been proposed. High-throughput approaches – which involve trying many conditions chosen essentially at random – do not succeed in generating generic hosts since proteins that are not produced using this format are discarded without understanding why. We suggest that a 'smart-throughput' approach should enable a more focused, strategic method of recombinant eukaryotic membrane protein production, through the identification and quantitation of the parameters critical for success, thereby allowing production on a milligram scale. In this study we have specifically identified the importance of a functional secretion pathway in host cells grown under tightly-controlled conditions. This should ultimately contribute to understanding the critical parameters that define a successful membrane protein production experiment.

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