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Screening for novel genes of *Saccharomyces cerevisiae* involved in recombinant antibody production

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*Running title: Contrasting secretory capacity in yeast*

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Abstract:

Cost-effective manufacturing of biopharmaceuticals in non-mammalian hosts still requires tremendous efforts in strain development. In order to expedite identification of novel leads for strain engineering, we used a transposon-mutagenized yeast genomic DNA library to create a collection of *Saccharomyces cerevisiae* deletion strains expressing a full-length IgG antibody. Using a high-throughput screening, transformants with either significantly higher or lower IgG expression were selected. The integration site of the transposon in three of the selected strains was located by DNA sequencing. The inserted DNA lay within the *VPS30* and *TAR1* open reading frame, and upstream of the *HEM13* open reading frame. The complete coding sequence of these genes were deleted in the wild-type strain background to confirm the IgG expression phenotypes. Production of recombinant antibody was increased 2-fold in the Δ*vps30* strain, but only mildly affected secretion levels in the Δ*tar1* strain. Remarkably, expression of endogenous yeast acid phosphatase was increased 1.7- and 2.4-fold in Δ*vps30* and Δ*tar1* strains. The study confirmed the power of genome-wide high-throughput screens for strain development and highlights the importance of using the target molecule during the screening process.
Introduction

The yeast *Saccharomyces cerevisiae* has been widely used as the expression host for the production of heterologous proteins mostly for industrial applications but also for production of biopharmaceuticals. Although the yeast has proven its value for large-scale industrial production processes, for many commercially interesting proteins such as biopharmaceuticals the production titers are too low to compete with existing mammalian cell based production platforms. Most notably, this gap can be seen in the production of recombinant full-length antibodies.

Reduced production rates can be the consequence of shortcomings in various processes including protein folding, N-glycosylation, trafficking steps such as ER to Golgi transport, Golgi to plasma membrane transport, vacuolar sorting, or secretory vesicle fusion to the plasma membrane, and missorting, or proteolytic degradation (Idiris *et al.* 2010). Up to date, only few systematic global surveys have been pursued to identify and overcome the rate limiting steps in protein production in fungal hosts (Graf *et al.* 2009).

These shortcomings can be tackled from two sides, by modifying either the target protein through protein engineering, or by engineering of the expression host (Hou *et al.* 2012; Kazemi Seresht *et al.* 2013; Wu *et al.* 2013; Xu *et al.* 2014; de Ruijter, Koskela and Frey 2016). Whereas the first approach is applicable for industrial enzymes, protein engineering is no option for the production of therapeutic proteins as the protein sequence defines their structure and specificity. However, the second strategy, to tailor the expression host, can be applied more widely. To optimize the yeast as a heterologous protein production cell factory, one of the current focal points in research is the adaptation of the secretory pathway for high-level protein expression, either through overexpression of genes promoting secretion efficiency, or through deletion of genes with an inhibitory role. In both cases, rational or non-targeted approaches can be used.

In the rationally designed approach, either prior knowledge of the bottlenecks and the genes causing them is required, or a sound hypothesis of the possible limiting steps has to be available. Recent examples include the overexpression of the yeast peptidyl-prolyl-isomerase CPR5 in the ER to aid folding (de Ruijter, Koskela and Frey 2016), the overexpression of SSO1, SLY1 and *SEC1* genes involved in vesicular transport steps (Hou *et al.* 2012; Xu *et al.* 2014), and the deletion of selected proteases to diminish proteolytic degradation or deletion of vacuolar sorting
receptors like VPS10 to prevent missorting of proteins to the vacuole for degradation (Wu et al. 2013; Xu et al. 2014). And even though the targeted approach has been successfully used to improve the secretion of various heterologous proteins, like cellobiohydrolase (Van Zyl, Den Haan and Van Zyl 2014; Xu et al. 2014), amylases (Liu et al. 2014; Rodríguez-Limas, Tannenbaum and Tyo 2015), and single chain and full length antibodies (Xu et al. 2005; Rakestraw et al. 2009), its applications are limited.

In contrast, in the non-targeted approach, libraries of yeast strains are screened for clones that show an improved production of the target protein, after which the responsible genetic element is analyzed. Examples include the screening of cDNA overexpression libraries (Wentz and Shusta 2007), deletion strain libraries, and random mutagenized strains (Davydenko et al. 2004; Liu et al. 2014) for improved production of the target protein. The value of these approaches lies in the opportunity to find new, unexpected targets for cellular engineering. This can include uncharacterized genes, genes with a function not directly linked to the secretory pathway, and other genes providing an accessory function. It’s noteworthy to keep in mind that only around 80 % of all 6604 ORFs of Saccharomyces cerevisiae are characterized, and still close to 20 % are without any known function or a dubious (Peña-Castillo and Hughes 2007).

Often, the positive results obtained in the rationale and non-targeted approaches are highly specific for the used target protein, and so far it proved difficult to find genes that stimulate heterologous protein secretion in a general way. Despite this strong connection between successful genetic modification of the strain and the protein to be overexpressed, a large fraction of the available screening research is done using an artificial product as model protein, instead of using the protein of interest. Most convenient for screening setups are readily measurable proteins such as fluorescent proteins like GFP or luciferase (Kanjou et al. 2007), or conveniently measurable enzymes like β-galactosidase or acid phosphatase (Wingfield and Dickinson 1992). Additionally, fusion proteins have been studied in order to ease the measurement of the expressed protein of interest, for example in the use of yeast surface display systems (Sun et al. 2014). Although in some cases there appears to be a good correlation between surface display of fusion proteins and the secretion levels of the native proteins, the structure of the protein that targets the fusion construct to the membrane can be of influence. It can for example influence the folding or activity of the target protein, so that the modifications of a strain with a high surface
display are not necessarily beneficial for the native structure of the target protein. Overall, these methods need a second round of confirmation to verify the suitability in a real production strain.

The ease of genetic manipulation of microbial host systems, and especially of *S. cerevisiae*, permits to straightforwardly screen genome-wide libraries for proteins affecting secretory capacity. In this study, we present the screening of a randomly generated deletion strain collection created using a transposon-inactivated yeast genomic DNA library for the identification of strains with altered full-length human IgG antibody production. Interesting clones were contrasted for IgG and acid phosphatase secretion, after which the genetic disruption caused by the transposon is determined. Finally, targeted deletion strains are made of two of these clones to confirm the expression phenotypes.

**Materials and Methods**

All used FastDigest - restriction enzymes were obtained from Thermo Fisher Scientific (Waltham, MA, USA). All media components and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Yeast nitrogen base without amino acids (YNB) was obtained from BD (Vantaa, Finland).

**Strains and plasmids**

*S. cerevisiae* strain W303α (*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) was used for all experiments. The transposon mutagenized yeast genomic DNA library has been kindly provided by Anuj Kumar (Kumar *et al.* 2004). The human IgG expressing plasmids pAX550 and pEK5, and yeast acid phosphatase (AP) expressing plasmids pAX466 and pAX469 have been described elsewhere (Parsaie Nasab *et al.* 2013; de Ruijter and Frey 2015; Frey and Aebi 2015). All plasmids are derived from either pRS415 or pRS416 plasmid series and contain CEN/ARS sequences and either *LEU2* or *URA3* selection markers (Mumberg, Müller and Funk 1995). Expression of IgG and AP are under control of the galactose inducible *GAL1* promoter.

Directed gene deletion strains were constructed using the method from Hegemann and Heick (Hegemann and Heick 2011). The target gene loci were deleted by replacement with a PCR
product containing the KanMX4 cassette and flanking target gene coding sequences. Plasmid pRS305K (Taxis and Knop 2006) was used as template for amplification of kanamycin resistance (KanMX4) marker cassettes. After transformation, cells were grown on medium supplemented with 200 µg/mL G418. Deletions were confirmed using colony PCR. A genetic cassette containing a GFP gene under control of the UPR control elements (UPRE-GFP) was introduced into TRP1 locus of W303α and VPS30 deletion strain after linearization of plasmid pDEP107 (Pincus et al. 2010). The lithium acetate method was used for all transformation of yeast cells (Gietz and Schiestl 2007).

Library amplification and yeast transformation
The Tn7 mutagenized yeast insertional library was propagated in Escherichia coli TOP10 as described previously (Kumar et al. 2004). 100 ng of DNA pool was used to transform E. coli using electroporation. Transformants were selected on solid LB medium (5 g/l Bacto yeast extract, 10 g/l Bacto tryptone and 5 g/l NaCl) supplemented with 1.5 g/l agarose, tetracycline (3 µg/ml) and kanamycin (40 µg/ml) on plates 14 cm in diameter. Approximately 10,000 transformants were obtained per pool following overnight growth at 37°C. Transformants were washed from selective agar plates using 6 ml LB medium preparing a homogenous suspension. An aliquot of this suspension was diluted into 45 ml LB medium supplemented with tetracycline (3 µg/ml) and kanamycin (40 µg/ml) to yield a culture of nearly saturated cell density. The culture was incubated at 37 °C with aeration for 2-3 hours. Plasmid DNA was isolated using Qiagen Midi plasmid isolation kit (Qiagen, Germany).

1 µg of the purified DNA library was digested with NotI and the released transposon mutagenized yeast genomic DNA was isolated after separation of DNA on 1% agarose gel using the NucleoSpin® Gel and PCR Clean up kit (Macherey-Nagel, Switzerland).

Competent yeast W303α cells carrying pAX550 were transformed with 500 ng of linearized DNA using the lithium acetate method. Transformants were plated on square plates with agar-solidified synthetic dropout medium lacking uracil and leucine (SD-Ura-Leu) and supplemented
with 2% glucose and incubated at 30 °C for 48 hours. The amount of cells spread per plates was adjusted in order to obtain approximately 500 colonies per plate.

**Primary screening**

Colonies were picked from rectangular SD-Ura-Leu plates using a HAMILTON Star line liquid handling station. 96-well deep well plates (VWR, Finland) were filled, for inoculation with single colonies, with 1 ml of SD-Ura-Leu and supplemented with 2% raffinose, 20 mM sodium phosphate buffer, pH 6.5 and 50 µg/ml BSA. Plates were incubated at 30 °C and 250 rpm for 24 hours. 50 µl of saturated precultures were transferred into 950 µl fresh media. Two rounds of preculturing were performed to even out the cell amounts and growth phase of the cells. After the preculturing procedure, individual cultures were spotted onto solid SD-Ura-Leu square plate and plates were grown for 72 hours at 30 ºC. These plates served as master clone banks.

Expression cultures were inoculated with 50 µl of the precultures, grown for 5.5 hours at 30 ºC, 250 rpm and IgG expression was induced by addition of galactose to a final concentration of 0.5%. For expression, the plates were incubated at 20 ºC for 24 hours.

Samples for IgG determination were collected after 24 hours of growth. Cultures were mixed with 50 µl of 20x PBT solution (1x PBT (PBS (135 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄, 1.75 mM KH₂PO₄) containing 0.5% Tween-20)), culture supernatants were cleared by centrifugation of plates at 5,000 g for 10 minutes, 4 ºC and 3 aliquots of 300 µl cleared culture supernatant were transferred to fresh 96-well plates and stored at -20 ºC until analysis.

**Secondary screening**

Strains A, B and C were selected for the secondary screening. In addition, four random colonies were picked from the master plates and used as controls. Lead and control strains were restreaked several times on non-selective media to remove plasmids. Plasmid loss was verified by absence of growth on SD-Leu plates.

Growth of strains was analyzed using Bioscreen analyzer. Precultures of strains were grown in 3 ml of YPD medium at 30 ºC, 250 rpm. Precultures were diluted to A₆₀₀ = 0.2 and allowed to grow to A₆₀₀ =1. These exponentially growing cultures were diluted to A₆₀₀ = 0.1. 200 µl of the diluted cultures were transferred to the multi-well plates. Plates were grown for 24 hours at
30 °C with constant shaking, $A_{600}$ was monitored every 30 minutes. Eight replicate cultures were grown per strain.

For confirmation of screening results, the empty strains were transformed with plasmid pAX466 and pAX550 as described above and transformants were selected on SD-Leu plates. Precultures were grown in 4 ml media at 30 °C, 250 rpm for 16 hours. Cells were collected and cell density was adjusted to $A_{600} = 5$. Deep-well plates containing 0.9 ml of media were inoculated with 0.1 ml of preculture reaching at starting $A_{600}$ of 0.5. Deep well plates were grown for 3 hours at 30 °C and 250 rpm. Temperature was adjusted to 20 °C and 30 °C and expression of proteins was induced by addition of galactose to final concentrations of 0.5% and 2.0%, respectively. 24 hours after induction, samples for IgG determination were cleared by centrifugation and the supernatant was adjusted to 1 x PBT and stored at -20°C until analysis. Samples for acid phosphatase activity measurements were immediately processed and analyzed.

The strain harbouring the transposon in the regulatory region of hem13 gene and transformed with plasmid pAX550 was grown in the presence of hemin. A 4 mM hemin stock solution was prepared in 100 mM NaOH. Serial dilutions of media containing 50 to 0.11 mg/l of hemin were prepared. 3 replicate culture per hemin concentrations were prepared and cells were grown as above. IgG titers were determined after 24 hours of growth.

**Identification of integration site using vectorette PCR**

Genomic DNA was isolated from clones using Qiagen Genomic DNA isolation kit. 1-2 μg of yeast genomic DNA was digested with FastDigest AluI in a total volume of 20μl according to manufacturers’ instructions. Primers ABP2 (5’-GACTCTCCCTTCTCGTAACCGTTCGTACGAGAATCGCTGTCCTCTCCTTC-3’) and ABP3 (5’-AAGGAGAGGACGCTGTCTCGAAGGTAAAGGAACGGACGAGAAGGGAGAG-3’) were annealed to each other to form the adaptor anchors by mixing 1 pmole of each primer in 200 μl of annealing buffer containing 10 mM Tris, 10 mM MgCl2, 50 mM NaCl. The primer mixture was heated for 5 minutes at 95 °C and allowed to slowly cool to 37 °C. 1 μl of the annealed primers were ligated to 20 μl of restricted DNA. The ligation reaction was allowed to
proceed for 2 hours at 22 °C. The DNA region between the inserted transposon and annealed primers were amplified using primer pair UV (5’-
CGAATCGTAACGTTCGTACGAGAATCGCT-3’) and M13-47 (5’-
CGCCAGGGTTTTCCAGTCACGAC-3’) using 1 µl from the ligation mixture as template.

PCR program consisted of one cycle of 2 minutes at 92 °C, followed by 35 cycles of 20 seconds at 92 °C, 30 seconds at 67 °C and 90 seconds at 72 °C with a final extension of 150 seconds at 72 °C. Each PCR product was gel purified using standard protocols into a final volume of 30 µl water. 50 ng of the purified product was used for one sequencing reaction from SEQCRR primer (5’-CGACGGGATCCAACGC-3’).

Characterization of TAR1 and VPS30 deletion strains

The Δtar1, Δvps30 and the wild-type strain were transformed with plasmids pEK5 and pAX469. For expression in 96 deep well plates, 6 to 8 different transformants were inoculated in triplicates into 1 ml of SD-Ura, supplemented with 2% raffinose, 20 mM sodium phosphate buffer, pH 6.5, and 50 µg/ml BSA, and grown for 21 hours at 30 °C, 250 rpm. The culture was diluted 1:4 into fresh media in a new 96 deep-well plate and grown at 30 °C, 250 rpm for 5.5 hours before protein expression was induced by addition of 0.5% galactose. For the shake flask expression, 6 clones of the preculture plates of each strain were used to inoculate 10 ml liquid cultures in 100 ml shake flasks at a starting A600=0.2 and were grown at 30 °C, 250 rpm for 5.5 hours before protein expression was induced by adding 0.5% galactose. For all experiments, protein expression was continued for 24 hours after galactose addition, followed by analysis of AP activity or IgG titers as described above.

Analytical procedures

The enzyme linked immunoassay (ELISA) for determination of IgG titers was run on a HAMILTON Star line liquid handling station as described before (de Ruijter and Frey 2015). Capture (anti-human IgG, Fc specific, I2136) and detection (anti-human IgG, Fc specific, HRP conjugate, A0170) antibodies were obtained from Sigma-Aldrich, the human IgG1 standard (#400120) was obtained from Calbiochem (Espoo, Finland).
AP activity was measured from cleared culture supernatant using an endpoint method as described before (Frey and Aebi 2015). Absorbance was read at 405 nm using a BioTek Synergy 2 spectrophotometer. Data evaluation was done with Gen5 software (BioTek).

Reporter strains were cotransformed with IgG expression plasmids (pAX550 and pEK5) and an empty plasmid with the complementary marker (pRS415 and pRS416) enabling growth in the identical medium. For UPR induction measurements strains harbouring the UPRE-GFP construct and the IgG expression plasmids were grown overnight and diluted to an $A_{600} = 0.1$ in fresh media. 100 µl of cell suspension was transferred to a well of a transparent round-bottom 96 well plate. $A_{600}$ and GFP fluorescence levels were measured using a Cytation 3 Microplate spectrophotometer. DTT was added to a final concentration of 5 mM. Induction of IgG expression was achieved by addition of 5 µl of 10% galactose solution to each well.

**Results and Discussion**

*Primary screening*

In order to assess the influence of technical effects on the variability of the screening procedure, we conducted experiments using the screening workflow, but, instead of using different transformants, 72 replicates of the wild-type strain transformed with the IgG expression plasmid were inoculated into deep-well plates. The average IgG titer in the replicate culture was $34.8 \pm 7.50$ µg/l after 24 hours of expression and most of the standard deviation was due to two clones deviating more than average from the mean. The calculated z-score over the plate was $3.17 \times 10^{-5}$. Therefore, we considered that the variations due to sample handling and slightly differing growth conditions were small enough for the screen.

For the initial screen we picked around 900 uracil auxotrophic colonies, which we obtained after transformation of the wild-type strain harboring the IgG expression plasmid with the transposon mutagenized yeast genomic DNA library. After colony picking, two rounds of preculturing were performed in order to synchronize growth phase and cell densities.

After 24 hours of expression, the IgG titers were determined from the cleared culture supernatants using an ELISA assay. In order to compare the results of the strains grown on
different plates z-scores were calculated from the IgG titers (Figure 1A). We defined a z-score of 2.0 as criteria for inclusion of hits into confirmatory tests. Both transformants with an increased and with a decreased IgG expression were included in the confirmation tests. In addition, randomly selected transformants were included. The transformants were grown as above and IgG titers were determined (Figure 1B). After the confirmation of the primary screening results, which included 80 transformants, three transformants were selected, designated as strains A, B, and C, and these strains were used for further experiments. Transformants A and B displayed improved production of IgG reaching titers of 101 µg/l and 122 µg/l, respectively, and, in contrast, expression of IgG in strain C was very low.

Initial characterization of strains

For the initial characterization, the previously selected transformants A, B, and C, and 4 randomly picked strains without a clear IgG expression phenotype in the primary screen, were selected as controls. After ensuring loss of the original expression plasmids, the strains were assessed for growth phenotypes through the recording of growth for 24 hours (Figure 2). As can be seen, only transformant A showed a significant lower growth rate.

Next, all 7 strains were transformed with either the IgG expression plasmid, or with the identical plasmid but encoding the yeast endogenous acid phosphatase (AP). The transformants were tested in the similar growth format as in the primary screening and expression of the proteins was studied with two different concentrations of the inducer galactose at 20 and 30 ºC. Samples were taken 24 hours after induction and IgG titers and acid phosphatase activity were determined (Figure 3 and 4, respectively). Absence of IgG production of transformant C was confirmed under all tested conditions. However, although strongly reduced, AP production in transformant C was still substantial, indicating that the expression of the endogenous protein was less affected in this strain. The original phenotype of transformant A and B was most strongly conserved when the IgG expression was induced with 0.5 % galactose and cells were grown at 20 ºC (Figure 3A), i.e. the experimental conditions used in the initial identification. Generally, their positive effects were lower at 30 ºC. Overall, the secondary screening could confirm the phenotypes of transformants A, B and C.

Identification of disrupted genes
Sequencing of the produced fragments from the selected clones through vectorette PCR revealed the integration sites as shown in Figure 5. The site of integration in transformant A was located in the ORF of the \textit{VPS30} gene, in transformant B it was located in the ORF of the \textit{TAR1} gene, and in strain C it was located just upstream of the \textit{HEM13} ORF. A summary of the functions of the disrupted genes is shown in Table 1. Whereas the biological functions of Vps30p and Hem13p are well established, the role of Tar1p is more elusive. The \textit{TAR1} gene is located on the anti-sense strand of the multi-copy 25S rRNA gene and the protein product localizes to the mitochondria. The \textit{TAR1} gene is conserved among hemiascomycetous species (Coelho \textit{et al.} 2002; Galopier and Hermann-Le Denmat 2011).

\textit{Verification of phenotypes using defined deletions of target genes:}

For verification of the phenotypes of the identified transformants A, B, and C, yeast strains with the complete deletion of the open reading frames of \textit{TAR1}, \textit{VPS30}, and \textit{HEM13} genes were prepared. However, transformations of four commonly used laboratory strains (W303, SS328, CEN.PK113-7D and BJ3500) with the deletion cassette for the \textit{HEM13} gene did not result in viable colonies. It has been shown previously that \textit{Δhem13} strains display very slow growth at all temperatures or are not viable at all (Giaever \textit{et al.} 2002; Ben-Aroya \textit{et al.} 2008). As mutations and partial deletion have shown to be viable and show less severe growth defects, this indicates that the integration of the transposon upstream of the coding region of the gene led only to a partial inactivation of the \textit{HEM13} gene (Zagorec \textit{et al.} 1988).

Therefore, we examined whether the putatively reduced heme biosynthesis in strain C could be restored by supplementation of exogenous heme to the culture medium, which in turn could rescue IgG expression in this strain. Surprisingly, when complementing the media of the initial strain C with up to 50 mg/l of hemin, the observed IgG expression defect was not restored and secreted IgG titers were still very low (data not shown). However, a 60% increase in final cell density was observed. Normally, heme biosynthesis is under tight control of the transcription factor Rox1p, which prevents cellular heme accumulation via repression of \textit{HEM13}. Rox1p itself is controlled by Hap1p activity, which in turn is activated by high heme levels (Martínez \textit{et al.} 2016). Interestingly, overexpression of \textit{HAP1} gene has recently been shown to have a profound
effect on growth and to some extent protein expression (Martínez et al. 2015) putatively by
induction of oxidative stress response and expression of enzymes involved in respiration. Thus,
the observed growth effect might be related to enhanced activation of Hap1p resulting from
heme supplementation, but the connection between HEM13 and IgG expression remains obscure.

The Δvps30 and Δtar1 yeast strains were compared with the wild-type strain for their secretion
capacity of acid phosphatase and IgG in 96-deep-well plate and shake flask format (Figure 6). As
can be seen in the figure, the data follows the same trends for both plate- and shake flask
cultivations. For the IgG production (Figure 6A), there is a clear increase in secretion in the
Δvps30 strain, with an on average 100 % increase in final IgG titers. In contrast, the Δtar1 strain
only produced slightly more in shake flasks, but not in the plate cultivations. For the AP
production (Figure 6B), both deletion strains showed more enzyme activity in the supernatant
than was found in supernatants of the control strain. Remarkably, in the expression of the
endogenous AP the Δtar1 strain even produced significantly more than the Δvps30 strain,
reaching final yields 2.4- and 1.7-fold higher than the wild-type strain in shake flask and deep-
well plate format, respectively. This difference highlights again the importance of contrasting the
secretion levels of different types of proteins to get a more general idea of the effects of the
mutation.

From these two deletion strains, part of the phenotypes can be explained by the nature of the
deleted genes. The gene VPS30 encodes a protein of the vacuolar protein sorting receptor family.
The gene has been shown to be involved in the sorting of proteins from the Golgi apparatus to
the vacuole, as mutations in or deletions of the gene led to missorting and secretion of the
vacuolar protein carboxypeptidase Y (CPY) (Seaman et al. 1997). Besides the effect of deleting
the VPS30 gene on protein sorting, its deletion was also reported to lead to a low constitutive
activation of the cellular unfolded protein response in unstressed conditions. However, its
deletion did not further accentuate the UPR levels in tunicamycin stressed cells compared to
wild-type controls (Kruse, Brodsky and McCracken 2006). This preactivation of the cellular
UPR might help to prime cells for the following unfolded protein stress imposed by the
overexpression of the antibody. Therefore, we created a wildtype and a Δvps30 strain where a
genetic construct containing four repeats of the UPR transcriptional control elements fused to a
GPF gene were integrated as a single copy into the genome of the strains (Pincus et al. 2010).
The measured basal fluorescence levels in both strains was similar, indicating comparable levels of UPR activation under unstressed conditions of wild-type and Δvps30 strains. In order to verify functionality of the reporter, we tested its response to induction of the UPR by addition of 5 mM DTT three hours after initiation of the cultivation. This resulted in GFP expression in both strains, however, the GFP signal in the Δvps30 background reached lower levels than in the wild type background (Figure 7 A). Next, two different IgG expression vectors were introduced into the two UPR-GFP reporter strains, and the fluorescence signals were recorded for 40 hours. The GFP signal increased strongly in the wild-type strain, but only after a lag-phase it also started to increase in the Δvps30 background and reached again lower levels (Figure 7 B). However, parallel cultivations of strains reproduced the previously observed expression phenotypes (Figure 7 C). In contrast to what was reported before, we could not detect any preactivation of the UPR in our assay. This could be due to genetic differences in the strain background or to the nature of the used reporter construct, using GFP as opposed to the previously used beta-galactosidase, and using a single integrated copy versus the previously used high-copy number plasmid for expression of the reporter. Overall, this data indicate that the improved IgG secretion observed in the Δvps30 strain is solely due to its role in protein trafficking and not due to its previously reported effect of pre-activating the UPR.

Additionally, it has been shown that also the ERAD target A1PiZ (the Z variant of the human α1-proteinase inhibitor), which upon strong overexpression was evading ERAD and leaving the ER, was escaping vacuolar degradation when the VPS30 gene is inactivated (Kruse, Brodsky and McCracken 2006). In this case it had been suggested that the vacuolar degradation serves as a rescue system when ERAD was overloaded. In murine hybridoma and B cells, IgG molecules are a target for ERAD and also in S. cerevisiae the IgG secretion levels are influenced by ERAD activity (Lee et al. 2012; de Ruijter and Frey 2015). Although also more AP was secreted in the Δvps30 strain, the IgG molecules seemed to be more sensitive to missorting and degradation in cells that do have a normal vacuolar sorting. The growth defect observed in the initial characterization of transformant A with the inactivated VPS30 gene (Figure 2) might be explained by the observation that deletion of several autophagy related genes among which is VPS30 that is also annotated as ATG6 negatively affects mitochondria maintenance and thus can affect respiratory growth (Zhang et al. 2007).
Contrastingly, the underlying mechanism that improves protein secretion in the case of the \textit{TAR1} deletion is unclear. The gene is located on the anti-sense strand of the multi-copy 25S rRNA gene and the protein product localizes to the mitochondria (Coelho et al. 2002; Galopier and Hermann-Le Denmat 2011). To date, little is known about the exact function of the protein, other than that it is involved in maintaining the cells oxidative phosphorylation capacity under respiratory conditions (Bonawitz \textit{et al.} 2008). To the best of our knowledge, the function of the \textit{TAR1} gene has so far not been connected with protein secretion.

Overall, the expression phenotypes of the \textit{Δvps30} and \textit{Δtar1} deletion strains were more prominent in the final confirmatory experiments, than during the screen. The reason for this could be that in the former experiments the deletion strains have been compared with their true wild-type strain background (W303α). In contrast, the four strains selected from the primary screening for use as control strains in the secondary screening, contained themselves as well the transposon insertions. And even though they exhibited no clear IgG expression phenotype, it is still possible that they were slightly more efficient secretors than the wild-type strain background and thereby influenced the apparent expression phenotype of the strains selected for characterization.

For the characterization of the \textit{Δvps30} and \textit{Δtar1} deletion strains we used deep-well plates and shake flasks for cultivation. As the cultivation conditions between deep-well plate and shake flasks are different it is important to consider if the deletion phenotypes are preserved in the different growth formats. In general the relative increase of both deletion strains compared to the wild-type is comparable in both expression formats, indicating that the deletions are also relevant in a slightly larger production scale. Overall, the specific IgG titer and specific AP activity was significantly higher for all the strains in the shake flask than in the deep well plate cultivations.

In this study we have established a high-throughput method for screening a randomly generated yeast deletion strain library. Our results confirmed that by using a screening set-up and the protein of interest as the primary readout of the screen, random deletion libraries can be screened and the mutations can be efficiently identified using DNA sequencing. This indicates that the approach could be successfully extended to a larger scale and used identify novel secretion-
related genes. Overall, our data demonstrate that deletion of $VPS30$ improves the secretion of IgG, while deletion of $TAR1$ improves the secretion of the IgG and AP.

Author contributions

Jorg de Ruijter created the high-throughput colony-picking method, created the clean deletion strains and verified their expression phenotype, and helped drafting the manuscript. German Jurgens created the yeast deletion library, carried out the primary screening experiments and verified the transposon insertion sites. Alexander Frey conceived the study, carried out the secondary screening experiments, the experiments with UPR reporter and the heme complementation study and drafted the manuscript.

Conflict of interests

The authors declare that they have no conflict of interests.
References


Kanjou N, Nagao A, Ohmiya Y et al. Yeast mutant with efficient secretion identified by a novel


de Ruijter JC, Koskela E V., Frey AD. Enhancing antibody folding and secretion by tailoring the *Saccharomyces cerevisiae* endoplasmic reticulum. *Microb Cell Fact* 2016:87.


# Table 1. Genes with an effect on antibody production.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Disrupted gene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Function&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Clone A</td>
<td><em>VPS30/YPL120W</em></td>
<td>Subunit of the phosphatidylinositol 3 kinase complexes I and II, required for overflow degradation of misfolded proteins when ERAD is saturated.</td>
</tr>
<tr>
<td>Clone B</td>
<td><em>TAR1/YLR154W-C</em></td>
<td>Protein potentially involved in regulation of respiratory metabolism, located in the mitochondria</td>
</tr>
<tr>
<td>Clone C</td>
<td><em>HEM13/YDR044W</em></td>
<td>Coproporphyrinogen III oxidases, oxygen requiring enzyme that catalyzes the sixth step in the heme biosynthetic pathway</td>
</tr>
</tbody>
</table>

<sup>a</sup>Annotation from the *Saccharomyces* Genome Database