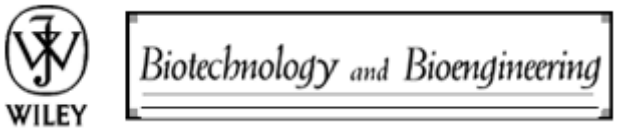

Deregulation of methanol metabolism reverts transcriptional limitations of recombinant Pichia pastoris (Komagataella spp) with multiple expression cassettes under control of the AOX1 promoter

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Deregulation of methanol metabolism reverts transcriptional limitations of recombinant *Pichia pastoris* (*Komagataella* spp) with multiple expression cassettes under control of the *AOX1* promoter

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Running title: Deregulation of methanol metabolism reverts *P. pastoris* limitations

Abstract

The methanol-regulated alcohol oxidase promoter (P_{AOX1}) of *Pichia pastoris* (syn. *Komagataella spp.*) is one of the strongest promoters for heterologous gene expression. Although increasing the gene dosage is a common strategy to improve recombinant protein productivities, *P. pastoris* strains harbouring more than two copies of a *Rhizopus oryzae* lipase gene (*ROL*) have previously shown a decrease in cell growth, lipase production and substrate consumption, as well as a significant transcriptional downregulation of methanol metabolism. This pointed to a potential titration effect of key transcriptional factors Mxr1 and Mit1 regulating methanol metabolism caused by the insertion of multiple expression vectors.

To proof this hypothesis, a set of strains carrying one and four copies of *ROL* (1C and 4C, respectively) were engineered to co-express one or two copies of *MXR1**, coding for a Mxr1 variant insensitive to repression by 14-3-3 regulatory proteins, or one copy of *MIT1*. Small scale cultures revealed that growth, Rol productivity and methanol consumption were improved in the 4C- MXR1* and MIT1 strains growing on methanol as sole carbon source, whereas only a slight increase in productivity was observed for re-engineered 1C strains. We further verified the improved performance of these strains in glycerol/methanol-limited chemostat cultures.

Keywords: *Pichia pastoris (Komagataella spp.)*, Mxr1, Mit1, *AOX1* promoter, methanol metabolism, heterologous gene dosage, recombinant protein production

Introduction

The methylotrophic yeast *Pichia pastoris* (*Komagataella spp.*) is a widely used recombinant protein host and one of the most preferred yeast system for the production of a wide range of

proteins, ranging from biopharmaceuticals and industrial enzymes to membrane proteins and complex nanostructures (Corchero et al., 2013; Bill, 2015; Byrne, 2015; Rabert et al., 2013; Spohner et al., 2015). Key features, established tools and methodologies for protein production in *P. pastoris* have been extensively reviewed (Ahmad et al., 2014; ~~Cos et al., 2006;; Gasser et al., 2013~~; ~~Vogl et al., 2013;~~ Yang and Zhang, 2018; Zahrl et al., 2017). One of the most compelling factors in this host is the presence of the strong and tightly regulated promoter of alcohol oxidase 1 (P_{AOX1}), the most frequently used option to drive the expression of foreign genes (Vogl and Glieder, 2013). Catabolite repression of P_{AOX1} and other methanol utilization (Mut) genes by glucose and glycerol, among other C-sources, has been reported since long time (Tschopp et al., 1987). However, the molecular mechanisms of P_{AOX1} transcriptional regulation have been unveiled only over the past 10 years. Lin-Cereghino et al. (2006) identified Mxr1 (*methanol expression regulator 1*), a transcription factor (TF) with a zinc finger DNA-binding domain, homologous to the TF Adr1 from *Saccharomyces cerevisiae*. Mutations in this gene resulted in a strain unable to grow in methanol and oleate and induce the transcription of alcohol oxidase 1 (*AOX1*), dihydroxyacetone synthase (*DAS*), peroxin 8 (*PEX8*) and peroxin 14 (*PEX14*). Further investigations by Kranthi et al. (2009 and 2010) revealed several Mxr1-binding sites in the promoter of *PEX8*, *AOX1* and *DAS*. Subsequently, Parua et al. (2012) characterized a 14-3-3 family protein (regulator of numerous biological processes in many eukaryotes, Fu et al., 2000) in *P. pastoris*. Furthermore, these authors described a highly conserved yeast 14-3-3 binding motif in Mxr1, revealing that the interaction between both proteins was due to the phosphorylation of Mxr1 Ser215 in a carbon-source dependent manner, leading to the repression of Mxr1-dependent genes. Interestingly, this study suggested that 14-3-3 might be functioning at a post-DNA binding step. Recently, Mit1 (*methanol-induced transcription factor*), Rop (*repressor of phosphoenolpyruvate carboxykinase*) and Trm1 have also been described as transcription factors responsible for

methanol regulation (Kumar and Rangarajan, 2012; Sahu et al., 2014; Wang et al., 2016). Wang et al. (2016) suggested a transmission of the methanol induction signal among Mxr1, Mit1 and Trm1 through a cascade, being Mxr1 the responsible for the derepression of P_{AOX1}, whereas methanol presence would induce Trm1 and, subsequently, Mit1 expression. Finally, it has been demonstrated that Rop (Kumar and Rangarajan et al., 2012) and Nrg1 (Wang et al., 2015), are repressors of methanol metabolism, competing with Mxr1 for the same binding sites in P_{AOX1}. New insights in this complex regulatory circuit can provide innovative strategies for recombinant protein production using $P_{AOX1.}$ For instance, Vogl and co-workers ~~et al. (2018)~~ have successfully overexpressed *MXR1* and *MIT1* for activation of P_{AOX1} under methanol-free carbon-limiting conditions (Vogl et al., 2018).

In this context, our recent study (Cámara et al., 2017) led to ~~supports~~ the hypothesis that P_{AOX1} faces a transcriptional limitation when ~~*P. pastoris* strains expressing~~ multiple copies of a *Rhizopus oryzae* lipase (Rol) enconding gene are expressed ~~more than one copy of a lipase of *Rhizopus oryzae* (Rol) encoding gene~~ under the control of this promoter. This also ~~P_{AOX1} leads to~~ results in a ~~transcriptional~~ limitation of ~~in~~ the methanol assimilation capacity of such strains. Because of *MXR1* is constitutively expressed at low levels (Lin-Cereghino et al., 2006), our observations suggested that the presence of multiple P_{AOX1} copies resulted in an insufficient number of Mxr1 molecules to fully induce ~~the expression~~ transcription of methanol-regulated genes in multi-copy expression strains, including the recombinant *ROL*. Coherently with this hypothesis, Takagi et al. (2009) reported an increase in recombinant protein production due to *MXR1* overexpression. In addition, *MIT1*-deficient strains have marginal *AOX1* mRNA when exposed to methanol, highlighting Mit1 as an essential TF for growth on methanol (Wang et al., 2016).

In this study, we have co-expressed a deregulated *MXR1* variant, *MXR1** (Parua et al., 2012), and *MIT1* in a series of *P. pastoris* strains harbouring one and four copies of *ROL* (named 1C

and 4C, respectively, Cámara et al., 2016). Mxr1* contains a mutation in the Ser215 that completely prevents the binding of the 14-3-3 proteins to this TF, including any background binding activity in the presence of methanol, thereby ensuring a constant action of Mxr1 independent of carbon source (Parua et al., 2012). We hypothesised that, by expressing the mutant form of MXR1 we would reinsure its full activity as transcriptional activator of PAOX1. The effect of *MXR1** and *MIT1* co-overexpression on cell growth and RoI productivity of the re-engineered 1C and 4C strains was tested in shake flasks. Furthermore, a comparative transcriptional analysis of 8 key genes involved in different relevant cellular processes between the reference strain 4C and two strains with two additional copies of Mxr1* and one additional copy of Mit1, respectively, was performed in chemostat cultures.

Methods and materials

Strains and plasmids

P. pastoris strains carrying 1 and 4 copies of the lipase of *Rhizopus oryzae* (named 1C and 4C strains, respectively) were constructed previously by Cámara et al. (2016), and together with the non-producing strain X-33 (named 0C), were used as a reference strains. These two strains were used as starting strains for this study. pGAPHA, a pGAPZA-derived plasmid in which the Zeocin resistance marker is replaced by the Hygromycin resistance one (Adelantado, 2016), was used as *MXR1** and *MIT1* expression vector.

*Construction of expression vector pGAPHA_MXR1** and *pGAPHA_MIT1*

The mutant *MXR1** gene, with the Ser215 replaced by an Ala, was amplified by PCR from the recombinant plasmid pG213 (a gift from E.T. Young, University of Washington, USA) using MXR1*_PmlI and MXR1*_KpnI primers (listed in Supplementary file I) and the Q5®Hot Start High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). After

amplification, PCR product was sequenced using MXR1*seq_1-5 primers (Supplementary file I). Afterwards, *MXR1** was cloned into the pGAPHA vector between the PmlI and KpnI sites, under the control of the *GAP* promoter (P_{GAP}). The resulting plasmid, pGAPHA_MXR1* (Figure 1A), was sequenced using the primers pGAP_fw, MXR1*_int_rev and MXR1*seq_5-6. Plasmid and PCR product sequencing was carried out by the Servei de Genòmica i Bioinformàtica of the UAB.

MIT1 gene was amplified by PCR from *P. pastoris* genomic DNA using MIT1 fw(inf) and MIT1 rev(inf) primers (Supplementary file I). pGAPHA vector was amplified using pGAPHA fw and pGAPHA rev primers (Supplementary file I). Then, pGAPHA_*MIT1* was constructed by using *Infusion cloning* (*Clontech* Laboratories, Becton Dickinson, New Jersey, USA) and sequenced using Mit1Seq1, Mit1Seq2, Mit1Seq3 and Mit1Seq4 primers (Supplementary file I).

Transformation of P. pastoris and clone selection

Competent cells were prepared following the protocol described by Cregg (2007). 100 ng of XbaI-linearized pGAPHA_MXR1* and AvrII-linearized pGAPHA_MIT1 were introduced into *P. pastoris* competent cells by electroporation, using a Gene PulserXcell™ Electroporation System (Bio-Rad, Hercules, CA, USA). Instruments settings were 1500 V, 25 µF, and 200 Ω. Subsequently, transformants were selected on Yeast Extract Peptone Dextrose (YPD) plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) containing 250 µg/mL Hygromycin (Invivogen, San Diego, CA, USA). Positive clones were confirmed by PCR (after genomic DNA extraction with Wizard® Genomic DNA Purification Kit, Promega, Madison, WI, USA), by means of the amplification of *MXR1**and *MIT1* insert using the pGAP and MXR1*_int and pGAP and MIT1_int primers, respectively (Supplementary file I).

Growth conditions

Shake flask cultures

The *P. pastoris* shake flask cultures were performed in triplicate as described in Cámara et al., 2016. Briefly, strains were first grown in 0.5 L-volume baffled Erlenmeyer flasks containing 50 mL of fresh Buffered Minimal Glycerol (BMG) medium. After 20 h, cells were harvested, and used to inoculate 0.25-L volume baffled flasks with membrane screw caps (Duran Group, Mainz, Germany) with 35 mL of Buffered Minimal Methanol (BMM) medium and further incubated for 70 h. One pulse of pure methanol to a final concentration of 0.5% v/v was added to the cultures each 24 h. All media were supplemented with Hygromycin (150 µg/mL).

Chemostat cultures

Chemostat cultures of selected strains were carried out at a working volume of 1 L in a 1.5-L vessel bioreactor (Biostat B Plus, Sartorius AG, Göttingen, Germany) as described in Cámara et al., 2016. Briefly, cells were grown under carbon-limited conditions at a dilution rate (D) of 0.1 h^{-1} by feeding a defined growth medium containing a glycerol/methanol mixture (60%/40%, w/w) as a carbon source. The bioreactor off-gasses were passed through a condenser cooled at 4°C by means of the cryostat Ecoline Star edition RE 106 (Lauda Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany), further dried with two silica gel columns and subsequently analysed with mass spectrometer Omnistar TM GSD 300 02 (Balzers Instruments, Balzers, Liechtenstein). Samples were taken after a minimum of five residence times, once the steady state conditions were reached (Jordà et al., 2013). The initial batch medium contained: 19.95 g/L glycerol, 0.9 g/L citric acid, 6.3 g/L $(NH_4)_2HPO_4$, 0.01 g/L $CaCl_2$, 0.45 g/L KCl, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 1 mL/L Biotin (0.2 g/L; Sigma, Munich, Germany), 0.3 mL antifoam (Glanapon, Bussetti & Co GmbH, Wien, Austria) and 2.3 mL/L PTM1 trace salts stock solution. Chemostat medium contained: 15 g/L glycerol, 10 g/L methanol, 0.42 g/L citric acid, 2.18 g/L $(NH_4)_2HPO_4$, 5.5 mg/L $CaCl_2 \cdot 2H_2O$, 0.85 g/L KCl,

0.32 g/L $MgSO_4 \cdot 7H_2O$, 0.5 mL Biotin (0.2 g/L), 50 µL antifoam (Glanapon) and 0.8 mL PTM1 trace salts stock solution. The pH was set to 5.0 with 25% HCl in both media. PTM1 trace salts stock solution was the same as previously described by Baumann et al. (2008).

Analytical procedures

Biomass determination

Cell biomass was monitored by measuring the optical density at 600nm (OD_{600}). For cellular dry weight, the method previously reported by Jordà et al. (2012) was used. All determinations were performed in triplicate.

Lipase activity assay

The lipolytic activity determination was performed in triplicate samples using the Lipase colorimetric assay (kit 1821792 from Roche Diagnostics), as previously described by Resina et al. (2004).

Metabolite quantification

Glycerol, methanol and other potential extracellular compounds in the shake flask and chemostat cultures were analysed by HPLC as previously reported by Cámara et al. (2016). Analyses were performed in duplicate for each independent experiment.

Flow cytometry analysis

Cell counting and viability was measured using the methods previously described by Cámara et al. (2016). To determine the cell population size, the Flow Cytometry Size Calibration Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used. Briefly, microspheres suspensions of 1 µm, 2 µm, 4 µm, 6 µm, 10 µm and 15 µm of diameter were sonicated for 5 s at 50 W with the ultrasonic processor VC-5 (Vibracell, Sonics & Materials, Newtown, CT, USA) before being subjected to analysis. After that, different thresholds were established for each range in

the analysis software to define the interval size of the processed samples. Experiments were performed using the Guava EasyCyte Mini cytometer (Millipore, Jaffrey, NH, USA), with a 488 nm Argon laser. An amount of 5,000 cells were measured per analysis at a flow rate of 0.59 mL/s. Viability assays were carried out in duplicates, whereas cell counting and cell size determination were performed in triplicate for each shake flask and chemostat culture.

Gene copy number determination by digital droplet PCR (ddPCR)

Gene dosage quantification of *ROL, MXR1* and *MIT1* was analysed by ddPCR using the method previously described (Cámara et al., 2016), using the reference gene β-actin (*ACT1*) as endogenous control for data normalization and the set of primers for *MXR1*, *MIT1, ACT1* and *ROL* genes described in Supplementary file I for DNA amplification. To verify the *ROL* and *MXR1* gene dosage stability, biomass samples of the chemostat cultures were taken after five residence times, once the steady state was achieved. Reagents for ddPCR were purchased to Bio-Rad (Hercules, CA, USA), whereas primers were synthesized by Sigma Aldrich (St Louis, MO, USA).

Measurement of transcriptional levels by ddPCR

For transcript quantification, 5 mL of collected pellets at the end of the shake flask culture were previously treated with phenol (5% v/v) and further stored at -80°C. Total RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) whereas cDNA was synthesized by using the iScript™ cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. RNA quality was tested by measuring the ratio of absorbance at 260 and 280 nm using the Nanodrop 1000 (Thermo Fisher Scientific). ddPCR reactions contained 10 µL of QX200™ ddPCR™ EvaGreen Supermix, 200 nM of forward primer, 100 nM of reverse primer, 0.4 ng of cDNA and the required amount of DNAse/RNAse-free water up to 20 µL of final volume. Reactions were incubated at 95°C for 10 min, followed by a

denaturation (94°C, 30 s) and an annealing/extension step (60.2°C, 1 min for the *KAR2* and *ROL* primers; 56.5°C for the rest) during 40 cycles. Positive droplets of each PCR reaction were normalized in relation to *ACT1* signal. Primer list is shown in Supplementary File I.

Statistics

Data are expressed as the mean ± standard deviation (SD). Statistical analyses of the data were performed using the unpaired Student's t-test using Microsoft's Excel software. A *P*-value lower than 0.05 was considered statistically significant.

Results and Discussion

Construction and characterization of Rol-producing strains co-expressing MXR1* or MIT1

In order to increase the Mxr1 levels in the Rol-producing strains, as well as ensuring a constant activation of Mxr1-dependent promoters under induction conditions, an expression vector harboring *MXR1** (encoding for the Mxr1 variant carrying the mutation in the position Ser215) was introduced in the Rol-producing reference strains 1C and 4C. Because previous studies had reported a lethal effect of *MXR1* overexpression under the control of inducible promoters (P_{AOX1} and P_{PEX8}, Lin-Cereghino et al., 2006), but permissive growth when using constitutive promoters (Takagi et al., 2012; Sahu and Rangarajan, 2015), P_{GAP} was selected as the promoter to drive *MXR1** expression. Five and eleven *MXR1** transformants were isolated for 1C and 4C strains, respectively. All 1C-MXR1* and 3 out of 11 for the 4C-MXR1* clones resulted to have the MXR1* expression cassette integrated in their genome. Intriguingly, only three clones of the 1C-MXR1*strain ($1C_1$, $1C_2$ and $1C_3$) were capable of growing in liquid media. We further determined the total *MXR1* (*MXR1*+*MXR1**) copy number for each clone by ddPCR (Figure 1B), revealing a population with one *MXR1** copy ($1C_2$-MXR1* and $1C_3$-MXR1* ~~1C_MXR1*a and 1C_MXR1*b~~ derived from the 1C strain, and $4C_1$-MXR1* and $4C_2$-MXR1*

~~4C_MXR1*a and 4C_MXR1*b~~ derived from the 4C strain), or two copies of *MXR1** (1C_1-2MXR1* and 4C_6-2MXR1*) ~~1C_2MXR1* and 4C_2MXR1*)~~, demonstrating that P_{GAP} was a suitable option to express *MXR1**. The fact that only clones with low gene dosages of *MXR1** were capable of growth in liquid media could be due to the deleterious effect of higher gene dosages of this gene, i.e. suggesting that clones that can only grow on agar plates might harbor~~ing~~ a higher number of ~~two copies of~~ *MXR1** copies ~~can only grow on agar plates~~.

Concerning *MIT1* co-overexpression, no fitness-related problems were observed in agar plates and liquid medium growth steps during the isolation of these strains, allowing to isolate a series of 1C- and 4C Rol-producing clones containing one extra copy of *MIT1*.

Overexpression of MXR1* or MIT1 increases Rol production levels and reverts detrimental effect of ROL multi-copy expression on methanol assimilation

As previously reported (Cámara et al., 2016; Cámara et al., 2017), *P. pastoris* strains carrying more than one copy of a P_{AOX1}-*ROL* expression cassette exhibit a reduced growth and methanol assimilation capacity. A first series of shake flask cultivations was carried out to test the effect of *MXR1** and *MIT1* co-overexpression on cell growth, methanol consumption and Rol production after 70 h of cultivation. As expected, biomass levels of the reference 4C-Rol-producing strain were 3-fold lower than those observed for its 1C counterpart (Figure 2A). In addition, significant levels of residual methanol were detected at the end of 4C strain culture, while no substrate accumulation was observed in the 1C strain case (Figure 2C). Moreover, while virtually all cells in the non-producer strain (0C) culture were viable (97%), a decrease of 20% in viability was observed in 1C and 4C cultures (Supplementary File II~~2~~). *ROL* overexpression further impacted cell size: whereas a slight (but significant) change in population size profile was observed between strains 0C and 1C, a remarkable increase of

larger cell fractions (6 µm and ≥10 µm) was measured for the 4C strain, compared with the other reference strains (Supplementary File II).

Co-expression of *MXR1** or *MIT1* in 1C strains had either no or slightly negative impact on cell growth compared to the reference 1C strain, also showing no residual methanol at the end of the culture. In contrast, the average biomass levels of both MXR1* and MIT1 series of 4C clones presented 1.73- and 1.75-fold increase, respectively. Consistently, these clones also showed significantly lower residual methanol levels at the end of the cultivation (2.12- and 3.48-fold reduction, respectively). Nonetheless, MXR1* strains still showed a 10-20% of non-viable cells at the end of cultivations, and this value was even higher (around 40% non-viable cells) in the case of the $4C_1$-MXR1* ~~_MXR1*a~~ strain.

As previously reported by Cámara et al. (2017), the increase in *ROL* gene dosage led to a higher lipase specific productivity in the 4C strain compared to the 1C strain. Interestingly, for all the 1C-MXR1* strains, Rol production was between two- and three-fold higher than the 1C control strain (Figure 2B). However, Rol production in 1C-MIT1 strains was comparable to the reference strain, or even slightly (not significantly) reduced (Figure 3B). Concerning the re-engineered 4C strains, 4C-MXR1 strains showed an average 1.2-fold increase and 4C-MIT1 strains a 2-fold increase. Although the average Rol production in MXR1* clones was not significantly improved, a MXR1 clone showed a 1.8-fold increase. Notably, this clone was determined to have 2 ~~extra~~ copies of the *MXR1** gene instead of one (clone $4C_6$-2MXR1*, Figure 1). As shown in Figure 2C and 3C, this increase in the lipase production was concomitant with a sharp decrease in the residual methanol in the medium, reflecting a methanol consumption rate similar to the 1C strains. In fact, strains with higher methanol consumption rate (0C and 1C) presented an average population size lower than those with reduced methanol assimilation capacities (4C and $4C_1$-MXR1*~~a~~). Notably, significant differences were also observed in terms of cell size distribution between the strains $4C_2$-

MXR1*~~b~~ and 4C$_6$-2MXR1* referred to the 4C strain control (Supplementary file II ~~Figure 2B~~). More specifically, a 20% increase was detected for the 4µm-cell population in the 4C-MXR1* strains, while the 6µm-cell fraction was reduced around 15%, altogether resulting in a cell distribution size similar to the 1C strain control, thus indicating a possible reversion of the detrimental effects of *ROL* overexpression on the methanol assimilation capacity of the 4C strain by means of the expression of *MXR1**.

Chemostat cultures reveal a shift in the ~~preference change of~~ C-source consumption pattern depending on the strain

To validate the results obtained in the shake flask cultures, the 4C reference strain, a 4C strain expressing 2 copies of MRX1* and a representative clone of a 4C strain overexpressing MIT1 (4C$_6$-2MXR1* and 4C-MIT1, respectively) were further grown in chemostat cultures at a D of 0.09 h^{-1} using a mixed glycerol:methanol feed (60/40% v/v), i.e. under conditions analogous to our previous studies (Cámara et al., 2016; Cámara et al., 2017). In terms of extracellular lipase activity, a 30% higher volumetric productivity was reached for the 4C$_6$-2MXR1* strain in comparison to 4C, corresponding to a slight but significant increase of 10% in terms of specific lipase productivity (Table I). Unexpectedly, a reduction of 13% and 28% in extracellular lipase activity levels and specific lipase productivity, respectively, was observed for the 4C-MIT1 strain compared to the reference 4C strain. This might be due to higher tendency to foaming observed for this strain under the tested chemostat conditions, since gas-liquid interphases can influence lipases adsorption and activity. Nonetheless, the cultivations confirmed the positive physiological impact of both *MXR1** and *MIT1* overexpression in the 4C strain. In particular, biomass levels were about 30% and 20% higher than the 4C strain for 4C$_6$-2MXR1* and 4C-MIT1, respectively, also reflected by an increase of 15% in the biomass/substrate yield in both cases (Table I). Under the established culture conditions, chemostats were ~~are~~ carbon limited when growing the reference strains 0C and 1C, but residual methanol was ~~is~~ observed in the

fermentation broth when growing multi-copy strains (i.e. 4C strain). Notably ~~In this experiment~~, a substantial change was observed regarding the specific C-source consumption rate ~~between~~ when comparing 4C *vs* ~~and~~ the $4C_6$-2MXR1* and 4C-MIT strains: Despite glycerol concentration in chemostat cultures of all 4C strains was below detection limit, *MXR**- and *MIT1*-overexpressing 4C strains showed significantly higher biomass yields. Consequently, specific glycerol consumption decreased about a 30%, from 1.96 mmol g^{-1} DCW h^{-1} ~~19.59~~ for 4C to 1.52 mmol g^{-1} DCW h^{-1} for the $4C_6$-2MXR1* strain and 1.48 mmol g^{-1} DCW h^{-1} for the 4C-MIT1 strain. ~~Such decrease, because despite that no glycerol accumulation was observed in the bioreactor, the increase in the biomass levels modified the assimilation rate.~~ In contrast, the specific methanol consumption rate ~~appeared to~~ increased for both modified strains, from 1.44 to 1.71 and 1.70 mmol g^{-1} DCW h^{-1}, when comparing the 4C with the $4C_6$-2MXR1* and 4C-MIT1 strain, respectively. Nevertheless, some residual methanol (3.7 g/L) was still observed in the cultivation broths ~~ure media~~ (not observed in analogous conditions for the reference strains 0C and 1C), which may pose ~~indicate~~ the possibility of further improvement of the methanol assimilation capacity~~, deserving further studies~~. Interestingly, this ~~might also indicate a~~ variation in the C-source preference of the *MXR**- and *MIT1*-overexpressing 4C strains may reflect ~~pointing out a lower metabolic constraints of in the~~ a methanol metabolism with lower constrains ~~that could be also reflected~~ at transcriptional level.

Transcriptional analysis reveals an upregulation of methanol metabolism key genes in the $4C_6$-2MXR1* and 4C-MIT1 strains

To further evaluate if the physiological changes observed in the strains $4C_6$-2MXR1* and 4C-MIT compared to the reference strain 4C (i.e. higher biomass levels, increase in the methanol specific consumption rate), corresponded to a transcriptional alteration (or deregulation) of the methanol metabolic pathway, several key genes of this route were analyzed in cells grown in

chemostat cultures (Figure 3). Specifically, transcriptional levels of *AOX1*, the gene encoding the enzyme alcohol oxidase I were 2.3-fold and 2.1-fold higher for 4C$_6$-2MXR1* and 4C-MIT1 than for the reference strain 4C. Similarly, *DAS1* and *FDH1*, encoding for key enzymes of the methanol assimilatory and dissimilatory pathways (Figure 4) were also upregulated 3 and 1.9-fold in the strain 4C$_6$-2MXR1*, and 1.75 and 1.52-fold in the 4C-MIT1 strain. This was coherent with the increased methanol assimilation capacity of these strains observed in both shake flasks and chemostat cultures. ~~In relation to~~ *ROL* transcriptional levels were increased ~~by, a~~ 5-fold and 3.2-fold ~~increase was observed with~~ in the 4C$_6$-2MXR1* strain and ~~a 3.2-fold with~~ 4C-MIT1 strain, respectively (Figure 3). Interestingly, this rise in mRNA did not result in a similar increment in extracellular lipase production for 4C$_6$-2MXR1*, where the specific lipase productivity was only a 10% higher than the reference strain for 4C$_6$-2MXR1* and a 30% for 4C-MIT1 (Table I), pointing out to a translational (Cámara et al 2017) and/or posttranslational limitation (Resina et al., 2007; Resina et al., 2009). Moreover, increased foaming occurring during 4C-MIT1 chemostats could also partially explain the lower specific product production. In addition, as our previous transcriptomic study revealed a high impact of the overexpression of *ROL* on the peroxisomal pentose phosphate regeneration pathway (PPP) and peroxisome biogenesis in *ROL* multi-copy strains (Cámara et al., 2017), the mRNA levels of fructose 1,6-bisphosphate aldolase (*FBA1-2*) and peroxine 6 (*PEX6*) were selected as markers of these pathways. In the case of *FBA1-2*, an increase of 40% and 36% was detected for 4C$_6$-2MXR1* and 4C-MIT1 strains, respectively, supporting the role of the peroxisomal PPP in methanol assimilation (Ruβmayer et al., 2015). Regarding *PEX6* (coding for an ATPase involved in the peroxisomal protein import and crucial for peroxisomal biogenesis, Saffian et al., 2012), no significant regulation was detected comparing both strains. However, to completely discard any regulation of peroxisomal biogenesis it would be interesting to further

analyze whether there were any changes in the expression of *PEX8* and *PEX14*, also coding for peroxines implied in the protein import machinery and directly regulated by *MXR1*.

Concerning the mRNA levels of native *MXR1* and *MIT1*, no changes were observed between both modified 4C strains and the reference 4C strain. Hence, the observed increases in total (i.e. native+heterologous) *MXR1* or *MIT1* expression levels can be assigned to the transcription factors encoding genes heterologously expressed under control of GAP promoter. Specifically, the $4C_6$-2MXR1* and 4C-MIT1 strains showed an increase of 55% and 50% in total *MXR1* and *MIT1* transcript levels, respectively. Therefore, the deregulation of methanol metabolism observed in these strains could be directly related to the significant increase in both transcription factors mRNA levels.

~~Remarkably, no changes in the *MXR1* mRNA levels of $4C_6$-2MXR1* were detected relative to the 4C strain.~~ The self-regulation of transcription factors has been extensively reviewed in prokaryotes and eukaryotes (MacPherson et al., 2006; Stekel and Jenkins, 2008), and even described for Trm1 (Wang et al., 2016), a TF also involved in methanol metabolism regulation. In this respect, our results ~~hypothesis~~ cannot totally exclude the possibility that ~~points out to~~ an auto regulatory feedback loop by Mxr1 to control the pool of available molecules occurs under ~~in~~ these ~~se~~ tested culture conditions. Despite this possibility, the significant upregulation detected in the methanol-related genes could be explained by the fact that, even though the transcriptional efficiency per copy (i.e. the ratio between mRNA levels and the number of copies of a certain gene) of *MXR1* decreased, the mutation introduced in a subpopulation of ~~molecules of~~ Mxr1 molecules (e.g. ensuring a total derepression of P_{AOX1}) was enough to trigger an overexpression of the~~ose~~ monitored genes. Although Parua et al. (2012) demonstrated that there was no 14-3-3 dependent inhibition of methanol utilization (Mut) genes in *P. pastoris* cultures growing on ~~with~~ glycerol or glucose as a C-source, basal phosphorylation of Mxr1 (i.e. promoting the union of 14-3-3 proteins) was~~ere~~ observed ~~in the analysis published,~~ even

in fully inducible conditions (methanol as the sole C-source). Due to the low levels of Mxr1, this basal phosphorylation might severely affect the transcriptional levels of methanol-dependent genes in the multi-copy strains. ~~Hence,~~ In this context, our results strongly support our initial hypothesis ~~points~~ that mutation in the Ser215 of Mxr1 (i.e. Mxr1*), which prevents its union to ~~of~~ 14-3-3 proteins ~~to Mxr1*~~, allows for the higher transcriptional levels of Mut genes observed in the strains expressing ~~this~~ *MXR1**, resulting in a better methanol assimilation capacity. This is further reflected in the reduced ~~Altogether, it could explain the decrease in~~ methanol residual levels observed in ~~for the~~ $4C_6$-2MXR1* chemostat cultures. ~~strain.~~

Overall, the results described above allow us to confirm the main role of Mxr1 in P_{AOX1}-driven recombinant protein production in *P. pastoris*. Due to the constitutive low expression of *MXR1* (Lin-Cereghino et al., 2008; Vogl and Glieder, 2013), a limited number of Mxr1 molecules has multiple binding sites available in several genes (*AOX1*, *DAS*, *PEX8*, *ACS1*) (Kranthi et al., 2009; Kranthi et al., 2010; Sahu and Rangarajan, 2015). In the case of the *ROL* single copy strain, this Mxr1 pool can be sufficient to accomplish an appropriate methanol assimilation concomitant with a proper recombinant protein expression (Figure 5, top). However, in the case of recombinant *ROL* multi-copy strains (i.e. with an increased number of P_{AOX1} sequences) the number of available molecules of Mxr1 might be insufficient to deal with the regulation of all the Mxr1-dependent genes and thereby negatively affecting the corresponding transcriptional levels, including *ROL* expression (Figure 5, middle). Hence, this assumption could explain the plateau-like trend observed between gene copy number and mRNA levels of recombinant genes under P_{AOX1} control in other studies (Liu et al., 2014; Sha et al., 2013; Huang et al., 2014; Yang et al, 2016). Our results strongly suggest that *MXR1** overexpression can at least partially revert Mxr1 limiting levels in *ROL* multi copy strains (Figure 5, bottom). Furthermore, the underlying mechanisms of P_{AOX1} repression in glycerol culture conditions allowed for maintaining an inducible recombinant protein system, while taking advantage of

the *MXR1** expression. Moreover, as Mit1 and Trm1 cooperate with Mxr1 in the regulation of methanol metabolism (Wang et al. (2016)) and, considering that *TRM1* is also expressed at constitutive low levels as *MXR1*, the *MXR1** overexpression effect might be further improved by enhancing the Trm1 expression levels.

Finally, as a typical Unfolded Protein Response (UPR) marker gene, *KAR2* mRNA levels were also measured. Previous transcriptomic analysis of the reference 4C strain did not show a dramatic impact of *ROL* overexpression on the folding and secretory pathways (Cámara et al., 2017). Interestingly ~~Strikingly~~, the $4C_6$-2MXR1* strain showed an upregulation of about 50% in *KAR2* transcription compared to the reference strain, whereas no significant changes were observed for the 4C-MIT1 strain ~~no significant changes were observed in *KAR2* mRNA levels~~. This might point out at a possible direct induction of UPR by *MXR1** overexpression or, indirectly, as a result of enhanced of *ROL* expression. Further analyses beyond the scope of this study would be necessary to investigate whether the folding machinery of the strain $4C_6$-2MXR1* is the limiting step in the production of a functional lipase as a result of the increased *ROL* transcriptional levels.

Conclusions

Knowledge about the underlying regulation mechanisms of the *AOX1* promoter of *P. pastoris* has increased significantly over past recent years, opening the door to develop ~~subsequent~~ engineering strategies ~~developments~~ overcoming transcriptional limitations and enhance ~~ing~~ the performance of P_{AOX1}-based expression systems.

Notably, in this study we show that overexpression of one of the major transcription factors strongly activating P_{AOX1} in *P. pastoris* (*MXR1* or *MIT1*) was sufficient to reverse the transcriptional attenuation of methanol metabolism caused by the insertion of multiple P_{AOX1}-based expression cassettes, further supporting our initial hypothesis that these transcription

factors are limiting. Furthermore, partial rewiring of P_{AOX1} transcriptional circuits by overproducing a deregulated form of Mxr1 can still preserve a basic output in terms of regulation pattern (derepression under glycerol-limiting conditions and induction by methanol) in strains harboring multiple P_{AOX1}-based expression cassettes, making these engineered strains suitable for bioreactor-scale cultivations based on mixed carbon source feeding strategies. Notably, Vogl et al. 2018 have shown that, by overexpressing transcription factors such as *MIT1* in P_{AOX1} based expression strains, it was sufficient to transform such methanol-dependent strains into a glucose/glycerol regulated system. This allowed developing fed-batch cultivation strategies for regulated, methanol-free, recombinant protein production. Our study further illustrates the potential of engineering regulatory circuits for enhanced protein production processes.

Finally, the proposed regulatory model of P_{AOX1} by Mxr1 and Mit1 in the multi-copy strains, brings light to better understand the transcriptional bottlenecks previously observed in several studies on multi-copy strains using methanol-based systems. Further quantitative physiology studies of these new strains could shed light about the global impact of these modifications in *P. pastoris* and, in particular, to novel bottlenecks that may appear downstream transcription still preventing a fully linear increase of secreted product as a function of heterologous gene dosage.

Acknowledgements

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FIGURE LEGENDS

Figure 1 Characterization of *MXR1* copy number. Determination of total *MXR1* (*MXR1*+*MXR1**) copy number by ddPCR. Because only one copy of *MXR1* is present in the genome of *P. pastoris* (De Schutter et al., 2009; Mattanovich et al., 2009), clones with two or three copies of *MXR1* consequently harbor one or two copies of *MXR1**, respectively. In bold, clone nomenclature used in the *MXR1** verification by PCR amplification. In brackets, final nomenclature based on the results of *MXR1** quantification by ddPCR.

Figure 2 Screening of *MXR1 transformants in shake flask cultures.** A) Biomass levels measured by OD_{600}. B) Specific lipase activity in shake flask cultures. C) Residual methanol quantified by HPLC at the end of the cultures. Samples were taken after 70 h of culture. Values for reference strains 1C and 4C are marked as horizontal thick black lines, whereas 1C and 4C strains expressing *MXR1** were represented as green and reddish plot boxes, respectively. Black dots inside plot boxes indicate values of individual clones of each population. Corresponding confidence intervals are shown in the Supplementary file III.

Figure 3 Screening of *MIT1* transformants in shake flask cultures. A) Biomass levels measured by OD_{600}. B) Specific lipase activity in shake flask cultures. C) Residual methanol quantified by HPLC at the end of the cultures. Samples were taken after 70 h of culture. Values for reference strains 1C and 4C are marked as horizontal thick black lines, whereas 1C and 4C strains expressing *MIT1* are represented as green and purple plot boxes, respectively. Black dots inside plot boxes indicate values of individual clones of each population. Corresponding confidence intervals are shown in the Supplementary file IV.

Figure 4 Transcriptional levels of selected genes. Comparative of mRNA levels of 4C (blue) and $4C_6$-2MXR1* (maroon) strains. Relative amounts of mRNA were calculated comparing to *ACT1* expression levels of each strain. Thicker black lines represent the routes directly

controlled by measured genes. DHA, dihydroxyacetone; E4P, erythrose-4-phosphate; HCOOH, formic acid; Form, formaldehyde; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; S1,7BP, sedoheptulose-1,7-bisphosphate; S7P, sedoheptulose-7-phosphate; GS-CH_2OH, S-(hydroxymethyl)gluthiatione, X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; Rul5P, ribulose-5-phosphate; *MXR1*,methanol expression regulator 1; *MIT1*, methanol-induced transcription factor 1; *AOX1,* alcohol oxidase 1; *DAS1*, dihydroxyacetone synthase; *FDH1,* formate dehydrogenase 1; *FBA1-2*, fructose 1,6 –bisphosphate aldolase; *ROL, Rhizopus oryzae* lipase*; KAR2*, endoplasmic reticulum chaperone BiP ~~karyogamy gene 2~~; *PEX6,* peroxisomal biogenesis factor 6.*$P<$ 0.05, **$P<$ 0.01, comparing with 4C control strain.

Figure 5 Regulatory model of *MXR1* in P_{AOX1}-driven recombinant protein production ~~**driven by P_{AOX1}**~~. Proposed regulatory model, where the presence of multiple TF binding sites contributes to a higher transcriptional levels of the gene as the number of attached TF increases, due to a synergistic stimulation of gene expression. **Top figure**: Scenario for recombinant protein production in single copy strains. Under methanol-growth conditions, the ~~number of limited~~ molecules of Mxr1, which are present in a limited number, ~~are available, which~~ are distributed among the multiple binding sites of the P_{AOX1} and P_{DAS}, as well as ~~among~~ other Mxr1-regulated promoters. Given the limited Mxr1 binding sites, methanol presence promotes high level induction of P_{AOX1}-driven recombinant expression. There may be also some basal binding of Mxr1 molecules to P_{AOX1} in the presence of methanol. **Middle figure**: Scenario for recombinant protein production in a 4C copy strain. As the number of Mxr1 binding sites increases, the number of Mxr1 molecules available per PAOX1 binding site decreases, resulting in reduced transcription levels of endogenous genes controlled by this transcription factor ~~Mxr1decreased~~. **Bottom figure**: Scenario for recombinant protein production in 4C strain expressing Mxr1*. Overexpression of MXR1* leads to an increase in the total number

of Mxr1 molecules. Moreover, replacement in the position Ser215 of Mxr1 by an Ala prevents its phosphorylation and the subsequent union of 14-3-3 proteins. Consequently, the number of occupied Mxr1 binding sites increases, with an important fraction of mutant Mxr1* molecules also being bound, thereby obtaining ~~Consequently,~~ higher transcriptional levels of Mut ~~methanol related~~ genes and *ROL* ~~are obtained~~. Number of Mxr1 sites on the P_{AOX1} is depicted according to Kranthi et al. (2009) and Kranthi et al. (2010).

Supplementary file I ~~1~~ List of primers used for cloning and droplet digital PCR. Tm, melting temperature; %GC, percentage of guanosine+cytosine.

Supplementary file II ~~2~~ Flow cytometric analysis of screening samples. A) Viability of the cells at the end of the screening. B) Cell size distribution of yeast cells.

Supplementary file III Screening of *MXR1 transformants in shake flask cultures**. A) Biomass levels measured by OD_{600}. B) Specific lipase activity in shake flask cultures. C) Residual methanol quantified by HPLC at the end of the cultures. Samples were taken after 70 h of culture. Values for reference strains 1C and 4C are marked as horizontal black lines. Black dots indicate values of individual clones of each population. Error bars show an interval with a 95% confidence level.

Supplementary file IV Screening of *MIT1* transformants in shake flask cultures. A) Biomass levels measured by OD_{600}. B) Specific lipase activity in shake flask cultures. C) Residual methanol quantified by HPLC at the end of the cultures. Samples were taken after 70 h of culture. Values for reference strains 1C and 4C are marked as horizontal black lines. Black dots inside plot boxes indicate values of individual clones of each population. Error bars show an interval with a 95% confidence level.

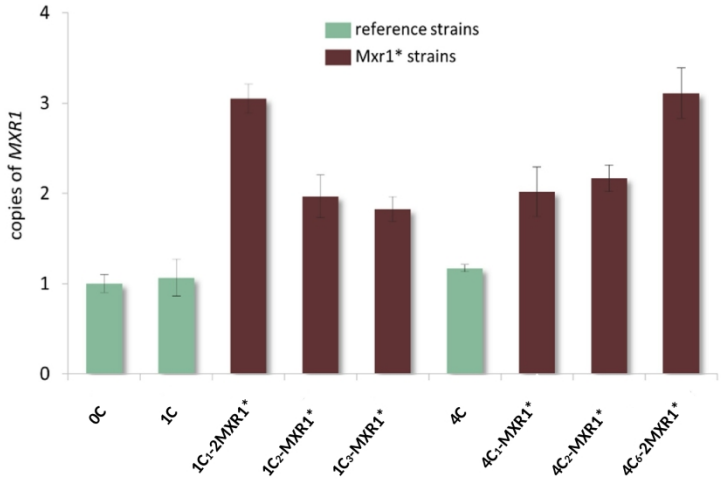

Figure 1. Characterization of *MXR1* copy number. Determination of total *MXR1* (*MXR1*+*MXR1**) copy number by ddPCR. Because only one copy of MXR1 is present in the genome of P. pastoris (De Schutter et al., 2009; Mattanovich et al., 2009), clones with two or three copies of MXR1 consequently harbor one or two copies of MXR1*, respectively. In bold, clone nomenclature used in the MXR1* verification by PCR amplification. In brackets, final nomenclature based on the results of *MXR1** quantification by ddPCR.

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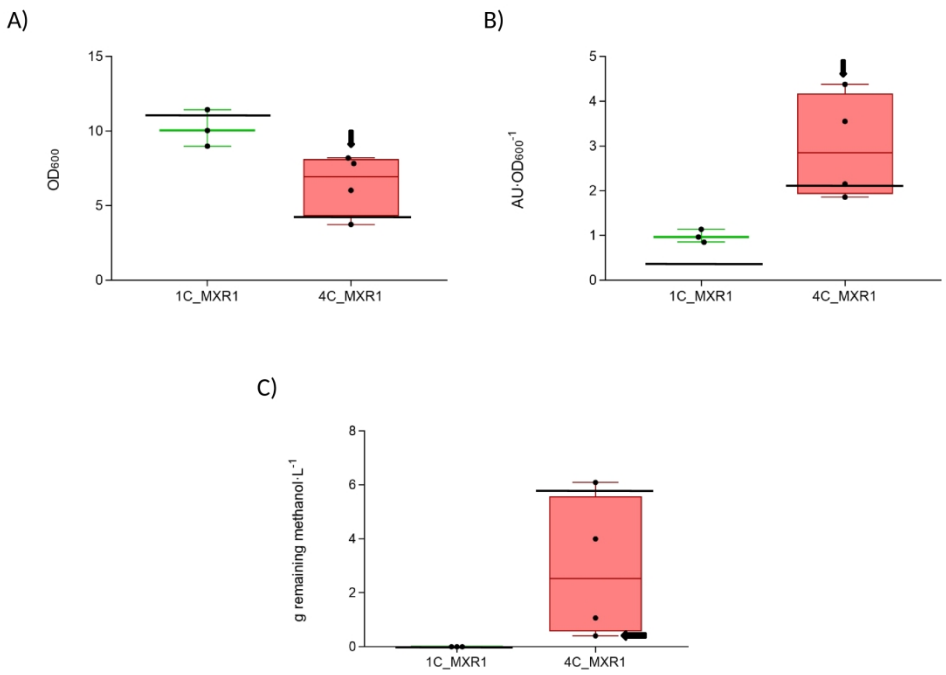

Figure 2. Screening of MXR1* transformants in shake flask cultures. A) Biomass levels measured by OD_{600}. B) Specific lipase activity in shake flask cultures. C) Residual methanol quantified by HPLC at the end of the cultures. Samples were taken after 70 h of culture. Values for reference strains 1C and 4C are marked as horizontal thick black lines, whereas 1C and 4C strains expressing *MXR1** were represented as green and reddish plot boxes, respectively. Black dots inside plot boxes indicate values of individual clones of each population. Corresponding confidence intervals are shown in the Supplementary file III.

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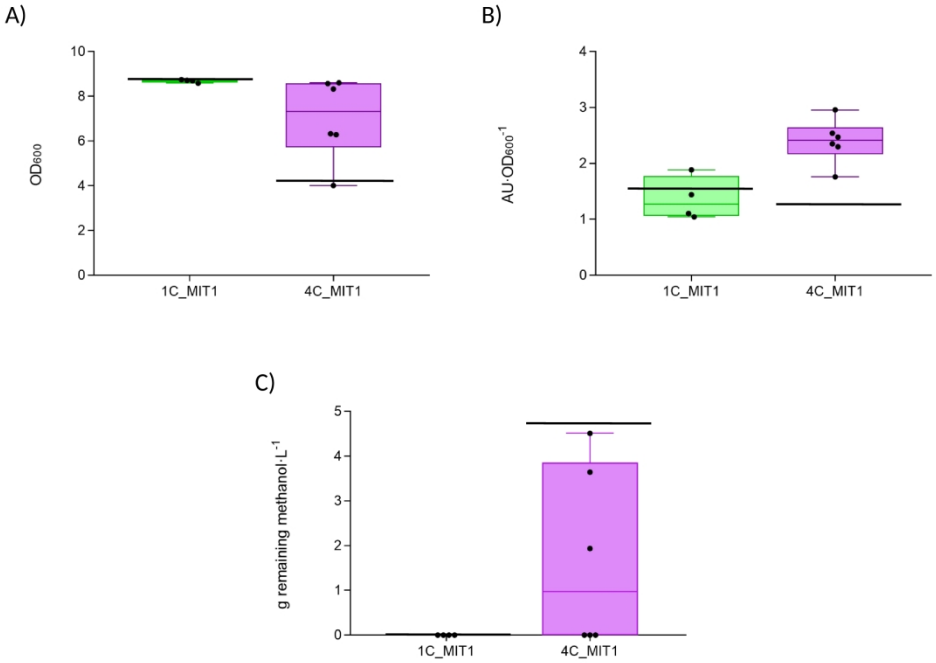

Figure 3. Screening of *MIT1* transformants in shake flask cultures. A) Biomass levels measured by OD_{600}. B) Specific lipase activity in shake flask cultures. C) Residual methanol quantified by HPLC at the end of the cultures. Samples were taken after 70 h of culture. Values for reference strains 1C and 4C are marked as horizontal thick black lines, whereas 1C and 4C strains expressing *MIT1* are represented as green and purple plot boxes, respectively. Black dots inside plot boxes indicate values of individual clones of each population. Corresponding confidence intervals are shown in the Supplementary file IV.

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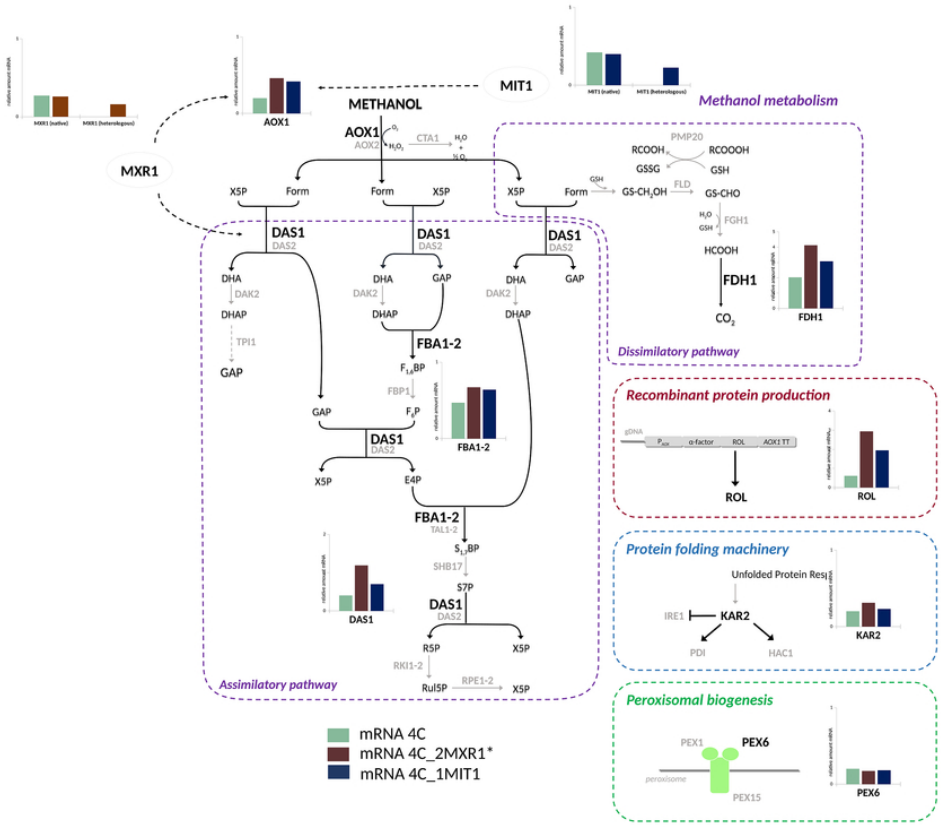

Figure 4 Transcriptional levels of selected genes. Comparative of mRNA levels of 4C (blue) and 4C6-2MXR1* (maroon) strains. Relative amounts of mRNA were calculated comparing to ACT1 expression levels of each strain. Thicker black lines represent the routes directly controlled by measured genes. DHA, dihydroxyacetone; E4P, erythrose-4-phosphate; HCOOH, formic acid; Form, formaldehyde; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; S1,7BP, sedoheptulose-1,7-bisphosphate; S7P, sedoheptulose-7-phosphate; GS-CH2OH, S-(hydroxymethyl)gluthiatione, X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; Rul5P, ribulose-5-phosphate; MXR1,methanol expression regulator 1; MIT1, methanol-induced transcription factor 1; AOX1, alcohol oxidase 1; DAS1, dihydroxyacetone synthase; FDH1, formate dehydrogenase 1; FBA1-2, fructose 1,6 –bisphosphate aldolase; ROL, Rhizopus oryzae lipase; KAR2, endoplasmic reticulum chaperone BiP; PEX6, peroxisomal biogenesis factor 6.*P< 0.05, **P< 0.01, comparing with 4C control strain.

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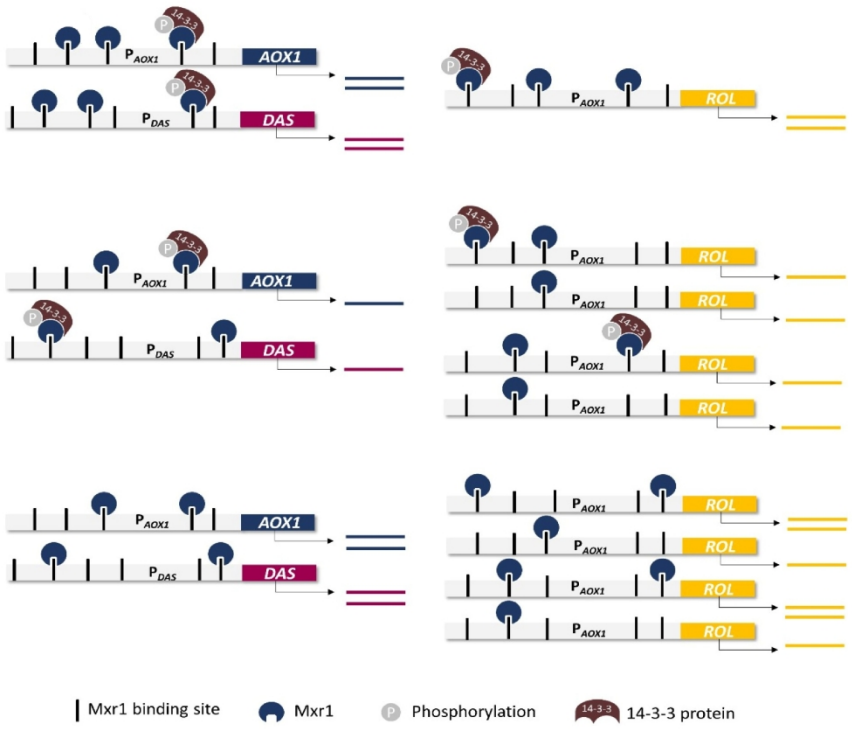

Figure 5. Regulatory model of *MXR1* in P*AOX1*-driven recombinant protein production. Proposed regulatory model, where the presence of multiple TF binding sites contributes to a higher transcriptional levels of the gene as the number of attached TF increases, due to a synergistic stimulation of gene expression. Top figure: Scenario for recombinant protein production in single copy strains. Under methanol-growth conditions, the molecules of Mxr1, which are present in a limited number, are distributed among the multiple binding sites of the P_{AOX1} and P_{DAS}, as well as other Mxr1-regulated promoters. Given the limited Mxr1 binding sites, methanol presence promotes high level induction of P_{AOX1}-driven recombinant expression. There may be also some basal binding of Mxr1 molecules to PAOX1 in the presence of methanol. Middle figure: Scenario for recombinant protein production in a 4C copy strain. As the number of Mxr1 binding sites increases, the number of Mxr1 molecules available per P_{AOX1} binding site decreases, resulting in reduced transcription levels of endogenous genes controlled by this transcription factor. Bottom figure: Scenario for recombinant protein production in 4C strain expressing Mxr1*. Overexpression of *MXR1** leads to an increase in the total number of Mxr1 molecules. Moreover, replacement in the position Ser215 of Mxr1 by an Ala prevents its phosphorylation and the subsequent union of 14-3-3 proteins. Consequently, the number of occupied Mxr1 binding sites increases, with an important fraction of mutant Mxr1* molecules also being bound, thereby obtaining higher transcriptional levels of Mut genes and *ROL*. Number of Mxr1 sites on the P_{AOX1} is depicted according to Kranthi et al. (2009) and Kranthi et al. (2010).

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Table 1. Macroscopic growth parameters of *P. pastoris* strains growing on glycerol-methanol in chemostat cultures

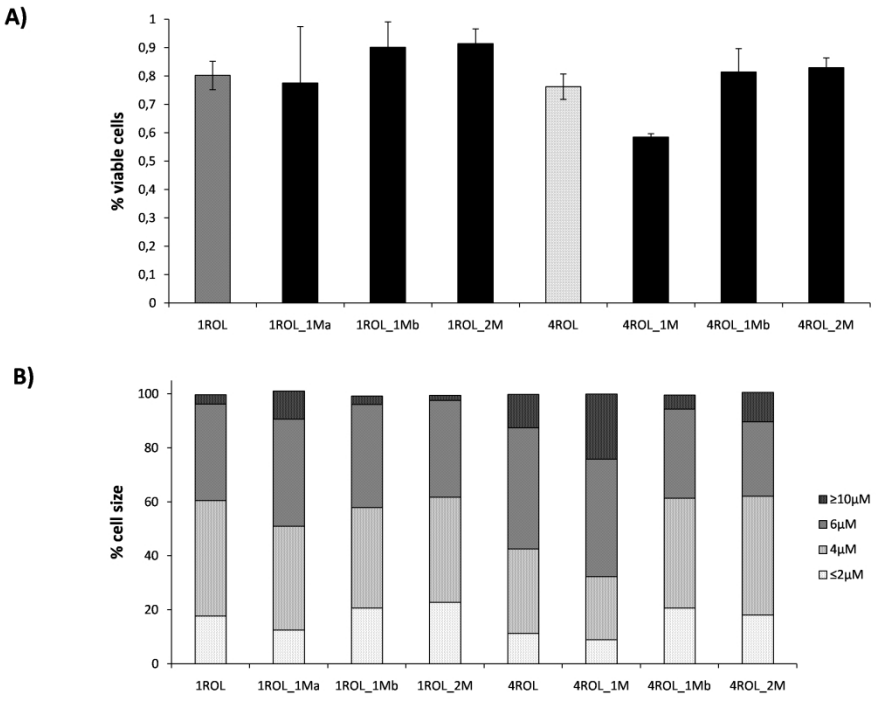
	DCW ($g\ L^{-1}$)	Biomass ($mmol\ g^{-1}$ DCW h^{-1})	$Y_{x/s}$ ($g\ C\text{-}mol^{-1}$)	$q_{s,\ glycerol}$ ($mmol\ g^{-1}$ DCW h^{-1})	$q_{s,\ methanol}$ ($mmol\ g^{-1}$ DCW h^{-1})	Lipase activity ($UA\ mL^{-1}$)	Lipase activity ($UA\ g^{-1}$ DCW)
4C	8.64 ± 0.24	3.37 ± 0.17	13.66 ± 0.38	-1.96± 0.20	-1.44 ± 0.10	40.13 ± 0.02	4644.33 ± 129.04
$4C_6$-2MXR1*	11.12 ± 0.11	3.37 ± 0.17	15.94 ± 0.16	-1.52± 0.20	-1.71 ± 0.20	57.57 ± 1.23	5177.23 ± 121.89
4C-1MIT1	10.32 ± 0.18	3.02 ± 0.12	16.00 ± 0.16	-1.48 ± 0.07	-1.70 ± 0.03	35.21 ± 0.97	3344.41 ± 286.65

DCW, dry cell weight, Biomass, specific biomass production rate, $Y_{x/s}$ biomass yield, q_s, specific substrate consumption rate.

Supplementary file 1. List of primers used for cloning and droplet digital PCR.

PRIMER	SEQUENCE (5'-3')	Tm	%GC	AMPLICON LENGHT (bp)
ROL probe	CCGGTCACTCACTCGGTGGTGCA	75	65	-
ACT probe	TCCGTATGGATCGGTGGTTCTATCCTCGCT	75	53	
ROL fwd1	CCTGTCGTCCAAGAACAAC	62	52	164
ROL rev1	GAGGACCACCAACAGTGAAG	62	53	
ACT1 fw	TGTCCGGTGGTACTACTATGTTCC	65	50	199
ACT1 rev	GATTCGTCGTACTCTTGCTTTGA	62	43	
AOX1 fw	GACATTCACGGTTTCGAAGG	61	40	78
AOX1 rev	CCTCAAGAAGTCCTGGCAAAC	63	42	
DAS1 fw	TTGAACTGGGACGGAGTG	60	44	135
DAS1 rev	CAAACCGTTGGCAATAGCAC	62	40	
FAB1-2 fw	CCCTTGGTTTGACGGAATG	60	42	97
FAB1-2 rev	TTCCTCCGACAGGTCTAAC	60	42	
FDH1 fw	GGTGCTGGAAGAATTGG	57	52	135
FDH1 rev	GACAGTGTCGACTCTTC	55	52	
KAR2 fw	GATGAAGTCGGGTCGTGTAC	61	55	110
KAR2 rev	TCTTAGCAGCATCACCCAACC	62	50	
MIT1 fw	CGTGAATCTGCAACAACAGC	57	50	
MIT1 rev	CGGATCTGAACTGCCAGAG	58	58	151
MIT1 fw(inf)	CGAGGAATTCACCGAAACGATGAGTACCGCAGCCCCAATC	71	55	2710
MIT1 rev(inf)	CATGTCTAAGGCTACAAACGATTTCTATTCTTCAACATTCCAG TAG	67	37	
MIT1 seq_1	GGGAAGTTGCATTGCTGAC	57	53	-
MIT1 seq_2	GTCAGCAATGCAACTTCCC	56	53	-
MIT1 seq_3	CTCTGACAAGTTCTCATTGAG	55	43	-
MIT1 seq_4	GGAATTGAACAAACCCTTGACC	56	45	-
MXR1 fw	TTCGCCCATTCTACTCG	57	52	101
MXR1 rev	GGGCTTGACTCATTTCAG	57	52	
pGAP_fw	GATTATTGGAAACCACCAGAATCG	61	41	450
MXR1*_int_rev	AGTCTTGTTATGGCCGCATC	62	50	
MXR1*_PmlI fw	GGCGACACGTGATGAGCAATCTACCCCCAAC	75	48	3490
MXR1*_KpnI rev	CGGCAGGTACCCTAGACACCACCATCTAGTCGG	76	60	
MXR1* seq_1	CCGGCTGTATCTGGATTTAG	59	50	-
MXR1* seq_2	CTCTCGATGTGAACAGGAAC	59	50	-
MXR1* seq_3	GTGGCTACGATAGGTGCAG	61	57	-
MXR1* seq_4	CACAGTTGGGATAGGATG	56	50	-
MXR1* seq_5	CTTTCAGCTGCGGCATTTG	62	52	-
MXR1* seq_6	GCAAAATGGCATTCTGACATCC	62	47	-
pGAPHA fw	GTTTGTAGCCTTAGACATGAC	55	43	3389
pGAPHA rev	CGTTTCGGTGAATTCCTCGTTTC	58	48	
PEX6 fw	GGTTTGGATGTGGTCAAG	57	50	148
PEX6 rev	TGGCTTTAGCAAGCAGAG	59	50	
ROL fwd	AAGTGGGACTGTGTCCAATG	61	50	158
ROL rev	GCACTTCTGAAGGAGTTG	57	50	

Tm, melting temperature; %GC, percentage of guanosine+cytosine.



Supplementary file 2. Flow cytometric analysis of screening samples. A) Viability of the cells at the end of the screening. B) Cell size distribution of yeast cells.

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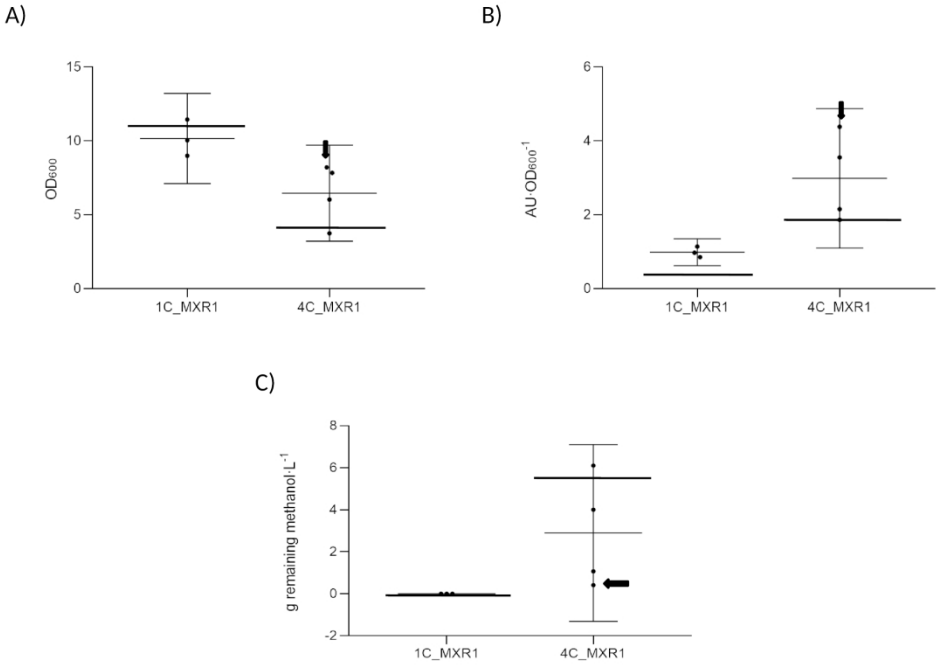

Suplementary file 3

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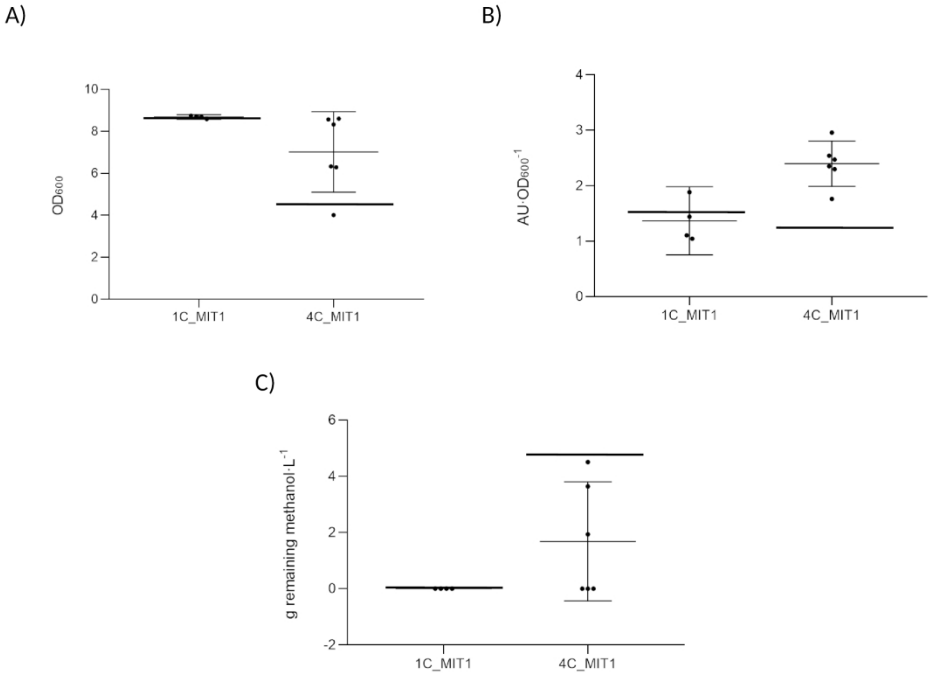

Supplementary file 4

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