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Effects of Deletions in *Pichia pastoris RTG* Genes on Phenotype and *AOX*1 Expression

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Abstract

Methylotrophic yeast *Pichia pastoris* is an object of modern biotechnology. Decisive understanding of gene regulation mechanisms is essential for successful protein production. In this study, we investigated the effect of deletions in *P. pastoris* genes encoding proteins, homologous to *S. serevisiae* Rtg1p, Rtg 2p, Msn2p and Msn4p. It was shown, that deletion in *PpRTG*1 gene results in inability of *P. pastoris* to grow on medium with methanol as a carbon source and ammonium sulfate as a source of nitrogen. We also demonstrate that deletions in *PpRTG*1 and *PpRTG*2 decrease activity of *AOX*1 promoter.

Keywords

Pichia pastoris, *AOX*1, Tor-Kinase, Retrograde Regulation Pathway

1. Introduction

Methylotrophic yeast *Pichia pastoris* is widely used in modern biotechnology as a recombinant protein production host [1]. However, from the genetic point of view, this type of yeast has not been studied enough. Most studies were devoted to specific recombinant proteins production and fermentation strategies. Sequencing of *P. pastoris* genome significantly accelerated research in the field of genetics and physiology of this species [2].

All methylotrophic yeasts have a similar mechanism of methanol utilization, which is called "MUT pathway" (methanol utilization pathway) [3]. At the first stage, methanol is oxidized to formaldehyde by specific enzyme—alcohol oxidase (*AOX* EC 1.1.3.13). A toxic byproduct of this reaction—hydrogen peroxide is neutralized by catalase (Cat EC 1.11.1.6). Both of these processes occur in peroxisomes [4]. Formaldehyde either enters assimilation pathway by condensation with xylulose-5-phosphate, or is oxidized by dehydrogenases to produce

energy [3]. *P. pastoris* is an obligate aerobe and its energy metabolism strictly depends on mitochondria. Thus, during methanol utilization peroxisomes and mitochondria function in *P. pastoris* should be coordinated by a regulatory system.

Promoter of the alcohol oxidase 1 (*AOX*1) gene is extremely strong and is widely used in biotechnology for heterologous genes expression [5]. That is why the regulation of this gene is of particular interest. *AOX*1 and other genes of MUT pathway (*MUT*-genes) are repressed when *P. pastoris* are grown on glucose or glycerol and are induced when methanol is used as the sole carbon source [6].

Previously we have shown that proline or glutamate as the sole sources of nitrogen lead to a decrease in the level of *AOX*1 and *MUT*-genes transcription if compared with ammonium sulfate or glutamine [7]. Genes involved in peroxisome biogenesis and functioning (*PEX*-genes) are regulated in the same manner [8]. Addition of rapamycin to the culture media reduces the negative effect of proline on *AOX*1 expression, which implies that Tor-kinase plays a key role in establishing this regulation [8].

In *S. cerevisiae* cells there are two Tor-kinase complexes (TORC), with Tor1p and Tor2p being their main components [9]. TORC1 plays key role in regulation of nutrient uptake and intermediary metabolism [10]. Nitrogen catabolite repression (NCR) is established by TORC1 via regulation of GATA-family transcription factors such as Gln3p. If preferred nitrogen sources (glutamine or ammonia) are present in the media, TORC1 activity leads to association of Gln3p with its cytoplasmic anchor Ure2p. If only poor nitrogen sources (e.g. proline or urea) are available, Gln3p is released from Ure2p and transported to the nucleus, where it activates expression of NCR sensitive genes [10] [11].

TORC1 is also involved in retrograde regulation pathway providing interorganelle communication between mitochondria, peroxisomes, and nucleus. In this pathway basic helix-loop-helix (bHLH) transcription factors Rtg1p and Rtg3p regulate expression of genes encoding enzymes required for tri-carboxylic acid cycle intermediates synthesis [12] [13]. When inactive, Rtg1p/Rtg3p complex is sequestered in cytoplasm by Mks1p. Activation of another regulatory protein Rtg2 leads to release of Rtg1p/Rtg3p complex, its transport to the nucleus and expression of Rtg-dependent genes [12].

TORC1 activity plays a key role in environmental stress response (e.g. nutrient limitation) via regulation of Zn-finger transcription factors Msn2p, Msn4p and Gis1p. Also in *S. cerevisiae* TORC1 is involved in regulation of protein synthesis, ribosome biogenesis, cell cycle, cell size and autophagy [14].

In the present work we searched for transcription factors that are involved in regulation of *AOX*1 by Tor-kinase in *P. pastoris*.

2. Materials and Methods

2.1. Plasmids

*pJET*1.2/*blunt* plasmid (Thermo Fisher scientific) was used for cloning of

*PpRTG*1, *PpRTG*2 and *PpMSN*2/4 PCR products that were amplified using Rtg1F/Rtg1R, Rtg2F/Rtg2R and MsnF/MsnR primers respectively. Resulting *pJET*1.2-*PpRTG*1, *pJET*1.2-*PpRTG*2 and *pJET*1.2-*PpMSN*2/4 plasmids contained 941 bp fragment with *PpRTG*1 coding sequence, 1655 bp fragment with *PpRTG*2 coding sequence and 944 bp fragment with *PpMSN*2/4 coding sequence respectively. pPICZ*α*A plasmid (Thermo Fisher scientific) was used as a template for amplification of zeocin resistant gene *ZeoR* using ZeoF/ZeoR primers. *SacI* and *EcoRI* sites were introduced within the primers, while *BamHI* and *BsrGI* sites already existed within the amplified sequence. *ZeoR* PCR product was also cloned in *pJET*1.2/*blunt*. Than it was cloned into 1) *pJET*1.2-*PpRTG*1 and pJET1.2-*PpRTG*2 using *SacI* and *BsrGI* restriction sites and into 2) pJET1.2-PpMSN2/4 using *BamHI* and *EcoRI* sites. Resulting *pJET*1.2-*PpRTG*1*ΔZeoR*, *pJET*1.2-*PpRTG*2*ΔZeoR* and *pJET*1.2-*PpMSN*2/4*ΔZeoR* plasmids contain *ZeoR* gene flanked by parts of *PpRTG*1, *PpRTG*2 and *PpMSN*2/4 sequences respectively (**Figure 2(a)**).

2.2. Strains

P. pastoris strains presented in **Table 1** were used. tr2-1-GS115 was derived previously from the original *P. pastoris* strain GS115 (*his*4) (Invitrogen). This strain lacks native ACP activity and carries a reporter acid phosphatase (ACP) *PHO*5 gene of *S. cerevisiae* under the control of *AOX*1 gene promoter [7] [8]. Other strains presented in this study were derived from tr2-1-GS115 by transformation with deletion cassettes.

The bacterial *E. coli* strain DH5*α* [F'phi80d*lacZ* delta (*lacZ*YA_*arg*F) U169 *deo*R recA1 *end*A1 *hsd*R17 (rK– mK+) *pho*A *sup*E44 lambda_*thi*_1 *gyr*A96 *rel*A1/F' *pro*AB+ *lac*IqdeltaM15 Tn10 (*tetr*)] was used for the construction of plasmids.

2.3. Culture Media and Conditions

Synthetic media MN, MP, GN, GP and P were used in this study. All variations of synthetic media contained per 1 L: 100 ml of 0.1 M Na-citrate buffer pH4.5; 0.5g $MgSO_4 \cdot 7H_2O$; 0.4 g $CaCl_2$; 1g KH_2PO_4; vitamins and trace metal. GN, GP contained 1% glycerol as a sole carbon source, and MN, MP contained 1% methanol. Ammonium sulfate was added to MN, GN media in concentration 0.46 g/L. MP, GP and P contained proline in concentration 0.46 g/L. For ACP activity

Table 1. *P. pastoris* strains used in this study.

Strain	Genotype	Source of strain
tr2-1-GS115	*PAOX*1-*PHO*5 *HIS*4 *phox*	[7]
*Δ*rtg2-GS115	*PAOX*1-*PHO*5 *HIS*4 *phox Pprtg*2::*ZEO*^{R}	This study.
*Δ*msn2/4-GS115	*PAOX*1-*PHO*5 *HIS*4 *phox Ppmsn*2/4::*ZEO*^{R}	This study.
*Δ*rtg1-GS115	*PAOX*1-*PHO*5 *HIS*4 *phox Pprtg*1::*ZEO*^{R}	This study.

measurements *P. pastoris* cells were grown at 25˚C in 30 ml volumes. For plates 24 g/L of agar was added to the media. Plates were incubated at 30˚C.

YPDS media containing 20 g of glucose, 20 g of peptone and 10 g of yeast extract and 182 g of sorbitol per 1 L was used for electroporation.

LB medium was used to cultivate bacterial strains. *E. coli* strains were grown at 37˚C.

2.4. Oligonucleotides

All oligonucleotides used in this study are presented in Table 2. The Primer 3 program was used to select primers for PCR (http://primer3.sourceforge.net/).

2.5. Molecular Methods

The bacterial transformation and plasmid isolation from *E. coli* was carried out in accordance with standard methods [15]. The isolation of DNA and yeast transformation was carried out according to [16] and [17].

PCR was performed according to the recommendations of the manufacturer of reagents (Thermo Fisher scientific). *Pfu*-polymerase was used when fragments were amplified for further cloning. *Taq*-polymerase was used for PCR analysis. Reactions were set in 25 uL volumes. 20 ng of plasmid DNA or 200 ng of gDNA was used as a template. Primer annealing step was performed at 52˚C.

DNA hydrolysis with restriction endonucleases was performed using the buffers and conditions recommended by the manufacturer of the enzymes (Thermo Fisher scientific). Dephosphorylation of vectors was done using FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher scientific). DNA ligation was performed using T4 DNA Ligase (Thermo Fisher scientific). Electrophoresis of DNA was performed in 1% agarose gel according to [15]. Purification of DNA from agarose gels was performed using Cleanup Standard kit according to the recommendations of the manufacturer (Evrogen).

ACP activity was determined qualitatively [18] and quantitatively [19]. The specific activity of ACP was designated as the ratio of the optical density at 410

Table 2. Oligonucleotides used in this study.

Primer	Sequence 5'-3'
ZeoF	GAGCTCAGACCTTCGTTTGTGCG
ZeoR	GAATTCTAAAGCCTTCGAGCGTC
Rtg1F	TTCCTCTCCACTAGAATCAG
Rtg1R	AAAAGGTGGAGTAGTGTGG
Rtg2F	ACTCCGTACTCTATTTGCCAGC
Rtg2R	TTGGTTGTAAACTGGCAATTCTG
MsnF	GTAGCCTGTTCCAGCAG
MsnR	CTCATTCGTGTCTATGGAC

nm to the density of cell suspension at 600 nm.

Statistical analysis was performed using the Past3 program.

3. Results

1) Nitrogen catabolite repression and *AOX*1 regulation by proline

Our previous results allowed suggesting that *AOX*1 and other *MUT*-genes are repressed via Tor-signaling pathway in media with methanol and proline as sole nitrogen source [8]. Here we investigated if NCR mechanisms are involved in such regulation. In *S. cerevisiae* NCR occurs if glutamine or ammonium are present in the media and results in repression of genes involved in metabolism of poor nitrogen sources, such as proline.

P. pastoris tr2-1-GS115 strain (*PAOX*1-*PHO*5 *HIS*4 *phox*), which was constucted earlier, contains *S. cerevisiae* acid phosphatase (ACP) *PHO*5 reporter gene under the control of the *AOX*1 gene promoter. This strain was grown in media with methanol. Media also contained ammonium sulfate at standard concentration (0.46% w/v) and proline at different concentrations (0.11%, 0.23%, 0.34% and 0.46%). After 40 hours of cultivation ACP specific activity was measured in the yeast culture (**Figure 1(a)**).

The figure shows that *AOX*1 promoter is repressed by proline even at low concentrations, despite the fact that rich nitrogen source, ammonium sulfate, is present in the media. These results allow suggesting that the mechanisms of nitrogen catabolite repression are not involved in the observed regulation of the *AOX*1 promoter by proline.

Recent studies demonstrated that *P. pastoris* can utilize some amino acids (e.g. glutamate) as sole source of carbon and nitrogen [20]. To study if proline can be used by *P. pastoris* in such way, tr2-1-GS115 strain was placed in 10 µl of cell suspensions (10^6, 10^5, 10^4, and 10^3 cells per ml) on the plates with mineral media (P) containing only proline as carbon and nitrogen source (**Figure 1(b)**).

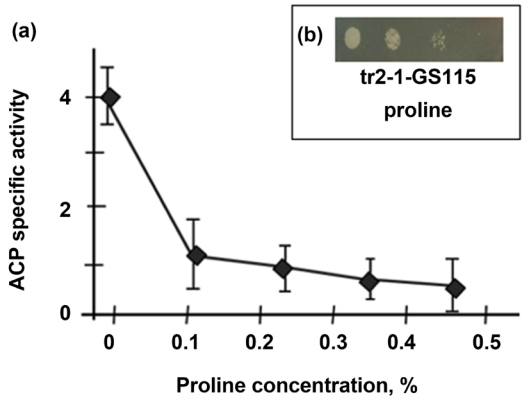


Figure 1. (a) ACP specific activity of *P. pastoris* strain tr2-1-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox*) grown in media with methanol, ammonium sulfate (0.46% w/v) and different concentrations of proline. All measurements were done at least in 4 replicates with 2 ACP specific activity measurements for each. SEM is plotted as error bars; (b) Growth of tr2-1-GS115 strain on media with proline as sole source of carbon and nitrogen (P).

Results demonstrate that *P. pastoris* are able to grow only on proline. Thus, repression of *MUT*-genes by this amino acid may be caused by its utilization as a carbon source.

2) Identification of *P. pastoris* and *S. cerevisiae* homologous proteins, which are targeted by Tor-signalling

To investigate, which other pathways downstream of Tor-kinase are involved in regulation of *AOX*1 and *MUT*-genes, we searched for *P. pastoris* proteins homologous to *S. cerevisiae* proteins acting in retrograde regulation pathway and nutrient limitation. A bioinformatic analysis of *P. pastoris* genome was carried out. Using the BLAST algorithm, the amino acid sequences of proteins homologous to the *S. cerevisiae* Rtg1p, Rtg2p, Msn2p, Msn4p, Tor1p, Tor2p were identified (**Table 3**).

It was shown that *P. pastoris* Tor protein has a high degree of identity with the sequences of the *S. cerevisiae* Tor1p and Tor2p. The observation that only one protein is found in *P. pastoris*, can be explained by the fact that yeast *S. cerevisiae* underwent genome duplication during evolution [21]. Similarly, for the Msn2p and Msn4p, only one homologous protein PpMsn2/4 was detected in *P. pastoris*. Proteins homologous to *S. cerevisiae* Rtg1p and Rtg2p were detected. Analysis didn't give any results for Rtg3p when genome assembly described in [2] was used. A protein was identified using another genome assembly [22].

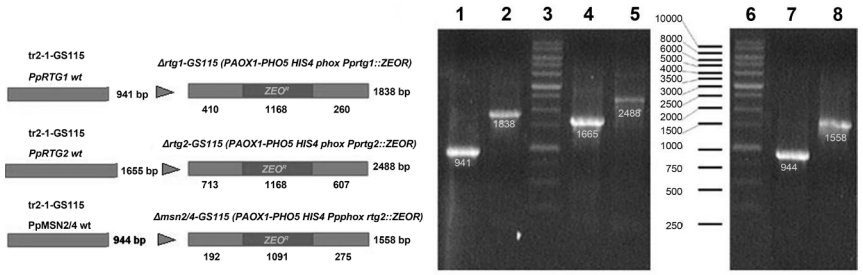
3) Construction of plasmids for introducing deletions into *P. pastoris* genome

To determine the effect of deletions in *PpRTG*1*, PpRTG*2*, PpMSN*2/4 genes, on the regulation of the *AOX*1 gene in *P. pastoris*, plasmids *pJET*1.2-*PpRTG*1*ΔZeoR, pJET*1.2-*PpRTG*2*ΔZeoR* and *pJET*1.2-*PpMSN*2/4*ΔZeoR* were constructed. The coding sequences of *PpRTG*1, *PpRTG*2, *PpMSN*2/4 genes were amplified by PCR using Rtg1F/Rtg1R, Rtg2F/Rtg2R, MsnF/MsnR primers. Chromosomal DNA of tr2-1-GS115 *P. pastoris* strain was used as the template. The obtained DNA fragments were cloned in *pJET*1.2/*blunt* plasmid.

ZeoR gene coding sequence was PCR amplified using ZeoF/ZeoR primers and *pPICZaA* plasmid as a template. *ZeoR* was cloned into *pJET*1.2-*PpRTG*1, *pJET*1.2-*PpRTG*2 and *pJET*1.2-*PpMSN*2/4 causing deletions in coding sequences of target genes (**Figure 2(a)**). Resulting *pJET*1.2-*PpRTG*1*ΔZeoR*,

Table 3. Comparison of *P. pastoris* and *S. cerevisiae* protein sequences.

S. cerevisiae protein	*P. pastoris* protein	Query cover, %	Identity, %	Protein reference (UniProtKB/TrEMBL)	Gene reference (EnsemblGenomes)
Rtg1	PpRtg1	72	48	C4QWX5	PAS_chr1-1_0371
Rtg2	PpRtg2	99	52	C4R4L3	PAS_chr3_0452
Rtg3	PpRtg3?	26	44	AOA70166.1 (GenBank)	PAS_chr2-1_0723
Msn2	PpMsn2/4	17	49	C4R1J8	PAS_chr2-1_0723
Msn4		15	51		
Tor1	PpTor	97	55	C4R117	PAS_chr2-1_0557
Tor2		96	57		



(a) (b)

Figure 2. (a) Schematic representation of deletions in *Δ*rtg1-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox Pprtg*1::*ZEO*^{R}), *Δ*rtg2-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox Pprtg*2::*ZEO*^{R}) and *Δ*msn2/4-GS115 (*PAOX*1-*PHO*5 *HIS*4 *Ppphox rtg*2::*ZEO*^{R}) strains; (b) Electropherogram of PCR products (strain gDNA used for PCR/primers): 1 lane—control strain tr2-1-GS115/Rtg1F-Rtg1R primers; 2—*Δ*rtg1-GS115/Rtg1F-Rtg1R; 3.6—Ladder 1 kb (Evrogen, Russia); 4—tr2-1-GS115/Rtg2F-Rtg2R; 5—*Δ*rtg2-GS115/Rtg2F-Rtg2R; 7—tr2-1-GS115 / MsnF-MsnR; 8—*Δ*msn2/4-GS115/MsnF-MsnR.

*pJET*1.2-*PpRTG*2*ΔZeoR* and *pJET*1.2-*PpMSN*2/4*ΔZeoR* plasmids were analyzed using PCR and Sanger sequencing.

4) Generation of *P. pastoris* strains with deletions of *PpRTG*1, *PpRTG*2 and *PpMsn*2/4 genes

Plasmids *pJET*1.2-*PpRTG*1*ΔZeoR, pJET*1.2-*P pRTG*2*ΔZeoR* and *pJET*1.2-*PpMSN*2/4*ΔZeoR* were used as a template for PCR amplification of the *PpRTG*1::*ZeoR*, *PpRTG*2::*ZeoR* and *PpMSN*2/4::*ZeoR* fragments with Rtg1F/Rtg1R, Rtg2F/Rtg2R and MsnF/MsnR primers. In the resulting DNA fragments, the zeocin resistance gene *ZeoR* is flanked by *PpRTG*1, *PpRTG*2 and *PpMSN*2/4 gene sequences. Transformation of *P. pastoris* with these cassettes resulted in the replacement of *PpRTG*1, *PpRTG*2 and *PpMSN*2/4 with the zeocin resistance gene through homologous recombination. tr2-1-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox*) was used as the recipient strain for transformation by electroporation. Transformants were selected on a YPDS medium with zeocin. Presence of deletions in resulting *Δ*rtg1-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox Pprtg*1::*ZEO*^{R}), *Δ*rtg2-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox Pprtg*2::*ZEO*^{R}) and *Δ*msn2/4-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phoxPprtg 2*::*ZEO*^{R}) strains was proved using PCR (**Figure 2(b)**) and Sanger sequencing.

5) Phenotypic characteristic of *Δ*rtg1-GS115, *Δ*rtg2-GS115 and *Δ*msn2/4-GS115 strains

Phenotype of strains with deletions in the *PpRTG*1, *PpRTG*2 and *PpMSN*2/4 genes was evaluated. Strains were grown on solid media GN, GP, MN and MP, containing glycerol (G) or methanol (M) as a carbon source and ammonium sulfate (N) or proline (P) as a nitrogen source, respectively. *P. pastoris* tr2-1-2-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox*) was used as a control. 10 μl of cell suspensions (10^6, 10^5, 10^4, and 10^3 cells per ml) were placed on the Petri dishes with these media and incubated for 4 days at 30˚C (**Figure 3**).

On media with glycerol as a carbon source, growth of *Δ*rtg1-GS115,

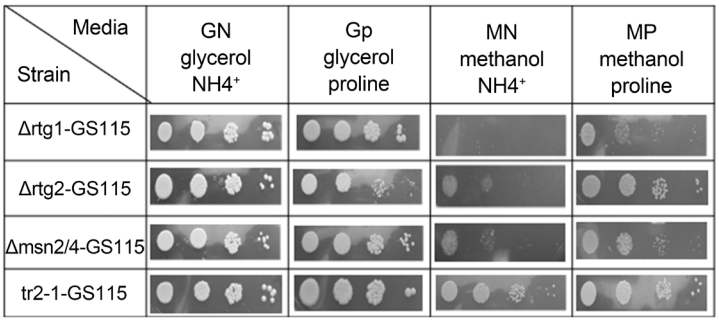


Figure 3. Growth of *Δ*rtg1-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox Pprtg1*::*ZEO*^{R}), *Δ*rtg2-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox Pprtg2*::*ZEO*^{R}), *Δ*msn2/4-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox Ppmsn*2/4::*ZEO*^{R}) and tr2-1-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox*) on media with different carbon and nitrogen sources.

*Δ*rtg2-GS115, *Δ*msn2/4-GS115 strains is similar to demonstrated by control strain tr2-1-GS115 either on ammonium sulfate (GN), or on proline (GP). On the other hand, *Δ*rtg1-GS115 strain is unable to grow on a medium with methanol and ammonium sulfate (MN). *Δ*rtg2-GS115 and *Δ*msn2/4-GS115 demonstrate slower growth under these conditions. On medium with methanol and proline (MP) growth of *Δ*rtg1-GS115 strain is slightly slower than of control tr2-1-GS115 strain. And growth of *Δ*rtg2-GS115 and *Δ*msn2/4-GS115 strains practically does not differ from the control.

6) Effect of deletions in *PpRTG*1, *PpRTG*2 and *PpMsn*2/4 genes on *AOXI* promoter activity

The addition of proline to the medium affects both the growth of *P. pastoris* strains on media with methanol and the activity of genes whose products are involved in the methanol utilization in these yeasts. Therefore, at this stage of the work, we investigated the effect of the deletions in *PrRTG*1, *PrRTG*2 and *PpMsn*2/4 genes on the expression of main methanol metabolism gene *AOXI*.

The initial strain tr2-1-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox*) and obtained transformants contain the ACP reporter gene *PHO*5 under the control of the *AOXI* promoter. These strains were first grown in GN medium with glycerol and ammonium sulfate for 48 hours, after which the biomass of the cells was transferred to media with methanol and various sources of nitrogen: proline and ammonium sulfate. In this case, the synthesis of acid phosphatase was induced by activation of the *AOXI* promoter with methanol. After 40 hours of incubation, the specific activity of acid phosphatase was determined (**Figure 4**).

As shown in **Figure 4** in a medium with ammonium sulfate, the level of *AOX*1 gene expression is higher than in a medium with proline in all studied strains. Deletions in the *PpRTG*1 and *PpRTG*2 genes result in a decrease in *AOXI* promoter activity in both proline and ammonium sulfate media. In *S. cerevisiae* Rtg1p-Rtg3p transcription factors bind to the sequence GGTCAC (R-box) in the promoter of its target genes [23]. Direct search using YEASTRACT database [24] did not show *S. cerevisiae* Rtg1p-Rtg3p binding sites in *AOX*1 promoter sequence.

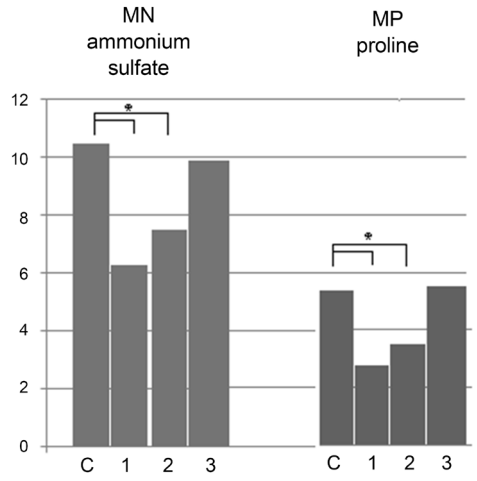


Figure 4. Specific activity of acid phosphatase synthesized by strains C. tr2-4-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox*), 1) *Δ*rtg1-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox Pprtg1*::*ZEO*^{R}), 2) *Δ*rtg2-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox Pprtg2*::*ZEO*^{R}) and 3) *Δ*msn2/4-GS115 (*PAOX*1-*PHO*5 *HIS*4 *phox Ppmsn*2/4::*ZEO*^{R}) after cultivation in media MN and MP with methanol and ammonium sulfate or proline respectively. All measurements were done at least in 4 replicates with 2 ACP specific activity measurements for each. *$-p < 0.02$ (Mann-Whitney U-test).

Deletions of the *PpMSN*2/4 gene do not affect the activity of the *AOX*1 promoter in media with methanol, and proline or ammonium sulfate as a nitrogen source.

4. Discussion

We show that *AOX*1 promoter is repressed by proline even if ammonium sulfate is present in the media. Recent studies revealed that some amino acids (e.g. glutamate) can be used by *P. pastoris* as sole source of carbon and nitrogen [20]. We demonstrate that *P. pastoris* utilize proline in such way. It may be proposed, that when grown on media with methanol *P. pastoris* utilize proline as complex carbon and nitrogen source. This explains the repression of *MUT*-genes to optimal levels by Tor signaling pathway and also means that nitrogen regulation, especially NCR, differs in *P. pastoris* from one known for *S. cerevisiae*.

Deletion in *PpRTG*1 gene results in inability of *P. pastoris* to grow on medium with methanol and ammonium sulfate. Deletions in *PpRTG*2 and *PpMSN*2/4 slow the growth of *P. pastoris* on such medium. These effects are compensated when *Δ*rtg1-GS115, *Δ*rtg2-GS115 and *Δ*msn2/4-GS115 strains are grown on glycerol instead of methanol, or when proline is used as sole nitrogen source. It should be noted that effects of these mutations on *P. pastoris* phenotype were not studied yet. In *S. cerevisiae rtg* mutants demonstrate growth requirement for glutamate which itself is a precursor for synthesis of other amino acids and nucleotides [25]. In yeast, there are three known pathways for glutamate synthesis that use a-ketoglutarate as a common precursor of glutamate. *S. cerevisiae* can also degrade proline into glutmate via the proline utilization pathway in the mitochondria [26]. Thus, it may be proposed, that in *P. pastoris* metabolism of

glutamate or it's precursor a-ketoglutarate changes when different carbon sources are used. Glutamate synthesis depends on retrograde regulation when cells are grown in media with ammonium sulfate and methanol, but is regulated in different way when glycerol is used as a carbon source. When proline is present in the media it is metabolized to glutamate, thus negating the effects of deletions in *PpRTG*1 and *PpRTG*2 genes.

We demonstrate that deletions in *PpRTG*1 and *PpRTG*2 decrease activity of *AOX*1 promoter. Protein products of these genes may be involved in regulation of *AOX*1 and their presence is required for full induction of *AOX*1 and other *MUT*-genes in *P. pastoris*. Absence of Rtg1p-Rtg3p binding sites known for *S. cerevisiae* in *AOX*1 promoter may allow suggesting, that in *P. pastoris* either Rtg-binding sites are different, or Rtg proteins are indirectly involved in regulation of *AOX*1 promoter. For example, in *S. cerevisiae* Rtg1p-Rtg3p interact with negative retrograde regulators Bmh1p and Bmh2p. And in *P. pastoris* activity of Mxr1p, which is known to be the main inductor of *MUT*-genes [27], is regulated by 14-3-3 protein that shows similarity to *S. cerevisiae* Bmh1p [28].

Neither of the deletions changed *AOX*1 regulation by proline. Thus, there are some other proteins downstream of Tor-kinase which establish such regulation. It may be proposed that Tor-kinase complex may modify activity of proteins involved in *AOX*1 regulation by carbon source, for example, Mxr1p and 14-3-3 protein homologous to *S. cerevisiae* Bmh1p. A model of such interactions was presented earlier [8].

5. Conclusion

P. pastoris is able to use proline as a sole source of carbon and nitrogen. *AOX*1 promoter is repressed by proline even at low concentrations (0.11% w/v), regardless of ammonium sulfate presence in the media. *PpRTG*1 gene is essential for growth on media with methanol as a carbon source and ammonium sulfate as a source of nitrogen. Deletions in *PpRTG*1 and *PpRTG*2 genes cause decrease in activity of *AOX*1 promoter.

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