Research Article

Effect of Nitrogen Source and Inorganic Phosphate Concentration on Methanol Utilization and PEX Genes Expression in *Pichia pastoris*

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Received 30 July 2014; Accepted 5 December 2014; Published 24 December 2014

Academic Editor: Jing Hua Zhao

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Methylotrophic yeast *Pichia pastoris* has proved to be especially useful for production of various heterologous proteins. In biotechnology it is very important to maintain the balance between high levels of heterologous gene expression and cell viability. Decisive understanding of gene regulation mechanisms is essential for reaching this goal. In this study, we investigated the effect of different nitrogen sources and phosphate concentration in media on methanol utilization. It was shown that expression levels of main genes, which are involved in methanol utilization (*MUT* genes) and in functioning of peroxisomes (*PEX* genes), are maximal when ammonium sulphate is used as a nitrogen source. Expression of these genes is decreased in media with poor nitrogen sources, such as proline. Addition of rapamycin to the media completely removed repression of *AOX1* promoter in media with proline, which allows proposing that Tor-kinase is involved in establishing of nitrogen regulation of this gene. It was also shown that *MUT* genes expression levels get higher, when the phosphate concentration in media is increased.

1. Introduction

Microorganisms adaptation to different environment changes is primarily established on transcriptional level. Hence, the expression of most enzyme encoding genes changes dramatically in nutrient deficiency conditions. Yeast *Pichia pastoris* represent the eukaryotic group capable of utilizing methanol as a sole carbon source. Methanol utilization pathway is common in all methylotrophic yeasts and involves several unique enzymes [1]. Alcohol oxidase (Aox EC 1.1.3.13) catalyzes methanol oxidation to formaldehyde. Hydrogen peroxide, which is also produced in this reaction, is degraded by catalase (Cat EC 1.11.1.6) to oxygen and water. Hydrogen peroxide formation is very dangerous for the living cell; thus alcohol oxidase and catalase are sequestered within special organelles—peroxisomes [2]. Proteins involved in peroxisome biogenesis are called peroxines (or *PEX* proteins) and are encoded by *PEX* genes [3].

A portion of formaldehyde generated by alcohol oxidase leaves peroxisomes and is further oxidized by formaldehyde dehydrogenase (Fld EC 1.2.1.1) and formate dehydrogenase (Fdh EC 1.2.1.2) providing energy for cells. S-formylglutathione hydrolase (Fgh EC 3.1.2.12) participates in the detoxication of formaldehyde and regenerates glutathione.

Another portion of formaldehyde is condensed with xylulose 5-phosphate by the third peroxisome enzyme—dihydroxyacetone synthase (Dhas EC 2.2.1.3) resulting in generation of glyceraldehyde 3-phosphate and dihydroxyacetone. These two tricarboxylic compounds are further involved in xylulose 5-phosphate regeneration resulting in one novel molecule of glyceraldehyde 3-phosphate for every three cycles [1, 2].

Expression of alcohol oxidase genes is strictly regulated by the type of carbon source presented in the media. In *P. pastoris* cells grown on glucose (or glycerol) alcohol oxidase is not detectable. However, in methanol-grown cells alcohol
oxidase levels increase dramatically, compromising up to 30% of total soluble protein [4].

P. pastoris alcohol oxidase has two isoforms which are the products of AOX1 and AOX2 genes. The coding regions of AOX1 and AOX2 genes have more than 90% similarity. But promoter regions are significantly different, and product of AOX1 gene provides about 90% of alcohol oxidase activity [4]. Regulation of AOX1 gene expression is established on transcriptional level and consists of two separate mechanisms, providing repression in the presence of various carbon sources and methanol induction. In repressing conditions, while glucose or glycerol are presented in the media, AOX1 expression is practically not detectable. In derepressing conditions, for example, carbon starvation, AOX1 gene expression levels increase 1000-fold in comparison with repressing conditions, while glucose or glycerol are represented in separate mechanisms, providing repression in the presence of nitrogen starvation [17].

In S. cerevisiae phosphate limitation induces dramatic changes in cell metabolism and modifies expression of large number of genes [18]. A key role in establishing phosphate regulation in S. cerevisiae is played by another serine-threonine kinase [19].

In this study, we demonstrated the effect of different nitrogen sources and phosphate concentration in media on expression of genes involved in methanol utilization and peroxisome biogenesis. It was shown that rapamycin treatment influences AOX1 gene expression in media with different nitrogen sources.

2. Materials and Methods

2.1. Plasmids. pPIC9-PAOX1-PHO5 plasmid contains AOX1 promoter and the PHO5 gene of S. cerevisiae, which encodes yeast acid phosphatase. It was created by cloning of a PCR amplified PHO5 gene into pPIC9 vector (Invitrogen) using BamHI and EcoRI sites [10].

pPIC9-delSacI-PAOX1-PHO5 and pPIC9-delNsiI-PAOX1-PHO5 carry 201 bp and 671 bp truncations of AOX1 promoter. To create these plasmids pPIC9-PAOX1-PHO5 was cut in AatII site and one of the unique sites within AOX1 promoter SacI or NsiI. The resulting fragments were blunted using Pfu polymerase and self-ligated [10].

pPIC9-PAOX2-PHO5 plasmid was created using pPIC9-PAOX1-PHO5 and contains PHO5 gene under control of AOX2 promoter. A PCR amplified AOX2 promoter fragment was cloned into pPIC9-PAOX1-PHO5 using AatII and BamHI restriction sites, so it replaced the AOX1 promoter.

pPIC9-delA-PAOX1-PHO5 plasmid contains a 80 bp deletion in AOX1 promoter. pPIC9-delB-PAOX1-PHO5 contains a 34 bp deletion, pPIC9-delC-PAOX1-PHO5 a 160 bp deletion, and pPIC9-delD-PAOX1-PHO5 a 80 bp deletion. Deletions in four regions of AOX1 promoter named A, B, C, and D (Figure 4(a)) were generated using site-specific mutagenesis. In the first round of PCR two fragments flanking the desired deletion were amplified. Reverse primer for promoter region upstream of deletion and forward primer for downstream region were designed to contain complementary sequences on their 5’ ends. This allowed combining the fragments together in the second round of PCR. Resulting AOX1 promoter fragments with desired deletions were cloned using AatII and BamHI restriction sites into pPIC9-PAOX1-PHO5 plasmid instead of native promoter.

All plasmids were verified using PCR, restriction, and sequencing analysis.
These strains were obtained by transforming of 1-GS115 of \( AOX1 \) PHO5 were created by transforming of 1-GS115 strain with delSacI-PAOX1-PHO5 strains carry a StuI torst that were cut in his4 fact that plasmids were integrated into pho gene under the control of AOX1 gene promoter. It was created by transforming of 1-GS115 strain with pPIC9-PAOX1-PHO5 vector that was cut in Stud site.

2.2. Strains. \( P. \) \( pastoris \) strains presented in Table 1 were used. All strains were derived from the original \( P. \) \( pastoris \) strain GS115 (his4) (Invitrogen). 1-GS115 strain lacks ACP activity, tr2-1-GS115 strain carries a reporter acid phosphatase (ACP) gene of \( S. \) \( cerevisiae \). His4 strain, which carries \( AOX1 \) gene promoters that were truncated using \( SacI \) and \( NsiI \) restriction sites. These strains were created by transforming of 1-GS115 strain with pPIC9-delSacI-PAOX1-PHO5 and pPIC9-delNsiI-PAOX1-PHO5 vectors that were cut in Stud site.

| Strain     | Genotype   | Source        |
|------------|------------|---------------|
| GS115      | his4       | Invitrogen    |
| 1-GS115    | Phox his4  | [10]          |
| tr2-1-GS115| Phox PAOX1-PHO5 HIS4 | [10]          |
| tr3-1-GS115| phox PAOX2-PHO5 HIS4 | This study   |
| tr\( \Delta \)SacI-1-GS115 | phox PAOX1\( \Delta \)SacI-PHO5 HIS4 | [10]          |
| tr\( \Delta \)NsiI-1-GS115 | phox PAOX1\( \Delta \)NsiI-PHO5 HIS4 | [10]          |
| trA-1-GS115 | phox PAOX1\( \Delta \)-PAOX1-PHO5 HIS4 | This study   |
| trB-1-GS115 | phox PAOX1\( \Delta \)-B-PHO5 HIS4 | This study   |
| trC-1-GS115 | phox PAOX1C-PHO5 HIS4 | This study   |
| trD-1-GS115 | phox PAOX1D-PHO5 HIS4 | This study   |

In this study standard rich YPD and synthetic MP media were used. YPD medium contained 20 g of glucose, 20 g of peptone, and 10 g of yeast extract per 1 L. All variations of MP media contained the following per 1 L: 100 mL of 0.1 M Na-citrate buffer pH 4.5; 0.5 g \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \); 0.4 g \( \text{CaCl}_2 \); vitamins and trace metals; 20 mg of histidine (for GS115 strain) 1% glycerol or 1% methanol as a sole carbon source. Three modifications of MP media were used: (1) MPN contained ammonium sulphate in concentration 0.46 g/L and \( \text{KH}_2\text{PO}_4 \) in concentration 1 g/L, and (3) MPL contained ammonium sulphate in concentration 0.46 g/L and \( \text{KH}_2\text{PO}_4 \) in concentration 0.03 g/L. \( P. \) \( pastoris \) cells were grown at 25 C. 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. Probes for real-time PCR were modified with ROX-BHQ2. The Primer 3 program was used to select primers for real-time PCR (http://primer3.sourceforge.net/).

| Primer     | Sequence \(^{5’-3’}\) |
|------------|----------------------|
| PpACT1F    | AGTTGTCACATCGGTCTTAG |
| PpACT1R    | GGTTCACTGGAGGCTAGTC  |
| PpDHASP    | TACGAGTGGGAGATATGCG  |
| PpDHASR    | GTGGTTGTTGGTTCTCCTCA  |
| PpDAKF     | TGCCCGAGAATAAGCAGAAG |
| PpDAKR     | TTCACCAATCACTACATCCTCC |
| PpPMM1F    | CGGAGGAGCAGATAGG     |
| PpPMM1R    | AAAGTCTCTTTGGGATTCCTC |
| PpFLDF     | GAATCTGCCACAGGGTTCAG |
| PpFLDR     | TGCTTTTGATGGTTGCCAG  |
| PpFGIF     | ATCTCCAAACCACTAAAAG |
| PpFGIR     | CAAGTGAATCATAAAAGTC |
| PpPMMX8F   | TTATGGTTAGATGCCCCTCCT |
| PpPMMX8R   | CCGGGTGGTTGTLAAGTAGC |
| PpPMMXI4F  | AATGGTCTGCTCAGTGGCTG |
| PpPMMXI4R  | TAAAGCCCAAGAACCAACCC |
| PpPMMXI5F  | CGGACAAGGAGCAAAGGAAG |
| PpPMMXI5R  | TCCCGCATGACACATTAGG |
| ACTproto   | CACCACACCTTCTAACAAGCAGTGGGTG |
| DHA5probe  | GAGAAAGTGGTGAAGATGGTTCAG |
| DAKprobe   | TATCTGCACTCCGTGAGTGCAG |
| PEXsprobe  | GGCGAGATAAAGGGGCCTTGCAAC |
| FLDprobe   | ATCTCCACGCTCCCTCCTTGCAGG |
| PpPGHprobe | GACTCACTAGCACCAACCAAGATTG |
| PpPEX8probe | TGGCCTTGGATTACGTTGAAGT |
| PpPEXI4probe | GCCATCCTCCTCCTTCCTTCC |
| PpPEXI5probe | GCTGTGACAAGGAAGGATTTACC |
| PpFLD2F   | TTGCGACTGCTCTCCTCCT |
| PpFLD2R   | TTTGCGATTCTTCAAGGGTATT |
| PpFLDF     | ACCTGAGCTTAAAGACCAT |
| PpFLDR     | CACCGGGGTCTCCCTTCC |
| delAF      | AAACGCAAATAGCCCAAAG |
| delAR      | TTTGCGATTTGGTGTGTTG |
| delBF      | GGAATCTGTTGCTAACCC |
| delBR      | GAAACGTTATCCCAAG |
| delCF      | GCCTAAGCACAATTTGAG |
| delCR      | AAGTTGCTGTAGGCTACATCAG |
| delDR      | GGAGTTCACTCCTCCT |

2.5. Molecular Methods. The transformation of bacteria and isolation of plasmid DNA from \( E. \) \( coli \) was carried out in...
accordance with standard methods [20]. The isolation of DNA and yeast transformation was carried out according to [21, 22]. All plasmids that were used in this work carry HIS4 gene as a selective marker; consequently media without histidine were used for selection of transformed clones.

PCR was performed according to the protocol of the manufacturer of reagents (Thermoscientific).

During real-time PCR experiments total RNA was isolated from an equal number of cells in each culture according to [23]. RNA was treated with DNase; then cDNA was synthesized by reverse transcription and used as a template for real-time PCR. ACT1 was used as a reference gene. Real-time PCR was performed using an ANK-32 nucleic acids amplifier (Synthol, Russia) and TaqMan technology as follows: 3 min at 95°C, followed by 40 cycles for 30 s at 95°C and 30 s at 60°C. The annealing temperature for all primers was 60°C. All real-time PCR experiments were set at least in triplicate.

DNA hydrolysis with restriction endonucleases, dephosphorylation of vectors, and DNA ligation were performed using the buffers and conditions recommended by the manufacturer of the enzymes (Thermoscientific). Electrophoresis of DNA and purification of DNA from agarose gels were performed according to [20].

ACP activity was determined qualitatively [11] and quantitatively [24]. The specific activity of ACP was designated as the ratio of the optical density at 410 nm to the density of cell suspension at 550 nm.

Statistical analysis was performed using the Statistic program.

3. Results

3.1. Effect of Nitrogen Source and Phosphate Concentration on Expression of Genes Involved in Methanol Utilization (MUT Genes). In previous study it was found that type of nitrogen source presented in the medium influences the expression levels of AOX and PpcAT1 genes, which encode enzymes involved in first steps of methanol utilization [10]. In this study we investigated regulation of other enzyme coding genes: PpDAK, PpDHAS, PpFLD, and PpFGH. Another group of studied genes consisted of PpPEX5 and PpPEX14, encoding peroxisomal structural proteins, and PpPEX1 and PpPEX8 genes encoding proteins, involved in biogenesis and degradation of peroxisomes. Real-time PCR, which enables a quantitative analysis of gene expression levels, was used to investigate the effect of the nitrogen source and phosphate concentration on expression of chosen MUT genes.

To study the effect of the nitrogen source cells of the parental GS115 P. pastoris strain were grown for 20 hours to the log phase in MPN and MPL media containing methanol as a carbon source. Ammonium sulfate was chosen as nitrogen source. KH2PO4 concentrations used were 0.03 g/L (MPL) and 1 g/L (MPN), because they demonstrated the most contrasting results in previous experiments.

Investigation of MUT and PEX genes regulation by carbon source [25] and our experiments with nitrogen sources allowed proposing that their activity is controlled by common transcription factors and is regulated in similar way under different nutrient conditions. That is why in this experiment studied genes were limited to PpDAK, PpDHAS, and PpFLD genes for MUT genes and PpPEX5 for PEX genes. Results of a quantitative analysis of the relative expression levels of PpDAK, PpDHAS, PpFLD, and PpPEX5 genes depending on the phosphate concentration in the medium are shown in Figure 2.

The expression of all studied genes in medium with ammonium sulphate was significantly higher than in medium with proline as a nitrogen source. This goes together with the results obtained in previous studies for AOX and PpcAT1 genes. Thus, it was demonstrated that regulation of main MUT and PEX genes expression depends on the type of nitrogen source in the media and is established at the transcriptional level.

To study the effect of phosphate concentration on expression of MUT genes cells of the parental GS115 strain were grown for 20 hours to the log phase in MPN and MPL media containing methanol as a carbon source. Ammonium sulfate was chosen as nitrogen source. KH2PO4 concentrations used were 0.03 g/L (MPL) and 1 g/L (MPN), because they demonstrated the most contrasting results in previous experiments.

Investigation of MUT and PEX genes regulation by carbon source [25] and our experiments with nitrogen sources allowed proposing that their activity is controlled by common transcription factors and is regulated in similar way under different nutrient conditions. That is why in this experiment studied genes were limited to PpDAK, PpDHAS, and PpFLD genes for MUT genes and PpPEX5 for PEX genes. Results of a quantitative analysis of the relative expression levels of PpDAK, PpDHAS, PpFLD, and PpPEX5 genes depending on the phosphate concentration in the medium are shown in Figure 2.

Thus, it was demonstrated that regulation of PpDAK, PpDHAS, PpFLD, and PpPEX5 genes also depends on phosphate concentration in the medium and is established at the transcriptional level.

3.2. Effect of Nitrogen Source and Phosphate Concentration on Activity of AOX1 and AOX2 Promoters. The protein-coding regions of the AOX1 and AOX2 genes for 90% [26] are identical. Thus it is very difficult to analyze their regulation separately using real-time PCR. Creation of convenient
reporter systems was essential for solving this problem. tr2-1-GSI15 P. pastoris strain carrying a reporter acid phosphatase (ACP) PHO5 gene of S. cerevisiae under the control of AOX1 gene promoter was constructed in previous studies [10]. A similar strain tr3-1-GSI15 carrying PHO5 gene under control of AOX2 promoter was generated in this study.

To investigate the effect of nitrogen source on the activity of AOX2 promoter tr2-1-GSI15 and tr3-1-GSI15 P. pastoris strains were grown for 40 hours to the stationary phase in MPN and MPP media containing methanol as a carbon source. tr2-1-GSI15 strain carrying PHO5 gene under the control of AOX1 gene promoter was used as a control. The specific activity of ACP was measured in the yeast culture (Figure 3(a)).

The figure shows that the expression of a reporter gene in both strains depends on nitrogen source. The highest ACP activity for both strains was detected in media with high phosphate concentration (1 g/L). ACP activity was almost two times lower in media with only 0.03 mg/L of KH$_2$PO$_4$. These data suggest that activity of AOX1 and AOX2 promoters depends on phosphate concentration in medium and is regulated in similar way.

### 3.3. Deletion Analysis of AOX1 Promoter

In previous studies deletion analysis of the promoter was carried out to identify AOX1 promoter regions involved in nitrogen regulation. In trΔNsrI-1-GSI15 strain, containing PHO5 gene under the control of AOX1 promoter carrying a 671-nucleotide truncation, regulation of expression ACP activity by nitrogen source still remained [10].

Thus a 267-nucleotide region was found to be sufficient to establish AOX1 promoter regulation by the nitrogen source. In this study this region was covered with a series of four deletions: A from −296 to −216, B from −222 to −188, C from −205 to −45, and D from −80 to −0. P. pastoris strains trA-1-GSI15, trB-1-GSI15, trC-1-GSI15, and trD-1-GSI15 which carry a reporter PHO5 gene under the control of AOX1 gene promoter with desired deletions were obtained (Figure 4(a)). These strains were grown for 40 hours to the stationary phase in MPN and MPP media containing methanol as a carbon source. tr2-1-GSI15 strain carrying PHO5 gene under the control of native AOX1 promoter was used as a control.

Figure 4(b) shows that for trA-1-GSI15, trB-1-GSI15, and trD-1-GSI15 strains the highest ACP specific activity was observed in medium with ammonium sulfate. The ACP specific activity of all three strains was significantly lower in medium with poor nitrogen source proline, suggesting that these deletions do not influence regulation of AOX1 promoter by nitrogen source. In the case of trC-1-GSI15 strain ACP specific activity was extremely low and did not allow us to get reliable results. This can be explained by that fact that the deletion in C fragment of AOX1 gene carried by this strain contains the main binding site for Mxr1 protein which is known to be the main regulator of MUT genes and is essential for methanol induction [27].

To study if the effect of phosphate concentration on regulation of AOX1 promoter is affected by different deletions we used trΔSacI-1-GSI15 and trΔNsrI-1-GSI15 strains that were created previously. These strains contain 207- and 671-nucleotide truncations in AOX1 promoter, respectively (Figure 4(a)). They were grown for 40 hours to the stationary phase in MPN and MPP media containing methanol as a carbon source. tr2-1-GSI15 strain carrying PHO5 gene under the control of native AOX1 promoter was used as a control.

Figure 4(c) shows that the overall ACP specific activity of trΔSacI-1-GSI15 strain is increased in media with both high and low phosphate concentrations in comparison with control strain. This fits with the data obtained in study, where it was shown that this region may contain a regulatory
element involved in AOX1 promoter repression. The ACP specific activity level of trΔSacI-1-GS115 in medium with high concentration of phosphate did not show statistically significant difference with the one observed in medium with low concentration. This allows proposing that regions of AOX1 promoter affected by SacI truncation may be involved in establishing its regulation by phosphate concentration in medium. ACP activity level of trΔNsiI-1-GS115 strain was significantly lower than the one observed for control strain and did not show statistically significant differences in MPN and MPL media.

3.4. Effect of Rapamycin on AOX1 Promoter Regulation. Kinase inhibitors can provide crucial information about these regulation pathways. In this study we analyzed effect of rapamycin on P. pastoris growth and on regulation of AOX1 gene. To determine active concentrations of rapamycin 10-fold dilutions of P. pastoris GS115 culture were placed on YPD medium with different concentrations of rapamycin. It was found that 10 nM concentration is more than enough to inhibit cell growth (Figure 5(a)). These results totally fit with ones used for S. cerevisiae [28].

On the next step rapamycin effect on AOX1 regulation by nitrogen source was investigated. Cultivation was done in two stages due to the fact that rapamycin inhibits P. pastoris growth. On the first stage cells of P. pastoris tr2-1-GS115 strain were grown for 40 hours to the stationary phase in MPN and MPP media containing 1% glycerol as a carbon source to obtain biomass. After that cultures were centrifuged and transferred into MPN and MPP media containing 1% methanol for induction of AOX1 promoter and 10 nM rapamycin. ACP specific activity was measured after 40 hours of induction (Figure 5(b)). Addition of rapamycin to the media completely removed repression of AOX1 promoter in medium with proline.

4. Discussion

We show here that expression of main genes involved in methanol utilization (e.g., AOX1, AOX2, PpDAK, PpDHAS, PpFLD, and PpFGH) and peroxisomal genes (PpPEXI, PpPEX5, PpPEX8, and PpPEX14) is tightly regulated depending on nitrogen source. This regulation is established on transcriptional level. In medium with ammonium sulphate expression levels of studied genes were notably higher than in medium with proline as a nitrogen source.

Deletions in AOX1 promoter affected its activity but did not change promoter regulation by nitrogen source. A deletion in C region of promoter (from −205 to −45) leads to such loss of activity that we were not able to retrieve statistically significant results. This region contains one of binding sites for Mxr1 protein and is essential for induction of AOX1 gene in medium with methanol [27, 29, 30]. It can be proposed that regulation of AOX1 promoter is established not by binding a separate transcriptional factor, involved in nitrogen regulation but on the higher level by modulation of Mxr1p activity. Our results obtained from experiment with ACP reporter systems using AOX1 and AOX2 promoter may serve as an indirect proof of this proposal. Although the promoter
regions of these genes are absolutely different, they are regulated by nitrogen source in the same manner.

Rapamycin treatment completely removed regulation of AOX1 promoter by nitrogen source, which implies that Tor-kinase plays a key role in establishing this regulation. Addition of rapamycin slightly decreased AOX1 promoter activity levels when cells were grown in medium with ammonium sulphate, while rapamycin treatment of cells grown in medium with proline increased PAOX1 activity to the level compared with the one observed in medium with ammonium sulphate. It may be proposed that AOX1 gene expression is regulated depending on a nitrogen source by a repression mechanism. Recently a 14-3-3 protein that mediates Mxr1p activity and inhibits the expression of genes involved in methanol utilization was found in P. pastoris [31,32]. This protein shows similarity to S. cerevisiae Bmh1p, which is involved in regulation of exocytosis, vesicle transport, Ras/MAPK, and rapamycin-sensitive signaling [33].

A model based on our observations is presented in Figure 6. P. pastoris cells discriminate the type of nitrogen source in the medium and modify gene expression via Tor-signalling pathway. Tor-kinase complex modifies activity of transcriptional factors involved in AOX1 regulation, conceivably 14-3-3 protein that was found to interact with Mxr1p and repress AOX1. This mechanism provides maximum induction of AOX1 promoter in medium with ammonium sulphate and methanol. When proline is used as a nitrogen source AOX1 activity is repressed to optimum level. It is known that proline can be used by some fungi as a sole carbon source [34]. The ability of P. pastoris to use proline as a carbon source is a subject for further investigation.

Apart from nitrogen regulation of genes involved in methanol utilization and peroxisome biogenesis, it was found that these genes also respond to phosphate limitation. Expression levels of PpDAK, PpDAS, PpFLD, and PpPEX5 genes are increased in media with high concentrations of phosphate.

**Figure 4:** (a) Strains created in this study carry PHO5 reporter gene under control of different variants of AOX1 promoter: tr2-1-GS115 carries a native promoter, trΔSacI-1-GS115—a variant truncated in SacI site (−737), trΔNsiI-1-GS115— a variant truncated in NsiI site (−267), trA-1-GS115 carries AOX1 promoter with deletion from −296 to −216, trB-1-GS115 from −222 to −188, trC-1-GS115 from −205 to −45, and trD-1-GS115 from −80 to −0 nucleotides. (b) ACP specific activity of P. pastoris strains trA-1-GS115, trB-1-GS115, trC-1-GS115, trD-1-GS115, and tr2-1-GS115 in media with different nitrogen sources (MN with ammonium sulphate and MP with proline). (c) ACP specific activity of P. pastoris strains trΔSacI-1-GS115, trΔNsiI-1-GS115, and tr2-1-GS115 in media with different concentrations of KH$_2$PO$_4$ (MN with 1 g/L and in ML with 0.03 g/L).
Deletion analysis of *AOX1* promoter has shown that a truncation in *SacI* site changes its regulation by phosphate concentration. Further investigation of this promoter region should be carried out to find if it is involved in establishing of *AOX1* phosphate regulation.

Methylotrophic yeast *P. pastoris* is widely used for production of various heterologous proteins. In biotechnology it is very important to maintain the balance between high levels of heterologous gene expression and cell viability. Decisive understanding of gene regulation mechanisms is essential for reaching this goal. Our result shows that *P. pastoris* have developed a regulation system which coordinates expression of main genes involved in methanol utilization and biogenesis of peroxisomes under different nutrient conditions. This system provides optimal transcriptional levels of *MUT* and *PEX* genes in media with different nitrogen sources or during phosphate limitation.

5. Conclusions

Expression levels of main genes involved in methanol utilization (e.g., *AOX1*, *AOX2*, *PpDAK*, *PpDHAS*, *PpFLD*, and *PpFGH*) and peroxisomal genes (*PpPEX1*, *PpPEX5*, *PpPEX8*, and *PpPEX14*) in *P. pastoris* are notably higher when ammonium sulphate is used as nitrogen source in comparison to proline. Rapamycin treatment removes repression of *AOX1* promoter in medium with proline.

Expression levels of *PpDAK*, *PpDAS*, *PpFLD*, and *PpPEX5* genes are increased in medium with high concentrations of phosphate.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research has been supported by Russian Federation president’s grant for leading scientific institutions no. 1.10.359.2014 and by Saint Petersburg State University under Grants nos. 1.0.131.2010 and 1.38.229.2014.

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