https://doi.org/10.17816/ecogen17447-55

THE ANALYSIS OF THE POLYAMINE OXIDASE GENES IN THE METHYLOTROPHIC YEAST *KOMAGATAELLA PHAFFII*

© A.V. Ivanova, A.V. Sidorin, E.V. Sambuk, A.M. Rumyantsev

St. Petersburg State University, Saint Petersburg, Russia

Cite this article as: Ivanova AV, Sidorin AV, Sambuk EV, Rumyantsev AM. The analysis of the polyamine oxidase genes in the methylotrophic yeast *Komagataella phaffii*. *Ecological genetics*. 2019;17(4):47-55. https://doi.org/10.17816/ecogen17447-55.

Received: *17.06.2019* Revised: *02.12.2019* Accepted: *17.12.2019*

❀ Polyamines are present in all living cells and regulate a wide range of biological processes. In *Saccharomyces cerevisiae* the polyamine oxidase Fms1p converts spermine to spermidine and 3-aminopropionaldehyde, which is necessary for the synthesis of pantothenic acid and hypusination. This paper shows that *S. cerevisiae FMS1* gene orthologs are present in all major representatives of the *Saccharomycotina* subdivision, but their copy numbers are different. In the *Komagataella phaffii* (*Pichia pastoris*) yeast, two polyamine oxidase genes (*KpFMS1* and *KpFMS2*) were identified, and the regulation of their promoters activity was studied.

❀ **Keywords:** polyamine oxidases; methylotrophic yeast; *Komagataella phaffii*; *Pichia pastoris*.

АНАЛИЗ ГЕНОВ ПОЛИАМИНОКСИДАЗ У МЕТИЛОТРОФНЫХ ДРОЖЖЕЙ *KOMAGATAELLA PHAFFII*

© А.В. Иванова, А.В. Сидорин, Е.В. Самбук, А.М. Румянцев

ФГБОУ ВО «Санкт-Петербургский государственный университет», Санкт-Петербург

Для цитирования: Иванова А.В., Сидорин А.В., Самбук Е.В., Румянцев А.М. Анализ генов полиаминоксидаз у метилотрофных дрожжей *Komagataella phaffii* // Экологическая генетика. – 2019. – Т. 17. – № 4. – С. 47–55. https://doi.org/10.17816/ecogen17447-55.

Поступила: *17.06.2019* Одобрена: *02.12.2019* Принята: *17.12.2019*

❀ Полиамины присутствуют во всех живых клетках и регулируют широкий спектр биологических процессов. У дрожжей *Saccharomyces cerevisiae* полиаминоксидаза Fms1p превращает спермин в спермидин и 3-аминопропаналь, что необходимо для синтеза пантотеновой кислоты и гипузинирования. В данной работе показано, что ортологи гена *FMS1* дрожжей *S. cerevisiae* присутствуют у всех основных представителей подотдела *Saccharomycotina*, однако их копийность различна. У дрожжей *Komagataella phaffii* (*Pichia pastoris*) идентифицированы два гена полиаминоксидаз (*KpFMS1* и *KpFMS2*) и изучена регуляция активности их промоторов.

❀ **Ключевые слова:** полиаминоксидазы; метилотрофные дрожжи; *Komagataella phaffii*; *Pichia pastoris*.

INTRODUCTION

Polyamines are a group of aliphatic polycations which are present in prokaryotes and eukaryotes. Putrescine, spermidine and spermine are the most commonly found polyamines in higher eukaryotes, including fungi. The functions of polyamines are determined by their chemical structure. Polyamines are positively charged at physiological pH, and due to electrostatic and hydrophobic interactions they can act as ligands, binding to DNA, RNA, proteins, phospholipids and nucleoside triphosphates [1, 2]. Polyamines are considered to be involved in the regulation of gene expression by altering the DNA structure, modulating the signal pathways or binding to the transcription factors. Therefore, polyamines are redundant in all living organisms. Polyamine deficiency leads to the cessation of cell growth, however excessive accumulation of these compounds can be cytotoxic, and therefore strict regulation of polyamine intracellular levels is needed [2, 3]. The present interest in polyamines is connected with their possible use as antiproliferative compounds.

The intracellular level of polyamines is controlled by a complex of biosynthetic (ornithine decarboxylase, S-adenosylmethionine decarboxylase, spermidine and spermine synthases) and catabolic (spermidine/spermine acetyltransferase, FAD-containing polyamine oxidase and copper-containing diamine oxidase) enzymes [2]. Polyamine oxidase is likely to be of particular interest, since it catalyzes the convertion of spermine to spermidine and 3-aminopropanal. The oxidation of 3-aminopropanal is required for *S. cerevisiae* for the biosynthesis of pantothenic acid [4], while spermidine is involved in the reaction of hipusination (the essential modification of the translation factor eIF-5A) [5].

The phylogenetic analysis of polyamine oxidases was performed for animals and plants. It has been discovered that during the evolution of animals, the ancestral gene of polyamine oxidase (PAO) was duplicated, and as a result, two protein paralogs of vertebrates emerged, encoded by SMO and APAO genes [6]. Additionally, the genome of *Arabidopsis thaliana* encodes for at least five polyamine oxidases. The analysis of the substrate specificity revealed that polyamine oxidase AtPAO13 oxidizes spermidine and spermine producing H_2O_2. Equally, it has been shown that polyamine oxidases of *A. thaliana* AtPAO2, AtPAO3 and AtPAO4 are localized in peroxisomes. One of the animal PAOs carries a peroxisomal targeting signal and is likely to be localized in these organelles.

In fungi polyamines regulate a wide range of biological phenomena: dimorphism, spore germination and the formation of appresorium. In several cases they control the virulence of fungal pathogens of animals and plants [8]. FAD – dependent (flavin adenine dinucleotide) polyamine oxidase Fms1p of *Saccharomyces cerevisiae* is studied in detail [9], and shown to be involved in β-alanine and pantothenate biosynthesis. Yeast mutants with *FMS1* deletion are unable to grow on media without pantothenic acid or β-alanine, while the overexpression of *FMS1* gene under the control of *ADH1* promoter leads to the secretion of pantothenic acid into the growth media [4]. At the same time, the number of polyamine oxidase genes varies among other members of the *Saccharomycotina* subdivision, and the correlation between the gene copy number of polyamine oxidase and the metabolism rate is not clear.

This work analyzes the orthologs of polyamine oxidases among the spp. of the *Saccharomycotina* subdivision. In this report we show that the methylotrophic yeasts *Komagataella phaffii* (*Pichia pastoris*) has two polyamine oxidase genes – *KpFMS1* and *KpFMS2*. The regulation of these genes have been studied.

MATERIALS AND METHODS

Comparison of the polyamine oxidase amino acid sequences

The orthologs of the *S. cerevisiae* Fms1 protein were found among the spp. of the *Saccharomycotina* group, using BLASTp algorithm with standard parameters [10]. The multiple alignment of the polyamine oxidase sequences was performed in MEGA X computer software [11]. The found sequences were aligned in CLUSTAL W [12]. The Maximum Likelihood (ML) method was used for the phylogenetic tree construction. The most appropriate model for this method was LG+G [13]. The construction of the ML tree was carried out using LG+G model with 500 bootstrap iterations. The final tree was rooted with the *Neurospora crassa* outgroup, and the tree was visualised in FigTree v1.4.3(http://influenza.bio.ed.ac.uk/software/Figtree/).

Primers

Primers used are listed in Table 1.

Table 1

Sequences of primers used in this work

Name	Sequence (5' → 3')
KpFMS1F	AAAGACGTCAGGGTACACGGTATTGTGAGA
KpFMS1R	AATGGATCCTGATAGCCGATTGCAATGTT
KpFMS2F	AAAAAGACGTCGTTTCGAATAATTAGTTGTT
KpFMS2R	AATGGATCCGTTGGTATTGTGAAATAGACG
PHO5R	CGGAATTCCAAAACTATTGT

Plasmids

pAL2-T (Evrogen, Russia) and pPIC9-AOX1-PHO5 [14] vectors were used in this work.

Strains

Yeast strains *K. phaffii* 4-GS115 (his4 phox) and tr2-4-GS115 (*phox PAOX1-PHO5*) [14], and the bacterial strain *E. coli* DH5α, (*fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44* Φ*80*Δ (*lacZ*)*M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*) were used in this work.

Media and culture conditions

For the cultivation of the yeast strains the following media were used. YEPD: 2% glucose, 2% peptone, 1% yeast extract, 2.4% agar; MPO (MPhO): 0.73 M $MgSO_4 \cdot 7H_2O$, 0.18 mM $CaCl_2$, 20 mM Sodium Citrate Buffer (pH 4.6), vitamins, trace elements. Carbon source (glycerol, methanol) – 1%, nitrogen source (ammonium sulfate) – 0.2%. For the cultivation of bacteria LB medium was used: 1% tryptone, 0.5% yeast extract, 170 mM NaCl, benzylpenicillin – $5 \cdot 10^5$ units of activity/l. The *K. phaffii* strains were cultivated at 30 °C, the *E.coli* – at 37 °C.

Construction of pPIC9-PKpFMS1-PHO5 and pPIC9-PKpFMS2-PHO5 plasmids

To construct pPIC9-PKpFMS1-PHO5 and pPIC9-PKpFMS2-PHO5 plasmids, polymerase chain reaction (PCR) was carried out with primers designed to specifically anneal to the promoter regions of *KpFMS1* (KpFMS1F and KpFMS1R) and *KpFMS2* (KpFMS2F and KpFMS2R) genes, with the chromosomal DNA of the *K. phaffii* 4-GS115 strain used as a template. The extraction of the chromosomal DNA from the yeast cells was carried out as described in [15]. The promoter sequences were cloned into pPIC9-PHO5 vector [14] us-

ing AatII and BamHI restriction sites, thus the promoter of alcohol oxidase gene (*AOX1*) was replaced by the promoters of the polyamine oxidase genes *KpFMS1* and *KpFMS2*. The transformation of bacteria was carried out according to [16]. As a result, the constructed plasmids contained the sequence of acid phosphatase (AP) *PHO5* reporter gene from *S. cerevisiae* under the control of *KpFMS1* and *KpFMS2* gene promoters. Moreover, these plasmids hold *HIS4* gene as a selective marker.

The structure of the final plasmids pPIC9-PKpFMS1-PHO5 and pPIC9-PKpFMS2-PHO5 was checked using PCR and restriction analysis.

The engineering of the PFMS1-4-GS115 and PFMS2-4-GS115 strains

The 4-GS115 strain was transformed with pPIC9-PKpFMS1-PHO5 and pPIC9-PKpFMS2-PHO5 plasmids. To that end, the plasmids underwent linearization by StuI restriction endonuclease, and the yeast cells were transformed via electroporation [17]. During electroporation, the genetic construction was incorporated into *K. phaffii* genome. The selection of transformants was performed by the restoration of histidine prototrophy. To analyze the integration of the plasmid into the genome, chromosomal DNA was extracted from the transformants, and PCR was carried out using *PAO* and *PHO5* specific primers (KpFMS1F and PHO5R for PFMS1-4-GS115; KpFMS2F and PHO5R for PFMS2-4-GS115).

Molecular methods

The hydrolysis of DNA was performed by BamHI, AatII and StuI restriction endonucleases following the manufacturer's recommendations (Thermo Fisher Scientific Inc., USA). The vectors were dephosphorylated using FastAP phosphatase (Thermo Fisher Scientific Inc., USA). The purification of DNA from agarose gels and reaction mixtures was accomplished with Cleanup Standard kits (Evrogen, Russia). The ligation of DNA fragments was performed by T4 DNA ligase (Evrogen, Russia). The plasmids were extracted using Plasmid Miniprep kits (Evrogen, Russia). To carry out PCR, Encyclo Plus PCR kits were used (Evrogen, Russia). The electrophoresis of DNA fragments was performed in the 0.7% agarose gel in TAE buffer [18].

Qualitative evaluation of the acid phosphatase activity

Paper filters were soaked in a solution containing α- naphthyl phosphate as the substrate and Fast Blue B Salt as a dye, diluted in 0.1 M citrate buffer pH 4.6 to 2 mg/ml. The wet filters were then placed on the surface of the medium containing grown yeast colonies. After 10 minutes the colony staining intensity was measured [19].

RESULTS

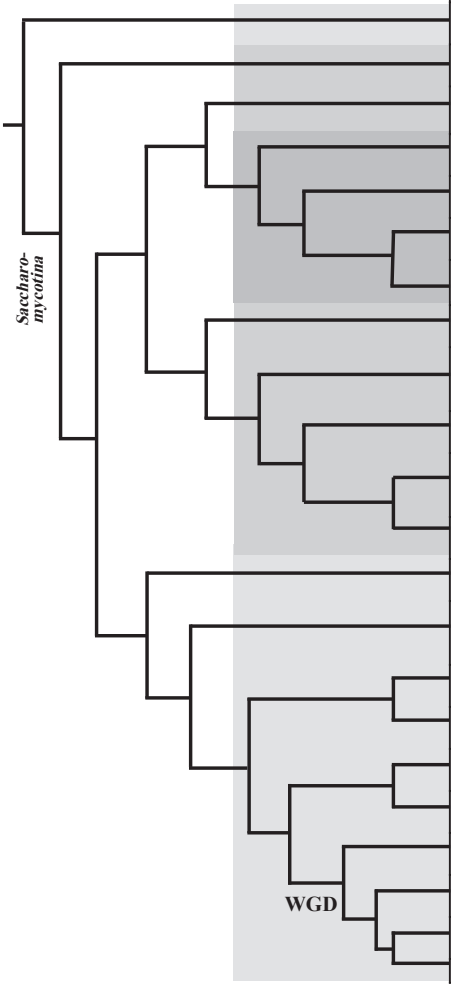
1. Bioinformatic analysis of *Saccharomycotina* yeast proteomic data

At the first stage of our work we studied the prevalence of polyamine oxidases among major representatives of *Saccharomycotina* subdivision (*Ascomycota* division). We searched for homologous proteins in the proteomes of 22 yeast species belonging to different families of this division and in one proteome of *Neurospora crassa* fungus (the representative of the *Pezizomycotina* subdivision, *Ascomycota* division). These data were obtained by means of BLAST analysis using the amino acid sequence of *S. cerevisiae* polyamine oxidase Fms1p as a template [9]. A similar approach was applied earlier in the search of polyamine oxidases in phytopathogenic fungi [8]. The results are shown in Fig. 1.

We identified that polyamine oxidases and corresponding genes are present in all investigated species from different families of the *Saccharomycotina* subdivision. Furthermore, the number of polyamine oxidase proteins and corresponding genes varies according to the systematic position of the yeast species: 1) the major representatives of *Ascoideaceae*, *Phaffomycetaceae*, *Saccharomycetaceae* families possess only one gene, 2) the representatives of *Debaryomycetaceae*, *Metschnikowiaceae* families and *Yarrowia* clade have two genes, 3) the representatives of *Pichiaceae* family possess three genes. An exception to this family is the yeast species *Komagataella phaffii* (better known as *Pichia pastoris*), who contains two genes. This yeast species is a representative of *Komagataella* clade regarded as the paraphyletic family *Pichiaceae* in the report [20].

Carrying out phylogenetic analysis with the use of the polyamine oxidase gene sequences was complicated due to the large differences among nucleotide sequences of the found genes. Hence, multiple comparisons of polyamine oxidase amino acid sequences were performed for all the investigated yeast species (Fig. 2).

The results enabled us to divide the amino acid sequences of polyamine oxidases into groups (PAO) based on their similarity. PAO1 group contains proteins found in representatives of the *Ascoideaceae*, *Phaffomycetaceae*, *Saccharomycetaceae* families, with only one polyamine oxidase gene identified in their proteomes. Whereas in PAO2.1 and PAO2.2 groups comprising proteins discovered in the representatives of the *Metschnikowiaceae* family, two polyamine oxidase genes were recognized. Finally, in PAO3.1, PAO3.2 and PAO3.3 groups comprising proteins found in the representatives of the *Pichiaceae* family, three genes were identified. The *Babjeviella inositovora* proteins (*Debaryomycetaceae*, two genes) also belong to these groups (PAO3.1 and PAO3.2). The results of the sequence multiple alignments within PAO



Species, strain	Num. of prot.	Protein 1	Protein 2	Protein 3	Family
Neurospora crassa	1	XP_960607.2	–	–	*Sordariaceae*
Yarrowia lipolytica	2	XP_501266.1	XP_503341.1	–	Clade *Yarrowia*
Komagataella phaffii GS115	2	XP_002493159.1	XP_002494272.1	–	Clade *Komagataella*
Kuraishia capsulata CBS 1993	3	XP_022459189.1	XP_022461193.1	XP_022459070.1	*Pichiaceae*
Ogataea parapolymorpha	3	XP_013933011.1	XP_013933775.1	XP_013933434.1	
Pichia membranifaciens NRRL Y-2026	3	XP_019019768.1	XP_019018091.1	XP_019016158.1	
Pichia kudriavzevii	3	XP_020546691.1	XP_020543008.1	XP_020542396.1	
Babjeviella inositovora NRRL Y-12698	2	XP_018983323.1	XP_018986710.1	–	*Debaryomycetaceae* *Metschnikowiaceae*
Metschnikowia bicuspidata var. bicuspidata NRRL YB-4993	2	XP_018713474.1	XP_018713421.1	–	
Scheffersomyces stipitis CBS 6054	2	XP_001383524.2	XP_001387500.2	–	
Candida albicans SC5314	2	XP_722661.1	XP_716457.1	–	
Candida tropicalis MYA-3404	2	XP_002546433.1	XP_002545464.1	–	
Ascoidea rubescens DSM 1968	1	XP_020047881.1	–	–	*Ascoideaceae*
Cyberlindnera jadinii NRRL Y-1542	1	XP_020072648.1	–	–	*Phaffomycetaceae*
Lachancea thermotolerans CBS 6340	1	XP_002553058.1	–	–	*Saccharomycetaceae*
Kluyveromyces lactis	1	XP_002999399.1	–	–	
Torulaspora delbrueckii	1	XP_003679995.1	–	–	
Zygosaccharomyces rouxii	1	XP_002499197.1	–	–	
[Candida] glabrata	1	XP_449679.1	–	–	
Kazachstania africana CBS 2517	1	XP_003954787.1	–	–	
Saccharomyces eubayanus	1	XP_018220082.1	–	–	
Saccharomyces cerevisiae	1	NP_013733.1	–	–	

Fig. 1. The prevalence of polyamine oxidases among the main representatives of the *Saccharomycotina* subdivision. Phylogenetic relationships are shown based on the results obtained in [21]. WGD refers to the whole-genomic duplication that the ancestors of *S. cerevisiae* and related species underwent [28]

groups generally reflect the phylogenetic relationships in corresponding families of the *Saccharomycotina* subdivision.

2. Bioinformatic analysis of the polyamine oxidase subcellular localization

A highly toxic by-product hydrogen peroxide is generated during reactions mediated by polyamine oxidases. Hence, several polyamine oxidases in plants and animals are directed to peroxisomes [7]. For all the investigated polyamine oxidases, we searched for the signal sequences that provide their transport to various cell organelles, primarily to peroxisomes. Two main types of signals are known to implement protein targeting to peroxisomes – PTS1 and PTS2 (Peroxisomal Targeting Signals). PTS1 is prevalent, it is found in 95% of the peroxisomal matrix proteins. Initially, the PTS1 signal was defined as a sequence comprising three amino acids at the C-terminus of a protein – SKL. However, it was subsequently shown that this signal can vary and thus it is a consensus sequence [21]. The polyamine oxidases were analyzed for having peroxisomal targeting signals, and the results are shown in Fig.2.

We report that two groups PAO2.2 and PAO3.2 can be distinguished among the studied polyamine oxidases, and their representatives often (in 6 out of 9 cases) possess a consensus C-terminal PTS1 signal. Meanwhile, three proteins have a sequence at the C-terminal end, not corresponding to the consensus signal but highly comparable. The polyamine oxidase sequences were analyzed in subcellular localization predictor server DeeLoc-1.0 (http://www.cbs.dtu.dk/services/DeepLoc/). In order to predict subcellular protein localization, this program uses machine learning methods instead of searching for known signal sequences in databases [22]. Aided by this algorithm we identified that the most probable subcellular localization outcome of *K. phaffii* polyamine oxidase XP_002494272.1 (*KpFMS2* gene) is the peroxisomes.

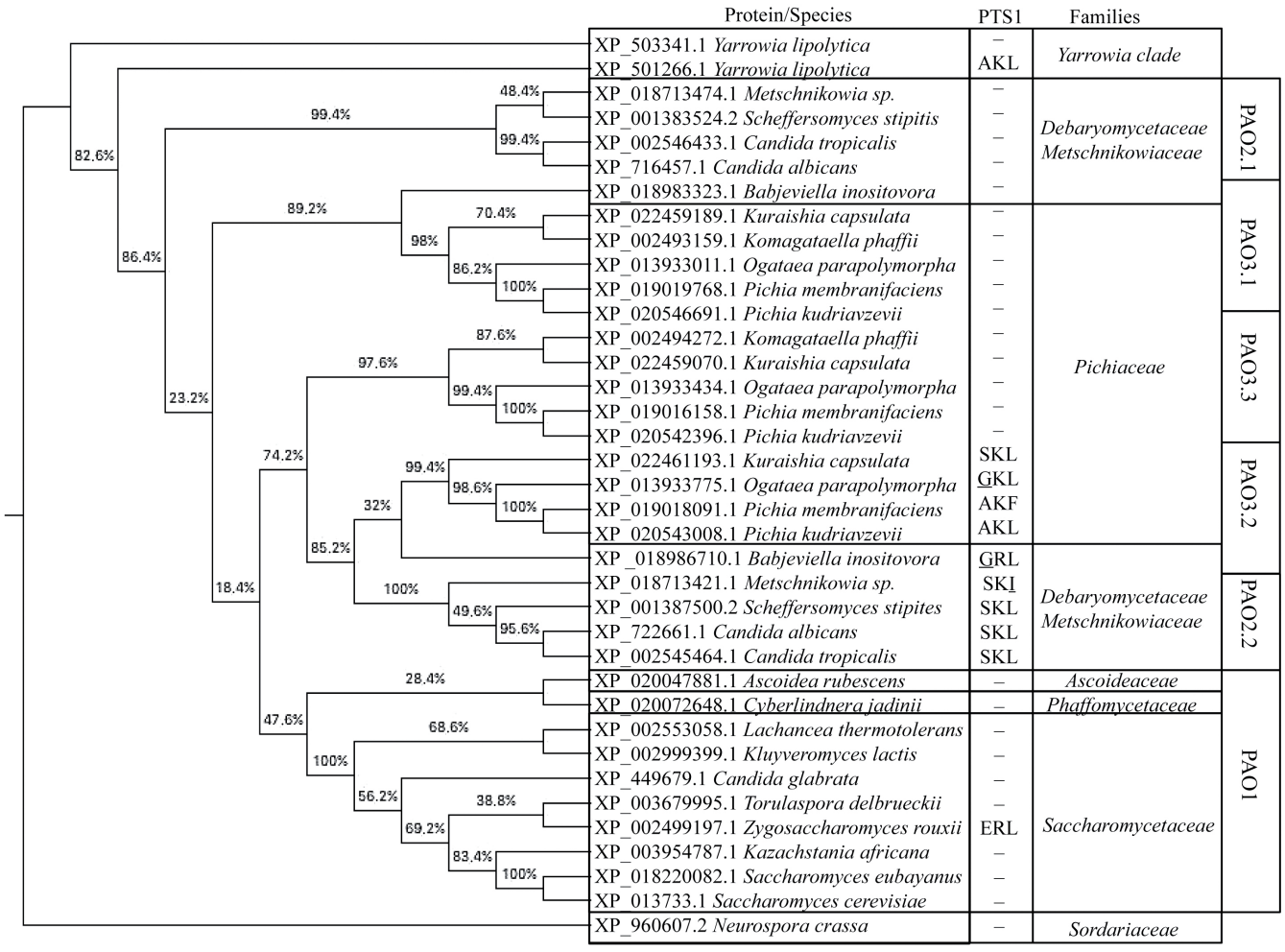


Fig. 2. The results of multiple comparisons of polyamine oxidases amino acid sequences from the studied yeast species. The table shows the PTS1 sequences at the C-terminus of polyamine oxidases, which provide the peroxisomal localization of proteins. The underlined amino acids differ from the consensus sequence known for *S. cerevisiae* [22]

K. phaffii yeasts studied in the current work are methylotrophic microorganisms, i. e. they are capable of utilizing methanol as the only carbon and energy source. The first steps in methanol metabolic pathway occur in peroxisomes, which is also connected with the generation of hydrogen peroxide by alcohol oxidases during methanol oxidation to formaldehyde [23]. The analysis of *K. phaffii* genome has shown two unlinked polyamine oxidase genes *KpFMS1* and *KpFMS2*, with *KpFMS2* located close to alcohol oxidase 1 gene (*AOX1*). Besides, *KpFMS2* and *AOX1* genes are arranged head to head, and their promoter regions may partially share a sequence (Fig. 3), feasibly affecting the regulation of *KpFMS2* transcription.

3. Comparative study of the *KpFMS1* and *KpFMS2* gene expression in *K. phaffii* yeast

In order to study the regulation of the activity of polyamine oxidase gene promoter, we developed PFMS1-4-GS115 and PFMS2-4-GS115 strains, each containing the reporter gene sequence of acid phosphatase *PHO5* under the control of polyamine oxidase gene promoters. To compare *KpFMS1* and *AOX1* gene promoter activi-

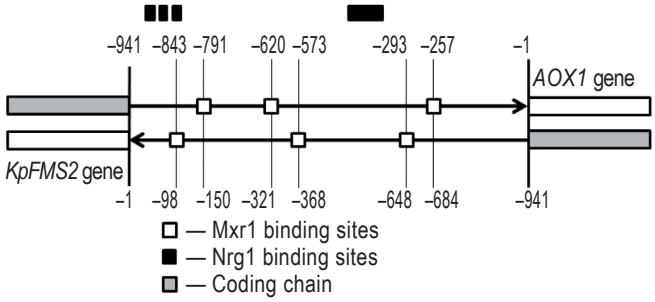


Fig. 3. The arrangement of *KpFMS2* and *AOX1 K. phaffii* genes. The binding sites of the transcription factors Mxr1p and Nrg1p (the main regulators of *AOX1* gene) are shown

ties, we used tr2-4-GS115 strain [14], which contains *PHO5* gene under the control of *AOX1* gene promoter. Serial dilutions of the strains were prepared and plated on agar media with different carbon and nitrogen sources. Glycerol and methanol were used as the sole carbon source. Ammonium sulfate was used as a nitrogen source. Besides, YEPD medium was used, containing glucose, yeast extract and peptone. The results of the qualitative analysis of the reporter gene *PHO5* activity in PFMS1-4-GS115, PFMS2-4-GS115 and tr2-4-GS115 strains are shown in Fig. 4.

The activity of *KpFMS2* gene promoter was demonstrated on YEPD medium, where glucose was used as a carbon source, and the nitrogen source was a mixture of amino acids and oligopeptides. We did not observe the activity of *KpFMS1* gene promoter in these conditions, and as it was expected [24], *AOX1* gene was not expressed.

Polyamine oxidase genes promoters are not active in media with glycerol as a carbon source. The expression of *AOX1* gene was repressed by glycerol in the studied conditions.

KpFMS1 and *AOX1* gene promoters are active in media with methanol as a carbon source. The activity of *KpFMS1* gene promoter was not observed.

DISCUSSION

It is established that duplications play a significant role in the evolution of living organisms. Duplications create additional copies of the genetic material and contribute to genome diversity, and the fate of duplicated genes can vary. Mutations can accumulate whether in a coding or a regulatory region. As a result, duplicated genes can undergo the following processes throughout evolution: 1) conservation, during which both copies maintain functions of an ancestral gene, 2) neofunctionalization, during which one gene develops a new function, while another preserves ancestral functions, 3) subfunctionalization, during which both copies acquire different functions and work together in order to compensate functions of an ancestral gene, and 4) specialization, when two copies gain different functions, and their common function is not similar to the ancestral one (in other words, it is a combination of neofunctionalization and subfunctionalization processes) [25, 26]. Moreover, structural genes can become a part of other regulons due to the changes in regulatory regions, developing the capability of responding to new signals via interactions with new regulatory factors.

In the current work, for the first time, we performed a bioinformatic analysis of the polyamine oxidase distribution among major representatives of the *Saccharomycotina* subdivision (*Ascomycota* division). We showed that all the studied species have polyamine oxidases and corresponding genes; however, their copy number varies. Besides, we demonstrated that the copy number correlates with the systematic position of the investigated yeast species. It is interesting that representatives of the *Saccharomycetaceae* family (*S. cerevisiae* yeast species) and the related *Ascoideaceae*, *Phaffomycetaceae* families contain a single polyamine oxidase gene. It should be noted that the ancestors of *S. cerevisiae* underwent the whole-genome duplication [27]. Accordingly, one of the two polyamine oxidase genes evolved after duplication in *S. cerevisiae*, and was subsequently lost, nevertheless, a single copy is sufficient to maintain the function.

Meanwhile, in other families of the *Saccharomycotina* subdivision representatives retained two or even three polyamine oxidase genes. The sequences of these genes diverged significantly, while the expressed proteins are supposed to have different subcellular localization. One polyamine oxidase (PAO2.2 and PAO3.2 groups) was found in most of the studied species (representatives of the *Debaryomycetaceae*, *Metschnikowiaceae*, *Pichiaceae* families and *Yarrowia* clade) containing a peroxisome targeting sequence. Polyamine oxidases from *B. inositovora*, *Metschnikowia spp.* and *Ogataea parapolymorpha*, (PAO2.2 and PAO3.2 groups), bear at their C-terminal end similar sequences to PTS1 of *S. cerevisiae* but not corresponding with consensus. Further analysis of polyamine oxidase subcellular localization will expand the set of peroxisomal targeting signals known for yeasts.

The differences discovered in the frequency of polyamine oxidase genes may be associated with peculiarities of metabolism in different yeast species. The study

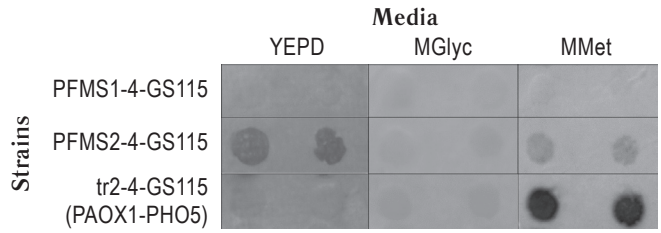


Fig. 4. The activity of reporter acid phosphatase synthesized by *K. phaffii* PFMS1-4-GS115, PFMS2-4-GS115 and tr2-4-GS115 strains during their growth on the media with different carbon and nitrogen sources

of the polyamine oxidase gene regulation in *K. phaffii* methylotrophic yeasts is of great interest in this regard. This yeast species is capable of using methanol as the only source of carbon and energy, and alcohol oxidase catalyzes the first chemical reaction of methanol metabolism. This enzyme functions within peroxisomes, since its activity is associated with the generation of the cytotoxic by-product hydrogen peroxide. Alcohol oxidase 1 gene is strictly regulated by the carbon source in the medium. *AOX1* gene is repressed in media containing glucose and glycerol, while *AOX1* promoter is induced in media with methanol [24]. The Mxr1p transcription factor is the main regulator of the *AOX1* gene promoter activity [28].

In this work we showed that polyamine oxidase *KpFMS2* gene of *K. phaffii* is located close to *AOX1* gene, and they are arranged head to head. The binding sites of Mxr15'-CYCC-3' protein are distributed throughout the region between the coding sequences of *KpFMS2* and *AOX1* genes on both strands (Fig. 3). Nrg1p protein acts as a repressor of *AOX1* gene in *K. phaffii* in the presence of either glycerol or glucose in the media. The first region with which Nrg1p interacts is situated close to *KpFMS2* coding sequence, while the second one is located close to *AOX1* gene (Fig. 3). Therefore, the promoter regions of these genes may partially share a sequence, i. e. they may share similar elements of regulation.

In this work it was shown for the first time that *KpFMS2* gene promoter (sharing a sequence with *AOX1* gene promoter and providing transcription in the opposite direction) functions in media with methanol. The level of its activity was noticeably lower than of *AOX1* gene promoter. *KpFMS2* gene promoter, as well as *AOX1* gene promoter, was inactive in media with glycerol as a source of carbon. However, at the same time *KpFMS2* gene promoter was active in YEPD medium. In other words, unlike *AOX1* gene promoter its activity was not suppressed by glucose in case of *K. phaffii* growth on this medium.

Thus, *KpFMS2* gene appeared to be linked with *AOX1* gene as a result of chromosomal rearrangements. While this gene preserves its own regulation features, its promoter is regulated similarily as *AOX1* gene promoter and the other genes of the methanol metabolic pathway that form *MUT*-regulon. On the one hand, it can be a side effect of the remarkably high activity of *AOX1* gene promoter. In fact, eukaryotic promoters can provide transcription of long noncoding RNAs [31]. On the other hand, the involvement of *KpFMS2* gene into the *MUT*-regulon may be established during the course of evolution. The fact supporting this idea is that PAO3.3 group of polyamine oxidases including KpFMS2 protein is close to PAO3.2 group containing proteins with PTS1 signals. In spite of the fact that KpFMS2 protein does not possess such a sequence, its targeting to peroxisomes is predicted with means of DeepLoc-1.0 server. The *MUT*-regulon genes are expressed when *K. phaffii* is growing in methanol containing media during which, peroxisomes are actively formed and functional. The involvement of *KpFMS2* gene into the MUT-regulon probably emerge to be beneficial and was selected throughout evolution.

The activity of *KpFMS1* gene promoter was not observed in the studied conditions. It may be the consequence of the promoter complete inactivity or its putative activity under specific conditions. Further investigation of *K.phaffii* polyamine oxidase gene regulation and function is important both from a theoretical point of view in order to understand the evolution of duplicated genes, and practically, according to the immense significance of these genes and the MUT-regulon promoters for biotechnology.

Acknowledgements

This work was supported by RFBR № 18-34-00750 mol_a grant.

REFERENCES

1. Wallace HM, Fraser AV, Hughes A. A perspective of polyamine metabolism. *Biochem J*. 2003;376(Pt 1):1-14. https://doi.org/10.1042/BJ20031327.
2. Miller-Fleming L, Olin-Sandoval V, Campbell K, Ralser M. Remaining mysteries of molecular biology: the role of polyamines in the cell. *J Mol Biol*. 2015;427(21):3389-3406. https://doi.org/10.1016/j.jmb.2015.06.020.
3. Wallace HM. Polyamines: specific metabolic regulators or multifunctional polycations? *Biochem Soc Trans*. 1998;26(4):569-571. https://doi.org/10.1042/bst0260569.
4. White WH, Gunyuzlu PL, Toyn JH. *Saccharomyces cerevisiae* is capable of de novo pantothenic acid biosynthesis involving a novel pathway of beta-alanine production from spermine. *J Biol Chem*. 2001;276(14):10794-10800. https://doi.org/10.1074/jbc.M009804200.
5. Chattopadhyay MK, Tabor CW, Tabor H. Spermidine but not spermine is essential for hypusine biosynthesis and growth in *Saccharomyces cerevisiae*: spermine is converted to spermidine *in vivo* by the FMS1-amine oxidase. *Proc Natl Acad Sci USA*. 2003;100(24):13869-74. https://doi.org/10.1073/pnas.1835918100.
6. Polticelli F, Salvi D, Mariottini P, et al. Molecular evolution of the polyamine oxidase gene family in Metazoa. *BMC Evol Biol*. 2012;12:90. https://doi.org/10.1186/1471-2148-12-90.
7. Reumann S, Ma C, Lemke S, Babujee L. AraPerox. A database of putative Arabidopsis proteins from plant

peroxisomes. *Plant Physiol*. 2004;136(1):2587-2608. https://doi.org/10.1104/pp.104.043695.
8. Valdes-Santiago L, Cervantes-Chavez JA, Leon-Ramirez CG, Ruiz-Herrera J. Polyamine metabolism in fungi with emphasis on phytopathogenic species. *J Amino Acids*. 2012;2012:837932. https://doi.org/10.1155/2012/837932.
9. Landry J, Sternglanz R. Yeast Fms1 is a FAD-utilizing polyamine oxidase. *Biochem Biophys Res Commun*. 2003;303(3):771-776. https://doi.org/10.1016/s0006-291x(03)00416-9.
10. Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. *J Mol Biol*. 1990;215(3):403-410. https://doi.org/10.1016/S0022-2836(05)80360-2.
11. Kumar S, Stecher G, Li M, et al. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol*. 2018;35(6):1547-1549. https://doi.org/10.1093/molbev/msy096.
12. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 1994;22(22):4673-4680. https://doi.org/10.1093/nar/22.22.4673.
13. Le SQ, Gascuel O. An improved general amino acid replacement matrix. *Mol Biol Evol*. 2008;25(7):1307-20. https://doi.org/10.1093/molbev/msn067.
14. Rumjantsev AM, Bondareva OV, Padkina MV, Sambuk EV. Effect of nitrogen source and inorganic phosphate concentration on methanol utilization and PEX genes expression in *Pichia pastoris*. *Scientific World Journal*. 2014;2014:743615. https://doi.org/10.1155/2014/743615.
15. Guthrie C, Fink GR. Guide to yeast genetics and molecular biology. *Methods Enzymol*. 1991;194:1-863. https://doi.org/10.1016/s0076-6879(00)x0276-5.
16. Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol*. 1983;166(4):557-580. https://doi.org/10.1016/s0022-2836(83)80284-8.
17. Wu S, Letchworth GJ. High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol. *Biotechniques*. 2004;36(1):152-154. https://doi.org/10.2144/04361DD02.
18. Остерман Л.А. Методы исследования белков и нуклеиновых кислот. Электрофорез и ультрацентрифугирование (практическое пособие). – М.: Наука, 1981. – 288 с. [Osterman LA. Metody issledovaniya belkov i nukleinovykh kislot. Elektroforez i ul'tratsentrifugirovaniye (prakticheskoye posobiye). Moscow: Nauka; 1981. 288 p. (In Russ).]
19. Самсонова М.Г., Падкина М.В., Краснопевцева Н.Г. Генетико-биохимическое изучение кислых фосфатаз дрожжей *Saccharomyces cerevisiae* // Генетика. – 1975. – Т. 11. – № 9. – С. 104-115. [Samsonova MG, Padkina MV, Krasnopevtseva NG. Genetiko-biokhimicheskoye izucheniye kislykh fosfataz drozhzhey *Saccharomyces cerevisiae*. *Genetika*. 1975;11(9):104-115. (In Russ.)]
20. Shen XX, Zhou X, Kominek J, et al. Reconstructing the backbone of the Saccharomycotina yeast phylogeny using genome-scale data. *G3 (Bethesda)*. 2016;6(12):3927-3939. https://doi.org/10.1534/g3.116.034744.
21. Notzel C, Lingner T, Klingenberg H, Thoms S. Identification of new fungal peroxisomal matrix proteins and revision of the PTS1 consensus. *Traffic*. 2016;17(10):1110-1124. https://doi.org/10.1111/tra.12426.
22. Almagro Armenteros JJ, Sonderby CK, Sonderby SK, et al. DeepLoc: prediction of protein subcellular localization using deep learning. *Bioinformatics*. 2017;33(21):3387-3395. https://doi.org/10.1093/bioinformatics/btx431.
23. Ellis SB, Brust PF, Koutz PJ, et al. Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast *Pichia pastoris*. *Mol Cell Biol*. 1985;5(5):1111-21. https://doi.org/10.1128/mcb.5.5.1111.
24. Tschopp JF, Brust PF, Cregg JM, et al. Expression of the *lacZ* gene from two methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Res*. 1987;15(9):3859-3876. https://doi.org/10.1093/nar/15.9.3859.
25. Assis R, Bachtrog D. Neofunctionalization of young duplicate genes in *Drosophila*. *Proc Natl Acad Sci*. 2013;110(43):17409-17414. https://doi.org/10.1073/pnas.1313759110.
26. He X, Zhang J. Rapid subfunctionalization accompanied by prolonged and substantial neofunctionalization in duplicate gene evolution. *Genetics*. 2005;169(2):1157-1164. https://doi.org/10.1534/genetics.104.037051.
27. Kellis M, Birren BW, Lander ES. Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*. *Nature*. 2004;428(6983):617-624. https://doi.org/10.1038/nature02424.
28. Lin-Cereghino GP, Godfrey L, de la Cruz BJ, et al. Mxr1p, a key regulator of the methanol utilization pathway and peroxisomal genes in *Pichia pastoris*. *Mol Cell Biol*. 2006;26(3):883-897. https://doi.org/10.1128/MCB.26.3.883-897.2006.
29. Kranthi BV, Kumar R, Kumar NV, et al. Identification of key DNA elements involved in promoter recognition by Mxr1p, a master regulator of methanol utilization pathway in *Pichia pastoris*. *Biochim Biophys Acta*. 2009;1789(6-8):460-468. https://doi.org/10.1016/j.bbagrm.2009.05.004.

30. Wang X, Cai M, Shi L, et al. PpNrg1 is a transcriptional repressor for glucose and glycerol repression of AOX1 promoter in methylotrophic yeast *Pichia pastoris*. *Biotechnol Lett*. 2016;38(2):291-298. https://doi.org/10.1007/s10529-015-1972-4.

31. Wilkinson D, Váchová L, Hlaváček O, et al. Long noncoding RNAs in yeast cells and differentiated subpopulations of yeast colonies and biofilms. *Oxid Med Cell Longev*. 2018;2018:4950591. https://doi.org/10.1155/2018/4950591.

❀ Authors and affiliations

Alina V. Ivanova – 4th year Student of the Department of Genetics and Biotechnology. Saint Petersburg State University, Saint Petersburg, Russia. E-mail: alinalans@gmail.com.

Anton V. Sidorin – Bachelor of Science (BSc) in the Genetics and Biotechnology Department. Saint Petersburg State University, Saint Petersburg, Russia. E-mail: antonsidorin@list.ru.

Elena V. Sambuk – Doctor of Science, Professor of the Department of Genetics and Biotechnology. Saint Petersburg State University, Saint Petersburg, Russia. SPIN: 8281-8020. E-mail: e.sambuk@spbu.ru.

Andrei M. Rumyantsev – PhD, Senior Researcher of the Department of Genetics and Biotechnology. Saint Petersburg State University, Saint Petersburg, Russia. SPIN: 9335-1184. E-mail: rumyantsev-am@mail.ru.

❀ Информация об авторах

Алина Владиславовна Иванова — студентка 4-го курса кафедры генетики и биотехнологии. ФГБОУ ВО «Санкт-Петербургский государственный университет», Санкт-Петербург. E-mail: alinalans@gmail.com.

Антон Витальевич Сидорин — бакалавр кафедры генетики и биотехнологии. ФГБОУ ВО «Санкт-Петербургский государственный университет», Санкт-Петербург. E-mail: antonsidorin@list.ru.

Елена Викторовна Самбук — д-р биол. наук, профессор кафедры генетики и биотехнологии. ФГБОУ ВО «Санкт-Петербургский государственный университет», Санкт-Петербург. SPIN: 8281-8020. E-mail: e.sambuk@spbu.ru.

Андрей Михайлович Румянцев — канд. биол. наук, младший научный сотрудник кафедры генетики и биотехнологии. ФГБОУ ВО «Санкт-Петербургский государственный университет», Санкт-Петербург. SPIN: 9335-1184. E-mail: rumyantsev-am@mail.ru.