Short Communication

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New polyamine oxidases from *Ogataea parapolymorpha* DL-1: expanding view on non-conventional yeast polyamine catabolism

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Abstract: Polyamines are ubiquitous and essential for cellular physiology, yet their metabolic pathways and functions remain only partially understood. Polyamine oxidases (PAO) are key to elucidating their physiological roles. In the methylotrophic yeast *Ogataea parapolymorpha*, we identified three putative PAO-encoding genes. Biochemical characterization showed that two of them function as PAOs, whereas the third has unknown substrate specificity. In contrast to previously studied yeasts, including *Saccharomyces cerevisiae*, which contain only a single PAO, *O. parapolymorpha* harbors multiple and functionally distinct PAOs. These findings highlight an unexpected diversification of polyamine catabolism in yeast and suggest previously unrecognized roles of PAOs in cellular physiology.

Keywords: metabolic diversification; methylotrophic yeast; polyamine catabolism

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Yeasts are among the most relevant microorganisms in biotechnology, with methylotrophic species such as *Komagataella phaffii* and *Ogataea parapolymorpha* widely used for heterologous protein production (Manfrão-Netto et al. 2019) and metabolic engineering (Löbs et al. 2017). A deeper understanding of their metabolism can reveal new opportunities for biotechnological applications (Qin et al. 2021). Given the important roles of polyamines in fungal physiology, including involvement in translation (Chattopadhyay et al. 2008), morphogenesis (Guevara-Olvera et al. 1997), β-alanine and pantothenate biosynthesis (White et al. 2001), virulence (Augustyniak et al. 2024; Mayer et al. 2012; Schaefer et al. 2020), and even lifespan (Hofer et al. 2022), their metabolism represents a potential target for enhancing beneficial traits and suppressing detrimental ones. However, yeast genomes are highly divergent (Riley et al. 2016), and insights from the most studied model yeast *Saccharomyces cerevisiae* cannot always be generalized.

Polyamine biosynthesis is quite conserved, with process starting from ornithine decarboxylation and involving spermidine and spermine synthases (Michael 2016), while catabolism is mediated by polyamine oxidases (PAOs) and spermidine/spermine acetyltransferases. In mammals and plants, PAOs are well characterized and associated with polyamine homeostasis and oxidative damage (Wang and Casero 2006) and stress responses (Wang et al. 2019). In contrast, yeast PAO functions remain poorly understood: in *S. cerevisiae* FMS1 links spermine catabolism to β-alanine biosynthesis (Landry and Sternglanz 2003), while PAOs from *Candida boidinii* (Nishikawa et al. 2000) and *Debaryomyces hansenii* (Bakke et al. 2007) show distinct and unclear roles.

Genome analysis of *O. parapolymorpha* DL-1 revealed three putative PAO genes – an unusual feature among yeasts, more reminiscent of plants and mammals. This raises the possibility of novel functions and broader roles in polyamine catabolism. In this study, we combined phylogenetic analysis and biochemical characterization of these enzymes to clarify their evolutionary and metabolic significance.

Based on the annotation of the Uniprot, sequences of *O. parapolymorpha* genome labeled as FAD-containing oxidase

were selected. Among them three genes (Uniprot ID W1QA33, W1QAC1, W1Q987, which were named OpaPAO1, OpaPAO2 and OpaPAO3 respectively) were annotated as amine and polyamine oxidase. Deep search with PSI-BLAST and FoldSeek (van Kempen et al. 2024) shows that these genes have relatively small amino acid identity (about 30 %) toward polyamine oxidase FMS1 from *S. cerevisiae* and high degree of structural similarity.

Recent phylogenetic analyses of polyamine oxidases place fungal sequences into two major clades, with our enzymes belonging to the fungal-specific group (Salvi and Tavladoraki 2020). To clarify Ascomycota specific phylogeny, we obtained phylogenetic tree for different Ascomycota species, including mycelial fungi and yeasts (the detailed algorithm is presented in Supplementary Material). This led to a clear separation of two branches of yeast genes. The Branch 1 consists of genes encoding proteins with PTS1-like sequences on the C-terminus, including CbPAO from *C. boidinii* (Nishikawa et al. 2000) and OpaPAO1, and of specific Pichiales sub-branch (e.g., OpaPAO2), while the Branch 2 contains sequences of FMS1 (Landry and Sternglanz 2003), OpaPAO3 and other genes lacking PTS1 sequence, such as DhaPAO from *D. hansenii* (Bakke et al. 2007). Topology of Branch 2 is highly similar to those of whole-genome yeast phylogeny (Riley et al. 2016) which hints at the preservation of the non-peroxisomal gene in the genomes of yeasts of each main clade during their evolution (Figure 1A).

According to the phylogenetic tree, the duplication of polyamine oxidase gene seems to occur in ascomycetous yeast common ancestor into peroxisomal and non-peroxisomal enzyme, while branching into OpaPAO1- and OpaPAO2-containing clades seems to occur at class level. This presence of two PAO genes (with and without PTS1) is conserved in some Serinales and Pichiales. In contrast, Saccharomycetales have lost their peroxisomal PAO as a consequence of so-called reductive evolution. Such removing of genes in different metabolic pathways is not unusual for budding yeasts and have occurred, for instance, in case of nitrate assimilation (Shen et al. 2018). OpaPAO2-like sequences are presented only for Pichiales, including the other biotechnology relevant methylotrophic yeast *K. phaffii* (*Pichia pastoris*) (Additional File S1, Uniprot ID C4R918). Nevertheless, bioinformatics only answers the question about the moment when this duplication occurred but does not answer why it happened. So, a thorough biochemical investigation of these genes is necessary in order to elucidate their functions and, consequently, the causes of this phenomenon.

Structural comparison of known and uncharacterized enzymes enables prediction of their catalytic features and may uncover novel biochemical functions. So, we compared the model structures of OpaPAOs produced by AlphaFold and the experimental structure of FMS1 with bound N^1-acetyspermine (PDB ID 3CND). Both conserved and altered residues were found (Figure 1B–supplementary Table S1), the first of which include His67, Asp56-Trp61 loop, Leu294, Lys296 (numeration of residues here and further corresponds to FMS1). The polyamine binding pocket of OpaPAO1 is different from that of FMS1 only by the presence of Asp in place of Asn195, so that enzyme is suggested to retain similar specificity for polyamines (including acetylated ones), although it may have slightly variable catalytic parameters due to local negative charge. In case of OpaPAO2 the differences are much more significant: Trp174 is replaced by Pro, Tyr450 by Val, and Gly418 by Thr. It should be noted that each sequence of the clade containing OpaPAO2 has a different residue in the position of Trp174, while all sequences of the other clades contain Trp in this position (Supplementary Figure S2). Such changes can disrupt the configuration of the active site and, accordingly, greatly alter or completely change the substrate specificity. In the active site of OpaPAO3, a few altered residues were identified, including Ala instead of Cys488, Asn instead of His191, and Phe instead of Leu375 (Figure 1B). Also, these amino acid residues are conserved in their positions in Pichiales genes without PTS1 (Supplementary Figure S3), while corresponding residues of FMS1 are conserved for other sequences, except for OpaPAO2 clade. These observations suggest that OpaPAO3 constitutes a divergently evolved PAO form specific to this subbranch, potentially exhibiting distinct regulatory properties or substrate interactions compared with FMS1.

Bioinformatics and structural analyzes provide valuable predictive insights but are insufficient for a comprehensive characterization of previously unstudied enzymes, necessitating experimental validation. To this end, OpaPAO genes were cloned into pET-24a with a C-terminal His6-tag for expression in *E. coli* BL21(DE3) Codon+ pLysS cells. OpaPAO1 was expressed in soluble and active form, while OpaPAO2 was soluble but inactive towards tested polyamines. OpaPAO3 initially formed inclusion bodies, but co-expression with bacterial chaperonins in *E. coli* ArcticExpress strain improved solubility. All proteins were purified to >95 % homogeneity by Ni-NTA chromatography (Supplementary Figure S4, Figure S5).

All enzymes contained FAD, migrated as monomers on gel-filtration (Supplementary Figure S6), and showed moderate thermal stability with ThermoFAD assay (melting temperature (T_m) 54–63 °C) (Supplementary Figure S7). OpaPAO1 and OpaPAO3 were most active at pH ~9.0, stable at pH 6.5–7.5 (Figure 2C and D), and retained activity up to 75–80 °C (Figure 2A). It should be noted that temperature of inactivation of OpaPAO3 (Figure 2B) is close to T_m measured

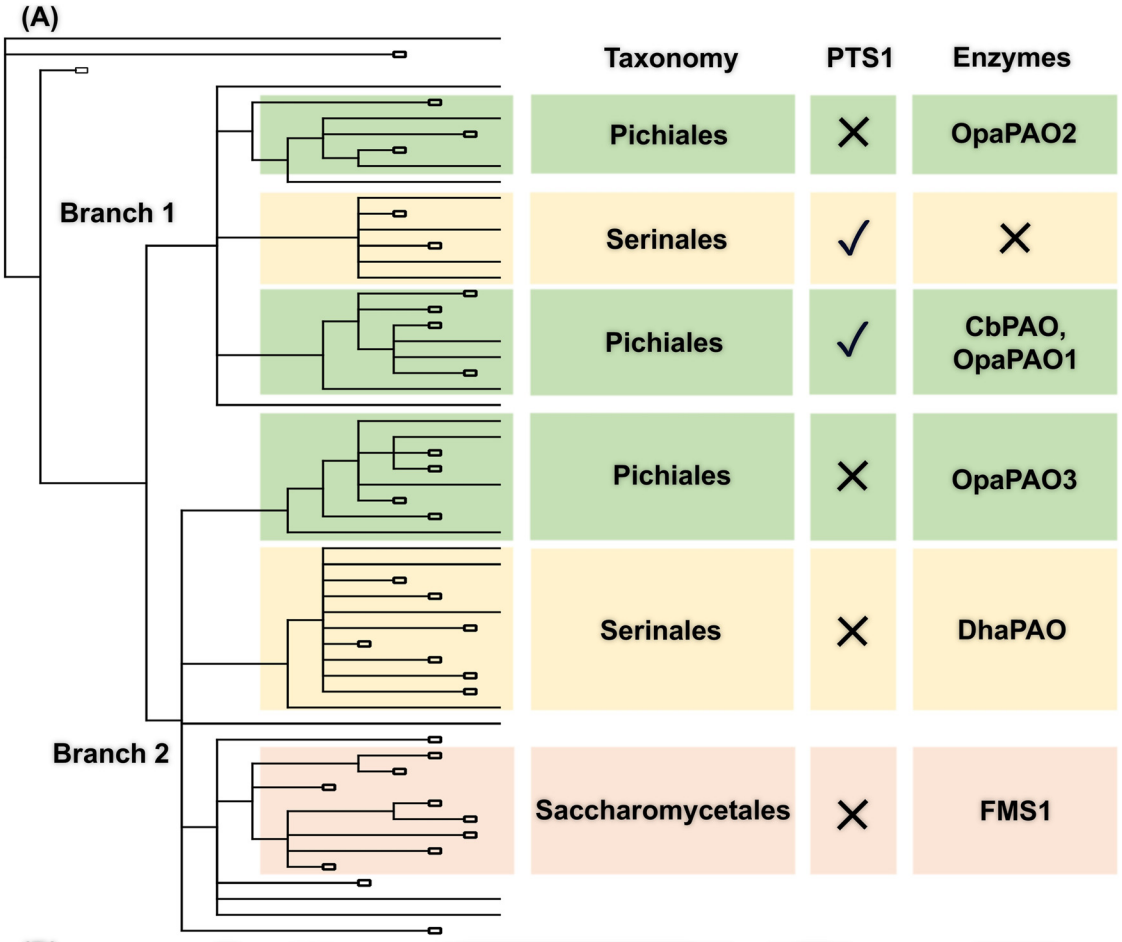

Figure 1: Bioinformatic analysis of yeast PAOs. (A) schematic representation of maximum likelihood phylogenetic tree of yeast PAOs. Full phylogenetic tree is presented on additional file S1. Branch 2 of yeast PAO genes can be matched with yeast whole-genome phylogeny (Riley et al. 2016). Main clades, in which conservativity of active site residues is observed, marked by color with additional commentaries (B) Active site residues of FMS1 (black line) and corresponding residues of OpaPAOs (color line). Conserved residues are shown in green, OpaPAO1 residues in grey, OpaPAO2 residues in bleu, and OpaPAO3 residues in red.

by releasing FAD, so we can suggest that the main way of inactivation of OpaPAO3 is FAD dissociation.

Next, we examined the capacity of studied enzymes to catalyze the oxidation of spermidine (Spd), spermine (Spm), and their acetylated derivatives, which were synthesized in this study (the detailed synthetic procedures are presented in the Supplementary Material): N^1-acetylspermidine (N^1-AcSpd), N^8-acetylspermidine (N^8-AcSpd), N^1,N^8-diacetylspermidine ($N^{1,8}$-diAcSpd) N^1-acetylspermine (N^1-AcSpm), N^1,N^{12}-diacetylspermine ($N^{1,12}$-diAcSpm).

Kinetic analysis (Table 1) revealed that OpaPAO1 demonstrates the highest catalytic efficiency of all the described enzymes (Table S4), particularly toward N^1-AcSpm, while in general it has catalytic parameters similar to FMS1. In contrast, OpaPAO3 displayed lower catalytic constant (more typical for plant enzymes, Supplementary Table S4) but similar yeast μM k_M values.

The difference in activity may be explained by the localization of these enzymes. Polyamine oxidases catalyze the oxidation of polyamines to form hydrogen peroxide and aldehydes, which are toxic to the cell. Peroxisomes contain catalase and other enzymes for the rapid decomposition of H_2O_2 and the neutralization of aldehydes. Peroxisomal localization of highly active OpaPAO1 protects the cytoplasm from oxidative stress. Similarly, low activity of OpaPAO3, which does not contain PTS1, may also reduce the negative impact of reaction products on the cell.

As noted above, OpaPAO2 does not demonstrate activity towards the studied polyamines, from which it can be

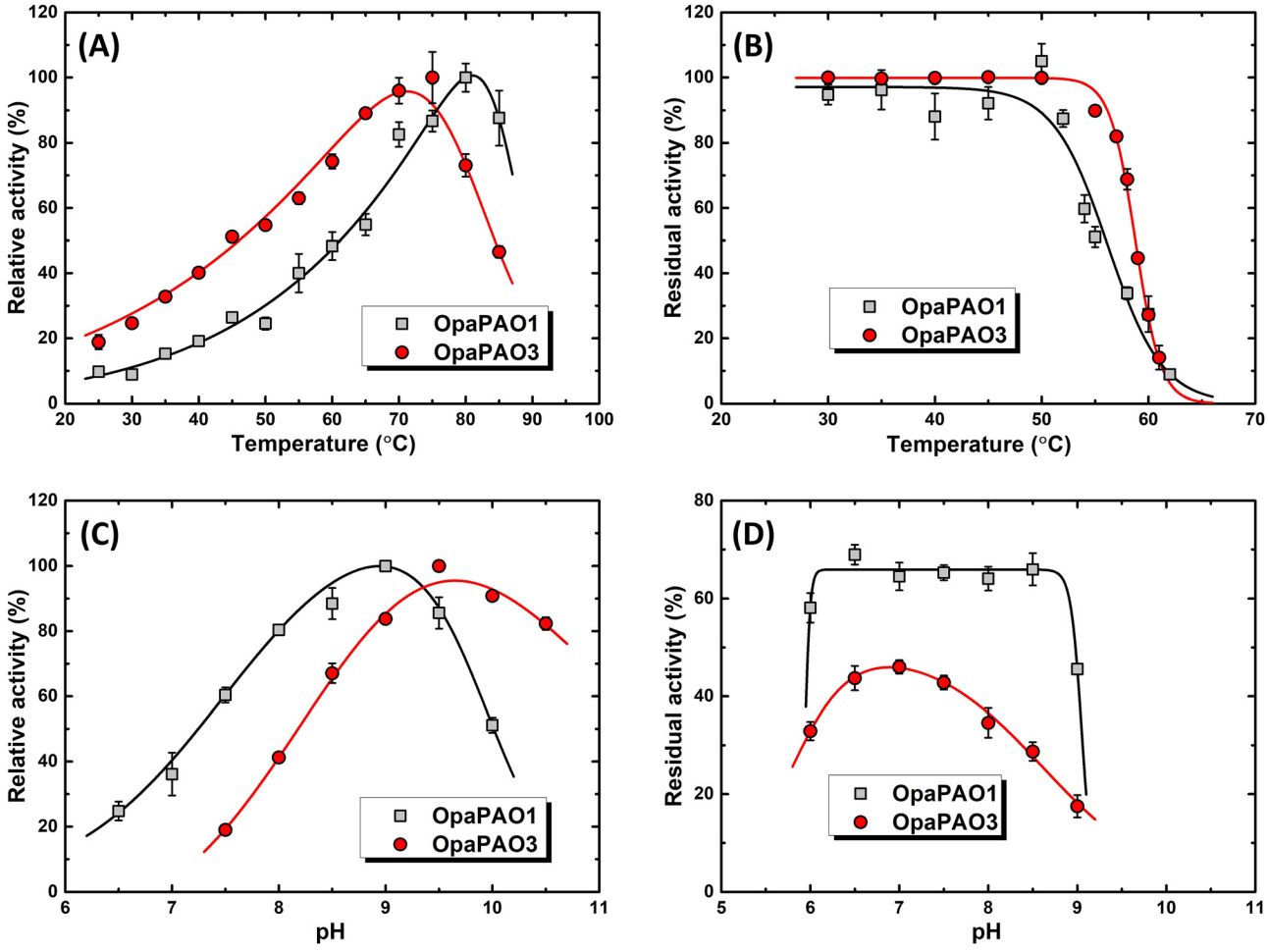

Figure 2: Activity and stability of OpaPAO1 and OpaPAO3 at different pH and temperature. All measurements were performed with HRP-coupled assay and 0.1 mM N^1-AcSpm as substrate. Further experimental details, equations and parameters for fitting are given in Supplementary Material (A) Dependence of activity on temperature, measured in 50 mM Tris-HCl buffer, pH 9.0; (B) residual activity after incubation of 0.05 mg/ml enzyme for 10 min in 100 mM sodium phosphate buffer, pH 7.0 (C) pH-activity profile, measured in phosphate-tris-carbonate buffer (50 mM each) at 25 C with 0.1 mM N^1-AcSpm (D) pH-stability profile, residual activity was measured after incubating of 0.05 mg/ml enzyme for 10 min in phosphate-tris-carbonate buffer (50 mM each) at 49 °C and 59 °C for OpaPAO1 and OpaPAO3 respectively.

Table 1: Kinetic parameters of OpaPAO1 and OpaPAO3. All measurements were performed at 25 °C, with HRP-coupled reaction of AmplexRed oxidation in 20 mM Tris-HCl buffer, pH 9.0. Further experimental details are given in the online Supplementary Material.

Enzyme	Substrate	k_{cat}, s^{-1}	K_M, μM	k_{cat}/K_M, $s^{-1}μM^{-1}$
OpaPAO1	N^1-AcSpd	350 ± 20	4.9 ± 0.5	71
	N^8-AcSpd	110 ± 5	14 ± 2	7.9
	N^1-AcSpm	200 ± 30	1.0 ± 0.3	200
	$N^{1,12}$-diAcSpm	260 ± 30	3.6 ± 0.7	72
OpaPAO3	N^1-AcSpd	6.3 ± 0.1	15 ± 1	0.4
	N^8-AcSpd	1.3 ± 0.02	68 ± 4	0.02
	$N^{1,8}$-diAcSpd	1.3 ± 0.05	378 ± 52	0.003
	N^1-AcSpm	6.2 ± 0.2	2.6 ± 0.4	2.4
	$N^{1,12}$-diAcSpm	11.6 ± 0.2	7.1 ± 0.4	1.6

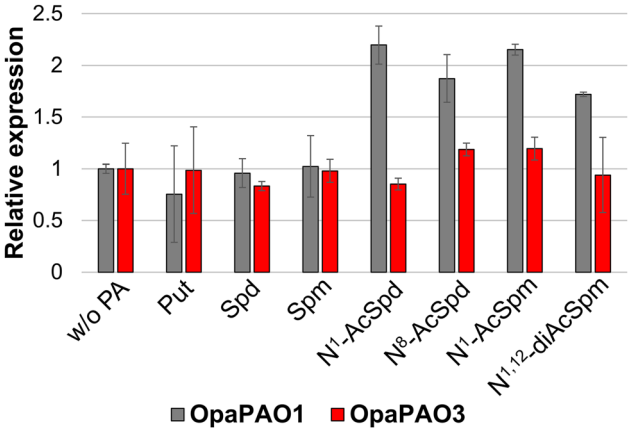

Figure 3: Quantitative rt-PCR analysis of OpaPAO1 and OpaPAO3 expression levels in the presence of exogeneous polyamines. Further experimental details are given in Supplementary Material. The sample w/o PA refers to control experiment without added polyamines. Put – putrescine.

concluded that this enzyme may exhibit activity towards specific polyamines or is not a polyamine oxidase.

Both, OpaPAO1 and OpaPAO3 oxidized the C–N bond adjacent to the acetyl group, yielding non-acetylated amines: putrescine in case of N^1-AcSpd, 1,3-diaminopropane in case of N^8-AcSpd and spermidine in case of N^1-AcSpm (Supplementary Table S3). Noteworthily, the oxidation of $N^{1,12}$-diAcSpm seemed to occur in two steps through the initial formation of N^1-AcSpd followed by its conversion to putrescine, in contrast to the oxidation of this substrate by DhaPAO from *D. hansenii* where N^1-AcSpd is the final product (Bakke et al. 2007).

RT-qPCR analysis revealed that acetylated polyamines (N^1-AcSpd, N^8-AcSpd, N^1-AcSpm, $N^{1,12}$-diAcSpm) induced an approximately 2-fold upregulation of OpaPAO1 expression, whereas non-acetylated polyamines had no effect. In contrast, OpaPAO3 expression remained unchanged under all conditions (Figure 3). These results suggest that OpaPAO1 is specifically responsive to acetylated polyamines, whereas OpaPAO3 appears to be constitutively expressed and not regulated by polyamine availability, indicating a potential functional divergence between the two enzymes.

Thus, all three enzymes show unique characteristics compared to one another. The most cryptic of obtained enzymes, OpaPAO2, was obtained as a soluble FAD-containing protein but showed no activity with tested polyamines, suggesting a divergent substrate, possibly non-polyamine. However, other yeast enzymes from clade of OpaPAO2 have not been described, so we cannot confidently assume the properties of this enzyme.

In contrast, OpaPAO1 is peroxisomal, highly active on acetylpolyamines, and transcriptionally inducible by adding substrates, resembling known yeast PAOs. These observations suggest that in *O. parapolymorpha* OpaPAO1 may mediate rapid oxidation of a large amounts of acetylated polyamines, which may occur, for example, when SSAT activity is increased under stress conditions (Fuller et al. 1990; Gerner et al. 1993).

OpaPAO3 shares substrate specificity with OpaPAO1 but shows non-peroxisomal localization, is less active, and not regulated by exogenous polyamines, implying a housekeeping role in maintaining cytosolic polyamine balance to provide normal hypusination of elF5A and regulation of translation or controlling the level of ROS (Chattopadhyay et al. 2006, 2008). The inability of OpaPAO1 to substitute for this role may be explained by its peroxisomal localization and the lack of efficient transport systems for relevant substrates or products across the peroxisomal membrane.

To sum up, all three studied enzymes differ from each other and from known yeast PAOs, so yeast data alone cannot predict their functions. Unlike other yeasts, *O. parapolymorpha* carries multiple PAO genes. Humans and plants also possess several PAOs, but comparisons are inconclusive due to differences in enzyme localization, substrate specificity, and regulatory mechanisms (Supplementary Table S4).

It appears that the features of the polyamine metabolism in *O. parapolymorpha* are quite unique among organisms with studied polyamine catabolism. Despite the presence of polyamines in all living organisms, the functions of these ubiquitous molecules seem to vary alongside their metabolism, and attempts to generalize experimental results from one organism to another are likely to fail.

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