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GENETIC INACTIVATION OF D-AMINO ACID OXIDASE GENES IN METHYLOTROPHIC YEAST *HANSENULA POLYMORPHA*

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Abstract

Cellular D-amino acid oxidases (ODA-FAD containing flavoproteins) are widely used in biotechnology. In this regard, it is of particular interest to investigate the functional role of the composition and regulation of individual ODA genes. The aim of this work was to investigate physiological and biochemical characteristics of ODA genes of strain DL1x *Hansenula (O.) polymorpha in vivo* using gene knockout methodology as well as to determine the nature of these genes expression and regulation of ODA activity as a function of nitrogen and carbon source composition in the cultivation medium. *H. polymorpha* is a thermotolerant methylotrophic yeast. They are used to study the mechanisms of peroxisomal biogenesis and degradation, regulation of methanol metabolism, nitrate assimilation and stress response. A serial dilution method has been used for rapid assessment of strain growth and stress tolerance. Vector design for genetic inactivation of ODA genes in *H. polymorpha* was performed using yeast vector pAM773. Selection of "knockout" cell clones was performed using PCR analysis. To complete the transformation process, *H. polymorpha* was deleted from the pAM773 vector and the obtained DNA was used in the experiment. *In vitro* cultivation of knockout strains of *H. polymorpha* was found to exhibit substrate-specificity of the ODA. According to the authors, the HP2914 gene is important for D-alanine oxidation,

Keywords

D-amino acid oxidase (DAAO), Hansenula polymorpha knock-out, D-alanine, L-alanine, glycerol, methanol

while the gene complex 2400 and 2914 is important for ODA activation in the presence of D-Phe in the medium. It is likely that the gene complex 2082 and 2165 regulates ODA activation when cells are cultured in medium with D-Ala and D-Asp. It was found that in the absence of 2165, 2400, 2914 genes, increased ODA activity to D-Ala only was observed in cell culture medium. The presence of D-alanine combined with 1 % glycerol and 1 % methanol in the culture medium stimulated the activity of the three major ODAs of *H. polymorpha* through the expression of the HP2914 gene, while the presence of glucose and L-alanine in the culture medium suppressed their activity

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Introduction. It is known that some cellular functions of D-amino acid oxidase (ODA-FAD-containing flavoproteins, DAAO) are widely used in biotechnology. The functions of ODA are diverse and include both direct participation in metabolism and in intercellular signaling [1]. For ODAs from different sources, the values of specific activity to substrates vary [2]. Note that microbial ODAs have the maximum activity with respect to the D-Ala substrate [3]. Thanks to efficient gene promoters, a number of ODA expression systems have been created and the fermentation processes of recombinant ODA-producing strains that are non-toxic to *E. coli* cells have been optimized. In this regard, studies of the physiological role of the composition and regulation of individual ODA genes are of particular interest. It is known that the cre-lox system allows multiple use of the same LEU2 marker for multiple consecutive knockouts of the ODA structure. In this work, this system was used to create constructs and subsequent genetic inactivation of the HP2082, HP2165, HP2400 genes [4].

The *aim of this work* was to study the physiological and biochemical characteristics of the ODA genes of the DL1 *H. polymorpha* strain in vivo using the gene knockout methodology, as well as to determine the character of expression of these genes and the regulation of ODA activity depending on the composition of nitrogen and carbon sources in the cultivation medium.

Materials and methods for solving problems. For *E. coli* cultivation, SOB or LB cultivation media were used (Table 1), and for the cultivation of *H. polymorpha* — YPD, YNB and YP media (Table 2). *Hansenula (O.) polymorpha* is a thermotolerant methylotrophic yeast. They are used to study the mechanisms of peroxisomal biogenesis and degradation, regulation of methanol

metabolism, nitrate assimilation, and stress response [5, 6]. In particular, SOB or LB media were used for cultivating *E. coli*: the first medium contained SOB (superoptimal broth) (per liter): 20 g of bacto-tryptone (*Difko*, Detroit, USA), 5 g of yeast extract (*Difko*, Detroit, USA), 0.5 g NaCl (pH = 7.5), 2.4 g $MgSO_4$, 186 mg KCl. The second medium included LB (Lysogeny Broth) (per liter): 5 g of yeast extract (*Difko*, Detroit, USA), 10 g of bacto-tryptone (*Difko*, Detroit, USA) and 10 g of NaCl (pH = 7.5). For the cultivation of bacterial transformants, ampicillin (50 μg/ml) and tetracycline (25 μg/ml) were added to LB or SOB media. To obtain solid media, 1.5 % bacto-agar was added to the corresponding liquid medium.

For the cultivation of *H. polymorpha*, we used YPD, YNB, and YP media with 0.5 % methanol and 1.5 % glycerol. Composition of YP: 10 g yeast extract, 20 g peptone. Composition of YPD: 1 l of YP, 20 g of glucose. To obtain a solid YPD medium, 20 g of agar, 100 ml of 10 × YNB: 6.7 g of YNB (yeast nitrogen base) in 100 ml of H_2O were added to the liquid medium (per liter). To prepare a solid medium, 2.5 % agar was used. When, after autoclaving, the temperature of the liquid agar had dropped to 50 °C, 20 ml of 10×YNB and 20 ml of 20 % glucose were added to 160 ml of agar. Composition of YP with 0.5 % methanol and 1.5 % glycerin: for 956 ml of YP 6.25 ml of 80 % methanol and 37.6 ml of 40 % glycerin.

Liquid and solid media for the cultivation of knockout strains with various nitrogen sources contained 1×YNB (without amino acids and ammonium sulfate, 0.17 % w/v), 30 mg/l leucine and, depending on the task, 0.5 % ammonium sulfate, 30 mM D, L-Ala or 30 mM D-Ala, 2 % glucose, or a mixture of 1 % glycerol and 1 % methanol.

Table 1

***Coli* culture medium composition (SOB and LB)**

Medium name	Composition and content per liter
SOB	20 g of bacto-tryptone (*Difko*, Detroit, USA), 5 g of yeast extract (*Difko*, Detroit, USA), 0.5 g of NaCl (pH = 7.5), 2.4 g of $MgSO_4$, 186 mg of KCl
LB	5 g of yeast extract (*Difko*, Detroit, USA), 10 g of bacto-tryptone (*Difko*, Detroit, USA) and 10 g of NaCl (pH = 7.5)

For rapid assessment of the growth and the degree of stress resistance of the strains, the method of serial dilutions was used: cultures of individual strains grown on a minimal medium (see Table 2) were diluted in distilled water to OD 600 = 1 (the optical density of the sample measured at a wavelength

of 600 nm was 1) and 100 ml of culture were transferred into the wells of a 96-well plate.

The experimental scheme was as follows: the resulting suspension was diluted (90 μl of water was added to 10 μl of the suspension in the well), and then 3 μl of the resulting suspension (10-4.10-3.10-2.10-1) was applied to Petri dishes containing dense medium YNB and various sources of nitrogen (ammonium sulfate, D-alanine, D, L-alanine), carbon. The medium was also supplemented with 0.5 M NaCl (for the formation of resistance to osmotic stress in culture cells) and 2 mM H_2O_2 (for the formation of resistance to oxidative stress in culture cells). The cultures were incubated for 48 hours at 37 °C and photographed.

Table 2

Composition of media for cultivation and selection of *H. polymorpha*, and medium for cultivation of knockout strains with various sources of nitrogen

Medium name	Composition and content per liter
For cultivation H. polymorpha	
YP	10 g yeast extract, 20 g peptone
YPD	1 l YP, 20 g glucose
YNB (10×)	6.7 g YNB (yeast nitrogen base) in 100 ml dH_2O
Selection medium H. polymorpha (solid medium)	
160 ml 2.5 % agar + 20 ml 10 × YNB + + 20 ml 20 % glucose	YP with 0.5 % methanol and 1.5 % glycerin (in 956 ml YP + + 6.25 ml 80 % methanol + + 37.6 ml 40 % glycerin
Minimum media for the cultivation of knockout strains with various nitrogen sources	
1 × YNB (no amino acids and ammonium sulfate, 0.17 % w/v) + + 30 mg/l leucine depending on the task to add: 0.5 % ammonium sulfate	
30 mM D, L-Ala	30 mM D-Ala,
2 % glucose	mixture of 1 % glycerin and 1 % methanol

Vectors for the genetic inactivation of ODA genes in *H. polymorpha* were constructed using the yeast pAM773 vector [7, 9, 10]. Isolation of plasmid DNA, construction of plasmids and their analysis were carried out using a number of methods of genetic engineering [3, 11, 12]. The selection of "knockout" cell clones was carried out using PCR analysis, and to complete the transformation

of *H. polymorpha*, vectors pAM773 were removed and the resulting DNAs were used in the experiment.

Results. It is known that the genes HP02082, HP2165, HP2194, HP2400 of the DL1 strain encode proteins from 332 a.a. up to 359 a.k. and the complex affects the expression of ODA [13, 14]. In the course of the study, the following strains were obtained — "knockouts" of cells: single strain (D2400::loxP), double strain (D2165::Zeo D2082::loxP, D2914::Zeo D2400::loxP, D2400::loxP D2082::loxP) and triple strain (D2914::Zeo D2400::loxP D2082::loxP). Strains D2165::Zeo D2914::Zeo were used to study their biochemical and physiological characteristics [12].

1. Study of the growth of single, double and three strains of H. polymorpha under standard conditions. When studying the growth of the obtained strains on liquid media containing glucose or methanol as a carbon source, it was found that the introduced genetic modifications do not lead to a decrease in the cell proliferation of the strains as compared to the cell proliferation of the recipient strain (DL1) or the strain with single knockouts D2165::Zeo, D2914::Zeo. Serial dilutions did not reveal significant differences in the growth patterns of the strains on a minimal medium with ammonium sulfate and glucose and methanol.

At the same time, differences were revealed in the growth parameters of these strains on solid media with D, L-alanine. Thus, the growth of cells of the strain (double knockouts) D2165::Zeo D2082::loxP and D2400::Zeo D2082::loxP was slightly better than the cells of other strains (Fig. 1). Since the HP2914 gene did not change in the cells of these strains, it is likely that it is this gene that is important for the oxidation of D-alanine [12, 15, 16].

2. Investigation of the growth of strains on various media. In this series of experiments, differences in the growth parameters of the strains were revealed during prolonged cultivation of *H. polymorpha* in a liquid medium (Fig. 2). During the experiment, changes were revealed in such parameters as the ratio of the density of cultures (DIC) grown for 72 hours on the following liquid media: (1) a medium containing D-alanine, 1 % glycerol and 1 % methanol and on (2) a medium containing sulfate ammonium and glucose. It was found that the original strain (DL1) with single knockouts for the HP2165 and HP2400 genes grew much better on a medium containing D-Ala, glycerol, and methanol (DIC > 1), and the cells of the strain were knocked out for the HP2914 gene, on the contrary grew better on a medium containing ammonium sulfate and glucose. The data obtained probably indicate that the HP2914 gene, encoding the synthesis of oxidases, ensures efficient cell growth on this substrate (DIC < 1).

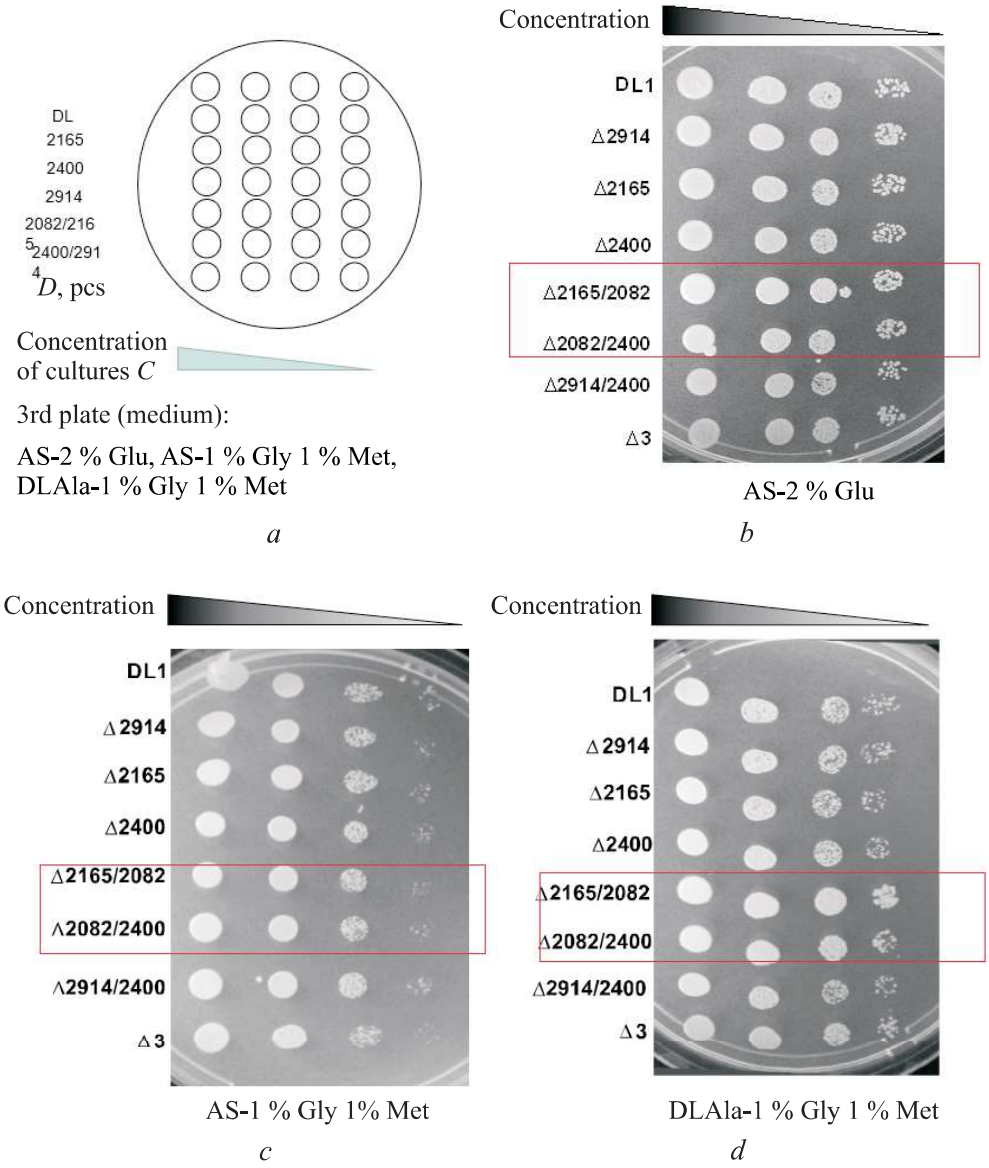
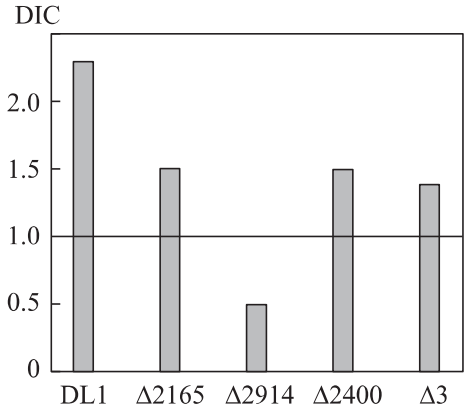


Fig. 1. Comparison of the growth of strains (DL-wild strain *H. Polymorpha*; Δ2914 — gene knockout 2914; Δ2165 — gene knockout 2165; Δ2400 — gene knockout 2400; Δ2165/2082 — genes knockout 2165, 2082; Δ2082/2400 — knockout for genes 2082, 2400; Δ2914/2400 — knockout for genes 2914, 2400; Δ3 — knockout for genes 2165, 2400, 2914); *a*) dilution method under standard conditions (plate test); *b*) growth of knockout strains on AS medium (0.5 % ammonium sulfate) with 2 % glucose; *c*) growth of knockout strains on AS medium containing 1 % glycerin and 1 % methanol; *d*) growth of knockout strains on DL-Ala medium (30 mM DL-alanine) with 1 % glycerol and 1 % methanol



$$DIC = \frac{\text{Growth parameters of strains on medium with D-Ala, mixture of 1 \% glycerol and 1 \% methanol}}{\text{Growth on media with ammonium sulfate and 2 \% glucose}}$$

Fig. 2. Changes in the value of DIC (the ratio of the DIC, differences in the growth parameters of strains on media with different sources of nitrogen and carbon) of different strains (DL-wild strain *H. Polymorpha*; Δ2914 — knockout for gene 2914; Δ2165 — knockout for gene 2165; Δ2400 — knockout for gene 2400; Δ3 — knockout for genes 2165, 2400, 2914)

3. Induction of oxidase activity by carbon and nitrogen sources. In the course of the next series of experiments, we investigated changes in ODA in the cells of the strains by monitoring the spectrophotometric changes in the maximum absorption spectrum of the suspension (at a wavelength of 590 nm) [11, 12]. It was found that when the DL1 strain was cultured on a medium containing the amino acid D-Ala and a mixture of 1 % glycerol and methanol, the ODA increased from 10 to 25 times in relation to the ODA activity when cultivated in a medium containing only D-Ala, D-Asp, D-Phe.

Under similar conditions, the double mutant (Δ2400–Δ2914) decreased the OPA value when cultured in a medium with D-Ala and D-Asp, but increased in a medium with D-Phe. On the contrary, for the double mutant (Δ2165–Δ2082), when cultivated on D-Phe, the value of ODA decreased, but when cultivated on the medium with D-Ala and D-Asp, it did not change. Note that during the cultivation of the triple mutant (Δ3), the ratio of the ODA activities decreased in the medium with D-Asp and D-Phe (Fig. 3).

4. Analysis of the efficiency of activity induction during cultivation in a medium with various sources of nitrogen and carbon. It is known that in the yeast *Rhodotorula gracilis*, the Dao 1 gene expression is activated by D-Ala and inhibited by L-Ala [8]. In this series of experiments, we investigated the nature of the induction of activity towards D-Ala when growing cells of the DL1 strain on media with various sources of nitrogen (D-Ala, DL-Ala, ammonium sulfate) and carbon (Fig. 4). It was found that the presence of D-Ala in the cultivation medium can be an effective inducer of ODA activity during cell culti-

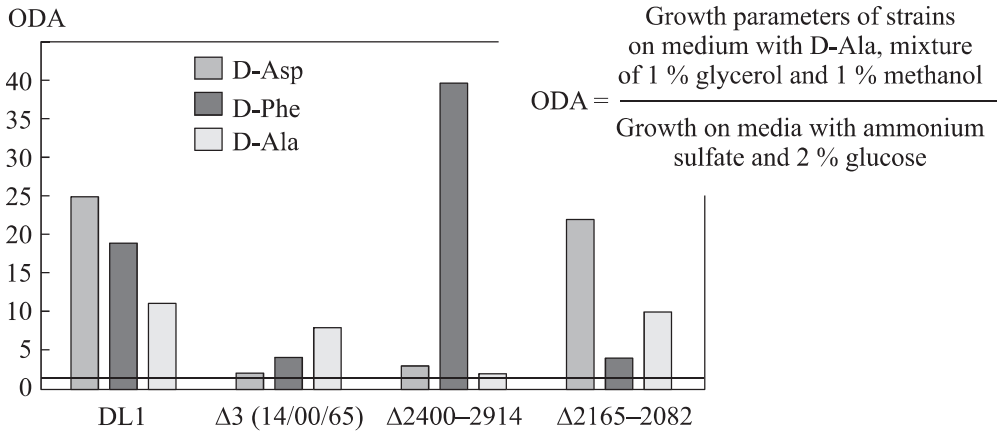


Fig. 3. Changes in the ratio of ODA activities (differences in the growth parameters of strains on media with different sources of nitrogen and carbon) of different strains (DL — wild strain *H. polymorpha*; Δ2165/Δ2082 — knockout for genes 2165, 2082; Δ2914/Δ2400 — knockout for genes 2914, 2400; Δ3 — knockout for genes 2165, 2400, 2914)

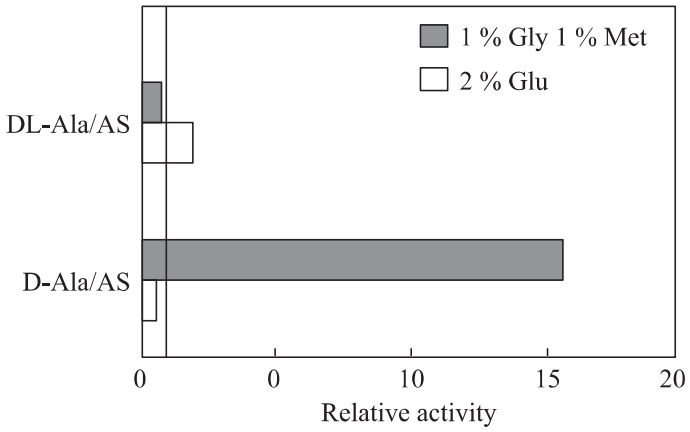


Fig. 4. Change in the relative activity of ODA in the DL1 strain upon cultivation for 72 hours in media with different sources of azote and carbon

vation on a medium with glycerol and methanol. The presence of glucose and ammonium sulfate in the cell incubation medium suppresses the activity of ODA, while DL-Ala is a weak inducer for the activation of ODA. Thus, it is important that the nature of the induction of ODA activities in *H. polymorpha* and *R. gracilis* is practically the same [8, 17, 18].

Discussion of the obtained results and conclusions. So, in the course of the study, it was found that during in vitro cultivation in "knockout" strains of *H. polymorpha*, the specificity of ODA to substrates is manifested. Probably,

the HP2914 gene is important for the oxidation of D-alanine, and the complex of the 2400 and 2914 genes plays an important role in the activation of ODA in the presence of D-Phe in the medium. Thus, the 2082 and 2165 gene complex play an important role in the activation of ODA when cells are cultured in a medium with D-Ala and D-Asp [14–18]. It was found that in the absence of gene 2165, 2400, 2914, an increased activity of ODA only to D-Ala was observed in the cell culture. The presence of D-alanine in the cultivation medium in combination with 1 % glycerol and 1 % methanol stimulates the activity of the three main ODAs of *H. polymorpha* due to the expression of the HP2914 gene, while the presence of glucose and L-alanine in the cultivation medium, on the contrary, suppresses their activity.

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