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EXPERIMENTAL ARTICLES

Deletion of the *FLD* Gene in Methylotrophic Yeasts *Komagataella phaffii* and *Komagataella kurtzmanii* Results in Enhanced Induction of the *AOX1* Promoter in Response to Either Methanol or Formate

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Abstract—Apart from the toxic methanol, relatively safe formic acid and its salts (formates) were shown to act as efficient inducers of the *AOX1* promoter in methylotrophic yeasts *Komagataella kurtzmanii* and *K. phaffii*. In order to investigate the mechanism of formate induction in the cells of these species, deletion of the formaldehyde dehydrogenase (FLD) gene was carried out, resulting in disruption of the metabolic pathway of methanol dissimilation and thus preventing the intracellular reduction of formate to methanol. The AOX1-lacZ reporter cassette was used to demonstrate that the *fld* mutation resulted in enhanced induction of the *AOX1* by both methanol and formate. This result casts doubt on the hypothesis that methanol is the real inducer of the *AOX1* promoter. Alternative mechanisms of induction are discussed.

Keywords: methylotrophic yeasts, *AOX1* promoter, methanol induction, formate induction, *Komagataella phaffii*, *K. kurtzmanii*

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The scheme of the metabolic pathways of methanol assimilation and dissimilation in methylotrophic yeasts of the genus *Komagataella* (*Pichia*) was described more than ten years ago and has not been substantially changed [1].

The initial reaction of methanol oxidation is catalyzed by the peroxisome alcohol oxidase Aox1p. This reaction results in the formation of formaldehyde and hydrogen peroxide. Part of the formaldehyde remains in peroxisomes and enters into the condensation reaction with xylulose-5-phosphate, which is the first reaction of the assimilation pathway. The other part of formaldehyde passes into the cell cytoplasm to be further oxidized via the dissimilation pathway. In the dissimilation pathway, formaldehyde obtained as a result of methanol oxidation is translocated into the cytoplasm in the form of S-hydroxymethyl glutathione, which is formed as a result of a noncatalytic reaction of reduced glutathione with formaldehyde. In the cytoplasm, S-hydroxymethyl glutathione undergoes further oxidation by NAD-dependent formaldehyde dehydrogenase. The resultant S-formyl glutathione is hydrolyzed to formic acid and glutathione by S-formyl glutathione hydrolase [2]. The last step of methanol dissimilation is NAD-dependent oxidation of formate to carbon dioxide, which is catalyzed by formate dehydrogenase.

Methanol-induced *AOX1*, a powerful alcohol oxidase promoter, is often used for heterologous expression [3]. The high efficiency of the expression systems developed depends on the strict regulation of the *AOX1* promoter and its high activity under conditions of induction. The promoter is characterized by complete repression in *K. phaffii* cells growing on most carbon substrates, including glucose or glycerin, and by induction under growth on methanol [4]. Certain carbon substrates (sorbitol, alanine, trehalose, etc.) were shown to support the growth of *K. phaffii* cells without repressing the *AOX1* when methanol was used as an inducer [5].

At the same time, despite considerable progress [6, 7], a detailed mechanism of *AOX1* induction is not completely understood. In particular, it remains unclear what compound is directly involved in induction of this promoter and in what way.

In our previous work, not only methanol but also formic acid (the last metabolite in the methanol dissimilation pathway) and its salts, formates, were shown to be efficient inducers of the *AOX1* promoter. The measurements showed that, depending on the yeast species, the level of induction by formate may reach 70–90% of the level of induction by methanol [8].

The fact that formic acid has an induction potential allowed us to suggest that a certain amount of methanol acting as a direct *AOX1* inducer is formed inside the cell from exogenous formic acid as a result of the action of the formaldehyde dehydrogenase and alcohol oxidase enzymes.

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The goal of the present work was to experimentally test the hypothesis that methanol is an inevitable intermediate in effecting formate-mediated induction of the *AOX1* promoter.

MATERIALS AND METHODS

Strains and media. The strains of methylotrophic yeasts *K. phaffii* GS115 (Invitrogen, United States) and *K. kurtzmanii* Y727his4 were used for investigation of the mechanisms of formate induction [9, 10]. The strains were grown on the YNM agar medium containing 0.17% Yeast Nitrogen Base, Amresco; 0.5% ammonium sulfate; and 1% (vol/vol) methanol), YNF (0.17% Yeast Nitrogen Base, Amresco; 0.5% ammonium sulfate; and 1% (wt/vol) potassium formate), or in the YP liquid medium (1% yeast extract, 2% peptone) containing 1% (vol/vol) methanol (YPM medium) or 1% (wt/vol) potassium formate (YPF medium).

Obtaining the mutant strains. In order to delete the *FLD* gene with simultaneous insertion of the AOX1-lacZ reporter cassette, two similar plasmid vectors, pFLD-AOX1$_{GS115}$-lacZ and pFLD-AOX1$_{Y727}$-lacZ, carrying the different *AOX1* promoter regions were constructed (Fig. 1). Each of the vectors contained the *AOX1* promoter of the strain for the transformation of which the vector was designed.

The transformation of recipient yeast strains with the linearized fragments of the constructed vectors was carried out by means of electroporation using the Invitrogen protocol. As a result of transformation of each of the strains (*K. phaffii* GS115 and *K. kurtzmanii* Y727his4Δ) with the linearized fragments of the constructed vectors pFLD-AOX1$_{GS115}$-lacZ and pFLD-AOX1$_{Y727}$-lacZ, the transformants *K. phaffii* GS115fldΔ and *K. kurtzmanii* Y727fldΔ, respectively, were selected. In the transformant cells, deletion of the *FLD* genes and simultaneous integration of the AOX1-lacZ reporter modules were verified by PCR.

Strains *K. phaffii* GS115FLD and *K. kurtzmanii* Y727FLD were used as negative controls. The control strains contained the native *FLD* genes and the AOX1$_{GS115}$-lacZ and AOX1$_{Y727}$-lacZ reporter cassettes integrated in the *AOX1* loci, respectively.

The activity of the *AOX1* promoter in the cells of the strains studied was assessed by the level of expression of β-galactosidase encoded by lacZ gene of the reporter modules.

Qualitative assessment of the activity of the *AOX1* promoter. The cells of the strains studied were grown for 24 h as streaks on a polyester membrane upon agar minimal medium containing 1% (vol/vol) methanol (YNM medium) or 1% (wt/vol) potassium formate.

After the streaks had grown, the cells on the membrane were permeabilized by freezing–thawing thrice. The membrane was then incubated for 60 min at room temperature in the buffer ($Na_2HPO_4 \cdot 7H_2O$, 16.1 g; $NaH_2PO_4 \cdot H_2O$, 5.5 g; KCl, 0.75 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; 2-mercaptoethanol, 2.7 mL; distilled water up to 1 L; pH 7.0) containing X-gal (1 mg/mL). Induction of the *AOX1* promoter (lacZ gene expression) was determined by the streaks turning blue by the action of β-galactosidase on the X-gal substrate.

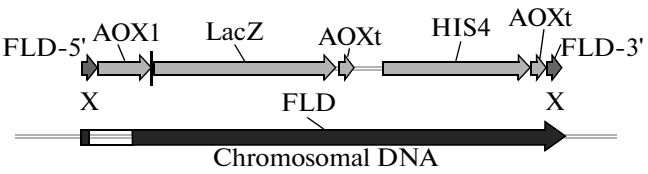

Fig. 1. Scheme of gene FLD deletion. FLD-5' and FLD-3' are the arms directing integration; AOX1 is the *AOX1* gene promoter of strains Y727/GS115; lacZ, the β-galactosidase gene; AOXt, the *AOX1* gene terminator; *HIS4*, histidinol dehydrogenase gene with its own regulatory region (strain Y727/GS115) complementing histidine-mediated auxotrophy of strains *K. kurtzmanii* Y727 and *K. phaffii* GS115.

Quantitative assessment of the level of induction of the *AOX1* promoter. In order to quantify the activity of the *AOX1* promoter, the cells of the mutant strains *K. kurtzmanii* Y727fldΔ, *K. phaffii* GS115fldΔ, as well as the cells of the control strains *K. kurtzmanii* Y727FLD and *K. phaffii* GS115FLD, were grown in the liquid media YP, YPM, and YPF. The cultures were grown to the mid-exponential growth phase (OD_{600} = 1.5–2.0) in flasks on a rotary shaker at 250 rpm and 29°C. The β-galactosidase activity in the grown cells was measured in cell lysates as described [11].

RESULTS AND DISCUSSION

The idea of our experimental approach consisted of artificially disrupting the metabolic pathway of methanol dissimilation by deleting the *FLD* (formaldehyde dehydrogenase) gene and, thus, according to the metabolic scheme, in ruling out the possibility of intracellular methanol reduction from exogenous formate. The *AOX1* promoter was expected to retain the capacity for induction by methanol in the cells of the mutant strain, whereas exogenous formate would not be reduced to methanol and, as a result, will completely lose its induction potential. In this case, the hypothesis that methanol is directly involved in *AOX1* induction would have been verified.

However, qualitative analysis of induction showed that, in the cells of the wild-type strain and in the cells of the strain with the *FLD* gene deletion, the *AOX1* promoter was induced on both the medium with methanol and the medium with potassium formate. This result was completely confirmed by the qualitative analysis data on the activity of the *AOX1* promoter in cell lysates (Fig. 2). Moreover, these data showed that, contrary to the expectations, the level of β-galactosidase expression in the cells of the mutant strains

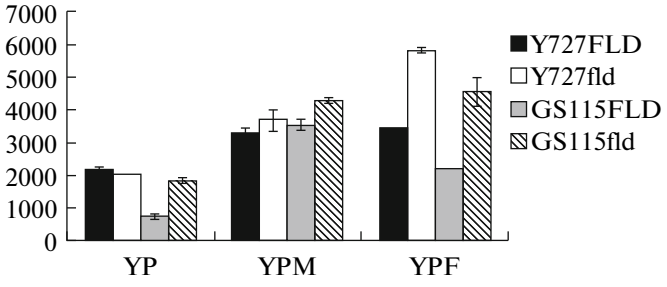

Fig. 2. β-Galactosidase activity in the cell lysates of the yeast strains. The yeast strains were grown up to the mid-exponential growth phase on the YP, YPM, and YPF media. Vertical axis: activity scale in Miller units [11]. In the diagram, the rods show the standard deviation by the results of measurements of the activity of two clones.

grown on medium with formate did not decrease, but rather increased almost twofold, having exceeded in the final analysis the level of methanol induction in the cells of nonmutant strains.

Thus, we may conclude that the data obtained are a refutation of the methanol mechanism of the formate induction of the *AOX1* promoter. Thus, direct involvement of methanol in the realization of the process of *AOX1* induction is questioned.

There is no doubt that we cannot completely exclude the possibility that, under conditions of inactivation of the known dissimilation pathway, a certain amount of methanol may be formed in the cells from exogenous formate as a result of a nonspecific action of endogenous oxidoreductases. However, this suggestion is not consistent with the elevation of the level of induction by formate observed in the cells of the mutant strains (Fig. 2) and seems to be unlikely in this context.

Thus, a certain presently unidentified intermediate product X (or products) of the metabolic pathway of methanol dissimilation, which may be formed in the yeast cells not only from methanol but also from formate, should be proposed to take on the role of the most probable intracellular compound causing induction of the *AOX1* promoter. In this case, the positive role of *fld* deletion in enhancing the methanol or formate induction can be explained as retarding the metabolism of this product and, hence, increasing its intracellular concentration.

This effect agrees with an increase in the level of *AOX1*-controlled gene expression in the mutant strains of *K. phaffii* with the mut^S phenotype in whose cells the alcohol oxidase *AOX1* gene is inactivated [12]. *AOX1*, similar to *FLD* deletion, also results in slowing down methanol metabolism and can therefore mediate an increase in the intracellular concentration of the hypothetical product and respond with an increase in the level of induction of the *AOX1* promoter.

Considering the alternative mechanisms of increasing the *AOX1* induction in the cells of the mutant strains, it is possible to note a significant decrease in the growth rate of the mutant cultures on the media with methanol or formate. According to the data available, slowing down the cell cycle may have a considerable effect on chromatin decompactization due to the action of histone acetylases and may lead to an activation of different promoters [13]. However, the role of this effect in relation to the *AOX1* promoter cannot be decisive, because it was shown that trehalose or sorbitol, which provide a lower rate of culture growth than methanol, do not cause induction of the *AOX1* promoter in the cells of *K. phaffii* [5]. These data, as well as the data on the low activity of the promoter under catabolite derepression [14], indicate that the principal mechanism of induction of the *AOX1* promoter is of substrate nature.

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