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Metabolome, transcriptome and metabolic flux analysis of arabinose fermentation by engineered *Saccharomyces cerevisiae*

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ABSTRACT

One of the challenges in strain improvement by evolutionary engineering is to subsequently determine the molecular basis of the improved properties that were enriched from the natural genetic variation during the selective conditions. This study focuses on *Saccharomyces cerevisiae* IMS0002 which, after metabolic and evolutionary engineering, ferments the pentose sugar arabinose. Glucose- and arabinose-limited anaerobic chemostat cultures of IMS0002 and its non-evolved ancestor were subjected to transcriptome analysis, intracellular metabolite measurements and metabolic flux analysis. Increased expression of the GAL-regulon and deletion of *GAL2* in IMS0002 confirmed that the galactose transporter is essential for growth on arabinose. Elevated intracellular concentrations of pentose-phosphate-pathway intermediates and upregulation of *TKL2* and *YGR043c* (encoding transketolase and transaldolase isoenzymes) suggested an involvement of these genes in flux-controlling reactions in arabinose fermentation. Indeed, deletion of these genes in IMS0002 caused a 21% reduction of the maximum specific growth rate on arabinose.

1. Introduction

Fermentation of lignocellulosic hydrolysates is a promising strategy for environmentally sustainable and cost-effective production of fuel ethanol from plant biomass (Aristidou and Penttilä, 2000). For economical feasibility at the industrial scale, also smaller carbohydrate fractions of lignocellulosic hydrolysates, such as L-arabinose, need to be converted at high yields and rates together with the most abundant sugars, such as glucose and xylose (Hahn-Hägerdal et al., 2007).

Saccharomyces cerevisiae, currently the organism of choice for fermentative production of ethanol in industry, ferments hexoses at high rates and yields, but wild-type strains cannot grow on the pentose sugars xylose and arabinose. Large efforts have been made to expand its substrate range to include these sugars (Hahn-Hägerdal et al., 2007; Jeffries and Jin, 2004; van Maris et al., 2007). Research initially focused on xylose, the most abundant pentose in plant biomass. By expression of either yeast xylose reductase and xylitol dehydrogenase (XR/XDH) genes or a heterologous xylose isomerase gene, combined with further metabolic and evolutionary engineering approaches, *S. cerevisiae* strains capable of rapidly fermenting xylose have been developed (Jeppsson et al., 2002; Kuyper et al., 2005a; Sedlak and Ho, 2004; Sonderegger and Sauer, 2003). For arabinose fermentation, both bacterial and fungal arabinose utilization pathways have been introduced in *S. cerevisiae* (Becker and Boles, 2003; Bera et al., 2010; Bettiga et al., 2009; Richard et al., 2003; Sedlak and Ho, 2001).

S. cerevisiae IMS0002, the first *S. cerevisiae* strain capable of efficient, fully anaerobic growth and ethanol production on arabinose, was based on expression of the L-arabinose pathway from *Lactobacillus plantarum* in a *S. cerevisiae* strain that had previously been engineered and evolved for xylose fermentation (Kuyper et al., 2005a). In addition to targeted genetic modification, efficient arabinose fermentation required extensive evolutionary engineering in sequential batch cultures grown on L-arabinose (Wisselink et al., 2007).

Evolutionary engineering is a powerful approach for improving industrially relevant properties of microorganisms. However,

Abbreviations: PPP, pentose phosphate pathway; TCA, tricarboxylic acid; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; T6P, trehalose-6-phosphate; G1P, glucose-1-phosphate; F1, 6BP, fructose-1,6-bisphosphate; PYR, pyruvate; 2, 3PG, 2- and 3-phosphoglycerate; PEP, phosphoenol pyruvate; R5P, ribose-5-phosphate; RBU5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; RBU, ribulose; E4P, erythrose-4-phosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; PYR, pyruvate; FUM, fumarate; SUC, succinate; MAL, malate; OXG, oxoglutarate; CIT, citrate; AcCoA, acetyl-CoA; OAA, oxaloacetate; AcALD, acetaldehyde

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once improved strains have been isolated, identification of the genetic and metabolic changes responsible for the new phenotypes is challenging. In this, chemostat cultures offer clear advantages for comparative analysis of evolved and parental strains because they enable tight control of specific growth rate and other culture parameters. Thus, changes in metabolism or gene expression can be more clearly attributed to the strain background or carbon source (Daran-Lapujade et al., 2009).

Several physiological and molecular studies, using either chemostat or batch cultivation, have been performed on (evolved) xylose-fermenting strains expressing XR and XDH. Many of the observed changes in gene expression were linked to NADPH and NAD^+ metabolism, probably as a consequence of redox constraints imposed upon the engineered cells by the non-matching cofactor preferences of XR and XDH (Jin et al., 2004; Pitkänen et al., 2003, 2005; Sonderegger et al., 2004; Wahlbom et al., 2001, 2003; Zaldivar et al., 2002).

In contrast to XR/XDH-based pathways for xylose fermentation, the bacterial arabinose pathway in *S. cerevisiae* IMS0002 does not impose redox cofactor constraints (Wisselink et al., 2007) and ethanol and carbon dioxide yields are the same for glucose and arabinose. Nevertheless, although major differences between growth on glucose and arabinose can be anticipated in catabolism and in sugar transport, changes in metabolism and gene expression in arabinose-fermenting *S. cerevisiae* strains have not been studied in detail.

The aim of the present study is to identify key genetic changes contributing to efficient arabinose utilization by the evolutionary engineered *S. cerevisiae* strain IMS0002. To this end, strain IMS0002 and its non-evolved ancestor IMS0001 were characterized during anaerobic growth in chemostat cultures, by a combination of transcriptome analysis, extensive intracellular metabolite measurements and metabolic flux analysis. Hypotheses generated by this integrated analysis were tested by deleting involved genes in strain IMS0002.

2. Methods

2.1. Strains and maintenance

S. cerevisiae strains used in this study are listed in Table 1. After addition of 30% (v/v) glycerol, samples from shake-flask cultures were stored in 2 ml aliquots at −80 °C.

2.2. Media and shake-flask cultivation

Cultivation in shake flasks and anaerobic fermenters was performed at 30 °C in synthetic medium (MY), containing 5 g l^{-1} $(NH_4)_2SO_4$, 3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.05 ml l^{-1} silicon antifoam and trace elements (Verduyn et al., 1992). For shake flask cultivation, medium pH was adjusted to 6.0 with 2 M KOH prior to sterilization. After heat sterilization (121 °C, 20 min), a filter-sterilized vitamin solution (Verduyn et al., 1992) and sugar(s) were added. Shake-flask cultures were prepared by inoculating 100 ml medium containing the appropriate sugar with a frozen stock culture, and were incubated at 30 °C in an orbital shaker (200 rpm). Solid MY plates containing 20 g l^{-1} glucose (MYG) were prepared by adding 2% agar. Plates were incubated at 30 °C until growth was observed.

2.3. Anaerobic chemostat cultivation

Anaerobic chemostat cultivation was carried out at 30 °C in 2 l fermenters (Applikon, Schiedam, the Netherlands) with a working volume of 1 l. Cultures were performed in MY supplemented with 0.01 g l^{-1} ergosterol and 0.42 g l^{-1} Tween 80 dissolved in ethanol (Andreasen and Stier, 1953, 1954), silicon antifoam, vitamin solution and trace elements (Verduyn et al., 1992), and 20 g l^{-1} glucose (MYG) or arabinose (MYA), and was maintained at pH 5.0 by automatic addition of 2 M KOH. Cultures were stirred at 800 rpm and sparged with 0.5 l min^{-1} nitrogen gas (<10 ppm oxygen). To minimize oxygen diffusion, fermenters were equipped with Norprene tubing (Cole Palmer Instrument Company, Vernon Hills, USA). Absence of oxygen was verified with an oxygen electrode (Applisens, Schiedam, the Netherlands). After inoculation and completion of the batch phase, chemostat cultivation on MYA or MYG was initiated at a dilution rate of 0.03 h^{-1}. The working volume of the culture was kept constant using an effluent pump controlled by an electric level sensor. Chemostats were assumed to be in steady state when, after at least five volume changes, dry weight and specific CO_2 production rate changed by less than 2% over two further volume changes. Samples for microarray, biomass dry weight, extra- and intracellular metabolite analyses were taken between 7 and 9 volume changes after the onset of continuous cultivation.

Table 1
S. cerevisiae strains constructed and used in this study.

Strain	Characteristics	Reference
IMS0001	*MATa ura3-52 HIS3 leu2-3,112 TRP1 MAL2-8c SUC2 loxP-P_{TPI}::(−266, −1)TAL1 gre3::hphMX pUGP$_{TPI}$-TKL1 pUGP$_{TPI}$-RPE1 loxP-P_{TPI}::(−40, −1)RKI1* {pRW231, pRW243} Strain constructed for growth on arabinose,; promoters of *TKL1*, *TAL1*, *RPE1* and *RKI1* replaced by strong *TPI* promoter; transformed with plasmids pRW231 and pRW243, containing *Lactobacillus plantarum AraA*, *AraB* and *AraD*	Wisselink et al. (2007)
IMS0002	As IMS0001; selected for anaerobic growth on L-arabinose	Wisselink et al. (2007)
IMS0012	As IMS0002; *gal2Δ::loxP-KanMX-loxP*	This work
IMS0013	As IMS0002; *ygr043cΔ::loxP-KanMX-loxP*	This work
IMS0014	As IMS0002; *tkl2Δ::loxP-KanMX-loxP*	This work
IMS0019	As IMS0002; *ygr043cΔ::loxP*	This work
IMS0020	As IMS0002; *ygr043cΔ::loxP (1-516)ygr043c::loxP-KanMX-loxP*	This work
IMS0021	As IMS0002; *ygr043cΔ::loxP (1-516)ygr043c::loxP*	This work
IMS0022	As IMS0002; *ygr043cΔ::loxP (1-516)ygr043c::loxP tkl2Δ::loxP-KanMX-loxP*	This work

2.4. Anaerobic sequential batch cultivation

Inocula for anaerobic batch cultures were pregrown at 30 °C in shake flasks containing MYG or MYA. Anaerobic sequential batch cultures of IMS0002 deletion mutants were performed in 1 l of MYA or MYG, using similar fermenter setup and settings as for chemostat cultivation. New cycles of batch cultivation were initiated by manually or computer-controlled replacement of ca. 98% of the culture with fresh medium. In each cycle, maximum specific growth rate was estimated from the CO_2 production profile in the exponential growth phase. To determine the probability (p) that the observed specific growth rates for different IMS0002 deletion mutants were identical, an unpaired Student's T-test was performed, assuming a two-tailed distribution and equal variances. For p-values below a threshold of 0.05, the growth rates were considered significantly different.