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Alleviation of feedback inhibition in *Saccharomyces cerevisiae* aromatic amino acid biosynthesis: Quantification of metabolic impact

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Abstract

A quantitative analysis of the impact of feedback inhibition on aromatic amino acid biosynthesis was performed in chemostat cultures of *Saccharomyces cerevisiae*. Introduction of a tyrosine-insensitive allele of *ARO4* (encoding 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase) caused a three-fold increase of intracellular phenylalanine and tyrosine concentrations. These amino acids were not detected extracellularly. However, an over 100-fold increase of the extracellular levels of phenylacetate, phenylethanol and their para-hydroxyl analogues was observed. The total increase of the flux through the aromatic pathway was estimated to be over four-fold. Individual overexpression of either the wild-type or feedback insensitive allele of *ARO7* (encoding chorismate mutase had no significant impact. However when they were combined with the Tyr-insensitive *ARO4* allele in combination with the Tyr-insensitive *ARO4* allele, extracellular concentrations of aromatic compounds were increased by over 200-fold relative to the reference strain, corresponding to a 4.5-fold increase of the flux through the aromatic amino acid biosynthesis pathway. Elimination of allosteric control on these two key reactions in aromatic amino acid metabolism significantly affected intracellular concentrations of several non-aromatic amino acids. This broader impact of amino acid biosynthesis presents a challenge in rational optimization of the production of specific amino acids and derived flavour compounds.

Keywords: Chemostat culture; *Saccharomyces cerevisiae*; Aromatic amino acid biosynthesis; Chorismate mutase; DAHP synthase; Feedback inhibition

1. Introduction

The aromatic amino acids phenylalanine and tryptophan are mainly used in food and feed applications. They are produced on an industrial scale via bacterial fermentation processes using *Escherichia coli* and *Corynebacterium glutamicum* (Pittard, 1996; Hermann, 2003). Interest in phenylalanine has increased proportionally with the increased demand for low caloric food and soft drinks, as phenylalanine is a precursor for the low caloric sweetener aspartame (Gunby, 1983; Sprenger, 2007). While *Saccharomyces cerevisiae* is not under consideration for the industrial production of amino acids, aromatic amino acid metabolism by this yeast is of interest for other industrial applications.

In *S. cerevisiae* and other yeasts (Fabre et al., 1998; Wittmann et al., 2002) the phenylalanine biosynthesis pathway is involved in the synthesis of phenylethanol. This molecule has interesting sensory properties (including a rose-like aroma) and is of increasing economical interest. Phenylethanol can be produced via bio-transformation of phenylalanine with *S. cerevisiae* (Etschmann et al., 2002). This conversion involves the reactions of the Ehrlich pathway (Ehrlich, 1907), which is involved in the catabolism of several amino acids by *S. cerevisiae* (Fig. 1). In the case of phenylalanine, the Ehrlich pathway is initiated by its transamination to phenylpyruvate. This 2-oxo acid is then decarboxylated to phenylacetaldehyde (Vuralhan

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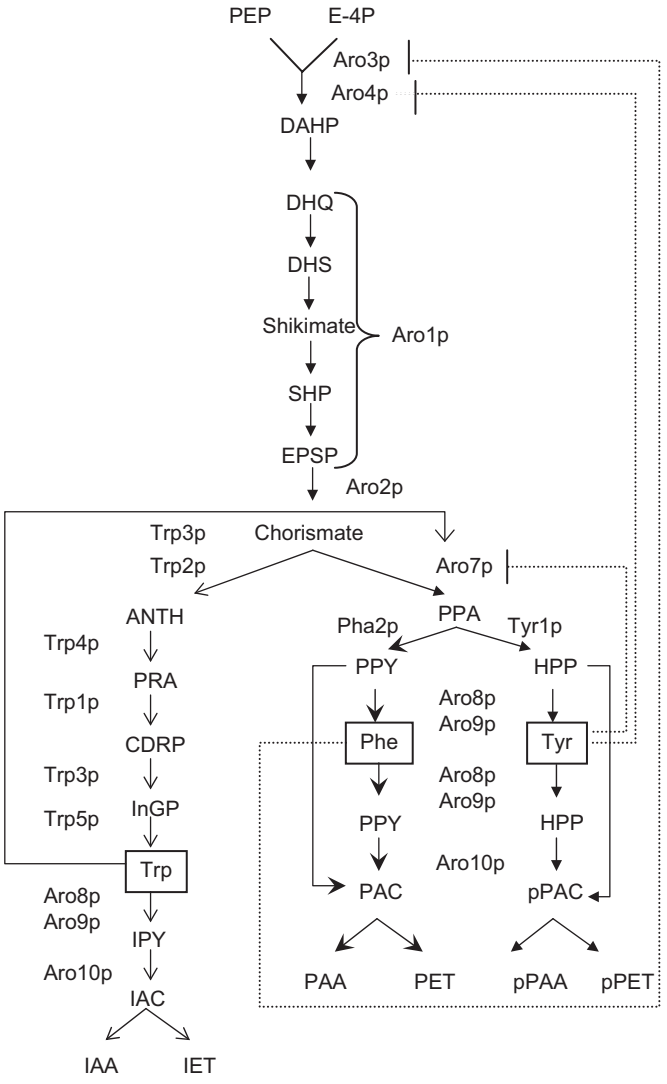

Fig. 1. Pathways of aromatic amino acid biosynthesis and catabolism in *S. cerevisiae*. The dashed lines indicate feedback inhibition of Aro4p and Aro7p by tyrosine and feedback inhibition of Aro3p by phenylalanine. The solid line indicates activation of Aro7p by tryptophan. E-4P: erythrose 4-phosphate, PEP: phosphoenol pyruvate, DAHP: 3-deoxy-D-arabino-heptulosonate-7-phosphate, DHQ: 3-dehydroquinate, DHS: 3-dehydro-shikimate, SHP: shikimate-3-phosphate, EPSP: 5 enolpyruvoylshikimate 3-phosphate, ANTH: anthranilate, PRA:phosphoribosyl anthranilate, CDRP: 1-(o-carboxyphenylamino-1-deoxyribulose 5-phosphate, InGP: indole 3-glycerol-phosphate, PPA: prephenate, PPY: phenylpyruvate, HPP: *para*-hydroxy-phenylpyruvate, PAC: phenylacetaldehyde, PAA: phenylacetate, PET: phenylethanol, *p*PAC: *para*-hydroxy-acetaldehyde *p*PAA:para-hydroxy-acetate, *p*PET: *para*-hydroxy-phenylethanol, IAC: indole-acetaldehyde, IAA: indole-acetate, IET: indole-ethanol, Trp: tryptophan, Phe: phenylalanine, Tyr: tyrosine.

et al., 2005). Depending on the redox status of the cells, phenylacetaldehyde is then reduced by alcohol dehydrogenases (yielding phenylethanol) or oxidized to phenylacetic acid by aldehyde dehydrogenases (Vuralhan et al., 2003). Phenylpyruvate, the precursor for phenylethanol production by *S. cerevisiae*, is also an intermediate in the *de novo* biosynthesis of phenylalanine from sugars.

In bacteria, extensive strain improvement programmes, involving a combination of random mutagenesis and targeted metabolic engineering, have been applied to improve aromatic amino acid biosynthesis (Ikeda and Katsumata, 1992; Ikeda, 2003; Sprenger, 2007; Lütke-Eversloh et al., 2007). In these strain improvement programmes, a first and essential step is invariably the elimination of feedback inhibition on key enzymes in the biosynthetic pathway. In *S. cerevisiae*, two reactions in the phenylalanine biosynthesis pathway are known to be subject to feedback inhibition (Fig. 1). The first committed step in aromatic amino acid metabolism is catalysed by 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase, for which two isoenzymes exist in *S. cerevisiae*, encoded by the *ARO3* and *ARO4* genes (Teshiba et al., 1986; Kunzler et al., 1992). Aro3p and Aro4p are feedback inhibited by phenylalanine and tyrosine, respectively. Besides, considerable tryptophan regulation of DAHP synthase has recently been reported (Helmstaedt et al., 2005). A single lysine-to-leucine substitution in Aro4p at position 229 results in a deregulated enzyme that is no longer feedback inhibited by tyrosine (Hartmann et al., 2003). Chorismate mutase, encoded by *ARO7*, has been identified as a second reaction subject to allosteric regulation (Ball et al., 1986; Brown and Dawes, 1990), its activity is inhibited by tyrosine and stimulated by tryptophan. Substitution of the serine residue 141 by a glycine abolished effects of both tyrosine and tryptophan, thus leading to a non-allosterically regulated chorismate mutase (Schnappauf et al., 1998; Krappmann et al., 2000). Although considerable knowledge is available on the molecular basis for feedback inhibition of aromatic amino acid metabolism (Helmstaedt et al., 2001), no quantitative studies have yet been performed on its impact on product formation by growing *S. cerevisiae* cultures.

The aim of this study is to quantify the combinatorial effects of deregulation of DHAP synthase and chorismate synthase in *S. cerevisiae*. To this end, a tyrosine-feedback insensitive DAHP synthase and a non-allosterically regulated chorismate mutase were expressed in an *aro3Δ* genetic background. Subsequently, the production of aromatic amino acids and the corresponding fusel alcohols and 'fusel acids' were quantified in aerobic, glucose-limited chemostat cultures. To assess the specificity of this approach to deregulate aromatic amino acid metabolism, we also analysed intracellular concentrations of other amino acids in reference and engineered strains.

2. Materials and methods

2.1. Strain construction and maintenance

The *S. cerevisiae* strains used in this study are listed in Table 1. Stock cultures were grown at 30 °C in shake flasks on synthetic medium (SM) (Luttik et al., 1998) supplemented with glucose (20 g l^{-1}) (for composition see growth condition section). When stationary phase was reached, sterile glycerol was added to 30% (vol/vol), and 2-ml aliquots were stored in sterile vials at −80 °C.