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Combinatorial pathway analysis for improved L-tyrosine production in *Escherichia coli*: Identification of enzymatic bottlenecks by systematic gene overexpression

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Abstract

Combinatorial overexpression of aromatic amino acid biosynthesis (AAAB) genes in the L-tyrosine producing *Escherichia coli* strains T1 and T2 was employed to search for AAAB reactions limiting L-tyrosine production. All AAAB genes except *aroG* and *tyrA*, which were substituted by their feedback resistant derivatives in the host strains, were cloned and overexpressed. A total of 72 different strains overexpressing various AAAB gene combinations were generated and from those strains with improved phenotype, enzymatic bottlenecks of the AAAB pathway could be inferred. The two major gene overexpression targets for increased L-tyrosine production in *E. coli* were *ydiB* and *aroK*, coding for a shikimate dehydrogenase and a shikimate kinase, respectively, and the combination of *ydiB* and *aroK* for overexpression resulted in the best L-tyrosine producing strains in this study, yielding 45% for strain T1 and 26% for strain T2, respectively, higher L-tyrosine titers. Interestingly, overexpression studies with combinations of more than one gene revealed that new gene targets could be identified when overexpessed together with other genes but not alone as single gene overexpression. For example, *tyrB* encoding the last enzyme of the AAAB pathway, an aromatic amino acid transaminase, improved L-tyrosine production significantly when co-overexpressed together with *ydiB* or *aroK*, but not when overexpressed alone. It is also noteworthy that *E. coli* T1, which generally yielded less L-tyrosine, was amenable to greater improvements than strain T2, i.e. *E. coli* T1 exhibited generally more space for phenotype improvement.

Keywords: *Escherichia coli*; L-tyrosine; Aromatic amino acid; Metabolic engineering; Gene overexpression

1. Introduction

The biotechnological production of aromatic amino acids from renewable biomass feedstocks represents an important route for the provision of valuable compounds by means of green chemistry. Aromatic amino acids are used as food and feed additives, dietary supplements, pharmaceuticals and neutraceuticals (Bongaerts et al., 2001). Diverse metabolic engineering strategies have been applied to microbial L-phenylalanine and L-tryptophan production yielding titers of 50–60 g/l, but much less attention has been paid to the fermentative production of L-tyrosine (Ikeda, 2003; Leuchtenberger et al., 2005; Lütke-Eversloh et al., 2007). Aromatic amino acid producing bacteria have been generated by removal of enzymatic feedback-inhibition and transcriptional repression, reduction of by-product formation and overexpression of genes encoding known rate-limiting enzymes (Frost and Draths, 1995; Pittard, 1996; Bongaerts et al., 2001; Krämer et al., 2003; Ikeda, 2003, 2006; Sprenger, 2007). Very recently, these approaches have also been applied in the engineering of *Escherichia coli* for overproducing L-tyrosine (Lütke-Eversloh and Stephanopoulos, 2005, 2007a; Takai et al., 2005; Olson et al., 2007; Lütke-Eversloh et al., 2007). To this end, feedback-inhibition resistant mutants of the the *aroG* gene, which encodes the L-phenylalanine-sensitive 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, and of the *tyrA* gene, coding for the chorismate mutase/prephenate dehydrogenase, were overexpressed in an *E. coli ΔtyrR* strain lacking the

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TyrR-mediated transcriptional control for various AAAB (aromatic amino acid biosynthesis) genes (Lütke-Eversloh and Stephanopoulos, 2005, 2007a; Takai et al., 2005). A different strategy was applied in another study, where the L-phenylalanine producing *E. coli* strains NST37 and NST74 were converted into L-tyrosine producers by replacing the *pheA* gene, which codes for the L-phenylalanine branch point enzyme chorismate mutase/prephenate dehydratase, with a *tyrA*-kanamycin cassette harboring the strong constitutive *trc* promotor (Tribe, 1987; Olson et al., 2007).

To date, all metabolic engineering approaches employed for bacterial amino acid production were based on rational methods of pathway design whereby pathway engineering strategies capitalized on prior knowledge of pathway topology, kinetics and regulation. Overexpression of AAAB genes to increase the production of aromatics in *E. coli* and other bacteria has been performed previously with *aroL*, encoding a shikimate kinase, being a common overexpression gene target (Ito et al., 1990; Krämer et al., 2003; Takai et al., 2005; Ikeda 2006). An interesting study on putative rate-limiting AAAB enzymes was performed using ^{1}H-NMR analysis of culture supernatant samples from different L-phenylalanine overproducing strains of *E. coli* (Dell and Frost, 1993). Based on measurements of the extracellular accumulation of AAAB intermediates, *aroB*, *aroL*, *aroA* and *aroC* and their combinations were targeted for overexpression and were found to remove the impediments of the AAAB pathway in L-phenylalanine producing *E. coli* strains (Dell and Frost, 1993, Frost et al., 1998). Chromosomal integration of a multi-gene cassette comprising *aroACB* and a kanamycin resistance gene under the control of the *tac* promotor into an *E. coli tyrR* mutant resulted in a stable L-phenylalanine producer, which would circumvent fermentation problems due to plasmid losses (Snell et al., 1996). More recently, limitations of L-phenylalanine production in *E. coli* caused by insufficient gene expression of *aroB*, *aroL* and *aroA* were inferred from metabolic profiling of intracellular AAAB intermediates in glucose pulse experiments (Oldiges et al., 2004).

In this study, we were also interested in rate-limiting enzymes of the AAAB pathway, but in contrast to the above-mentioned investigations, we initially did not consider any experimental data on intermediate concentrations or metabolic, biochemical or regulatory knowledge on microbial physiology. The combinatorial gene overexpression approach was intended to exploit the entire AAAB pathway for possible bottlenecks, and interesting results on the metabolic impediments to L-tyrosine production in *E. coli* were obtained from this approach.

2. Materials and methods

2.1. Bacterial strains and cultivation conditions

The generation of the L-tyrosine producing strains *E. coli* T1 and T2 has been described earlier (Lütke-Eversloh and Stephanopoulos, 2007a). The strains were grown in Luria Bertani (LB) or MOPS-buffered minimal medium (Neidhardt et al., 1974) comprising 5 g/l glucose and 2 g/l NH_4Cl. For the maintenance of plasmids, respective antibiotics were added (Sambrock et al., 1989) and for the induction of the P_{lac} promoter, 0.5 mM isopropyl-β-D-thiogalctopyranoside (IPTG) was added. L-tyrosine production experiments were performed at 37 °C as 40 ml cultures in 250 ml Erlenmeyer flasks, as 2 ml cultures in 15 ml test tubes or as 200 µl cultures in 96-well microtiter plates.

2.2. Isolation, manipulation and transfer of DNA

Plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (Qiagen, Valencia CA). Chromosomal DNA from *E. coli* K12 was prepared by using the Wizard Genomic DNA Purification Kit (Promega, Madison WI). Agarose gel purification of DNA fragments was done with the Geneclean Spin Kit (Q-Biogene, Carlsbad CA). Restriction enzymes, ligases and other DNA-manipulating enzymes were used according to the manufacturer's manual. All plasmid constructs were verified by DNA sequencing. Plasmid DNA was transferred to chemically competent cells of *E. coli* DH5α (Invitrogen, Carlsbad CA) and to *E. coli* T1 and T2 by electroporation (Sambrock et al., 1989), respectively.

2.3. Construction of plasmid pTL1

For the generation of plasmid pTL1, a multiple cloning site (MCS) and a constitutive promotor were introduced into the medium-copy vector pBR322 (Bolivar et al., 1977). The MCS was obtained by polymerase chain reaction (PCR) of a synthetic oligonucleotide (5′-aaa aag aat tca gct ctc ata tcc cgg gcc cta ggt acc gcg gcc gct aag agc tct cta gat tat cga tac aca-3′) using the primers MCS_fw (5′-aaa aag aat tca gct c-3′) and MCS_rv (5′-tgt gta tcg ata atc t-3′) (Invitrogen, Carlsbad CA). After digestion with *Eco*RI and *Cla*I, the MCS was ligated into *Eco*RI-*Cla*I-digested pBR322. The $P_{LtetO-1}$ promoter was obtained from plasmid pZE21-MCS1 (Lutz and Bujard, 1997) by *Aat*II and *Eco*RI digestion, agarose gel purified and cloned into *Aat*II-*Eco*RI-digested pBR322/MCS, resulting in plasmid pTL1.

2.4. Cloning and overexpression of AAAB genes

The nine AAAB genes *aroB*, *aroD*, *aroE*, *ydiB*, *aroK*, *aroL*, *aroA*, *aroC* and *tyrB* were amplified by PCR from genomic DNA of *E. coli* K12 using primers listed in Table S1 (Supplementary material). Each forward primer comprised a ribosome binding site with a distance of nine bases towards the start codon and an *Eco*RI restriction site; each reverse primer included a *Sma*I/*Xma*I restriction site. The PCR products were digested with *Eco*RI and *Xma*I and cloned into *Eco*RI-*Xma*I-digested pTL1 after agarose gel purification. For the plasmid constructs comprising two AAAB genes, *aroK* was amplified from pTL1::*aroK* using primers with *Kpn*I and *Xma*I restriction sites and