Available online at www.sciencedirect.com
ScienceDirect
Metabolic Engineering 10 (2008) 69–77
METABOLIC ENGINEERING
www.elsevier.com/locate/ymben

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

Tina Lütke-Eversloh, Gregory Stephanopoulos*

Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Room 56-469, Cambridge MA, USA

Received 22 September 2007; received in revised form 29 November 2007; accepted 3 December 2007
Available online 1 February 2008

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

*Corresponding author. Fax: +1 617 253 3122.
E-mail address: gregstep@mit.edu (G. Stephanopoulos).

1096-7176/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2007.12.001

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

cloned into the respective constructs harboring single AAAB genes via *Kpn*I and *Xma*I. Analogously, *ydiB* and *aroA* were cloned via *Kpn*I and *Sac*I restriction sites (Table S1).

All ligation mixtures were first transformed to chemically competent cells of *E. coli* DH5α (Invitrogen, Carlsbad CA) and after controlling by DNA sequencing, the plasmid constructs were transferred by electroporation to *E. coli* T1 and T2.

2.5. Analysis of L-tyrosine

For quantification of L-tyrosine, cell-free culture supernatant samples were filtered through 0.2 μm PVDF membrane filters (Acrodisc LC 13 mm syringe filters, PALL Life Sciences, East Hills NY) and used for HPLC analysis with a Waters 2690 Separations module connected with a Waters 996 Photodiode Array (PDA) detector (Waters Corp., Milford MA), set to a wavelength of 278 nm. The samples were separated on a Waters Resolve C18 column with 0.1% (v/v) trifluoroacetic acid (TFA) in water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B) as mobile phase. The following gradient was used at a flow rate of 1 ml/min: 0 min, 95% solvent A + 5% solvent B; 8 min, 20% solvent A + 80% solvent B; 10 min, 80% solvent A + 20% solvent B; 11 min, 95% solvent A + 5% solvent B. The L-tyrosine concentrations in the samples were determined according to a standard, the free acid of L-tyrosine (Sigma-Aldrich, St. Louis MO). Culture samples containing >500 mg/l L-tyrosine were diluted accordingly and alkalized with potassium hydroxide to a final concentration of 0.25 M KOH and incubated for 30 min at 37 °C to dissolve all L-tyrosine. The mixture was then centrifuged for 5 min at 14,000 rpm and the supernatant was filtered through a 0.2 μm syringe filter before applying to the HPLC column.

For the analysis of large numbers of samples, i.e. from cultivations in microtiter plates, L-tyrosine was determined by the fluorescence-based nitrosonaphthol assay (Waalkes and Udenfriend, 1957; Lütke-Eversloh and Stephanopoulos, 2007b). For this, 100 μl of undiluted or 1:10 (v/v) culture samples were mixed with 100 μl of the reaction solution consisting of a 1:1 (v/v) mixture of 0.1% (w/v) 1-nitroso-2-naphthol in ethanol and 20% (v/v) nitric acid containing 0.5 g/l (w/v) $NaNO_2$ and incubated for 45 min at 55 °C. After incubation for 120–240 min at room temperature, fluorescence was measured in a Packard Fusion microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 590 nm.

2.6. Analyses of cell growth, glucose and acetate

Cell growth was monitored by measurement of the Optical Density at 600 nm (OD_{600}) on an Ultrospec 2100 *pro* UV/VIS spectrophotometer (Amersham Biosciences, Piscataway NJ). For determination of the cellular dry weight (CDW) per liter, the weight of cell pellets of 25 ml of culture was measured after drying at 75 °C for 12–16 h.

Glucose concentrations were measured in culture supernatant samples with a YSI 2700 SELECT biochemistry analyzer (Yellow Springs Instruments Inc., Yellow Springs OH, USA).

Acetate was quantified by HPLC in cell-free culture supernatant samples filtered through 0.2 μm PVDF membrane filters (Acrodisc LC 13 mm syringe filters, PALL Life Sciences, East Hills NY) with a Waters 2690 Separations module connected with a Waters 996 Photodiode Array (PDA) detector (Waters Corp., Milford MA), set to a wavelength of 209 nm. Separation was performed on an Aminex organic acid column Bio-Rad HPX87H equipped with a guard column (Bio-Rad Laboratories, Hercules CA) at 50 °C for 30 min with 14 mM sulfuric acid at a flow rate of 0.7 ml/min.

3. Results and discussion

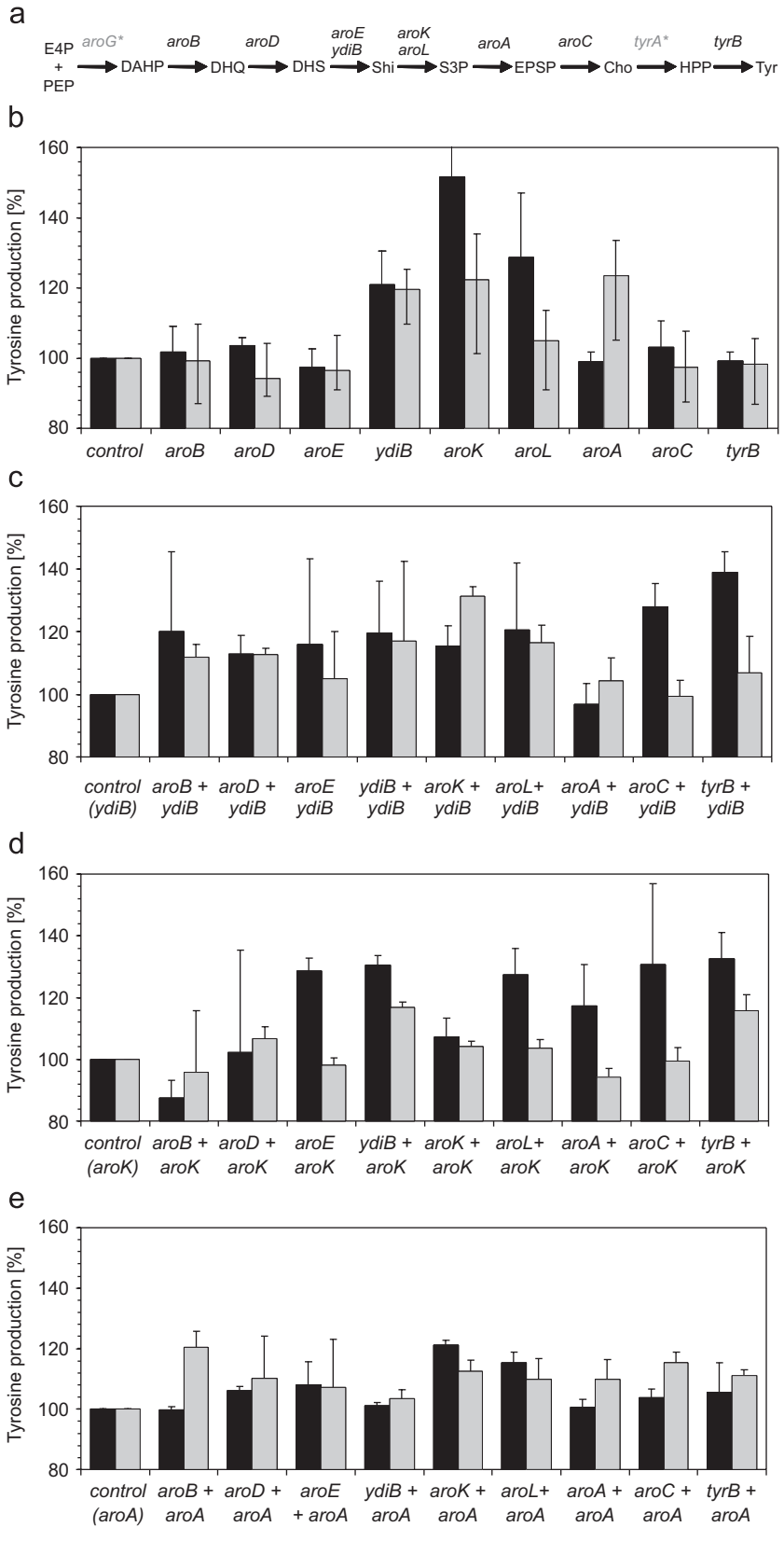
In *E. coli*, the AAAB pathway starts with the condensation of phospho*enol*pyruvate (PEP) and erythrose-4-phosphate (E4P), catalyzed by the 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, which occurs as three isoforms, each feedback-regulated by L-phenylalanine (AroG), L-tyrosine (AroF) or L-tryptophan (AroH). Subsequently, DAHP is converted to chorismate via dehydroquinate, dehydroshikimate, shikimate, shikimate-3-phosphate and 5-enol-pyruvylshikimate-3-phosphate (EPSP). From chorismate, different pathways diverge and L-tyrosine biosynthesis is catalyzed by the bifunctional enzyme chorismate mutase/prephenate dehydrogenase and an aromatic amino acid transaminase (Pittard, 1996). A simplified schematic of the AAAB pathway for the production of L-tyrosine from PEP and E4P and the genes encoding the respective enzymes are illustrated in Fig. 1a.

Recently, we have generated L-tyrosine excreting strains of *E. coli* based on the deregulation of the AAAB pathway. *E. coli* T1 harbored feedback-inhibition resistant (fbr) derivatives of the DAHP synthase ($aroG^{fbr}$) and chorismate mutase/prephenate dehydrogenase ($tyrA^{fbr}$) genes, which were overexpressed in an *E. coli* K-12 *ΔtyrR* strain. The second L-tyrosine producer, *E. coli* T2, was engineered such as to also overexpress the *ppsA* and *tktA* genes encoding for PEP synthetase and a transketolase, respectively, in the strain T1 background in order to increase the AAAB precursor supply (Lütke-Eversloh and Stephanopoulos, 2007a). These two *E. coli* strains were used in this study to further investigate limiting enzymatic reactions of the AAAB pathway in order to improve L-tyrosine production.

3.1. Identification of ydiB, aroK and aroA as putative bottlenecks by combinatorial overexpression of single AAAB genes

All AAAB genes except *aroG* and *tyrA*, whose fbr-derivatives were already overexpressed in the host strains, i.

e. *aroB*, *aroD*, *aroE*, *ydiB*, *aroK*, *aroL*, *aroA*, *aroC* and *tyrB* were cloned into the pBR322-derived plasmid pTL1. The AAAB genes were overexpressed in both *E. coli* T1 and T2 and L-tyrosine production was determined and compared to the respective control strain harboring only the vector pTL1. Significant improvement of L-tyrosine production by *E. coli* T1 was observed in strains overexpressing *ydiB*, *aroK* or *aroL*, whereas *E. coli* T2 exhibited increased



L-tyrosine production when *ydiB*, *aroK* or *aroA* were overexpressed (Fig. 1b).

The paralogous genes *aroE* and *ydiB* both code for shikimate dehydrogenases, but only overexpression of *ydiB*, not *aroE*, exhibited an increase in L-tyrosine production of about 20% in both *E. coli* T1 and T2. In contrast to *ydiB*, the *aroE*-encoded shikimate dehydrogenase is feedback-inhibited by shikimate, explaining the different effects of overexpression on product formation (Dell and Frost, 1993). Although *ydiB* and *aroE* have only low DNA sequence similarity, the crystal structure of both enzymes revealed a very similar architecture. Since *ydiB* is located between AAAB-related genes on the *E. coli* chromosome, but not *aroE*, one may speculate that the *ydiB*-encoded enzyme was the first shikimate dehydrogenase of the AAAB pathway from the evolutionary point of view (Michel et al., 2003). However, if only the *aroE*-encoded feedback-inhibited shikimate dehydrogenase would be available in *E. coli*, generation of a shikimate-insensitive version of this enzyme would possibly overcome the shikimate dehydrogenase bottleneck and lead to improved L-tyrosine production.

In previous studies, increased shikimate kinase activity, achieved by *aroL* overexpression, has been shown to improve the flux towards chorismate formation through the AAAB pathway (Ito et al., 1990; Dell and Frost, 1993; Oldiges et al., 2004; Ikeda 2006). The *aroL*-encoded shikimate kinase II is the dominant isoenzyme in wild-type *E. coli* with high substrate affinity and tight regulation. The *aroK*-encoded shikimate kinase I does not presumably play a major role under normal growth conditions. It may also be involved in other so far unknown metabolic reactions, and its K_M value of 20 mM is hundred-fold higher than the K_M value of the shikimate kinase II (Ely and Pittard, 1979; DeFeyter, 1987). We found that overexpression of both, *aroK* and *aroL* elevated L-tyrosine production, but *aroK* overexpression led to a much higher increase, particularly in *E. coli* T1 (Fig. 1b). According to AAAB metabolite analyses in culture supernatants and overexpression experiments, *aroL* has been shown to improve aromatic amino acid production, but the effect of *aroK* overexpression has never been investigated before—presumably because it was not known in early studies and subsequent investigations focussed on the well-known *aroL*-encoded shikimate kinase II (Dell and Frost, 1993; Frost et al., 1998; Krämer et al., 2003; Takai et al., 2005, Ikeda, 2006). More recently, activities of both shikimate kinase I and II were proposed in L-phenylalanine producing *E. coli* cells with high AAAB intermediate levels, but the authors suggested *aroL* as the preferred metabolic engineering target because of the higher K_M of the shikimate kinase II (Oldiges et al., 2004). However, shikimate kinase II is inhibited at high substrate concentrations, as shown *in vitro* and in *aroL* mutants, but *aroK*-encoded shikimate kinase I supposedly functions well in an uninhibited fashion under these conditions (DeFeyter and Pittard, 1986; DeFeyter, 1987). Considering the high intracellular AAAB metabolite concentrations, it is quite possible that shikimate kinase II activity is limiting due to substrate inhibition in L-tyrosine producing *E. coli* T1 and T2. Under this hypothesis, overexpression of *aroK* would catalyze efficient shikimate kinase I-mediated conversion of shikimate and thus improve phenotype. Since *aroK* and *aroL* code for enzymes catalyzing the same AAAB reaction and overexpression of *aroL* revealed not as much improved product formation, only *aroK* was considered for further overexpression experiments.

Besides shikimate dehydrogenase and shikimate kinase, the *aroA*-encoded EPSP synthase was identified as third enzymatic bottleneck for L-tyrosine production by single AAAB gene overexpression. Interestingly, overexpression of *aroA* led to increased L-tyrosine production only in *E. coli* T2, but not in strain T1. The pre-engineered *E. coli* T2 overexpressed *tktA* and *ppsA* to increase the availability of the AAAB presursors PEP and E4P, which has been demonstrated as one of the major impediments of the AAAB pathway (Patnaik and Liao, 1994; Patnaik et al., 1995; Chandran et al., 2003). Since the conversion of shikimate-3-phosphate to EPSP requires another PEP molecule, elevated EPSP synthase activity achieved by *aroA* overexpression only became relevant if sufficient PEP was available, which was supposedly realized in *E. coli* T2, but not in strain T1.

3.2. Combinatorial overexpression analysis of AAAB genes combined with ydiB, aroK and aroA

Following identification of *ydiB*, *aroK* and *aroA* as metabolic engineering targets, we next undertook the combinatorial overexpression of pairs of AAAB genes in

Fig. 1. Combinatorial overexpression of AAAB genes in *E. coli* T1 and T2. Schematic overview of L-tyrosine production via AAAB pathway in *E. coli* (a), overexpression of single (b) and AAAB genes combined with *ydiB* (c), *aroK* (d) and *aroA* (e), respectively, in *E. coli* T1 (black bars) and *E. coli* T2 (grey bars). The strains were cultivated for 20–24 h at 37 °C in MOPS-buffered minimal medium containing 5 g/l glucose, 2 g/l ammonium chloride, 0.5 mM IPTG, 100 mg/l ampicillin and 50 mg/l spectinomycin, respectively. The L-tyrosine concentrations in the culture supernatants were converted to percent increase referring to the respective control strain (b, pTL1; c, pTL1::*ydiB*; d, pTL1::*aroK*; e, pTL1::*aroA*). The cultivation experiments were done in multiple replicates, and the error bars represent the data range. Metabolites and genes (a): E4P, erythrose-4-phosphate; PEP, phospho*enol*pyruvate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; Shi, shikimate; S3P, shikimate-3-phosphate; EPSP, 5-enol-pyruvylshikimate-3-phosphate; Cho, chorismate; HPP, 4-hydroxyphenylpyruvate; Tyr, L-tyrosine; *aroG**, fbr-DAHP synthase; *aroB*, DHQ synthase; *aroD*, DHQ dehydratase; *aroE*, shikimate dehydrogenase; *ydiB*, quinate/shikimate dehydrogenase; *aroK*, shikimate kinase I; *aroL*, shikimate kinase II; *aroA*, EPSP synthase; *aroC*, chorismate synthase; *tyrA**, fbr-chorismate mutase/prephenate dehydrogenase; *tyrB*, aromatic amino acid transaminase.

Table 1
L-Tyrosine production of *E. coli* T1 and T2 overexpressing *ydiB*, *aroK* and *aroA* as well as combinations thereof

E. coli T1					*E. coli* T2				
	−/−	*ydiB*	*aroK*	*aroA*		−/−	*ydiB*	*aroK*	*aroA*
−/−	173±7	207±8	234±18	186±6	−/−	258±12	297±4	309±6	283±11
ydiB	207±8	219±13	270±13	216±10	*ydiB*	297±4	296±11	354±4	309±15
aroK	234±18	262±11	227±11	234±4	*aroK*	309±6	361±14	301±10	279±6
aroA	186±6	207±3	225±17	168±5	*aroA*	283±11	315±12	283±14	249±8

The first overexpression genes are listed in columns, the second overexpression genes are listed in rows. The values are shown in milligram L-tyrosine per gram cellular dry weight. The strains were cultivated for 20 h at 37 °C in 40 mL MOPS-buffered minimal medium containing 5 g/L glucose, 2 g/L ammonium chloride, 0.5 mM IPTG, 100 mg/L ampicillin and 50 mg/L spectinomycin, respectively. The results were obtained from four independent cultivation experiments and the standard deviations are given.
Abbreviation: −/−, no gene.

order to unveil further bottlenecks of L-tyrosine production after overcoming the first AAAB limitation. For this goal, more than 50 new strains were constructed and L-tyrosine production was determined for each strain and compared with the respective control strains expressing only *ydiB* (Fig. 1c), *aroK* (Fig. 1d) and *aroA* (Fig. 1e), respectively.

It was not surprising that combining the overexpression of *ydiB* and *aroK* yielded the overall best L-tyrosine producers for both strains *E. coli* T1 and T2. However, other overexpression targets were also found, which were not obvious from the first instance of single AAAB gene overexpression.

Among the gene combinations tested, *aroC* for *E. coli* T1 and *tyrB* for both strain emerged as the most striking overexpression targets, because these genes code for enzymes at the very end of the L-tyrosine biosynthesis pathway (Fig. 1a). Overexpression of *aroB* showed increased L-tyrosine production in both strains only in combination with *ydiB*, but not with *aroK*, and additionally with *aroA* in strain T2. Surprisingly, AAAB combinations with *aroA* did not reveal as many improved phenotypes in comparison with the *ydiB* and *aroK* combinations, whereas no significcant increases at all were detected in *E. coli* T1 (Fig. 1e).

Overall, the results of this study are in good agreement with previous studies based on AAAB metabolite analyses and rational gene overexpression predictions, but here we also identified novel gene overexpression targets due to the broader and more exhaustive nature of the combinatorial approach (Dell and Frost, 1993; Oldiges et al., 2004).

The results presented in Fig. 1 were based on approximately 500 cultivation experiments conducted in mircrotiter plates, test tubes and Erlenmeyer flasks. Since L-tyrosine production is associated with biomass formation (Lütke-Eversloh and Stephanopoulos, 2007a) and cell densities varied considerably according to the fermentation scales, data are presented as total increases in L-tyrosine production with respect to the corresponding control cultures. As such, results were well comparable within each set of cultivation experiments. Those strains exhibiting significantly improved production phenotypes were subjected to further experimental validation. As shown in Table 1, L-tyrosine production by strains overexpressing *ydiB*, *aroK* and *aroA*, as well as combinations thereof, were investigated and overexpression of *ydiB* and *aroK* was confirmed to result in the best L-tyrosine producers of this study, yielding 270 and 360 milligrams L-tyrosine per gram cell dry weight, resepectively. Moreover, overexpression of two copies of either gene did not further improve L-tyrosine production and the sequence of the cloned genes, e. g. *ydiB-aroK* vs. *aroK-ydiB*, did not have a significant influence on L-tyrosine production. In addition, overexpression of *aroA*-combinations were confirmed to not being interesting metabolic engineering targets in the second instance (Table 1). Further experimental validation of relevant AAAB gene overexpression candidates was obtained by monitoring L-tyrosine production during fermentation experiments of various engineered strains (Figs. 2 and S2).

3.3. Characterization of engineered vs. parental L-tyrosine producing strains

As shown above, *E. coli* T1 exhibited generally greater increases of L-tyrosine compared to strain T2, indicating that this, initially lower L-tyrosine producer, has more room for phenotype improvement (Figs. 1 and S1). We chose *E. coli* T1 and T2 overexpressing *ydiB* and *aroK* for detailed analyses and compared these engineered strains, T1-YK and T2-YK, with the parental strains harboring only the vector pTL1 (Fig. 2). Overexpression of *ydiB* and *aroK* improved the final L-tyrosine titer by 45% in *E. coli* T1 and by 26% in *E. coli* T2, respectively. As shown previously, the bulk of L-tyrosine production occurred in the exponential growth phase (Lütke-Eversloh and Stephanopoulos, 2007a), and additional glucose feeding did not improve L-tyrosine production (Fig. 2, grey circles).

In an approach aiming to uncouple L-tyrosine biosynthesis from biomass formation, experiments with resting cells were conducted. To this end, cell growth was arrested in the middle of the exponential growth phase by addition of

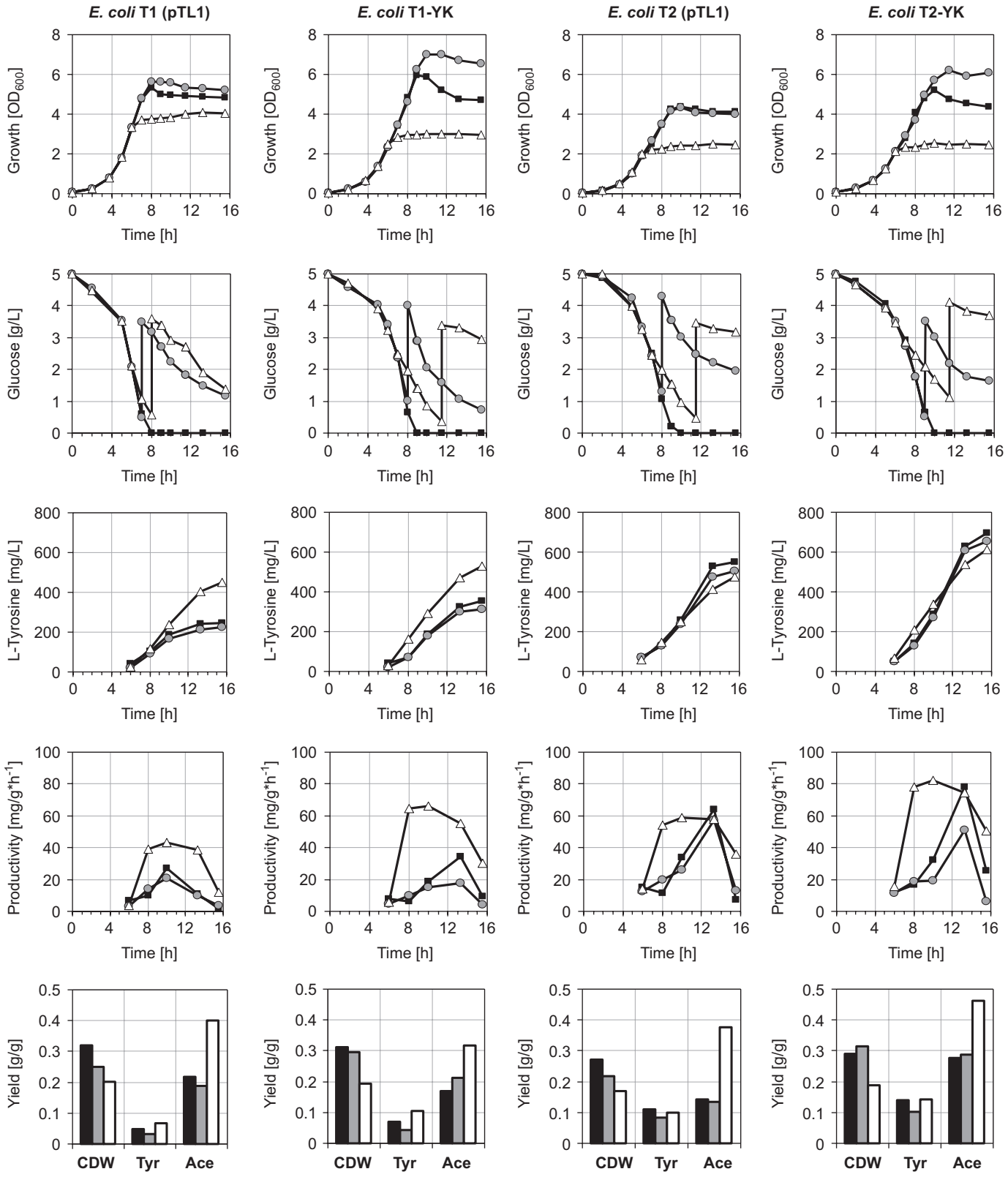


Fig. 2. Characterization of engineered and parental L-tyrosine production strains. *E. coli* T1 (pTL1, vector control), *E. coli* T1-YK (pTL1::*ydiBaroK*, engineered strain), *E. coli* T2 (pTL1, vector control), and *E. coli* T2-YK (pTL1::*ydiBaroK*, engineered strain) were cultivated for 16 h at 37 °C in 40 ml MOPS-buffered minimal medium (5 g/l glucose, 2 g/l ammonium chloride, 0.5 mM IPTG, 100 mg/l ampicillin and 50 mg/l spectinomycin) under different conditions: batch culture (black squares), fed-batch with 3 g/l glucose (grey circles) and growth inhibition after 6 h of cultivation by addition of 30 mg/l chloramphenicol (empty triangles). Cell growth and glucose consumption were monitored during fermentation, L-tyrosine concentrations and specific productivities were determined as well as the final yields of cell dry weight (CDW), L-tyrosine (Tyr) and acetate (Ace) per gram glucose.

chloramphenicol after 6 h which inhibited further growth but not directly the function of the AAAB pathway (Fig. 2, empty triangles). In comparison to the batch and fed-batch cultures, L-tyrosine concentrations and productivities of the non-growing cells increased rapidly immediately after approximately two hours following the introduction of the growth inhibitor with the maximum specific productivity occurring at 10 h. The L-tyrosine concentrations increased linearly before leveling after 13 h, whereas the batch and fed-batch cultures revealed an exponential L-tyrosine formation pattern in accordance with the growth curve. Interestingly, these cultivation conditions, which also resulted in higher acetate formation, improved L-tyrosine production very clearly in *E. coli* T1 and T1-YK, whereas *E. coli* T2 and T2-YK showed their maximum L-tyrosine titers in batch cultures, and *E. coli* T1-YK even outperformed the *E. coli* T2 strain (Fig. 2).

In general, phenotypic characterization of engineered L-tyrosine producers revealed that higher L-tyrosine formation was accompanied by reduced growth rates. Specifically, growth rates were determined as $\mu = 0.62\,h^{-1}$ for *E. coli* T1, $\mu = 0.57\,h^{-1}$ for *E. coli* T1-YK, $\mu = 0.52\,h^{-1}$ for *E. coli* T2, and $\mu = 0.46\,h^{-1}$ for *E. coli* T2-YK. The negative impact of L-tyrosine production on the growth rate was also observed in various other engineered strains of this study (Fig. S3).

Overall, greater improvements in L-tyrosine production were achieved in initially lower L-tyrosine producers. As revealed by the resting cell experiments, varying the cultivation conditions had a much greater impact on lower L-tyrosine producers than on higher producers. Finally, a maximum of ~700 mg/l L-tyrosine was achieved by engineered *E. coli* T2 strains in shaken flasks experiments (Figs. 2 and S2). However, high-cell density fermentations of the parental *E. coli* T1 and T2 strains carried out in controlled bioreactors yielded up to 9.7 g/l L-tyrosine production (Lütke-Eversloh and Stephanopoulos, 2007a). More detailed evaluation of the bioreactor performance of the engineered strains described in this study is in order.

3.4. Conclusions and perspectives

The metabolic engineering approach presented here explored the combinatorial overexpression of an entire gene set encoding a specific metabolic pathway in *E. coli*, namely the AAAB pathway for biosynthesis and overproduction of L-tyrosine. By constructing various AAAB gene overexpression combinations in *E. coli* T1 and T2 strains the L-tyrosine production of each strain was monitored, and combinations of *ydiB*, *aroK* and *tyrB* were identified as major overexpression targets for improved L-tyrosine production. Increased shikimate kinase (*aroK*/*aroL*) activity for improved aromatics production has been described in previous publications (Dell and Frost, 1993; Snell et al., 1996; Oldiges et al., 2004), but shikimate dehydrogenase (*ydiB*) and aromatic amino acid transaminase (*tyrB*) were found here as unexpected new metabolic engineering targets. This combinatorial overexpression approach can easily be applied to other systems to identify enzymatic bottlenecks of metabolic pathways.

The construction of 72 strains of this study evaluated the overexpression of single and double AAAB genes in two stages. First, *ydiB*, *aroK* and *aroA* were identified in the first round and subsequently, all possible AAAB genes plus *ydiB*, *aroK* and *aroA*, respectively, combinations were examined. In the second round, various new and unexpected AAAB gene combinations, such as *aroB* plus *ydiB* or *tyrB* plus *aroK*, were found to significantly improve the phenotype. Hence, it would be an intriguing challenge to reveal which other AAAB gene combinations would lead to increased L-tyrosine production—considering not only the other possible 135 *E. coli* T1 and T2 strains with AAAB gene pairs or the 1458 possible triple AAAB gene combinations, but the entire space of gene combinations. We demonstrated that systematic gene overexpression can be a powerful tool for the identification of metabolic pathway impediments, but the obtained results also indicated only the tip of the iceberg and further extension of this approach may become limiting due to elaborate cloning work. Therefore, random AAAB gene combinations would eventually exploit the full capacity of AAAB gene overexpression for improving L-tyrosine production in *E. coli*, but a high-throughput screening method would be required. Since L-tyrosine is a colorless and diffusible compound, two screening techniques combining L-tyrosine production with fluorescence or melanin pigment synthesis were developed very recently (Lütke-Eversloh and Stephanopoulos, 2007b; Santos and Stephanopoulos, 2007), and future work on random metabolic engineering approaches such as shuffled AAAB gene overexpression libraries will possibly reveal further new gene targets for improved L-tyrosine production.

Acknowledgments

The authors thank the Deutsche Forschungsgemeinschaft (DFG) for providing the research fellowship LU 893/2-1 and the DuPont-MIT Alliance for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at 10.1016/j.ymben.2007.12.001.

References

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heynecker, H.L., Boyer, H.W., 1977. Construction and characterization of new cloning vehicles. 2: multipurpose cloning system. Gene 2, 95–113.

Bongaerts, J., Krämer, M., Müller, U., Raeven, L., Wubbolts, M., 2001. Metabolic engineering for microbial production of aromatic amino acids and derived compounds. Metab. Eng. 3, 289–300.

Chandran, S.S., Yi, J., Draths, K.M., von Daeniken, R., Weber, W., Frost, J.W., 2003. Phosphoenolpyruvate availability and the biosynthesis of shikimic acid. Biotechnol. Prog. 19, 808–814.

DeFeyter, R.C., 1987. Shikimate kinases from *Escherichia coli* K12. Methods Enzymol. 142, 355–361.

DeFeyter, R.C., Pittard, J., 1986. Purification and properties of shikimate kinase II from *Escherichia coli* K-12. J. Bacteriol. 165, 331–333.

Dell, K.A., Frost, J.W., 1993. Identification and removal of impediments to biocatalytic synthesis of aromatics from D-glucose: rate-limiting enzymes in the common pathway of aromatic amino acid biosynthesis. J. Am. Chem. Soc. 115, 11581–11589.

Ely, B., Pittard, J., 1979. Aromatic amino acid biosynthesis: Regulation of shikimate kinase in *Escherichia coli* K-12. J. Bacteriol. 138, 933–943.

Frost, J.W., Draths, K.M., 1995. Biocatalytic synthesis of aromatics from D-glucose: renewable microbial sources of aromatic compounds. Annu. Rev. Mircobiol. 49, 557–579.

Frost, J.W., Snell, K.D., Frost, K.M., 1998. Deblocking the common pathway of aromatic amino acid synthesis. United States Patent No. US 5,776,736.

Ikeda, M., 2003. Amino acid production processes. In: Scheper, T., Faurie, R., Thommel, J. (Eds.), Advances in Biochemical Engineering/biotechnology, vol. 79. Springer, Berlin, Heidelberg, New York, pp. 1–35.

Ikeda, M., 2006. Towards bacterial strains overproducing L-tryptophan and other aromatics by metabolic engineering. Appl. Microbiol. Biotechnol. 69, 615–626.

Ito, H., Sato, K., Enei, H., Hirose, Y., 1990. Improvement in microbial production of L-tyrosine by gene dosage effect of *aroL* gene encoding shikimate kinase. Agric. Biol. Chem. 54, 823–824.

Krämer, M., Bongaerts, J., Bovenberg, R., Kremer, S., Müller, U., Orf, S., Wubbolts, M., Raeven, L., 2003. Metabolic engineering for microbial production of shikimic acid. Metab. Eng. 5, 277–283.

Leuchtenberger, W., Huthmacher, K., Drauz, K., 2005. Biotechnological production of amino acids and derivatives: current status and prospects. Appl. Microbiol. Biotechnol. 69, 1–8.

Lutz, R., Bujard, H., 1997. Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I_1-I_2 regulatory elements. Nucleic Acids Res 25, 1203–1210.

Lütke-Eversloh, T., Stephanopoulos, G., 2005. Feedback inhibition of chorismate mutase/prephenate dehydrogenase (TyrA) of *Escherichia coli*: generation and characterization of tyrosine-insensitive mutants. Appl. Environ. Microbiol. 71, 7224–7228.

Lütke-Eversloh, T., Stephanopoulos, G., 2007a. L-Tyrosine production by deregulated strains of *Escherichia coli*. Appl. Microbiol. Biotechnol. 75, 103–110.

Lütke-Eversloh, T., Stephanopoulos, G., 2007b. A semi-quantitative high-throughput screening method for microbial L-tyrosine production in microtiter plates. J. Ind. Microbiol. Biotechnol. 34, 807–811.

Lütke-Eversloh, T., Santos, C.N.S., Stephanopoulos, G., 2007. Perspectives of biotechnological production of L-tyrosine and its applications. Appl. Microbiol. Biotechnol. 77, 751–762.

Michel, G., Roszak, A.W., Sauvé, V., Maclean, J., Matte, A., Coggins, J.R., Cygler, M., Lapthorn, A.J., 2003. Structure of shikimate dehydrogenase AroE and its paralog YdiB. J. Biol. Chem. 278, 19463–19472.

Neidhardt, F.C., Bloch, P.L., Smith, D.F., 1974. Culture medium for enterobacteria. J. Bacteriol. 119, 736–747.

Oldiges, M., Kunze, M., Degenring, D., Sprenger, G.A., Takors, R., 2004. Stimulation, monitoring, and analysis of pathway dynamics by metabolic profiling in the aromatic amino acid pathway. Biotechnol. Prog. 20, 1623–1633.

Olson, M.M., Templeton, L.J., Suh, W., Youderian, P., Sariaslani, F.S., Gatenby, A.A., Van Dyk, T.K., 2007. Production of tyrosine from sucrose or glucose achieved by rapid genetic changes to phenylalanine-producing *Escherichia coli* strains. Appl. Microbiol. Biotechnol. 74, 1031–1040.

Patnaik, R., Liao, J.C., 1994. Engineering of *Escherichia coli* central metabolism for aromatic metabolite production with near theoretical yield. Appl. Environ. Microbiol. 60, 3903–3908.

Patnaik, R., Spitzer, R.G., Liao, J.C., 1995. Pathway engineering for production of aromatics in *Escherichia coli*: confirmation of stoichiometric analysis by independent modulation of AroG, TktA and Pps activities. Biotech. Bioeng. 46, 361–370.

Pittard, J., 1996. Biosynthesis of aromatic amino acids. In: Neidhardt, F.C. (Ed.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, vol. 1. American Society of Microbiology, Washington, DC, pp. 458–484.

Sambrock, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Santos, C.N.S., Stephanopoulos, G., 2007. Methods for identifying bacterial strains that produce L-tyrosine. United States Patent Application No. US 60/965,149.

Snell, K.D., Draths, K.M., Frost, J.W., 1996. Synthetic modification of the *Escherichia coli* chromosome: enhancing the biocatalytic conversion of glucose into aromatic chemicals. J. Am. Chem. Soc. 118, 5605–5614.

Sprenger, G.A., 2007. From scratch to value: engineering *Escherichia coli* wild type cells to the production of L-phenylalanine and other fine chemicals derived from chorismate. Appl. Microbiol. Biotechnol. 75, 739–749.

Takai, A., Nishi, R., Joe, Y., Ito, H., 2005. L-tyrosine producing bacterium and a method for producing L-tyrosine. United States Patent No. 2005/0277179 A1.

Tribe, D.E., 1987. Novel microorganism and method. United States Patent No. US 4,681,852.

Waalkes, T.P., Udenfriend, S., 1957. A fluorometric method for the estimation of tyrosine in plasma and tissues. J. Lab. Clin. Med. 50, 733–736.