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MINI-REVIEW

From scratch to value: engineering *Escherichia coli* wild type cells to the production of L-phenylalanine and other fine chemicals derived from chorismate

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Abstract Recombinant strains of *Escherichia coli* K-12 for the production of the three aromatic amino acids (L-phenylalanine, L-tryptophan, L-tyrosine) have been constructed. The largest demand is for L-phenylalanine (L-Phe), as it can be used as a building block for the low-calorie sweetener, aspartame. Besides L-Phe, an increasing number of shikimic acid pathway intermediates can be produced from appropriate *E. coli* mutants with blocks in this pathway. The last common intermediate, chorismate, in *E. coli* not only serves for production of aromatic amino acids but can also be used for high-titer production of non-aromatic compounds, e.g., cyclohexadiene-transdiols. In an approach to diversity-oriented metabolic engineering (metabolic grafting), platform strains with increased flux through the general aromatic pathway were created by suitable gene deletions, additions, or rearrangements. Examples for rational strain constructions for L-phenylalanine and chorismate derivatives are given with emphasis on genetic engineering. As a result, L-phenylalanine producers are available, which were derived through several defined steps from *E. coli* K-12 wild type. These mutant strains showed L-phenylalanine titers of up to 38 g/l of L-phenylalanine (and up to 45.5 g/l using in situ product recovery). Likewise, two cyclohexadiene-transdiols could be recovered.

Introduction

During the last two decades, industrial microbiology has seen a dramatic increase in productivities using recombinant microorganisms as producers of amino acids, vitamins, and other fine chemicals. Bacteria such as *Corynebacterium glutamicum* and *Escherichia coli* have been successfully developed for the production of amino acids to meet an ever-increasing nutritional demand for L-glutamic acid, L-lysine, L-threonine, and others. The annual worldwide production of amino acids has long surpassed the 1 million ton mark with present gross sales of several billion US dollars (Eggeling and Sahm 1999; Hermann 2003; Ikeda 2003; Leuchtenberger et al. 2005; Eggeling et al. 2006; Wendisch 2007; and chapters therein). Most amino acids are currently used as food ingredients for human consumption (monosodium glutamate and glycine) or feed additives (L-lysine and L-threonine; Leuchtenberger et al. 2005; Eggeling et al. 2006; Wendisch 2007).

The aromatic amino acid L-phenylalanine (next to L-glutamate, D,L-methionine and L-lysine) is among the most important commercially produced amino acids (Bongaerts et al. 2001; Ikeda 2006; Sprenger 2007) with annual sales of its derivatives approaching US$1 billion (Hermann 2003; Sprenger 2007). L-phenylalanine is used as food or feed additive, in infusion fluids, or for chemical syntheses of pharmaceutically active compounds (Bongaerts et al. 2001). Interestingly, the demand for L-phenylalanine stems mainly from being a building block in the synthesis of a dipeptide, the low-calorie sweetener aspartame (L-aspartyl-L-phenylalanine methyl ester), which is 160–180 times sweeter than sucrose (Ager et al. 1998) and is increasingly being used in diet drinks or food (Ager et al. 1998; Bongaerts et al. 2001). The estimated total market volume was 18,000 tons in 2006 (Ajinomoto 2006). Up to 20,000 tons more may be produced in China (Sprenger 2007). Several companies are manufacturing the sweetener based on different processes. L-Phenylalanine can be obtained by chemical, enzymatic, or microbial methods. Chemical

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synthesis of amino acids delivers racemic mixtures, for instance D,L-phenylalanine of which the methylester can be used in the DSM/Holland Sweetener/Tosoh process for aspartame using a metalloprotease, thermolysine, as coupling enzyme for the selective L,L-coupling with L-aspartic acid (Schmid et al. 2001; Bongaerts et al. 2001).

Enzymatic steps for L-phenylalanine synthesis are either by amination of *trans*-cinnamic acid, transamination, or reductive amination of phenylpyruvate (see Sprenger 2007). Microbial production involves either biotransformations of phenylpyruvate and aspartate with recombinant *E. coli* cells with elevated levels of aminotransferases and phospho*enol*pyruvate (PEP) carboxykinase or microbial fermentations from glucose, sucrose, or molasses. Especially, recombinant strains of *C. glutamicum* and *E. coli* are in industrial use for fermentative production of phenylalanine (Bongaerts et al. 2001; Ikeda 2003, 2006; Eggeling et al. 2006; Sprenger 2007 and refs therein).

This mini-review summarizes approaches to use recombinant *E. coli* as producer of building blocks from the aromatic amino acid biosynthesis pathway with emphasis on strain constructions for the synthesis of L-phenylalanine and more recent developments in production of chorismate-derived fine chemicals. This latter work started at the Institutes of Biotechnology at the Research Center in Jülich (Germany) under the auspices of Hermann Sahm (and his colleague Christian Wandrey) in the late 1990s, where he was a mentor in collaboration projects with DSM Biotech GmbH (Jülich) to explore the rational construction of *E. coli* strains, which produce compounds derived from the aromatic amino acid (AAA) pathway, L-phenylalanine, and other chorismate-derived fine chemicals.

Biosynthesis and regulation of L-phenylalanine production

To develop a commercially viable industrial process for large-volume manufacture of a given biological compound, improvements in producer strain(s) and process technology must occur (Berry 1996; Grinter 1998). For L-phenylalanine, as with other cases, a fundamental knowledge of the underlying biosynthesis pathway (AAA pathway) and especially of its genes, enzymes, and regulatory circuits is essential for microbial strain development and improvement (Berry 1996; Eggeling and Sahm 1999; Bongaerts et al. 2001; Ikeda 2003, 2006; Eggeling et al. 2006; Sprenger 2007).

Classical genetic breeding of microbial strains usually has been performed by iterative rounds of mutagenesis and selection and/or screening for useful strains, which often turned out either to be auxotrophic or regulatory mutants (Ikeda 2003). Tyrosine-auxotrophic mutants are of interest for L-phenylalanine production, as tyrosine biosynthesis up to prephenate runs in parallel to L-Phe whence the two pathways diverge. Overproduction of L-Phe could inadvertently lead to tyrosine production and thus form an unwanted by-product, which raises problems of its removal during downstream processing. Moreover, it would be a drain of carbon source and energy (which limits the productivity). On the other hand, a tyrosine-auxotroph mutant requires a source of tyrosine during the growth and maintenance phase (for examples see Backman et al. 1990; Takagi et al. 1996; Gerigk et al. 2002a, b; Rüffer et al. 2004).

As indicated already, during classical strain improvement, random mutagenesis and appropriate screening or selection methods were used. Antimetabolites (for example, 5-methyltryptophan or 5-fluorotryptophan for the case of L-tryptophan producers, Sahm and Zähner 1971) were helpful tools, as they allow the selection of feedback inhibition resistant mutants. For L-Phe, these compounds (e.g., fluoro- or chlorophenylalanine) exert the same feedback inhibition features as the natural amino acids on, e.g., 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase or the first committed steps of the terminal Phe pathway (references in Sprenger 2007). Thus, these antimetabolites induce starvation for L-phenylalanine and microbial cells are unable to grow in the absence of L-Phe. Mutants that are resistant against the antimetabolite(s) are able to grow and can easily be selected on suitable agar plates (De Boer and Dijkhuizen 1990). In many cases, the resistance relies on altered allosteric binding sites of the pacemaker enzymes or leads to deregulation of a pathway and product accumulation in the culture supernatant. The approaches of feedback deregulation and knockout of competing metabolic pathways via classical strain improvement, however, were not sufficient for industrial productions (Ikeda 2006).

Before I discuss rational strain development, a few comments on the biosynthesis of L-Phe. The biosynthesis of L-phenylalanine in bacteria occurs via the common (or general) AAA biosynthetic pathway, which is also called shikimate pathway (Bentley 1990; Haslam 1993; Herrmann 1995; Pittard 1996). Figure 1 gives a representation of the general AAA biosynthesis and a model of its regulation in *E. coli*. The initial step of the aromatic biosynthetic pathway consists of the condensation of PEP (from the glycolytic pathway) and of erythrose 4-phosphate (Ery4P; from the pentose phosphate pathway) to form DAHP. DAHP is further metabolized via shikimate to chorismate (including incorporation of an additional molecule of PEP). The bifunctional enzyme chorismate mutase/prephenate dehydratase (PheA) converts chorismate into phenylpyruvate, whereupon, through transamination, L-phenylalanine is formed (Pittard 1996).

Chorismate is the branching point from where not only the precursors of AAAs (Gibson et al. 1962) but also of isochorismate (biosynthesis of menaquinone and sidero-

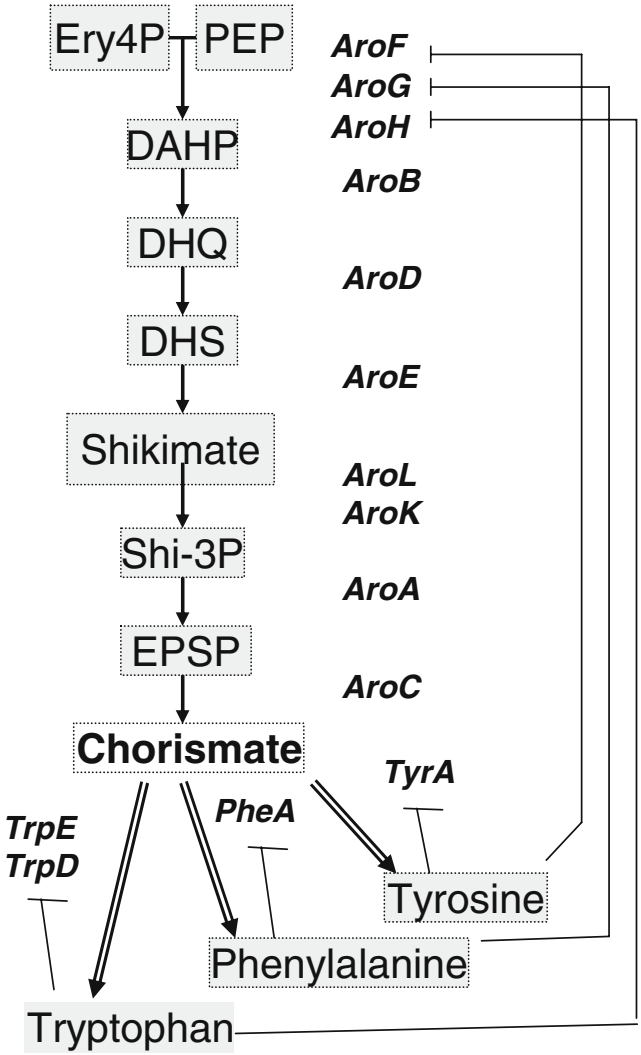

Fig. 1 Feedback regulation of the aromatic amino acid pathway in *E. coli.* The aromatic amino acid (AAA) pathway in *E. coli* is regulated at the levels of transcription, translation, and enzyme activity. The end products inhibit the first committed steps of the general pathway (DAHP synthase isoenzymes AroF, AroG, AroH) individually (indicated by *straight lines*) and the first steps of the terminal pathways (e.g., PheA is inhibited by phenylalanine). *DAHP* 3-deoxy-D-arabino-heptulosonate 7-phosphate, *DHQ* 3-dehydroquinate, *DHS* 3-dehydroshikimate, *EPSP* 5-enolpyruvoylshikimate 3-phosphate, *Ery4P* erythrose 4-phosphate, *PEP* phospho*enol*pyruvate, *Shi-3P* shikimate 3-phosphate

phores like enterobactin), *p*-aminobenzoate (folic acid biosynthesis), and *p*-hydroxybenzoate (ubiquinone biosynthesis) are formed (Dosselaere and Vanderleyden 2001; Fig. 2). All enzymes of the aromatic pathway, their corresponding genes, and metabolic intermediates are known and well studied, especially from *E. coli* (Pittard 1996; Sprenger 2007).

Regulation of the L-Phe pathway has been studied in detail, and three layers of regulation can be distinguished: feedback inhibition of pacemaker enzymes, repression at the transcriptional level and attenuation at the transcriptional/translational interface (Pittard 1996).

For L-phenylalanine, the first committed step of aromatic biosynthesis (DAHP synthase) and the step of chorismate mutase/prephenate dehydratase (PheA) are controlled by feedback inhibition exerted by the final product, L-phenylalanine (Fig. 1). DAHP synthase, as the first committed step of general AAA synthesis, controls carbon flow into the pathway (Ogino et al. 1982). In *E. coli*, three isoenzymes of DAHP synthase occur, encoded by the genes *aroF* (tyrosine-sensitive DAHP synthase AroF), *aroG* (phenylalanine-sensitive enzyme AroG), and *aroH* (tryptophan-sensitive enzyme AroH), respectively (Pittard 1996). Both the Phe- and Tyr-sensitive isoenzymes can be inhibited by about 0.1 mM of the corresponding amino acid (K_i values of 13 and 82 μM, respectively; McCandliss et al. 1978). Mutations conferring feedback resistance upon DAHP synthases (Kikuchi et al. 1997) or chorismate mutase-prephenate dehydratase (PheA; Nelms et al. 1992) have been described (some of them leading to single amino acid exchanges), and feedback-resistant mutant forms are being used in biotechnology for the production of L-Phe (for references, see Ikeda 2006; Sprenger 2007).

The intracellular level of the three DAHP synthase(s) in *E. coli* is controlled by transcriptional repression through the repressors TyrR and TrpR, which bind the AAAs (Pittard 1996; Herrmann and Weaver 1999). Deletion of the *tyrR* and *trpR* genes alleviates the transcriptional control (Berry 1996). Attenuation is a regulatory element in the biosynthesis of phenylalanine where gene *pheL* encoding a leader region is found upstream of *pheA.* The availability of phenylalanine-charged transfer RNAs is crucial for this type of regulation (Pittard 1996). Genes of the common AAA pathway and for Phe and Tyr biosynthesis are scattered over the chromosome of *E. coli*, few of them are clustered with genes of the common aromatic pathway (e.g., *aroK-aroB* and *aroF-tyrA-pheA*; Sprenger 2007).

Genetic and metabolic engineering

After decades of classical strain improvements by mutagenesis and selection, economically sufficient product titers for L-Phe had not been achieved (Ikeda 2006). Through the genetic engineering of regulatory circuits, an amplification and overexpression of genes of the general AAA and the terminal L-Phe pathway was possible, along with improvements in the precursor supply (see below). Metabolic engineering of a given pathway requires first to alleviate existing control levels (repression, attenuation, and feedback inhibition), and to identify and remove rate-limiting steps by the appropriate overproduction of enzymes of the general AAA pathway (Dell and Frost 1993; Ikeda 2003; Rüffer et al. 2004), and then to reduce competing pathways

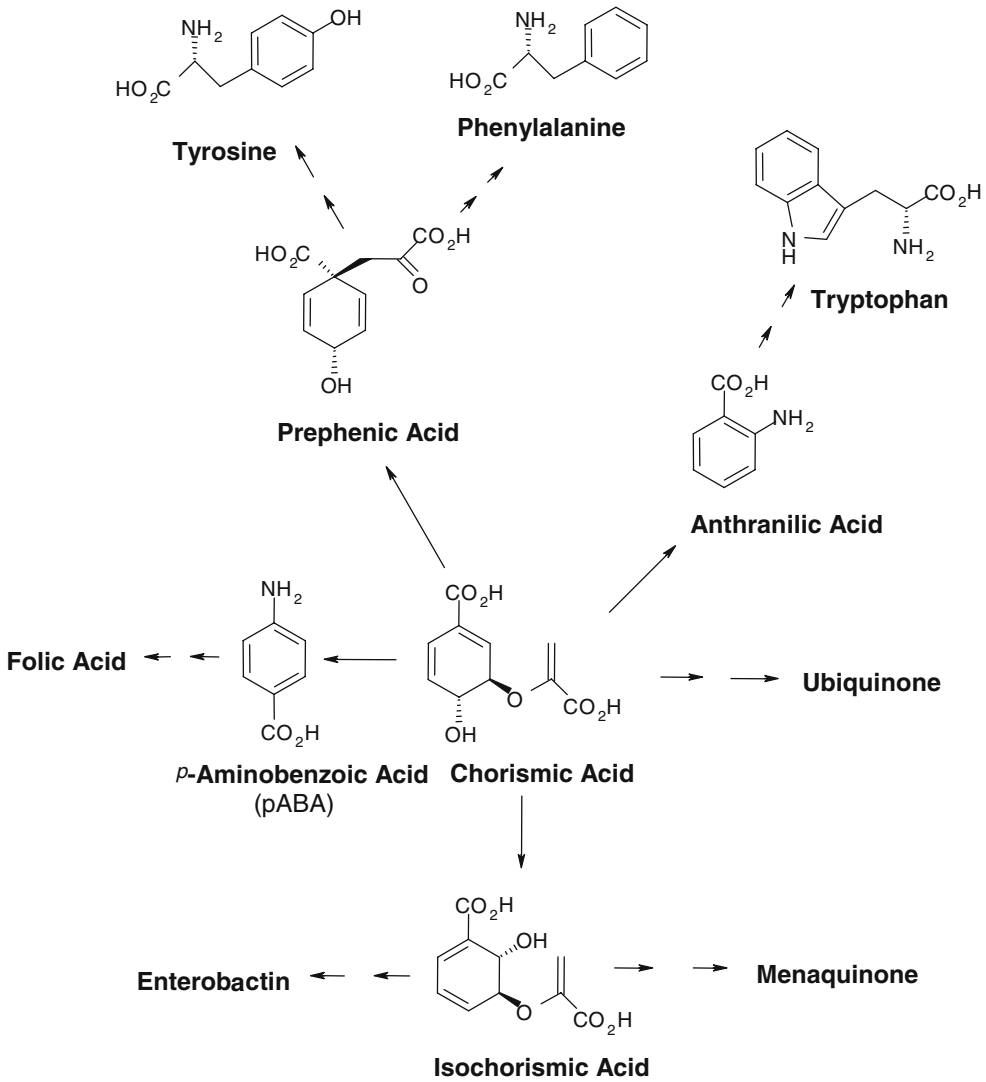

Fig. 2 Chorismic acid as branching point in *E. coli*

and to improve and balance precursor supply both in the common pathway as well as in the specific branch (reviewed in Bongaerts et al. 2001; Ikeda 2003, 2006). To overcome regulatory circuits, knockout of repressor genes (for *E. coli*: *trpR*, *tyrR*; Berry 1996), removal or alteration of operator and/or attenuator sequences and the use of genes encoding feedback-resistant (fbr) variants of pacemaker enzymes have been very useful (see above). In summary, product titers of L-Phe are now up to 50 g/l, and current production yields towards sugar are 20–25 wt% (Ikeda 2003, 2006). However, further improvements might be possible, as these yields are still lagging behind those of L-lysine or L-threonine (Ikeda 2006).

Metabolic engineering (Bailey 1991) for the production of AAA and other products of the shikimate pathway has been reviewed recently (Bongaerts et al. 2001; Ikeda 2003, 2006; Krämer et al. 2003; Sprenger 2007). Already during the early 1980s, microbial strains were developed, which carried extra gene copies of *pheA* or of genes for the general AAA pathway (e.g., DAHP synthase and shikimate kinase; Ikeda 2006). These gene–dosage effects alone were already useful to (partially) overcome regulations and limitations and to improve yields. Use of alternative promoter systems (e.g., of the bacteriophage lambda) allowed controllable expression of recombinant genes and overproduction of phenylalanine in *E. coli* (Sugimoto et al. 1987). Competing pathways could be knocked out by gene disruptions or deletions introducing auxotrophies (Miller et al. 1987; Konstantinov et al. 1991). Moreover, genes encoding feedback-resistant pacemaker enzymes have been cloned and overexpressed (for reviews, see Bongaerts et al. 2001; Ikeda 2006; Sprenger 2007). Combinations of genes on expression plasmids allowed overcoming limitations in pathways (Backman et al. 1990). Nowadays, strain improvement in *E. coli* is possible by precise "surgical molecular genetics" where genes of interest can be cloned, amplified, deleted, rearranged, or altered in their base sequence by site-directed mutagenesis on purpose (Link et al. 1997; Datsenko and Wanner 2000). To avoid genetic instability due to difficulties in plasmid maintenance,

examples for synthetic modification of the *E. coli* chromosome for the shikimate pathway intermediates have been given by Frost (Dell and Frost 1993; Snell et al. 1996; Krämer et al. 2003). This allows to work without antibiotics and with no (or fewer) plasmid-borne genes, and the strategy can also be applied for L-Phe production strains.

The FAME approach: rational construction of *E. coli* L-phenylalanine producers

The Fermentation And Metabolic Engineering (FAME) project at the DSM Biotech GmbH and Research Center Jülich was undertaken to study whether the knowledge of the literature on biosynthesis of AAAs and in general of L-Phe could be directly implemented into strain constructions. The starting strain *E. coli* W3110 was chosen as (1) its genomic structure was known, (2) genetic tools (gene cloning, expression plasmids, deliberate gene deletions) were available, and (3) it can be handled relatively easy in biofermentors using defined mineral salts media with glucose as sole carbon source. The strain W3110 (alias LJ110 or F1) is prototrophic and fast growing. As expected, it produces only as much L-phenylalanine as needed for biomass production, and no L-phenylalanine is excreted due to the various layers of metabolic regulation as discussed above. To avoid any random mutagenesis and selections, only rational steps of strain improvement were allowed.

First, through a precise chromosomal deletion, the gene cluster *pheA-aroF-tyrA* was removed together with the TyrR repressor site and *pheL* attenuator region to result in the basic double-auxotrophic (L-phenylalanine and L-tyrosine) strain F4, which served as host for various plasmid constructs (Bongaerts et al. 2006). Deletion of *pheA* and *aroF* genes was done to avoid any recombination with homologous, plasmid-borne versions of *pheA* and *aroF* genes. Deletion of *tyrA* rendered the strain auxotrophic for L-tyrosine. Tyrosine is both an unwanted by-product but may also be used to limit biomass growth (see below). Next, to allow and possibly enhance L-phenylalanine production, two genes in altered forms ($aroF^{fbr}$ and $pheA^{fbr}$) were introduced on a medium-copy-number vector under control of a $lacI^{q}$/Ptac control (pJF119EH, Fürste et al. 1986). Isopropyl-beta-D-thiogalactopyranoside (IPTG) was used as inducer (100 μM final concentration). Expression of this bicistronic artificial operon in strain F4 rendered the strain prototrophic for L-phenylalanine again (this feature also allows selection for plasmid maintenance without antibiotic selection) while retaining L-tyrosine auxotrophy. In shake-flask experiments, L-phenylalanine formation was found; therefore, trial fermentations in a 20-l fermenter (initial volume of 7.5 l; 37°C) followed (Gerigk et al. 2002a). This first expression strain (F4/pF20) displays several features, which reflect ideas of metabolic engineering. The regulatory levels of transcriptional repression (TyrR repression of *pheA* gene expression) and attenuation (*pheL* attenuator gene) had been removed by surgical genetic methods. The level of feedback inhibition was circumvented by expression of both a DAHP synthase (AroF), which is feedback-resistant to tyrosine (gene *aroF* encoding tyrosine-sensitive AroF was used instead of the L-Phe-sensitive AroG), and by a phenylalanine-resistant form of PheA. First attempts in glucose-based fermentation already gave L-phenylalanine yields of up to 25–30 g/l after 50 h of fermentation at 37°C. This result showed that the gene products were rendering a defined strain with a precise deletion and the expression of two feedback-resistant enzymes into a useful L-phenylalanine producer strain (Gerigk et al. 2002a). This strain served then both for a further analysis of fermentation side-products as well as for bioprocess improvements (downstream processing and reactive extraction of product).

Two prominent by-products of these fermentations were shikimic acid and 3-dehydroshikimic acids, which accumulated up to 6.2 and 1.5 g/l (Sprenger and team of CHORUS 2006), indicating that the flux through the aromatic pathway was impeded, most likely by insufficient activity of the shikimate kinases. Therefore, the *aroL* gene (encoding the major shikimate kinase) was cloned as third gene under the control of Ptac promoter to yield plasmid pF26 (Ptac-$aroF^{fbr}$-$pheA^{fbr}$-*aroL*). Indeed, the expression in strain F4 led to reduced production of shikimic acid and 3-dehydroshikimic acid, thereafter. However, L-phenylalanine yields were not improved, pointing to still other limitations in the pathway. Eventually, DAHP and its dephosphorylated derivative DAH were found to constitute up to 9 g/l in culture supernatants (Sprenger and team of CHORUS 2006). This now pointed to a suboptimal activity of the second step of aromatic biosynthesis catalyzed by dehydroquinate synthase (AroB). Therefore, *aroB* was added to the gene construct. Expression of the extra *aroB* gene abolished formation of DAHP and DAH (Rüffer et al. 2004; Oldiges et al. 2004) in line with earlier findings (Ogino et al. 1982; Frost and Knowles 1984).

Yet, while addition of plasmid-borne extra copies of *aroL* and *aroB* significantly lowered formation of by-products, there was no concomitant increase in overall yields of L-phenylalanine as could have been predicted. Further analysis of the production system showed that the initial step of DAHP synthesis performed by the tyrosine-resistant AroF isoenzyme ($AroF^{fbr}$) could be the Achilles' heel. The use of $AroF^{fbr}$ allowed tyrosine feeding and tyrosine limitation of cell growth of plasmid-hosting strains and for maintenance during the production phase (Gerigk et al. 2002a, b). However, enzyme activity of $AroF^{fbr}$ showed a maximum after 13 h of fermentation at 37°C, while after 30 h, no enzyme activity could be detected (Gerigk et al.

2002a). This pointed to a severe instability of AroF^{fbr} throughout the process. In a separate study (Jossek et al. 2001), the recombinant AroF^{fbr} enzyme was compared in vitro with the wild-type form (AroF). AroF^{fbr} bears two major alterations in comparison to AroF: an altered C terminus of the protein with the five last amino acid residues differing and at position 8 a lysyl residue instead of the wild-type asparagine residue (N8>K; Jossek et al. 2001). In fact, the single amino acid residue change N8K was solely responsible for feedback-inhibition resistance and constituted a new feedback-resistant form of a DAHP synthase. In vitro, AroF was inhibited to 50% in the presence of 20 μM L-tyrosine, whereas the AroF^{fbr} and AroF^{N8K} mutant forms showed no sensitivity towards L-tyrosine in a range of 0–100 μM (Jossek et al. 2001). Purified AroF wild type and AroF^{N8K} mutant protein showed similar DAHP synthase activities at 30°C and similar K_M values for PEP and erythrose-4-phosphate. However, at 37°C, AroF^{N8K} mutant protein lost its enzyme activity rapidly (50% in 20 min) underlining the concomitant resistance to feedback inhibition and the decreased thermostability in the AroF^{N8K} mutant. These in vitro data and the observations during long-term fermentations led to the idea that the feedback-resistant DAHP synthase should be exchanged with the wild-type AroF form. This, however, is against classical knowledge of strain improvement, which states that the pacemaker enzymes should be feedback-resistant (see above). Expression plasmids that carried the wildtype *aroF* allele (*aroF*^{wt}) instead of the allele encoding the feedback-resistant enzyme (gene *aroF*^{fbr}) were constructed and compared (plasmid pF69 *aroF*^{wt}-*pheA*^{fbr}-*aroL*) with the feedback-resistant form (plasmid pF49 *aroF*^{fbr}-*pheA*^{fbr}-*aroL*). Of course, when the tyrosine feedback-sensitive form was used (Foerberg and Haeggstroem 1988; Takagi et al. 1996), L-tyrosine feeding had to be strictly limited in the production phase. Actually, if the prechosen upper limit of 20 mg/l tyrosine (<110 μM) was used, production of L-phenylalanine was optimal (Gerigk et al. 2002a). From an analysis of seven fed-batch fermentations, it became evident that the longest L-Phe production periods correlated with the lowest tyrosine-feeding rates and resulted in the highest L-Phe titer (34 g/l), whereas the *aroF*^{fbr} control did not exceed 28 g/l of L-Phe (Gerigk et al. 2002a). DAHP synthase activities after 30-h process duration were still higher than the maximal values of the feedback-resistant form that was used. The maximum process selectivity for L-Phe from glucose was 21% (mol/mol). Given a suitable tyrosine-feeding control, the wild-type DAHP synthase can thus be used for L-phenylalanine in the presence of L-tyrosine (Gerigk et al. 2002a). If the *aroB* gene was combined (strain F4/ pF81) with this protocol, even higher L-Phe titers (up to 38 g/l) could be reached with virtually no by-products from the aromatic biosynthesis pathways and with low acetate formation after 50-h process time (Rüffer 2004; Rüffer et al. 2004). Using a new reactive extraction procedure for integrated in situ product recovery (ISPR) for amino acids with liquid–liquid centrifuges, L-phenylalanine could be removed from the aqueous fermentation medium of a 20-l fed-batch process and extracted in a by-pass into an organic phase (kerosene with a cation-selective carrier and sulfuric acid). This phenylalanine process allowed an extended product formation period and an improved phenylalanine yield. Thus, a final maximum titer of 45.5 g/l [space-time yield 0.91 g/(l×h)] was achieved, which compares to the best published L-Phe process results published so far (46 g/l, Konstantinov et al. 1991; 50 g/l with a 25% molar yield; Backman et al. 1990). However, the genetic features of these other L-Phe producers have not been completely revealed.

In conclusion, combining defined genetic elements (including the unusual feedback-sensitive pacemaker enzyme) with bioprocess engineering (continuous glucose feeding to avoid unwanted acetate formation, tyrosine feeding to allow biomass formation and limitation) and fully integrated separation of L-phenylalanine through reactive extraction allowed a competitive process for L-phenylalanine production starting from a wild-type *E. coli* strain (Rüffer 2004; Rüffer et al. 2004). In the future, various techniques to follow changes in the genome, transcriptome, proteome, and metabolome of industrial strains may help to further improve L-phenylalanine strains. First results on the effects of L-phenylalanine on the transcriptome of *E. coli* strains have been already reported (Polen et al. 2005). For other amino acids, e.g., for L-lysine or L-threonine, the various "omics" approaches have already led to a better understanding of producer strains and therefrom to the rational genetic improvement of wild-type strains (for references, see Hayashi et al. 2006; Rieping and Hermann 2007). It may be envisaged that similar approaches may also be helpful with strains that produce AAAs.

Fine chemicals from the aromatic biosynthetic pathway in *E. coli*

Apart from the AAA (L-Phe, L-Trp, and L-Tyr), other derivatives of the shikimate pathway have been produced by recombinant *E. coli* strains, which had lesions in genes of the general aromatic biosynthesis pathway. For example, *aroB*-mutants excrete DAHP (or mainly the dephosphorylated form, DAH) into the culture supernatant (Frost and Knowles 1984; Frost and Draths 1995). Appropriately engineered mutants with lesions in the genes for one or both shikimate kinases (AroL and AroK) excreted shikimate (up to 80 g/l; Yi et al. 2002). Shikimate is currently being used as a precursor in a multistep synthesis of the

influenza drug Tamiflu™ by Roche (Krämer et al. 2003; Johansson et al. 2005). Especially the group of John W. Frost has been successful in providing a long list of compounds, which can be obtained by metabolic engineering of *E. coli* cells; the various processes allowed yields of up to 80 g/l of product (for reviews, see Frost and Draths 1995). By introduction of genes from other microbial genera as *Klebsiella*, *Acinetobacter*, or *Pseudomonas* into engineered strains of *E. coli*, interesting quinoid organic compounds (quinic acid and hydroquinone; Draths et al. 1992), catechol, and derivatives (protocatechuate, catechol, *cis*,*cis*-muconic acid, and adipic acid; Frost and Draths 1995) and tryptophan pathway derivatives (indoleacetic acid, indigo) have been shown (for reviews, see Frost and Draths 1995; Berry 1996; Krämer et al. 2003). D-Phenylglycine and D-*p*-hydroxyphenylglycine (for semi-synthetic penicillins and cephalosporins) can be obtained from recombinant *E. coli* strains via an artificial pathway including a deregulated AAA biosynthesis up to phenylpyruvate and additional genes from *Amycolatopsis* and *Streptomyces*, which allow the formation of (hydroxy)phenylglyoxylate and eventually D-(hydroxy)phenylglycine through a stereoinverting aminotransferase derived from *Pseudomonas* (Assema et al. 2000; Müller et al. 2006).

In this regard, I like to propose the term "metabolic grafting" for this sort of metabolic engineering (Bailey 1991). Metabolic grafting thus describes the introduction ("grafting") of gene(s) encoding one or several metabolic steps from the same or from other microorganisms into a producer strain ("platform strain") that has been engineered for an enhanced common metabolic pathway (here, the aromatic biosynthesis pathway, metaphorically "a strong fruit-bearing tree as the understock") by genetic alterations as deletions or rearrangements (cutting of existing twigs or branches). Metabolic grafting thus results in novel products. In this respect, the microbial host (*E. coli*) is the platform strain, which has genetically been improved to allow unimpeded flux of precursor metabolites into and through the AAA pathway. Elimination of competing branches and enhancement of desired features lead to novel building blocks, which are excreted into the culture medium. Two examples are given below.

Chorismate as a universal synthon (CHORUS)

Chorismate is not only the precursor of AAAs or quinones (ubiquinone and menaquinone) but also of *p*-aminobenzoic acid (a folate precursor) of bacterial siderophores as enterobactin or of secondary metabolites as phenazine (in *Pseudomonas aeruginosa*; Kozak 2006). The group of Leistner at the University of Bonn had already shown that mutant strains of *Klebsiella pneumoniae* (with blocks in all three AAA terminal pathways) can be utilized to produce the transdiol compound 2,3-*trans*-CHD (*S,S*-2,3-dihydroxy-2,3-dihydrobenzoic acid) from an interrupted enterobactin biosynthetic pathway (see Fig. 3). In *Klebsiella* mutants, however, titers of 2,3-*trans*-CHD never exceeded 200 mg/l (Müller et al. 1996). Nonetheless, this small and highly functionalized molecule is a valuable building block for a wide variety of target molecules as aminocarbasugars (valienamine) or crotepoxide (Fig. 4; for references, see Franke et al. 2001; Lorbach et al. 2004). We (Sprenger and team of CHORUS 2006) decided to use *E. coli* mutants for the production of 2,3-*trans*-CHD for its genetic amenability and to avoid working with potentially pathogenic microorganisms. *E. coli* mutants with blocks in the *entA* gene were used as hosts for the expression of cloned *entBC* genes. The gene products of *entBC* encode an isochorismate synthase (EntC) and an isochorismatase (EntB). EntA leads to the aromatization of 2,3-*trans*-CHD to 2,3-dihydroxybenzoic acid and had to be eliminated. Expression of *entBC* genes in various *entA*-mutants of *E. coli* led to the formation of up to 4.6 g/l of 2,3-*trans*-CHD in the culture medium of a 20-l fermentation. The 2,3-*trans*-CHD could be easily removed by ion exchange chromatography and was stable at room temperature over weeks (Franke et al. 2001). Reactive extraction with cationic carriers in organic solvents as octan-1-ol could also be successfully performed. The 2,3-*trans*-CHD was applied in a short organic synthesis (five steps) of the enantiomer of a plant-growth inhibitor, *ent*-streptol (Franke et al. 2003a, b) or *iso*-crotepoxide, *ent*-senepoxide, and *ent*-valienone (Lorbach et al. 2002; Lorbach et al. 2004). Figure 4 depicts a range of possible chemical reactions with 2,3-*trans*-CHD as starting compound. To show the versatility of this approach, biosynthesis of another compound was attempted. In a side reaction, EntB directly cleaves chorismate into pyruvate and 3,4-*trans*-CHD (Fig. 3). This creates a molecule that has not yet been described in biosynthetic pathways. Indeed, overexpression of the *entB* gene in a strain with mutations in the pathways competing for chorismate (Tyr, Phe, Trp, and *p*-aminobenzoic acid) led to formation and excretion of up to 0.79 g/l of 3,4-*trans*-CHD. It should be mentioned that enantiomerically pure 3,4-*trans*-CHD from organic synthesis is an extremely difficult task, which could be solved only very recently by a chemoenzymatic approach (Boyd et al. 2006). Thus, microbial access to this building block is a great improvement. To allow even higher titers of both new substances, the metabolic grafting approach was followed. Novel *E. coli* strains were constructed, which lacked *pheA* and *tyrA* genes, to avoid drain of chorismate into these pathways. Enterobactin biosynthesis was blocked either at *entA* (expression of *entBC* genes; accumulation of 2,3-*trans*-CHD) or at *entC* (expression of entB, accumulation of 3,4-*trans*-CHD).

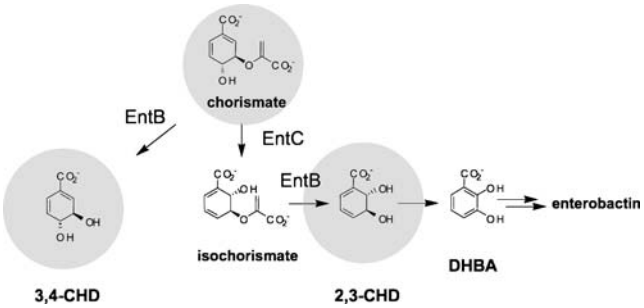

Fig. 3 Diversity-oriented metabolic engineering. Transdiols (2,3-CHD and 3,4-CHD) can be obtained by overexpression of genes *entB* or *entB* + *entC* in appropriately tailored *ent*-mutant strains of *E. coli* (see text for details). *DHBA* dihydroxybenzoate; for other abbreviations, see text

These tyrosine- and phenylalanine auxotrophs were then transformed with plasmids bearing the *aroF*-, *aroB*- and *aroL* genes to enhance flux into and through the general aromatic pathway up to chorismic acid. These modifications greatly improved performance of the transdiol producer strains.

Outlook: increase of precursor supply

So far, I have mainly discussed engineering of the general AAA pathway and of the terminal pathways leading to L-Phe and other chorismate derivatives. However, the precursors for the general AAA pathway, PEP and Ery4P, derive from glycolysis and from the pentose phosphate pathway, respectively (Frost and Draths 1995). Each may become limiting for the production of L-Phe or chorismate derivatives once there are no more limits in the common or specific pathways. Therefore, attempts to improve supply of either PEP or Ery4P or of both have been reported (for recent reviews, see Bongaerts et al. 2001; Ikeda 2003, 2006; Sprenger 2007). These approaches include successfully increasing the PEP supply by knocking out the genes of the competing PEP carboxylase or pyruvate kinases in *E. coli* phenylalanine producers (Miller et al. 1987; Patnaik and Liao 1994; Flores et al. 1996; Gosset et al. 1996; Liao et al. 1996; Grinter 1998) or combining PEP carboxylase gene mutations with overproduction of the gluconeogenic PEP carboxykinase for improvement of carbon flux into the shikimate pathway (Patnaik et al. 1995). Cloning and overexpression of the transketolase gene increased intracellular Ery4P and led to a raised production of DAHP (Draths et al. 1992). Overproduction of enzymes from the pentose phosphate pathway, such as transketolase or transaldolase, was found to improve Ery4P supply (Draths et al. 1992; Frost and Draths 1995; Liao et al. 1996; Lu and Liao 1997; Sprenger et al. 1998b). Enzymes from glycolysis, gluconeogenesis, or the glyoxylate cycle (Koehn et al. 1994) were studied for their contribution to the balanced supply of PEP and Ery4P (for a discussion, see Berry 1996; Grinter 1998; Ikeda 2006; Sprenger 2007). The most limiting factor in PEP supply in *E. coli*, however, is the PEP-dependent sugar phosphotransferase system (PTS), which is responsible for glucose uptake. Fifty percent of PEP generated in glycolysis during growth on glucose has to be spent for uptake of glucose and is concomitantly converted into pyruvate (Postma et al. 1993). Theoretically, with an intact PTS, less than 50% of the available PEP from C-sources such as glucose can be funneled into the AAA pathway (Patnaik and Liao 1994). Thus, an exchange of the glucose–PTS for other transport systems, which do not depend on

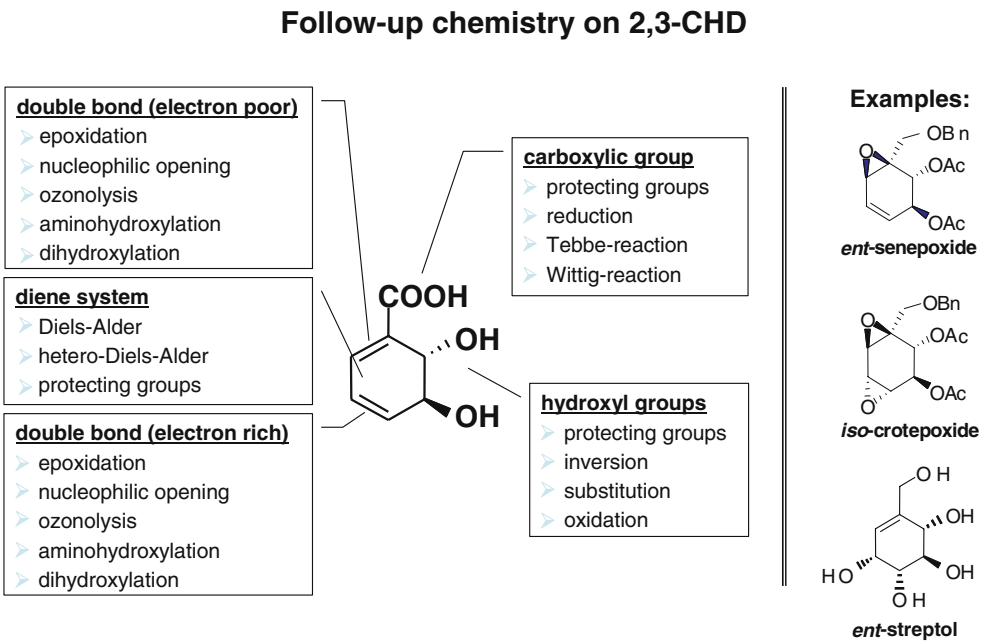

Fig. 4 Follow-up chemistry on 2,3-CHD. Possible organic syntheses reactions starting from the building block 2,3-CHD. (Michael Müller, Univ. Freiburg, personal communication). *Bn* Benzoyl group, *Ac* acetyl group

PEP, was studied in *E. coli* mutant strains deficient for the glucose–PTS (Flores et al. 1996; Berry 1996; Gosset et al. 1996; Chen et al. 1997). Indeed, several groups showed that PEP saving sugar uptake is possible. Either by activation of a galactose permease, which transports glucose independently from PEP (Gosset et al. 1996; Berry 1996), or by importing a glucose facilitator from *Zymomonas mobilis*, *E. coli* mutants with a PEP-independent glucose uptake have been reported (Snoep et al. 1994; Weisser et al. 1995; Parker et al. 1995; Sprenger et al. 1998a, b; Krämer et al. 1999). Positive effects on the production of a variety of products from the AAA pathway have been reported (Gosset et al. 1996; Berry 1996; Chandran et al. 2003; Baez-Viveros et al. 2004; Yi et al. 2002). Finally, pathway engineering by combinations of improved Ery4P and/or PEP supplies has already succeeded in improving phenylalanine formation in *E. coli* strains (Flores et al. 1996; Berry 1996; Gosset et al. 1996; Grinter 1998; Baez-Viveros et al. 2004).

Future prospects of the biotechnological use of *E. coli* as biological factory for building blocks are in further improvement of the precursor supply (see above) and in carbon flux analysis (for example, see Wahl et al. 2004), as well as transcriptome analysis on the influence of high titers of phenylalanine on gene expression of *E. coli* (Polen et al. 2005) may help to determine rate-limiting steps and metabolic overflow systems. Introduction of additional heterologous genes may also increase the diversity of building blocks, which may be produced by metabolically grafted *E. coli* cells.

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