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Masato Ikeda

Towards bacterial strains overproducing L-tryptophan and other aromatics by metabolic engineering

Masato Ikeda (✉)

Department of Bioscience and Biotechnology
Faculty of Agriculture
Shinshu University
Minami-minowa, Nagano 399-4598, Japan
Fax: +81-265-77-1629
E-mail: m_ikeda@shinshu-u.ac.jp

Abstract

The aromatic amino acids, L-tryptophan, L-phenylalanine, and L-tyrosine, can be manufactured by bacterial fermentation. Until recently, production efficiency of classical aromatic amino acid-producing mutants had not yet reached a high level enough to make the fermentation method the most economic. With the introduction of recombinant DNA technology, it has become possible to apply more rational approaches to strain improvement. Many recent activities in this metabolic engineering have led to several effective approaches, which include modification of terminal pathways leading to removal of bottleneck or metabolic conversion, engineering of central carbon metabolism leading to increased supply of precursors, and transport engineering leading to reduced intracellular pool of the aromatic amino acids. In this review, advances in metabolic engineering for the production of the aromatic amino acids and useful aromatic intermediates are described with particular emphasis on two representative producer organisms, *Corynebacterium glutamicum* and *Escherichia coli*.

Introduction

Breeding of amino acid-producing strains has now reached a state of very high development. This advance currently enables industrial production of most L-amino acids by microbial fermentation from cheap sugar and ammonia (Leuchtenberger 1996; Ikeda 2003; Hermann 2003). The aromatic amino acids, L-tryptophan, L-phenylalanine, and L-tyrosine, are also manufactured by fermentation while other methods, such as extraction from natural protein-rich resources and enzymatic synthesis from specific substrates, have led to industrial processes as well. Accompanied by development of mass production technology, various uses of the aromatic amino acids have been exploited. For example, L-tryptophan serves as medicine to improve the sleep state and mood and as a feed additive, while L-phenylalanine and L-tyrosine are used as the materials for the production of the low-calorie sweetener aspartame and of the anti-Parkinsion's disease drug L-DOPA, respectively. The major manufacturers of the aromatic amino acids, their uses and listed price, and the estimated amounts of production have been summarized in reviews (Frost 1994; Leuchtenberger 1996; Bongaerts et al. 2001).

The aromatic amino acids are a group where production efficiency by fermentation

remains relatively low. L-Tryptophan, in particular, is one of the amino acids that have been difficult to realize a high level of production yield. Due to its rather high production costs, the market for L-tryptophan is still limited whereas the amino acid has a large application field as a supplement of animal feed. On the basis of literatures reported mainly during the last decade, the current production yields towards sugar (wt%) can be estimated 20-23 for L-tryptophan, and around 25 for L-phenylalanine. In contrast, far higher yields have been reported for many other amino acids, e.g., L-lysine HCl, 40-50; L-glutamate, 45-55; L-arginine, 30-40;, L-threonine, 40-50, L-valine, 30-40, and L-alanine, 45-55 (Leuchtenberger 1996; Ikeda 2003).

Previous attempts at strain improvement for amino acid production relied on classical mutagenesis and screening procedures, aiming at deleting competing pathways and eliminating feedback regulations in the biosynthetic pathways. However, the classical approach has the limited usefulness since complete deregulation of regulatory steps and enhancement of an appropriate biosynthetic enzyme activity are difficult to achieve. The availability of recombinant DNA techniques in representative amino acid-producing organisms such as *Corynebacterium glutamicum* and *Escherichia coli* has enabled the introduction of such genetic modifications. This review first describes the essence of aromatic amino acid biosynthesis and its regulation in *C. glutamicum* and *E. coli*. The recent advances in strain development for the production of the aromatic amino acids and useful aromatic intermediates are then summarized. In addition, the progress in genomics begins to exert profound effects on the strategy of strain development, which is finally discussed. An overview of *C. glutamicum* and its properties regarding genomics, genetics, biochemistry, physiology, and applications have been reviewed recently (Eggeling and Bott 2005).

Biosynthesis of aromatic amino acids

Biosynthesis of the aromatic amino acids in all organisms begins with the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), then proceeds to chorismate, from which the pathways to L-tryptophan, L-tyrosine, and L-phenylalanine branch (Fig. 1). Accordingly, the overall aromatic pathways after diverging from central metabolism is best considered in two parts: the common pathway leading to chorismate, and the branched pathways from chorismate to L-tryptophan, L-tyrosine, and

L-phenylalanine.

The common pathway

In both *C. glutamicum* and *E. coli*, carbon flow through the common pathway up to chorismate is primarily controlled at the first reaction of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DS). In *C. glutamicum*, two types of DSs with different subunit sizes exist (Fig. 1A). One is an L-tyrosine-sensitive DS with a predicted molecular mass of 39 kDa (type I-DS; the *aro* product; NCgl0950) and an L-phenylalanine- and L-tyrosine-sensitive DS with a predicted molecular mass of 51 kDa (type II-DS; the *aro* II product; NCgl2098). The type II-DS forms a polypeptide complex with chorismate mutase (CM; the *csm* product; NCgl0819), which converts chorismate to prephenate (Sugimoto and Shiio 1980a, 1980b). The type II-DS exhibits its activity by itself while CM activity requires the presence of the type II-DS protein (Sugimoto and Shiio 1980a, 1980b). Due to this specific protein interaction in *C. glutamicum*, simultaneous overproduction of the DS and CM proteins is necessary to achieve overexpression of CM activity (Ikeda and Katsumata 1992). It has been reported that an amino acid residue, Ser-187, of the type I-DS played a crucial role for the feedback inhibition (Liao et al. 2001).

In *E. coli*, three genes, *aroG* , *aroF* , and *aroH* , encode DS isozymes that are sensitive to L-phenylalanine, L-tyrosine, and L-tryptophan, respectively (Fig. 1B) (Umbarger 1978; Herrmann 1983). In wild-type *E. coli* grown in minimal medium, about 80%, 20%, and 1% of the total DS activities are contributed by the *aroG*, *aroF*, and *aroH* products, respectively (Herrmann 1983). The amino acid residues 147 through 149 and its neighboring regions are considered to be involved in the allosteric regulation of each DS because amino acid changes at positions Val-147 and Gly-149 led to L-tryptophan-insensitive *aroH* products (Ray et al. 1988), changes at Pro-148 and Gln-152 led to L-tyrosine-insensitive *aroF* products (Edwards et al. 1987; Weaver and Herrmann 1990), and changes at Asp-146 and Pro-150 led to L-phenylalanine-insensitive *aroG* products (Kikuchi et al. 1997).

The branched pathways

In *C. glutamicum*, the control of carbon flow in the branched pathways occurs mainly

through end-product inhibition at five enzymatic steps shown in Fig. 1A. The concentrations of end-product(s) giving 50% inhibition are about 0.0015 mM for anthranilate synthase (ANS) (Shiio et al. 1972) and 0.15 mM for anthranilate phosphoribosyltransferase (PRT) (Sugimoto and Shiio 1983), 7.7 mM for tryptophan synthase (TS) (Sugimoto and Shiio 1982), 0.0023 mM for CM (Sugimoto and Shiio 1980b), and 0.0025 mM for prephenate dehydratase (PD) (Sugimoto and Shiio 1974). Specific mutations causing desensitization of these key enzymes has been reported: e.g., a Ser38Arg substitution leading to a desensitized ANS (Matsui et al. 1987a), an Ala162Glu substitution that rendered PRT less sensitive to feedback inhibition (O'gara and Dunican 1994), and a Ser99Met substitution leading to a desensitized PD (Hsu et al. 2004).

In addition to the inhibition control, formation of all enzymes in the L-tryptophan branch is several-fold repressed by L-tryptophan (Hagino and Nakayama 1975; Shiio et al. 1972, Sugimoto and Shiio 1977) through an attenuation control (Fig. 1A)(Sano and Matsui 1987). A point mutation in this attenuator region resulted in increased ANS and PRT activities in *C. glutamicum* ssp. *lactofermentum* (Matsui et al. 1987a). A nonsense mutation in the attenuator region that could be responsible for a constitutive antitermination response is also known from *C. glutamicum* (Heery and Dunican 1993).

In *E. coli*, the main control of metabolic flow occurs by the end-product inhibition of the bifunctional enzyme CM-PD specified by *pheA,* the other bifunctional enzyme CM-prephenate dehydrogenase specified by *tyrA*, and ANS (Fig. 1B). The CM-PD and ANS have been extensively studied in *E. coli* and *Salmonella typhimurium*, revealing regulatory domains of the enzymes as well as mutation sites resulting in desensitization to feedback inhibition (Gething et al. 1976; Caligiuri and Bauerle 1991; Nelms et al. 1992; Tonouchi et al. 1997; Zhang et al. 1998; Pohnert et al. 1999). In addition to the inhibition control, expression of genes relevant to aromatic amino acid biosynthesis and transport are regulated by the protein specified by *tyrR* or *trpR*, and in some cases, by an attenuation control (Umbarger 1978; Herrmann 1983; Somerville 1983; Pittard1996; Bongaerts et al. 2001). The genes regulated by *trpR* include *aroH*, the *trp* operon, *aroLM*, and *mtr*, while the *tyrR* regulon includes *aroF-tyrA*, *aroG*, *aroLM*, *tyrB*, *aroP, mtr*, and *tyrP*. It is noteworthy that whereas the *trpR* gene product mediates only repression, the *tyrR* gene product can act both as a repressor and as an activator of transcription, which has been reviewed recently (Pittard et al. 2005). Expression control of *pheA*, the *trp* operon, and *pheST* involves attenuation.

Genes for aromatic amino acid biosynthesis and transport

The whole-genome sequence of *C. glutamicum* has been deposited in the DDBJ/GenBank/EMBL database under the accession number BA000036 (Ikeda and Nakagawa 2003). The genome sequence identified almost all genes constituting the common pathway, the branched pathways, and the transport of the aromatic amino acids. Regarding the *aroE* gene (NCgl0409), there are two additional paralogs (NCgl1087, NCgl1567), but no functional analyses of them have been reported. A paralog (NCgl2010) of *trpC* (NCgl2930) can be found on the genome although also in this case the function of the gene product remains unclear. The *pat* gene (NCgl0215) was recently shown to be responsible for the last aminotransferase reaction in the L-phenylalanine branch (Fig. 1A) (McHardy et al. 2003). The distribution and organization of these biosynthetic genes on the genome are different from those in *E. coli*, whereas the gene arrangement of the *trp* operon (NCgl2927-2932) of *C. glutamicum* closely resembles that of *E. coli*. The *trp* operons of both bacteria have been extensively studied for its organization and regulation (Umbarger 1978; Somerville 1983; del Real et al. 1985; Katsumata and Ikeda 1993; Matsui et al. 1986; Matsui et al. 1987a, 1987b, 1987c; Sano and Matsui 1987).

The uptake of L-tryptophan, L-phenylalanine, and L-tyrosine occurs mainly via the general aromatic amino acid uptake system specified by *aroP* (NCgl1062) in *C. glutamicum* (Fig. 1A) (Ikeda and Katsumata 1994; Wehrmann et al. 1995). It has been shown that a mutant defective in the transport system has considerably reduced the transport activities of L-tryptophan, L-phenylalanine, and L-tyrosine, and that plasmid-mediated amplification of the *aroP* gene confers on *C. glutamicum* strains a simultaneous increase in the uptake activities of all three aromatic amino acids (Ikeda and Katsumata 1994). In *E. coli*, at least five different systems are known for the aromatic amino acid transport (Fig. 1B) (Pittard1996). These include the specific transport systems for L-tyrosine (the *tyrP* gene product), L-phenylalanine (the *pheP* gene product), or L-tryptophan (the *mtr* and *tnaB* gene products) in addition to the general transport system (the *aroP* gene product). Also in *C. glutamicum*, the possibility of the existence of a second low-affinity system cannot be excluded. Such a candidate gene could be the *tyrP* gene (NCgl0464) in the *C. glutamicum* genome, the product of which shows about 26% homology to the *tyrP* product of *E. coli*.

Metabolic engineering for L-tryptophan production

Biosynthesis of L-tryptophan is strictly controlled at several steps both in *E. coli* and *C. glutamicum*. Therefore, overproduction of L-tryptophan requires, first of all, genetic removal of all the metabolic controls existing both in the common pathway and in the L-tryptophan branch. In addition, amplification of DS, which initiates the common pathway, should be an important strategy to increase net carbon flow down the common pathway. Then, balanced supply of precursors has to be addressed to achieve efficient production of the amino acid, considering that biosynthesis of 1 mol of L-tryptophan from glucose requires 1 mol each of E4P and PEP as starting precursors, and in addition, consumes 1 mol each of PEP, L-glutamine, phosphoribosyl-5-pyrophosphate, and L-serine on its biosynthetic pathways (Umbarger 1978). Due to these several hurdles, the classical mutagenesis and screening approach does not realize a high level of L-tryptophan production although the efforts have been continuing near four decades. Remarkable progress in production efficiency has been made after more rational approaches using recombinant DNA technology was introduced into strain improvement. In the following, the advances in metabolic engineering for L-tryptophan production are highlighted.

Engineering of the common pathway and L-tryptophan branch

The first application of recombinant DNA technology to strain improvement for L-tryptophan production was performed with *E. coli* by Tribe and Pittard (1979). They reported increased production by amplification of the *trp* operon with a deregulated *trpE* gene, though the final titer of about 1 g/l was low. On the other hand, Aiba et al. (1982) constructed a genetically engineered strain by introducing a plasmid containing the *trp* operon with deregulated *trpE* and *trpD* genes into an *E. coli trpR* and *tnaA* mutant. By cultivating the strain in glucose medium to which anthranilic acid was continuously fed, they obtained 6.2 g/l of L-tryptophan after 27 h. This process of L-tryptophan fermentation from both glucose and anthranilic acid was further improved to a high production efficiency by Azuma et al. (1993). They isolated a 6-fluorotryptophan- and 8-azaguanine-resistant mutant from the recombinant strain constructed by Aiba et al (1982). In the process based on this strain, more than 50 g/l L-tryptophan accumulated

after 91 h with non-ionic detergents added to the culture to cause crystallization of L-tryptophan. In this process, about 30 g/l L-tryptophan was derived from exogenous anthranilic acid whereas the remaining 20 g/l L-tryptophan was derived from glucose.

In another development for a recombinant *E. coli*, Chan et al. (1993) derived a stable L-tryptophan-producing *E. coli* strain with chromosomally integrated three copies of the *trp* operon and reported 9.2 g/l of L-tryptophan with 13% conversion yield on glucose. In this process, the *trp* operon was shown to be stably maintained during fermentation without selective pressure, suggesting that gene amplification on the chromosome could be a useful strategy. Other approaches to improve L-tryptophan production by *E. coli* through amplification of the deregulated *trp* operon have also been described by Berry (1996) and Camakaris et al. (1997).

A microorganism belonging to *Bacillus* sp. was also used for the production of L-tryptophan from glucose and anthranilic acid (Yajima et al. 1990). *Bacillus amyloliquefaciens* was genetically engineered so that the recombinant strain carried 2-4 copies of both the *trp* operon and the phosphoribosyl-5-pyrophosphate synthetase gene (*prs*) on the chromosome as well as a plasmid containing the *serA* gene. When the engineered strain was cultured in a medium with glucose and anthranilic acid, a final L-tryptophan titer of 14.2 g/l was obtained.

In the mid 1980s, the first host-vector systems were developed for the coryneform bacteria (Katsumata et al. 1984; Santamaria et al. 1984; Miwa et al. 1985; Yoshihama et al. 1985). Since then, there have been many attempts to engineer existing L-tryptophan-producing strains of the coryneform bacteria with the use of recombinant DNA technology. In this molecular approach, rational pathway engineering was demonstrated (Katsumata and Ikeda 1993; Ikeda et al. 1994). The strategy was amplification of the first enzyme DS in the common pathway diverging from central metabolism to increase carbon flow down that pathway, followed by sequential removal of bottlenecks discerned by the accumulation of intermediates. This strategy was applied for further improvement of a classically derived L-tryptophan-producing *C. glutamicum* strain, resulting in a 61% increase in L-tryptophan production, to approximately 50 g/l. As this exceeds the solubility of L-tryptophan, more than half of the product crystallizes in the medium.

The remarkable improvement involves not only systematic genetic modifications to efficiently channel carbon towards L-tryptophan via plasmid-mediated amplification of all together eight genes of the pathways leading to L-tryptophan and L-serine, that is,

aro II (NCgl2098), *trpEGDCBA* (NCgl2927-2932), and *serA* (NCgl1235), but also construction of a plasmid stabilization system based on the presence of the *serA* gene on the plasmid and the gene's absence from the chromosome (Fig. 2). This plasmid stabilization system has been shown to provide selection for plasmid maintenance even in complex media containing L-serine presumably due to high demand and rapid consumption of L-serine by *C. glutamicum* (Ikeda et al. 1994; Katsumata et al. 1997).

Engineering of the L-serine-biosynthetic pathway

In *C. glutamicum*, genetic modifications leading to increased carbon flux down the L-tryptophan pathway results in the overflow of indole, the last intermediate in the pathway, due to a limiting supply of L-serine to convert indole to L-tryptophan (Ikeda et al. 1994). Therefore, increasing the availability of L-serine is another requisite for efficient L-tryptophan production. One such strategy can be amplification of the *serA* gene (NCgl1235) coding for 3-phosphoglycerate dehydrogenase, the key enzyme in the L-serine biosynthesis (Ikeda et al. 1994). In the *C. glutamicum* enzyme, it was reported that amino acid changes at position Gly-325 or deletion of the C-terminal domain resulted in deregulation from feedback inhibition by L-serine (Suga et al 2000; Peters-Wendisch et al. 2002). In this context, Suga et al. (2000) derived an L-serine-producing *C. glutamicum* ssp. *flavum* strain deficient in L-serine-degradation activity and with multicopies of deregulated *serA* (NCgl1235) as well as *serB* (NCgl2436) and *serC* (NCgl0794). When the engineered strain was cultured in a medium with glucose, a final L-serine titer of 35.2 g/l was obtained with 32% conversion yield on glucose. The enzyme from *E. coli* has also been characterized in detail, revealing not only the regulatory and substrate binding domains but specific mutations causing desensitization of the enzyme (Grant et al. 2000, 2001, 2002; Bell et al. 2002).

Engineering of central metabolism

After bottlenecks in the common pathway and the L-tryptophan branch are removed, the next target is central metabolism to increase the availability of the precursors for L-tryptophan biosynthesis. Thus, increasing the availability of PEP and E4P is a key issue towards achieving the maximum flow of carbon into the common pathway. In *E. coli*, several different approaches to overcome precursor limitations have been developed

(Fig. 3).

Strategies for increasing the PEP availability include:

(i) Inactivation of enzymes that compete for PEP, such as PEP carboxylase (Backman 1992) and pyruvate kinases (Gosset et al. 1996)

(ii) Recycling of pyruvate formed by either the PEP-dependent glucose phosphotransferase system (PTS) or pyruvate kinases back to PEP using PEP synthase (Liao 1996) or PEP carboxylase (Yurievich et al. 2004)

(iii) Elimination of the PTS followed by increased glucokinase activity to phosphorylate glucose with ATP instead of PEP (Sprenger et al. 1998a)

(iv) Induction of the glyoxylate cycle under glycolytic conditions (Koehn et al. 1994)

(v) Disruption of the *crsA* gene, carbon storage regulator (Tatarko and Romeo 2001)

Strategies for increasing the E4P availability include:

(i) Overexpression of the enzymes in the non-oxidative pentose phosphate pathway such as transketolase (Frost JW 1992) and transaldolase (Lu and Liao 1997; Sprenger et al. 1998b)

(ii) Inactivation of phosphoglucose isomerase (Mascarenhas et al. 1991)

Although limitation of each precursor could be relieved by such genetic approaches to some extent, effective yield improvement requires a balanced supply of each precursor to the common pathway by the combination of these approaches. To this end, various combinations were studied for the production of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) with an *E. coli aroB* mutant (Patnaik et al. 1995; Flores et al. 1996; Gosset et al. 1996; Liao et al. 1996). In a representative example, DAHP production with near theoretical yield of 6 mol DAHP from 7 mol glucose (86% molar yield) was achieved by increasing the supply of both precursors via simultaneous overexpression of transketolase and PEP synthase as well as DS (Patnaik and Liao 1994).

Also in *C. glutamicum*, a further improvement was obtained by engineering the central metabolism to increase the availability of PEP and E4P. To overcome the limitation of PEP availability, a PEP carboxylase mutant that had lost about 75% of the enzyme activity was isolated (Fig. 2), which was shown to produce more L-tryptophan than its parent (Katsumata and Kino 1989). A few-fold amplification of the *tkt* gene (NCgl1512) encoding transketolase further raised L-tryptophan production in the recombinant hyperproducing *C. glutamicum* strain mentioned above (Fig. 2), attributed to an increased availability of the other precursor E4P (Ikeda and Katsumata 1999). In this engineered recombinant strain combining the favorable modifications in the central

metabolism with those of the downstream pathways leading to L-tryptophan and L-serine (Fig. 2), a final titer of 58 g/l was obtained in fed-batch cultivations with sucrose as a substrate and without the need of antibiotics.

Engineering of transport systems

In addition to many examples representing successful application of pathway engineering for L-tryptophan production, strain improvement by modifying transport systems for the aromatic amino acids have been reported in *C. glutamicum* (Ikeda and Katsumata 1994, 1995). A finding that forwarded the work was that the presence of multicopies of the *aroP* gene (NCgl1062) responsible for the aromatic amino acid uptake in an L-tryptophan-producing *C. glutamicum* strain resulted in a drastic decrease in L-tryptophan production, indicating that the uptake activities negatively affected L-tryptophan production. Following this, the strategy of prevention of the uptake was applied to the L-tryptophan producer, an L-phenylalanine and L-tyrosine double auxotroph deficient in CM (Fig. 2). The resulting transport mutants that were impaired in the uptake were shown to accumulate10 to 20% more L-tryptophan than their parent in fed-batch jar fermentor cultivation (Ikeda and Katsumata 1995). Although these mutants require high concentrations of L-phenylalanine and L-tyrosine, they could grow well in the medium containing corn steep liquor because their demand for both amino acids would be compensated for peptides in the cheap natural material. This work indicates that prevention of amino acid uptake can be a useful strategy to bypass remaining regulatory steps and thereby to increase production.

Metabolic engineering for L-phenylalanine production

L-Phenylalanine-producing strains of *E. coli* and *C. glutamicum* have also been constructed with the use of recombinant DNA technology. The strategies used for improved production include amplification of possible rate-limiting enzyme(s) and/or the first enzyme in the common pathway, the concepts of which are basically the same as those for L-tryptophan production. However, some modified strategies have been applied for L-phenylalanine production. Sugimoto et al. (1987) used a temperature-controllable expression vector to conditionally express the deregulated *aroF* and *pheA* genes in an

L-tyrosine-auxotrophic *E. coli* strain, and obtained a maximal L-phenylalanine titer of 16.8 g/l at the optimal temperature 38.5˚C. This process was further improved by other groups, which resulted in titers of around 50 g/l of L-phenylalanine (Konstantinov et al. 1991; Konstantinov and Yoshida 1992; Takagi et al. 1996). Backman et al. (1990) also constructed a genetically modified *E. coli* strain with respect to L-phenylalanine-biosynthetic pathways and developed an efficient process with a titer of 50 g/l with 23% conversion yield on glucose after 36 h. In addition to these strain constructions, central metabolism was also modified for improved L-phenylalanine production. For example, inactivation of PEP carboxylase has been shown to be effective for L-phenylalanine production by *E. coli*, although such a modification was accompanied by unwanted by-products such as acetate and pyruvate (Miller et al. 1987). The potential methods of metabolic engineering applied to the production of L-phenylalanine or other aromatics in *E. coli* are summarized in Fig. 3.

Likewise, large increases in L-phenylalanine production have been achieved by amplifying the deregulated enzyme(s) in existing *C. glutamicum* producers (Ozaki et al. 1985; Ikeda et al. 1993; Ito et al. 1900b, 1990c). Primary targets for engineering have been the three key enzymes DS, CM, and PD (Fig. 1). While most cases have used endogenous enzymes from the organisms themselves, the desired metabolic engineering could be attained by the use of enzymes from heterologous organisms, as exemplified by a study with a recombinant *C. glutamicum* strain overexpressing the *E. coli pheA* gene that encodes the bifunctional key enzymes CM and PD (Ikeda et al. 1993). For the L-phenylalanine production processes, it is desirable to minimize by-production of L-tyrosine to a level at which supplementary purification of L-phenylalanine is not necessary. In this sense, the *pheA* expression has an additional merit because of its effect on diminished by-production of L-tyrosine (Ikeda et al. 1993). Metabolic conversion from L-tryptophan production to L-phenylalanine production by amplifying the genes for the deregulated branch-point enzymes, that is, *aro* II (NCgl2098), *csm* (NCgl0819), and *pheA* (NCgl2799), was also reported (Fig. 4) (Ikeda and Katsumata 1992). This approach has been shown to result in a marked accumulation of L-phenylalanine (28 g/l) with almost no by-production of L-tryptophan and L-tyrosine. The usefulness of decreased activity of PEP carboxylase or amplification of transketolase has been demonstrated also for *C. glutamicum* strains suitable for L-phenylalanine production (Katsumata and Kino 1989, Ikeda et al. 1999). Positive effect of prevention of the aromatic amino acid uptake on production has also been reported for L-phenylalanine-producing *C. glutamicum*

(Ikeda and Katsumata 1994).

Tatarko and Romeo (2001) have reported a unique approach to engineer a global regulatory network in central carbon metabolism for improved L-phenylalanine production by *E. coli*. They disrupted a global regulatory gene, *csrA*, to cause both increased flux of gluconeogenesis and decreased flux of glycolysis, thus to elevate the intracellular PEP pool for L-phenylalanine biosynthesis (Fig. 3). By this "global metabolic engineering", a twofold increase of L-phenylalanine was obtained.

It is known that high L-phenylalanine concentrations inhibit growth of *E. coli* (Grinter 1998). This growth inhibition by L-phenylalanine might have negative effects on process performance of L-phenylalanine production by *E. coli*. Polen et al. (2005) employed DNA microarrays to analyze the effects of L-phenylalanine on global gene expression of *E. coli*. On the basis of the observed gene expression profiles, they reported that the L-phenylalanine inhibition was related to a *tyrR*-mediated perturbation of aromatic amino acid metabolism and that disruption of *tyrR* resulted in a strain no longer inhibited by L-phenylalanine at the concentrations tested (5 g/l), thus being a useful strategy for improved L-phenylalanine production.

When L-tyrosine-auxotrophic strains of *E. coli* are used for industrial processes, L-tyrosine and glucose supply become key problems in process development. A general approach for the on-line control of the two key parameters of L-phenylalanine production was presented, which would allow a successful process scale-up without an overflow metabolism leading to acetate or L-alanine (Gerigk et al. 2002a, 2002b).

Metabolic engineering for L-tyrosine production

Classical methods have developed not only L-tryptophan-, or L-phenylalanine-producing mutants, but also L-tyrosine-producing mutants. The majority of these efforts for L-tyrosine production have been focused on *C. glutamicum*, *E. coli*, and *Bacillus subtilis* (Maiti et al. 1995).

With the introduction of recombinant DNA technology, improved strains for L-tyrosine production have been generated mostly from *C. glutamicum* and its subspecies. As was the case of L-phenylalanine production, the focuses of metabolic engineering has directed to PEP carboxylase and transketolase in central metabolism (Katsumata and Kino 1989, Ikeda et al. 1999), as well as the key regulatory enzymes DS

and CM. Simultaneous amplification of *aro* II (NCgl2098) encoding the desensitized type II-DS, together with *csm* (NCgl0819) encoding the branch-point enzyme CM, in an L-tryptophan-producing mutant of *C. glutamicum* has led to a shift from 18 g/l of L-tryptophan to 26 g/l of L-tyrosine (Fig. 4) (Ikeda and Katsumata 1992). In addition to these common targets, importance of shikimate kinase for improved L-tyrosine production has been demonstrated by the study with a *C. glutamicum* ssp. *lactofermentum* strain overexpressing the *aroK* gene (NCgl1560, initially named as *aroL*) (Ito et al. 1990a). By this modification, the bottleneck in the common pathway was removed, which resulted in an increase in L-tyrosine titer from 17.5 g/l to 21.6 g/l.

Metabolic engineering for other aromatic production

The aromatic pathway includes several useful intermediates to serve as suitable starting materials for the synthesis of industrially useful chemicals and medicines. For examples, 3-dehydroshikimic acid and shikimic acid can be used for the renewable production of a variety of industrial chemicals such as catechol, vanillic acid, and adipic acid (Li and Frost 1999; Li et al. 1999) and for the synthesis of the neuramidase inhibitor GS4140 that was developed for the treatment of influenza under the name of Tamiflu® (Kim et al. 1997), respectively. In order to meet the current market volumes at competitive prices, microbial production processes of these aromatics have been developed. Production strains have been constructed basically by blocking the common aromatic pathway at the next step of the desired intermediates and by metabolic engineering leading to increased carbon flow from central metabolism into the common pathway. For the production of 3-dehydroshikimic acid, a titer of 69 g/l was obtained in 30% yield (mol/mol) from glucose by using an *E. coli aroE* mutant overexpressing the transketolase gene (*tktA*) and the feedback-insensitive DS gene (*aroF*) (Li and Frost 1999; Li et al. 1999). Likewise, efficient shikimic acid production, titers of 50-90 g/l in around 30% yield (mol/mol) from glucose, was achieved by using an *E. coli aroL* and *aroK* mutant overexpressing the aromatic genes (*aroF*, *aroB*, and *aroE*) as well as the relevant genes in central metabolism such as *tktA* and *ppsA* (Draths et al. 1999; Knop et al. 2001; Chandran et al. 2003). Such metabolic engineering of shikimic acid production have been reviewed (Krämer et al. 2003).

Conclusions and perspectives

This review has summarized the strategies useful for increasing carbon flow to aromatics chiefly in two representative producer organisms *C. glutamicum* and *E. coli*. Reviewing the strategies, it is found that increasing the availability of PEP and E4P has attracted much attention as a common strategy in metabolic engineering for all aromatic production, leading to several different approaches. However, in accessing the central metabolism, we should note that previous attempts to drastically redirect carbon flux into a desired pathway resulted in growth impairment and/or formation of unwanted by-products (Miller et al. 1987; Mascarenhas et al. 1991; Kamada et al. 2001; Marx et al. 2003). The junction between glycolysis and TCA cycle, in particular, must be given special attention because the junction consists of several enzyme reactions carrying fluxes that connect PEP with oxaloacetate or pyruvate, pyruvate with oxaloacetate, and pyruvate with malate, and are thus important for sharing metabolic fluxes with not only aromatic biosynthesis but also various biosynthetic pathways. The set of enzymes and the metabolic fluxes at the junction in bacteria has been reviewed recently, with a particular focus on *C. glutamicum*, *E. coli*, and *B. subtilis* (Sauer and Eikmanns 2005). Engineering of the pentose phosphate pathway also may require delicate modifications of the activities of the key enzymes, as pointed out by the study in which only a moderate increase in transketolase activity resulted in further improvement of L-tryptophan in the recombinant hyperproducing *C. glutamicum* strain (Ikeda and Katsumata 1999).

Despite the progress already obtained with aromatic production, further improvements can be expected by engineering targets that yet remain to be identified. One of these targets is the efflux of the aromatic amino acids which seems to be of key importance. Although at present little is known about the efflux mechanism for the aromatic amino acids in bacteria, active carrier-mediated systems may be related to the excretion of them, as has already been established for several amino acids, such as L-glutamate, L-lysine, L-isoleucine, and L-threonine, in *C. glutamicum* (Krämer 1994; Krämer et al. 1994; Eggeling and Sahm 2001). Considering the finding that the deficiency in the aromatic amino acid uptake resulted in an improved production (Ikeda and Katsumata 1994, 1995), additional engineering of the excretion processes may have an impact on efficiency of aromatic amino acid production.

Due to the availability of the genome sequences of the amino acid-producing organisms (Ikeda and Nakagawa 2003; Kalinowski et al. 2003; Nishio et al. 2003), genomic approaches offer an additional possibility for the development of aromatic amino acid

production strains. Indeed, it has already been demonstrated for L-lysine production that the identification of beneficial mutations in a classical producer and their subsequent assembling in the wild-type background resulted in a defined producer with superior production properties (Ohnishi et al. 2002, 2003; Ikeda et al. 2005). *C. glutamicum*, in particular, has a long history of classical breeding, which results in a huge variety of industrially useful mutants. Once their beneficial genetic properties are exploited by genomics, it becomes possible to share the genetic properties with other strains to be engineered. For example, the availability of a *gnd361* mutation that was recently identified in the genome of an L-lysine-producing *C. glutamicum* strain will provide useful addition to the future metabolic engineering for improved aromatic production, because the mutation has been shown to be responsible for diminished allosteric regulation of the enzyme and contribute to redirection of more carbon to the pentose phosphate pathway (Ohnishi et al. 2005).

The development of genomics has also broadened the methodologies of *in silico* approaches for modeling and optimization of metabolite production (Patil et al. 2004). As an example of this *in silico* biology, Schmid et al. (2004) have designed the metabolic network model of L-tryptophan biosynthesis from glucose in *E. coli* and discussed the improvements in L-tryptophan production obtained by optimization *in silico*. Such work will serve as an important guideline in identifying metabolic engineering targets. By being fused with genomics as well as the rich property of useful mutants and accumulated knowledge so far, metabolic engineering technologies can be expected to further extend the performance of existing production strains.

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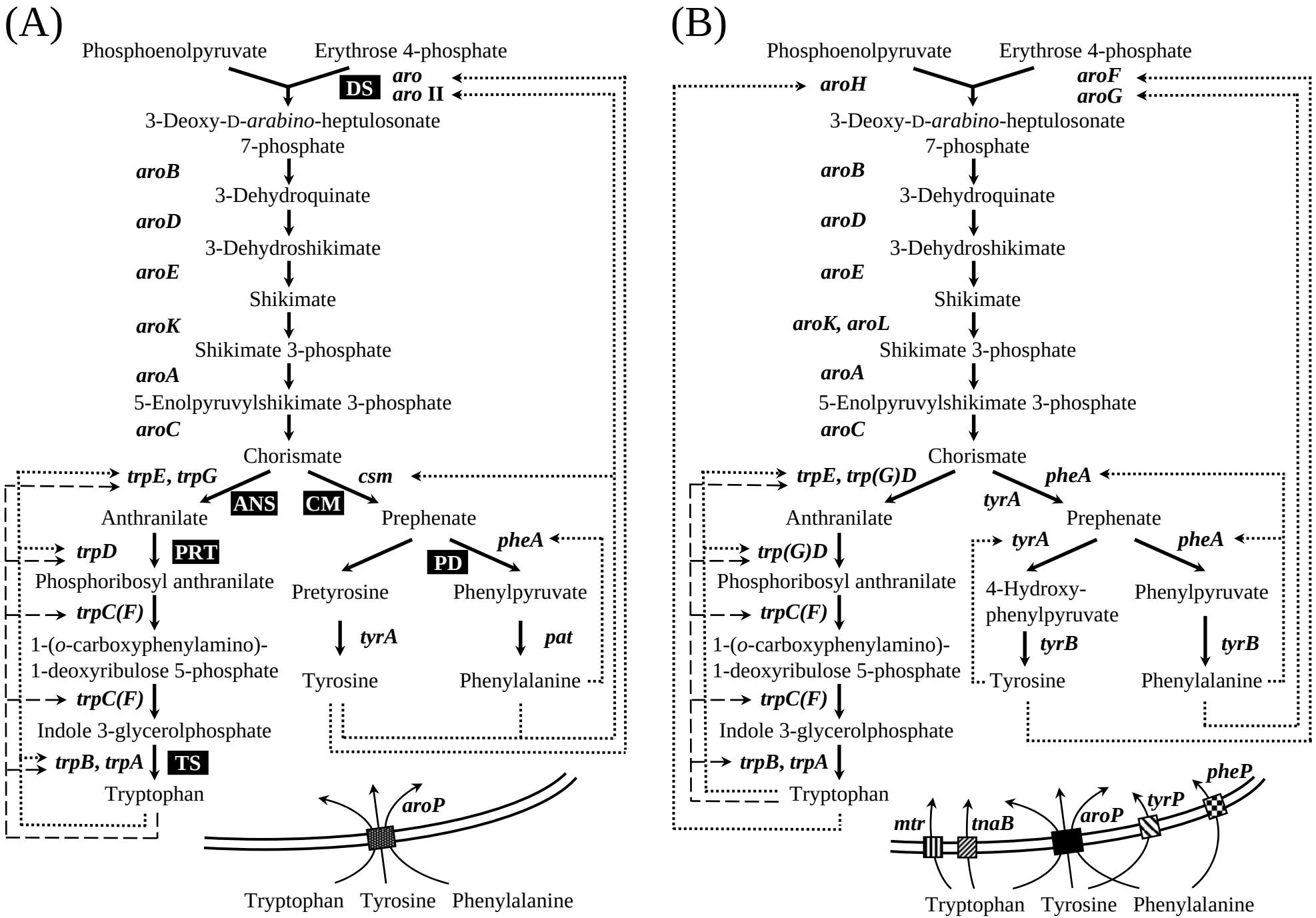
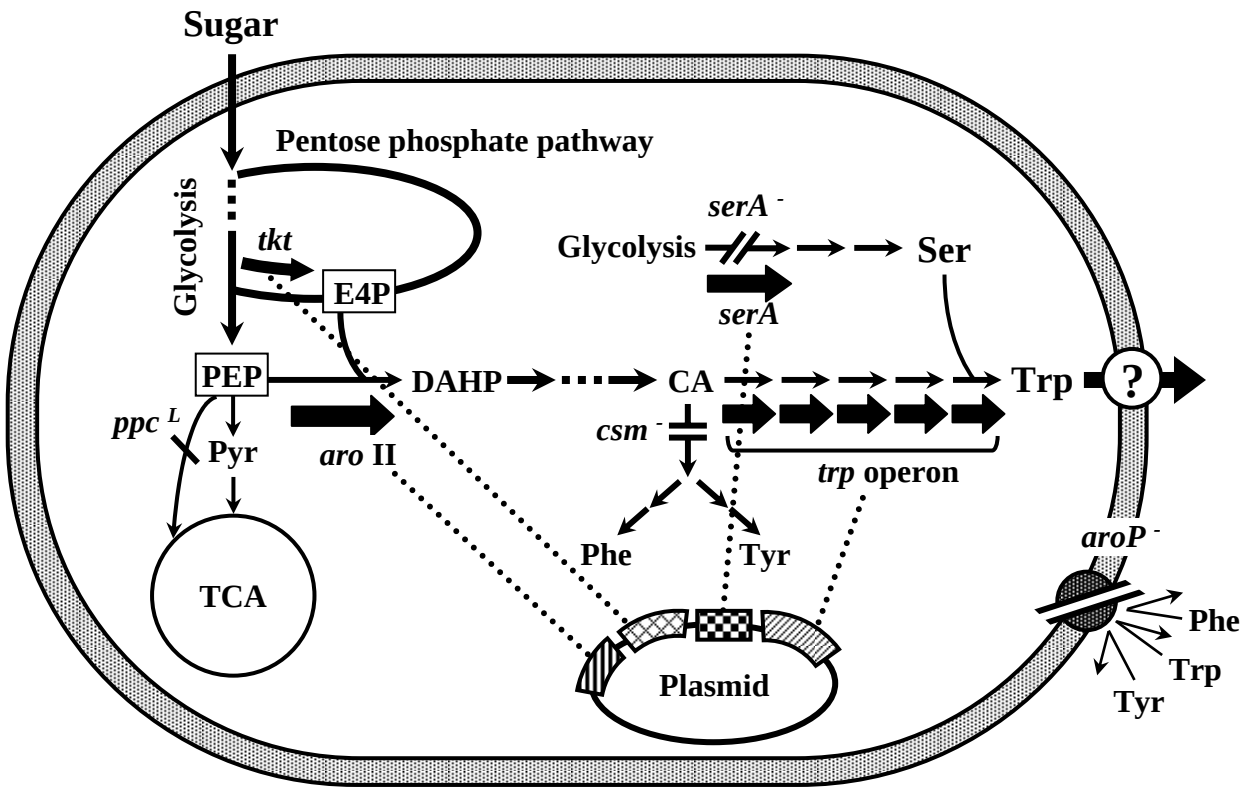
Fig. 1. Biosynthetic pathways, regulations, and transport systems of aromatic amino acids in *C. glutamicum* (A) and *E. coli* (B). The dotted lines and the dashed lines indicate feedback inhibition and repression, respectively. Symbols for genes follow mostly the *E. coli* K-12 linkage map. *DS* 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase, *CM* chorismate mutase, *PD* prephenate dehydratase, *ANS* anthranilate synthase, *PRT* anthranilate phosphoribosyltransferase, *TS* tryptophan synthase

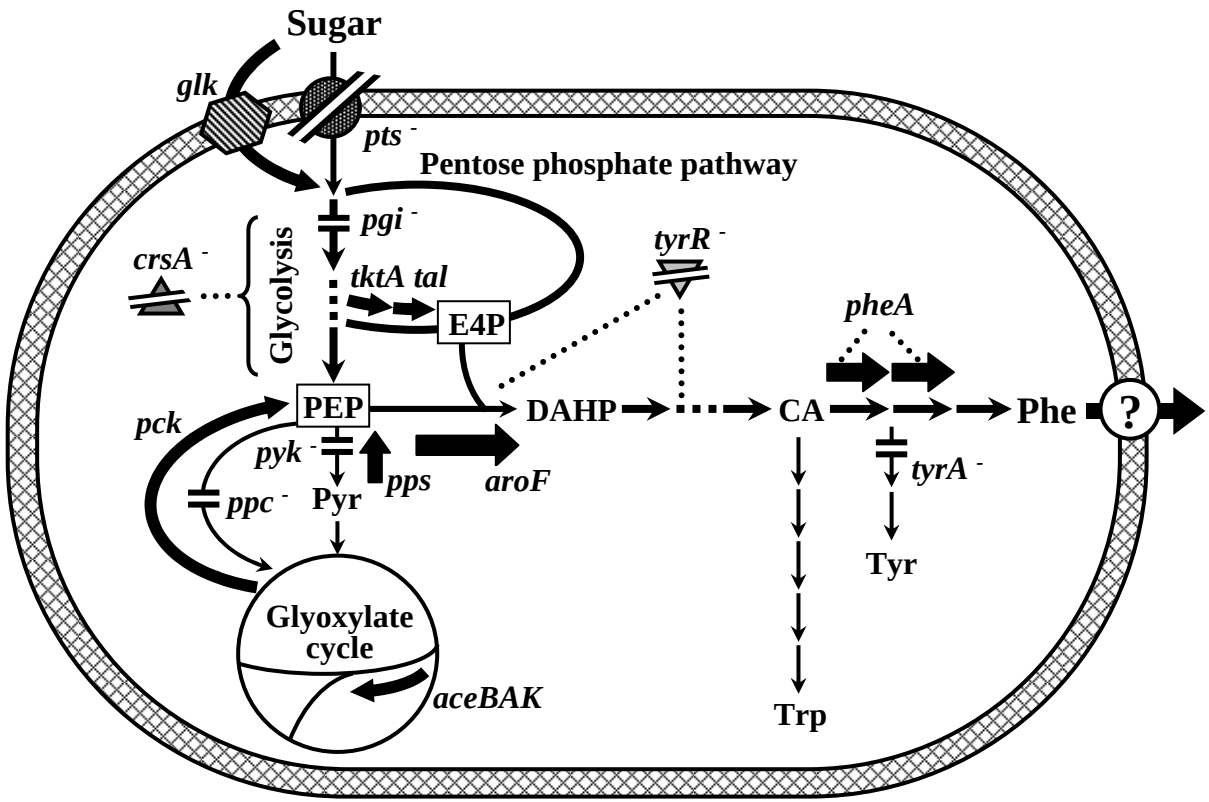
Fig. 2. Outline of metabolic engineering for L-tryptophan production demonstrated in *C. glutamicum*. The excretion process of L-tryptophan remains to be elucidated. *PEP* phosphoenolpyruvate, *E4P* erythrose 4-phosphate, *Pyr* pyruvate, *DAHP* 3-deoxy-D-*arabino*-heptulosonate 7-phosphate, *CA* chorismate

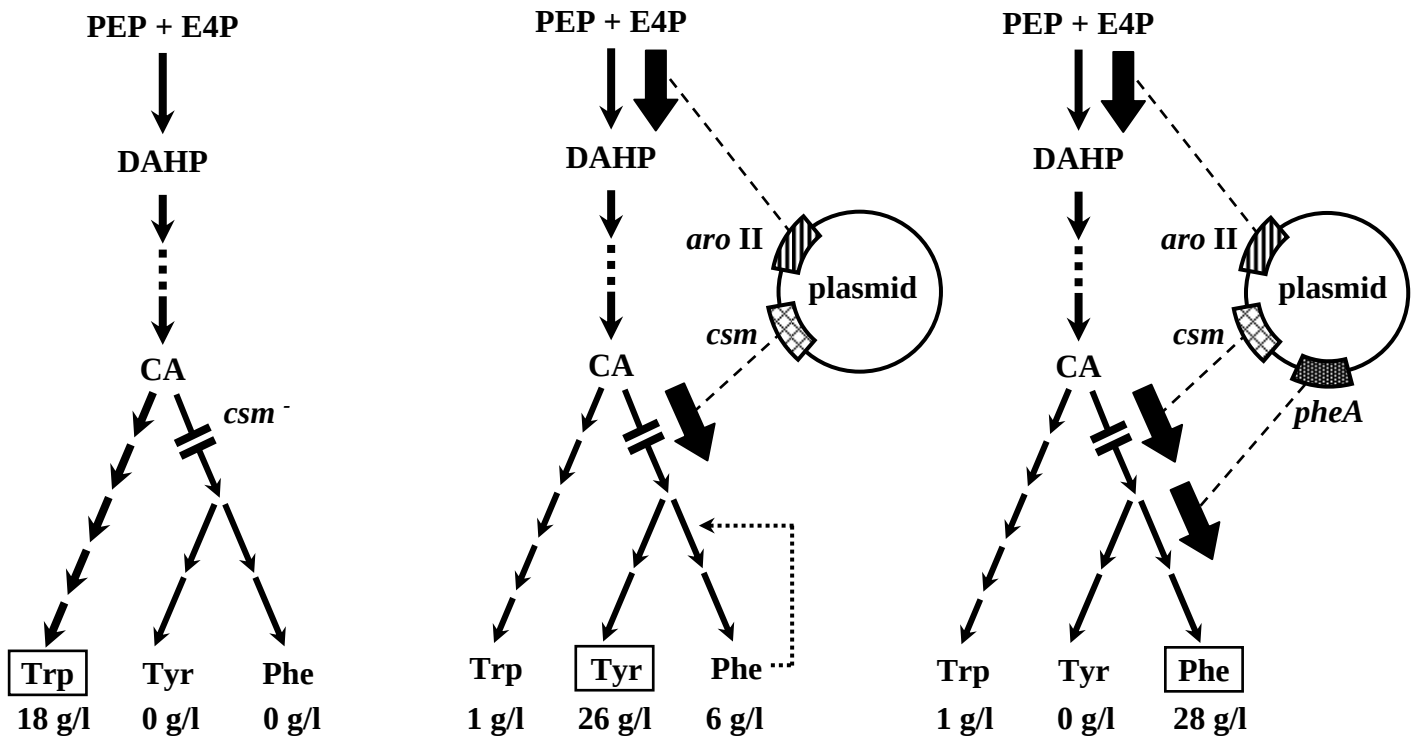
Fig. 3. Different approaches of metabolic engineering applied to the production of L-phenylalanine or other aromatics in *E. coli*. The excretion process of L-phenylalanine remains to be elucidated. Abbreviations are as described in the legend to Fig. 2

Fig. 4. Outline of metabolic conversion from L-tryptophan to L-phenylalanine and L-tyrosine in *C. glutamicum*. The dotted line indicates feedback inhibition. Abbreviations are as described in the legend to Fig. 2


(A)
Phosphoenolpyruvate
Erythrose 4-phosphate
DS
aro
aro II
3-Deoxy-D-arabino-heptulosonate 7-phosphate
aroB
3-Dehydroquinate
aroD
3-Dehydroshikimate
aroE
Shikimate
aroK
Shikimate 3-phosphate
aroA
5-Enolpyruvylshikimate 3-phosphate
aroC
Chorismate
trpE, trpG
ANS
csm
CM
Anthranilate
Prephenate
trpD
PRT
pheA
PD
Phosphoribosyl anthranilate
Pretyrosine
Phenylpyruvate
trpC(F)
1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate
tyrA
pat
Tyrosine
Phenylalanine
trpC(F)
Indole 3-glycerolphosphate
trpB, trpA
TS
Tryptophan
aroP
Tryptophan
Tyrosine
Phenylalanine
(B)
Phosphoenolpyruvate
Erythrose 4-phosphate
aroH
aroF
aroG
3-Deoxy-D-arabino-heptulosonate 7-phosphate
aroB
3-Dehydroquinate
aroD
3-Dehydroshikimate
aroE
Shikimate
aroK, aroL
Shikimate 3-phosphate
aroA
5-Enolpyruvylshikimate 3-phosphate
aroC
Chorismate
trpE, trp(G)D
pheA
tyrA
Anthranilate
Prephenate
trp(G)D
tyrA
pheA
Phosphoribosyl anthranilate
4-Hydroxy-phenylpyruvate
Phenylpyruvate
trpC(F)
tyrB
tyrB
1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate
Tyrosine
Phenylalanine
trpC(F)
Indole 3-glycerolphosphate
trpB, trpA
Tryptophan
pheP
mtr
tnaB
aroP
tyrP
Tryptophan
Tyrosine
Phenylalanine


Sugar
Pentose phosphate pathway
Glycolysis
tkt
E4P
PEP
DAHP
CA
Trp
?
ppc L
Pyr
aro II
TCA
serA -
Glycolysis
Ser
serA
csm -
trp operon
Phe
Tyr
Plasmid
aroP -
Phe
Trp
Tyr


Sugar
glk
pts -
Pentose phosphate pathway
pgi -
crsA -
Glycolysis
tktA tal
E4P
tyrR -
pheA
PEP
DAHP
CA
Phe
?
pck
pyk -
pps
aroF
ppc -
Pyr
tyrA -
Tyr
Glyoxylate cycle
aceBAK
Trp


PEP + E4P
DAHP
CA
csm -
Trp
Tyr
Phe
18 g/l
0 g/l
0 g/l
PEP + E4P
DAHP
aro II
plasmid
csm
CA
Trp
Tyr
Phe
1 g/l
26 g/l
6 g/l
PEP + E4P
DAHP
aro II
plasmid
csm
pheA
CA
Trp
Tyr
Phe
1 g/l
0 g/l
28 g/l