

**MOLECULAR BREEDING OF AROMATIC
AMINO ACID - PRODUCING
*CORYNEBACTERIUM GLUTAMICUM***

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INTRODUCTION

Corynebacterium glutamicum, an aerobic, nonsporeforming Gram-positive organism, was isolated by Kinoshita et al. in the mid-fifties in the course of screening for efficient glutamic acid-producing bacteria (5). After that, its relatives shown in Fig. have been isolated from nature. These coryneform bacteria all closely resemble the first isolated strain C. glutamicum in physiological and chemotaxonomical characteristics (1, 4), and so are called generically "glutamic acid bacteria" associated with their intrinsic ability to produce this amino acid directly from sugar. The pioneering work of Kinoshita and co-workers represents the introduction of bacterial fermentations in the industrial production of amino acids.

In the fermentation process, improvement involves strains capable of producing amino acids in higher yields. While glutamic acid fermentation was established based on the characteristic that glutamic acid bacteria excrete

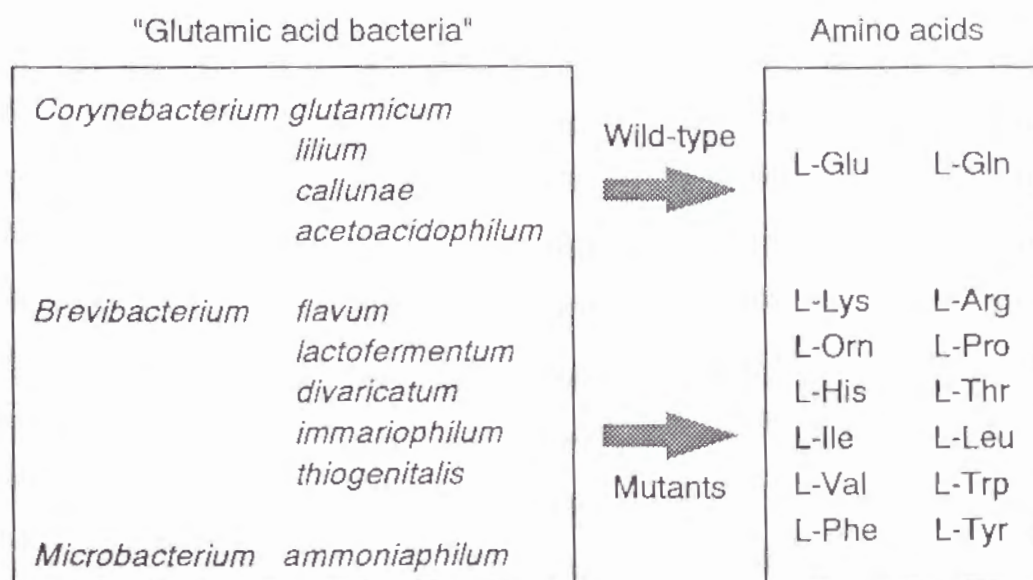


FIG. Glutamic acid bacteria isolated from nature and amino acids overproduced by fermentation with these organisms.

the intracellularly synthesized amino acid into the medium under the special culture conditions, other amino acids are not normally overproduced due to rigid controls of their biosyntheses by multiple regulatory mechanisms such as feedback inhibition and repression. Therefore, a series of other amino acid fermentations have been developed with the use of their mutants (6). The strain constructions have been done exclusively on the basis of mutation programs that aim at deregulating the corresponding biosynthetic pathway and blocking side branch pathways. At present, many amino acids listed in Fig. are being produced by such strains on an industrial scale. However, further improvement of the productivity of amino acids is desired to meet the increasing demands for amino acids.

In recent years, gene cloning systems were developed for *C. glutamicum* and its relatives (3, 7-9). These advances include: the establishment of a polyethylene glycol-mediated protoplast transformation system, and the construction of indigenous or shuttle vectors. In *C. glutamicum*, these systems have been further completed by the development of a lysozyme-sensitive and restriction-deficient host strain that is greatly advantageous for efficient recovery of recombinant DNA (2). All these developments have allowed the use of recombinant DNA technology in breeding of industrially important amino acid-producing strains of these organisms. This recombinant DNA approach to strain improvement has several potential advantages over classical mutagenesis including its feasibility of amplifying the rate-limiting enzymes through increased gene dosage.

In this study, the author describes metabolic engineering for hyperproduction of the aromatic amino acids (chapter 1-4), demonstrating the usefulness of molecular breeding in further improvement of highly developed amino acid producers of *C. glutamicum*. Furthermore, the author shows that prevention of amino acid transport as well as deregulation of biosynthetic pathways is one of the crucial factors in amino acid overproduction (chapter 5).

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INTRODUCTION

Biosynthesis of the aromatic amino acids in microorganisms proceeds via a common pathway to chorismate, from which the pathways to phenylalanine, tyrosine and tryptophan branch (25). In *Corynebacterium glutamicum* and its closely related strains (9, 24), control of metabolic flow on these aromatic pathways occurs primarily through end-product inhibition in four enzymatic steps (Fig. 1). The first enzyme in the common pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DS), is synergistically inhibited by phenylalanine and tyrosine. Similarly, the activity of chorismate mutase (CM) which converts chorismate to prephenate exhibits sensitivity to phenylalanine and tyrosine. Prephenate dehydratase (PD) and anthranilate synthase, which initiate the pathways to phenylalanine and tryptophan, respectively, are subject to inhibition by each end product.

Each regulation can be altered by a mutation resulting in auxotrophy for the aromatic amino acid(s) or resistance to its structural analog(s). Regulatory mutants of *C. glutamicum* that carry a combination of these mutations have been induced as potent producers of tyrosine, phenylalanine or tryptophan (6-8).

Besides classical mutagenesis, the recent development of host-vector systems for *C. glutamicum* and its related bacteria (12, 17, 20, 26) has allowed us to use recombinant DNA technology for further strain improvement. Some attempts to genetically engineer existing mutants which produce the aromatic amino acid resulted in significant improvement in yields (11, 18). In such studies, the strategy used aimed at amplifying the gene coding for the rate-limiting enzyme, thereby eliminating the bottleneck in the biosynthetic pathway.

However, considering the fact that the aromatic amino acids are synthesized via a common pathway, it is reasonable to expect that if a branch-point enzyme is amplified in a certain aromatic amino acid-producing strain, the strain would be altered to produce the corresponding aromatic amino acid. It is also predictable that simultaneous amplification of DS together with the branch-point enzyme(s) might accelerate carbon flow into the overall pathway and thus substantially increase the yield of the desired amino acid. Based on these assumptions, the author undertook the conversion of a tryptophan-producing strain to a tyrosine or phenylalanine producer. This novel strain construction is presented in this chapter.

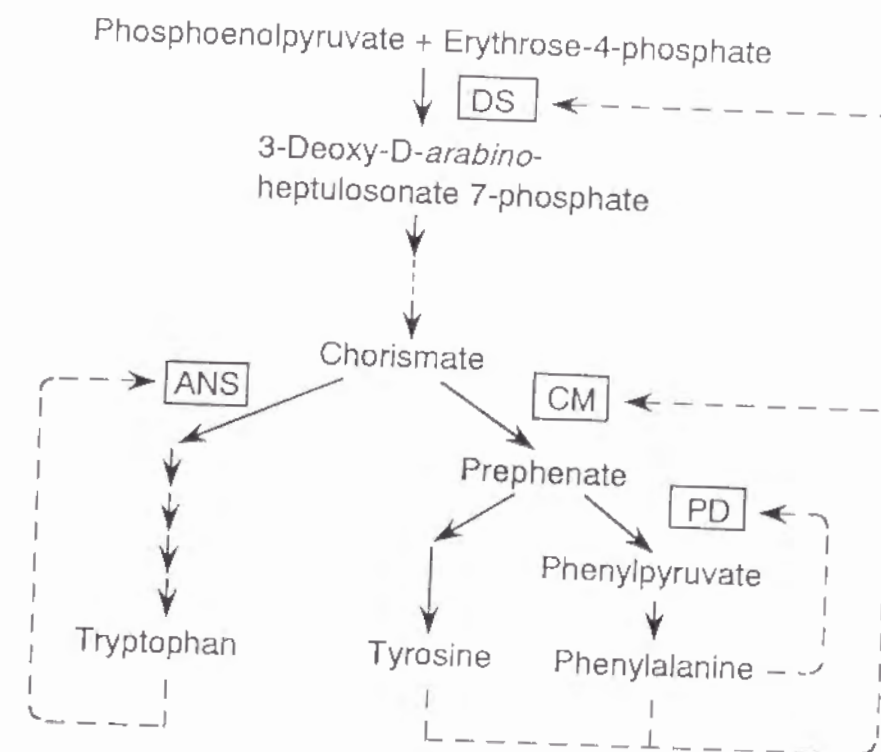


FIG. 1. Pathway and primary regulations of aromatic amino acid biosynthesis in *C. glutamicum*. Broken arrows indicate feedback inhibition. ANS, anthranilate synthase.

Bacterial strains and plasmids.

C. glutamicum KY10693 and KY10694, phenylalanine-producing mutants derived from strain 31-PAP-20-22 (7), were used as the DNA donors for cloning of the deregulated biosynthetic genes. The former has a phenylalanine-insensitive PD, and the latter has a DS and CM highly desensitized to synergistic feedback inhibition by phenylalanine and tyrosine. *C. glutamicum* KY10865, used as the host strain for production of the aromatic amino acids, is a tryptophan-producing mutant derived from strain Px-115-97 (8). This strain is a CM-deficient, phenylalanine and tyrosine double auxotroph whose DS is wild-type and whose anthranilate synthase is partially desensitized to inhibition by tryptophan. Strains KLS4, KY9457 and KY9182 are lysozyme-sensitive, phenylalanine and tyrosine double auxotrophic (CM-deficient) and phenylalanine auxotrophic (PD-deficient) mutants, respectively, derived from *C. glutamicum* wild-type strain ATCC 13032.

Plasmid pCG115, a derivative of the *C. glutamicum* vector pCG11 (13), has the streptomycin-spectinomycin resistance gene and the polylinker present in M13mp18 RF DNA (16). Plasmid pCE53 (17) is a *C. glutamicum*-*Escherichia coli* shuttle vector and carries the kanamycin, chloramphenicol and tetracycline resistance genes derived from the *E. coli* vector pGA22 (5), which are usable as selectable markers.

Media.

Complete medium BY (12), minimal medium MM (18) and enriched minimal medium MMYE (12) were used for cultivation of *C. glutamicum*. Solid plates were made by the addition of Bacto-Agar (Difco) to 1.6%. RCCA medium (12) was used for regeneration of *C. glutamicum* protoplasts. When required, supplements or antibiotics were added at the following final concentrations: spectinomycin, 400 µg/ml for RCCA plates or 100 µg/ml for BY and MM plates;

kanamycin, 200 µg/ml for RCCA plates or 10 µg/ml for BY and MM plates; phenylalanine and tyrosine, 100 µg/ml. TP1 medium used for production in test tubes contained (per liter) glucose, 60 g; KH_2PO_4 , 1 g; K_2HPO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g; $(\text{NH}_4)_2\text{SO}_4$, 20 g; corn steep liquor, 10 g; $\text{MnSO}_4 \cdot 4\sim 6\text{H}_2\text{O}$, 10 mg; D-biotin, 30 µg; and CaCO_3 , 20 g (pH 7.2). TS1 medium used for second-seed culture in jar fermentation contained (per liter) sucrose, 50 g; KH_2PO_4 , 2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $(\text{NH}_4)_2\text{SO}_4$, 5 g; urea, 0.6 g; corn steep liquor, 40 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg; $\text{MnSO}_4 \cdot 4\sim 6\text{H}_2\text{O}$, 10 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 4 mg; L-phenylalanine, 362 mg; L-tyrosine, 266 mg; D-biotin, 100 µg; thiamine-HCl, 5 mg; CaCO_3 , 20 g (pH 7.2). TP2 medium used for production in 2-liter jar fermentors contained (per liter) sucrose, 60 g; KH_2PO_4 , 2 g; K_2HPO_4 , 1.2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.7 g; $(\text{NH}_4)_2\text{SO}_4$, 17 g; corn steep liquor, 66 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 13 mg; $\text{MnSO}_4 \cdot 4\sim 6\text{H}_2\text{O}$, 13 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 6 mg; L-tyrosine, 310 mg; L-phenylalanine, 650 mg; D-biotin, 230 µg; and thiamine-HCl, 450 µg (pH 6.8).

Cultivations for production of amino acids.

(i) Test tubes. Cells grown on a BYG (containing 1.0% glucose in medium BY) plate were inoculated into 3 ml of BYC medium containing phenylalanine and tyrosine (200 µg/ml each). After 24 h of cultivation at 30°C, 0.5 ml of the seed culture was transferred to a large test tube containing 5 ml of TP1 medium. Cultivation was carried out aerobically at 30°C for 72 h.

(ii) Two-liter jar fermentors. A 2.4 ml amount of the first-seed culture in BYG medium was inoculated into 120 ml of TS1 medium in a 1-liter flask. After 24 h of cultivation at 30°C on a rotary shaker, the second-seed broth was transferred into a 2-liter jar fermentor containing 550 ml of TP2 medium. After the sugar initially added was consumed, solution containing 44% sucrose was continuously fed until the total amount of sugar supplied in the medium reached 20%. The culture was agitated at 800 rpm and aerated at 1 liter/min at 30°C, and the pH was maintained at 6.1 with NH_4OH . Cultivations of recombinant strains were carried out in medium with spectinomycin (100 µg/ml).

Preparation and manipulation of DNA.

Chromosomal DNA was extracted from protoplasts of *C. glutamicum* KY10693 and KY10694 by the method of Saito and Miura (19). The protoplasts were prepared as described previously (12). Plasmid DNA was isolated by the alkaline lysis method (15) and, if necessary, purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (12). DNA digestion and ligation were carried out as described by Maniatis *et al.* (15). Restriction enzymes and T4 DNA ligase were obtained from Takara Shuzo Co., Ltd., Kyoto, Japan.

Transformations.

The protoplast transformation method used has been described previously (12).

Enzyme assays.

Cells were grown at 30°C in 40 ml of MMYE medium, harvested in exponential phase, washed with cold 40 mM potassium phosphate buffer (pH 7.0), and suspended in 5 ml of the same buffer. Then, the cells were continuously sonicated for 15 min on ice with an ultrasonic disruptor (model UR-200P; Tomy Seiko Co., Ltd.), and cellular debris was removed by centrifugation at 10,000 rpm for 20 min at 4°C to obtain the supernatants. The crude extracts were dialyzed against the above potassium phosphate buffer for 6 h and used for the enzyme assay. Protein was determined by the method of Bradford (1) with the Bio-Rad kit. Enzyme activities in crude cell extracts were measured by the method of Sprinavasan and Sprinson (22) for the DS assay and by the method of Cotton and Gibson (2) for the CM and PD assay except that the assays were carried out at 30°C.

Analysis.

Cell growth was monitored by measuring the OD₆₆₀ with a spectrophotometer

(model 100-20; Hitachi Co., Ltd.). Sugar concentration was measured by an Auto Analyzer (model AA-II; Technicon Co., Ltd.). The aromatic amino acids were analyzed by high-performance liquid chromatography (Shimazu Co., Ltd.) after derivatization with *o*-phthalaldehyde.

RESULTS

Cloning of the DS, CM, and PD genes.

As a first step to metabolic engineering, we cloned the three biosynthetic genes encoding the first enzyme in the common pathway, DS, and the branch-point enzymes CM and PD. Cloning of the desensitized DS gene from a regulatory mutant, *C. glutamicum* KY10694, was carried out as follows. Chromosomal DNA of strain KY10694 and vector pCG115 were completely digested with *Sal*I, mixed, and treated with T4 DNA ligase. The ligation mixture was used to transform the protoplasts of prototrophic strain *C. glutamicum* KLS4. Transformants were selected on RCGA plates containing spectinomycin (400 µg/ml). Regenerated spectinomycin-resistant colonies were transferred by replica-plating to MM agar plates containing spectinomycin (100 µg/ml) and *p*-fluorophenylalanine (PFP) (800 µg/ml). Twelve PFP-resistant clones were obtained among about 10⁵ spectinomycin-resistant transformants. All plasmids purified from these clones were shown to have a common 6.7 kb *Sal*I fragment by restriction enzyme cleavage analysis. Retransformation of KLS4 with pCA1, one of the resulting recombinant plasmids, resulted in the concomitant spectinomycin- and PFP-resistant phenotype. Cells carrying pCA1 showed an eightfold-higher level of DS activity than the plasmidless cells. The overexpressed DS was insensitive to synergistic inhibition by phenylalanine and tyrosine, indicating that the cloned segment had the desensitized DS gene.

To clone the desensitized CM gene from the same strain KY10694, its chromosomal DNA and vector pCE53 were completely digested with *Sal*I, and ligated. As a result of transforming the protoplasts of a CM-deficient,

phenylalanine and tyrosine auxotroph, *C. glutamicum* KY9457, with the ligation mixture, over 10^4 transformants were generated on RCGA plates containing kanamycin (200 μ g/ml). By replica-plating these colonies to MM agar plates, eight clones with the prototrophic phenotype were obtained. All clones contained plasmids with a common 1.9 kb *Sal*I fragment, one of which was designated pCA2. Similarly, cloning of the desensitized PD gene from another regulatory mutant, *C. glutamicum* KY10693, was carried out by complementation of a PD deficient, phenylalanine auxotroph, *C. glutamicum* KY9182. After transformation with the ligation mixture of *Bam*HI-digested KY10693 DNA and pCE53 and subsequent screening for prototrophy, six kanamycin-resistant and phenylalanine-independent clones were selected. The plasmids isolated from the clones carried a common 4.9 kb *Bam*HI insert at the corresponding site of the vector. One plasmid was designated pCA3.

Construction of recombinant plasmids containing multiple biosynthetic genes.

The three genes cloned separately were joined stepwise onto one plasmid as shown in Fig. 2. First, pCA1 partially digested with *Sal*I was ligated with completely *Sal*I-digested pCA2. The ligation mixture was used to transform protoplasts of strain KY9457. Transformants were isolated on RCGA plates containing spectinomycin. By transferring these spectinomycin-resistant colonies to MM agar plates containing PFP, phenylalanine- and tyrosine-independent and PFP-resistant clones were selected. Restriction cleavage analysis of the plasmids isolated from these clones confirmed that they contained the DS and CM genes. One of these plasmids was designated pKY1.

Next, the *Bam*HI fragment containing the PD gene on pCA3 was ligated with pKY1 digested with *Bgl*II which did not cut within the DS gene (data not shown). The ligation mixture was used to transform protoplasts of strain KY9182. Spectinomycin-resistant, phenylalanine-independent, and PFP-resistant clones were isolated. From restriction cleavage analysis, the resulting plasmid isolated from one of the clones was shown to possess all the three

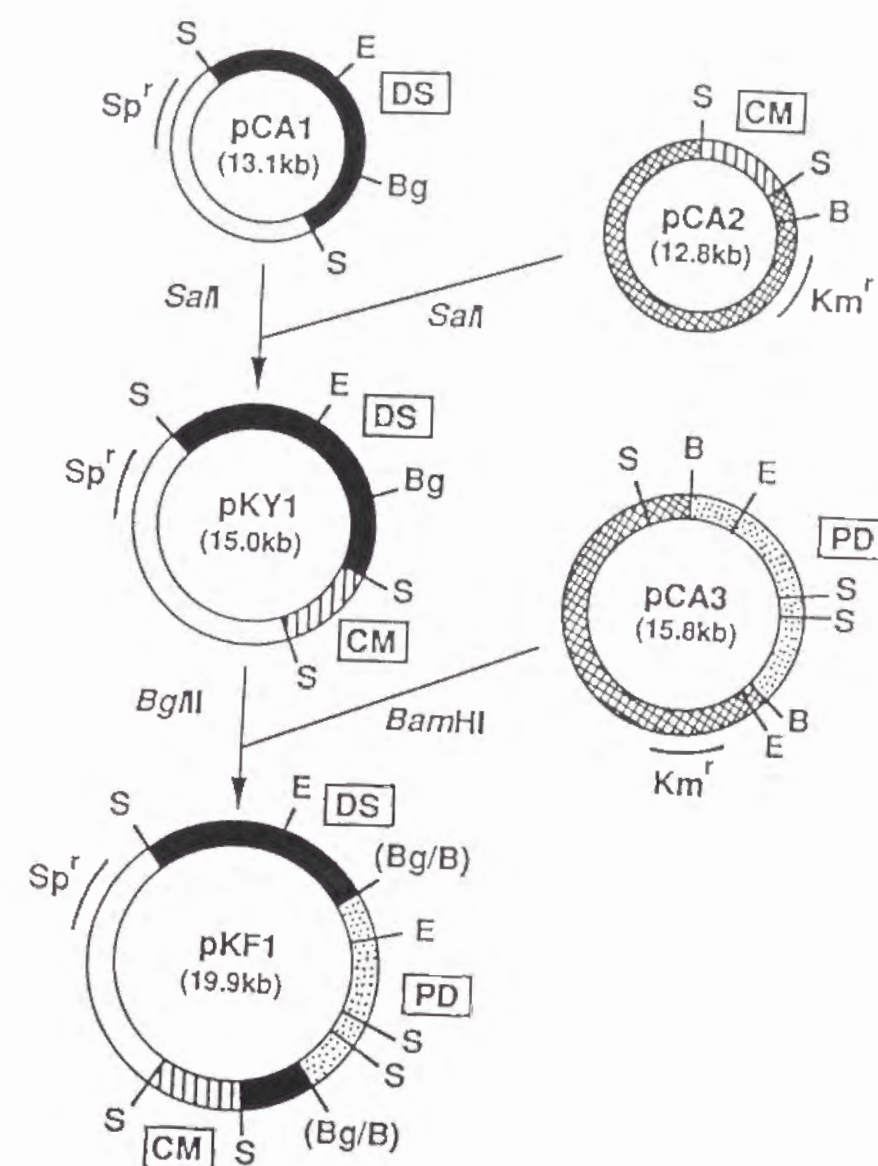


FIG. 2. Construction of recombinant plasmids containing aromatic amino acid-biosynthetic genes. Symbols: solid bars, *C. glutamicum* KY10694 chromosomal DNA fragment containing the DS gene; hatched bars, *C. glutamicum* KY10694 chromosomal DNA fragment containing the CM gene; stippled bars, *C. glutamicum* KY10693 chromosomal DNA fragment containing the PD gene; open bars, pCG115; cross-hatched bars, pCE53. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RV; S, *Sal*I; Km^r, kanamycin resistance; Sp^r, spectinomycin resistance.

genes and was designated pKF1.

DS, CM and PD activities of the recombinant strains.

To confirm the expression of the biosynthetic genes on pKY1 and pKF1, the relevant enzyme activities in the crude cell extract of strain KLS4 carrying each plasmid were measured (Table 1). The presence of pKY1 in this host strain elevated the level of DS and CM activities about eightfold, while the level of PD was also elevated eightfold in the pKF1-carrying strain. Each of the overexpressed biosynthetic enzymes was insensitive to end product inhibition to the same extent as the donor strain used for shotgun cloning. On the other hand, in the case of the pCA2-carrying strain, CM activity increased only threefold compared with that of the host as shown in Table 2. However, when this extract was mixed with the extract of the pCA1 carrier, CM activity increased to approximately sixfold (Table 2).

TABLE 1. Activities of DS, CM and PD encoded by recombinant plasmids

Strain (plasmid)	Amplified gene products	DS		CM		PD	
		Sp. act. ^a	Inhibition ^b	Sp. act.	Inhibition ^b	Sp. act.	Inhibition ^c
KLS4		16.0	72	5.3	74	2.9	81
KLS4 (pKY1)	DS, CM	131.2	6	39.8	5	3.8	74
KLS4 (pKF1)	DS, CM, PD	137.6	7	44.5	6	23.2	28
KY10694		19.2	3	5.8	3		
KY10693						2.9	22

^a Expressed as nanomoles of product per milligram of protein per minute.

^b Phenylalanine and tyrosine were added at 30 mM and 3 mM, respectively.

^c Phenylalanine were added at 30 mM.

TABLE 2. Expression of DS and CM activities in *C. glutamicum* KLS4 carrying recombinant plasmids

Strain (plasmid)	Amplified gene product(s)	Relative activity ^a	
		DS	CM
KLS4		1.0	1.0
KLS4 (pCA1)	DS	9.3	3.9
KLS4 (pCA2)	CM	1.0	3.0
KLS4 (pCA1) + KLS4 (pCA2) ^b	DS, CM	5.5	6.2

^a Relative to the activity in KLS4 with no plasmid.

^b Each crude extract was mixed at an equal concentration of protein and used for the enzyme assay.

Effects of the recombinant plasmids on metabolic flow in a tryptophan-producing strain.

It was examined whether the recombinant plasmids had effects on the metabolic flow in a tryptophan-producing strain KY10865 (Table 3). This strain is auxotrophic for phenylalanine and tyrosine due to a defect in CM and has an anthranilate synthase partially desensitized to inhibition by tryptophan. It produces a considerable amount of tryptophan under limited conditions of phenylalanine and tyrosine, where the synergistic inhibition of the DS can be bypassed.

Introduction of pCA2 into KY10865 did not result in remarkable accumulation of phenylalanine or tyrosine despite largely decreased production of tryptophan. The total yield of the aromatic amino acids in the recombinant strain decreased to below half of the tryptophan yield of the host strain.

By contrast, when pKY1 was introduced into KY10865, the transformed strain acquired the ability to produce tyrosine (predominantly) and phenylalanine, although a small amount of tryptophan was concomitantly accumulated. This preferential synthesis of tyrosine appeared to occur, because the PD of the host cells is wild-type and sensitive to phenylalanine formed intracellularly while the carbon flow from chorismate to tyrosine is uncontrolled. On the other hand, pKF1 allowed the same host to produce a large amount of phenylalanine almost without by production of tryptophan and tyrosine.

Strain KY10865 and its plasmid-carrying strains were tested for production of the aromatic amino acids in jar fermentors. Fig. 3 shows the time courses of fed batch cultures in sucrose medium to which sugar was added initially at 6% and subsequently fed to 20% total. While KY10865 produced 18 g of tryptophan per liter, the pKY1 carrier and the pKF1 carrier produced 26 g of tyrosine and 28 g of phenylalanine per liter, respectively.

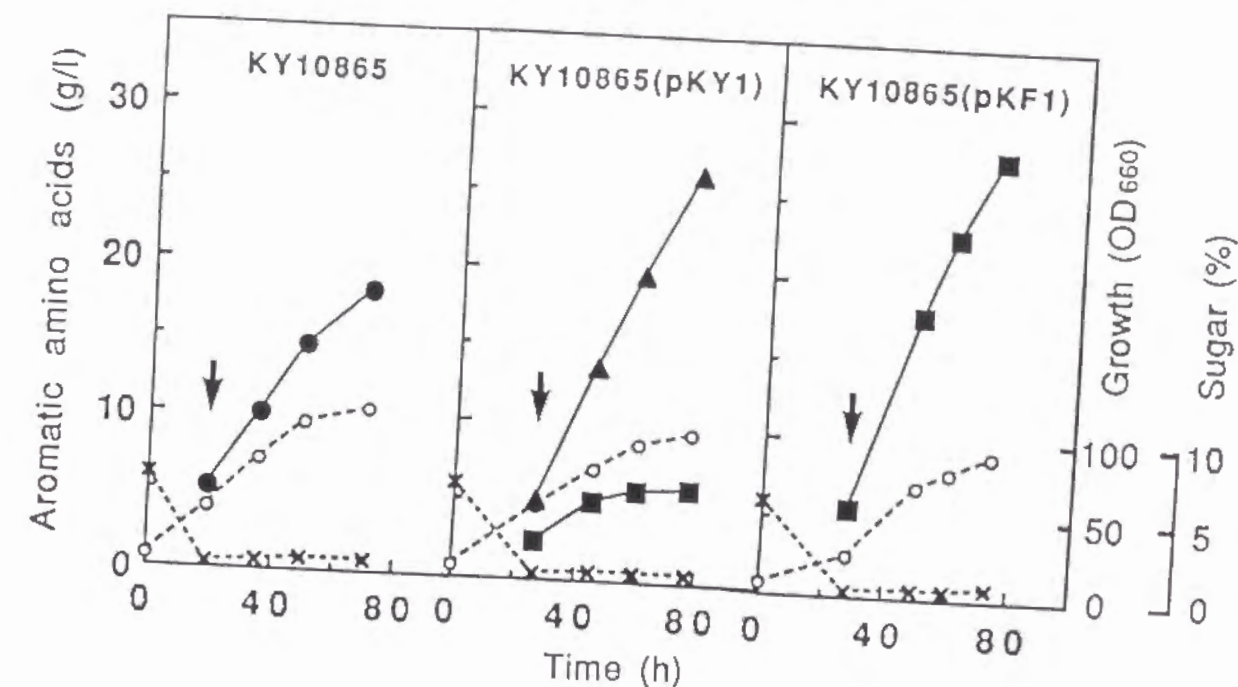


FIG. 3. Time course of aromatic amino acid production by strain KY10865 and its plasmid-carrying strains in jar fermentors. Symbols: ●, tryptophan; ▲, tyrosine; ■, phenylalanine; ○, growth (OD₆₆₀); x, sugar. Arrows indicate the points at which solution containing 44% sucrose began to be fed continuously.

TABLE 3. Production of aromatic amino acids by *C. glutamicum* KY10865 carrying recombinant plasmids

Plasmid	Amplified gene product(s)	Amino acids produced ^a (g/liter)			
		Tryptophan	Phenylalanine	Tyrosine	Total
None		6.8	0.0	0.0	6.8
pCA2	CM	1.0	0.8	0.9	2.7
pKY1	DS, CM	0.7	3.8	7.0	11.5
pKF1	DS, CM, PD	0.5	10.3	0.1	10.9

^a Production was carried out in test tubes as described under Materials and Methods.

DISCUSSION

The author succeeded in converting a tryptophan-producing strain, *C. glutamicum* KY10865, into a potent tyrosine or phenylalanine producer by introducing pKY1 or pKF1, respectively, which contains the genes for DS and the branch-point enzyme(s) desensitized to end-product inhibition. There seems to be no doubt that high activity of the branch-point enzyme(s) produced by the recombinant plasmids rechannelled the common intermediate chorismate into the branched pathway, thereby allowing marked production of phenylalanine or tyrosine in strain KY10865. Compared with the tryptophan yield given by the host strain, the total yield of the aromatic amino acids in these recombinant strains increased 1.5- to 1.8-fold (Table 3, Fig. 3). This

increase might be ascribed to the amplification of pKY1- or pKF1-specified DS, which would accelerate carbon flow into the aromatic pathway. On the contrary, when pCA2 containing only the CM gene was introduced into strain KY10865, no notable metabolic conversion was brought about despite largely decreased production of tryptophan. The yield of the aromatic amino acids in total in the pCA2 carrier decreased to below half that of tryptophan produced by the host strain. This decreased accumulation of the metabolites would arise, because phenylalanine and tyrosine formed endogenously by the pCA2-specified CM reaction inhibited the wild-type DS of the host strain to restrict the carbon flow towards the aromatic pathway.

Plasmids pCA2, pKY1, and pKF1 consist of the common replication origin derived from pCG1 (13) and have almost the same copy number in *C. glutamicum* KLS4 (data not shown). Nevertheless, CM activity of the pCA2 carrier was significantly lower than that of the strain carrying either of the other two plasmids (Table 1 and 2). In relation to this phenomenon, a mechanism for expression of CM and DS activities in *Brevibacterium flavum*, closely related to *C. glutamicum*, was studied by Shiio and Sugimoto (21, 23). They reported that two components responsible for CM and DS activities form a complex. They also confirmed that the CM component exerts its activity upon association with the DS component, but not alone. The CM activity of *C. glutamicum* may be expressed in the same manner since the enzyme activity in the crude extract of the pCA2 carrier increased after being mixed with the extract of the pCA1 carrier (Table 2). Therefore, a fewfold increase in CM activity in the pCA2 carrier (Table 2) would occur because the CM component overproduced by pCA2 might facilitate its association with the DS component produced by the host. Similarly, amplification of the DS component by pCA1 also could aid formation of the complex with the CM component produced by the host, thus increasing the CM activity (Table 2). In the case of pKY1 and pKF1, the high level of CM activity might surely arise from simultaneous overproduction of both components. Based on the mechanism for the expression of CM activity,

amplification of DS and CM could allow the enhancement not only of carbon flow into the aromatic pathway but also of rechanneling of chorismate towards tyrosine or phenylalanine, leading to successful metabolic conversion in strain KY10865 carrying pKY1 or pKF1.

Previously, Ozaki *et al.* cloned the *C. glutamicum* CM gene and assumed that since the cloned fragment had the ability to restore the CM and PD deficient mutant *C. glutamicum* KY9456 to prototrophy, the CM and PD genes might constitute an operon or be fused (18). However, the author showed by a separate experiment that the fragment could not complement another PD deficient mutant, KY9182 (data not shown). Presumably, this inconsistency would have arisen from the presence of a trace of PD activity in strain KY9456; it might operate to proceed with the PD reaction when the substrate prephenate is oversupplied through amplified CM activity. In this context, Follettie and Sinskey (4) reported the cloning of the *C. glutamicum* PD gene and showed that the gene was unable to complement CM-deficient mutants of *C. glutamicum*. From these findings and the present results that both genes could be isolated as separate fragments, the author concludes that the CM and PD reactions in *C. glutamicum* are catalyzed by separate enzymes, not by a bifunctional enzyme which is known to be carried by *E. coli* (10) and *Salmonella typhimurium* (3).

Like the aromatic amino acids, the aspartate family of amino acids, including lysine and threonine, are synthesized *via* a common pathway. Katsumata *et al.* previously reported that amplified activities of the threonine-biosynthetic enzymes in a high lysine-producing strain of *C. glutamicum* redirected metabolic flow from the intermediate aspartate β -semialdehyde and resulted in a shift to marked production of threonine (14). In addition to this example, the present study demonstrates that metabolic conversion by amplifying the branch-point enzyme(s) is useful in allowing production of another metabolite in high yield and with ease.

SUMMARY

The aromatic amino acids are synthesized via a common biosynthetic pathway. A tryptophan-producing mutant of Corynebacterium glutamicum was genetically engineered to produce tyrosine or phenylalanine in abundance. To achieve this, three biosynthetic genes encoding the first enzyme in the common pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DS), and the branch-point enzymes chorismate mutase and prephenate dehydratase were individually cloned from regulatory mutants of C. glutamicum which have either of the corresponding enzymes desensitized to end product inhibition. These cloned genes were assembled one after another onto a multicopy vector of C. glutamicum to yield two recombinant plasmids. One plasmid, designated pKY1, contains the DS and chorismate mutase genes, and the other, designated pKF1, contains all three biosynthetic genes. The enzymes specified by both plasmids were simultaneously overexpressed approximately sevenfold relative to the chromosomally encoded enzymes in a C. glutamicum strain. When transformed with pKY1 or pKF1, tryptophan-producing C. glutamicum KY10865, with the ability to produce 18 g of tryptophan per liter, was altered to produce a large amount of tyrosine (26 g/liter) or phenylalanine (28 g/liter), respectively, because the accelerated carbon flow through the common pathway was redirected to tyrosine or phenylalanine.

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INTRODUCTION

Corynebacterium glutamicum and its closely related bacteria are industrially important microorganisms, since they are widely used in fermentative production of various amino acids. A fermentative process for production of phenylalanine has also been developed by using mutants of the same organisms (15, 28, 29), as well as *Escherichia coli* (4, 16) and *Bacillus subtilis* (22). Phenylalanine-producing mutants of these coryneform bacteria have been constructed by repeated mutagenesis, resulting in release of the feedback regulations, as shown in Fig. 1. Besides the classical genetic approaches, the recent development of host-vector systems for these bacteria (19, 24, 27, 31) enabled further strain improvement with the use of recombinant DNA technology. Large increases in phenylalanine production have been achieved by amplifying the rate-limiting enzymes in existing phenylalanine producers (18, 25). Metabolic conversion from tryptophan production to phenylalanine production by amplifying the branch-point enzymes was also reported (17). In either case, the procedure used was amplification of endogenous enzymes from the organisms themselves.

However, the desired metabolic engineering should be attained also by the use of enzymes from heterogeneous organisms. In some microorganisms, examples of multifunctional enzymes are encountered in the aromatic pathway in which a single polypeptide product catalyzes two or more consecutive biosynthetic steps (30). These enzymes are of particular interest, since they probably catalyze sequential reactions more efficiently than separate enzymes as demonstrated in aromatic amino acid biosynthesis of *Neurospora crassa*, a phenomenon termed "catalytic facilitation" (8).

Based on the above point of view, the author took an interest in

utilizing the *E. coli pheA* gene as a potentially favorable genetic material for improving a phenylalanine-producing mutant of *C. glutamicum*, since it encodes the bifunctional key enzyme that catalyzes the two successive reactions in the pathway, i.e., chorismate mutase (CM) and prephenate dehydratase (PD) (Fig. 1). It is reasonable to expect that the bifunctional enzyme that converts chorismate, presumably via the enzyme-bound intermediate, prephenate, to phenylpyruvate would be advantageous over the separately existing enzymes of *C. glutamicum* (7, 17) in accelerating both the reactions.

Regulatory mechanisms of the *pheA* gene and kinetic properties of its specified CM-PD have been extensively investigated (9). Expression of this gene is regulated by an attenuation mechanism. Recent studies (10, 11) revealed that *pheR*, a *trans*-acting regulatory gene for *pheA*, encodes a tRNA^{Phe}

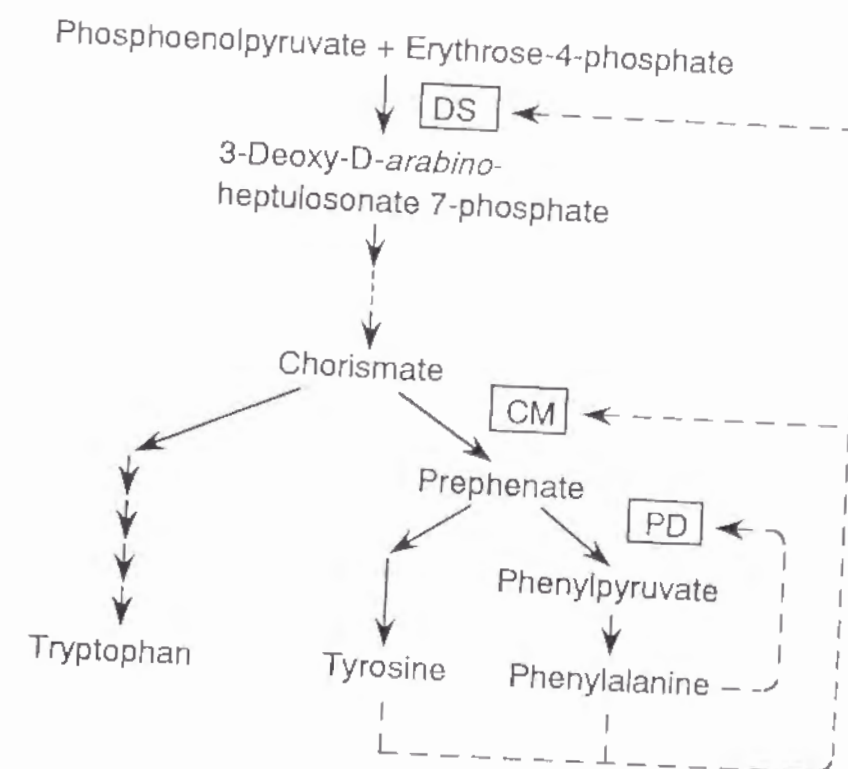


FIG. 1. Pathway and primary regulations of phenylalanine biosynthesis in *C. glutamicum*. Broken arrows indicate feedback inhibition.

molecule, not a repressor protein as previously suggested (13), and regulates *pheA* expression via attenuation control of transcription. Besides the transcriptional control, enzymatic studies (6, 12) have also shown that both the activities of CM-PD are inhibited by phenylalanine up to about 40% and 94% at maximum, respectively, by binding of phenylalanine to a single allosteric site of the bifunctional enzyme. This knowledge will be helpful in using the *pheA* gene for metabolic engineering.

In this chapter, the author has attempted to improve a phenylalanine producer of *C. glutamicum* by introducing the *pheA* gene, which was mutated to eliminate feedback inhibition. Its effect on phenylalanine production is described.

MATERIALS AND METHODS

Bacterial strains and plasmids.

E. coli strains MM294 (1) and its phenylalanine-auxotrophic derivative, KY14168 [*pheA*], were used as the DNA donor and the recipient, respectively, for cloning of the *pheA* gene. The phenylalanine-producing strain to be improved in this chapter was *C. glutamicum* KY10694 derived from strain 31-PAP-20-22 (15). Strain KLS4, a lysozyme-sensitive mutant of *C. glutamicum* wild-type strain KY9002 (ATCC 13032), was used as the host for examining the expression of the *pheA* gene and for selection of *p*-fluorophenylalanine (PFP)-resistant mutation on the gene. Strain KY10437, a shikimate dehydrogenase-deficient mutant of *C. glutamicum* another wild-type strain KY10025, was used as the host for examining the regulation of the *pheA* gene.

Plasmids pBR322 and pCE51 (24) were used as the vectors. The latter is a *C. glutamicum*-*E. coli* shuttle vector and carries the kanamycin resistance gene from the *E. coli* vector pGA22 (14), which is usable as a selectable marker in *C. glutamicum*.

Media.

Complete medium BY (19), minimal medium MM (25), and enriched minimal medium MMYE (19) were used for cultivation of *C. glutamicum*. For growth of *E. coli*, LB and M9 media (21) were used. Solid plates were made by the addition of Bacto-Agar (Difco) at the concentration of 1.6%. RCGA medium (19) was used for regeneration of *C. glutamicum* protoplasts. When required, antibiotics were added at the following final concentrations: ampicillin, 100 µg/ml for LB and M9 plates; tetracycline, 20 µg/ml for LB and M9 plates; kanamycin, 20 µg/ml for LB and M9 plates, 10 µg/ml for BY and MM plates or 200 µg/ml for RCGA plates. PPI medium used for production in test tubes contained (per liter): cane molasses, 100 g (as sugar); (NH₄)₂SO₄, 20 g; KH₂PO₄, 0.5 g; K₂HPO₄, 0.5 g; MgSO₄ · 7H₂O, 0.25 g; corn steep liquor, 2.5 g; L-tyrosine, 100 mg; and CaCO₃, 20 g (pH 7.2). PSI medium used for second-seed culture in jar fermentation contained (per liter): cane molasses, 50 g (as sugar); yeast extract, 10 g; polypeptone, 10 g; corn steep liquor, 5 g; urea, 3 g; NaCl, 2.5 g; D-biotin, 50 µg; thiamine-HCl, 5 mg; L-tyrosine, 200 mg; and CaCO₃, 10 g (pH 7.2). PP2 medium used for production in 2-liter jar fermentors contained (per liter): cane molasses, 50 g (as sugar); KH₂PO₄, 0.5 g; K₂HPO₄, 0.5 g; MgSO₄ · 7H₂O, 0.25 g; (NH₄)₂SO₄, 20 g; MnSO₄ · 4~6H₂O, 10 mg; FeSO₄ · 7H₂O, 10 mg; corn steep liquor, 2.5 g; D-biotin, 50 µg; thiamine-HCl, 5 mg; and L-tyrosine 200 mg (pH 7.2).

Cultivations for phenylalanine production.

(i) Test tubes. Cells grown on a BYG (containing 1.0% glucose in medium BY) plate were inoculated into 3 ml of BYG medium. After 24 h of cultivation at 30°C, 0.5 ml of the seed culture was transferred to a large test tube containing 5 ml of PPI medium. Cultivation was carried out aerobically at 30°C for 72 h.

(ii) Two-liter jar fermentors. A 2.4-ml amount of the first-seed culture in BYG medium was inoculated into 120 ml of PSI medium in a 1-liter flask.

After 24 h of cultivation at 30°C on a rotary shaker, the second-seed broth was transferred into a 2-liter jar fermentor containing 550 ml of PP2 medium. After the sugar initially added was consumed, solution containing 36% (as sugar) cane molasses was continuously fed until the total amount of sugar supplied in the medium reached 20%. The cultivation was agitated at 800 rpm and aerated at 1 liter/min at 30°C, and the pH was maintained at 6.3 with NH₄OH. Cultivations of recombinant strains were carried out in medium with kanamycin (100 µg/ml).

Preparation and manipulation of DNA.

Chromosomal DNA was extracted from *E. coli* MM294 by the method of Saito and Miura (26). Plasmid DNA was isolated by the alkaline lysis method (21) and, if necessary, purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (19). DNA digestion and ligation were carried out as described by Maniatis *et al.* (21). Restriction enzymes and T4 DNA ligase were obtained from Takara Shuzo Co., Ltd., Kyoto, Japan.

Transformations.

Transformation procedures used were the protoplast method (19) for *C. glutamicum* and the CaCl₂ method (21) for *E. coli*.

Mutagenesis.

Induction and isolation of the mutant plasmid from pEAll were performed in the following *in vivo* mutagenesis. Strain KLS4 carrying pEAl were grown in 3 ml of BY medium containing kanamycin, harvested in exponential phase, and washed in 3 ml of 50 mM Tris-maleate buffer (pH 6.0). Then, the cells were resuspended in 3 ml of the same buffer containing of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (400 µg/ml), and incubated for 30 min at 30°C. The mutagenized cells were harvested, washed twice in 3 ml of the above buffer, resuspended in 40 ml of BY medium containing kanamycin, and cultivated

overnight at 30°C. Plasmid DNA was isolated from the cells and was used to transform fresh cells of strain KLS4. After selection for resistance to PFP, mutant plasmids were isolated from the resistant clones.

Enzyme assays.

Crude cell extracts were prepared by sonic disruption of washed cells in 40 mM potassium phosphate buffer (pH 7.0) as described previously (17). Cellular debris was removed by centrifugation at 10,000 rpm for 20 min at 4°C to obtain the supernatants. The crude extracts were dialyzed against the above potassium phosphate buffer for 6 h and used for the enzyme assay. Protein was determined by the method of Bradford (3) with the Bio-Rad kit. CM and PD activities in crude cell extracts were measured by the method of Cotton and Gibson (5) except that the assays were carried out at 30°C.

Analysis.

Cell growth, sugar, and phenylalanine were analyzed as described previously (17).

RESULTS

Cloning of the *E. coli* pheA gene.

It was shown that the *E. coli* *pheA* gene is located on a 2.1 kb *EcoRI*-*Bam*HI DNA fragment (32). According to this knowledge, cloning of the *pheA* gene was performed as depicted in Fig. 2. Chromosomal DNA from *E. coli* MM294 and vector pBR322 were digested with *EcoRI* and *Bam*HI, and ligated. The genomic library was used to transform *E. coli* KY14168, a phenylalanine auxotroph with a lesion in the *pheA* gene. The cell suspension was plated onto an M9 plate containing ampicillin (100 µg/ml). Eight colonies with a phenylalanine-independent, ampicillin-resistant, and tetracycline-sensitive phenotype were selected. All plasmids purified from these clones were shown

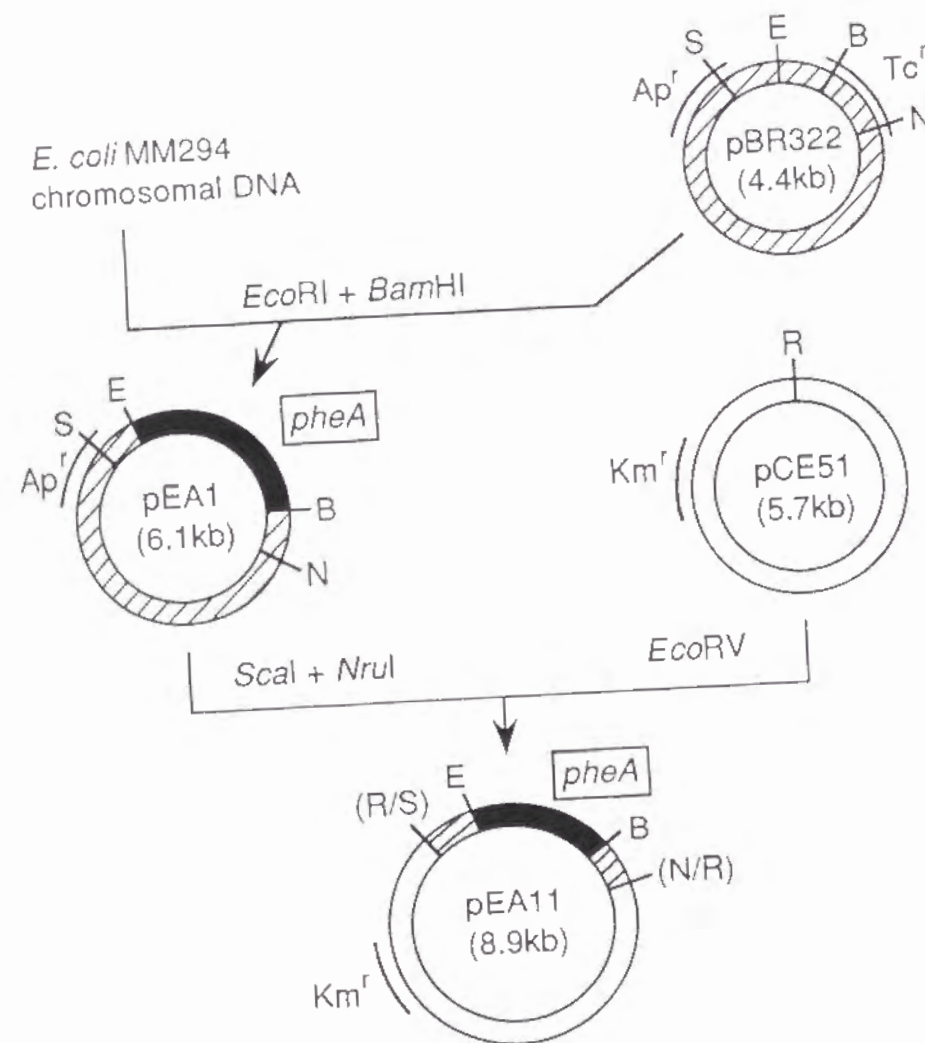


FIG. 2. Construction of plasmid pEA11. Symbols: solid bars, *E. coli* MM294 chromosomal DNA containing the *pheA* gene; S, *ScaI*; E, *EcoRI*; B, *BamHI*; N, *NruI*; R, *EcoRV*; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance.

to have a common 2.1 kb *EcoRI*-*BamHI* fragment by restriction enzyme cleavage analysis. Retransformation of strain KY14168 with pEA1, one of the resulting recombinant plasmids, resulted in the concomitant ampicillin-resistant and phenylalanine-independent phenotype, indicating that the cloned segment had the *pheA* gene.

Subsequently, its *ScaI* and *NruI*-generated 3.2 kb fragment containing the *pheA* gene was ligated with the *EcoRV* site of pCE51, a *E. coli*-*C. glutamicum* shuttle vector, since pEA1 was unable to replicate in *C. glutamicum*. Transformation of strain KY14168 with the ligation mixture and selection for kanamycin resistance and phenylalanine independence yielded transformants with the plasmids designated pEA11 and pEA12, differing only in the orientation of the 3.2 kb insert with respect to the vector.

Expression and regulation of the *pheA* gene in *C. glutamicum*.

To examine whether the *E. coli* *pheA* genes on pEA11 and pEA12 were expressed in Gram-positive *C. glutamicum*, both the plasmids were introduced into a prototrophic strain, *C. glutamicum* KLS4, by protoplast transformation and the relevant enzyme activities of each transformant were measured (Table 1). The presence of each plasmid in the host strain elevated the level of CM and PD activities 10- to 14-fold, indicating the functional expression of the *pheA* gene in *C. glutamicum*.

The *pheA* gene is known to be subject to an attenuation control in *E. coli*. The author, therefore, examined how the expression of the gene was affected by phenylalanine in *C. glutamicum*. For this purpose, pEA11 was introduced into a shikimate dehydrogenase-deficient mutant, *C. glutamicum* KY10437, which requires the aromatic amino acids and *p*-aminobenzoate for its growth. When strain KY10437 carrying pEA11 was grown under limited condition of phenylalanine, about threefold increase was observed in both the activities relative to cells grown under excess condition of the amino acid (Table 2).

TABLE 1. Expression of the *pheA* gene in *C. glutamicum* KLS4

Strain (plasmid)	Sp. act. ^a	
	CM	PD
KLS4	6.5 (1.0) ^b	3.0 (1.0) ^b
KLS4 (pEA11)	85 (13.1)	39 (13.0)
KLS4 (pEA12)	78 (12.0)	32 (10.7)
KLS4 (pEA22)	82 (12.6)	38 (12.7)

^a Expressed as nanomoles of product per milligram of protein per minute.^b Relative to the activity in KLS4 with no plasmid.TABLE 2. Effect of phenylalanine on *pheA* expression in *C. glutamicum* KY10437

Strain (plasmid)	Phenylalanine added (μg/ml) ^a	Sp. act. ^b	
		CM	PD
KY10437	10	<10	<10
KY10437 (pEA11)	10	206 (1.0) ^c	64 (1.0) ^c
	200	66 (0.32)	23 (0.36)
KY10437 (pEA22)	10	226 (1.0)	84 (1.0)
	200	78 (0.35)	30 (0.36)

^a Cells were grown in MM medium supplemented with tryptophan (50 μg/ml), tyrosine (50 μg/ml), p-aminobenzoate (5 μg/ml) and phenylalanine (10 or 200 μg/ml).^b Expressed as nanomoles of product per milligram of protein per minute.^c Relative to the activity in cells grown under phenylalanine limitation.Desensitization of pEA11-specified CM-PD to inhibition by phenylalanine.

The wild-type CM-PD specified by pEA11, even if overproduced, might not serve my purpose due to its sensitivity to inhibition by phenylalanine. Thus, the author attempted to mutate the *pheA* gene on pEA11 to eliminate the feedback inhibition by *in vivo* mutagenesis. KLS4 cells carrying pEA11 were mutagenized and cultured overnight. Plasmid DNA isolated from the mutagenized cells was used to transform the protoplasts of the same strain. Transformants were selected on RCGA plates containing kanamycin (200 μg/ml). Regenerated kanamycin-resistant colonies were transferred by replica-plating to MM plates containing kanamycin (10 μg/ml) and PFP (1.6 mg/ml). Thirty-two PFP-resistant clones were obtained among about 10⁵ kanamycin-resistant transformants. Retransformation with the plasmid, named pEA22, purified from one of these clones resulted in co-transfer of PFP resistance and the vector marker, confirming the plasmid-linked mutation. Enzymatic analyses indicated that the mutant plasmid retained almost the same gene dosage effects for CM and PD

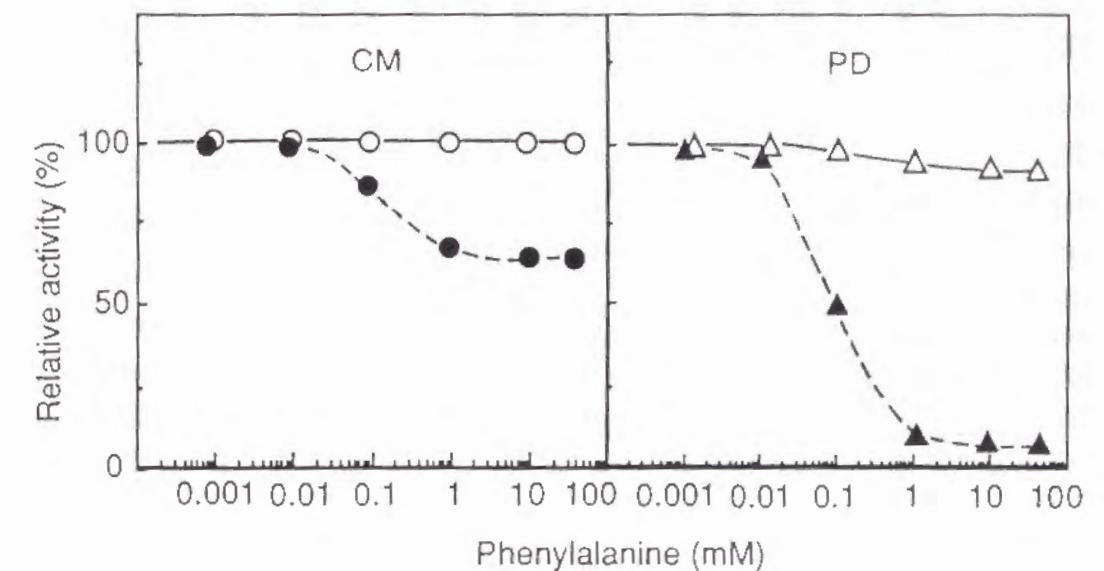


FIG. 3. Effect of phenylalanine on CM and PD activities. Symbols: ●, CM activity of KLS4 carrying pEA11; ○, CM activity of KLS4 carrying pEA22; ▲, PD activity of KLS4 carrying pEA11; △, PD activity of KLS4 carrying pEA22.

activities in strain KLS4 as those of the parental plasmid (Table 1), and that either activity was indeed desensitized to inhibition by phenylalanine (Fig. 3). However, as shown in Table 2, the expression of the mutant *pheA* gene on pEA22 was regulated in the same manner as that of the wild-type one on pEA11 in strain KY10437.

Phenylalanine production by strain KY10694 carrying pEA22.

The effects of pEA11 and pEA22 on phenylalanine production were investigated in a phenylalanine-producing strain, *C. glutamicum* KY10694 (Table 3). This strain has been constructed through repeated mutagenesis resulting in resistance to several phenylalanine analogs. The 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase and CM of this strain are highly desensitized to synergistic inhibition by phenylalanine and tyrosine. Another key enzyme, PD, is, although partially, desensitized to inhibition by phenylalanine. This strain has the ability to produce phenylalanine at a titer of 10 g/l in PP1 medium containing 10% (as sugar) cane molasses, although a small amount of tyrosine is concomitantly accumulated. Introduction of pEA11 into KY10694 resulted in only a little increase in phenylalanine production. The amount of tyrosine remained unchanged in the recombinant strain. By contrast, when pEA22 was introduced into KY10694, the transformed strain no longer accumulated tyrosine and produced 37% more phenylalanine than the control host strain.

Strain KY10694 and its pEA22-carrying strain were further tested for phenylalanine production in jar fermentors. Fig. 4 shows the time courses of fed batch cultures in cane molasses medium to which sugar was added initially at 6% and subsequently fed to 20% total. While KY10694 exhibited a phenylalanine titer of 17 g/l, the pEA22 carrier produced 23 g/l of phenylalanine in a 35% increased yield. Enzyme assays showed that both the activities of CM and PD in this recombinant strain were highly maintained throughout the cultivation (Fig. 4).

TABLE 3. Production of phenylalanine by *C. glutamicum* KY10694 carrying recombinant plasmids

Strain (plasmid)	Accumulation ^a (g/liter)	
	Phenylalanine	Tyrosine
KY10694	10.0	0.3
KY10694 (pEA11)	10.8	0.3
KY10694 (pEA22)	13.7	0.0

^a Production was carried out in test tubes as described under Materials and Methods.

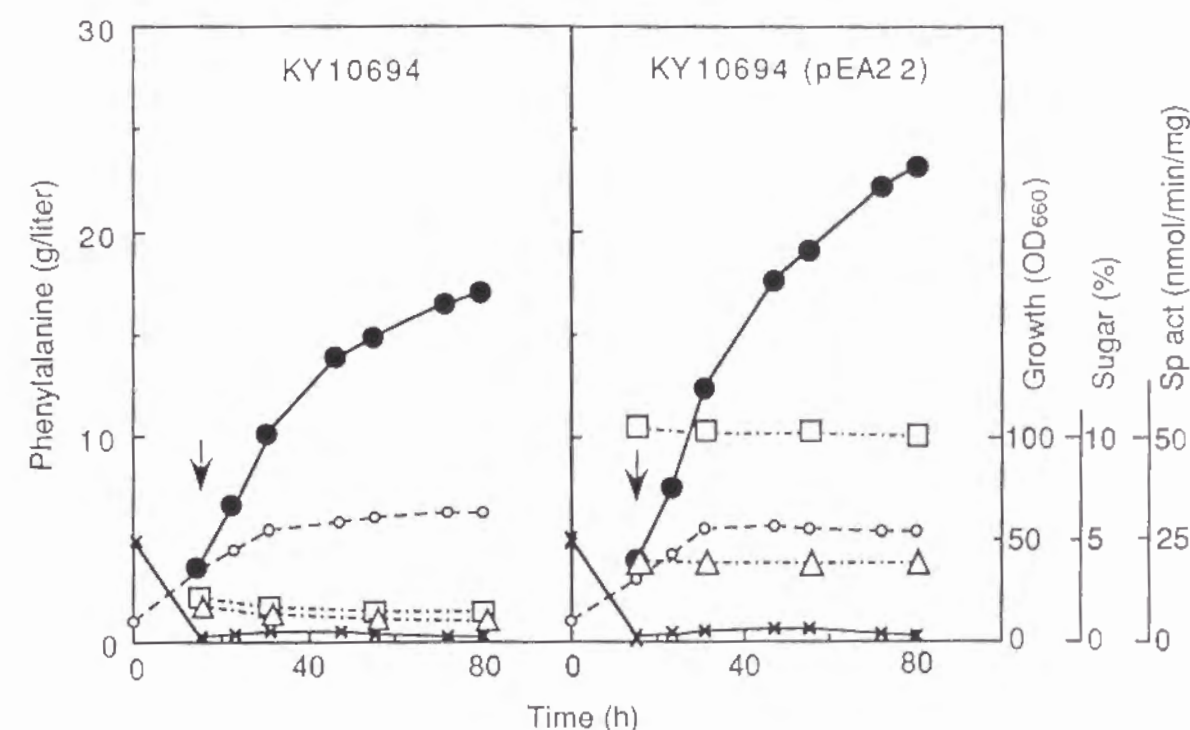


FIG. 4. Time course of phenylalanine production by strain KY10694 and its pEA22-carrying strain in jar fermentors. Symbols: ●, phenylalanine; ○, growth; ×, sugar; □, CM activity; △, PD activity. Arrows indicate the points at which solution containing 36% (as sugar) cane molasses began to be fed continuously.

DISCUSSION

The bifunctional enzyme CM-PD, which is encoded by the pheA gene of E. coli, could be successfully utilized to improve a phenylalanine-producing strain, C. glutamicum KY10694. It seems apparent that the phenylalanine-insensitive CM-PD specified by pEA22 facilitated the two successive reactions converting chorismate to phenylpyruvate, thereby allowing increased production of phenylalanine without any tyrosine in strain KY10694. The bifunctional enzyme would appear to serve to channel prephenate, the product of the first reaction, toward phenylalanine without being free to diffuse, thereby increasing the catalytic efficiency. On the other hand, pEA11, which specifies a wild-type enzyme, made a little contribution to the increase in phenylalanine production in the same host, whereas the level of tyrosine production remained unchanged. Since the CM activity of the wild-type enzyme is maximally inhibited by only 40% (Fig. 3), the small increase might be ascribed to the remaining CM activity, which would accelerate carbon flow from chorismate to prephenate. These results showed that both the CM and PD activities of the bifunctional enzyme could act on increased production of phenylalanine in strain KY10694 carrying pEA22.

In C. glutamicum KY10437, the level of pheA expression was varied about threefold under the excess and limited conditions of phenylalanine (Table 2). Since at present there is no experimental evidence to suggest an involvement of repression in the regulation of pheA, the variation seems to be attributable to the attenuation mechanism. It involves controlled transcription termination at a site, attenuator, located within the leader region of the gene in response to the level of charged tRNA^{Phe} which reflect the concentration of intracellular phenylalanine. Although it needs transcriptional analysis for verification, the finding presented here is of interest in indicating the possibility that the transcriptional and translational apparatus including the intracellular level of cognate tRNA is

basically similar between Gram-negative E. coli and Gram-positive C. glutamicum.

So far, a few kinds of pheA mutations that reduce the sensitivity of CM-PD to feedback inhibition have been described (2, 23). Nelms et al. (23) showed that the enzyme that had mutated within the codon 304 to 310 region of the peptide resulted in almost total resistance to the feedback inhibition at very high concentrations of phenylalanine. From the clustered nature of the mutations, they suggested alteration of allosteric binding site of phenylalanine. Since the CM-PD specified by pEA22 that was obtained by a single round of mutagenesis exhibited the similar feedback inhibition resistance profiles in both the activities (Fig. 3), the mutation on pEA22 seems to have occurred in the same region of the gene.

Katsumata et al. previously reported that a threonine-producing strain of C. glutamicum was significantly improved by introducing the E. coli thr operon which specified the bifunctional enzyme aspartokinase I-homoserine dehydrogenase I, highly desensitized to inhibition by threonine (20). Besides this example, the present study demonstrates that the use of intergeneric genes, especially well characterized E. coli genes, can be beneficial for the improvement of amino acid-producing C. glutamicum strains.

SUMMARY

The bifunctional enzyme chorismate mutase (CM)-prephenate dehydratase (PD), which is encoded by the pheA gene of Escherichia coli, catalyzes the two consecutive key steps in phenylalanine biosynthesis. To utilize the enzyme for metabolic engineering of phenylalanine-producing Corynebacterium glutamicum KY10694, the intact gene was cloned on a multicopy vector to yield pEA11. C. glutamicum cells transformed with pEA11 exhibited a more than tenfold increase in CM and PD activities relative to the host cells. Moreover, the level of pheA expression was further elevated a fewfold when

cells were starved of phenylalanine, suggesting that the attenuation regulation of pheA expression functions in heterogeneous C. glutamicum. Plasmid pEA11 encoding the wild-type enzyme was mutated to yield pEA22, which specified CM-PD exhibiting almost complete resistance to end product inhibition. When pEA22 was introduced into KY10694, both the activities of CM and PD were highly maintained throughout the cultivation, thus leading to a 35% increased production (23 g/liter) of phenylalanine.

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INTRODUCTION

L-Tryptophan is an essential amino acid required by man and monogastric animals, such as swine and chicken. Although this amino acid is now used for medicinal purposes, it potentially lends itself to some other uses, including the use as a feed supplement. However, these applications are still hampered due to high production costs of L-tryptophan. Therefore, the manufacturing process of L-tryptophan is strongly desired to be improved to the point at which its market price is low enough to enable the use of L-tryptophan for that purpose. At present, because chemical processes always possess the drawback of producing a mixture of DL-tryptophan, commercial production of L-tryptophan depends on two different microbial processes (14); production from cheap carbon and nitrogen sources by fermentation and conversion from chemically synthesized precursors by enzymatic reactions. In fermentation, process improvement involves strains capable of producing tryptophan in higher yields. This objective is most likely to be achieved with the limited kind of bacterial species, such as *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium glutamicum* and *Brevibacterium flavum*, since intensive studies of tryptophan production have so far been made with them (1, 7, 15, 23, 25, 30). In my laboratories, strain development in *C. glutamicum* has consistently been investigated in view of the fact that this organism is widely used for production of many other amino acids on an industrial scale (14).

Biosynthesis of the aromatic amino acids in all organisms proceeds via a common aromatic pathway to chorismate, from which the pathways to tryptophan, tyrosine and phenylalanine branch (31). In *C. glutamicum* and closely related isolate *B. flavum*, carbon flow over the whole pathway to tryptophan is known to be controlled mainly through the feedback inhibition and repression as

shown in Fig. 1 (5, 6, 27, 29). The first enzyme in the common pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DS), is synergistically inhibited by tyrosine and phenylalanine. Anthranilate synthase (ANS) and anthranilate phosphoribosyltransferase (PRT), which catalyze the first two reactions in the tryptophan pathway, are feedback-inhibited by the end product. The formation of all enzymes in this branch is repressed by tryptophan as well.

In addition to the regulatory systems, early studies also established that each regulation can be bypassed in mutants auxotrophic for the aromatic amino acid(s) (7) or resistant to its structural analog(s) (24). On the basis of these knowledges, there have been many attempts to assemble the beneficial mutations in one strain background, thereby constructing tryptophan producers of *C. glutamicum* or *B. flavum*. The strain constructions were carried out exclusively by repeated mutagenesis and strain selection for lack of useful genetic exchange systems in these bacteria. While these efforts have resulted in the creation of improved tryptophan producers, the efficiencies remain low compared with those attained for other amino acids derived by mutagenic procedures from the same organisms (14). The cause of this productivity

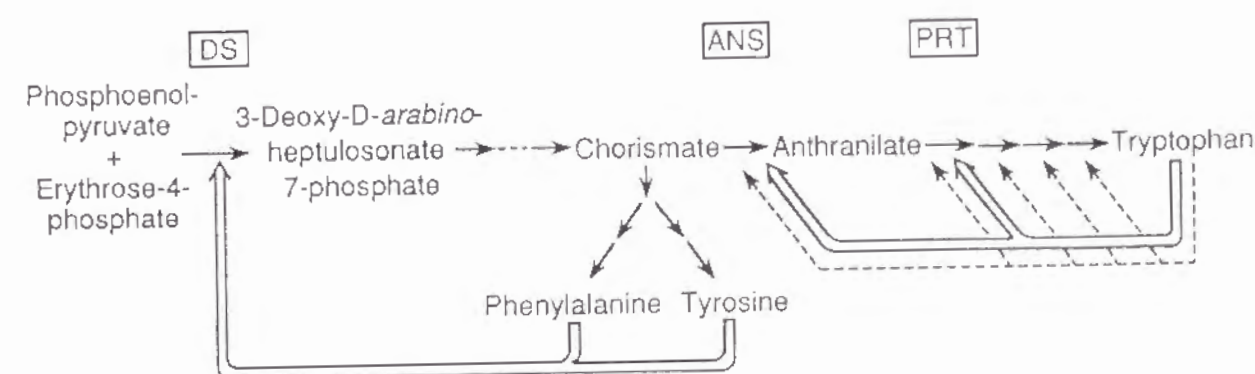


FIG. 1. Pathway and primary regulations of tryptophan biosynthesis in *C. glutamicum*. The open and broken arrows indicate feedback inhibition and repression, respectively.

limitation is reasonably likely to be attributed either to the incomplete deregulation in one or more feedback control systems or to the shortage of a certain enzyme involved in tryptophan biosynthesis. In either case, genetic alterations required for yield improvement in tryptophan production should be facilitated by recombinant DNA technology rather than classical mutagenesis. The availability of gene cloning systems for *C. glutamicum* and its relatives (13, 19, 22, 34) enables us to conduct the investigation as has already been applied to metabolic engineering in other amino acid producers (10, 11, 20).

The most effective approach to further strain improvement is to sequentially modify the biosynthetic pathway, discerning a rate-determining reaction. Using this strategy, the author evidenced that the biosynthetic pathway of an existing tryptophan-producing strain of *C. glutamicum* could be improved to increase the metabolic flux toward tryptophan ultimately by amplification of DS, ANS, PRT and other tryptophan-biosynthetic enzymes, among which the former three key enzymes were highly desensitized to the feedback effect. The present chapter describes the construction of a plasmid for the pathway engineering and the hyperproduction of tryptophan by the engineered strain with the plasmid. Both yield and titer of tryptophan achieved by the recombinant strain are the highest that have ever been obtained by fermentation with microorganisms.

MATERIALS AND METHODS

Bacterial strains and plasmids.

A tryptophan-producing strain used in this chapter is *C. glutamicum* KY10894 (Kino and Katsumata, unpublished) derived from strain Px-115-97 (7) through several rounds of mutagenesis. *C. glutamicum* ATCC 13032, a wild-type strain from which strain KY10894 is originated, was used as the control for enzymatic analyses. Strains TA101 and TA133 are tryptophan auxotrophs deficient in ANS and TS- β , respectively, of *C. glutamicum* wild-type strain

ATCC 31833, and were used as the recipient strains for cloning of the tryptophan-biosynthetic genes and for complementation tests. Strain KLS4, a lysozyme-sensitive mutant of strain ATCC 13032, exhibits high sensitivity to 5FT and was used as the host for selection of analog-resistant mutation on the plasmid containing the cloned genes. *E. coli* strains JA200 [*trpE63*, *thr-1*, *leuB6*], ATCC 23720 [*trpD*], CGSC 5889 [*trpC60*, *pyrF287*, *hisG1*, *lacZ53*, *rpsL8*], CGSC 6688 [*trpB83::Tn10*, *IN(rrnD-rrnE)1*] and CGSC 4456 [*trpA9761*, *sucA8*, *trpR47*, *gal-25*, *ic1R7*] were used as the host strains for complementation tests. These *E. coli* strains were obtained from the American Type Culture Collection or the *E. coli* Genetic Stock Center in U.S.A.

Plasmid pCA1 (20) contains the desensitized DS gene of phenylalanine-producing mutant, *C. glutamicum* KY10694, as a 6.7 kb *Sall* fragment in the corresponding site of pCG115. Plasmids pCG115 and pCG116, derivatives of the *C. glutamicum* vector plasmid pCG11 (12), have the streptomycin-spectinomycin resistance genes and the polylinkers present in M13mp18 RF DNA (18). Plasmid pCE53 (19) is a *C. glutamicum*-*E. coli* shuttle vector and carries the kanamycin, chloramphenicol and tetracycline resistance genes from the *E. coli* vector pGA22 (4), which are usable as selectable markers in *C. glutamicum*.

Media.

Complete medium BY (13), minimal medium MM (20), and enriched minimal medium MMYE (13) were used for cultivation of *C. glutamicum*. Solid plates were made by the addition of Bacto-Agar (Difco) to 1.6%. RCGA medium (13) was used for regeneration of *C. glutamicum* protoplasts. When required, supplements or antibiotics were added as described previously (10). TP1 medium (10) was used for production in test tubes. TS1 and TP2 media (10) were used for second-seed culture and production, respectively, in jar fermentation. For growth of *E. coli*, LB and M9 media (16) were used.

Cultivations for tryptophan production.

(i) Test tubes. A 0.5-ml amount of the seed culture grown on a BYG medium (containing 1.0% glucose in medium BY) containing phenylalanine and tyrosine (200 µg/ml each) was transferred to a large test tube containing 5 ml of TP1 medium and cultivated aerobically at 30°C for 72 h. All cultivations of recombinant strains were performed in the presence of spectinomycin (100 µg/ml).

(ii) Two-liter jar fermentors. A 120-ml amount of the second-seed culture prepared as described previously (10) was transferred into a 2-liter jar fermentor containing 550 ml of TP2 medium. After the sugar initially added was consumed, solution containing 44% sucrose was continuously fed until the total amount of sugar supplied in the medium reached 22%. The culture was agitated at 800 rpm and aerated at 1 liter/min at 30°C, and the pH was maintained at 6.1 with NH₄OH. Cultivations of recombinant strains were carried out in medium with spectinomycin (100 µg/ml).

Preparation and manipulation of DNA.

Chromosomal DNA was extracted from protoplasts of *C. glutamicum* KY10894 by the method of Saito and Miura (21). The protoplasts were prepared as described previously (13). Plasmid DNA was isolated by the alkaline lysis method (16) and, if necessary, purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (13). DNA digestion and ligation were carried out as described by Maniatis *et al* (16). Restriction enzymes and T4 DNA ligase were obtained from Takara Shuzo Co., Ltd., Kyoto, Japan. DNA fragment was purified from agarose gel by the glass-binding method (32).

Transformations.

Transformation procedures used were the protoplast method (13) for *C. glutamicum* and the CaCl₂ method (16) for *E. coli*.

Mutagenesis.

Induction and isolation of the mutant plasmid from pKW1 were performed as follows. Strain KLS4 carrying pKW1 were grown in 3 ml of BY medium containing spectinomycin, harvested in exponential phase, and washed in 3 ml of 50 mM Tris-maleate buffer (pH 6.0). Then, the cells were resuspended in 3 ml of the same buffer containing of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (400 µg/ml), and incubated for 30 min at 30°C. The mutagenized cells were harvested, washed twice in 3 ml of the above buffer, resuspended in 40 ml of BY medium containing spectinomycin, and cultivated overnight at 30°C. After retransformation of KLS4 protoplasts with plasmid DNA purified from the cells and selection for resistance to 5FT, mutant plasmids were isolated from the resistant clones.

Enzyme assays.

Crude cell extracts were prepared by sonic disruption of cells grown in MMYE medium containing phenylalanine and tyrosine (100 µg/ml each) as described previously (10). Only cells for determination of PRT activity were sonicated in the presence of 40% glycerol according to the method of Sugimoto and Shiio (29). Protein was determined by the method of Bradford (2) with the Bio-Rad kit. Enzyme activities in crude cell extracts were measured by the method of Sprinavasan and Sprinson (26) for the DS assay and by the method of Sugimoto and Shiio (27) for the ANS and PRT assay. All assays were carried out at 30°C.

Analysis.

Cell growth and sugar concentration were measured as described previously (10). Tryptophan and anthranilate were analyzed by HPLC (Shimazu Co., Ltd.) using a CLC-ODS column (6.0 X 150 mm, Shimazu Co., Ltd.) and a mobile phase of acetonitrile and phosphate buffer after derivatization with *o*-phthalaldehyde as described by Hill *et al* (9). In the case of the fermentation broth containing

the crystals of tryptophan, it was diluted with water to dissolve the crystals and used for analysis. Chorismate was also quantified by HPLC (UV detection at 275 nm) using the same column and mobile phase.

RESULTS AND DISCUSSION

Effect of amplified DS on tryptophan production.

C. glutamicum KY10894 is a tryptophan-producing strain that is derived through multiple rounds of mutagenesis from strain KY9456 (7), a phenylalanine and tyrosine double auxotroph with a lesion in the chorismate mutase gene. The producer is derepressed with respect to tryptophan repression and so overexpresses tryptophan-biosynthetic enzymes by a fewfold relative to the wild-type strain, *C. glutamicum* ATCC 13032 (Table 1). As compared with the wild-type enzymes, the first key enzyme ANS of the pathway is slightly desensitized to inhibition by tryptophan, whereas the second one PRT remains unchanged in sensitivity to the end product inhibition (Table 1). However, since the tryptophan sensitivity of PRT is still much lower than that of ANS, PRT is considered free substantially from the feedback inhibition within the cells. When strain KY10894 is cultivated under conditions of phenylalanine and tyrosine limitations, the synergistic inhibition of DS is bypassed and thus carbon is channelled into the common pathway to chorismate, thence to tryptophan through the genetically altered terminal pathway. This strain has the ability to produce tryptophan at a titer of approximately 8 g per liter in test-tube cultivation with TPI medium that contains 6% glucose (Table 1).

In improving strain KY10894, the author first attempted to identify a rate-limiting step by analyzing what intermediate along the overall pathway to tryptophan was excreted in the fermentation broth. However, no intermediates were detectable. One possible explanation for this result is that the metabolite flux toward tryptophan would have been restricted at the earliest step in the common pathway, namely, the DS reaction. If that is true, it is

expected that amplification of DS, which initiates the pathway by condensing phosphoenolpyruvate and erythrose-4-phosphate to yield 3-deoxy-D-arabino-heptulosonate 7-phosphate, could increase the net carbon flow down the pathway, thereby raising tryptophan production.

To explore this possibility, plasmid pCA1 that contains the gene for the feedback-insensitive DS, which had been cloned from the regulatory mutant *C. glutamicum* KY10694 (10), was introduced into strain KY10894 by protoplast transformation and the resultant transformant was examined in its DS activity and tryptophan productivity (Table 1). Enzyme assays with the crude cell extracts confirmed that the transformed cells overproduced DS by about eightfold relative to the control host cells. The transformed strain indeed exhibited an increased productivity of tryptophan but the production level was far lower than we expected. Instead chorismate, the terminal metabolite in

TABLE 1. Levels and feedback-sensitivities of the key enzymes and metabolite accumulation in *C. glutamicum* KY10894 and its recombinant derivatives.

Strain (plasmid)	Characteristics					Metabolite accumulation ^c		
	DS	ANS		PRT				
	Sp. act. ^a	Sp. act.	50% inhibition ^b (mM)	Sp. act.	50% inhibition (mM)	Tryptophan (g/l)	Chorismate (g/l)	Anthranilate (g/l)
ATCC13032	16.0	0.83	0.003	0.70	0.19	0.0	0.0	0.0
KY10894	18.2	2.7	0.008	1.3	0.19	8.1	0.0	0.0
KY10894 (pCA1)	141.9	2.4	0.008	1.3	0.19	9.0	1.2	0.0
KY10894 (pKW1)	150.2	29.5	0.008	12.9	0.19	6.1	0.4	2.6
KY10894 (pKW99)	146.8	26.1	4.0	11.7	4.8	11.4	0.1	0.0

^a Expressed as nanomoles of product per milligram of protein per minute.

^b Concentrations of tryptophan giving 50% inhibition.

^c Production was carried out in test tubes as described under Materials and Methods.

the common pathway, proved to be accumulated as well. This change in metabolite accumulation implies that the metabolic flux through the pathway to tryptophan in the recombinant strain was arrested at the ANS reaction, presumably because it was too weak to fully convert oversupplied chorismate to anthranilate. Despite the unfavorable result, the amplified DS activity appears to have more effect in increasing the carbon flow down the common pathway than estimated since chorismate appreciably decomposes itself spontaneously in an aqueous solution (3).

Cloning of the tryptophan-biosynthetic gene cluster.

The author used an approach to accelerate the above rate-limiting step further by amplifying ANS in the DS-overexpressing recombinant tryptophan producer. For this purpose, cloning of the ANS gene was performed as follows. Chromosomal DNA from strain KY10894 and multicopy vector pCE53, a *C. glutamicum*-*E. coli* shuttle plasmid, were digested with *Bam*HI and ligated. The genomic library was used to transform the protoplasts of an ANS-deficient tryptophan auxotroph, *C. glutamicum* TA101, and the protoplasts were plated out on RCGA plates with kanamycin (200 µg/ml). This semi-synthetic medium does not contain enough tryptophan to permit growth of the recipient cells so that only kanamycin-resistant and tryptophan-independent clones can be selected on the selective medium. All five colonies formed were ascertained to be prototrophic by subsequent replica-plating onto the defined medium. Restriction cleavage analysis of the plasmids carried by these transformants indicated that they all carried the same 11.0 kb *Bam*HI insert (Fig.2). One of the recombinant plasmids, designated pCA4 (Fig. 3), was chosen for subsequent analyses.

Since the tryptophan-biosynthetic genes are well known to be clustered on the genome in a variety of microorganisms (8, 17, 33), the author examined whether other *C. glutamicum* *trp* genes together with the ANS gene existed on pCA4 by complementation analyses. The plasmid was capable of complementing

another tryptophan-requiring *C. glutamicum* mutant, TA133, that carries a lesion in the gene for the β subunit of tryptophan synthase (TS- β). Furthermore, when pCA4 was introduced into *E. coli* *trp* mutants, complementation was also observed with all of *trpE*, *trpD*, *trpC*, *trpB* and *trpA* mutants, which are deficient in ANS, PRT, phosphoribosylanthranilate isomerase-indoleglycerol phosphate synthase, TS- β and TS- α , respectively (Fig. 2). These results indicated that the cloned 11.0 kb *Bam*HI fragment contained the *C. glutamicum* genes for not only ANS but the other enzymes responsible for tryptophan biosynthesis. Based on the restriction cleavage map of this fragment, the author further recloned its internal *Sal*I-, *Bgl*II- and *Eco*RI-fragments into pCE53 and examined for the ability of the resultant plasmids to complement *E. coli* *trp* mutants. The complementation results shown

Plasmid	Insert	Growth				
		JA200 (<i>trpE</i>)	ATCC 23720 (<i>trpD</i>)	CGSC 5889 (<i>trpC</i>)	CGSC 6688 (<i>trpB</i>)	CGSC 4456 (<i>trpA</i>)
pCA4		+	+	+	+	+
pCA41		+	-	-	-	-
pCA42		+	+	-	-	-
pCA43		+	+	+	-	-
pCA44		-	-	-	+	+

1kb

trpE D C (B A)

FIG. 2. Restriction cleavage maps of the cloned DNA fragments and complementation of *E. coli* *trp* mutants by its subclones. The results of complementation tests were shown as + (growth) and - (no growth). The most probable coding regions of the *trp* genes are shown at the bottom. (B A) indicates that the order of *trpB* and *trpA* is not determined. Abbreviations: B, *Bam*HI; E, *Eco*RI; S, *Sal*I; Bg, *Bgl*II.

in Fig. 2 ensured that the tryptophan-biosynthetic gene cluster of *C. glutamicum* was localized within a 7.0 kb *EcoRI*-*Bgl*III region.

Construction of pKW1 and its effect on tryptophan production.

The limitation of ANS reaction, encountered in the pCA1-carrying tryptophan producer that overexpresses DS, seems to be removed by additional amplification of only ANS activity. However, co-amplification of the other tryptophan-biosynthetic enzymes with ANS should be more favorable to overproduction of tryptophan. Accordingly, the tryptophan-biosynthetic gene cluster and the DS gene were assembled onto one multicopy vector as depicted

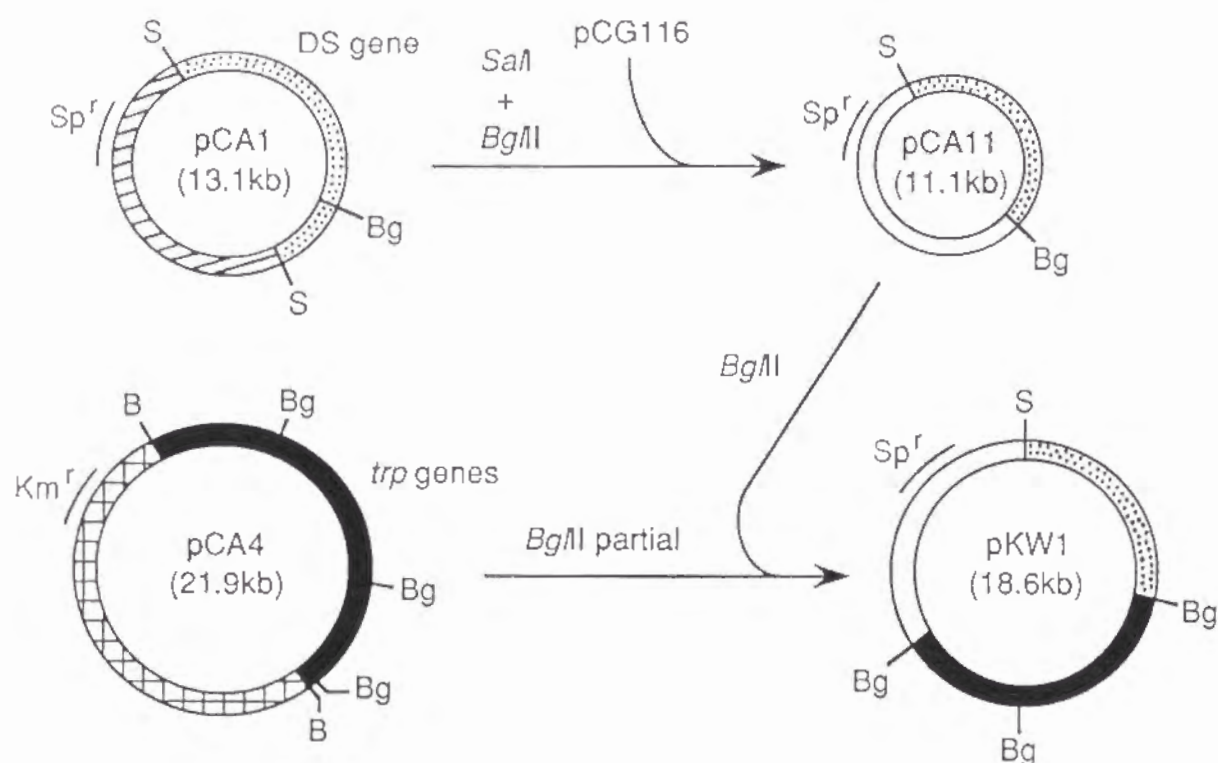


FIG. 3. Construction of pKW1 containing the DS gene and the tryptophan-biosynthetic gene cluster. Symbols: stippled bars, *C. glutamicum* KY10694 chromosomal DNA fragment containing the DS gene; solid bars, *C. glutamicum* KY10894 chromosomal DNA fragment containing the tryptophan-biosynthetic gene cluster; hatched bar, pCG115; cross-hatched bar, pCE53; open bars, pCG116. Abbreviations: S, *Sal*I; Bg, *Bgl*III; B, *Bam*HI; Sp^r, spectinomycin resistance; Km^r, kanamycin resistance.

in Fig. 3. As the larger 5.0 kb *Sal*I-*Bgl*III segment of the *Sal*I-insert originally cloned onto pCA1 was determined to contain the intact DS gene (data not shown), it was subcloned into pCG116 to generate pCA11. Separately, pCA4 that contains the entire tryptophan-biosynthetic gene cluster was partially digested with *Bgl*III and electrophoresed on agarose gel. The 7.5 kb fragment separated on the gel was purified and then ligated with *Bgl*III-digested pCA11. Transformation of the protoplasts of ANS-deficient *C. glutamicum* TA101 with the ligation mixture and selection for resistance to spectinomycin on RCGA plates yielded transformants with a prototrophic phenotype. The plasmid, which was obtained from one of the transformants and designated pKW1 (Fig. 3), had the ability to confer p-fluorophenylalanine resistance and prototrophy on strains KLS4 and TA133, respectively, indicating that it contained both the DS gene and the entire tryptophan-biosynthetic genes.

Introduction of pKW1 into KY10894 (Table 1) resulted in overexpression of the enzymes encoded by the plasmid. DS, ANS and PRT activities were elevated by 8- to 11-fold relative to the control host. Although the transformed strain accumulated three times less chorismate than the pCA1 carrier, tryptophan production was adversely reduced to a low level even relative to the host alone. It, however, was found that the pKW1-carrying strain excreted a considerable amount of another metabolite, anthranilate that is the first intermediate in the tryptophan branch. Moreover, because of the detrimental action of this intermediate on cells, growth of the recombinant strain was suppressed to below 75% of the control level, and carbon consumption was reduced by 20% (data not shown).

The excretion of anthranilate suggested that the PRT reaction would become a new rate-limiting step in the recombinant strain with pKW1. Apparently this prediction is inconsistent with the results of the enzyme assays, which showed that the ANS activity was inhibited by tryptophan at much lower concentrations than the PRT activity (Table 1). However, attention must be given to the fact that the inhibition of *C. glutamicum* ANS by tryptophan is

competitive with the substrate chorismate, as has been demonstrated by the previous study of Hagino and Nakayama (6). This competition appears to really occur within the cells, whose intracellular pool of chorismate is possibly elevated from the observation that the recombinant strain still excreted the intermediate (Table 1). It is therefore considered that relief of the ANS activity from tryptophan inhibition through competition with chorismate would result in the shift of a rate-limiting step to the next tryptophan-inhibitable PRT reaction. The predicted bottleneck in the tryptophan pathway appears to be removed if PRT is released from tryptophan inhibition.

Desensitization of pKW1-specified PRT to inhibition by tryptophan.

The author thus decided to desensitize the PRT, encoded on pKW1, to tryptophan inhibition by mutagenesis. *C. glutamicum* KLS4 cells carrying pKW1

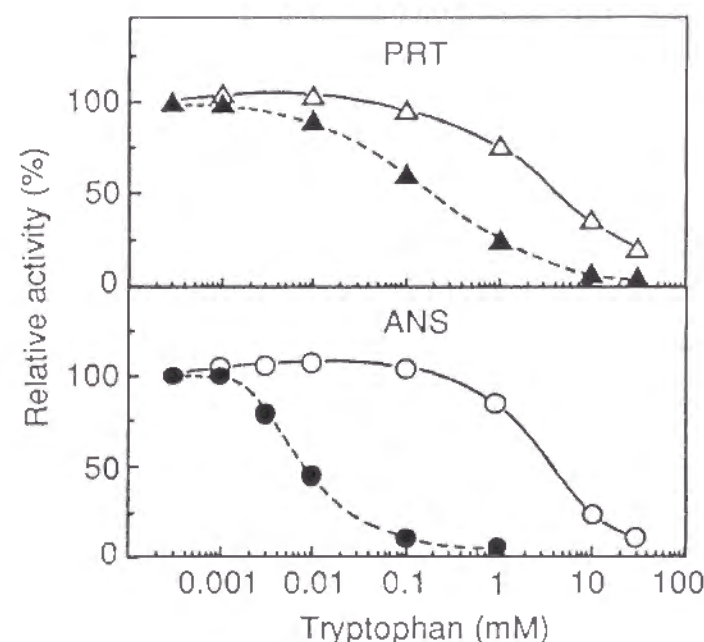


FIG. 4. Tryptophan-sensitivity of PRT and ANS encoded by pKW1 and pKW99. Symbols: $\blacktriangle$, PRT activity of KLS4 carrying pKW1; $\triangle$, PRT activity of KLS4 carrying pKW99; $\bullet$, ANS activity of KLS4 carrying pKW1; $\circ$, ANS activity of KLS4 carrying pKW99.

were mutagenized and cultured overnight. Plasmid DNAs were isolated from the whole of the cells collected and used to transform again protoplasts of strain KLS4. Transformants were selected on RCGA plates containing spectinomycin (400 μ g/ml) and 5-fluorotryptophan (5FT) (1 mg/ml). The resultant 163 transformants resistant to both drugs were then tested for their ability to excrete tryptophan. The plasmid, which was carried by the transformant with the highest productivity and designated pKW99, was shown by restriction cleavage analysis to retain the same physical structure as pKW1. Retransformation with pKW99 resulted in co-transfer of 5FT resistance and the vector marker, confirming that the mutation occurred on the plasmid.

Enzymatic analyses with the crude cell extracts of the KLS4 transformants (Fig. 4) indicated that the PRT activity specified by pKW99 was 25-fold less sensitive to tryptophan than that specified by the parental plasmid. Unexpectedly, it was also found that the ANS specified by pKW99 was more highly desensitized to the end product inhibition than the enzyme encoded by pKW1. The simultaneous desensitization of PRT and ANS seems to have been caused by co-mutation since these native enzymes are verified to be separable as independent tryptophan-sensitive ones by the study (29) with *B. flavum* that closely resembles *C. glutamicum*.

Tryptophan production by strain KY10894 carrying pKW99.

Lastly, the mutated plasmid pKW99 thus obtained was introduced into the tryptophan producer KY10894 and its effect on the carbon flow over the pathway to tryptophan was examined (Table 1). In this host pKW99 preserved almost the same gene dosage effects for the three key enzymes as pKW1. Production experiments in the test-tube cultivations showed that the pKW99-carrying strain produced 41% more tryptophan than the control host. In contrast with KY10894 strain carrying pCA1 or pKW1, the pKW99-carrying producer accumulated little chorismate and no anthranilate. This result implies that the highly desensitized ANS and PRT, coexpressed by pKW99, might promote the carbon

channelling through the tryptophan pathway, thereby allowing accumulation of the end product in a larger amount.

Tryptophan production by strain KY10894 and its pKW99-carrying recombinant strain was further investigated with jar fermentors. Fig. 5 shows the fermentation profiles obtained in fed batch cultures with sucrose medium to which sugar was added initially at 6% and subsequently fed to 22% total. While the untransformed host gave a tryptophan titer of 28 g per liter, the engineered strain with pKW99 produced 43 g of tryptophan per liter with an yield increased by 54%. As the latter titer is far over the solubility of tryptophan, about half of the product was crystallized in the medium. The crystals of tryptophan in the fermentation broth were observed under a microscope as photographed in Fig. 6.

The author has shown in this chapter that sequential improvement of the biosynthetic pathways of tryptophan-producing *C. glutamicum* KY10894 can result in the creation of a hyperproducer with highly increased tryptophan productivity. The successful metabolic engineering can be summarized to have been achieved by the following programme; (i) amplification of the first enzyme in a particular pathway diverging from central carbohydrate metabolism increases carbon flow down that pathway, and then (ii) sequential removal of metabolic bottlenecks in the following pathway, discerning rate-limiting steps based on intermediate accumulation, allows the efficient channelling of intermediates into a desired product. This strategy will be generally applicable to the metabolic engineering for yield improvement of primary metabolites.

Besides *C. glutamicum* and its relatives, there have been attempts to construct tryptophan-producing strains with either *E. coli* (30) or *B. subtilis* (15). Although some producers of these species are designed to carry a variety of beneficial mutations for tryptophan production, they give relatively low titers of tryptophan. Their fermentation profiles show that the rates of tryptophan production decline during exponential or stationary phase. In both cases, the declining is likely to be ascribed to the decay of

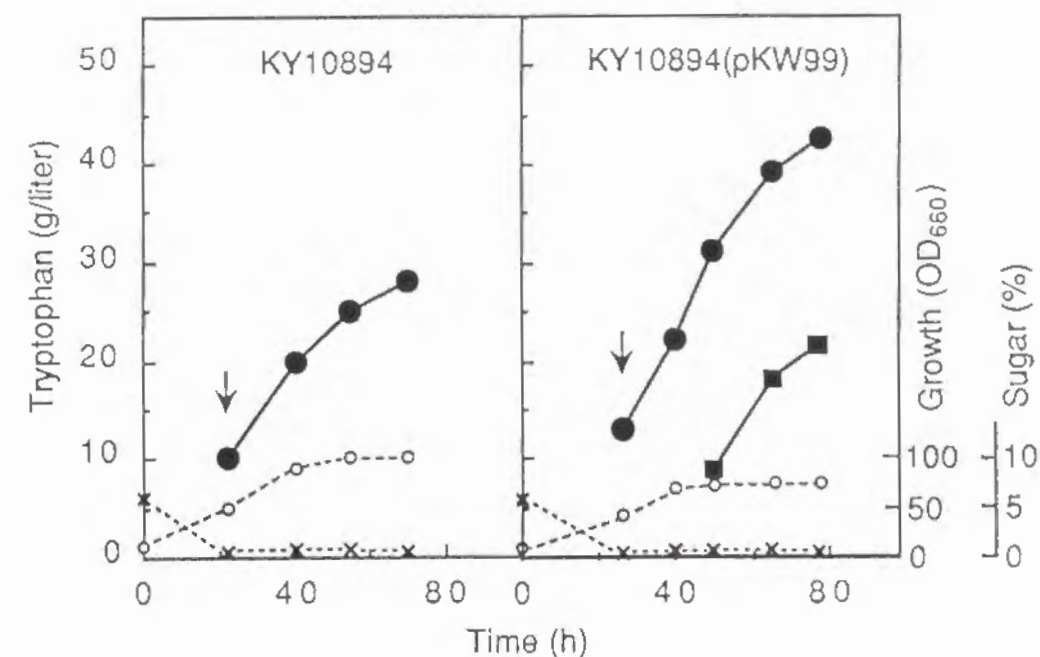


FIG. 5. Time course of tryptophan production by strain KY10894 with and without pKW9901 in jar-fermentor cultivations. Symbols: ●, tryptophan; ■, tryptophan crystallized in the medium; ○, growth; ×, sugar. Arrows indicate the points at which feeding with a 44% sucrose solution began.

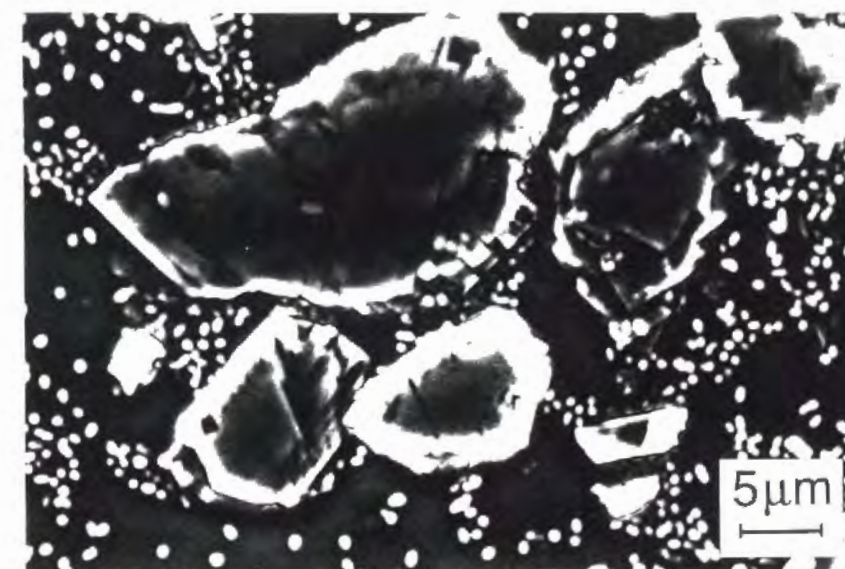


FIG. 6. Phase-contrast microphotograph of crystals of tryptophan and *C. glutamicum* cells with pKW99 in the jar-fermentation broth.

DS activity with the progress of fermentation. This seems to constitute the insuperable barrier to tryptophan production in *E. coli* or *B. subtilis*. On the other hand, the author confirmed that the engineered tryptophan producer of *C. glutamicum* mentioned above did not exhibit a significant decrease in DS activity until the end of fermentation (data not shown). This result is consistent with the result of biochemical analysis by Suguimoto and Shiio (28); they showed that the purified DS of *B. flavum*, closely related to *C. glutamicum*, was labile but extremely stabilized by tryptophan. This characteristic of DS convinces the author that *C. glutamicum* has a decided advantage over *E. coli* and *B. subtilis* for overproduction of tryptophan.

The recombinant *C. glutamicum* presented in this chapter has the capacity of producing tryptophan in much higher yield and titer than any other tryptophan-producing microorganisms so far obtained. However, high level of tryptophan production was realized in the presence of spectinomycin during fermentation. Unless antibiotic pressure was applied, there was a decrease in tryptophan production because a considerable proportion of cells lost the plasmid (data not shown). Plasmid stability must be ensured for practical production of tryptophan by the recombinant strain. A procedure to overcome this problem is described in the next chapter.

SUMMARY

Pathway engineering of a tryptophan-producing strain of *Corynebacterium glutamicum* was conducted using cloned homologous genes. Plasmid-mediated amplification of a feedback-insensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DS) in the strain augmented the carbon flow down the common aromatic pathway, but caused concurrent excretion of chorismate, the last metabolite. Alternatively, introduction of a plasmid coexpressing the set of tryptophan-biosynthetic enzymes along with DS resulted in formation of anthranilate, the first intermediate in the tryptophan branch, as another

by-product. However, mutational alterations of plasmid-encoded anthranilate synthase and anthranilate phosphoribosyltransferase, which rendered them insensitive to tryptophan inhibition, led to efficient channelling of carbon through the overall pathway to tryptophan. This engineered strain displays a 54% yield increase relative to its parent and produces 43 g of tryptophan per liter.

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Chapter 4. Fermentative Production of Tryptophan by a Stable Recombinant Strain of *Corynebacterium glutamicum* with a Modified Serine-Biosynthetic Pathway

INTRODUCTION

Fermentative production of amino acids has been done with mutants of *Corynebacterium glutamicum* and its related *Brevibacterium flavum* and *Brevibacterium lactofermentum* (12). Tryptophan-producing mutants of these coryneform bacteria have been constructed by repeated mutagenesis conferring auxotrophy for the aromatic amino acid(s) and/or resistance to nonmetabolizable structural analog(s) (6, 20). However, production costs of tryptophan by these mutants are still high due to low productivity, which hampers its newer applications such as the use as a feed supplement.

For further yield improvement, the author previously attempted to engineer an existing tryptophan-producing strain of *C. glutamicum* with the use of recombinant DNA technology (9). The strategy was to sequentially modify the biosynthetic pathways (Fig. 1), discerning rate-limiting steps based on intermediate accumulation. The successful metabolic engineering ultimately

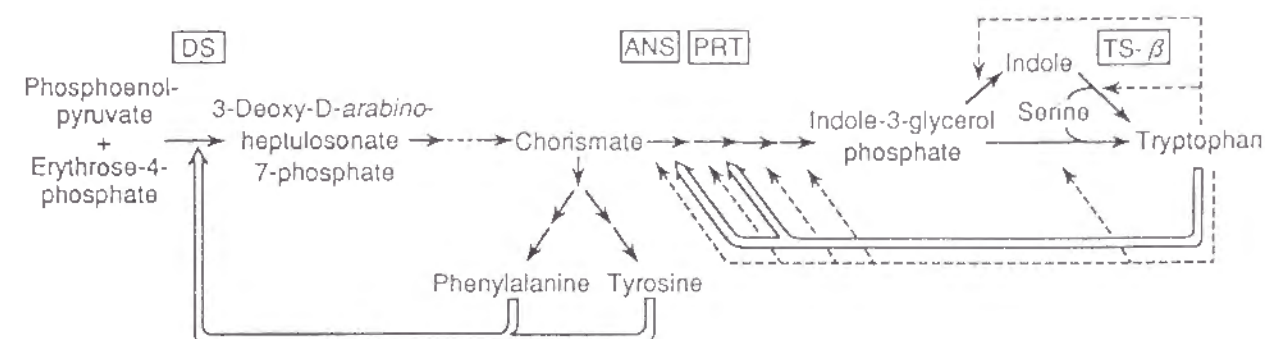


FIG. 1. Pathway and primary regulations of tryptophan biosynthesis in *C. glutamicum*. The open and broken arrows indicate feedback inhibition and repression, respectively.

conducted was plasmid-mediated amplification of the genes encoding 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DS), anthranilate synthase (ANS), anthranilate phosphoribosyltransferase (PRT) and other tryptophan-biosynthetic enzymes, among which the former three key enzymes were rendered insensitive to feedback inhibition. The recombinant producer thus engineered has acquired the ability to produce tryptophan in a significantly higher yield and titer than any tryptophan-producing microorganism previously reported. However, there were the following two problems to be solved for practical application of the recombinant strain. One was a decline in sugar consumption at the late stage of fermentation, which not only retarded fermentation but also reduced the production yield. The other was plasmid loss from the culture unless antibiotic selective pressure was applied. Although the use of an antibiotic can prevent outgrowth of plasmidless cells, it is not necessarily desirable. Therefore, another system for plasmid stabilization needs to be developed.

In this chapter, the author describes the construction of a tryptophan hyperproducer of *C. glutamicum* in which these two problems were both solved by manipulation of the serine-biosynthetic pathway.

MATERIALS AND METHODS

Bacterial strains and plasmids.

C. glutamicum KY10894 (9) is a tryptophan-producing mutant derived through multiple rounds of mutagenesis from a phenylalanine and tyrosine double auxotrophic strain KY9456 (6). *C. glutamicum* wild-type strain ATCC 31833 and its serine-auxotrophic derivative RS57 were used as the DNA donor and the recipient, respectively, for cloning of the 3-phosphoglycerate dehydrogenase (PGD) gene. *C. glutamicum* KY9218 is a PGD-deficient, serine auxotroph of KY10894 and was used for examination of the bacteriocidal action of indole and as the tryptophan-producing host for plasmid stabilization.

Plasmid pKW99 (9) is a mutated derivative of pKW1 which contains the DS

gene of a phenylalanine-producing mutant, *C. glutamicum* KY10694, and the tryptophan-biosynthetic gene cluster of *C. glutamicum* KY10894 in pCG116. The DS, ANS and PRT encoded on pKW99 are highly desensitized to feedback inhibition. Plasmid pCG116, a derivative of the *C. glutamicum* vector plasmid pCG11 (10), has the streptomycin-spectinomycin resistance gene and the polylinker present in M13mp18 RF DNA (14).

Media.

Complete medium BY (11), minimal medium MM (18), and enriched minimal medium MMYE (11) were used for cultivation of *C. glutamicum*. Solid plates were made by the addition of Bacto-Agar (Difco) to 1.6%. RCGA medium (11) was used for regeneration of *C. glutamicum* protoplasts. When required, supplements or antibiotics were added as described previously (7). TP1 medium (7) was used for production in test tubes. TS1 and TP2 media (7) were used for second-seed culture and production, respectively, in jar fermentation.

Cultivations for tryptophan production and for plasmid stability studies.

Cultivation in test tubes was carried out in TP1 medium containing 6% glucose as described previously (9).

For cultivation in 2-liter jar fermentors, a 2.4-ml amount of the first-seed culture in BYC medium (containing 1.0% glucose in medium BY) was inoculated into 120 ml of TS1 medium in a 1-liter flask. After 24 h of cultivation at 30°C on a rotary shaker, the second-seed broth was transferred into a 2-liter jar fermentor containing 550 ml of TP2 medium. After the sugar initially added was consumed, solution containing 44% sucrose was added intermittently or continuously until the total amount of sugar supplied in the medium reached 22% or 25%, respectively. The culture was agitated at 800 rpm and aerated at 1 liter/min at 30°C, and the pH was maintained at 6.1 with NH_4OH .

Preparation and manipulation of DNA.

Chromosomal DNA was extracted from protoplasts of *C. glutamicum* ATCC 31833 by the method of Saito and Miura (19). The protoplasts were prepared as described previously (11). Plasmid DNA was isolated by the alkaline lysis method (13) and, if necessary, purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (11). DNA digestion and ligation were carried out as described by Maniatis *et al* (13). Restriction enzymes and T4 DNA ligase were obtained from Takara Shuzo Co., Ltd., Kyoto, Japan. DNA fragment was purified from agarose gel by the glass-binding method (26).

Transformations.

The protoplast transformation method used has been described previously (11).

Enzyme assays.

Crude cell extracts were prepared by sonic disruption of cells grown in MMYE medium as described previously (7). Protein was determined by the method of Bradford (3) with the Bio-Rad kit. PGD activity in crude cell extracts was measured by the method of Sugimoto and Pizer (23) except that the assays were carried out at 30°C.

Determination of cell viability.

C. glutamicum KY9218 was grown in BYG medium containing serine (1 mg/ml) and harvested in the exponential phase. Then, the cells were washed with 0.1 M potassium phosphate buffer, suspended in the same buffer containing indole at the concentrations indicated in Fig. 3, and incubated aerobically at 30°C for 96 h. Samples were taken periodically and their appropriate dilutions were plated on BYG plates. Cell viability was determined by counting the number of colonies on the plate.

Plasmid stability.

Appropriate dilutions of cultures were plated on BYG plates. After incubation at 30°C for 3 days, 100 colonies from each culture were tested for the presence of spectinomycin resistance.

Analysis.

Cell growth and sugar concentration were measured as described previously (7). Tryptophan and serine were analyzed by HPLC after derivatization with *o*-phthalaldehyde. Indole was determined as follows. A 0.5-ml amount of culture broth was mixed well with 1 ml of toluene. Then, 0.5 ml of the toluene layer was separated and followed by the addition of 1 ml of ethanol and 0.5 ml of indole reagent (5 g of *p*-dimethylaminobenzaldehyde, 75 ml of *n*-amyl alcohol and 25 ml of concentrated HCl). After 20 min at room temperature, the absorption at 540 nm was measured and compared with that of standards.

RESULTS

Characteristics of tryptophan fermentation by strain KY10894 carrying pKW99.

C. glutamicum KY10894 carrying pKW99 has the ability to produce 43 g/liter of tryptophan with crystallization, a yield increase of 54% over the host strain, in fed-batch fermentor cultivation with sucrose medium to which sugar was added initially at 6% and subsequently increased to 22% (Fig. 2 (A) and (B)). However, one disadvantage was that the cultivation period was prolonged due to a decrease in sugar consumption at the late phase of growth. In the recombinant strain, the number of viable cells at the end of fermentation was found to be reduced to under one-tenth as compared with that of the control host. To clarify the cause of the loss of cell viability, by-products which existed mainly in the fermentation broth of the pKW99 carrier was searched. As such a by-product, a trace of indole, the last

intermediate of the tryptophan pathway, was detected. Since the chemical has been shown to exhibit biological toxicity (8), it was assumed that its accumulation might give rise to the loss of cell viability, resulting in the retardation of sugar consumption.

Effect of indole on cell viability.

To examine whether the trace amount of indole actually had a bacteriocidal action on *C. glutamicum* cells, changes in the number of viable cells were monitored in the presence of indole. For this experiment, the author used a serine-auxotrophic mutant, *C. glutamicum* KY9218, derived from KY10894, because its inability to biosynthesize serine prevented indole from being metabolized to tryptophan by the tryptophan synthase β (TS- β) reaction. As shown in Fig. 3, the number of viable cells decreased gradually in the

presence of 20 or 40 $\mu\text{g/ml}$ of indole, indicating its toxicity to cells of KY9218. The concentration of indole with toxicity was almost the same as that excreted into the fermentation medium by the pKW99 carrier. From these results, it was concluded that the toxic by-product caused the loss of cell viability during fermentation by the recombinant strain.

Effect of serine on indole formation.

The accumulation of indole implied that the TS- β reaction was the rate-limiting step for tryptophan biosynthesis in the recombinant strain. The author thus attempted to remove the predicted bottleneck. One possible reason for limitation of the TS- β reaction was a deficiency of serine, the other substrate for the enzyme. To explore this possibility, the effect of serine on indole formation was examined by a production experiment in jar fermentors.

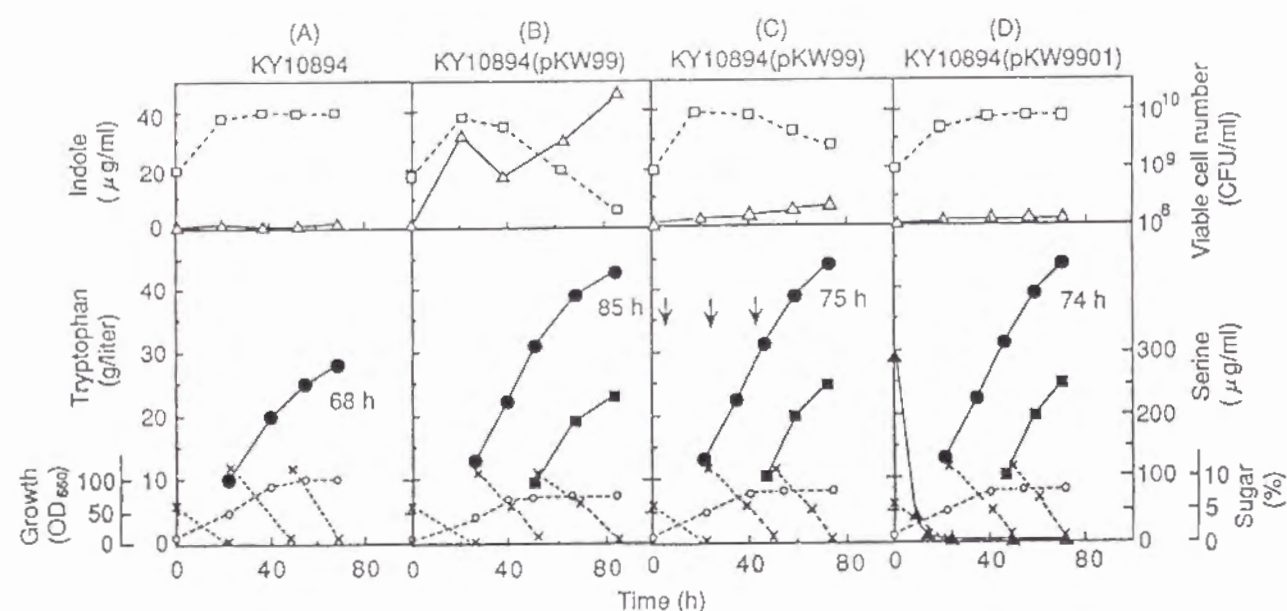


FIG. 2. Indole formation and cell viability in tryptophan production process with jar fermentors. Symbols: ●, tryptophan; ■, tryptophan crystallized in the medium; ○, growth; ×, sugar; △, indole; □, viable cell number; ▲, serine. Arrows shown in (C) indicate the points at which serine (5 g each) was added. Cultivations of recombinant strains were carried out in medium with spectinomycin (100 $\mu\text{g/ml}$).

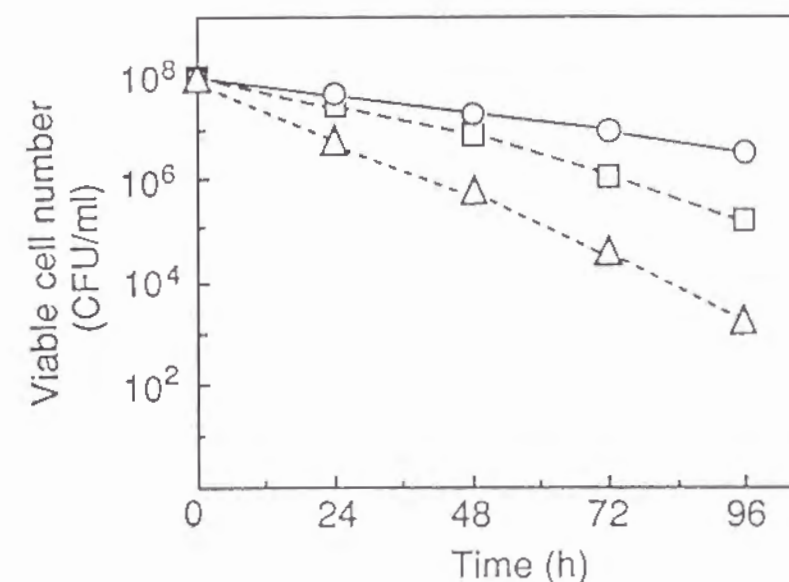


FIG. 3. Effect of indole on cell viability (Colony-Forming Units, CFU). The cells of *C. glutamicum* KY9218 were incubated as described under Materials and Methods in 0.1 M potassium phosphate buffer containing indole at the following concentrations: ○, 0 $\mu\text{g/ml}$; □, 20 $\mu\text{g/ml}$; △, 40 $\mu\text{g/ml}$.

As shown in Fig. 2 (C), the addition of serine to the culture of the recombinant strain led to a drastic decrease in indole formation and resulted in enhanced sugar consumption without a remarkable loss of cell viability.

Construction of pKW9901.

Considering that the accumulation of indole might be due to an insufficient supply of serine necessary to convert indole to tryptophan, it was reasonable to expect that enhancement of the metabolic flux toward serine would remove the limitation of the TS- β reaction and lead to a decrease in indole formation. In order to conduct such metabolic engineering through an increased gene dosage, the gene for 3-phosphoglycerate dehydrogenase (PGD), the first enzyme in the serine pathway, was cloned as follows. Chromosomal

DNA of *C. glutamicum* wild-type strain ATCC 31833 and vector pCG116 were digested with *Sal*I, ligated, and used to transform the protoplasts of a PGD-deficient serine auxotroph, *C. glutamicum* RS57. Transformants were selected on RCGA plates containing spectinomycin (400 μ g/ml). Regenerated spectinomycin-resistant colonies were transferred by replica-plating to MM agar plates containing spectinomycin (100 μ g/ml). Sixteen spectinomycin-resistant and serine-independent clones were obtained. All plasmids purified from these clones were shown to have a common 2.7 kb *Sal*I fragment by restriction cleavage analysis, and one of the plasmids was designated pCS571. The presence of pCS571 in strain ATCC 31833 elevated the level of PGD activity about 13-fold, indicating that the cloned segment had the PGD gene.

Next, the gene was joined onto pKW99 as shown in Fig. 4. As the 1.4 kb *Bam*HI fragment of pCS571 was determined to contain the intact PGD gene by subcloning analysis (data not shown), the fragment was purified from agarose gel and ligated with *Bam*HI-digested pKW99. The ligation mixture was used to transform the protoplasts of strain RS57. Spectinomycin-resistant and serine-independent clones were isolated. Restriction cleavage analysis of the plasmids isolated from these clones showed that they contained the PGD gene in addition to the DS gene and the entire tryptophan-biosynthetic genes. One of these plasmids, designated pKW9901, was further confirmed to have almost the same gene dosage effect for PGD activity as pCS571 in strain ATCC 31833.

Characteristics of tryptophan fermentation by strain KY10894 carrying pKW9901.

To investigate the effect of pKW9901 on tryptophan fermentation, this plasmid was introduced into the tryptophan producer KY10894, and the transformed strain obtained was tested for production in jar fermentors (Fig. 2 (D)). The recombinant strain with pKW9901 no longer produced indole throughout the cultivation. Due to this, the number of viable cells remained high and thus sugar consumption at the late phase of growth was improved. Consequently, slightly more tryptophan (45 g/liter) was accumulated in a

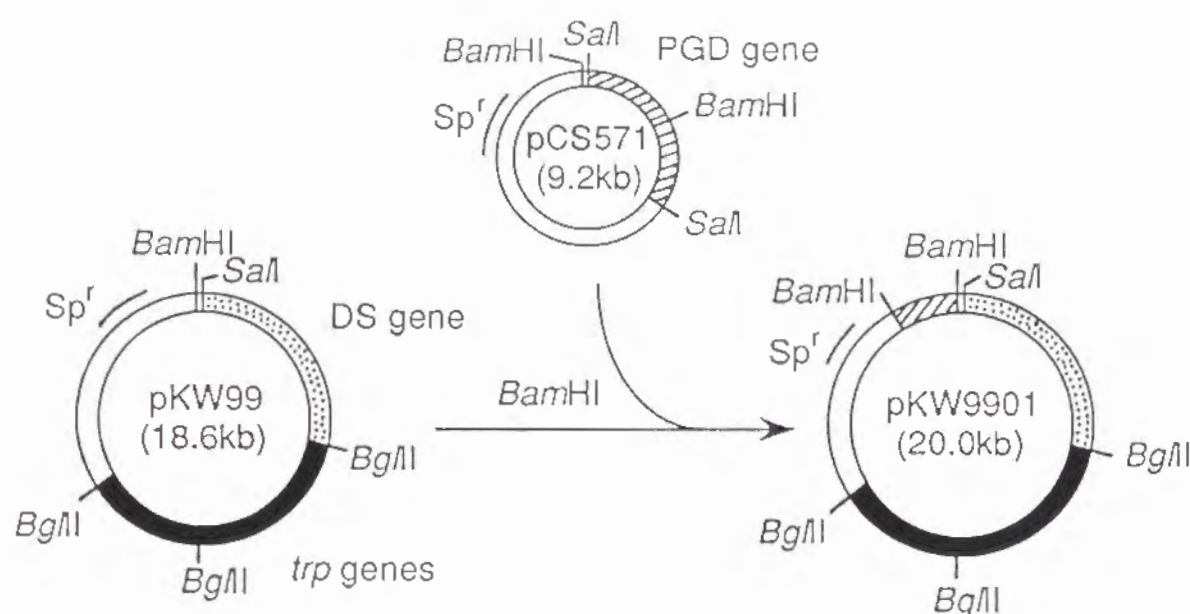


FIG. 4. Construction of plasmid pKW9901. Symbols: stippled bars, *C. glutamicum* KY10694 chromosomal DNA fragment containing the DS gene; solid bars, *C. glutamicum* KY10894 chromosomal DNA fragment containing the tryptophan-biosynthetic gene cluster; hatched bars, *C. glutamicum* ATCC 31833 chromosomal DNA fragment containing the PGD gene; open bars, pCG116; Sp^r, spectinomycin resistance.

shorter cultivation time (74 h) than in the cases of the pKW99-carrying strain. However, the recombinant plasmid pKW9901 showed a similar instability to pKW99 in strain KY10894 under cultivation without antibiotic pressure (Table).

Construction of a plasmid stabilization system.

In the culture of strain KY10894 carrying pKW9901, serine which was initially contained at the concentration of about 300 µg/ml in the medium was consumed rapidly and then depleted after 10 h of cultivation (Fig. 2 (D)). The author used this characteristic to establish a plasmid stabilization system by combination of a serine-auxotrophic host and a plasmid that complemented the serine auxotrophy. The probable mechanism was as follows. Serine non-requiring plasmid carriers can grow normally, but serine requiring

plasmid-free segregants cannot grow rapidly because of the depletion of serine in the early phase of growth and thus are diluted as minority in the total population. As a consequence, the population of plasmid carriers is preferentially maintained. This system was examined using the serine-auxotrophic host KY9218 and plasmid pKW9901 which complements the serine auxotrophy. After cultivation in antibiotic-free production media with test tubes, the stability of pKW9901 in host KY9218 was compared with that in the serine-independent host KY10894 (Table). The plasmid was stably maintained in KY9218; all of the colonies tested possessed the spectinomycin-resistant phenotype, whereas unstable in KY10894; about 80% of the colonies tested showed the spectinomycin sensitivity. On the other hand, remarkable loss of the plasmid from KY9218 was observed when cells were cultivated under the excess conditions of serine. These results indicated that the stable maintenance of pKW9901 in KY9218 was ensured by the mechanism suggested above.

TABLE. Plasmid stability after cultivation in antibiotic-free media

Strain (plasmid)	Serine (10 mg/ml)	Cells carrying plasmid (%)
KY10894 (pKW99)	-	31
	+	28
KY10894 (pKW9901)	-	16
	+	22
KY10894 (pKW9901)	-	100
	+	30

Plasmid stability was examined after test-tube cultivation in TP1 medium with (+) and without (-) serine. Cultivations in both seed and production media were carried out in the absence of spectinomycin.

Tryptophan production by strain KY9218 carrying pKW9901.

Strain KY10894 and the recombinant strain KY9218 carrying pKW9901 were tested for tryptophan production in fed-batch fermentor cultivation without the addition of antibiotics. Fig. 5 shows the fermentation profiles when cells were cultivated in sucrose medium to which sugar was added initially at 6% and subsequently increased to 25%. The pKW9901-carrying producer indeed exhibited high plasmid stability (96% of the cells carried the plasmid at the end of cultivation) and thus accumulated 61% more tryptophan than strain KY10894. The tryptophan titer finally obtained reached as much as 50 g/liter.

DISCUSSION

Recombinant DNA techniques are useful for enhancing the enzyme activities responsible for amino acid biosynthesis. However, it has been suggested that overexpression of certain genes sometimes causes a metabolic imbalance or

accumulation of intermediates, thus impairing the growth of cells (1, 2, 25). Similarly in the case of this study, introduction of pKW99 into tryptophan-producing *C. glutamicum* KY10894 resulted in a decrease in sugar consumption at the late phase of growth. The present results have revealed that the drawback was due to the accumulation of a toxic intermediate, indole, and a subsequent loss of cell viability.

At least, three possible reasons can be considered for the indole formation. One is feedback repression of TS- β by tryptophan, which has been found in *B. flavum* (24), closely related to *C. glutamicum*. But, this possibility can be ruled out because indole was formed even at the early phase of growth when the tryptophan concentration was still low. A second possibility is a decrease in TS- β activity as fermentation continues. This

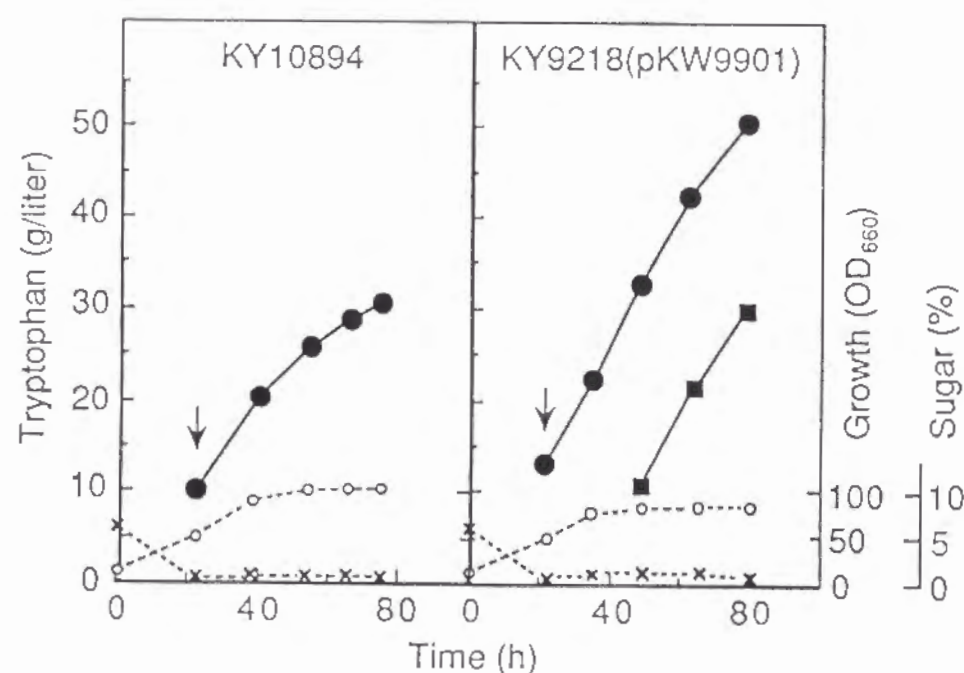


FIG. 5. Time course of tryptophan production by strain KY10894 and the recombinant strain KY9218 carrying pKW9901 with jar fermentors. Symbols: ●, tryptophan; ■, tryptophan crystallized in the medium; ○, growth; ×, sugar. Arrows indicate the points at which solution containing 44% sucrose began to be fed continuously. Cultivations in both seed and production media were carried out in the absence of spectinomycin.

explanation also seems unlikely because amplification of the TS- β gene by pKW99 indeed caused increased activity of the enzyme throughout fermentation in the recombinant strain (data not shown). On the other hand, the results presented in this chapter show that a third possibility, a deficiency of serine, is most likely. The indole formation of the recombinant strain can be explained in the following manner. Introduction of pKW99 into strain KY10894 might cause increased carbon flux down the tryptophan pathway, whereas the capacity of serine supply remained unchanged. Therefore, the increased indole supply might exceed the serine supply in the TS- β reaction, which resulted in the overflow of indole. This conclusion is reinforced by the observation that the serine auxotroph KY9218 derived from KY10894 produced indole if cultivated under the limited condition of serine (data not shown).

Plasmids pKW99 and pKW9901 were not stably maintained in strain KY10894 under cultivation without antibiotic selective pressure. Plasmid instability is one of the serious problems to be solved before practical applications of recombinant strains can be made. Several methods to stabilize plasmids in host strains have been reported: for example, the use of a vector stabilized by possession of the partition locus of a low copy number plasmid such as pSC101 (21), R1 (5), or F (17), and the use of a stabilization system by combination of a special host and vector such as a streptomycin-dependent mutant and a plasmid containing the streptomycin-independence gene (15), a temperature-sensitive *valS* mutant and a plasmid containing the wild-type *valS* gene (22), an *asd* mutant and a plasmid containing the wild-type *asd* gene (16), or a mutant lacking an alanine racemase and a plasmid containing the corresponding gene (4). Although these methods have been shown to ensure high plasmid stability in *Escherichia coli*, *Salmonella typhimurium*, or *Bacillus subtilis*, it is not so easy to apply these systems to *C. glutamicum* due to shortage of its genetic information. Therefore, the author developed an alternative new system to stabilize the recombinant plasmid in the tryptophan-producing *C. glutamicum* strain, based on the presence of the serine-

biosynthetic gene on the plasmid and its absence from the chromosome. The advantage of this system is that it provides selection for plasmid maintenance in complex media containing serine, because the amino acid is consumed rapidly by *C. glutamicum* cells. The data presented in this study strongly suggest that the presence of the serine-biosynthetic gene on the plasmid can be successfully used to provide selective pressure for maintenance of the plasmid even in large-scale industrial fermentation. Moreover, considering that serine is generally a central intermediate in cell metabolism, this plasmid stabilization system should be applicable to other genetically modified bacteria.

The best recombinant strain thus far constructed can produce tryptophan at a titer of 50 g/liter after 80 h in jar-fermentor cultivation without the addition of antibiotics. The specific productivity and the yield were 0.63 g/liter per hour and 20.0%, respectively. In any point of view, the ability of the strain to produce tryptophan directly from sugar is the highest that has ever been obtained by fermentation with microorganisms.

SUMMARY

Introduction of plasmid pKW99, which coexpresses the deregulated 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase and tryptophan-biosynthetic enzymes, into tryptophan-producing *Corynebacterium glutamicum* KY10894 resulted in a marked increase (54%) in yield of tryptophan production (43 g/liter), but incurred two problems. One was a decline in sugar consumption at the late stage of fermentation, and the other the loss of the plasmid in the absence of selective pressure. The retarded sugar assimilation was found to be attributed to the death of cells that arose from the detrimental action of indole, the last intermediate in the tryptophan pathway, accumulated as a by-product. A chain of these events simultaneously disappeared when serine, the other substrate of the final reaction by tryptophan synthase, was added.

These results indicated that a limiting supply of serine was the cause of the decline in the sugar consumption. Thus, to increase carbon flux into serine, the gene for 3-phosphoglycerate dehydrogenase (PGD), the first enzyme in the serine pathway, was cloned from wild-type *C. glutamicum* ATCC 31833 and joined onto pKW99 to generate pKW9901. Strain KY10894 transformed with pKW9901 favorably consumed sugar through fermentation with accumulating little indole. Furthermore, on the basis of the observation that serine in the medium was consumed rapidly by the recombinant cells, the author developed a unique plasmid stabilization system composed of KY9218 (a PGD-deficient serine-requiring strain of KY10894) and pKW9901. In its combination, cells lacking the plasmid should not proliferate in the fermentation medium which does not contain serine. Even if selective pressure was not applied, the modified strain KY9218 with pKW9901 stably maintained the plasmid during fermentation and produced 50 g/liter of tryptophan in a 61% increased yield relative to strain KY10894.

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Chapter 5. Transport of Aromatic Amino Acids and Its Influence on Overproduction of the Amino Acids in Corynebacterium glutamicum

INTRODUCTION

Biosynthesis of amino acids in microorganisms is strictly controlled by several regulatory mechanisms such as feedback inhibition and repression. Therefore, overproduction of a particular amino acid requires removal of the metabolic controls over the pathway directly associated with the amino acid biosynthesis. In Corynebacterium glutamicum and its related bacteria Brevibacterium flavum and Brevibacterium lactofermentum, which are widely used for the industrial production of various amino acids, extensive efforts on strain improvement for amino acid overproduction have been directed toward deregulation of the corresponding pathway by classical mutagenesis and screening procedures (16). Since each regulation can be removed by a mutation resulting in auxotrophy for a certain amino acid or resistance to a toxic structural analog, amino acid-producing strains of these coryneform bacteria have been constructed by assembling these beneficial mutations in one background (16). However, in certain amino acids such as the aromatic amino acids whose biosynthetic pathways are subject to multiple regulations at several steps (10, 29, 30), their productivity remains relatively low in spite of much efforts (16). This is probably due to the difficulty of removing all of the regulatory mechanisms over the entire pathway by means of repeated mutagenesis. In such cases, the remaining regulatory steps, even if not removed, should be bypassed by reducing the intracellular pools of the amino acids responsible for the regulations.

One probable strategy to reduce the intracellular pool of a certain amino acid would be accelerating the efflux of the intracellularly synthesized amino acid. This seems reasonable, but not feasible because the existence of efflux systems for amino acids has not yet been generally established in microorgani-

sms, although it has been suggested to exist by recent biochemical studies with C. glutamicum (17). On the other hand, since the intracellular pool of an amino acid is likely to depend on the uptake rate of the amino acid accumulated extracellularly as well as the efflux rate, the same goal seems to be achieved by prevention of the amino acid uptake. From this point of view, amino acid uptake is considered to be an important target for strain improvement.

In some Gram-negative bacteria such as Escherichia coli and Salmonella typhimurium, extensive research efforts have been devoted to biochemical and genetic analyses of amino acid uptake (1, 2, 26), which have revealed that multiple systems involve uptake of amino acids. Although considerable information is already available concerning basic uptake characteristics and transport mechanisms, very little investigation has been made on the relationship between uptake systems and amino acid productivity.

The focus of this study is to elucidate the influence of uptake on the productivity of the corresponding amino acid in amino acid-producing bacteria. For this purpose, aromatic amino acid uptake in C. glutamicum was analyzed, and its influence on aromatic amino acid productivity was examined. In this chapter, the author shows that the uptake activity negatively affects overproduction of the aromatic amino acids and that prevention of amino acid uptake can result in improved production of the amino acid.

MATERIALS AND METHODS

Bacterial strains and plasmids.

C. glutamicum KY9182, from which transport mutants defective in the uptake of aromatic amino acids were derived, is a prephenate dehydratase (PD)-deficient phenylalanine auxotroph of wild-type strain ATCC 13032. C. glutamicum ATCC 31833, another wild-type strain, was used as the DNA donor for cloning of a DNA fragment responsible for the uptake of aromatic amino acids.

C. glutamicum KY9225 is a highly developed tryptophan-producing mutant derived from strain Px-115-97 (9) through several rounds of mutagenesis. C. glutamicum SL64 is a tryptophan and lysine co-producing mutant which was selected as a S-(2-amino-ethyl)-cysteine-resistant strain (Araki, K. et al. unpublished) by mutagenesis of a tryptophan-producing strain, KY10865 (12). Both strains KY9225 and SL64 are auxotrophic for phenylalanine and tyrosine due to a defect in chorismate mutase (CM), and thus require these amino acids for their growth.

Plasmid pCE54 (23) is a C. glutamicum-E. coli shuttle vector and carries the kanamycin, chloramphenicol and tetracycline resistance genes from the E. coli vector pGA22 (8), which function as selective markers in C. glutamicum. Plasmid pKF1 (12) contains the desensitized 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DS), CM, and PD genes in the C. glutamicum vector pCG115. The former two genes were cloned from a regulatory mutant, C. glutamicum KY10694, and the latter gene was cloned from another regulatory mutant, C. glutamicum KY10693. Plasmid pKW9901 (13) contains the DS gene of strain KY10694, the 3-phosphoglycerate dehydrogenase gene of C. glutamicum wild-type strain ATCC 31833, and the tryptophan-biosynthetic gene cluster in the C. glutamicum vector pCG116. The last gene cluster was cloned from a tryptophan-producing strain, C. glutamicum KY10894, which underwent mutational alterations of its encoded anthranilate synthase and anthranilate phosphoribosyltransferase, rendering them insensitive to tryptophan inhibition (14).

Media.

Complete medium BY (15), minimal medium MM (24), and enriched minimal medium MMYE (15) were used for cultivation of C. glutamicum. Solid plates were made by the addition of Bacto-Agar (Difco) to 1.6%. RCCA medium (15) was used for regeneration of C. glutamicum protoplasts. When required, supplements or antibiotics were added as described previously (12), except where otherwise stated. TP1 medium (12) was used for growth in test tubes.

Chemicals.

L-[U-¹⁴C]phenylalanine (450 mCi/mmol), L-[U-¹⁴C]tyrosine (450 mCi/mmol) and L-[methylene-¹⁴C]-tryptophan (50-60 mCi/mmol) were purchased from Amersham Japan, Ltd. The isotopes were diluted appropriately with nonradioactive amino acids for use.

Assays for aromatic amino acid uptake.

Cells were grown at 30°C in 20 ml of MMYE medium supplemented with the required amino acids (0.5 mM). In the exponential phase, they were harvested, washed twice with 0.1 M Tris-phosphate buffer (pH 7.2) containing 1mM MgSO₄, and suspended in the same buffer to a final OD₆₆₀ of approximately 10. The uptake rates were measured using the cell suspension thus prepared according to the method of Araki *et al.* (3) with some modifications. The reaction mixture (1 ml) contained 100 μmol Tris-phosphate (pH 7.2), 1 μmol MgSO₄, 10 μmol glucose, 100 μg chloramphenicol, and 0.1 ml (approximately 0.4 mg dry cell weight) of the cell suspension. After preincubation at 30°C for 3 min, the reaction was started by the addition of ¹⁴C-labeled amino acid (0.01 μmol, 0.1-0.5 μCi). At the indicated intervals, 0.1 ml of the reaction mixture was withdrawn, vacuum-filtered using Millipore HA filter with a pore size of 0.45 μm, and immediately washed 5 times with 1 ml portions of cold 0.1 M Tris-phosphate buffer (pH 7.2). The filters containing cells were dried and their radioactivities were determined by a Packard Tri-Carb Liquid Scintillation Spectrometer. The uptake activity was expressed as nanomoles of amino acid taken up per milligram of dry cell weight.

Cultivations for production of amino acids.

A 0.5 ml amount of the seed culture grown on a BYG medium (containing 1.0% glucose in medium BY) containing the required amino acids (200 μg/ml) was transferred to a large test tube containing 5 ml of TP1 medium and cultivated aerobically at 30°C for 72 h. All cultivations of recombinant strains were

performed in the presence of spectinomycin (100 μg/ml) and/or kanamycin (100 μg/ml).

Preparation and manipulation of DNA.

Chromosomal DNA was extracted from protoplasts of *C. glutamicum* ATCC 31833 by the method of Saito and Miura (25). The protoplasts were prepared as described previously (15). Plasmid DNA was isolated by the alkaline lysis method (19) and, if necessary, purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (15). DNA digestion and ligation were carried out as described by Maniatis *et al.* (19). Restriction enzymes and T4 DNA ligase were obtained from Takara Shuzo Co., Ltd., Kyoto, Japan.

Transformations.

The protoplast transformation method used has been described previously (15).

Mutagenesis.

Strain KY9182 was grown in 3 ml of BYG medium, harvested in exponential phase, and washed in 3 ml of 50 mM Tris-maleate buffer (pH 6.0). Cells were resuspended in 3 ml of the same buffer containing N-methyl-N'-nitro-N-nitrosoguanidine (400 μg/ml), and incubated at 30°C for 30 min. The mutagenized cells were harvested, washed twice in 3 ml of the above buffer, and their appropriate dilutions were spread on the selection agar plates.

Analysis.

Cell growth and amino acid concentration were determined as described previously (12).

RESULTS

Specificity of aromatic amino acid uptake in wild-type cells.

The specificity of the uptake of L-phenylalanine, L-tyrosine, and L-tryptophan in *C. glutamicum* wild-type strain ATCC 13032 was examined by experiments in which 20-fold excess of various unlabeled compounds were allowed to compete with the transport of labeled aromatic amino acids (Table 1). The initial uptake rate of each aromatic amino acid was strongly inhibited by either of the other two aromatic amino acids, whereas a different family of six natural amino acids showed no effect. Toxic structural analogs, 5-fluoro-DL-tryptophan (5FT), *m*-fluoro-DL-phenylalanine (MFP), and *p*-fluoro-DL-phenylalanine (PFP) also caused significant inhibition of the uptake of three aromatic amino acids. In contrast, no or little inhibition was observed with phenylpyruvate (PPA) and anthranilate, which are biosynthetic intermediates of phenylalanine and tryptophan, respectively, and dipeptides, glycyl-L-phenylalanine (Gly-Phe), glycyl-L-tyrosine (Gly-Tyr), and glycyl-L-tryptophan (Gly-Trp). However, the intermediates and dipeptides were assumed to enter the cells since these compounds supported the growth of certain mutants auxotrophic for either of the three aromatic amino acids. These results suggested the following: (i) the uptake of aromatic amino acids and their analogs occurred mainly via a common transport system; and (ii) the biosynthetic intermediates and the dipeptides were taken up by distinct transport systems.

Isolation of mutants defective in the uptake of aromatic amino acids.

Studies with enteric bacteria such as *E. coli* have revealed that a certain mutant resistant to a toxic amino acid analog has a defect in the transport of the corresponding amino acid (18), and that the mutation alters its phenotype to require a large amount of the amino acid for growth, when introduced into an auxotroph for the amino acid (18, 31). Based on these findings, the author attempted to isolate *C. glutamicum* mutants defective in

TABLE 1. Effect of various substances on aromatic amino acid uptake in *C. glutamicum* ATCC 13032

Addition	Relative uptake rate (%)		
	[¹⁴ C]Phenylalanine	[¹⁴ C]Tyrosine	[¹⁴ C]Tryptophan
None	100	100	100
L-Phenylalanine	14	11	8
L-Tyrosine	33	20	25
L-Tryptophan	19	24	8
L-Histidine	105	88	80
L-Leucine	102	110	93
L-Alanine	98	109	105
L-Lysine	105	86	93
L-Glutamate	107	114	104
L-Methionine	103	111	104
Gly-Phe	111	90	95
Gly-Tyr	103	86	95
Gly-Trp	100	84	89
PPA	83	76	92
Anthranilate	87	91	94
5FT	38	32	27
MFP	45	25	27
PFP	45	23	38

After incubation for 3 min in the presence of each substance (200 μ M), the reaction was started by the addition of [¹⁴C]phenylalanine, [¹⁴C]tyrosine or [¹⁴C]tryptophan (10 μ M). Uptake was measured by the filter assay method as described in Materials and Methods. Uptake activities were determined from initial uptake rates (1.0 min).

the common transport system for the aromatic amino acids from a phenylalanine auxotroph KY9182. This strain, which lacks PD, can also grow on MM plate supplemented with PPA. Since the transport of PPA in *C. glutamicum* does not compete with those of the aromatic amino acids as demonstrated above, the use of strain KY9182 as the parent should facilitate selection of the desired transport mutants.

After mutagenesis of KY9182 cells, appropriate dilutions of the cell suspension were plated on MM plates containing PPA (0.2 mM) and 5FT (1 mM). 5FT-resistant colonies developed were transferred by replica-plating to MM plates supplemented with low (0.05 mM) and high (1 mM) concentrations of phenylalanine. Mutants that could grow on the phenylalanine-rich plate but not on the phenylalanine-poor plate were obtained at a relatively high

TABLE 2. Growth responses of strains KY9182, HCA1 and its transformants to phenylalanine, PPA, Gly-Phe and 5FT

Strain /Group	None	Phenylalanine (mM)			PPA (mM)	Gly-Phe (mM)	5FT ^a (mM)
		0.005	0.05	0.5	0.05	0.05	1
KY9182	-	-	+	++	+	+	-
HCA1	-	-	-	+	+	+	+
HCR101	-	-	+	++	+	+	-
TFS-1	++	++	++	++	++	++	+
TFS-2	-	+	++	++	+	+	-

^a In the presence of PPA (0.05 mM).

Growth on MM plate containing phenylalanine, PPA, Gly-Phe, or 5FT with PPA was assessed after 42 h of incubation at 30°C. Growth response is signified as follows: ++, good growth; +, weak growth; -, no growth.

frequency (approximately 30%). They retained the ability to grow in the presence of a low concentration of PPA or Gly-Phe. The phenotypic differences between strain KY9182 and one of the mutants, HCA1, are shown in Table 2. When spread on a phenylalanine-poor plate, HCA1 cells spontaneously yielded variants less dependent on phenylalanine at a frequency of about 10^{-8} . These variants concurrently reverted to being sensitive to 5FT as shown with one revertant, HCR101 (Table 2). This result indicated that a single mutation caused the two phenotypic changes of strain HCA1: resistance to 5FT and requirement for a larger amount of phenylalanine. The author then assessed whether the common transport system for the aromatic amino acids was impaired in mutant HCA1. As shown in Fig. 1, its transport activities of all three aromatic amino acids were found to be markedly reduced as compared with those of parent strain KY9182. These results confirmed that the uptake of aromatic amino acids in *C. glutamicum* was mediated by a common transport system, and that the transport system was destroyed in HCA1, the 5FT-resistant mutant requiring a high concentration of phenylalanine.

Cloning of a DNA fragment responsible for the uptake of aromatic amino acids.

In order to isolate a DNA fragment involved in the uptake of aromatic amino acids, a shotgun cloning experiment was carried out by using the transport mutant, HCA1, as a recipient. Genomic DNA of a *C. glutamicum* wild-type strain, ATCC 31833, and vector pCE54 were digested with *Eco*RI partially and completely, respectively, and ligated. The genomic DNA library was used to transform the protoplasts of mutant HCA1 requiring a high concentration of phenylalanine. Transformants were selected on RCGA plates containing kanamycin (200 µg/ml). Regenerated kanamycin-resistant colonies were transferred by replica-plating to MM agar plates containing kanamycin (10 µg/ml) and a low concentration of phenylalanine (0.05 mM). Fifteen colonies formed were classified in two groups (TFS-1 and TFS-2) based on their phenylalanine dependency (Table 2). One group TFS-1, phenylalanine-independent clones

(4 colonies), had plasmids with a common 6.2 kb EcoRI insert which contained an additional EcoRI site. The restriction enzyme cleavage analysis revealed that the insert of one of the plasmids contained the prephenate dehydratase gene cloned previously by the author (12). On the other hand, the other group TFS-2, phenylalanine-dependent clones (11 colonies), had plasmids with a common 6.0 kb EcoRI insert which contained unique XhoI and KpnI sites. Although these transformants of strain HCA1 maintained a phenylalanine-auxotrophic phenotype, they grew in the presence of a lower concentration of phenylalanine than that required for growth of strain KY9182 and also lost the 5FT-resistant phenotype (Table 2). These results suggested that the cloned segment had a gene region responsible for the uptake of aromatic amino acids. This was examined by measuring the transport activities of aromatic amino acids in strain HCA1 retransformed with pCA5, a plasmid which was isolated

from one of the clones. As illustrated in Fig. 1, its ability to take up the aromatic amino acids was completely restored, and the levels of transport activities were even higher than those observed in strain KY9182 which is wild-type with respect to the uptake of aromatic amino acids. These results confirmed that the 6.0 kb DNA fragment cloned onto pCA5 contained a gene region involved in the uptake of aromatic amino acids.

Influence of the uptake of aromatic amino acids on their productivity.

The author investigated how the productivity of aromatic amino acid-producing strains was influenced by their possession of the uptake activities of the same amino acids as follows.

First, strain KY9182 and its transport mutant HCA1, neither of which produced any amino acids, were engineered to produce the aromatic amino acids by introducing the recombinant plasmid pKF1 or pKW9901 which contains the genes for a set of deregulated enzymes involved in phenylalanine or tryptophan biosynthesis, respectively. As shown in Table 3, in either case of pKF1- and pKW9901-carrying strains, the production of aromatic amino acids was apparently higher in the HCA1 background than in the KY9182 background, indicating that the defect in the uptake of aromatic amino acids contributed to increased production of the aromatic amino acids.

Subsequently, plasmid pCA5 was introduced into tryptophan-producing mutants and the influence of increased uptake activities of aromatic amino acids on tryptophan production was examined (Table 3). Introduction of pCA5 into a tryptophan-producing strain, KY9225, resulted in a drastic decrease in tryptophan productivity, which was about one-tenth that of the host strain. However, the pCA5-induced inhibition of tryptophan production in this producing mutant was alleviated by further introduction of the compatible plasmid pKW9901 that coexpresses highly deregulated tryptophan-biosynthetic enzymes. When pCA5 was introduced into a tryptophan and lysine co-producer SL64, only tryptophan production was greatly inhibited in the transformed

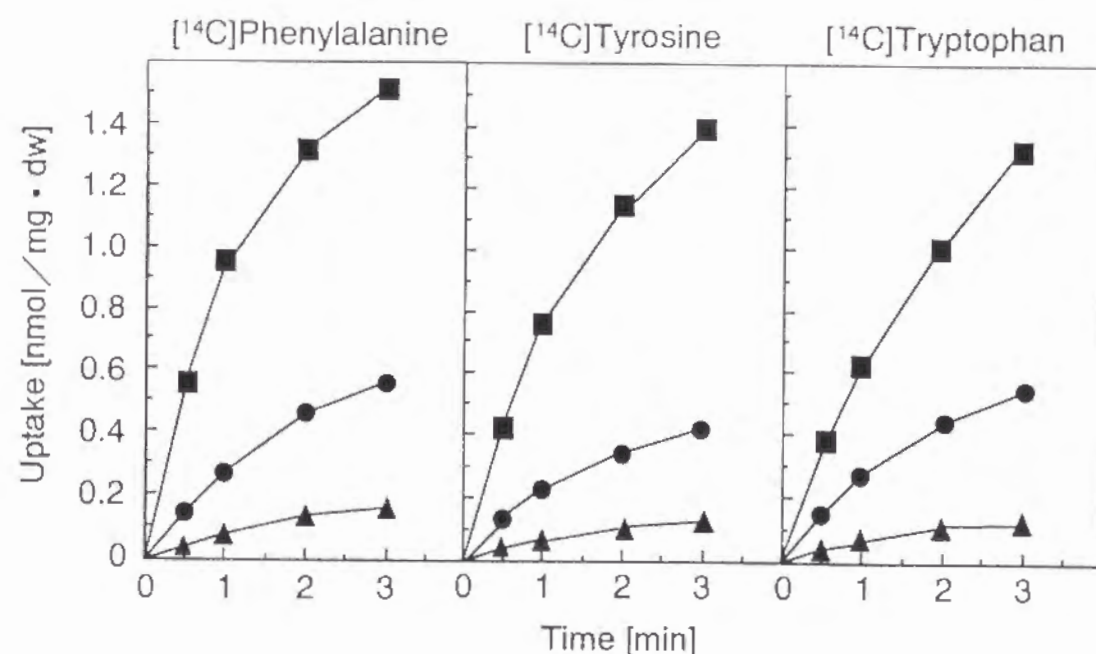


FIG. 1. Uptake of aromatic amino acids by strains KY9182, HCA1 and HCA1 carrying pCA5. Uptake by KY9182 (●), HCA1 (▲), and HCA1 carrying pCA5 (■) was measured as described under Materials and Methods.

strain, while lysine production remained unchanged. These observations clearly showed that increased uptake activities of aromatic amino acids negatively affected overproduction of tryptophan.

TABLE 3. Effect of aromatic amino acid uptake on productivity

Strain (plasmid)	Growth (OD660)	Amino acids produced ^a (g/liter)			
		Phenylalanine	Tyrosine	Tryptophan	Lysine
KY9182 (pKF1)	50	4.1	0.0	0.0	0.0
HCA1 (pKF1)	49	5.2	0.0	0.0	0.0
KY9182 (pKW9901)	40	0.0	3.3	1.1	0.0
HCA1 (pKW9901)	38	0.0	4.3	2.0	0.0
KY9225	20	0.0	0.0	8.5	0.0
KY9225 (pCA5)	21	0.0	0.0	0.9	0.0
KY9225 (pKW9901)	21	0.0	0.0	12.3	0.0
KY9225 (pCA5, pKW9901)	21	0.0	0.0	7.0	0.0
SL64	34	0.0	0.0	5.8	4.0
SL64 (pCA5)	33	0.0	0.0	0.6	4.0

^a Production was carried out in test tubes as described under Materials and Methods.

DISCUSSION

The uptake of aromatic amino acids in *C. glutamicum* was investigated in this study, which allowed the author to conclude that phenylalanine, tyrosine and tryptophan were taken up into cells mainly by a common transport system in this bacterium. This conclusion is based on the following observations: (i) the specificity of the uptake of aromatic amino acids was similar; (ii) the uptake of each aromatic amino acid was inhibited by either of the other two aromatic amino acids; and (iii) a point mutation resulted in the simultaneous reduction of the uptake activities of all three aromatic amino acids. In relation to the aromatic amino acid uptake in *C. glutamicum* and its relatives, two findings have so far been reported (21, 28). One is competition among the aromatic amino acids in growth experiments with amino acid-auxotrophs of *C. glutamicum* (syn. *M. glutamicus*) and the other is competition between phenylalanine and tyrosine in uptake experiments with *B. flavum*. Both observations can be reasonably explained by the common transport system for the aromatic amino acids.

A common transport system has also been found for the branched-chain amino acids in *C. glutamicum* by Ebbighausen *et al.* (6). They examined the properties of the uptake of branched-chain amino acids and showed that the uptake of these amino acids occurred via a common specific carrier. Similarly, the transport system for the aromatic amino acids appears to involve a single membrane-bound carrier without the participation of periplasmic binding proteins, because not only strain HCA1 but other transport mutants that had been isolated from strain KY9182 were complemented by plasmid pCA5 with respect to the phenotype of requirement for a high concentration of phenylalanine (data not shown). The transport system in question is considered to be the main system responsible for the uptake of aromatic amino acids in this bacterium since its mutants have lost the ability to take up the aromatic amino acids. Nevertheless, the possibility of the existence of a

second low-affinity system cannot be excluded.

Recent biochemical studies on the transport mechanism in C. glutamicum have suggested that there exists a specific efflux carrier system for each of lysine (4), glutamate (11) and isoleucine (7), which is not related to the individual uptake system. Furthermore, Seep-Feldhaus et al. reported the cloning of the lysine uptake gene of C. glutamicum and showed by a gene disruption experiment that the gene was not involved in lysine excretion (27). On the other hand, nothing is known about the efflux system for the aromatic amino acids in this bacterium. However, it is interesting to note that introduction of pKW9901 or pKF1 into strain HCA1 allowed the mutant to excrete the aromatic amino acids, because this indicates that excretion of the amino acids occurs normally even when their uptake system remains destroyed. Based on this finding, it can be assumed that the uptake system for the aromatic amino acids in C. glutamicum is independent of their excretion, although it is not clear whether this bacterium possesses the efflux system(s) for the aromatic amino acids or not.

In the experiment using KY9182 and HCA1 as host strains, it has been shown that the defect in the uptake of aromatic amino acids resulted in increased production of the aromatic amino acids. An explanation for this can be that prevention of the uptake of aromatic amino acids accumulated extracellularly caused a reduction of the steady-state levels of the intracellular aromatic amino acids, which would highly deregulate the corresponding biosynthetic pathways. In contrast, the reason why introduction of pCA5 into strains KY9225 and SL64 resulted in a drastic decrease in tryptophan production can be that higher activity of tryptophan uptake conversely caused an increase in the intracellular concentration of tryptophan, which would bring about severe feedback control of the tryptophan biosynthesis. These speculations are supported by the observation that the pCA5-induced inhibition of tryptophan production in KY9225 was alleviated under coexistence with pKW9901 which could render the tryptophan biosynthesis of the strain less

sensitive to feedback control. On the other hand, the lysine productivity of strain SL64 was not affected at all by introduction of pCA5. This observation is reasonably comprehensible because the plasmid is not related to the uptake of the aspartate family of amino acids which is known to regulate lysine biosynthesis in C. glutamicum (5, 22). Concerning uptake activity and amino acid productivity, Mori and Shiio reported that some of the B. flavum mutants, which were selected by their inability to grow on glutamate as the sole carbon and nitrogen source and also showed increased glutamate productivity, exhibited a lower rate of glutamate uptake than the parent strain (20). Based on this observation, they suggested the correlation between uptake activity and productivity of glutamate.

To date, there have been many examples representing successful applications of pathway engineering for strain improvement. In addition to the feasibility of these methods, the author has shown in this chapter that uptake systems for amino acids can also be important targets for genetic engineering. The author has now applied the strategy of prevention of amino acid uptake to further improve a tryptophan-producing strain. This strain construction will be described elsewhere.

SUMMARY

The uptake of phenylalanine, tyrosine and tryptophan by wild-type Corynebacterium glutamicum ATCC 13032 was demonstrated to occur mainly via a common transport system by competition experiments using these amino acids which were either ¹⁴C-labeled or unlabeled. Strain HCA1 defective in the common transport system was derived from a phenylalanine auxotroph, C. glutamicum KY9182, based on its resistance to 5-fluorotryptophan and requirement for a high concentration of phenylalanine. The wild-type gene responsible for the common transport system was cloned into a multicopy vector, based on its ability to make strain HCA1 less dependent on phenylalanine and sensitive to

5-fluorotryptophan. The resulting plasmid pCA5 conferred on strain HCA1 a fewfold increase in the uptake activities of all three aromatic amino acids relative to the wild-type levels. Although strains KY9182 and HCA1 acquired the productivity of aromatic amino acids by introducing a plasmid coexpressing a set of feedback-resistant enzymes involved in phenylalanine or tryptophan biosynthesis, the production levels were higher in the HCA1 strain background than in the KY9182 background. In contrast, introduction of pCA5 into tryptophan-producing *G. glutamicum* KY9225 resulted in a drastic decrease in the tryptophan production. However, when another compatible plasmid specifying feedback-resistant tryptophan-biosynthetic enzymes coexisted in the transformant, the tryptophan productivity was restored to an appreciable level. These results indicated that prevention of amino acid transport, as well as deregulation of biosynthetic pathways, was one of the crucial factors in amino acid overproduction.

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CONCLUSION

Strain construction for amino acid production in C. glutamicum and its relatives has relied entirely on random mutagenesis, selection and screening procedures. Despite the success with the development of a number of amino acid producers, the classical mutagenesis method has the limitations as a means of strain construction. It involves two disadvantages: its inability to increase the levels of particular biosynthetic enzymes and the difficulty of completely circumventing complex regulatory mechanisms in the biosynthetic pathways. Moreover, since mutants obtained by whole-cell mutagenesis often have unexpected additional mutations which result in physiological disruption, it is laborious and time-consuming to obtain a desired mutant suitable for practical use. The present study has demonstrated that recombinant DNA technology becomes a new useful means to overcome such barriers of classical mutagenesis in strain development.

The author has successfully employed recombinant DNA technology to carry out rational metabolic engineering for hyperproduction of the aromatic amino acids and to clarify the correlation between the uptake system and productivity of the aromatic amino acids in C. glutamicum. The conclusions of this study are summarized as follows.

- (1) metabolic conversion by amplifying branch-point enzyme(s) is useful in allowing production of another metabolite in high yield and with ease.
- (2) the use of intergeneric genes, especially well-characterized E. coli genes, is beneficial for desired metabolic engineering of C. glutamicum.
- (3) amplifying the first enzyme in a pathway diverging from central carbohydrate metabolism to increase carbon flow down that pathway, followed by sequential removal of bottlenecks discerned by the accumulation of intermediates, can be a useful strategy for yield improvement of primary metabolites.
- (4) a unique plasmid stabilization system, based on the presence of a serine-

biosynthetic gene on the plasmid and its absence from the chromosome, can be used to provide selective pressure for maintenance of the plasmid in large-scale fermentation.

- (5) prevention of amino acid transport is one of the crucial factors in amino acid overproduction.

The genetic engineering strategies presented here should be generally applicable to the yield improvement of amino acids. By combining these strategies, more advances will undoubtedly be achieved in amino acid production by these organisms.

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