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MOLECULAR BREEDING OF AROMATIC

AMINO ACID - PRODUCING

CORYNEBACTERIUM GLUTAMICUM

MASATO IKEDA

1994

CONTENTS

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 fermentat-
ions 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

"Glutamic acid bacteria"   Amino acids

| Corynebacterium glutamicum | | Wild-type | L-Glu L-Gln |
| | lilium callunae acetoacidophilum | | |

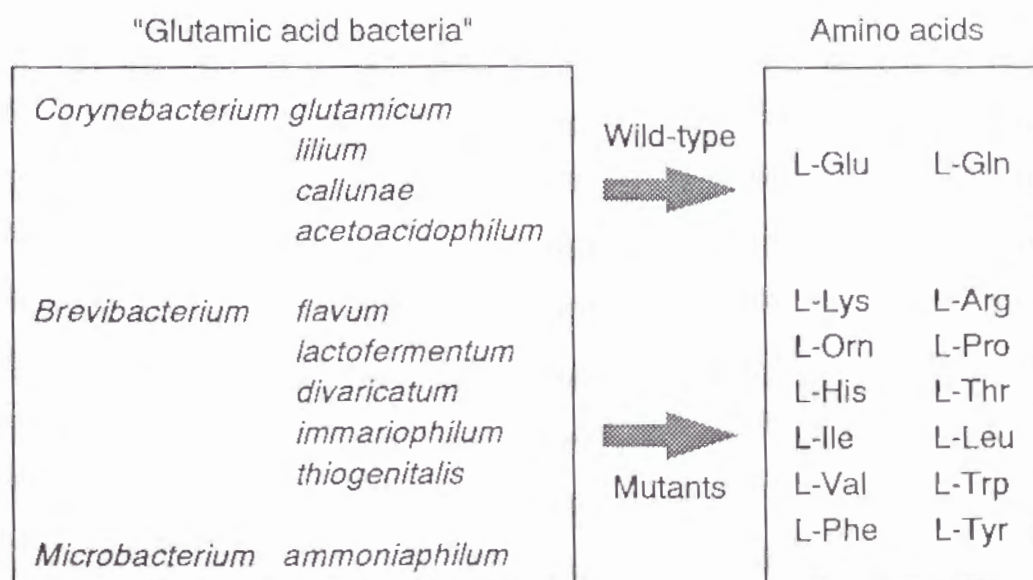| Brevibacterium | flavum lactofermentum divaricatum immariophilum thiogenitalis | Mutants | L-Lys L-Arg / L-Orn L-Pro / L-His L-Thr / L-Ile L-Leu / L-Val L-Trp / L-Phe L-Tyr |

| Microbacterium | ammoniaphilum | | |

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 indnstrial 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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Chapter 1. Metabolic Engineering to Produce Tyrosine or Phenylalanine in a
    Tryptophan-Producing Corynebacterium glutamicum Strain

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
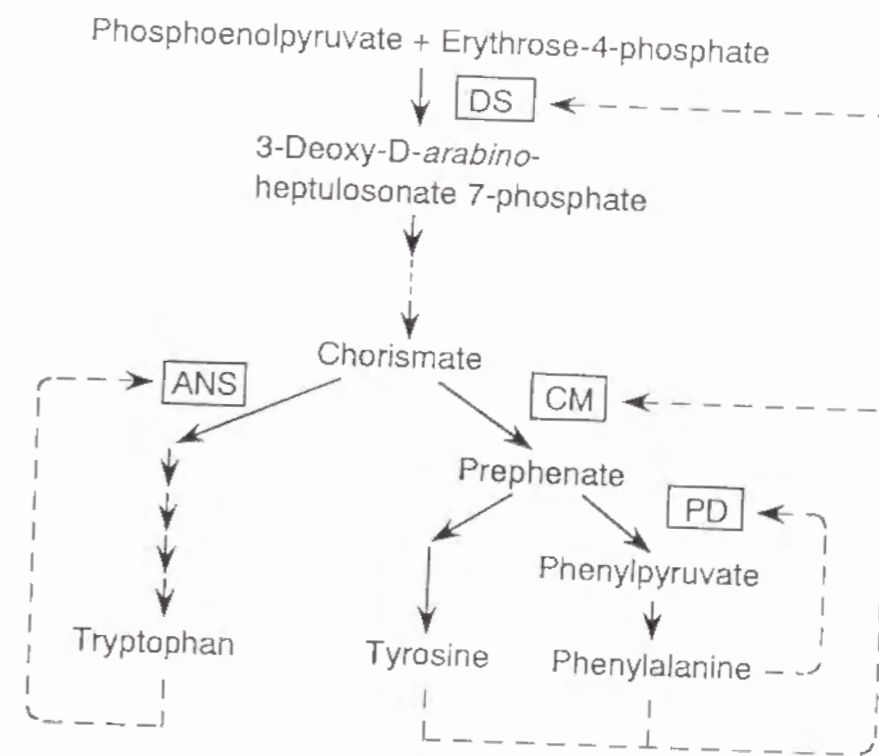construction is presented in this chapter.

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

MATERIALS AND METHODS

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 RCGA plates or 100 μg/ml for BY and MM plates;

kanamycin, 200 μg/ml for RCGA 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; $MgSO_4 \cdot 7H_2O$, 1 g; $(NH_4)_2SO_4$, 20 g; corn steep liquor, 10 g; $MnSO_4 \cdot 4 \sim 6H_2O$, 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; $MgSO_4 \cdot 7H_2O$, 0.5 g; $(NH_4)_2SO_4$, 5 g; urea, 0.6 g; corn steep liquor, 40 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $MnSO_4 \cdot 4 \sim 6H_2O$, 10 mg; $CuSO_4 \cdot 5H_2O$, 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; $MgSO_4 \cdot 7H_2O$, 1.7 g; $(NH_4)_2SO_4$, 17 g; corn steep liquor, 66 g; $FeSO_4 \cdot 7H_2O$, 13 mg; $MnSO_4 \cdot 4 \sim 6H_2O$, 13 mg; $CuSO_4 \cdot 5H_2O$, 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 BYG medium containing phenylalanine and tyrosine (200 μg/ml each). After 24 h of cultivation at 30℃, 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℃ 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℃ 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℃, 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℃ 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℃ 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℃.

Analysis.

Cell growth was monitored by measuring the OD_{660} 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 SalI, 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 10^5 spectinomycin-resistant transformants. All plasmids purified from these clones were shown to have a common 6.7 kb SalI 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 eightold-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 SalI, 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 10⁴ transformants were generated on RCGA plates containing kanamycin (200 μg/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 SalI 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 BamHI-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 BamHI 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 SalI was ligated with completely SalI-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 BamHI fragment containing the PD gene on pCA3 was ligated with pKY1 digested with BglII 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
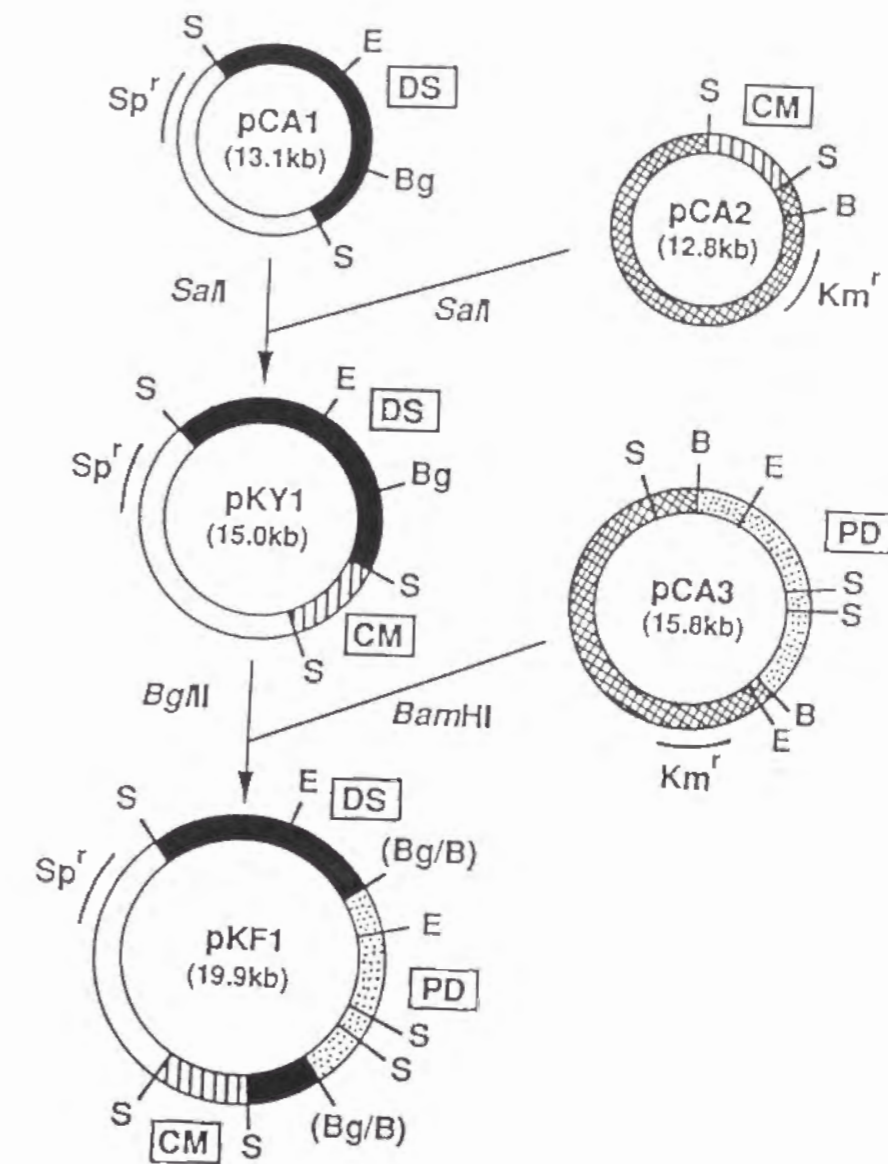

FIG. 2. Construction of recombinant plasmids containing aromatic amino acid-biosynthetic genes. Symbols: solid bars, C. glutamicum KY10694 chromosomal DNA fragment containing the DS gene; hatched bars, C. glutamicum KY10694 chromosomal DNA fragment containing CM gene; stippled bars, C. glutamicum KY10693 chromosomal DNA fragment containing the PD gene; open bars, pCG115; cross-hatched bars, pCE53. Abbreviations: B, BamHI; Bg, BglII; E, EcoRV; S, SalI; Km ͬ, kanamycin resistance; Sp ͬ, spectinomycin resistance.

genes and was designated pKFl.

<u>DS, CM and PD activities of the recombinant strains.</u>

　　To confirm the expression of the biosynthetic genes on pKYl and pKFl, the
relevant enzyme activities in the crude cell extract of strain KLS4 carrying
each plasmid were measured (Table 1). The presence of pKYl 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 pKFl-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 pCAl 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 <u>C. glutamicum</u> 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.

<u>Effects of the recombinant plasmids on metabolic flow in a tryptophan-
producing strain.</u>

　　It was examined whether the recombinant plasmids had effects on the
metabolic flow in a tryptophan-producing strain KYI0865 (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 KYI0865 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.
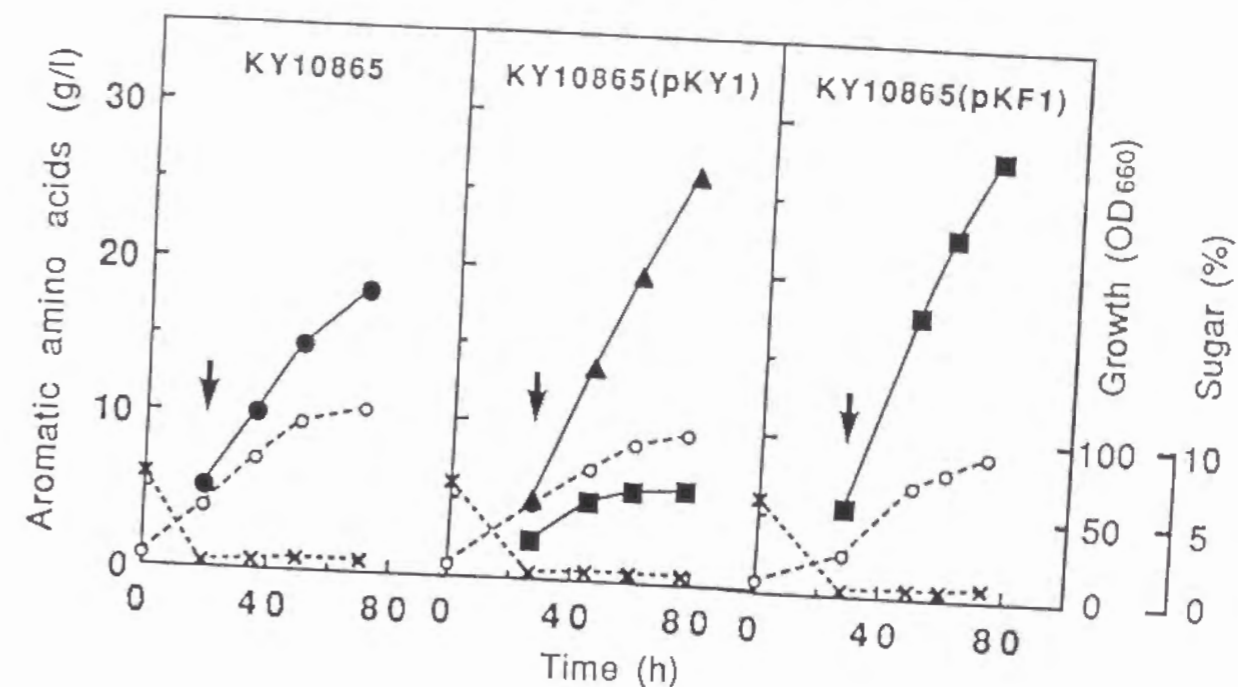

FIG. 3. Time course of aromatic amino acid production by strain KY10865 and its plasmid-carrying strains in jar fermentors. Symbols: ●, tryptophan; ▲, tyrosine; ■, phenylalanine; ○, growth (OD660); ×, sugar. Arrows indicate the points at which solution containing 44% sucrose began to be fed continuously.

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 rechanelled 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 rechannelling 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 ß-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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Chapter 2. Phenylalanine Production by Metabolically Engineered

Corynebacterium _glutamicum_ with the _pheA_ Gene of _Escherichia_ _coli_

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]
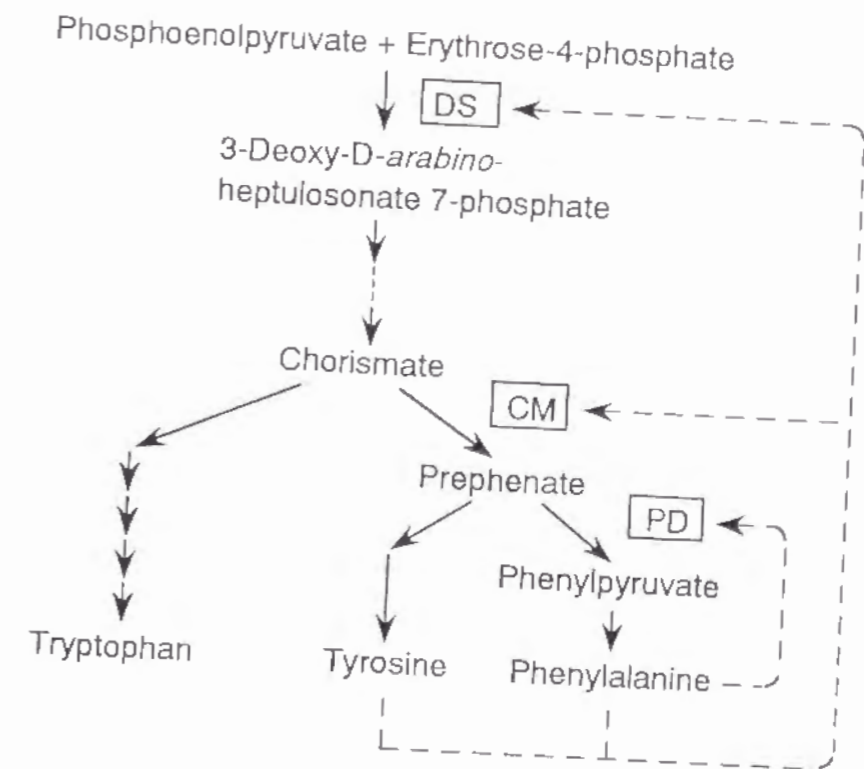

FIG. 1. Pathway and primary regulations of phenylalanine biosynthesis in _C._ _glutamicum_. Broken arrows indicate feedback inhibition.

molecule, not a repressor protein as previously suggested (13), and regulates pheA expression via attenuation control of transcription. Besides the transcriptonal 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 prodnction 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 tbe 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 tbe 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. PP1 medium used for production in test tubes contained (per liter): cane molasses, 100 g (as sugar); $(NH_4)_2SO_4$, 20 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; corn steep liquor, 2.5 g; L-tyrosine, 100 mg; and $CaCO_3$, 20 g (pH 7.2). PS1 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 $CaCO_3$, 10 g (pH 7.2). PP2 medium used for production in 2-liter jar fermentors contained (per liter): cane molasses, 50 g (as sugar); KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $(NH_4)_2SO_4$, 20 g; $MnSO_4 \cdot 4\sim6H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$, 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℃, 0.5 ml of the seed culture was transferred to a large test tube containing 5 ml of PP1 medium. Cultivation was carried out aerobically at 30℃ 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 PS1 medium in a 1-liter flask.

After 24 h of cultivation at 30℃ 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℃, 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 pEA11 were performed in the following in vivo mutagenesis. Strain KLS4 carrying pEA1 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℃. 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℃. 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℃ 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℃.

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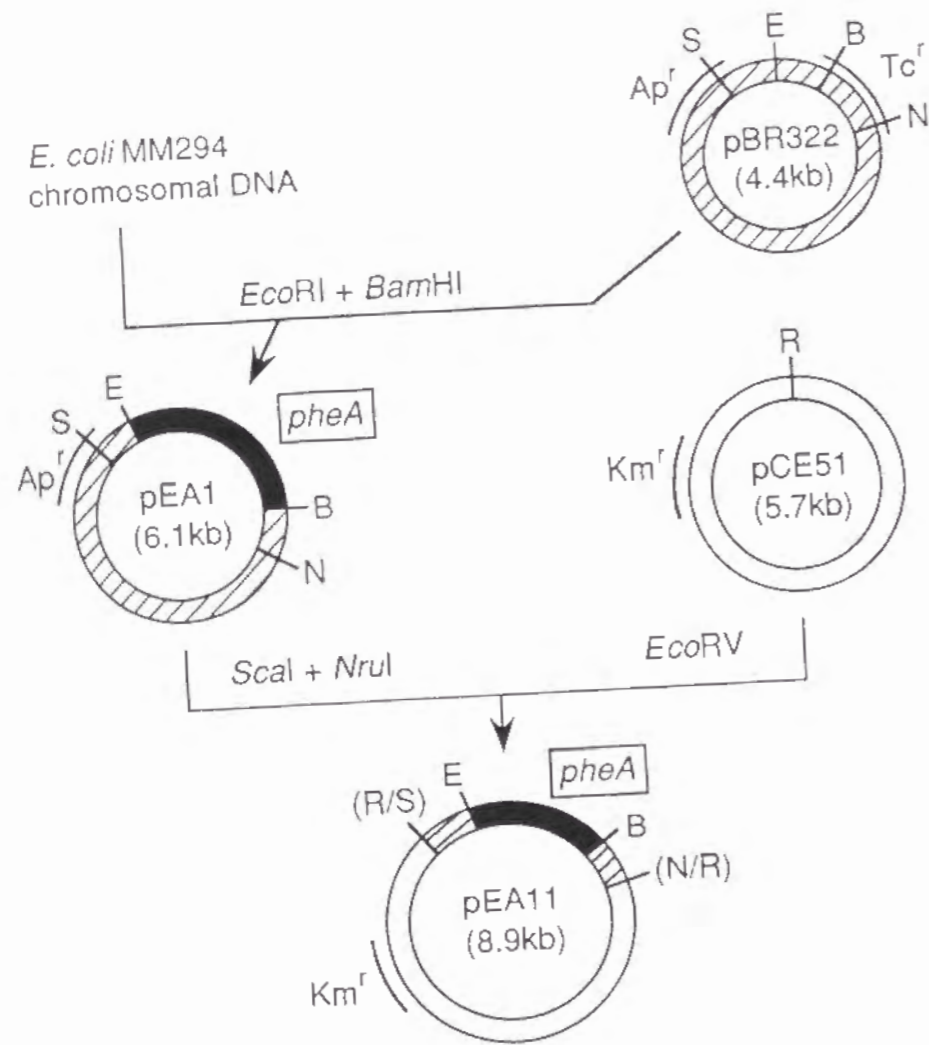
FIG. 2. Construction of plasmid pEA11. Symbols: solid bars, E. coli MM294 chromosomal DNA containing the pheA gene; S, ScaI; E, EcoRI; B, BamHI; N, NruI; R, EcoRV; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance.

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. glntamicum.

The pheA gene is known to be subject to an attenutation 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 tranferred 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
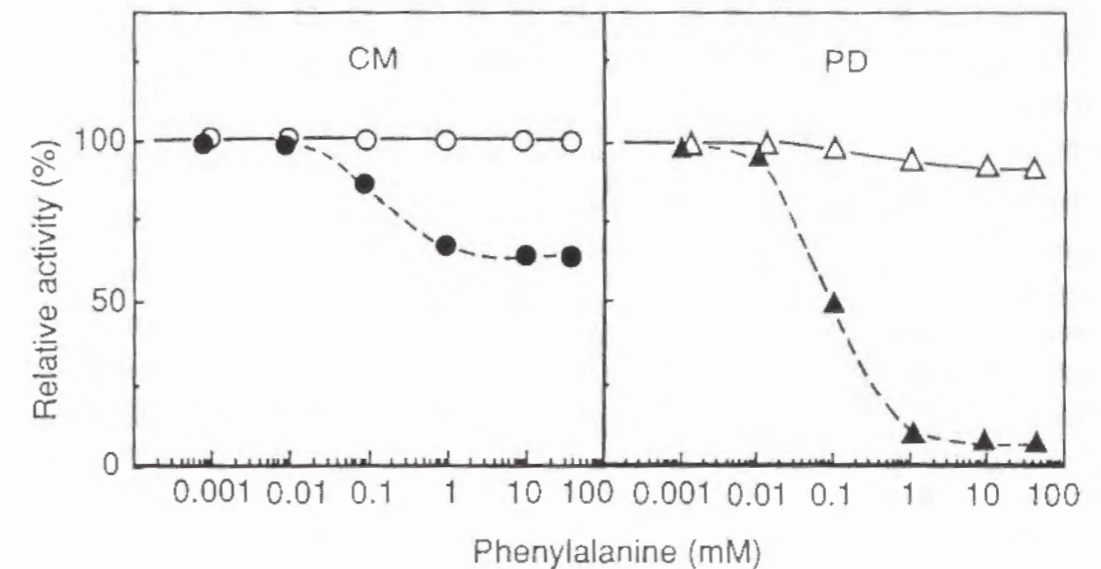

FIG. 3. Effect of phenylalanine on CM and PD activities. Symbols: ●, CM activity of KLS4 carrying pEA11; ○, CM activity of KLS4 carrying pEA22; ▲, PD activity of KLS4 carrying pEA11; △, PD activity of KLS4 carrying pEA22.

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.
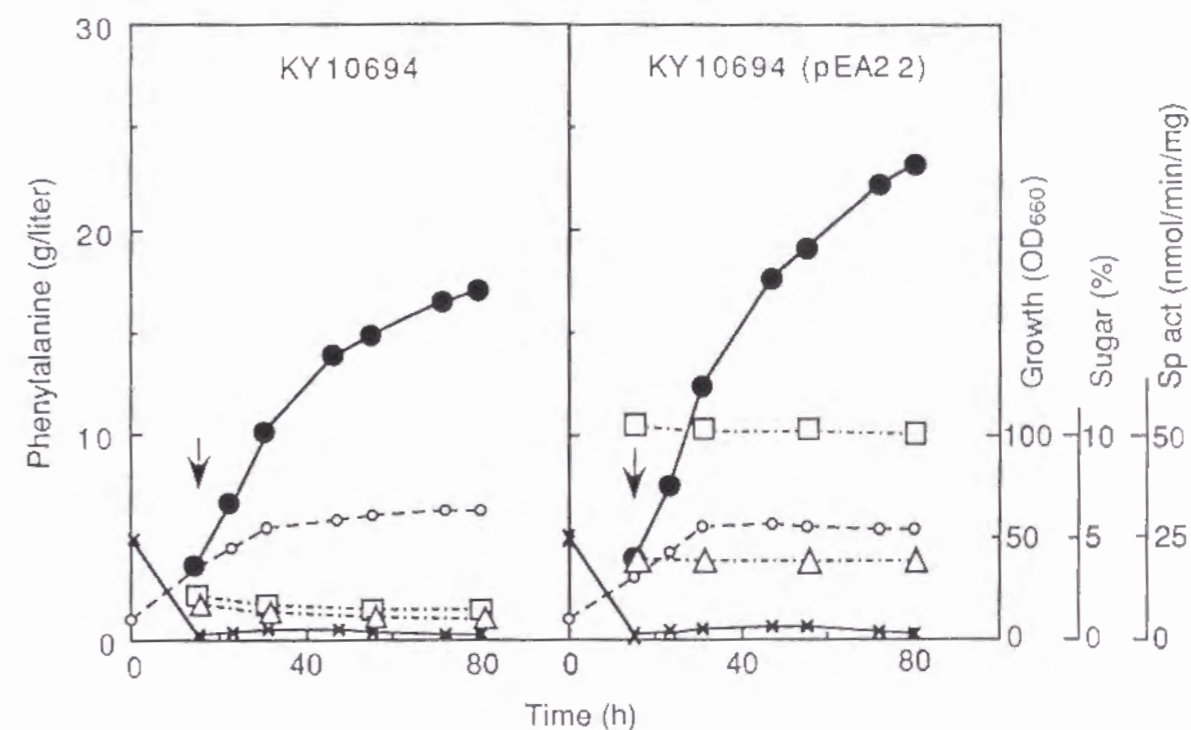

FIG. 4. Time course of phenylalanine production by strain KY10694 and its pEA22-carrying strain in jar fermentors. Symbols: ●, phenylalanine; ○, growth; ×, sugar; □, CM activity; △, PD activity. Arrows indicate the points at which solution containing 36% (as sugar) cane molasses began to be fed continuously.

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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Chapter 3. Hyperproduction of Tryptophan in Corynebacterium glutamicum by
 Pathway Engineering

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, Corynebacte-
rium 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
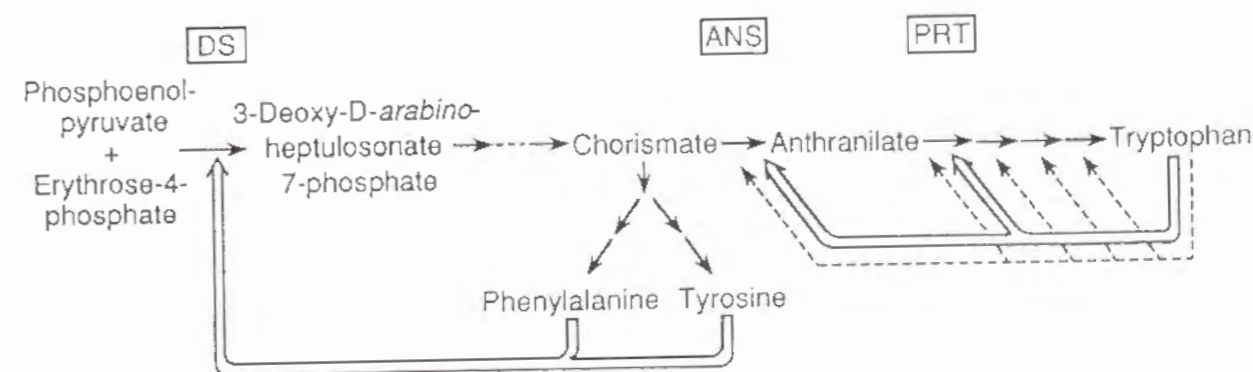procedures from the same organisms (14). The cause of this productivity

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 alterlations 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, iclR7] 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 SalI 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℃ 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℃, 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 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 $CaCl_2$ 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℃. 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℃. 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℃.

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 phoshate 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 TP1 medium that contains 6% glucose (Table 1).

In improving strain KY10894, the author first attemted 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 BamHI 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 BamHI 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 BamHI 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 SalI-, BglII- and EcoRI-fragments into pCE53 and examined for the ability of the resultant plasmids to complement E. coli trp mutants. The complementation results shown
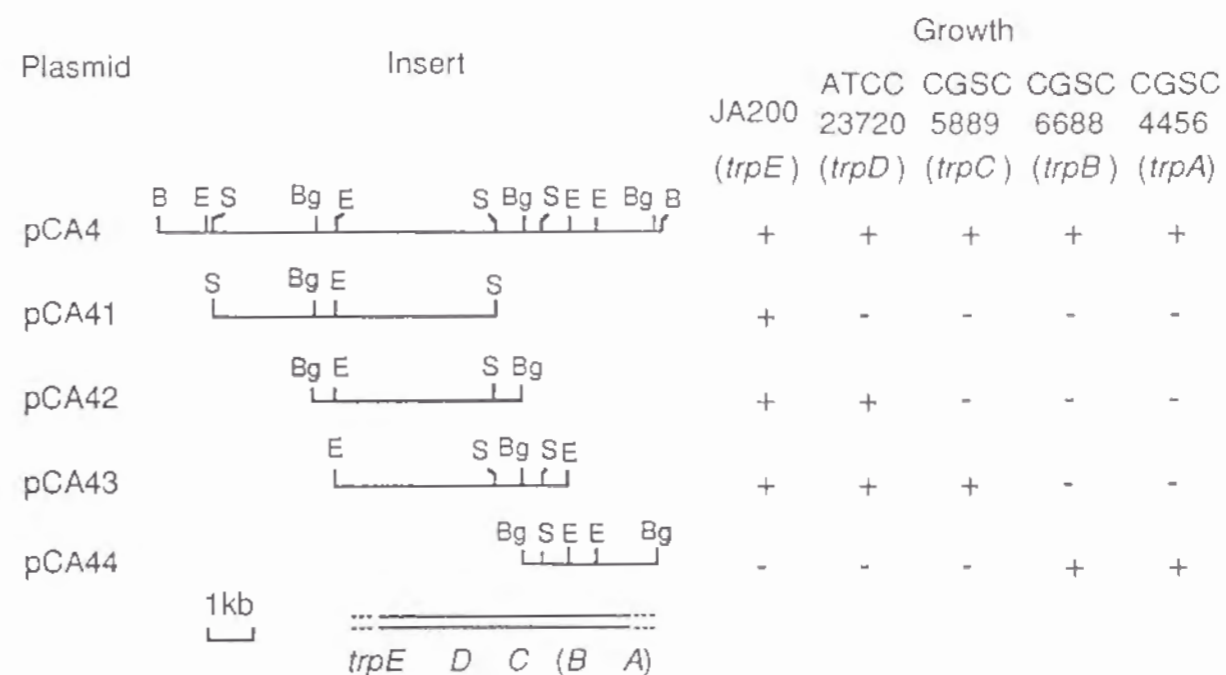

FIG. 2. Restriction cleavage maps of the cloned DNA fragments and complementation of E. coli trp mutants by its subclones. The results of complementation tests were shown as + (growth) and - (no growth). The most probable coding regions of the trp genes are shown at the bottom. (B A) indicates that the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

in Fig. 2 ensured that the tryptophan-biosynthetic gene cluster of C. glutami-
cum was localized within a 7.0 kb EcoRl-BglII region.

Construction of pKWl and its effect on tryptophan production.

The limitation of ANS reaction, encountered in the pCAl-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
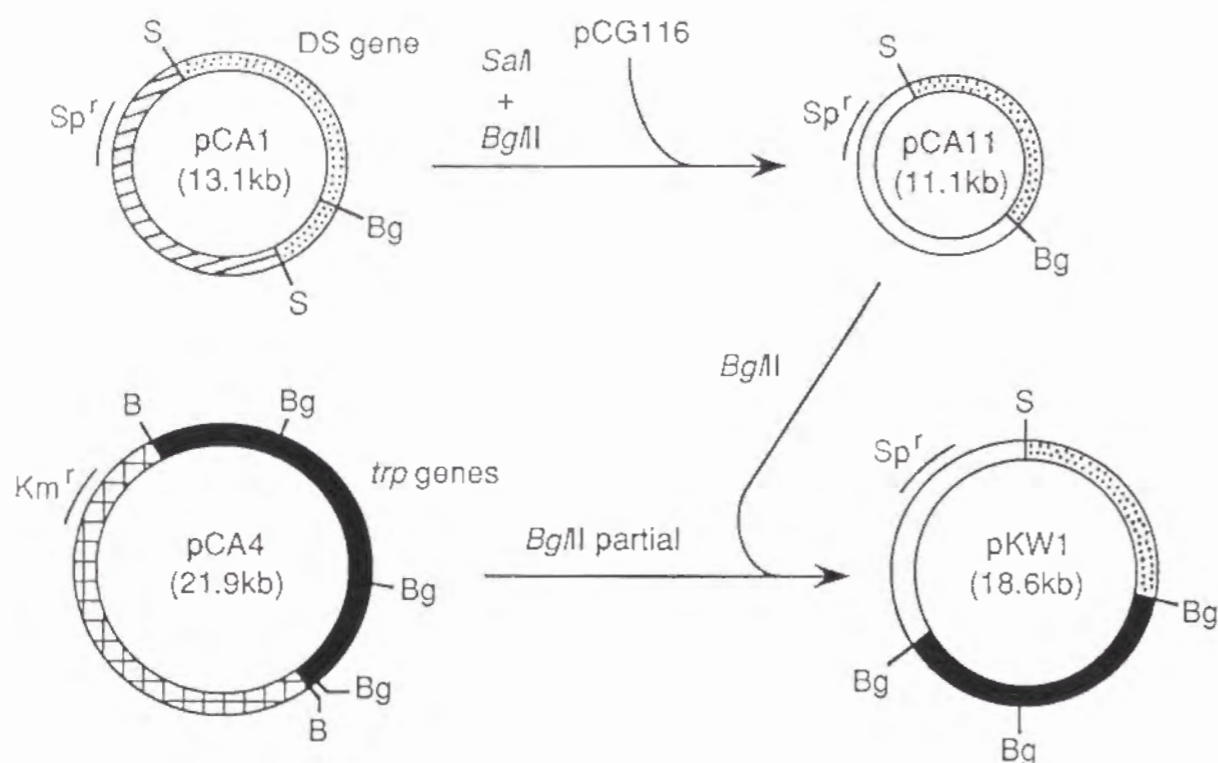cluster and the DS gene were assembled onto one multicopy vector as depicted

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, Sall; Bg, BglII; B, BamHI; Sp^r, spec-
tinomycin resistance; Km^r, kanamycin resistance.

in Fig. 3. As the larger 5.0 kb Sall-BglII segment of the Sall-insert
originally cloned onto pCAl was determined to contain the intact DS gene (data
not shown), it was subcloned into pCGl16 to generate pCAll. Separately, pCA4
that contains the entire tryptophan-biosynthetic gene cluster was partially
digested with BglII and electrophoresed on agarose gel. The 7.5 kb fragment
separated on the gel was purified and then ligated with BglII-digested pCAll.
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 pKWl (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 pKWl 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 pCAl carrier,
tryptophan production was adversely reduced to a low level even relative to
the host alone. It, however, was found that the pKWl-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
supressed 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 pKWl.
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
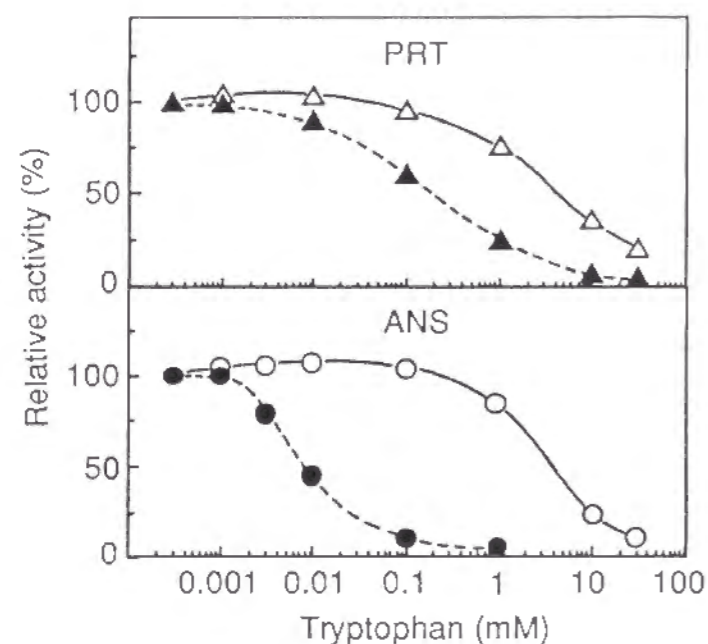

FIG. 4. Tryptophan-sensitivity of PRT and ANS encoded by pKW1 and pKW99. Symbols: ▲, PRT activity of KLS4 carrying pKW1; △, PRT activity of KLS4 carrying pKW99; ●, ANS activity of KLS4 carrying pKW1; ○, ANS activity of KLS4 carrying pKW99.

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 µg/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 summerized 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
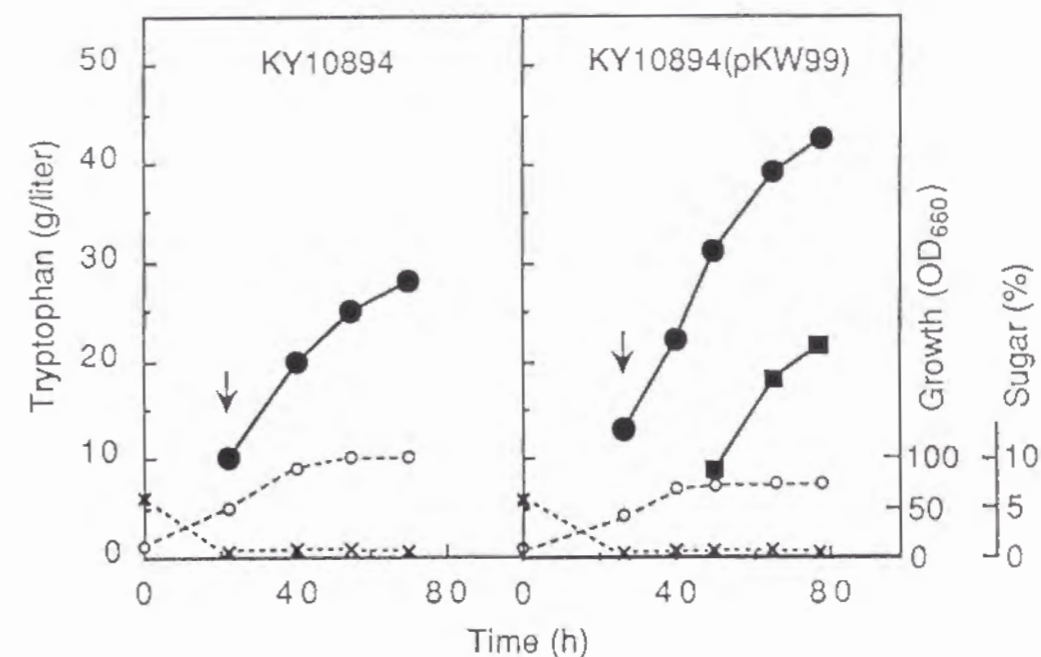

FIG. 5. Time course of tryptophan production by strain KY10894 with and without pKW9901 in jar-fermentor cultivations. Symbols: ●, tryptophan; ■, tryptophan crystallized in the medium; ○, growth; ×, sugar. Arrows indicate the points at which feeding with a 44% sucrose solution began.
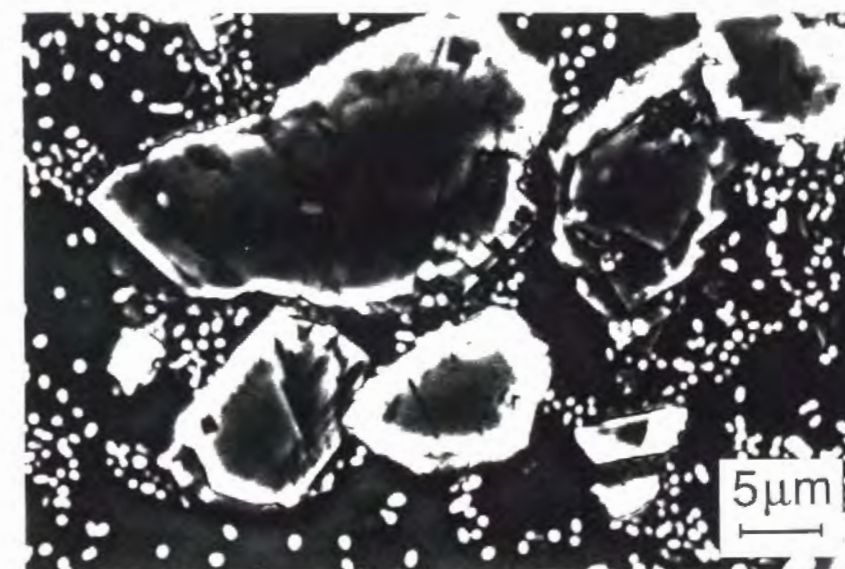

FIG. 6. Phase-contrast microphotograph of crystals of tryptophan and C. glutamicum cells with pKW99 in the jar-fermentation broth.

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 consistant 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 nonmetaboliza-
ble 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
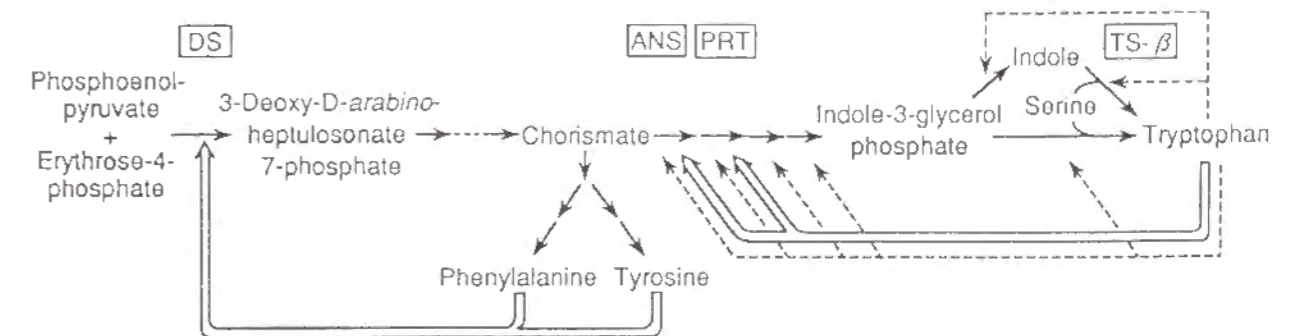intermediate accumulation. The successful metabolic engineering ultimately

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 BYG 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 NH₄OH.

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 bacterio- cidal 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 μg/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.
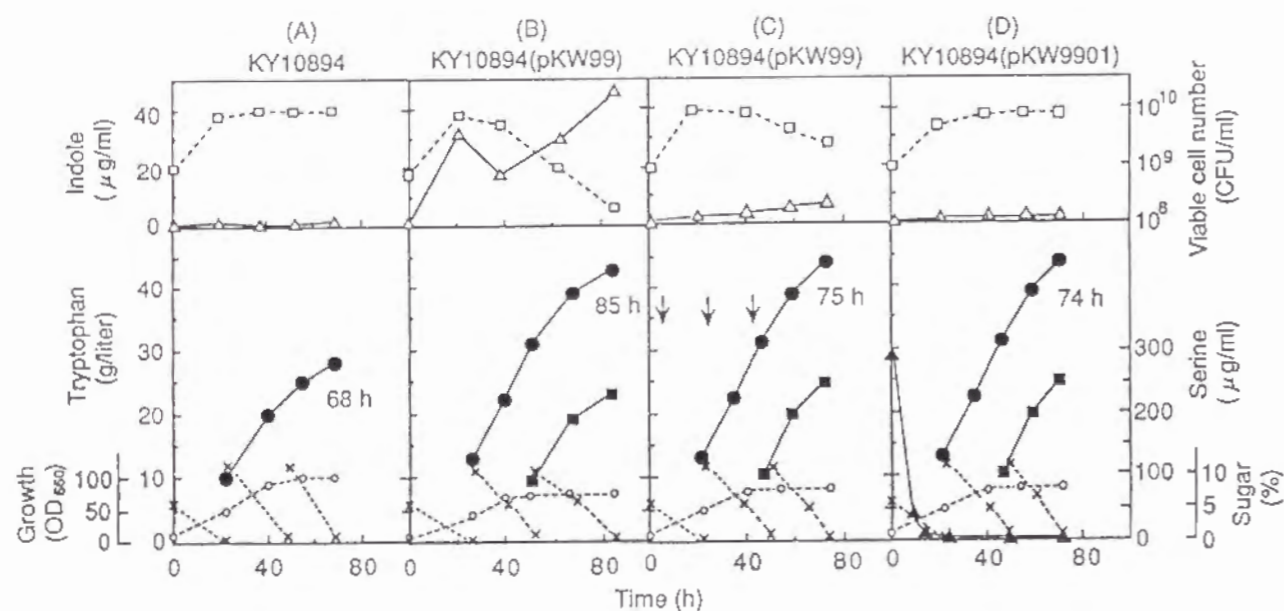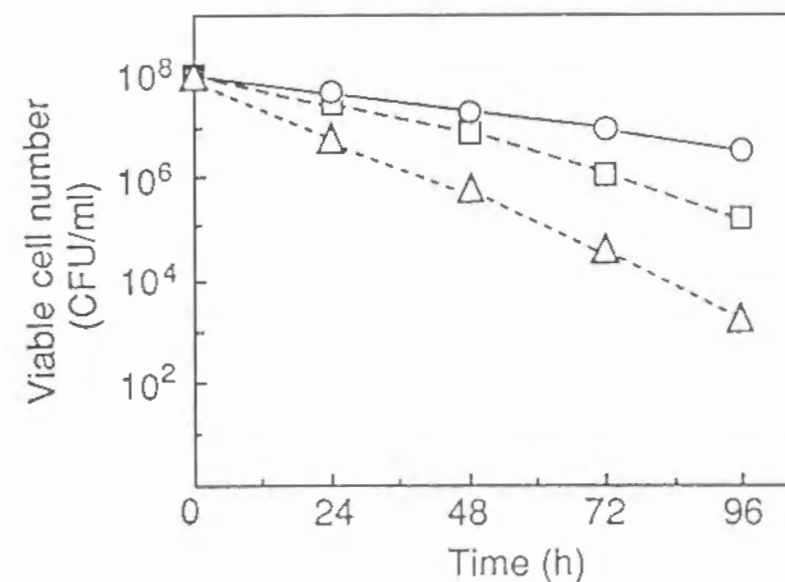

FIG. 2. Indole formation and cell viability in tryptophan production process with jar fermentors. Symbols: ●, tryptophan; ■, tryptophan crystallized in the medium; ○, growth; ×, sugar; △, indole; □, viable cell number; ▲, serine. Arrows shown in (C) indicate the points at which serine (5 g each) was added. Cultivations of recombinant strains were carried out in medium with spectinomycin (100 μg/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 μg/ml; □, 20 μg/ml; △, 40 μg/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

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.

DNA of C. glutamicum wild-type strain ATCC 31833 and vector pCG116 were digested with SalI, 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 μg/ml). Regenerated spectinomycin-resistant colonies were transferred by replica-plating to MM agar plates containing spectinomycin (100 μg/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 SalI 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 BamHI 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 BamHI-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
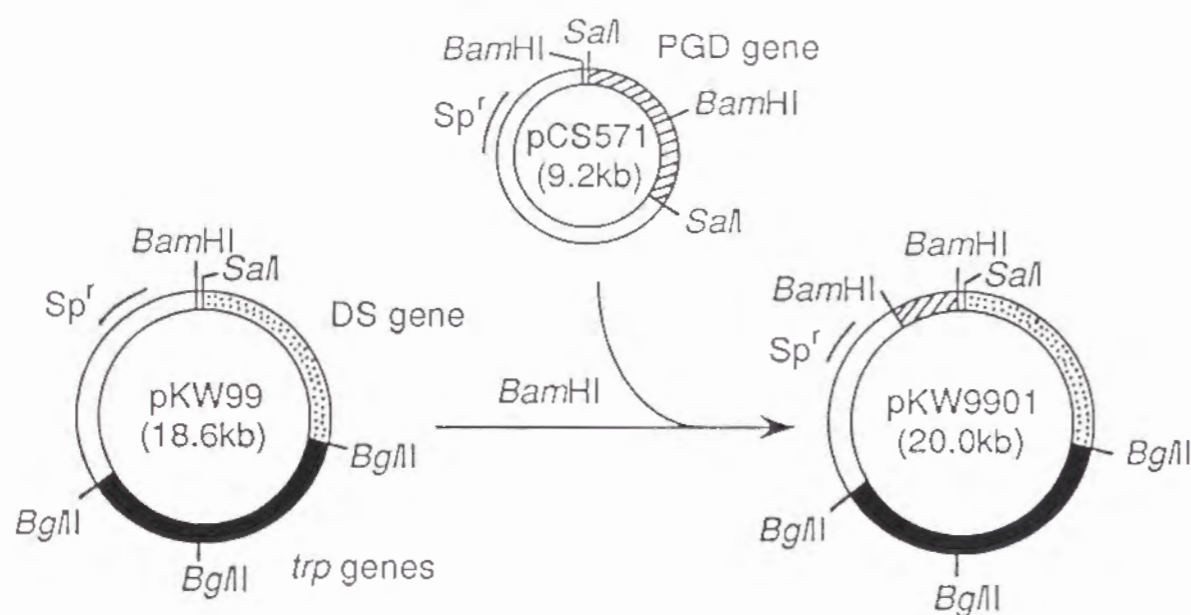
shorter cultivation time (74 h) than in the cases of the pKW99-carrying
strain. However, the recombinant plasmid pKW9901 showed a similar instability
to pKW99 in strain KY10894 under cultivation without antibiotic pressure
(Table).

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

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.

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.

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
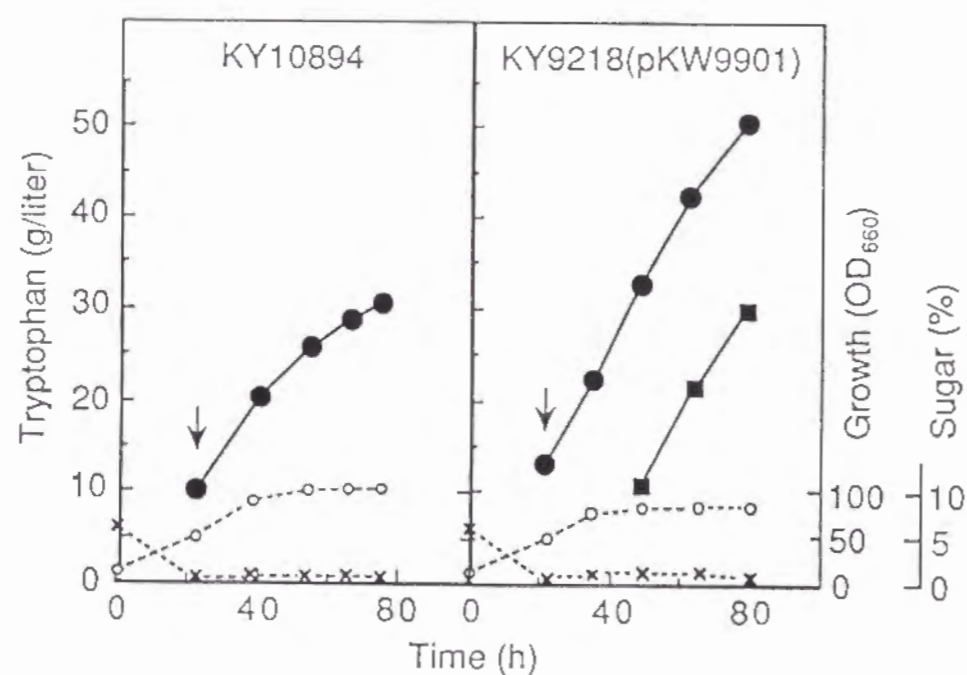

FIG. 5. Time course of tryptophan production by strain KY10894 and the recombinant strain
KY9218 carrying pKW9901 with jar fermentors. Symbols: ●, tryptophan; ■, tryptophan
crystallized in the medium; ○, growth; ×, sugar. Arrows indicate the points at which
solution containing 44% sucrose began to be 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 benefical 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%. RCGA 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-^{14}C]phenylalanine (450 mCi/mmol), L-[U-^{14}C]tyrosine (450 mCi/mmol) and L-[methylene-^{14}C]-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 MgSO$_4$, and suspended in the same buffer to a final OD$_{660}$ 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 MgSO$_4$, 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 ^{14}C-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 Packared 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-nitroso-guanidine (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).

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 glycyI-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 μM), the reaction was started by the addition of [^{14}C]phenylalanine, [^{14}C]tyrosine or [^{14}C]tryptophan (10 μM). 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 EcoRI 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
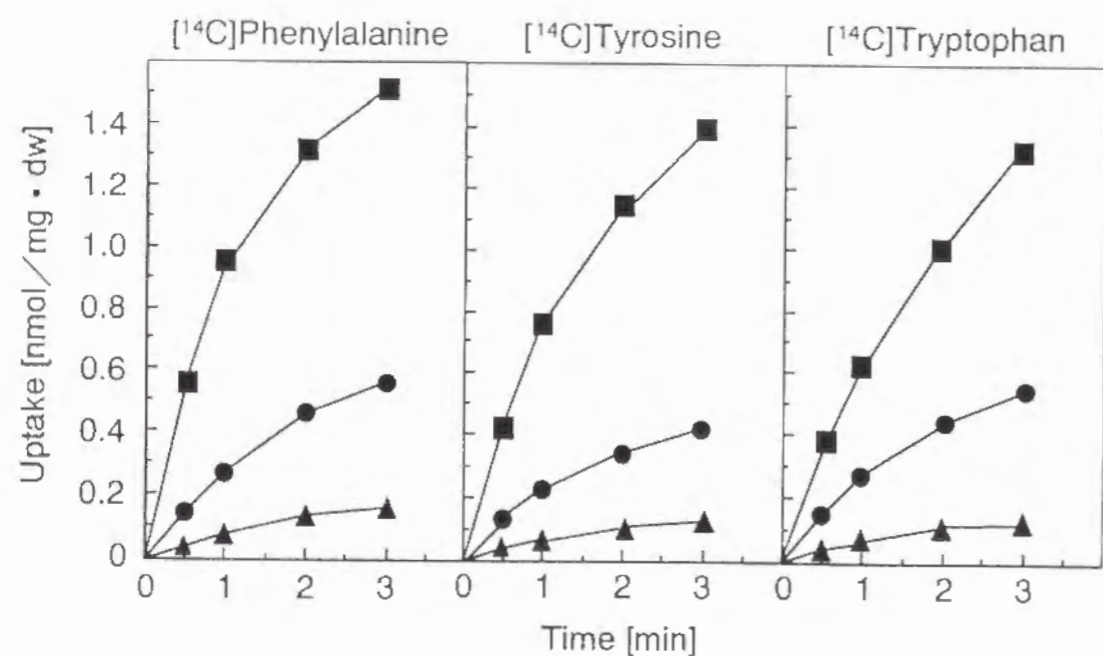

FIG. 1. Uptake of aromatic amino acids by strains KY9182, HCA1 and HCA1 carrying pCA5. Uptake by KY9182 (●), HCA1 (▲), and HCA1 carrying pCA5 (■) was measured as described under Materials and Methods.

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.

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.

<center>SUMMARY</center>

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 ^{14}C-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. Dispite 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 crutial 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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