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Metabolic Engineering To Produce Tyrosine or Phenylalanine in a Tryptophan-Producing *Corynebacterium glutamicum* Strain

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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.

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, 23), 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 (14, 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, we undertook the conversion of a tryptophan-producing strain to a tyrosine or phenylalanine producer. This novel strain construction is presented here.

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.

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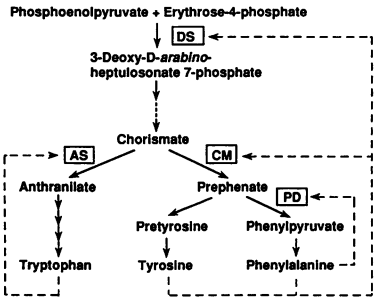

FIG. 1. Pathways and primary regulations of aromatic amino acid biosynthesis in *C. glutamicum*. Broken lines indicate feedback inhibition. AS, anthranilate synthase.

Media. Complete medium BY (14), minimal medium MM (18), and enriched minimal medium MMYE (14) were used for cultivation of *C. glutamicum*. Solid plates were made by the addition of Bacto-Agar (Difco) to 1.6%. RCGA medium (14) 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; $MnSO_4 \cdot 4\sim6H_2O$, 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; $MnSO_4 \cdot 4\sim6H_2O$, 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; and $CaCO_3$, 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; $MnSO_4 \cdot 4\sim6H_2O$, 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°C, 0.5 ml of the seed culture was transferred to a large test tube containing 5 ml of TP1 medium. Cultivation was carried out aerobically at 30°C for 72 h.

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

Preparation and manipulation of DNA. Chromosomal DNA was extracted from protoplasts of *C. glutamicum* KY10693 and KY10694 by the method of Saito and Miura (19). The protoplasts were prepared as described previously (14). Plasmid DNA was isolated by the alkaline lysis method (15) and, if necessary, purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (14). 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 (14).

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

Analysis. Cell growth was monitored by measuring the 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 derivation with *o*-phthalaldehyde.

RESULTS

Cloning of the DS, CM, and PD genes. As a first step to metabolic engineering, we cloned the three biosynthetic genes encoding the first enzyme in the common pathway, DS, and the branch-point enzymes CM and PD. Cloning of the desensitized DS gene from a regulatory mutant, *C. glutamicum* KY10694, was carried out as follows. Chromosomal DNA of strain KY10694 and vector pCG115 were completely digested with *Sal*I, mixed, and treated with T4 DNA ligase. The ligation mixture was used to transform the protoplasts of prototrophic strain *C. glutamicum* KLS4. Transformants were selected on RCGA plates containing spectinomycin (400 μg/ml). Regenerated spectinomycin-resistant colonies were transferred by replica-plating to MM agar plates containing spectinomycin (100 μg/ml) and *p*-fluorophenylalanine (PFP) (800 μg/ml). Twelve PFP-resistant clones were obtained among about 10^5 spectinomycin-resistant transformants. All plasmids purified from these clones were shown to have a common 6.7-kb *Sal*I fragment by restriction enzyme cleavage analysis. Retransformation of KLS4 with pCA1, one of the resulting recombinant plasmids, resulted in the concomitant spectinomycin- and PFP-resistant phenotype. Cells carrying pCA1 showed an eightfold-higher level of DS activity than the plasmidless cells. The overexpressed DS was insensitive to synergistic inhibition by phenylalanine and tyrosine, indicating that the cloned segment had the desensitized DS gene.

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

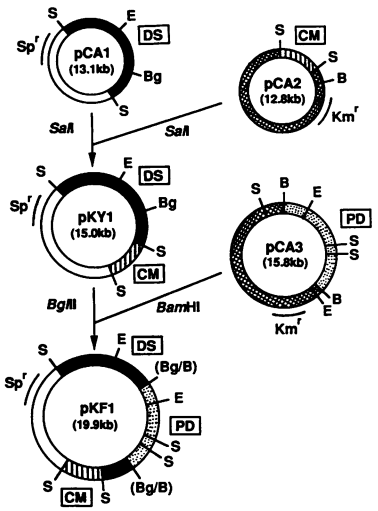

FIG. 2. Construction of recombinant plasmids containing aromatic amino acid-biosynthetic genes. Symbols: solid bars, *C. glutamicum* KY10694 chromosomal DNA fragment containing the DS gene; hatched bars, *C. glutamicum* KY10694 chromosomal DNA fragment containing the CM gene; stippled bars, *C. glutamicum*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

and tyrosine auxotroph, *C. glutamicum* KY9457, with the ligation mixture, over 10^4 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 *Sal*I fragment, one of which was designated pCA2. Similarly, cloning of the desensitized PD gene from another regulatory mutant, *C. glutamicum* KY10693, was carried out by complementation of a PD-deficient phenylalanine auxotroph, *C. glutamicum* KY9182. After transformation with the ligation mixture of *Bam*HI-digested KY10693 DNA and pCE53 and subsequent screening for prototrophy, six kanamycin-resistant and phenylalanine-independent clones were selected. The plasmids isolated from the clones carried a common 4.9-kb *Bam*HI insert at the corresponding site of the vector. One plasmid was designated pCA3.

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

Next, the *Bam*HI fragment containing the PD gene on pCA3 was ligated with pKY1 digested with *Bgl*II, which did not cut within the DS gene (data not shown). The ligation mixture was used to transform protoplasts of strain KY9182. Spectinomycin-resistant, phenylalanine-independent, and PFP-resistant clones were isolated. From restriction cleavage analysis, the resulting plasmid isolated from one of the clones was shown to possess all three genes and was designated pKF1.

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

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

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

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

[a] Relative to the activity in KLS4 with no plasmid.
[b] Each crude extract was mixed at an equal concentration of protein and used for the enzyme assay.

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 and 3 mM, respectively.
[c] Phenylalanine was added at 30 mM.

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	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.

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

Introduction of pCA2 into KY10865 did not result in remarkable accumulation of phenylalanine or tyrosine despite largely decreased production of tryptophan. The total yield of the aromatic amino acids in the recombinant strain decreased to below half of the tryptophan yield of the host strain. By contrast, when pKY1 was introduced into KY10865, the transformed strain acquired the ability to produce tyrosine (predominantly) and phenylalanine, although a small amount of tryptophan was concomitantly accumulated. This preferential synthesis of tyrosine appeared to occur because the PD of the host cells is wild type and sensitive to phenylalanine formed intracellularly, while the carbon flow from chorismate to tyrosine is uncontrolled. On the other hand, pKF1 allowed the same host to produce a large amount of phenylalanine almost without by production of tryptophan and tyrosine.

Strain KY10865 and its plasmid-carrying strains were tested for production of the aromatic amino acids in jar fermentors. Figure 3 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 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.

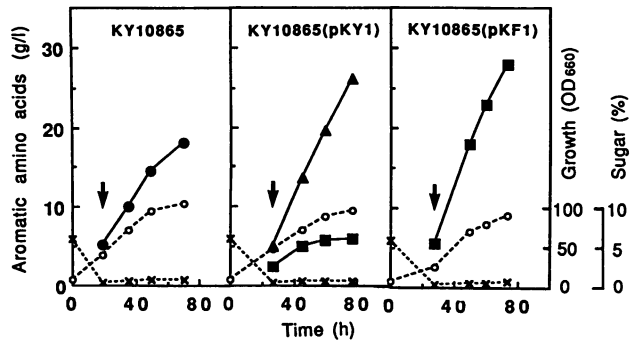

FIG. 3. Time course of aromatic amino acid production by strain KY10865 and its plasmid-carrying strains in jar fermentors. Symbols: ●, tryptophan; ▲, tyrosine; ■, phenylalanine; ○, growth (OD_{660}); ×, sugar. Arrows indicate the points at which solution containing 44% sucrose began to be fed continuously.

DISCUSSION

We 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 the 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 (Tables 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, 24). 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 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 the 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.

In a previous article (18), we 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. However, a separate experiment showed 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 our present results that both genes could be isolated as separate fragments, we conclude 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. We previously reported that amplified activities of the threonine-biosynthetic enzymes in a high lysine-producing strain of *C. glutamicum* redirected metabolic flow from the intermediate aspartate β-semialdehyde and resulted in a shift to marked production of threonine (12). 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.

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REFERENCES

1. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
2. **Cotton, R. G. H., and F. Gibson.** 1965. The biosynthesis of phenylalanine and tyrosine; enzymes converting chorismic acid into prephenic acid and their relationships to prephenate dehydratase and prephenate dehydrogenase. Biochim. Biophys. Acta **100:**76–88.
3. **Dayan, J., and D. B. Sprinson.** 1971. Enzyme alterations in tyrosine and phenylalanine auxotrophs of *Salmonella typhimurium*. J. Bacteriol. **108:**1174–1180.
4. **Follettie, M. T., and A. J. Sinskey.** 1986. Molecular cloning and nucleotide sequence of the *Corynebacterium glutamicum pheA* gene. J. Bacteriol. **167:**695–702.
5. **Gynheung, A., and J. D. Friesen.** 1979. Plasmid vehicles for direct cloning of *Escherichia coli* promoters. J. Bacteriol. **140:** 400–407.
6. **Hagino, H., and K. Nakayama.** 1973. L-Tyrosine production by analog-resistant mutants derived from a phenylalanine auxotroph of *Corynebacterium glutamicum*. Agric. Biol. Chem. **37:**2013–2023.
7. **Hagino, H., and K. Nakayama.** 1974. L-Phenylalanine production by analog-resistant mutants of *Corynebacterium glutamicum*. Agric. Biol. Chem. **38:**157–161.
8. **Hagino, H., and K. Nakayama.** 1975. L-Tryptophan production by analog-resistant mutants derived from a phenylalanine and tyrosine double auxotroph of *Corynebacterium glutamicum*. Agric. Biol. Chem. **39:**343–349.
9. **Hagino, H., and K. Nakayama.** 1975. The biosynthetic control in aromatic amino acid producing mutants of *Corynebacterium glutamicum*. Agric. Biol. Chem. **39:**351–361.
10. **Im, S. W. K., and J. Pittard.** 1971. Phenylalanine biosynthesis in *Escherichia coli* K-12: mutants derepressed for chorismate mutase P-prephenate dehydratase. J. Bacteriol. **106:**784–790.
11. **Ito, H., K. Sato, K. Matsui, K. Sano, H. Enei, and Y. Hirose.** 1990. Molecular breeding of a *Brevibacterium lactofermentum* L-phenylalanine producer using a cloned prephenate dehydratase gene. Appl. Microbiol. Biotechnol. **33:**190–195.
12. **Katsumata, R., T. Mizukami, Y. Kikuchi, and K. Kino.** 1986. Threonine production by the lysine producing strain of *Corynebacterium glutamicum* with amplified threonine biosynthetic operon, p. 217–226. *In* M. Alacevic, D. Hranueli, and Z. Toman (ed.), Genetics of industrial microorganisms. B. Pliva, Zagreb, Yugoslavia.
13. **Katsumata, R., T. Mizukami, A. Ozaki, Y. Kikuchi, K. Kino, T. Oka, and A. Furuya.** 1987. Gene cloning in glutamic acid bacteria: the system and its applications. Proc. 4th Eur. Congr. Biotechnol., Amsterdam, 14–19 June 1987, vol. 4, p. 767–776.
14. **Katsumata, R., A. Ozaki, T. Oka, and A. Furuya.** 1984. Protoplast transformation of glutamate-producing bacteria with plasmid DNA. J. Bacteriol. **159:**306–311.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
17. **Ozaki, A., R. Katsumata, T. Oka, and A. Furuya.** 1984. Functional expression of the genes of *Escherichia coli* in gram-positive *Corynebacterium glutamicum*. Mol. Gen. Genet. **196:** 175–178.
18. **Ozaki, A., R. Katsumata, T. Oka, and A. Furuya.** 1985. Cloning of the genes concerned in phenylalanine biosynthesis in *Corynebacterium glutamicum* and its application to breeding of a phenylalanine producing strain. Agric. Biol. Chem. **49:**2925–2930.
19. **Saito, H., and K. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72:**619–629.
20. **Santamaria, R., J. A. Gil, J. M. Mesas, and J. F. Martin.** 1984. Characterization of an endogenous plasmid and development of cloning vectors and a transformation system in *Brevibacterium lactofermentum*. J. Gen. Microbiol. **130:**2237–2246.
21. **Shiio, I., and S. Sugimoto.** 1979. Two components of chorismate mutase in *Brevibacterium flavum*. J. Biochem. **86:**17–25.
22. **Sprinavason, P. R., and D. B. Sprinson.** 1959. 2-Keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate synthetase. J. Biol. Chem. **234:**716–722.
23. **Sugimoto, S., M. Nakagawa, T. Tsuchida, and I. Shiio.** 1973. Regulation of aromatic amino acid biosynthesis and production of tyrosine and phenylalanine in *Brevibacterium flavum*. Agric. Biol. Chem. **37:**2327–2336.
24. **Sugimoto, S., and I. Shiio.** 1980. Purification and properties of bifunctional 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase-chorismate mutase component A from *Brevibacterium flavum*. J. Biochem. **87:**881–890.
25. **Umbarger, H. E.** 1978. Amino acid biosynthesis and its regulation. Annu. Rev. Biochem. **47:**533–606.
26. **Yoshihama, M., K. Higashiro, E. A. Rao, M. Akedo, W. G. Shanabruch, M. T. Follettie, G. C. Walker, and A. J. Sinskey.** 1985. Cloning vector system for *Corynebacterium glutamicum*. J. Bacteriol. **162:**591–597.