Short Communication

Cloning of a transaminase gene of *Citrobacter freundii* and the gene dosage effect on L-phenylalanine production through transaminase reaction*

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In a preceding paper (Anazawa et al., 1991), we reported a process for the enzymatic production of L-phenylalanine from phenylpyruvic acid and ammonium fumarate. In this process, the cells of *Citrobacter freundii* were used as the source of two enzymes for a coupling reaction with aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) and transaminase(s) (Fig. 1). Oxaloacetic acid produced as a byproduct in the transaminase reaction was rapidly decomposed enzymatically and/or nonenzymatically to pyruvic acid, after which the reaction inclined toward L-phenylalanine production. Under adequate conditions for production, the transaminase reaction was the limiting one rather than that of aspartase. We then tried to clone the transaminase gene with the aim to improve the process for L-phenylalanine production.

Some genes of *C. freundii* have been expressed in *Escherichia coli* (Blumenberg and Yanofsky, 1982; Karube et al., 1984; Yamamoto et al., 1983). Moreover, it is known that the *tyrB* gene-directed and *ilvE* gene-directed transaminases of *E. coli* have broad specificities on several amino acids including L-phenylalanine (Gelfand and Steinberg, 1977; Jensen and Calhoun, 1981) (Table 1) and are important for enzymatic L-phenylalanine production with this bacterium (Calton et al., 1986). Next, we considered that if *C.*

freundii had any transaminase of these types expressed in a *tyrB*[−] or *ilvE*[−] mutant of *E. coli*, the amino acid requirement of the *E. coli* mutant would be useful as a selection marker for cloning the gene of the transaminase functioning for L-phenylalanine production.

Chromosomal DNA was extracted from *C. freundii* ATCC 6750 (a wild strain) by the method of Murmer (Maniatis et al., 1982), digested with a restriction enzyme (*Pst*I) and ligated into the *Pst*I site [located on ampicillin (Ap) resistance gene] of a plasmid vector pACYC177 (Chang and Cohen, 1978) carrying Ap^r and kanamycin (Km) resistance genes. The enzymes and DNAs used in the genetic experiments were the products of Takara Shuzo Co., Ltd., Kyoto, Japan.

The ligation mixture was used to transform an *E. coli* recipient strain (TA784-6). This strain was constructed in our laboratory in order to simplify the selection of the transformant by F-mediated conjugation between Hfr784 (Bachmann et al., 1976) (kindly supplied by Dr. D. T. Mizukami) and a spontaneous rifampicin-resistant mutant of DG-30 (Gelfand and Steinberg, 1977) (kindly supplied by Dr. C. M. Berg) having genetic defects in three (*aspC*, *ilvE*, and *tyrB*; Fig. 2) of the four transaminase genes which are important for amino acid biosynthesis (Jensen and Calhoun, 1981) (Table 1). The conjugation was done according to the method of Curtiss (1981) by incubation in Penassay broth at 30°C for 100 min, and the TA784-6 thus selected exhibited a phenotype of *tyr*[−], *asp*[−], *pro*[−], *thi*[−], and *xyl*⁺ but not other DG-30 traits (*phe*[−], *ile*[−], *leu*[−], *arg*[−], *his*[−]). The auxotrophy and *xyl*⁺ of the transconjugant were explained by taking that it had a recombinant range from 22 [origin of chromosome transfer of

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Fig. 1. Enzymatic production of L-phenylalanine from phenylpyruvic acid and ammonium fumarate.

L-Aspartic acid supplied by an aspartase reaction (1) from fumaric acid and ammonia is used as the amino donor in the transaminase reaction (2), with phenylpyruvic acid as the amino acceptor to form L-phenylalanine and oxaloacetic acid. The oxaloacetic acid is rapidly converted to pyruvic acid enzymatically and/or nonenzymatically (3), and this makes the reaction irreversible and favours L-phenylalanine production.

Table 1. Genes and functions of transaminases in *Escherichia coli*.^a

Gene symbol ^b	Amino acids synthesized by the function of the gene						
<i>aspC</i>	Asp	Tyr	Phe				
<i>ilvE</i>			Phe	Leu	Ile	Val	
<i>tyrB</i>	Asp	Tyr	Phe	Leu			
<i>avtA</i>						Val	Ala

^a Jensen and Calhoun, 1981.

^b Bachmann, 1983.

Hfr784 (Bachmann et al., 1976) to around 89 min (site of *argE*) on chromosome (Bachmann, 1983) as shown in Fig. 2. Thus, it was expected that expression of the *tyrB* gene in TA784-6 would satisfy simultaneously the tyrosine and aspartic acid requirements if the *C. freundii* had a transaminase with a similar substrate specificity to that of *tyrB* of *E. coli* (Table 1).

The transformation was done by the CaCl_2 method of Cohen et al. (1972). The transformed cells were incubated on L broth agar (Maniatis et al., 1982) containing Km (20 $\mu\text{g/ml}$) and minimal agar medium (Maniatis et al., 1982) supplemented with L-proline but not L-tyrosine and L-aspartic acid, and incubated for 1 to 4 days at 37°C. The resultant colonies were screened for strains with the phenotypes of Km^r , Ap^s , tyr^+ , and asp^+ . As the result, we isolated two types of transformants (No. 31 and 32) with such phenotypes.

The plasmids, designated pTAC31 and pTAC32 for each transformant obtained above, were able to re-transform TA784-6. Moreover, these strains had 3.7 or 2.5 times higher activity of the transaminase functioning for L-phenylalanine formation than the control strain (TA784-6/pACYC177) carrying the primal plasmid (pACYC177), as shown in Table 2. This might be

Fig. 2. Recombination range in *Escherichia coli* TA784-6.

TA784-6 was constructed by a cross between Hfr784 and DG-30-5-1 (rifampicin-resistant spontaneously derived from DG-30 (Gelfand and Steinberg, 1977)). Inner arrow, origin, direction, and range of recombination in TA784-6; outer line, chromosome of TA784-6 in which the recombinant range is shadowed and defective markers important to this research are restrictedly given; for the details of other deficient markers originating from DG-30, see Reference, Gelfand and Steinberg, 1977.

Table 2. Plasmids carrying a transaminase gene of *Citrobacter freundii* which complements the *tyrB* defect in *Escherichia coli*.

Plasmid No.	<i>Pst</i> I-fragments of inserted part of DNA (kb)				Transaminase activity in transformants ^a
pTAC31	2.0	6.0	4.0		391
pTAC32			4.0	0.72	268
pACYC177 (control)					106

^a The transaminase activity (μmol of NADH consumed/mg-protein/min) with L-aspartic acid and phenylpyruvic acid as substrates in the cell-free extracts of the transformants from *E. coli* TA784-6. The recipient was determined in a system coupled with malate dehydrogenase reaction between NADH and oxaloacetic acid (the product of the transaminase reaction) by following a decrease in absorbance at 340 nm according to the method of Yagi et al. (1979). Protein concentration was determined by the method of Bradford (1976). For other details, see text.

the result of the expression of a transaminase gene of *C. freundii* commonly specifying L-tyrosine and L-phenylalanine as the substrate, and in that case, the first description of the expression of a transaminase gene of *C. freundii* in *E. coli*. The inserted parts of DNA in plasmids pTAC31 and pTAC32 were 12 and 4.72 kb in length, respectively. By cleaving with *Pst*I, it was suggested that the transaminase gene located on a 4 kb *Pst*I-fragment commonly exists in these plasmids (Table 2).

As pTAC32 was shorter than pTAC31 and the expressed transaminase activities were hardly different between these two plasmids in repeated experiments, pTAC32 was used in the succeeding experiments. Figure 3 shows a restriction map of pTAC32 in more detail.

Subsequently, we tried to transform *C. freundii*

Fig. 3. Restriction map of plasmid pTAC32 carrying a transaminase gene of *Citrobacter freundii*.

pTAC32 was constructed by the ligation of a *Pst*I-fragment of chromosomal DNA of *C. freundii* ATCC 6750 to the *Pst*I site of plasmid vector pACYC177. For details of the experiment, see text. Thin line, pACYC177 DNA in which the kanamycin^r gene is shadowed; boxed line, chromosomal DNA carrying a transaminase gene from *C. freundii* ATCC 6750. Numbers indicate the clockwise distance (kb) from a *Pst*I site (*Pst*I 0.00).

Table 3. Gene dosage effect on transaminase formation in *Citrobacter freundii*.

Transformant (recipient/plasmid)	Transaminase activity
CX-1/pTAC32	148
CX-1/pACYC177 (control)	50

For the transaminase activity, see the legend to Table 2. For other details, see text.

strain CX-1, which we thought to be adequate as a recipient, with pTAC32 by similar procedures as described above for *E. coli*. Strain CX-1 was derived from ATCC 6750 in our laboratory by curing a plasmid (pACYC177) with acridine orange (Hirota, 1960), which once expressed the feature of pACYC177 in the wild strain. Km-resistant colonies were tested for expression of the transaminase gene. As the result, one strain (CX-1/pTAC32) of these was determined to have three times higher the transaminase activity than the control strain (CX-1/pACYC177), as shown in Table 3. L-Phenylalanine production was compared between the cells of the transformant (CX-1/pTAC32) and the control strain using half [20 mg (wet weight) per milliliter] as much cells as the optimal for L-phenylalanine production with the wild-type strain. As can be seen in Fig. 4, the rate and amount of L-phenylalanine production by the transformant with the recombinant DNA were 1.5 times and twice higher than those of the control strain, respectively. The cells of CX-1/pTAC32 also showed higher activity than those of the control strain when compared with various cell concentrations, as shown in Fig. 5.

Fig. 4. Enhancement of L-phenylalanine productivity in the *Citrobacter freundii* cell transformed with a transaminase gene; L-phenylalanine production with a limited cell concentration.

The reaction mixture contained 350 mM phenylpyruvic acid, 600 mM ammonium fumarate, 0.4 mM pyridoxal phosphate, and a limited concentration [20 mg (wet weight) per milliliter] of the transformant cells (○, CX-1/pTAC32; ●, CX-1/pACYC177 (control)). The pH of the reaction mixture was adjusted to 8.5 with ammonia. Other details of the experiment are the same as those described in our previous paper (Anazawa et al., 1991).

Fig. 5. Enhancement of L-phenylalanine productivity in the *Citrobacter freundii* cell transformed with a transaminase gene; L-phenylalanine production with variable cell concentrations.

The reactions were done in a mixture containing 300 mM phenylpyruvic acid, 600 mM ammonium fumarate, 0.4 mM pyridoxal phosphate, and indicated concentrations (given as protein) of the cells of transformants for 4 and 24 h. ●, CX-1/pTAC32 for 4 h; ▲, CX-1/pACYC177 (control) for 4 h; ○, CX-1/pTAC32 for 24 h; △, CX-1/pACYC177 (control) for 24 h. Protein in the cell was extracted by boiling in 1 N NaOH for 10 min, and determined by the method of Bradford (1976). Other details for the experiment are the same as those described in the legend to Fig. 4.

It is reasonable that the activity for L-phenylalanine production was elevated in a transformant with an elevated level of transaminase which limits production in a wild-type strain. This will make a great contribution to the enzymatic production of L-phenylalanine

through transaminase reaction.

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