Threonine Production by Co-existence of Cloned Genes Coding Homoserine Dehydrogenase and Homoserine Kinase in *Brevibacterium lactofermentum*

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The homoserine kinase (HK) gene was cloned from *Brevibacterium lactofermentum* M-15, a threonine-producing mutant. Then, a trimethoprim-resistant hybrid plasmid pAJ212 expressing the HK gene was constructed. A threonine-producing recombinant strain harboring pAJ210, a chloramphenicol-resistant plasmid on which homoserine dehydrogenase (HD) gene was cloned, was transformed further with the obtained HK-expressing plasmid pAJ212. Plasmids pAJ210 and pAJ212 were compatible in *B. lactofermentum* because these two plasmids were derived from two different cryptic plasmids, pHM1519 and pAM330 respectively. Co-existence of the cloned HD and HK genes in M-15 resulted in more than a 30% increase of threonine production (33 g/liter) and a decrease of the by-products (lysine and homoserine) as compared with M-15 carrying HD recombinant plasmid alone. The activities of HD and HK in the strain harboring both HD- and HK-recombinant plasmids were elevated markedly by gene dosage effect, which brought about the apparent increase in threonine production.

So-called coryneform glutamic acid-producing bacteria, such as those belonging to *Brevibacterium*, are highly important, as they are used for the industrial fermentation production of various amino acids. We have developed host-vector systems for *B. lactofermentum*.^{1,2)} Further, versatile cloning vectors carrying the trimethoprim-resistant (Tp^r) marker gene was constructed. HD is known as the most important rate-limiting enzyme in threonine biosynthesis in *B. flavum* and *B.*

lactofermentum.^{4,5)} In a previous paper,⁶⁾ we described the improved threonine production by cloning of the HD gene in *B. lactofermentum*. It was evident that the increase of HD activity by gene dosage caused the accelerated conversion of aspartate- β -semialdehyde, a common intermediate of threonine and lysine biosynthesis, to homoserine and therefore threonine production was stimulated (Fig. 1). However, a problem was that this HD-recombinant strain produced consider-

FIG. 1. Pathway and Regulation of Threonine Biosynthesis in *B. lactofermentum*.

The following abbreviations are used in this figure: Asp, aspartate; Asp-P, β -aspartylphosphate; ASA, aspartyl- β -semialdehyde; Hse, homoserine; Thr, threonine; Ile, isoleucine; Lys, lysine; Met, methionine; HD, homoserine dehydrogenase; HK, homoserine kinase; $\rightarrow$, feedback inhibition; $-\rightarrow$, repression.

able amounts of lysine and homoserine besides threonine, mainly due to the incomplete conversion of homoserine to threonine. So, further improvement of threonine production was expected by cloning of the homoserine kinase (HK) gene which catalyzed the first step of homoserine conversion to threonine. In this case, the simultaneous expression of both HD and HK genes in the same host cells should be necessary. We will describe in this paper the cloning of the HK gene of *B. lactofermentum* and the development of a gene coexistence system using compatible vectors in *B. lactofermentum* to breed a potent amino acid producer, such as a threonine-producer.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were derivatives of *B. lactofermentum* AJ1511. A threonine-overproducing mutant M-15,⁵⁾ which was isoleucine and leucine double auxotroph and resistant to three antimetabolites, S-(2-aminoethyl) L-cysteine (AEC), α -amino- β -hydroxyvaleric acid (AHV) and S-methylcysteine sulfoxide (SMCS), was used as the DNA donor and the recipient of recombinant plasmids. The HK-deficient mutant S-2 was derived from the wild-type strain AJ12036 by NTG mutagenesis. Cm^r plasmid (pAJ1844)²⁾ and trimethoprim-resistant (Tp^r) plasmids (pAJ226 and pAJ224)³⁾ were used as the vectors. The composite plasmid pAJ210 was a derivative of pAJ1844 carrying the HD gene cloned from M-15 chromosomal DNA.⁶⁾

Media. The complete and minimal media had the following compositions: Complete medium (CM2G): 1% yeast extract, 1% peptone, 0.5% NaCl and 0.5% glucose, pH 7.0. Minimal medium (MM): 2% glucose, 1% (NH₄)₂SO₄, 0.25% urea, 0.1% KH₂PO₄, 0.04% MgSO₄·7H₂O, 200 μ g/liter thiamine-HCl, 50 μ g/liter *d*-biotin, 2 ppm Fe²⁺, and 2 ppm Mn²⁺, pH 7.0. The threonine-production medium contained 10% glucose, 4.5% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.1% MgSO₄·7H₂O, 300 μ g/liter thiamine-HCl, 100 μ g/liter *d*-biotin, 2 ppm Fe²⁺, 2 ppm Mn²⁺, Ajinomoto's "Mieki" (soybean meal acid hydrolysate) 15 ml/liter, 250 mg/liter L-isoleucine, 300 mg/liter L-leucine and 5% CaCO₂ (sterilized separately), pH 7.0. Protoplast cells were regenerated on RB agar plates.

Preparation of DNA. Chromosomal and plasmid DNA were prepared as described previously.¹⁾

Protoplast transformation. The preparation of protoplast cells and the transformation were performed as described previously.²⁾

Preparation and assay of enzymes. The cells were harvested after cultivation in the threonine production medium for 23 hr, washed twice with 0.2% KCl, suspended in 0.1 M Tris-HCl buffer of pH 8.0 containing 0.02 M β -mercaptoethanol, and disintegrated by treatment with a sonic oscillator (300 W) for 20 min under cooling at 4°C. The cell debris was removed by centrifugation at 14,000 $\times g$ for 30 min at 0–4°C and the supernatant was mixed at 0°C with 5 volumes of saturated ammonium sulfate solution. The precipitates were collected by 14,000 $\times g$ centrifugation and dissolved in the same buffer. The resultant solution was used as the enzyme solution. HD activity was determined by the procedure of Truffa-Bachi *et al.*⁷⁾ HK activity was determined according to Miyajima *et al.*⁸⁾

Production of amino acids. A loopful of cells grown at 30°C for 24 hr on CM2G agar (supplemented with 10 μ g/ml Cm and 100 μ g/ml Tp when necessary) was inoculated into 20 ml of the threonine production medium in a 500 ml flask, and cultured at 30°C for 3 days with shaking. The amount of L-threonine, L-lysine and L-homoserine in the culture broth was determined by microbioassay with *Leuconostoc mesenteroides* ATCC 8042 and by an amino acid analyzer.

RESULTS

Cloning of homoserine kinase (HK) gene

The strategy for the cloning of the HK gene is shown in Fig. 2. M-15 chromosomal DNA and pAJ1844 DNA were digested by *Pst*I, mixed and ligated, then the ligated mixture was used to transform the HK-deficient strain S-2. After incubation at 30°C on an RB regeneration medium containing 3 μ g/ml of Cm, regenerated Cm^r colonies were transferred on MM containing 10 μ g/ml of Cm and eight transformants were found to grow on MM. Two out of the eight transformants carried almost the same size plasmids, which were larger than the vector pAJ1844 (9.0 kb). To confirm the clone, plasmid DNA was extracted from the transformant and used for retransformation of S-2. Ten Cm^r transformants were selected and examined for their amino acid requirement and found to be prototroph. Thus, the resultant plasmid was concluded to

FIG. 2. Cloning Strategy of HK Gene.

Details are described in the text. The following abbreviations are used in this figure: AHV^r, α -amino- β -hydroxyvaleric acid resistant; AEC^r, S-(2-aminoethyl)-L-cysteine resistant; SMCS^r, S-methylcysteine sulfoxide resistant; Cm^r, chloramphenicol resistant; HK⁻, homoserine kinase deficient; P, *Pst*I; E, *Eco*RI; B, *Bam*HI; S, *Sma*I.

TABLE I. HK ACTIVITY OF THE RECOMBINANT STRAIN

Host	Plasmid	HK activity (nmol/min/mg protein)
M-15	None	2.9
S-2	None	Not detected
S-2	pAJ211	29.2

The cultures were grown to the late logarithmic phase (23 hr) in a threonine production medium. S-2 harboring pAJ211 was cultured in a medium containing 10 μ g/ml Cm.

be a pAJ1844-HK gene recombinant and designated as pAJ211. pAJ211 had a 2.9 kb *Pst*I insert carrying the HK gene in the *Pst*I site of pAJ1844 (Fig. 2).

Homoserine kinase activity

As shown in Table I, the HK activity of the strain carrying pAJ211 was ten times higher than that of M-15 due to the gene dosage ef-

FIG. 3. Effect of Threonine Addition on the Cloned HK Activity.

The procedures for the assay of HK activity are described in the text. The following symbols are used in this figure: ●, HK activity in the crude extract of the threonine-producing mutant, M-15; ○, HK activity in the crude extract of the HK-deficient mutant, S-2, harboring the HK-recombinant plasmid pAJ212; ◐, HK activity in the crude extract of the wild type strain AJ12036.

fect. Further, as shown in Fig. 3, HK activity from the wild type strain AJ12036 was inhibited about 50% by 10 mM threonine. However, the activity of the enzyme from the mutant strain M-15 and the recombinant strain harboring pAJ211 was scarcely inhibited even in the presence of 10 mM threonine. In a previous paper,⁵⁾ we reported that HD from strain M-15 was resistant to feedback inhibition by threonine. Thus the two important enzymes (HD and HK) in threonine biosynthesis in this threonine-producing mutant M-15 altered to become feedback resistant.

Transfer of cloned HK gene to *Tp*^r vectors

Previously we found that the introduction of the cloned HD gene into the threonine-overproducing mutant M-15 resulted in an increased production of threonine and a decrease of the by-product lysine.⁴⁾ However, this HD-recombinant strain still produced a considerable amount of homoserine, a precursor of threonine. Therefore, the elevation of the HK activity which catalyzes the first step of homoserine conversion to threonine is considered to be necessary for the improvement of threonine production. In order to get

the gene dosage effect of two individual genes in the same host cells, we used two different plasmids, compatible in *B. lactofermentum* cells, to manipulate the genes. As described above, the plasmids pAJ210 and pAJ211 in which the HD gene and HK gene were inserted respectively, were derived from the identical vector pAJ1844. Therefore it was difficult for these two plasmids to co-exist in the same recipient due to plasmid incompatibility. In order to let the HD and HK gene co-exist in the same host, it was necessary to transfer the once cloned HK gene into another vector, compatible with pAJ1844. The procedure is outlined in Fig. 4. The 2.9 kb *Pst*I fragment from pAJ211 containing the HK gene was inserted into the *Pst*I site of Tp^r plasmid vector pAJ226 which was compatible with pAJ1844. However, regardless of the orientation of the insertion, the expression of the HK gene was not observed (data not shown). One reason for this fact was presumed to be that a promoter region may not be cloned in the 2.9 kb fragment. The structural gene of HK in pAJ211, inserted in the *Pst*I site in the middle of the β -lactamase gene originated from pBR325,²⁾ seemed to be expressed by the read through from the β -lactamase promoter. In order to express the

HK gene using the Tp^r vector, we tried to insert the HK fragment just downstream of the promoter region originally existing in the vector by a tricky procedure shown in Fig. 4. Both ends of the 2.9 kb *Pst*I fragment excised from pAJ221 were altered to *Bam*HI cohesive ends using synthetic oligonucleotide adaptors, then the fragment was joined with the *Mbo*I partially digested pAJ228. The ligated DNAs were introduced into *B. lactofermentum* S-2 by protoplast transformation, and the Tp^r and thr^+ colonies were selected. Four transformants (Tp^r , thr^+) harboring different size plasmids about 14.9 kb, 12.2 kb, 9.6 kb and 6.6 kb each containing the 2.9 kb HK fragment commonly were obtained. The smallest one (6.6 kb), designated as pAJ212, was analyzed by restriction enzyme digestion. We found a promoter-like structure upstream of the *Pst*I site of this plasmid where the HK gene was inserted (data will be published elsewhere). After removing the 2.9 kb HK fragment from pAJ212 by *Pst*I digestion, the remaining DNA fragment (3.7 kb) was re-ligated and a novel versatile vector named pAJ224 was constructed as described elsewhere.³

Breeding of HD, HK-recombinant strain

The breeding of the recombinant strain har-

FIG. 4. Transfer of the HK Gene Cloned in the Cm^r Plasmid Vector to Tp^r Plasmid Vectors.

Details are described in the text. The following abbreviations are used in this figure: B, *Bam*HI; P, *Pst*I; S, *Sal*I; Tp^r, trimethoprim resistance; Cm^r, chloramphenicol resistance.

boring both pAJ210 (HD-recombinant plasmid) and pAJ212 (HK-recombinant plasmid) was performed as follows. The protoplasts of *B. lactofermentum* M-15 carrying pAJ210 (HD-recombinant) were transformed by pAJ212 DNA followed by regeneration on Tp,Cm-containing RB regeneration medium.²⁾ The candidates for the HD,HK-recombinant strains harboring both pAJ210 and pAJ212 were selected as clones resistant to both Cm and Tp. Twenty candidates were cultured in 3ml threonine production medium in test tubes. More than 70% of the candidates

produced more threonine than the HD-recombinant (Fig. 5). Furthermore, the amounts of by-products (lysine and homoserine) decreased significantly. The diversity of the threonine-producing abilities of the HD,HK-recombinants (Fig. 5) was possibly due to the diversity of the recipient cells. The environments surrounding the cells during protoplast transformation and regeneration might be toxic for the amino acid-producing cells which were genetically fragile. Therefore, the transformants exhibiting low threonine production (Fig. 5) were probably derived from the recipient cells which had genetically lowered threonine productivity. We selected the HD,HK-recombinant which showed the highest threonine accumulation in the test tube culture. Figure 6 shows the time course of amino acid production by the HD-recombinant and the HD,HK-recombinant in 20 ml threonine production medium. The HD,HK-recombinant accumulated 33 g/liter of threonine after 72 hr culture while the HD-recombinant accumulated 25 g/liter under the same conditions. All the 20 colonies isolated from this HD,HK-recombinant clone showed almost the same threonine accumulation. Thus, we demonstrated that this HD,HK-recombinant was very powerful for

FIG. 5. Distribution of Threonine Productivity in the HD, HK-Recombinant Strains Harboring both pAJ210 and pAJ212.

FIG. 6. Time Course of Threonine Production by Recombinant Threonine Producers.

Detailed procedures for culture are described in the text. 10 μ g/ml Cm (in the case of HD-recombinant) or both 10 μ g/ml Cm and 100 μ g/ml Tp (in the case of HD, HK-recombinant) were added to the medium. The following symbols are used in this figure: ○, L-threonine; ●, L-homoserine; △, L-lysine; ----, growth (OD_{562}).

threonine production.

Enzyme analysis of HD, HK-recombinant

We tested HD and HK activities in the recombinant strains at the logarithmic (24 hr) and stationary (48 hr) phase of growth. The HD level was stably maintained during the culture. The activity in the extract of the HD, HK-recombinant was 4 to 14 times higher than that in the host strain M-15 (Table II). Though the HK level was relatively unstable and decreased about 20~40% in the stationary-phase, the HD, HK-recombinant always showed an activity more than 20 times

higher as compared with that of M-15. Thus, both HD and HK activities increased clearly in the HD, HK-recombinant due to the gene dosage effect, which evidently supported the high threonine production.

Plasmid stability

The stability of pAJ210 and pAJ212 in M-15 was examined during cultivation in the threonine-producing medium. Single colonies were isolated on CM2G agar plates and transferred onto CM2G plates containing Cm or Tp. As shown in Table III, all the isolated clones tested carried pAJ212 (Tp^r) even from

TABLE II. ACTIVITIES OF HD AND HK OF THE RECOMBINANT THREONINE-PRODUCERS DURING THREONINE PRODUCTION CULTURE

Strain	Plasmid	HD activity (nmol/min/mg protein)		HK activity (nmol/min/mg protein)	
		24 hr	48 hr	24 hr	48 hr
Host (M-15)	None	0.72	0.56	3.3	2.5
HD-recombinant	pAJ210	2.26	3.83	3.3	2.5
HK-recombinant	pAJ212	0.39	0.42	10.4	8.3
HD, HK-recombinant	pAJ210, pAJ212	3.22	7.93	97.9	55.0

The cultures were grown to the late logarithmic phase (24 hr) and to the stationary phase (48 hr) in a threonine production medium. The HD-recombinant and HK-recombinant were cultured in a medium containing 10 µg/ml Cm and 100 µg/ml Tp, respectively. The HD, HK-recombinant was cultured in the presence of both 10 µg/ml Cm and 100 µg/ml Tp.

TABLE III. STABILITY OF PLASMIDS IN RECOMBINANT THREONINE-PRODUCERS

Strain	Plasmids	Drug added to medium (µg/ml)	Plasmids retaining cells (%)							
			pAJ210				pAJ212			
			24	48	72	96 hr	24	48	72	96 hr
HD-recombinant	pAJ210	Cm 10	72	84	72	68	0	0	0	0
HD, HK-recombinant	pAJ210, pAJ212	Cm 10, Tp 100	84	92	80	64	100	100	100	100
HD, HK-recombinant	pAJ210, pAJ212	None	72	88	60	72	100	100	100	100

The cultures were grown in a threonine production medium supplemented with the drugs indicated. At the indicated cultivation time, the colonies were isolated on CM2G plate without drug and tested for Cm (10 µg/ml) resistance and Tp (100 µg/ml) resistance. All of the ten Cm^r colonies selected at random from the HD-recombinant (M-15/pAJ210) culture were confirmed to harbor intact pAJ210. All of the ten colonies selected at random from the HD, HK-recombinant culture as simultaneously resistant to Cm and Tp carried both intact pAJ210 and pAJ212. The percentages of the pAJ210- and pAJ212-retaining cells in the cultures were estimated from the percentages of Cm^r cells and Tp^r cells in the total cells in the cultures, respectively.

the threonine-producing culture grown in the absence of Tp. On the other hand, pAJ210 (Cm^r) gradually disappeared during the culture in the HD, HK-recombinant strain, as well as in the HD-recombinant strain. Thus pAJ212 was confirmed to be very stable in the recombinant strain. However, it is not clear why pAJ210 was not so stable as pAJ212.

DISCUSSION

In this work, we attempted to develop a novel gene co-existence system using compatible plasmids in *B. lactofermentum* for the production of amino acids. When we wish to adopt recombinant DNA techniques to breed a potent microorganism for metabolite production, generally we will be faced with the problems that not only the key enzyme but also the other enzymes involved in the biosynthesis of the metabolite need to be elevated by gene dosage with a good balance in order to optimize the metabolic flow for the formation of the target metabolite. In such a case, the stable co-existence of several different genes carried on vectors, and the balanced expression of the genes in the host cells, are essential. There are two possible approaches to gene co-existence. First, each gene is joined in two or more compatible vectors, separately. Second, several genes or an operon are joined in a single vector as shown by Miwa *et al.*¹⁰⁾ for threonine operon in *E. coli*, Helinski *et al.*¹¹⁾ for tryptophan operon in *E. coli* and Gushima *et al.*¹²⁾ for γ -glutamyl-cysteine synthetase and glutathione synthetase. The former system has the profitable point that a large amount of passenger DNAs can be scattered over several vectors, however it requires different plural vectors which can co-exist stably in the same host cells. The latter system is rather simple, but it has a weak point that the amount of passenger DNAs which can be stably maintained in one vector is limited. In this study, we adopted the former system using two different plasmid vectors, *i.e.* Cm^r plasmid pAJ1844 and Tp^r plasmid pAJ224, which are compatible in *B. lactofermentum* cells. As for the genes to be

manipulated, we chose the two important genes for threonine biosynthesis, *i.e.* the HD and HK genes. We have already reported on the cloning of the HD gene from *B. lactofermentum* and observed the marked improvement of threonine production in the HD-recombinant strain.⁶⁾ However, the HD-recombinant strain produced a considerable amount of by-products, *i.e.* homoserine, an intermediate of threonine biosynthesis, and lysine. Restricted metabolic flow from homoserine to threonine was considered to cause the overproduction of homoserine, which led to the occurrence of the tributary of the metabolic flow to lysine. In order to prevent by-product accumulation, it seemed necessary to enhance the conversion of homoserine to threonine. HK catalyzes the first step of homoserine conversion to threonine. Therefore, the co-existence of the cloned HD and HK gene in *B. lactofermentum* was expected to be a powerful strategy for the further stimulation of threonine production. As shown in this study, the clear effect of the co-existence of both the HD and HK gene, joined in pAJ1844 and pAJ224 respectively, was observed. Threonine production increased by about 30% and the formation of by-products (homoserine and lysine) markedly decreased as compared with M-15 harboring only the HD-recombinant plasmid. The elevated levels of HD and HK activities in the HD, HK-recombinant strain apparently caused the increase of threonine production and the decrease of by-products (homoserine and lysine). Consequently, the high levels of HD and HK activities in the HD, HK-recombinant indicated that the gene co-existence system developed in this study worked effectively for the improvement of threonine production.

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