

Construction of a Proline-Producing Mutant of the Extremely Thermophilic Eubacterium *Thermus thermophilus* HB27

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Growth of *Thermus thermophilus* HB27 was inhibited by a proline analog, 3,4-dehydroproline (DHP). This result suggested that the γ -glutamyl kinase (the product of the *proB* gene) was inhibited by feedback inhibition in *T. thermophilus*. DHP-resistant mutants were reported previously for *Escherichia coli* (A. M. Dandekar and S. L. Uratsu, *J. Bacteriol.* 170:5943–5945, 1988) and *Serratia marcescens* (K. Omori, S. Suzuki, Y. Imai, and S. Komatsubara, *J. Gen. Microbiol.* 138:693–699, 1992), and their mutated sites in the *proB* gene were identified. Comparison of the amino acid sequence of *T. thermophilus* γ -glutamyl kinase with those of *E. coli* and *S. marcescens* mutants revealed that the DHP resistance mutations occurred in the amino acids conserved among the three organisms. For eliminating the feedback inhibition, we first constructed a DHP-resistant mutant, TH401, by site-directed mutagenesis at the *proB* gene as reported for the proline-producing mutant of *S. marcescens*. The mutant, TH401, excreted about 1 mg of L-proline per liter at 70°C after 12 h of incubation. It was also suggested that *T. thermophilus* had a proline degradation and transport pathway since it was able to grow in minimal medium containing L-proline as sole nitrogen source. In order to disrupt the proline degradation or transport genes, TH401 was mutated by UV irradiation. Seven mutants unable to utilize L-proline for their growth were isolated. One of the mutants, TH4017, excreted about 2 mg of L-proline per liter in minimal medium at 70°C after 12 h of incubation.

Thermus thermophilus, a gram-negative aerobic eubacterium, is one of the most widely studied species of extremely thermophilic microorganisms. We have been working on the molecular genetics and molecular reproduction of *T. thermophilus* HB27. We have already cloned and sequenced three proline biosynthetic genes, *proB*, *proA*, and *proC*, and reported that the *proB* and *proA* genes exist in tandem (7, 9).

We have also constructed physical maps of the HB27 chromosome and of a large plasmid, pTT27, and determined the locations of all proline biosynthetic genes on the chromosomal DNA (20, 21). We have already succeeded in overproducing carotenoids in *T. thermophilus* HB27 (6), but at present there is no report about extracellular production of amino acids in extreme thermophiles. We have elucidated the consensus sequences for strong promoters of *T. thermophilus* (11) and developed a thermostable antibiotic resistance gene (12). It is also easy to disrupt or mutate genes on chromosomal DNA in *T. thermophilus* HB27 (8). Among the extreme thermophiles, a host-vector system has been established only in *T. thermophilus*. Generally, the reaction rate of thermostable enzymes which are produced from *T. thermophilus* is higher than those of enzymes from mesophiles. In a fermentation process such as amino acid production, *T. thermophilus* may contribute to the improvement of amino acid productivity since fermentation at a high temperature eliminates the problems of contamination and cooling procedures. So, we decided to attempt excretion of proline at a high temperature with *T. thermophilus* mutants.

L-Proline is synthesized from glutamate by the sequential reaction of γ -glutamyl kinase, γ -glutamyl phosphate reductase, and pyrroline-5-carboxylate reductase in bacteria (1). Genes *proB* and *proA*, which encode γ -glutamyl kinase and γ -glutamyl phosphate reductase, respectively, were found to comprise an

operon in *T. thermophilus* (9), *Escherichia coli* (5), and *Serratia marcescens* (13). In *E. coli* and *S. marcescens*, γ -glutamyl kinase is subject to feedback control by L-proline (3, 13), but γ -glutamyl phosphate reductase and pyrroline-5-carboxylate reductase are not inhibited by proline (3, 15). Meanwhile, *E. coli* and *S. marcescens* rapidly degrade proline by proline dehydrogenase (proline oxidase), encoded by the *putA* gene (3, 14, 22). So far, it has been reported that *E. coli* mutants resistant to proline analogs, DL-3,4-dehydroproline and L-azetidine-2-carboxylic acid, excreted L-proline into the medium. But the amount of L-proline excreted was too small for practical use because of the existence of the proline degradation pathway (2). For *S. marcescens*, Sugiura et al. (18, 19) have constructed a proline-overproducing strain, SP126, as a double mutant resistant to 3,4-dehydroproline and thiazolidine-4-carboxylate and derived from a proline dehydrogenase-deficient mutant (18). Strain SP126 produced about 20 mg of L-proline per ml in the fermentation medium (18).

In *T. thermophilus*, the control system in proline biosynthesis has not been elucidated. However, we thought that the feedback control of proline biosynthesis in *T. thermophilus* should be similar to that of *E. coli* and *S. marcescens*, since the amino acid sequences of proline biosynthetic enzymes in *T. thermophilus* show a high similarity to sequences of those of *E. coli* and *S. marcescens* (7, 9). *E. coli* and *S. marcescens* mutants resistant to 3,4-dehydroproline have already been determined to be *proB* mutants (4, 14). The comparison of the amino acid sequences of γ -glutamyl kinases in *E. coli*, *S. marcescens*, and *T. thermophilus* showed that these mutations occurred in the positions conserved among the three microorganisms (Fig. 1). We thought that it was possible to construct a 3,4-dehydroproline-resistant mutant of *T. thermophilus* by introducing the same mutations into the *proB* gene found in the mutants of *E. coli* and *S. marcescens*. We determined the strategy for construction of a proline-producing strain of *T. thermophilus* by following two steps: first, construction of a 3,4-dehydroproline-resistant mutant by introduction of mutations into the *proB*

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FIG. 1. Comparison of the amino acid sequences of γ -glutamyl kinases in *E. coli*, *S. marcescens*, and *T. thermophilus*. The amino acid substitutions found in *E. coli* (4) and *S. marcescens* (14) are shown by arrows. Asterisks show the amino acid residues conserved in the three microorganisms.

gene, and second, isolation of a mutant which cannot utilize proline for its growth by mutagenizing the dehydroproline-resistant mutant.

MATERIALS AND METHODS

Chemicals. Restriction endonucleases and DNA modification enzymes were purchased from Toyobo (Tokyo, Japan) or Takara Shuzo (Kyoto, Japan). L-Proline and DL-3,4-dehydroproline were purchased from Sigma Chemical Co. (St. Louis, Mo.). All the other reagents used were of the purest grade available.

Bacterial strains and growth conditions. *T. thermophilus* HB27 (17) and its proline auxotrophic mutant, TH104 (7), were used. TM medium (10) was used for routine cultivation of *T. thermophilus*. Minimal medium (MM) (10) was also used. When necessary, 3,4-dehydroproline and L-proline were added to MM at the concentration of 1 mM. MM-proline plates which contained 10 mM L-proline instead of $(\text{NH}_4)_2\text{SO}_4$ were used to isolate proline-producing mutants. The growth curve of *T. thermophilus* was measured as follows. *T. thermophilus* HB27 was grown in 10 ml of TM medium at 70°C for 16 h. A 0.1-ml aliquot of the culture was inoculated into 10 ml of a fresh medium. The growth was monitored by measuring absorbance at 580 nm. *E. coli* JM109 (23) was also used in the cloning experiments.

Preparation of the fragment containing the mutated *proB* genes. Four primers, PROBDNF (5'-GTCCTCCTCACCGCCGAGAACCTC-3'), PROBDNR (5'-GCAGTTCTCGGCGGTGAGGAGGAC-3'), PROBAVF (5'-AGGAGCCGCTACCTGAACGTCAAG-3'), and PROBAVR (5'-CTTGACGTTCCAGGTAGCGCTCCT) (Fig. 2), were prepared to introduce the mutations into the *proB* gene. Two primers, PROB_F (5'-GGGAATTCCCAGGCCATGCCGAGGC-3') and PROB_R (5'-GAAGCTTTCATGCCTCCTCCTCAAGGC-3') (Fig. 2), were prepared to amplify the fragment containing the entire mutated *proB* gene. The primers contained restriction endonuclease sites for *EcoRI* (italics) and *HindIII* (underlined).

In the first PCR, the four DNA fragments which would be the DNA template for construction of the mutated *proB* gene were prepared (Fig. 2). For the preparation of fragments A, B, C, and D, primers PROB_F and PROBDNR, PROB_F and PROBAVR, PROB_R and PROBDNF, and PROB_R and PROBAVF were used, respectively. The PCR mixture contained PCR buffer with 2 mM MgCl_2 (Takara Shuzo), 0.2 mM deoxynucleoside triphosphates, 0.1 μM (each) primer, 0.1 μg of template (pUC-pro3,5⁺ [9]), and 2.5 U of *rTaq* DNA polymerase (Takara Shuzo). A DNA thermal cycler (Perkin-Elmer Cetus) was used with the following conditions: melting temperature, 96°C (30 s); annealing temperature, 62°C (1 s); and polymerization at 72°C (150 s). Twenty-five cycles were run with a subsequent polymerization period of 10 min at 72°C. The amplified fragments A (610 bp), B (637 bp), C (814 bp), and D (787 bp) were recovered from 1.0% agarose gels and used for the second PCR.

In the second PCR, three types of DNA fragments containing the mutated *proB* genes were prepared. For the amino acid change of Asp-115 to Asn in the *proB* gene, fragments A and C were used for the DNA template (Fig. 2, fragment 1). For the amino acid change of Ala-125 to Val (Fig. 2, fragment 2), fragments B and D were used. For the two amino acid changes of Asp-115 and Ala-125 to Asn and Val, respectively, fragments B and C were used (Fig. 2, fragment 3). The second PCR mixture contained PCR buffer with 2 mM MgCl_2 (Takara Shuzo), 0.2 mM deoxynucleoside triphosphates, 0.1 μM (each) primer PROB_F and PROB_R, 10 ng of each amplified fragment, and 2.5 U of *rTaq* DNA polymerase (Takara Shuzo). PCR was performed under the following conditions: 96°C for 30 s, gradual decrease of the temperature to 62°C for 45 s, and immediate increase of the temperature from 62 to 72°C, and maintenance for 150 s. Twenty-five cycles were run with a subsequent polymerization period of 10 min at 72°C. The three types of amplified DNA fragments were recovered from 1.0% agarose gels and filled and phosphorylated with T4 DNA polymerase and T4 polynucleotide kinase. Each fragment was ligated to *SmaI*-digested and dephosphorylated pUC19, and the ligation mixtures were used for *E. coli* JM109 transformation. Three types of plasmids which contained D115N, V125A, and D115N and

V125A replacements were prepared, and their nucleotide sequences were determined.

Construction of a 3,4-dehydroproline-resistant mutant. *T. thermophilus* HB27 was transformed with 3 μg each of three types of plasmid (containing fragment 1, 2, or 3 [Fig. 2]) as described previously (10). The transformed cells were washed with 0.85% NaCl (saline) and resuspended in saline. The diluted suspensions were spread on MM plates containing DL-3,4-dehydroproline at the concentration of 1 mM. When the plasmid containing fragment 2 which included the amino acid change in the *proB* gene of Ala-125 to Val was used for the transformation, many 3,4-dehydroproline-resistant mutants were obtained. Eight transformants among them were randomly selected, and total DNAs were prepared by the method of Saito and Miura (16). Total DNA from the eight transformants was digested with *SphI*, and DNA fragments including the *proBA* genes of 2.8 kb in size were recovered by agarose gel electrophoresis. Each DNA fragment was ligated with *SphI*-digested and dephosphorylated pUC19 and introduced into *E. coli* JM109. The clones containing the entire mutant *proB* genes were screened by colony hybridization using the inserted fragment of pUC-pro3,5⁺ as a probe. The entire *proB* genes of eight transformants were sequenced and checked for the amino acid change of Ala-125 to Val. The eight transformants showed the same growth curves in MM containing 1 mM 3,4-dehydroproline. We named one of the eight transformants TH401 and used it for further experiments.

Construction of a proline-producing strain. Strain TH401 was grown in TM medium for 3 h at 70°C. The culture was diluted 1/10 with saline and irradiated for 3 min under a 15-W UV lamp. Five milliliters of the culture was added to 5 ml of fresh TM medium and incubated at 70°C for 60 min in the dark. This culture was diluted and spread on TM plates. A total of 8,217 colonies on TM plates were replica plated onto MM and MM-proline plates. Seven strains which were able to grow on MM plates but not on MM-proline plates were isolated. We named them TH4011, TH4012, TH4013, TH4014, TH4015, TH4016 and TH4017.

Bioassay of proline production. An overnight TM culture of each strain was diluted 1/10 with saline (0.85% NaCl), and 5 μl was spotted onto the MM plates onto which TH104 had been spread. The plates were incubated at 70°C for 2.5 days. After incubation, the growth of TH104 around the spots was checked.

Analysis of amino acid production. Each strain was incubated in 10 ml of MM at 70°C for 12 h. All cells were removed by centrifugation (18,000 \times g, 10 min), and the amino acids in the culture supernatant were analyzed by ion-exchange chromatography with a Hitachi L-8500 amino acid analysis system (Hitachi, Tokyo, Japan).

Nucleotide sequence accession number. The nucleotide sequence of the *proBA* genes in *T. thermophilus* is in the EMBL, GenBank, and DDBJ databases under accession no. D29973.

RESULTS AND DISCUSSION

Analysis of the regulation system of proline biosynthesis in *T. thermophilus*. The growth of *T. thermophilus* in 3,4-dehydroproline-containing MM was first assayed. As shown in Fig. 3A, the growth of *T. thermophilus* was clearly inhibited by dehydroproline at the concentration of 1 mM. This result indicated that proline biosynthesis was controlled by feedback inhibition and that γ -glutamyl kinase was inhibited by proline in *T. thermophilus*.

Analysis of the existence of a proline degradation system in *T. thermophilus*. The proline degradation system in *T. thermophilus* was examined since it disturbs overproduction of proline. In *E. coli*, two proline degradative genes, the *putA* and *putP* genes, have been reported previously. The *putA* gene encodes proline dehydrogenase, which catalyzes the conversion of proline to pyrroline-5-carboxylate. The *putP* gene encodes proline permease, which is necessary for uptake of extracellular proline into the cell. If *T. thermophilus* has PutA and PutP activity, it is able to grow in MM which contains proline as a sole nitrogen source. We checked whether *T. thermophilus* was able to utilize proline for its growth. As shown in Fig. 3B, *T. thermophilus* was able to grow in MM containing proline as sole nitrogen source. This result indicated that *T. thermophilus* had a proline degradation system.

Site-directed mutagenesis of the chromosomal *proB* gene in *T. thermophilus*. The mutation sites of the proline-overproducing mutants have been determined previously in *E. coli* and *S. marcescens* (3, 4, 14). These proline-overproducing mutants showed 3,4-dehydroproline resistance, and their γ -glutamyl ki-

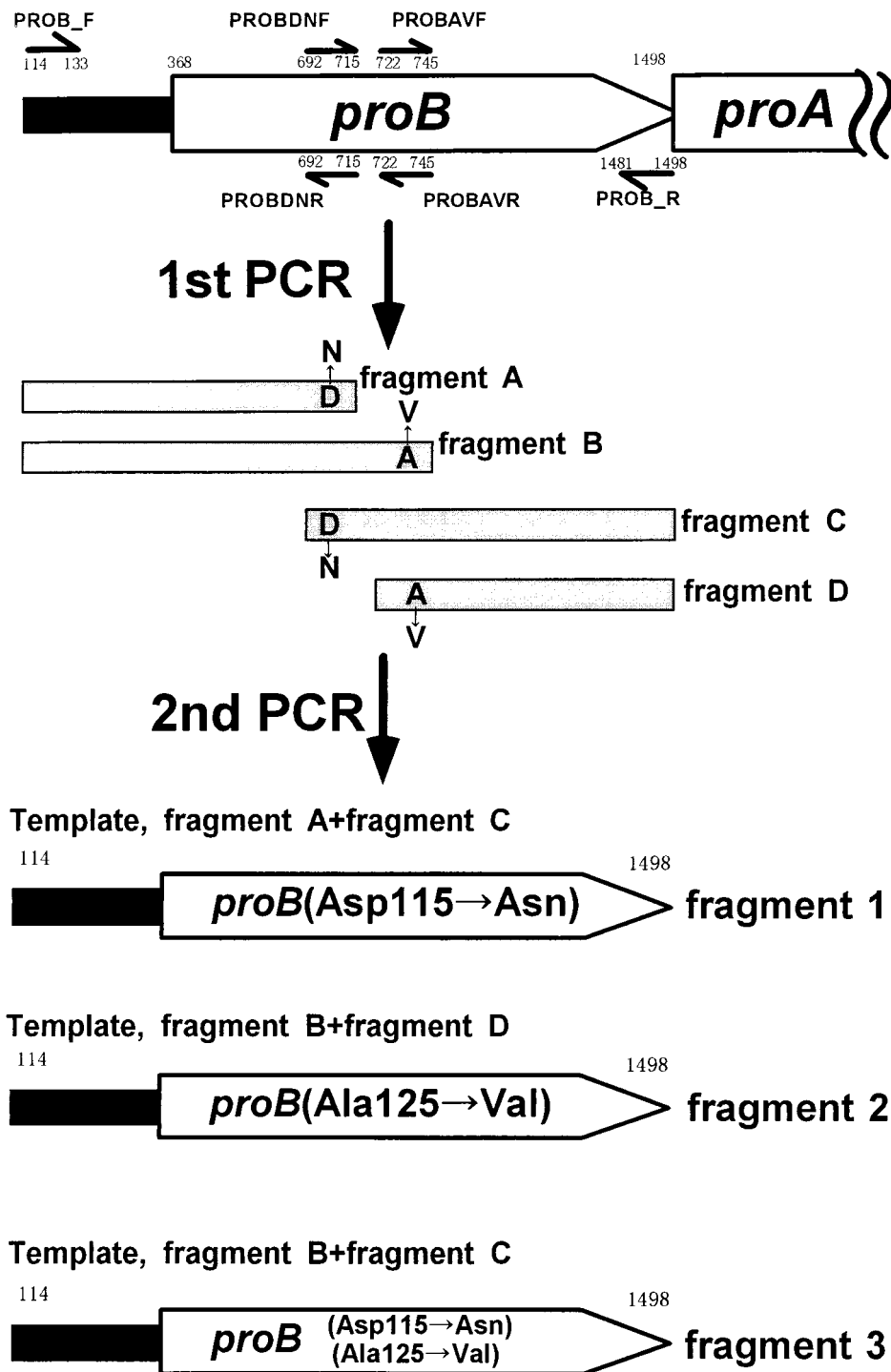


FIG. 2. Outline of the preparation of DNA fragments which contain three types of mutated *proB* genes. The detailed explanations are given in Materials and Methods. The numbers shown below or above each primer correspond to the nucleotide numbers of the *proBA* region.

nases were not inhibited by proline. We compared the amino acid sequences of γ -glutamyl kinases among *T. thermophilus* and two proline-overproducing mutants. As shown in Fig. 1, the mutations occurred at the sites which were conserved in the three γ -glutamyl kinases.

Three kinds of plasmids including the mutated *proB* gene were prepared by recombinant PCR (Fig. 2). Fragment 1 had

the amino acid substitution of Asn for Asp115 in the *proB* gene, fragment 2 had the amino acid substitution of Val for Ala125, and fragment 3 had both amino acid substitutions (Fig. 2). We transformed *T. thermophilus* HB27 by using these plasmids. Many dehydropoline-resistant transformants were obtained when the plasmid including fragment 2 was used for the donor DNA whereas no colony was obtained when the plasmid

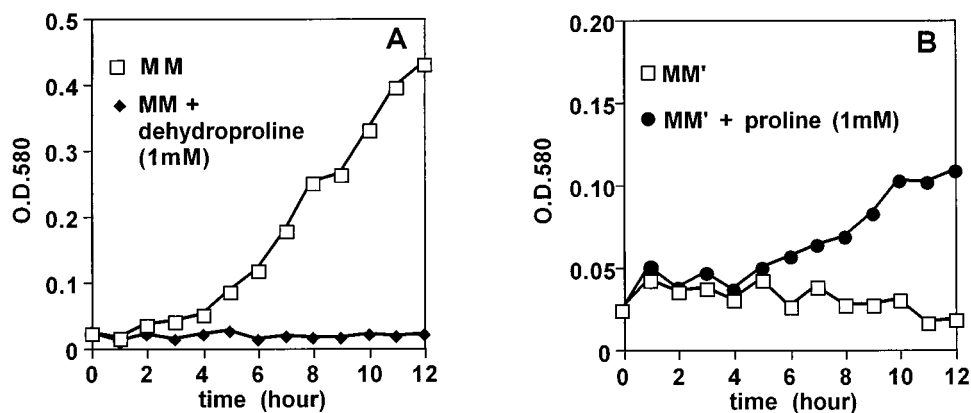


FIG. 3. Growth curves of *T. thermophilus* HB27. HB27 was grown in MM with or without 3,4-dehydroproline (A) and in $(\text{NH}_4)_2\text{SO}_4$ -minus MM (MM') with or without L-proline (B). O.D.580, optical density at 580 nm.

including fragment 1 or fragment 3 was used. No dehydroproline-resistant colonies appeared on the MM containing dehydroproline when no DNA was added. This result showed that only the A125V substitution led to dehydroproline resistance in HB27 and that the D115N substitution might impair the heat stability of γ -glutamyl kinase of *T. thermophilus*. The eight transformants were randomly selected, and their growth was assayed in MM containing 3,4-dehydroproline or proline as sole nitrogen source. Every mutant showed the same growth curve in MM containing 3,4-dehydroproline or proline as sole nitrogen source. From this result, we confirmed that the eight mutants had the same mutations only in the *proB* gene. One of the eight 3,4-dehydroproline-resistant mutants was named TH401. The growth of TH401 was not inhibited in MM containing 1 mM 3,4-dehydroproline. The growth curve of TH401 in MM containing 1 mM 3,4-dehydroproline was the same as that of wild type in MM (data not shown). This fact suggested that TH401 was not subjected to feedback inhibition in proline biosynthesis.

Construction of a proline-producing mutant. Strain TH401 was further mutated by UV irradiation. Seven strains, TH4011, TH4012, TH4013, TH4014, TH4015, TH4016, and TH4017, which were unable to grow on MM-proline plates containing proline as a sole nitrogen source were isolated. The growth curves of these seven mutants in MM were the same as those of TH401 and HB27 wild type. The proline production levels of seven mutants and TH401 were tested by bioassay (see Materials and Methods). The growth of the proline auxotrophic mutant was observed only around the spot of TH4017. This result showed that TH4017 excreted proline into the medium. The detailed amino acid production levels of *T. thermophilus* HB27, TH401, and TH4017 were measured. The amino acid production was measured twice, and a little difference was observed in two experiments. As shown in Table 1, TH4017 produced about 2 mg of proline per liter at 70°C after 12 h of incubation in MM but the wild type did not. TH401 also produced about 1 mg of proline per liter. The proline production levels of other mutants (TH4011, TH4012, TH4013, TH4014, TH4015, and TH4016) were the same as that of TH401 (data not shown). TH4017 showed the highest production of proline among the mutants. This result indicated that TH4017 was the first *T. thermophilus* strain excreting proline into the medium.

As shown in Table 1, TH4017 excreted various amino acids into the medium. The amounts of some of them were larger than that of proline. Since the production of various amino acids was also observed for TH401, it is highly likely that this

fact is due to the mutation in the *proB* gene. In *E. coli*, proline biosynthesis is linked to arginine biosynthesis through the interconversion of pyrroline-5-carboxylate to ornithine (1). No other linkages of proline biosynthesis have been reported. TH401 and TH4017 did not excrete arginine into the medium, but they produced relatively larger amounts of alanine, threonine, and valine. It will be interesting if proline biosynthesis in *T. thermophilus* has some relationship to these amino acid biosynthetic pathways.

Until now, the level of L-proline produced from TH4017 has been very low. We thought this was mainly due to the low expression of the proline biosynthetic genes and the remaining activity of the L-proline degradation system in *T. thermophilus*. We believed that TH4011, TH4012, TH4013, TH4014, TH4015, TH4016, and TH4017 were defective for proline uptake but not for proline dehydrogenase because all mutants could not grow in MM containing 10 mM proline as sole nitrogen source but could grow in MM containing 100 mM proline. As already

TABLE 1. Amino acids production levels of HB27, TH401, and TH4017

Amino acid	Production level (mg/liter) for strain:		
	HB27	TH401	TH4017
Proline		1.01	1.99
Aspartate		0.59	1.56
Threonine		1.86	3.44
Serine		0.28	0.73
Glutamate		0.43	1.35
Glycine		0.36	0.47
Alanine	0.11	6.38	4.81
Cystine			
Valine		1.75	3.86
Methionine		0.28	0.63
Isoleucine		0.23	0.44
Leucine		0.55	1.19
Tyrosine		0.52	1.33
Phenylalanine		0.40	0.62
Lysine	0.13	0.02	
Histidine			
Arginine	0.17		
Tryptophan			
Methionine sulfoxide			
Cysteic acid			
Total	0.41	14.7	22.4

reported, there were no strong promoter sequences upstream of the *proBA* and *proC* genes in *T. thermophilus* (7, 9). Increasing L-proline production by replacing the proline biosynthetic promoters should be effective. We also plan to attempt to increase L-proline production by disrupting the *putA* gene.

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