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Serial increase in the thermal stability of 3-isopropylmalate dehydrogenase from *Bacillus subtilis* by experimental evolution

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Abstract

We improved the thermal stability of 3-isopropylmalate dehydrogenase from *Bacillus subtilis* by an in vivo evolutionary technique using an extreme thermophile, *Thermus thermophilus*, as a host cell. The *leuB* gene encoding *B. subtilis* 3-isopropylmalate dehydrogenase was integrated into the chromosome of a *leuB*-deficient strain of *T. thermophilus*. The resulting transformant showed a leucine-autotrophy at 56 °C but not at 61 °C and above. Phenotypically thermostabilized strains that can grow at 61 °C without leucine were isolated from spontaneous mutants. Screening temperature was stepwise increased from 61 to 66 and then to 70 °C and mutants that showed a leucine-autotrophic growth at 70 °C were obtained. DNA sequence analyses of the *leuB* genes from the mutant strains revealed three stepwise amino acid replacements, threonine-308 to isoleucine, isoleucine-95 to leucine, and methionine-292 to isoleucine. The mutant enzymes with these amino acid replacements were more stable against heat treatment than the wild-type enzyme. Furthermore, the triple-mutant enzyme showed significantly higher specific activity than that of the wild-type enzyme.

Keywords: evolutionary molecular engineering; integration vector system; thermal stability; *Thermus thermophilus*; 3-isopropylmalate dehydrogenase

Enhancement of protein thermostability is one of the major subjects in protein engineering. Recently, many successful examples of protein stabilization have been reported, and several principles to increase the stability of proteins have been proposed, for example: (a) introducing disulfide bonds to reduce the difference in entropy between native and denatured states (Matthews et al., 1987; Matsumura et al., 1989), (b) increasing intramolecular hydrophobic interaction (Yutani et al., 1987), (c) changing specific residues to stabilize the dipoles of α-helices (Nicholson et al., 1988), and (d) introducing metal binding sites (Pantoliano et al., 1988; Kuroki et al., 1989). However, the application of these principles is limited to the proteins whose three-dimensional structures have been determined.

Evolutionary molecular engineering technique is an alternative approach to obtain mutant proteins with enhanced thermostability. The concept of the evolutionary technique was first proposed by Eighen and Gardiner (1984). Using the evolutionary engineering strategy, we can feasibly obtain stabilized proteins even in the absence of information on their three-dimensional structures. This strategy consists of two steps: (a) introduction of random or spontaneous mutations to a target gene, and (b) selection and reproduction of the mutant genes with the desired properties under selective pressure. We previously constructed an integration vector system that enables the expression of foreign genes in an extreme thermophile, *Thermus thermophilus* (Tamakoshi et al., 1995). This thermophilic species is able to grow at temperatures of up to 80 °C (Oshima & Imahori, 1974), and shows high efficiency of homologous recombination with foreign DNAs (Koyama et al., 1986). These characteristics provide us with an opportunity to screen for mutant proteins with enhanced thermostability.

3-Isopropylmalate dehydrogenase (IPMDH), a product of the *leuB* gene, catalyzes the oxidative decarboxylation of (2R,3S)-3-isopropylmalate to 2-oxoisocaproate, which is involved in leucine biosynthesis. The gene have been cloned from *Bacillus subtilis* (Nagahari & Sakaguchi, 1978) and *T. thermophilus* (Tanaka et al., 1981), and their sequences have been determined (Kagawa et al., 1984; Imai et al., 1987; Kirino et al., 1994). The enzyme from *T. thermophilus* has been well characterized in terms of its catalytic properties, thermal stability (Yamada et al., 1990), and tertiary structure (Imada et al., 1991). We have recently constructed several chimeric enzymes by combining parts of the enzymes of *T. thermophilus* and *B. subtilis* (Numata et al., 1995). The gene encoding one of such chimeric enzymes, designated as 2T2M6T, was integrated into the chromosome of a *leuB*-deficient strain of *T. ther-*

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mophilus by using the integration vector system for the thermophile (Tamakoshi et al., 1995). Because the chimeric enzyme is less thermostable than the thermophilic enzyme, the resulting transformant shows a temperature sensitive autotrophy; the strain can grow at 70 °C but not at 79 °C without leucine. However, a phenotypically thermostabilized strain was obtained by selecting a leucine-autotrophic mutant at 79 °C. It was confirmed that the variant of the chimeric enzyme isolated from the spontaneous mutant was more thermostable than the original chimeric enzyme (Tamakoshi et al., 1995).

In the present study, we attempted to stabilize a thermolabile IPMDH from *B. subtilis* by a similar evolutionary technique. The amino acid sequence of the enzyme shows 56% identity with that of *T. thermophilus* IPMDH, whereas thermal stability of the enzyme is less than that of *T. thermophilus* IPMDH by 40 °C (Numata et al., 1995). The enzyme has been purified and characterized (Numata et al., 1995), but crystallographic data have not been available. We applied the evolutionary technique to *B. subtilis* IPMDH to obtain mutant enzymes with enhanced thermostability.

In this article, mutant enzymes designated as I95L, M292I, and T308I represent those in which Ile95 is replaced by Leu, Met292 is replaced by Ile, and Thr308 is replaced by Ile, respectively. For designation of the mutant enzymes with double or triple mutations, the two or three mutations within a single molecule are indicated with a slash, i.e., I95L/T308I and I95L/M292I/T308I.

Results

Integration of B. subtilis leuB gene into T. thermophilus

Figure 1 shows a procedure for the integration of *B. subtilis leuB* gene into the chromosome of *T. thermophilus*. Plasmid pEB118-NdeI was constructed from pEB118 by generating *Nde*I site at the initiation codon of the *leuB* gene by means of the site-directed mutagenesis procedure. The 2.2-kb *Nde*I-*Hin*cII fragment containing the *B. subtilis leuB* gene was cut out from plasmid pEB118-NdeI and then ligated with the larger *Nde*I-*Eco*RV fragment of pITleuBNV. The *leuB*-deficient strain of *T. thermophilus* MT106 was transformed with the resulting plasmid pITBSleuB at 65 °C, followed by selection at 53 °C on a plate coated with *Thermus* minimum medium without leucine. A leucine-autotrophic transformant BTH5601 was isolated on the plate after 7 days of incubation. Strain BTH5601 could grow at 56 °C but not at 61 °C under the conditions lacking leucine, but it could grow at 75 °C with leucine, indicating that *B. subtilis* IPMDH retains the enzymatic activity up to 56 °C under the in vivo condition.

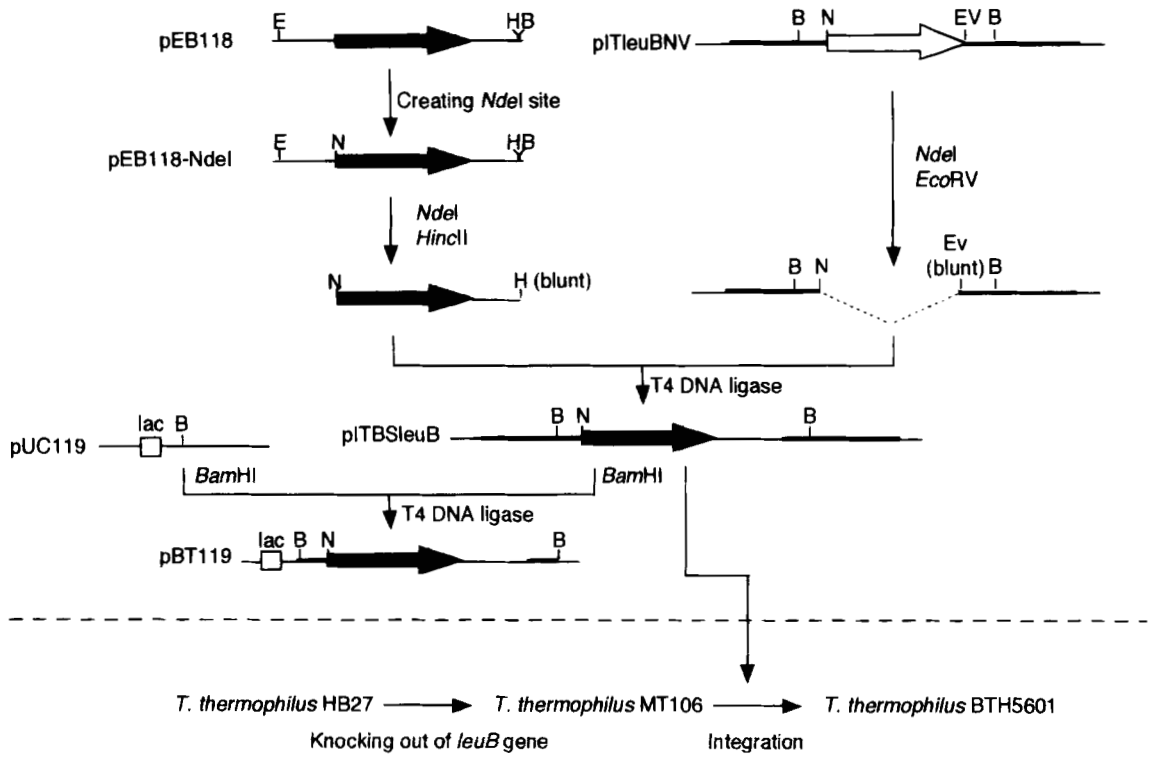

Fig. 1. Construction of plasmids. The recognition site for *Nde*I was generated at the initiation codon of the *leuB* gene carried by plasmid pEB118, and the resulting plasmid was designated as pEB118-NdeI. Plasmid pEB118-NdeI was digested with *Nde*I and *Hin*cII, and the 2.2-kb fragment containing *B. subtilis leuB* gene was isolated. Plasmid pITleuBNV was digested with *Nde*I and *Eco*RV to remove the 1.2-kb fragment containing *T. thermophilus leuB* gene. The resulting 5.7-kb fragment was ligated with the 2.2-kb fragment containing *B. subtilis leuB* gene. The resulting plasmid designated as pITBSleuB was used as an integration vector. *B. subtilis leuB* gene was integrated into the chromosomal DNA of *T. thermophilus* Δ*leuB* strain MT106 by homologous recombination. The 2.1-kb *Bam*HI fragment containing *B. subtilis leuB* gene was excised from pITBSleuB and inserted into the *Bam*HI site of pUC119. The resulting plasmid pBT119 and its derivatives were used for expression of the *leuB* genes in *E. coli*. Thick lines, franking sequences of the *leuB* gene from the wild-type strain of *T. thermophilus*; filled arrows, *B. subtilis leuB* gene; open arrow, *T. thermophilus leuB* gene; open box, lac promoter of pUC119. Restriction sites: E, *Eco*RI; H, *Hin*cII; B, *Bam*HI; N, *Nde*I; Ev, *Eco*RV.

Isolation of phenotypically thermostabilized strains

T. thermophilus mutant strains obtained in this study were summarized in Table 1. The strain BTH5601 was pre-cultured in 2 mL of *Thermus* minimum medium at 56 °C for 3 days. One hundred microliters of the culture containing about 10^7 cells was spread on each of three plates coated with *Thermus* minimum medium and then incubated at a restrictive temperature of 61 °C. After a 3-day incubation, about 1,000 colonies appeared on each plate. One colony was randomly selected from each plate and the selected colonies were named BTH6121, BTH6141, and BTH6151, respectively. These strains could grow at 61 °C but not at 66 °C on the minimum medium plate without leucine.

The strains BTH6121, BTH6141, and BTH6151 were subjected to screening at 66 °C. Each of BTH6121, BTH6141, and BTH6151 was pre-cultured at 61 °C in 2 mL of *Thermus* minimum medium for 3 days and each pre-culture containing about 10^7 cells was plated on *Thermus* minimum medium. Five colonies appeared after a 3-day incubation at 66 °C and were designated as BTH6622, BTH6623, BTH6642, BTH6643, and BTH6652, respectively. Similarly, the strains BTH7024 and BTH7044, leucine-autotrophic mutants at 70 °C, were obtained by screening 5×10^7 cells each of BTH6622 and BTH6642, respectively, on *Thermus* minimum medium at 70 °C for 3 days.

Sequence of mutant leuB genes

The chromosomal DNA of each mutant strain was isolated. The *leuB* gene was amplified by PCR, cloned, and sequenced. Table 1 shows the amino acid replacements revealed by the DNA sequencing of the *leuB* genes from the thermostabilized strains. The DNA sequence of the *leuB* gene from strain BTH6151 showed no nucleotide replacement, suggesting that mutations at positions outside of the *leuB* gene led the phenotype of leucine autotrophic growth at 61 °C. One possible explanation is that a mutation improving the promoter activity of the *leu* operon was generated, and thereby the expression efficiency of the *leuB* gene was increased.

The sequences of the *leuB* genes from BTH6121, BTH6141, and BTH6652 contained a same base substitution that resulted in Thr308 → Ile replacement. The *leuB* genes from BTH6622, BTH6623, BTH6642, and BTH6643 contained an additional base substitution that resulted in Ile95 → Leu replacement. Namely, these *leuB* genes encode the mutant enzyme with double replacements, Ile95 → Leu and Thr308 → Ile. The DNA sequence analyses of BTH7024 and BTH7044 revealed that the *leuB* genes of these strains contained three point mutations corresponding to Ile95 → Leu, Met292 → Ile, and Thr308 → Ile.

Thermal stabilities of mutant enzymes

The wild-type and mutant *leuB* genes were recloned into pUC119 and expressed in *E. coli* JA221. The cells were lysed by sonication and the soluble fractions were used for enzyme purification. The levels of thermal stability of the purified enzymes were estimated by measuring the remaining activities at 40 °C after heat treatment for 10 min at various temperatures ranging 40–63 °C. The thermal inactivation processes of the wild-type and mutant enzymes were irreversible under the conditions employed in the present study. Figure 2 shows that the mutant enzymes T308I, I95L/T308I, and I95L/M292I/T308I are more stable against heat treatment than the wild-type enzyme by 4.5, 8.0, and 10.0 °C, respectively, in terms of the half-inactivation temperature at which 50% inactivation is observed after heat treatment for 10 min.

To examine the effect of the single mutations Ile95 → Leu and Met292 → Ile on thermal stability of the enzyme, the mutant enzymes I95L and M292I were also purified and their thermal inactivations were analyzed. Figure 2 shows that the I95L and M292I mutants are more thermostable than the wild-type enzyme by 5.0 °C and 2.0 °C, respectively, in half-inactivation temperature. These results indicate that the three amino acid replacements independently contributed to the enhanced thermostability of the enzyme and additive effect of these amino acid replacements was observed in the variants with the double or triple mutations.

The thermal denaturation processes of the wild-type and mutant enzymes were also analyzed by measuring the change in ellipticity at 222 nm. Two-step unfolding profiles were observed with all of these enzymes (Fig. 3). The solution of the *B. subtilis* wild-type

Table 1. T. thermophilus *mutant strains obtained in this study*

	Parent	Selection temperature (°C)	Mutations found in *B. subtilis* IPMDH[a]
HB27			Wild-type
MT106	HB27		Δ*leuB*
BTH5601	MT106	53	*B. subtilis leuB*
BTH6121	BTH5601	61	Thr308 → Ile (ACA → ATA)
BTH6141	BTH5601	61	Thr308 → Ile (ACA → ATA)
BTH6151	BTH5601	61	—[b]
BTH6622	BTH6121	66	Thr308 → Ile (ACA → ATA), Ile95 → Leu (ATC → CTC)
BTH6623	BTH6121	66	Thr308 → Ile (ACA → ATA), Ile95 → Leu (ATC → CTC)
BTH6642	BTH6141	66	Thr308 → Ile (ACA → ATA), Ile95 → Leu (ATC → CTC)
BTH6643	BTH6141	66	Thr308 → Ile (ACA → ATA), Ile95 → Leu (ATC → CTC)
BTH6652	BTH6151	66	Thr308 → Ile (ACA → ATA)
BTH7024	BTH6622	70	Thr308 → Ile (ACA → ATA), Ile95 → Leu (ATC → CTC), Met292 → Ile (ATG → ATA)
BTH7044	BTH6642	70	Thr308 → Ile (ACA → ATA), Ile95 → Leu (ATC → CTC), Met292 → Ile (ATG → ATA)

[a]Codons with mutation are shown in parentheses.
[b]No mutation was found in *B. subtilis* IPMDH gene.

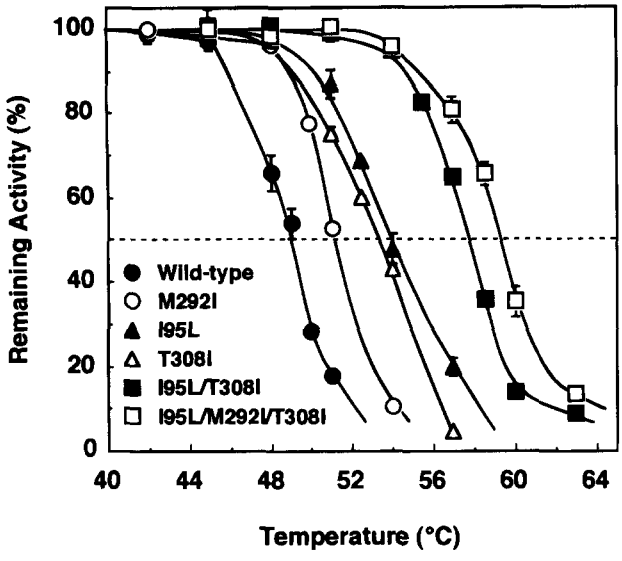

Fig. 2. Activities of *B. subtilis* IPMDHs after heat treatment. Each purified enzyme was diluted to 0.4 mg/mL with 20 mM potassium phosphate buffer (pH 7.6) containing 0.5 mM EDTA and treated at the indicated temperatures for 10 min. The remaining activities were expressed as percentages of the original activities. The values are the averages of three independent determinations.

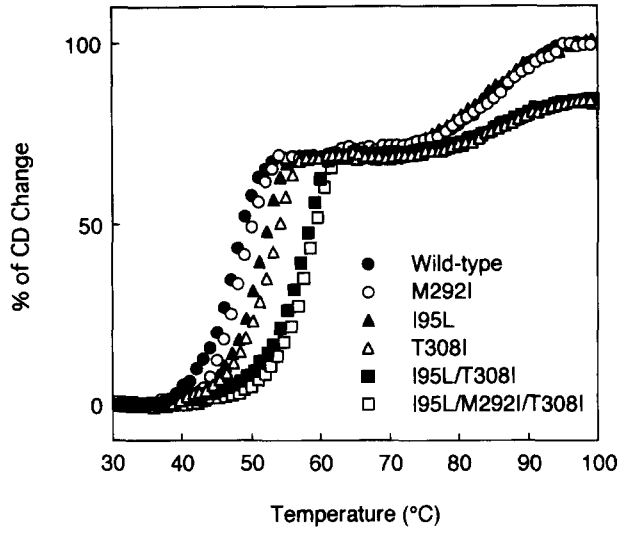

Fig. 3. Thermal melting profiles of *B. subtilis* IPMDHs at pH 7.6. The denaturation was monitored by change in CD at 222 nm. The melting curves were normalized as described by Iwasaki et al. (1996).

enzyme, heated up to 60 °C and then cooled down at 40 °C, showed no enzymatic activity, indicating that the intermediate is catalytically inactive. Figure 3 shows that the Thr308 → Ile replacement stabilizes the enzyme against the thermal denaturation in both of the biphasic steps. The melting profiles of the mutant enzymes with Ile at position 308 indicate the presence of the residual secondary structure even at 100 °C. On the other hand, the amino acid replacements, Ile95 → Leu and Met292 → Ile, increase the first denaturation temperature without affecting the second one.

Enzymatic activities of the mutant enzymes

The steady-state kinetic parameters of the wild-type and mutant enzymes are listed in Table 2. The Michaelis constants, K_m, values for D-3-isopropylmalate (D-3-IPM), a substrate of IPMDH, and for coenzyme NAD of the I95L mutant increased to 1.9- and 1.6-fold those of the wild-type enzyme, respectively. In contrast, the single Met292 → Ile replacement improves K_m values for both D-3-IPM and NAD; the both values decreased to 0.75-fold those of the wild-type enzyme. For the enzyme with the triple mutations, in contrast to the K_m value for D-3-IPM, which was almost similar to that of the wild-type enzyme, the K_m value for NAD was slightly improved compared with that of the wild-type enzyme. Slight increase in catalytic constant, k_{cat}, was also observed in the mutant enzyme with the triple-amino acid replacements.

The specific activities of the wild-type enzyme and its mutant with the triple-amino acid replacement were measured over temperature range 40–75 °C in the presence of fixed concentrations of D-3-IPM (0.4 mM) and NAD (8 mM). Figure 4 indicates that the mutant enzyme shows slightly higher temperature optimum for its activity than that of the wild-type enzyme. Moreover, although the mutant enzyme shows level of activity only 1.3-fold that of the wild-type enzyme at 40 °C, the mutant enzyme is 3.4-fold more active than the wild-type enzyme at 70 °C. These results clearly

Table 2. *Kinetic constants of* B. subtilis *IPMDH and its variants*

Variant	K_m (μM)[a]		k_{cat} (s^{-1})[a,b]	k_{cat}/K_m (M^{-1} s^{-1})	
	D-3-IPM	NAD		D-3-IPM	NAD
Wild-type	30.5 (±2.9)	333 (±3)	21.2 (±0.3)	6.95×10^5	6.36×10^4
M292I	22.6 (±0.2)	248 (±6)	22.0 (±0.3)	9.73×10^5	8.87×10^4
I95L	56.8 (±2.4)	546 (±34)	23.9 (±1.2)	4.21×10^5	4.38×10^4
T308I	32.8 (±2.2)	332 (±20)	23.2 (±0.8)	7.07×10^5	6.98×10^4
I95L/T308I	60.1 (±2.5)	433 (±35)	22.6 (±0.9)	3.76×10^5	5.21×10^4
I95l/M292I/T308I	37.4 (±1.8)	241 (±6)	26.0 (±0.6)	6.95×10^5	1.07×10^5

[a]The kinetic constants, K_m and k_{cat}, were determined in steady-state experiments at 40 °C in 100 mM potassium phosphate buffer containing 1 M KCl, 0.2 mM $MnCl_2$, and various concentrations of D-3-IPM and NAD. Each value is the average of three independent determinations. Standard deviations are shown in parentheses.

[b]k_{cat} values are expressed as reaction per dimer.

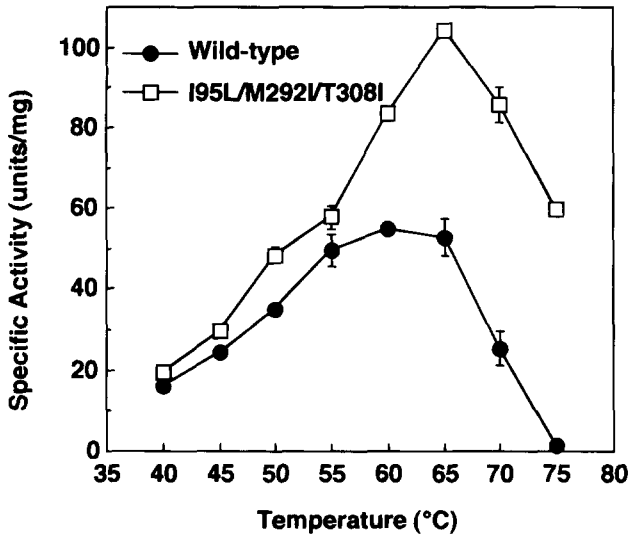

Fig. 4. Effect of temperature on activities of the wild-type and triple-mutant *B. subtilis* IPMDHs. The specific activities were measured in 100 mM potassium phosphate buffer (pH 7.6) containing 1 M KCl, 0.2 mM $MnCl_2$, 0.4 mM D-3-IPM and 8 mM NAD. The values are the averages of three independent determinations.

show that our evolutionary experiment generated the mutant IPMDH, which is more stable and active than the wild-type enzyme at higher temperatures.

Discussion

Evolutionary modification technique using a thermophile as a host cell is one of the useful approaches to improve thermal stability of proteins. The first in vivo selection of an enzyme with enhanced thermostability was carried out with kanamycin nucleotidyltranferase by Liao et al. (1986). We previously reported another evolutionary engineering experiment (Tamakoshi et al., 1995). We developed an integration vector system for *T. thermophilus* and introduced the gene encoding the chimeric IPMDH 2T2M6T into the extreme thermophile. This thermophilic species shows the highest growth temperature among microorganisms whose genetic manipulation system has been developed. The thermal stability of the chimeric enzyme was improved by selecting a spontaneous mutant at 79 °C . In the present study, we introduced *B. subtilis leuB* gene into *T. thermophilus* using the integration vector system and successfully thermostabilized the mesophilic IPMDH.

The contribution of a single amino acid replacement to protein stabilization is not so large in general and, therefore, the combination of multiple stabilizing mutations is necessary to obtain hyperthermostable mutant proteins (Stearman et al., 1988; Pantoliano et al., 1989; Akasako et al., 1995). In this study, the selection of mutations was repeated three times as selection temperature was increased from 61 to 66 and then to 70 °C and the mutant enzyme with the triple amino acid replacements was obtained. The thermal stability of the mutant enzyme was higher than that of the wild-type enzyme by 10 °C in half-inactivation temperature, indicating that the successive selection of thermostabilized variants is a powerful strategy to obtain mutant proteins with significant increase in thermal stability.

To further stabilize the mutant enzyme with the triple amino acid replacements, the strain BTH7024 has been cultured at 73 °C on the minimum medium plate without leucine. Unfortunately, no colony with improved thermal stability was obtained by this method. A limitation to the evolutionary modification technique used in this study is that the method rarely generates contiguous double-base replacements in one codon. Because of this rare incidence, several types of amino acid substitution cannot be practically produced. Recently, a saturation mutagenesis approach was used to identify amino acid residues critical for the function of β-lactamase (Huang et al., 1996). The strategy consisted of randomizing three contiguous codons to create a random library containing all possible amino acid substitutions for the region randomized. An application of this saturated randomization technique may be effective to identify additional stabilizing substitutions that are generated through two simultaneous base replacements within a codon.

Although stabilization of an enzyme is sometimes achieved with a cost of reduced activity (Shoichet et al., 1995), the evolutionary technique produces variants with increased stability without significant loss of catalytic activity because the variants are selected for their activities in vivo at a higher growth temperature. The variant with the triple mutations obtained in this study showed specific activity slightly higher than that of the wild-type enzyme at 40 °C (Fig. 4). Moreover, the maximum value of the specific activity of the variant at 65 °C was twofold higher than that of the wild-type enzyme at 60 °C (Fig. 4). These findings clearly show that our evolutionary experiment generated mutant enzymes with increased activity as well as increased stability compared to those of the original enzyme.

In the present study, we selected three mutations that stepwise increased thermal stability of *B. subtilis* IPMDH. The mutations were generated in the order of Thr308 → Ile (ACA → ATA, at 61 °C), Ile95 → Leu (ATC → CTC, at 66 °C), and Met292 → Ile (ATG → ATA, at 70 °C) in most cases of the independent experiments (Fig. 5). The increase in thermal stability by the Thr308 → Ile mutation is almost similar to that by the Ile95 → Leu mutation (Fig. 2). Nevertheless, the mutant enzyme with Thr308 → Ile substitution was selected as the first mutation, i.e., BTH6121, BTH6141, and BTH6652. The difference in the mutation rate of C → T (Thr308 → Ile) and A → C (Ile95 → Leu) base replacements may be responsible for that the Thr308 → Ile mutation was selected prior to the Ile95 → Leu mutation. Li et al. (1984) pre-

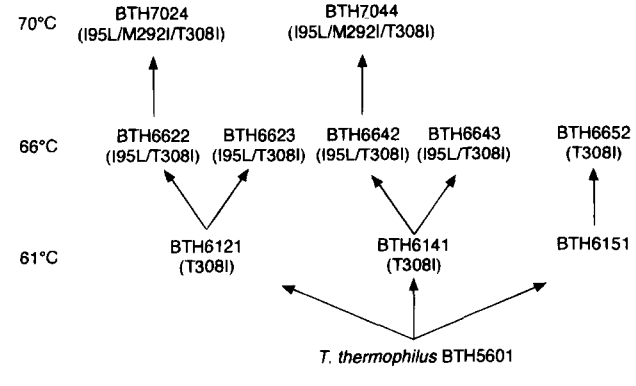

Fig. 5. Evolution pathway of *T. thermophilus* mutant strain, which carried *B. subtilis leuB* gene. Temperatures at which mutant strains were selected are shown.

viously reported that the frequency of C → T base replacement is significantly higher than that of A → C base replacement.

The increase in thermal stability by the Met292 → Ile mutation is less than that by the other two mutations. However, only the Met292 → Ile mutation significantly improves K_m values for D-3-IPM and NAD of the enzyme. Generally, K_m value of an enzyme for its substrate increases with increase in reaction temperature, indicating that production of a mutant enzyme functional at an unusually high temperature requires an improved K_m value as well as an enhanced thermal stability. Therefore, it is reasonably speculated that the in vivo substrate concentration is as such that the Met292 → Ile mutation effectively contributes to make the enzyme functional at 70 °C rather than the mutation does at 61 and 66 °C. This can be why the Met292 → Ile mutation was selected at 70 °C.

Figure 6 shows the amino acid sequence of *B. subtilis* IPMDH, which is composed of 365 amino acid residues, aligned with that of the thermophilic counterpart *T. thermophilus* IPMDH. The amino acid sequence of *T. thermophilus* IPMDH shows 56% identity to that of *B. subtilis* IPMDH. In addition to the types of amino acid replacement, the localization in the primary sequence of the amino acid residues with mutations that increase the protein stability are also shown in Figure 6. The replacements of the residues with the corresponding ones of *T. thermophilus* enzyme are found at positions 95 and 292. However, the residue at position 308 found in the thermostabilized variant of *B. subtilis* IPMDHs is different from that in the sequence of *T. thermophilus* enzyme: *T. thermophilus* IPMDH sequence contains His at this position, whereas it is Ile in the stabilized *B. subtilis* IPMDH. The result indicates that stabilization of thermolabile proteins does not always require to change a residue to a corresponding one found in the sequence of the thermophilic counterpart.

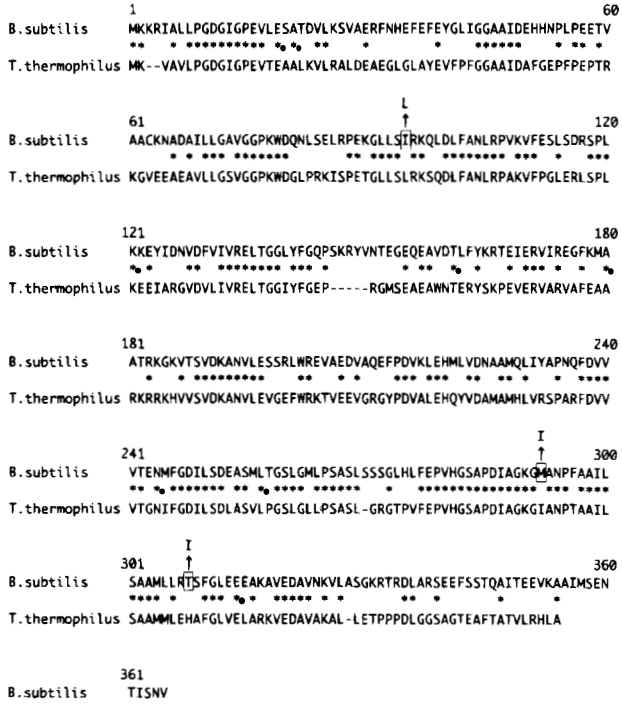

Fig. 6. Alignment of the amino acid sequences of *B. subtilis* and *T. thermophilus* IPMDHs. Numbers above the sequences are those of *B. subtilis* IPMDH. Identical amino acid residues between *B. subtilis* and *T. thermophilus* IPMDHs are indicated by an asterisk. The residues found in the mutants with enhanced thermostability are indicated by arrows, and the original residues are boxed.

The high-resolution three-dimensional structure of *T. thermophilus* IPMDH has been determined by X-ray crystallographic studies (Imada et al., 1991), showing that the enzyme is a dimeric protein composed of two identical subunit each consisting of two structural domains; one (domain 1) contains the N- and C-termini, and the other (domain 2) contains the subunit interface (Fig. 7). In contrast, the efforts to crystallize *B. subtilis* IPMDH have been unsuccessful so far, precluding any crystallographic analyses. However, the significant sequence homology between *B. subtilis* and *T. thermophilus* IPMDHs on their primary structure suggests that both enzymes have similar secondary and tertiary structures. The crystal structure of *T. thermophilus* IPMDH suggests that all of the mutated residues are located in domain 1, which is not involved in the subunit interaction. Iwasaki et al. (1996) previously studied the thermal unfolding process of the chimeric IPMDH 2T2M6T by differential scanning calorimetry and CD spectrophotometry. They reported the biphasic thermal unfolding transition of the chimeric enzyme; the first phase corresponds to the unfolding of domain 1 and the second to that of domain 2. Similar biphasic thermal denaturations were observed in the CD melting profiles of *B. subtilis* IPMDHs (Fig. 3), suggesting that the thermal denaturation of domain 1 has occurred prior to that of domain 2. Furthermore, all of the mutations found in this study stabilize the first step in the biphasic denaturation. Iwasaki et al. also suggested that the improvement of the thermal stability of domain 1 affects to the stability of the whole molecule more effectively than that of domain 2 does. Our present results support the previous finding that the mutations in the thermolabile domain (domain 1) can efficiently improve the thermostability of the whole enzyme.

Several authors have reported improvement of protein thermostability by increasing intramolecular hydrophobicity of the proteins (Yutani et al., 1987; Matsumura et al., 1988; Kellis et al., 1989). We have previously found a mutation Ala172 → Val in a thermostabilized *T. thermophilus* IPMDH variant that was obtained by means of a suppressor mutation method (Kotsuka et al.,

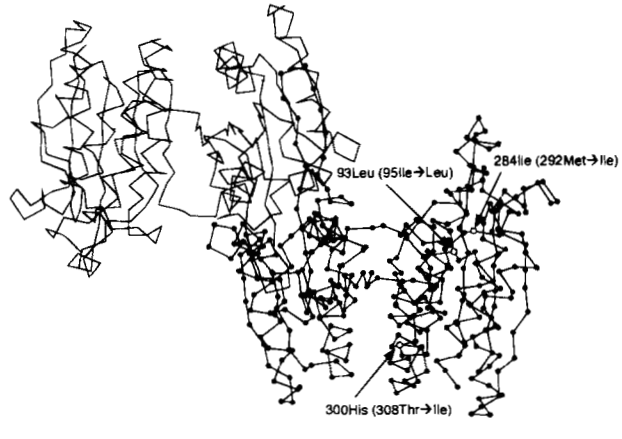

Fig. 7. Backbone structure of *T. thermophilus* IPMDH. α-Carbons in one subunit of the dimer are represented with filled circles. Amino acid substitutions found in the mutant IPMDHs are shown in parentheses with residue number in *B. subtilis* IPMDH. Corresponding residues in *T. thermophilus* IPMDH are represented by open circles and are indicated by an arrow with the position of the residue.

1996). A crystal structure of the thermostabilized variant revealed that the Ala172 → Val mutation filled up a gap between domains 1 and 2, which was surrounded by His300 and several hydrophobic residues (Qu et al., unpubl. obs.). Thereby, we believe that the enhanced thermal stability of the *T. thermophilus* IPMDH variant was caused by the improved hydrophobic interaction between the domains. Because the residue at position 308 in the sequence of *B. subtilis* IPMDH corresponds to His300 in that of the *T. thermophilus* enzyme (Fig. 6), the Thr308 → Ile substitution is also expected to improve the domain–domain interaction through the increased hydrophobicity. To test this possibility, it must be a useful approach to randomize the codon for the residue 308 to generate all possible amino acid substitutions. The study is under investigation.

On the other hand, the effect of the mutation at amino acid position 95 cannot be explained by hydrophobicity of the residues; the introduced residue, leucine, is slightly less hydrophobic than the original one, isoleucine. Previously, the same type of amino acid replacement was found in the thermostabilized variant of chimeric IPMDH (Tamakoshi et al., 1995), that was also obtained by the evolutionary experiment. The chimeric enzyme variant with enhanced thermostability had also leucine at 93, the site corresponds to Ile95 in the sequence of *B. subtilis* IPMDH, instead of the original residue isoleucine. The crystal structures of the original and mutant chimeric enzymes have been determined (Onodera et al., 1994; Sakurai et al., 1995), and the results suggest that the removal of the steric repulsion by the mutation plays a crucial role in the increased thermostability. A similar mechanism may be involved in the increased thermostabilities of *B. subtilis* IPMDHs with the Ile95 → Leu mutation.

The amino acid sequence of *T. thermophilus* IPMDH has Ile at position 284, which corresponds to Met292 in the sequence of *B. subtilis* IPMDH (Fig. 6). The residue is in a loop linking a β-strand and an α-helix in the crystal structure of the thermophile enzyme (Fig. 7). Although the detailed mechanism of the stabilization by the Met292 → Ile mutation has not been apparent yet, it is most likely that the improved hydrophobic interaction is responsible for the increased thermostability of the *B. subtilis* IPMDH variants with the Met292 → Ile mutation.

Materials and methods

Plasmids

The *Eco*RI-*Bam*HI fragment encoding the *leuB* gene of *B. subtilis* was previously cloned into pUC118, and the resulting plasmid was designated as pEB118 (Numata et al., 1995). Plasmid pITleuBNV (Tamakoshi et al., 1997) was also previously constructed by inserting the 1.2-kb *Bam*HI fragment containing *T. thermophilus leuB* gene into pIT1, which is an integration vector for *T. thermophilus* (Tamakoshi et al., 1995). The plasmid pITleuBNV has *Nde*I and *Eco*RV sites at the initiation codon and at immediately downstream of the termination codon of the *T. thermophilus leuB* gene, respectively.

Bacterial strains and media

The *leuB*-deficient strain of *T. thermophilus* MT106 was isolated by Tamakoshi et al. (1995). *Thermus* strains were cultured in *Thermus* nutrient medium (Oshima & Imahori, 1974) or *Thermus* minimum medium (Sakaki & Oshima, 1975) supplemented with 25 mg/L each of isoleucine and valine (Takada et al., 1993). Solidification of the media was performed by mixing the double-strength media and the Gelrite solution after autoclave sterilization. *Escherichia coli* JA221 (F^-, *hsdR*, *trpE5*, *leuB6*, *lacY*, *recA1*, λ^-) was used as a host for plasmid amplification and for expression of the *leuB* gene, and was cultured in 2YT medium (Sambrook et al., 1989) supplemented with 150 mg/L ampicillin.

DNA manipulation procedures

Recombinant DNA experiments were done by standard methods (Sambrook et al., 1989). Restriction endonucleases and modification enzymes were purchased from Takara shuzo, Toyobo, or New England Biolab. To generate a *Nde*I site at the initiation codon of the *B. subtilis leuB* gene harbored by plasmid pEB118, site-directed mutagenesis was carried out using an oligonucleotide, 5′-AAT ACG TTT CTT CAT ATG AAC CGT TCT CCT-3′, by the method of Kunkel et al. (1987). The resulting plasmid was designated as pEB118-NdeI.

Transformation of T. thermophilus

Integration of *B. subtilis leuB* gene into the *leuB*-deficient strain of *T. thermophilus* MT106 was carried out according to the transformation procedure described by Koyama et al. (1986), with slight modifications reported by Takada et al. (1993). The transformation mixture was incubated at 65 °C for 3 h and then plated on *Thermus* minimum medium without leucine. Five colonies appeared after a 7-day incubation at 53 °C. One of the colonies were designated as BTH5601 and used for the further study.

PCR and sequencing

The *leuB* genes of the spontaneous mutants were amplified by PCR from the genomic DNAs prepared as described previously (Kotsuka et al., 1996). Oligonucleotides 5′-ACC GCA CCG CTC CCC TCT TCA TCC-3′ and 5′-AGG CAA GAT CTT TTA TTT CCT CCC TCT AC-3′ were used as specific PCR primers for the *leuB* gene. The details of the PCR conditions were reported previously (Akanuma et al., 1996). The PCR products were cloned into M13mp18 or M13mp19 to prepare templates for DNA sequence analysis. The DNA sequences of the mutant *leuB* genes were determined by the dideoxy chain termination method (Sanger et al., 1977) with AutoRead sequencing kits (Pharmacia) and a Pharmacia ALF DNA Sequencer II.

Thermal stability measurement

For production of the wild-type and mutant *B. subtilis* IPMDHs, the respective *leuB* genes were recloned into *Bam*HI site of pUC119 according to Numata et al. (1995). Expression of the genes in *E. coli* and purification of the enzymes were also carried out as described by Numata et al. (1995). All the enzymes used in this study were purified to homogeneity as judged by SDS-gel electrophoresis. The remaining activities of the purified enzymes after heat treatment over temperature range 40–63 °C were measured at 40 °C as described previously (Kotsuka et al., 1996).

Thermal denaturation was measured with a JASCO J720C spectropolarimeter as described by Iwasaki et al. (1996). The enzymes were dissolved in 20 mM potassium phosphate buffer (pH 7.6) and

the enzyme concentration was 0.3 mg/mL. The temperature of the enzyme solution was controlled with a circulating bath and a programmable temperature controller. The precise temperature was monitored with a thermocouple and scan rate was 1.0 °C/min.

Enzyme assay

The enzymatic activities were measured in 100 mM potassium phosphate buffer (pH 7.6) containing 1 M KCl, 0.2 mM $MnCl_2$,0.4 mM D-3-IPM and 8 mM NAD over temperature range 40–75 °C by monitoring the production of NADH at 340 nm as described by Yamada et al. (1990). The enzyme unit was defined as 1 μmol NADH formed per min (Yamada et al., 1990).

The kinetic parameters, K_m for D-3-IPM and k_{cat}, were determined in steady state experiments at 40 °C in 100 mM potassium phosphate buffer (pH 7.6) containing 1 M KCl, 0.2 mM $MnCl_2$, and 4 mM NAD with varying concentrations of D-3-IPM, ranging 10–100 μM. To determine K_m for NAD, the concentrations of the coenzyme were varied in the range of 50–400 μM, employing a fixed D-3-IPM concentration (0.4 mM). Initial rates were measured as described by Yamada et al. (1990).

References

Akanuma S, Yamagishi A, Tanaka N, Oshima T. 1996. Spontaneous tandem sequence duplications reverse the thermal stability of carboxyl-terminal modified 3-isopropylmalate dehydrogenase. *J Bacteriol 178*:6300–6304.

Akasako A, Haruki M, Oobatake M, Kanaya S. 1995. High resistance of *Escherichia coli* ribonuclease HI variant with quintuple thermostabilizing mutations to thermal denaturation, acid denaturation, and proteolytic degradation. *Biochemistry 34*:8115–8122.

Eighen M, Gardiner W. 1984. Evolutionary molecular engineering based on RNA replication. *Pure Appl Chem 56*:967–978.

Huang W, Petrosino J, Hirsch M, Shenkin PS, Palzkill T. 1996. Amino acid sequence determinants of β-lactamase structure and activity. *J Mol Biol 258*:688–703.

Imada K, Sato M, Tanaka N, Katsube Y, Matsuura Y, Oshima T. 1991. Three-dimensional structure of a highly thermostable enzyme, 3-isopropylmalate dehydrogenase of *Thermus thermophilus* at 2.2 Å resolution. *J Mol Biol 222*:725–738.

Imai R, Sekiguchi T, Nosoh Y, Tsuda K. 1987. The nucleotide sequence of 3-isopropylmalate dehydrogenase gene from *Bacillus subtilis*. *Nucleic Acids Res 15*:4988.

Iwasaki YH, Numata K, Yamagishi A, Yutani K, Sakurai M, Tanaka N, Oshima T. 1996. A stable intermediate in the thermal unfolding process of a chimeric 3-isopropylmalate dehydrogenase between a thermophilic and a mesophilic enzymes. *Protein Sci 5*:511–516.

Kagawa Y, Nojima H, Nukiwa N, Ishizuka M, Nakajima T, Yasuhara T, Tanaka T, Oshima T. 1984. High guanine plus cytosine content in the third letter of codons of an extreme thermophile. DNA sequence of the isopropylmalate dehydrogenase of *Thermus thermophilus*. *J Biol Chem 259*:2956–2960.

Kellis JT, Nyberg K, Fersht AR. 1989. Energetics of complementary side-chain packing in a protein hydrophobic core. *Biochemistry 28*:4914–4922.

Kirino H, Aoki M, Aoshima M, Hayashi Y, Ohba M, Yamagishi A, Wakagi T, Oshima T. 1994. Hydrophobic interaction at the subunit interface contributes to the thermostability of 3-isopropylmalate dehydrogenase from an extreme thermophile, *Thermus thermophilus*. *Eur J Biochem 220*:275–281.

Kotsuka T, Akanuma S, Tomuro M, Yamagishi A, Oshima T. 1996. Further stabilization of 3-isopropylmalate dehydrogenase of an extreme thermophile, *Thermus thermophilus*, by a suppressor mutation method. *J Bacteriol 178*:723–727.

Koyama Y, Hoshino T, Tomizuka N, Furukawa K. 1986. Genetic transformation of the extreme thermophile *Thermus thermophilus* and of other *Thermus* spp. *J Bacteriol 166*:338–340.

Kunkel TA, Roberts JD, Zakour RA. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol 154*:367–382.

Kuroki R, Taniyama Y, Seko C, Nakamura H, Kikuchi M, Ikehara M. 1989. Design and creation of a Ca^{2+} binding site in human lysozyme to enhance structural stability. *Proc Natl Acad Sci USA 86*:6903–6907.

Li WH, Wu CI, Luo CC. 1984. Nonrandomness of point mutation as reflected in nucleotide substitutions in pseudogenes and its evolutionary implications. *J Mol Evol 21*:58–71.

Liao H, McKenzie T, Hageman R. 1986. Isolation of a thermostable enzyme variant by cloning and selection in a thermophile. *Proc Natl Acad Sci USA 83*:576–580.

Matsumura M, Becktel WJ, Levitt M, Matthews BW. 1989. Stabilization of phage T4 lysozyme by engineered disulfide bonds. *Proc Natl Acad Sci USA 86*:6562–6566.

Matsumura M, Becktel WJ, Matthews BW. 1988. Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3. *Nature 334*:406–410.

Matthews BW, Nicholson H, Becktel WJ. 1987. Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. *Proc Natl Acad Sci USA 84*:6663–6667.

Nagahari K, Sakaguchi K. 1978. Cloning of *Bacillus subtilis* leucina A, B and C genes with *Escherichia coli* plasmids and expression of the *leuC* gene in *E. coli*. *Mol Gen Genet 158*:263–270.

Nicholson H, Becktel WJ, Matthews BW. 1988. Enhanced protein thermostability from designed mutations that interact with α-helix dipoles. *Nature 336*:651–656.

Numata K, Muro M, Akutsu N, Nosoh Y, Yamagishi A, Oshima T. 1995. Thermal stability of chimeric isopropylmalate dehydrogenase genes constructed from a thermophile and a mesophile. *Protein Eng 8*:39–43.

Onodera K, Sakurai M, Moriyama H, Tanaka N, Numata K, Oshima T, Sato M, Katsube Y. 1994. Three-dimensional structures of chimeric enzymes between *Bacillus subtilis* and *Thermus thermophilus* 3-isopropylmalate dehydrogenases. *Protein Eng 7*:453–459.

Oshima T, Imahori K. 1974. Description of *Thermus thermophilus* (Yoshida and Oshima) comb. nov., a nonsporulating thermophilic bacterium from a Japanese thermal spa. *Int J Syst Bacteriol 24*:102–112.

Pantoliano MW, Whitlow M, Wood JF, Dodd SW, Hardman KD, Rollence ML, Bryan PN. 1989. Large increases in general stability for subtilisin BPN′ through incremental changes in the free energy of unfolding. *Biochemistry 28*:7205–7213.

Pantoliano MW, Whitlow M, Wood JF, Rollence ML, Finzel BC, Gilliland GL, Poulos TL, Bryan PN. 1988. The engineering of binding affinity at metal ion binding sites for the stabilization of proteins: Subtilisin as a test case. *Biochemistry 27*:8311–8317.

Sakaki Y, Oshima T. 1975. Isolation and characterization of a bacteriophage infectious to an extreme thermophile, *Thermus thermophilus* HB8. *J Virol 15*:1449–1453.

Sakurai M, Moriyama H, Onodera K, Kadono S, Numata K, Hayashi Y, Kawaguchi J, Yamagishi A, Oshima T, Tanaka N. 1995. The crystal structure of thermostable mutants of chimeric 3-isopropylmalate dehydrogenase, 2T2M6T. *Protein Eng 8*:763–767.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA 74*:5463–5467.

Shoichet BK, Baase WA, Kuroki R, Matthews BW. 1995. A relationship between protein stability and protein function. *Proc Natl Acad Sci USA 92*:452–456.

Stearman RS, Frankel AD, Freire E, Liu BS, Pabo CO. 1988. Combining thermostable mutations increases the stability of lambda repressor. *Biochemistry 27*:7571–7574.

Takada T, Akanuma S, Kotsuka T, Tamakoshi M, Yamagishi A, Oshima T. 1993. Recombination-deficient mutants of an extreme thermophile, *Thermus thermophilus*. *Appl Environ Microbiol 59*:2737–2739.

Tamakoshi M, Uchida M, Tanabe K, Fukuyama S, Yamagishi A, Oshima, T. 1997. A new *Thermus–E. coli* shuttle-integration vector system. *J Bacteriol 179*:4811–4814.

Tamakoshi M, Yamagishi A, Oshima T. 1995. Screening of stable proteins in an extreme thermophile, *Thermus thermophilus*. *Mol Microbiol 16*:1031–1036.

Tanaka T, Kawano N, Oshima T. 1981. Cloning of 3-isopropylmalate dehydrogenase gene of an extreme thermophile and partial purification of the gene product. *J Biochem (Tokyo) 89*:677–682.

Yamada T, Akutsu N, Miyazaki K, Kakinuma K, Yoshida M, Oshima T. 1990. Purification, catalytic properties, and thermal stability of threo-Ds-3-isopropylmalate dehydrogenase coded by *leuB* gene from an extreme thermophile, *Thermus thermophilus* strain HB8. *J Biochem (Tokyo) 108*:449–456.

Yutani K, Ogasahara K, Tsujita T, Sugino Y. 1987. Dependence of conformational stability on hydrophobicity of the amino acid residue in a series of variant proteins substituted at a unique position of tryptophan synthase alpha subunit. *Proc Natl Acad Sci USA 84*:4441–4444.