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Directed Evolution for Thermostabilization of a Hygromycin B Phosphotransferase from *Streptomyces hygroscopicus*

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To obtain a selection marker gene functional in a thermophilic bacterium, *Thermus thermophilus*, an *in vivo*-directed evolutionary strategy was conducted on a hygromycin B phosphotransferase gene (*hyg*) from *Streptomyces hygroscopicus*. The expression of wild-type *hyg* in *T. thermophilus* provided hygromycin B (HygB) resistance up to 60 °C. Through selection of mutants showing HygB resistance at higher temperatures, eight amino acid substitutions and the duplication of three amino acids were identified. A variant containing seven substitutions and the duplication (HYG10) showed HygB resistance at a highest temperature of 74 °C. Biochemical and biophysical analyses of recombinant HYG and HYG10 revealed that HYG10 was in fact thermostabilized. Modeling of the three-dimensional structure of HYG10 suggests the possible roles of the various substitutions and the duplication on thermostabilization, of which three substitutions and the duplication located at the enzyme surface suggested that these mutations made the enzyme more hydrophilic and provided increased stability in aqueous solution.

Key words: thermostabilization; hygromycin B phosphotransferase; *Thermus thermophilus*; circular dichroism analysis; reverse hydrophobic effect

Enzymes from thermophiles are of interest in many research fields as well as industry. In industry, the thermal stability of the enzymes and stability at ambient temperatures allows for their use at higher temperatures (*e.g.*, in faster catalytic conditions) and easier handling. In research fields, thermophilic enzymes are generally thought to be suitable for the analysis of three-dimensional structures, because they are relatively easy to crystallize as compared to mesophilic enzymes. Hence, the whole-cell project of *Thermus thermophilus* HB8 is ongoing in Japan.[1)] In addition, analysis of the thermostability of these enzymes is important to elucidate how they obtain their thermostability, and, based on the knowledge in these studies, how a mesophilic enzyme can be converted to a thermostable or thermophilic one through amino acid substitutions. Many studies have been conducted to design thermostable enzymes with the backbones of mesophilic enzymes based on their three-dimensional structures or amino acid sequence similarities, but they were unsuccessful in many cases. It is generally recognized that a single amino acid substitution only has a small effect on thermostability, while additional substitutions have a cumulative effect. This recognition together with the requirement of information on precise three-dimensional structures has made design approaches to thermostable enzymes difficult.

On the other hand, a directed evolutionary strategy is very convenient in generating a thermostable mutant enzyme, because no structural information is required. This can be accomplished by selecting a mutant gene from a pool of randomly mutagenized genes whose product is functional at higher temperatures. For this purpose, the host-vector system of *T. thermophilus* is the most suitable, because this bacterium has a higher and wider range of growth temperatures (50–82 °C) than *Escherichia coli*. It is also easy to transform. Several successful studies have been done by this host-vector system, such as HTK for the kanamycin (Km) resistance gene from *Staphylococcus aureus*,[2)] HTS for the bleomycin-resistance gene from *Streptomyces hindustanus*,[3)] and the *leuB* genes from *Bacillus subtilis*[4)] and *Saccharomyces cerevisiae*.[5)]

To select thermostable mutants, the development of a positive screening system is necessary. In this regard, antibiotic resistance genes are the best candidates,

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Abbreviations: the *hyg*, hygromycin B phosphotransferase gene from *Streptomyces hygroscopicus*; HygB, hygromycin B; Km, kanamycin; *hph*, hygromycin B phosphotransferase gene from *Escherichia coli*; T_{max}, maximum temperature to function as a selection marker; E_a, activation energy; $\Delta G^{\ddagger}$, Gibbs free energy change in activation; $\Delta H^{\ddagger}$, enthalpy change in activation; $\Delta S^{\ddagger}$, entropy change in activation; CD, circular dichroism; DSC, differential scanning calorimetry; ΔH_m, enthalpy change in unfolding at T_m; T_{half}, half-inactivation temperature; APH, aminoglycoside phosphotransferase

because thermostable mutant genes can be selected at higher temperatures in the presence of the respective antibiotics. Thermostable antibiotic resistance genes are also desired to develop host-vector systems in thermophilic and hyperthermophilic organisms. Recently, we reported the development of a thermostable hygromycin B resistance marker gene that is functional in *T. thermophilus* based on an *E. coli* hygromycin B phosphotransferase gene (*hph*) by introducing five amino acid substitutions obtained by natural mutations and selection at higher temperatures in *T. thermophilus*. The mutant gene obtained, *hph5*, was functional as a selection marker in *T. thermophilus* at up to 65 °C.[6] We have also reported that mutant protein HPH5 was thermostabilized at the enzyme level at 17 °C,[7] as well as crystallization and structural analysis of it.[8,9] Recently, another thermostable *hph* mutant was found to be functional at up to 82 °C in *T. thermophilus*,[10] but no precise enzymatic characterization of this mutant protein has been reported.

Here we describe the thermostabilization of another hygromycin B phosphotransferase (HYG; EC 2.7.1.119) from *Streptomyces hygroscopicus*. HPH and HYG show relatively low identity, of approximately 30%, and phosphorylate hygromycin B (HygB) at different sites.[11,12] The introduction of seven amino acid substitutions and a duplication of three amino acids, obtained by natural mutations, left the mutant gene (*hyg10*) functional as a selection marker at up to 74 °C in *T. thermophilus*. A precise enzymatic characterization of HYG10 is also given.

Materials and Methods

Bacterial strains, plasmids, and media. T. thermophilus HB27 TH104 (*proC4*),[13] harboring cryptic plasmid pTT8,[14] was used as host to screen thermostabilized mutant genes. The *E. coli* JM109 and BL21 (DE3) strains were used as hosts for genetic manipulation and protein production respectively. Plasmid pT8S-P31[15] was used to introduce the *hyg* gene into *T. thermophilus*. It can be replicated only in *E. coli*, but when introduced into *T. thermophilus* harboring pTT8, homologous recombination occurs between the two plasmids. Thus, by selecting Km-resistance (Km^r) colonies, it is possible to obtain a pTEV-P31 plasmid that is replicable only in *T. thermophilus*.[15] Plasmid pET21a(+) was used for protein production and purification. Plasmid pV1[16] was used as source of the *hyg* gene.

TM[17] and LB media were used in the cultivation of *T. thermophilus* and *E. coli* respectively. When necessary, Km, HygB, or ampicillin was added to the medium, at concentrations of 40, 40, and 100 μg/mL, respectively.

Transformation. Transformation of the *T. thermophilus* strain was conducted as previously described.[18] Transformation of *E. coli* strains was performed by electroporation by standard laboratory methods.

Construction of pTEV-P31-hyg. The wild-type *hyg* gene was PCR-amplified from pV1 with primers hyg-f and hyg-r (Table S1; see *Biosci. Biotechnol. Biochem.* Web site). The resulting DNA fragment was digested with *Nde*I and *Sph*I sites located at the initiation codon of the *hyg* gene in hyg-f and just downstream of the termination codon in hyg-r respectively, and then cloned into the respective sites of pT8S-P31. After proper construction of the plasmid was confirmed by sequencing with several primers, listed in Table S1, and a CEQ8000XL DNA sequencer (Beckman-Coulter, Brea, CA), the resulting plasmid, pT8S-P31-hyg, was transformed in *T. thermophilus*. The transformants were selected using 40 μg/mL of Km at 60 °C, and hence the plasmid was designated pTEV-P31-hyg.

Selection of in vivo *thermostabilized* hyg *mutants on solid medium.* Screening for thermostabilized *hyg* mutants was done through several steps. First, approximately 10,000 colonies of the strain harboring pTEV-P31-hyg were replica-plated onto TM containing HygB (TM-HygB) plates and cultivated at 65 °C for 2 d. The microcolonies that appeared were picked out and streaked onto fresh TM-HygB plates, and fast-growing colonies were selected after 1 d of cultivation at 65 °C. Plasmids were recovered from the cells, and were used to transform *T. thermophilus* TH104 (pTT8). After selection by Km^r at 60 °C, the transformants were cultivated on TM-HygB plates at 65 °C, and again the colonies that had formed after 1 d of cultivation were selected and considered thermostabilized mutants.

Besides the procedure described above, a DNA shuffling experiment was conducted. The plasmids were isolated from all of the microcolonies appearing at the first screening at 65 °C as a mixture (pTEV-P31-hyg65mix), and the *hyg* gene was PCR amplified with hyg-f and hyg-r primers. Then 2 μg of the product was treated with 0.15 U DNase I for 10 min and the degradation products of <150 bp were recovered from an agarose gel. The fragments were used as templates for two-step PCR amplification, first without primers and then with the hyg-f and hyg-r primers. The amplified *hyg* gene was cloned again into pT8S-P31, as described above, and the resulting plasmid was used to transform *T. thermophilus* TH104 (pTT8). Transformants carrying the thermostabilized mutants were selected directly onto TM-HygB plates at 70 °C. All of the mutants selected were assessed for the presence of mutations in the *hyg* gene by DNA sequencing, as described above.

Accumulation of identified thermostabilized mutations by overlap extension PCR. Based on the *hyg4* mutant, containing four amino acid substitutions of L79F, G189R, S252P, and L290M, which was isolated in the DNA shuffling experiment, the mutations identified in the other mutants were introduced. The *hyg5* gene, containing E237K in addition to the mutations in the *hyg4* gene, was constructed by overlap extension PCR[19] using primer pairs P31-f and hyg-E237K-r and E237K-f and mcs-r, together with pTEV-P31-hyg4 as template. Similarly, the *hyg6-1*, *hyg6-2*, *hyg6-3*, and *hyg7* genes, containing additional S83L/D285N, E237K/D285N, S83L/E237K, and S83L/E237K/D285N mutations based on *hyg4* gene, respectively, were constructed using the appropriate primer pairs, listed in Table S1. All of the mutant genes were cloned into the *Nde*I-*Sph*I sites of pT8S-P31, and after confirmation of the mutations by DNA sequencing, were introduced into *T. thermophilus* cells.

Isolation of further thermostabilized hyg *mutants by liquid culture.* The *T. thermophilus* strain harboring pTEV-P31-hyg6-2 was precultured in TM liquid medium at 72 °C. One mL of the culture was inoculated into 10 mL of TM-HygB liquid medium and cultured at 72 °C. After 30 h, cultures that reached an OD600 of >1.0 were appropriately diluted, plated onto TM-Km plates, and incubated at 60 °C. Several colonies were transferred onto TM-HygB plates, and growth was tested at 74 °C. At the same time, one mL of liquid culture at 72 °C was inoculated into 10 mL of fresh TM-HygB liquid medium and cultured at 74 °C. From this culture, the thermostabilized mutants were isolated by the procedure described above, but a growth test on TM-HygB plates was conducted at 76 °C. Likewise, serial cultivation in liquid TM-HygB medium was conducted at 76, 78, and 80 °C, and the colonies obtained from each culture were tested for the $HygB^r$ phenotype on TM-HygB plates at a temperature 2 °C higher than that for liquid cultivation.

Plasmids were isolated from the colonies obtained and re-introduced into the wild-type strain. The transformants were assessed for $HygB^r$ at the various temperatures.

Growth test in liquid culture. T. thermophilus cells harboring a pTEV-P31 plasmid containing the mutant *hyg* gene were cultivated in liquid TM-HygB medium at various temperatures, and cell growth was monitored by the optical density at 600 nm. The maximum growth temperature determined was defined as the maximum temperature, to function as a selection marker (T_{max}).

Production and purification of HYG and HYG10 in E. coli. The DNA fragments corresponding to the *hyg* and *hyg10* genes were PCR

amplified from pT8S-P31-hyg and pTEV-P31-hyg10 respectively with primers hyg-f and hyg-Xho-r, and the resulting fragments were cloned into the *Nde*I-*Xho*I sites of pET21a. This gave rise to pET21a-hyg and pET21a-hyg10 respectively. In these constructs, the 6 × His-tag in the plasmid was fused in-frame with these ORFs at the C-termini.

E. coli BL21 (DE3), harboring these plasmids, were cultured in LB medium containing ampicillin at 30 °C for 10 h. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 0.1 mM, and cultivation was continued for an additional 2 h. The cells were collected by centrifugation, and were washed with 100 mM Tris–HCl pH 8.0, containing 150 mM NaCl, and suspended with the same buffer. They were disrupted by sonication (Sonifier 250, Branson, Danbury, CT), and the cell debris were removed by centrifugation at 15,000 × *g* over 60 min at 4 °C. The supernatants obtained were applied to a Chelating Sepharose Fast Flow (GE Healthcare, Uppsala, Sweden) column pre-equilibrated with 100 mM Tris–HCl pH 8.0, containing 150 mM NaCl and 100 mM $NiCl_2$, and the column was washed with 100 mM Tris–HCl pH 8.0, containing 300 mM NaCl and 30 mM imidazole. The adsorbed proteins were eluted with 100 mM Tris–HCl pH 8.0, containing 300 mM NaCl and 300 mM imidazole.

Next, the eluted fractions were applied to a column of Resource Q (GE Healthcare) after dialysis against 10 mM HEPES pH 7.5, and were eluted by a 0–1 M NaCl gradient, with an ÄKTA purifier (GE Healthcare). The HYG and HYG10 proteins were then further purified by gel-filtration with Superose 6 10/300 GL (GE Healthcare) using a buffer of 20 mM Tris–HCl pH 8.0, and 150 mM NaCl.

The purification steps were monitored by SDS–PAGE, quantification of the proteins was done by the Bradford method (protein assay kit, BioRad, Hercules, CA) and a BCA protein assay kit (Thermo Scientific, Rockford, IL). The concentration of the purified proteins was calculated from the UV absorption at 280 nm using a molar extinction coefficient of 56,100 $M^{-1}\,cm^{-1}$ for both proteins and estimated molecular weights (37,979.8 and 38,348.3 daltons for HYG and HYG10 respectively).

Enzyme assays. The activities of HYG and HYG10 were measured by ATP consumption during the reactions. Two μg of purified enzymes was mixed with 50 mM sodium phosphate buffer pH 7.5, containing 10 mM $MgCl_2$, 20 mM KCl, 1 mM HygB, and 0.1 mM ATP, in a volume of 0.5 mL, and then incubated for 10 min at 30 °C. The reaction was stopped by the addition of EDTA at a final concentration of 20 mM. The solution was diluted 1,000-fold, and the remaining ATP was quantified by luciferin-luciferase assay with a CheckLite 250 Plus kit (Kikkoman, Noda, Japan). One unit of activity was defined as the amount of enzyme that consumes 1 μmol of ATP per min under the assay conditions.

For kinetic analysis, a coupled enzyme assay was used as previously described.[7] Two μg of the purified enzymes was mixed with 50 mM sodium phosphate buffer pH 7.5, containing 10 mM $MgCl_2$, 20 mM KCl, 0.0005–0.1 mM HygB, 0.005–0.5 mM ATP, 0.1 mM NADH, 5 mM phosphoenolpyruvate, and 12 U each of pyruvate kinase and lactate dehydrogenase (from rabbit muscle, Sigma-Aldrich, St. Louis, MO) in a total volume of 1 mL. The mixture was incubated at 45 °C. The reaction was monitored by following the absorbance at 340 nm, and the initial velocity for the HYG reaction was calculated, taking the molar extinction coefficient of NADH to be 6,220 $M^{-1}\,cm^{-1}$. The results were fitted to the basic Michaelis-Menten equation or one positing substrate inhibition,[20,21] using fitting tool Origin ver. 8.6 (OriginLab, Northampton, MA). By this equation, the K_m, k_{cat}, and K_i values were calculated.

For measurements of activation parameters, the kinetic parameters for ATP were measured under the above conditions with 5 mM HygB at 20–45 °C. The activation energy (E_a) was calculated by fitting the results to the Arrhenius equation,[22] and the values of changes in Gibbs free energy ($\Delta G^{\ddagger}$), enthalpy ($\Delta H^{\ddagger}$), and entropy ($T\Delta S^{\ddagger}$) of activation were also calculated.[22]

Measurements of thermal stability by circular dichroism (CD) and differential scanning calorimetry (DSC) analysis. The thermal denaturation processes for HYG and HYG10 were observed by measuring the change in ellipticity at 222 nm with a J-720W spectropolarimeter (Jasco, Tokyo). The enzymes (0.1 mg/mL) in 30 mM sodium phosphate buffer pH 7.5, were heat-treated at a rate of increase of 1.0 °C/min from 4 to 80 °C. The results were fitted to a conventional two-state equation of the thermal-unfolding process,[23] and the values of T_m and enthalpy change of unfolding at T_m (ΔH_m) were calculated.

For DSC analysis, purified HYG and HYG10 were dialyzed against 100 mM sodium phosphate buffer pH 7.5, and the protein concentrations were adjusted to 2 mg/mL. Then 1.5 mL of the samples was injected into a MCS-DSC apparatus (Microcal, Pittsburgh, PA) and heat-treated at a rate of increase of 30 °C/h from 10 to 90 °C. The results were analyzed by Origin software (OriginLab), and the T_m and ΔH_m values were calculated.

Prediction of three-dimensional structures by the Phyre2 program. The amino acid sequence of HYG10 was used to elucidate structurally similar proteins by the Phyre2 program,[24] and the predicted structure of HYG10 was constructed and visualized by UCSF-Chimera version 1.6.[25]

Results and Discussion

In vivo *directed evolution for thermostabilization of HYG*

The nucleotide sequencing of the cloned *hyg* gene in pT8S-P31-hyg showed a base substitution at the 63th codon (GAC to GGC), which resulted in an amino acid replacement of Asp by Gly. This substitution was also found in the original plasmid, pV1, indicating a sequence error in the database.

The introduction of pT8S-P31-hyg into *T. thermophilus* (pTT8) cells gave a $HygB^r$ phenotype up to 60 °C, but not at 65 °C. However, further cultivation of the transformants at 65 °C for 2 d gave microcolonies on TM-HygB plates, 145 colonies of which showed growth at 65 °C overnight after re-streaking onto TM-HygB plates. Therefore we assumed that some mutations occurred, either in *hyg* on the plasmid or in the chromosome, and these mutations gave a $HygB^r$ phenotype to the cells at 65 °C. Sixty colonies were selected randomly from the original 145 colonies, and the plasmids were recovered. Re-introduction of these plasmids independently into *T. thermophilus* (pTT8) indicated that 16 plasmids gave a $HygB^r$ phenotype at 65 °C, and six different mutations were identified by sequencing of the *hyg* regions in these plasmids: L79P (CTC to TTC), S83L (TCG to TTG), E237K (GAG to AAG), S252P (TCC to CCC), D285N (GAC to AAC), and L290M (CTG to ATG) (Table 1), suggesting that these mutations increased the thermostability of HYG. Cells harboring these mutant *hyg* genes showed T_{max} values at between 61 and 64 °C.

Next we did a DNA shuffling experiment with a plasmid mixture from the original 145 colonies as templates. Two colonies were obtained by screening at 70 °C, of which *hyg* harbored the same four mutations, L79F, S252P, and L290M found in the mutants obtained at 65 °C as well as a new mutation of G189R (GGG to AGG). The strain harboring this gene, *hyg4*, showed a T_{max} value of 68 °C (Table 1).

In general, mutations affecting the thermostability of proteins show cumulative effects.[4,26] To obtain additional thermostabilized mutants, we introduced the mutations identified in the first screening into *hyg4* and then tested them for thermostability. As expected, a small but distinct increase in thermostability was observed. In particular, the introduction of the E237K mutation gave an increase of T_{max} by 2 °C in *hyg5* (mutations in the *hyg4* + E237K), *hyg6-2* (+E237K and

Table 1. *hyg* Mutant Genes Obtained and Constructed in This Study

Mutants *hyg* (wt)	Selection temperature (°C)	Amino acid substitutions at position									T_{max} (°C)
		9-11 LDR	79 L	83 S	189 G	237 E	252 S	285 D	290 L	300 E	60
Genes obtained by in vivo *directed evolution on solid medium*											
hyg1-1	65						P				64
hyg1-2	65								M		61
hyg1-3	65		F								63
hyg1-4	65			L							62
hyg1-5	65							N			62
hyg1-6	65					K					63
hyg4	69		F		R		P		M		68
Genes constructed by in vitro *mutagenesis*											
hyg5			F		R	K	P		M		70
hyg6-1			F	L	R		P	N	M		69
hyg6-2			F		R	K	P	N	M		70
hyg6-3			F	L	R	K	P		M		70
hyg7			F	L	R	K	P	N	M		69
Genes obtained by in vivo *directed evolution in liquid culture*											
hyg9	74	duplication	F		R	K	P	N	M		72
hyg10	76	duplication	F		R	K	P	N	M	D	74

D285N), and *hyg6-3* (+S83L and E237K) mutants. On the other hand, co-introduction of the S83L and D285N mutations, together with the E237K mutation, showed a slight negative effect, because *hyg7*, containing all of the mutations, showed a T_{max} value lower than the *hyg6-2* and *hyg6-3* mutants by 1 °C.

Finally, we conducted *in vivo* directed evolution for thermostabilization with liquid culture with a strain harboring pTEV-P31-hyg6-2. Serial cultivation in liquid TM-HygB medium at increasing temperatures from 72 to 80 °C showed an increase in OD600 > 1.0 within 30 h at each temperature tested, from which colonies were isolated by plating on TM-Km plates. Because we have experienced in our previous studies of thermostabilization of HPH that the maximum selection temperature on TM-HygB plates was 2 °C higher than the T_{max} value in liquid culture,[6] we tested the growth of these colonies on TM-HygB plates at temperatures 2 °C higher than those temperatures. The plasmids were independently recovered from several isolated colonies and introduced again into the wild-type strain, and the transformants were tested for $HygB^r$ at the temperatures used for plate cultivation.

The plasmids from the colonies obtained from liquid culture at 72 °C produced few or no transformants on TM-Hyg plates at 74 °C, and nucleotide sequencing revealed that no additional mutations were present in the *hyg* genes. However, we obtained $HygB^r$ transformants with plasmids from colonies obtained from liquid culture at 74 °C and at 76 °C by selection on TM-Hyg plates at 76 °C and at 78 °C respectively. The transformation frequencies were comparable with those by selection with Km, which resides in the same plasmid. Sequencing of the *hyg* genes in the plasmids from the colonies obtained by liquid culture at 74 °C indicated that all of the mutant genes identified contained the same additional mutation, a duplication of the 9th to 11th amino acids (LDR), designated *hyg9*. Tandem duplication of the amino acids affecting the thermostability of a protein has been reported by Akanuma *et al.*, with a C-terminally truncated mutant of 3-isopropylmalate dehydrogenase from *T. thermophilus*.[27] Also, a possible molecular mechanism for generating mutations of tandem duplications was discussed by Kless and Vermaas.[28]

All of the genes in the plasmids isolated from the colonies obtained from liquid culture at 76 °C contained the E300D mutation (GAG to GAT) in addition to those in *hyg9*, which was designated *hyg10*. On the other hand, we did not obtain any transformants on TM-Hyg plates at 80 °C and 82 °C with the plasmids from the colonies obtained by liquid culture at 78 °C and 80 °C, respectively. These plasmids contained insertions or deletions in the *hyg* genes, indicating that some mutations occurred in the chromosome of the host strains to give the $HygB^r$ phenotype. The reason such mutations occurred in the *hyg* genes is not known.

Enzymatic characterization of HYG10

To determine whether HYG10 is thermostabilized at the protein level, we produced HYG10 and the wild-type HYG by an *E. coli* pET system and purified the protein by affinity chromatography with the 6 × His-tag attached to the C-termini, followed by anion exchange chromatography and gel filtration. The purified proteins showed single bands of approximately 38 kDa on SDS–PAGE (Fig. S1). The estimated molecular masses by gel filtration were 36.6 and 37.1 kDa for HYG and HYG10 respectively, indicating that these enzymes function as monomers. The specific activities of HYG and HYG10 at 30 °C were 0.70 and 1.04 units/mg protein respectively, showing a slight increase in activity for the HYG10 protein.

As shown in Fig. 1, an increment of thermostability was observed in HYG10. The half-inactivation temperature, at which 50% inactivation was observed after 30 min of incubation (T_{half}), of HYG10 was 48 °C, whereas that of HYG was 35 °C. These results indicate that the thermal stability of HYG10 increased by 13 °C, at least at the level of enzyme activity, and this increment almost coincided with that observed for the T_{max} values (14 °C).

The T_{half} of HYG10 was 26 °C lower than its T_{max}, and HYG10 was almost completely inactivated at its

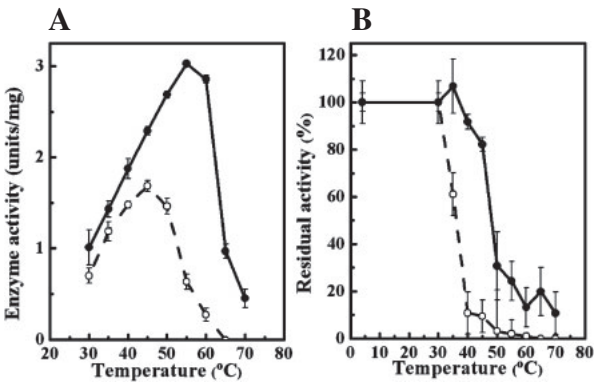

Fig. 1. Optimum Temperatures (A) and Thermal Stabilities (B) of HYG (hollow circles with broken line) and HYG10 (solid circles with solid line).

Measurements were conducted in triplicate, and means ± standard deviation (SD) are shown.

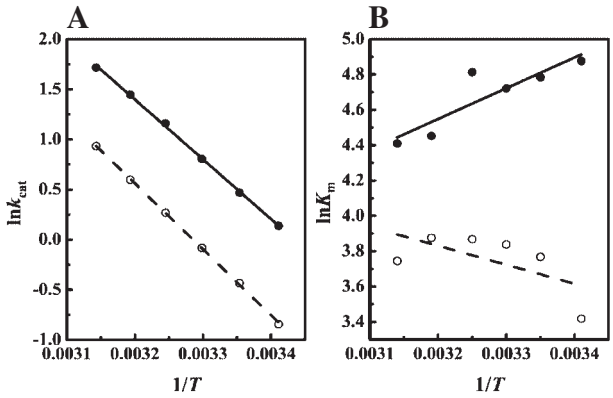

Fig. 2. Arrhenius Plot (A) and van't Hoff Plot against K_m Values (B) of HYG (hollow circles with broken line) and HYG10 (solid circles with solid line).

Measurements were conducted in triplicate, and average values are shown.

Table 2. Kinetic Parameters of HYG and HYG10 Measured at 45 °C

Kinetic parameters were measured in triplicate, and means ± standard deviations (SD) are shown.

Enzyme	K_m (μM)		k_{cat} (s^{-1})		K_i (mM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	
	HygB[a]	ATP[b]	HygB[a]	ATP[b]	HygB[a]	HygB[a]	ATP[b]
HYG	16.7 ± 4.2	42.3 ± 2.8	6.4 ± 0.7	2.5 ± 0.4	0.61 ± 0.17	3.9×10^5	6.0×10^4
HYG10	30.0 ± 6.6	82.2 ± 6.8	8.0 ± 0.8	5.6 ± 0.1	1.27 ± 0.41	2.7×10^5	6.8×10^4

[a,b]The kinetic parameters for HygB and for ATP were measured at fixed concentrations of ATP (5 mM) and of HygB (5 mM).

T_{max} (Fig. 1B). The fact that *T. thermophilus* cells harboring the *hyg10* gene showed the $HygB^r$ phenotype at T_{max} can be explained by continuous expression of the gene. Even at T_{max}, HYG10 phosphorylates a small portion of HygB before it is heat-inactivated, and a continuous supply of the newly-synthesized enzyme is enough for the cells to attain the $HygB^r$ phenotype.

The optimum temperature of HYG10 increased concomitantly, from 47 °C of HYG to 59 °C, and the specific activities of HYG and HYG10 at their optimum temperatures were 1.7 and 3.1 units/mg protein respectively. This result was contrary to our previous results for the thermostabilization of HPH, in which the mutant, HPH5, showed a 17 °C increment of thermal stability, while its optimum temperature increased by only 5 °C.[7)]

To analyze the effects of these mutations on enzyme activity in more detail, we compared the steady-state kinetics of HYG and HYG10 at temperatures between 20 °C and 45 °C by coupled enzyme assay with pyruvate kinase and lactate dehydrogenase from rabbit muscle, followed by thermodynamic analyses. It should be noted that the coupled enzymes were stable at up to 45 °C.

When the reaction was conducted at 45 °C, a decrease in the initial velocity of the reaction was observed at a HygB concentration of more than 0.1 mM for both enzymes (Fig. S2), indicating substrate inhibition by high concentrations of HygB. The K_m and k_{cat} values for ATP increased approximately 2-fold for HYG10, with very similar catalytic efficiencies (k_{cat}/K_m values) for HYG and HYG10 (Table 2). In addition, the K_m and k_{cat} values for HygB during HYG10-mediated catalysis increased by approximately 1.8- and 1.3-fold respectively. It is notable that the K_i value of HYG10 was twice the value of HYG, indicating that HYG10 was less inhibited by the substrate.

Table 3. Activation Parameters for HYG and HYG10 Calculated with Kinetic Parameters for ATP

Enzyme	E_a (kJ/mol)	$\Delta G^{\ddagger}$ (kJ/mol)	$\Delta H^{\ddagger}$ (kJ/mol)	$T\Delta S^{\ddagger}$ (kJ/mol)
HYG	54.7 ± 0.5	75.6	52.1	−23.6
HYG10	49.6 ± 0.8	73.6	47.0	−26.6

The Arrhenius plots of the k_{cat} values for the ATP of HYG and HYG10 (Fig. 2A) were linear, suggesting that the rate-determining step for the reaction does not change over temperatures ranging from 20 to 45 °C. The E_a value, determined from the slope of the plot for HYG, was 54.7 kJ/mol, whereas that for HYG10 was 49.6 kJ/mol, indicating a 5.1 kJ/mol reduction in HYG10. Moreover, the thermodynamic parameters, $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $T\Delta S^{\ddagger}$, calculated on the transition-state model described by Lonhienne *et al.*,[22)] showed slight decreases in all of the parameters in HYG10 (Table 3). The reduction of $\Delta G^{\ddagger}$ in HYG10, at 2 kJ/mol, consisted of 5 kJ/mol and 3 kJ/mol reductions in the $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$ values respectively.

Lonhienne *et al.* have reported that, from comparison of several sets of psychrophilic and mesophilic enzymes, a decrease in the $\Delta H^{\ddagger}$ value (and therefore in the E_a value) is a general feature for enzymes in adapting to low temperature.[22)] A similar tendency, a low $\Delta H^{\ddagger}$ value in psychrophilic enzymes and high $\Delta H^{\ddagger}$ value in thermophilic enzymes, was found by comparing psychrophilic, mesophilic, and thermophilic α-amylases.[29)] It was explained that psychrophilic enzymes evolved to adapt to low temperatures with a decrease in the $\Delta H^{\ddagger}$ value to reduce the temperature dependence of k_{cat}, whereas thermophilic enzymes did to adapt to high temperature to make efficient use of the thermal heat

energy of the environment by increases in reactivity (increases in the $\Delta H^{\ddagger}$ value). From this point of view, the observed differences in the thermodynamic parameters of HYG and HYG10 indicate that HYG10 adapted to low temperature, not high temperature. The reason for this discrepancy is not known, but it is possible that it reflects a difference between natural evolution and our directed evolution. However, lowering the E_a value might benefit by increasing enzyme activity even at high temperatures.

The K_m values of these enzymes showed the opposite change as temperature increased. The value of HYG increased approximately 1.5-fold, from 20 to 45 °C, whereas that of HYG10 decreased by 1.5-fold (Fig. 2B). In general, the K_m value of an enzyme increases as the reaction temperature rises, as in the case of HYG. It is thought that increasing temperature induces increased local flexibility of the enzyme, as at the substrate-binding site, and thereby increases the fraction of enzyme molecules that possess poor substrate-binding abilities.[30] On the other hand, some enzymes from thermophiles, such as inositol monophosphatase of *Archaeoglobus fulgidus*, show an opposite change in values, resulting in activation of the enzymes at higher temperatures.[31] Although there was a slight decrease in the K_m values, from 20 to 45 °C, this decrease, together with the lower $\Delta H^{\ddagger}$ value (and therefore the E_a value), might contribute to increased activity at higher temperatures, as observed for HYG10.

Biophysical analysis of HYG10

To analyze thermostabilization in HYG10 in more detail, we conducted thermal denaturation analyses with HYG and HYG10 by the methods CD and DSC. The far-UV CD spectra of HYG and HYG10 at 4 °C showed similar traces of an α-helical structure with minima at 208 nm and 222 nm, indicating that the secondary structure of the enzyme was not changed by these mutations (Fig. S3).

As shown in Fig. 3, CD analyses at 222 nm showed one-step unfolding profiles for both proteins. These thermal denaturation processes were irreversible, and the apparent T_ms of HYG and HYG10 were 35.9 and 53.0 °C respectively, based on the results of fitting to a conventional two-state equation of the thermal unfolding process.[23] These values coincided well with the T_{half} values. The ΔH_m values for HYG and HYG10 were calculated to be 223.4 and 410.5 kJ/mol respectively, showing an approximately 2-fold increase in HYG10. DSC analysis showed essentially the same results: the apparent T_m and ΔH_m values for HYG and HYG10 were calculated to be 40.4 and 52.6 °C and 326.3 and 702.9 kJ/mol respectively (Fig. S4). These results showed good correlation with the results of the CD analyses under the same buffer condition (39.5 and 54.2 °C and 377.8 and 470.8 kJ/mol for HYG and HYG10 respectively; data not shown). The data indicated that HYG10 was thermostabilized at the protein structure level. Based on the increase in the ΔH_m value, thermostabilization was perhaps achieved by strengthening the interactions between the side chains.

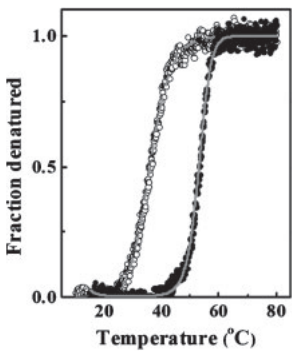

Fig. 3. Effects of Temperature on the Protein Structures of HYG and HYG10.

Thermal unfolding profiles of HYG (hollow circles with broken gray line) and HYG10 (solid circles with solid gray line), monitored by changes in the ellipticity at 222 nm. Lines indicate the results of fitting to a conventional two-state equation of the thermal unfolding process.

Three-dimensional modeling of HYG10

To identify the functions of these amino acid substitutions and duplication on the thermostabilization of HYG10, the precise crystal structure of HYG and/or HYG10 is necessary, but since this information is currently unavailable, we constructed a three-dimensional model of HYG10 with the Phyre2 program. It should be noted that unlike the crystal structure, three-dimensional modeling of a protein structure is not true to the real structure. However, in the case of the Phyre2 program, a confidence level of >90% indicates that the overall fold of the model is almost correct and that the central core of the model tends to be accurate, even if the sequence identity is low (<20%).[24] The results of the PSI-BLAST search based on the amino acid sequence of HYG10 showed similarities to several aminoglycoside phosphotransferases (APHs), including putative ones (Table S2). In particular, HPH of *E. coli* (3TYK)[32] showed 15% identity with 100% confidence. Moreover, multiple alignments of the amino acid sequence of HYG10 with those of selected APHs from the list generated by the Phyre2 program indicated that some conserved residues among APHs, such as the catalytic Asp198 and the residues for ATP binding (Arg49, Asn203, and Asp216) in the HPH numbering, are also conserved in HYG10 (Fig. S5). This suggests that the model is accurate to some extent, especially for the overall structure. On the other hand, the structures of the N-terminal 29 amino acids of HYG10, including the mutation of the three amino acid duplication, were modeled *ab initio*, indicating limited reliability of the structure in that region.

The final model of HYG10 is shown in Fig. S6. The overall structure of HYG10 was predicted to have a similar structure to other APH family proteins.[32–36] The three substitutions, L79F, S252P, and L290M, were located inside the protein (Fig. 4A). The L79F substitution provides a bulky side chain, and therefore it is possible that this substitution contributes to thermostabilization through the effect of cavity-filling. In addition, the S252P substitution may contribute by strengthening the hydrophobic core of the protein. The S252P substitution was located at the edge of an α-helix corresponding to α7 of HPH. Therefore, it is possible that this substitution contributes to thermostabilization as well by stabilizing the secondary structure. The effect of

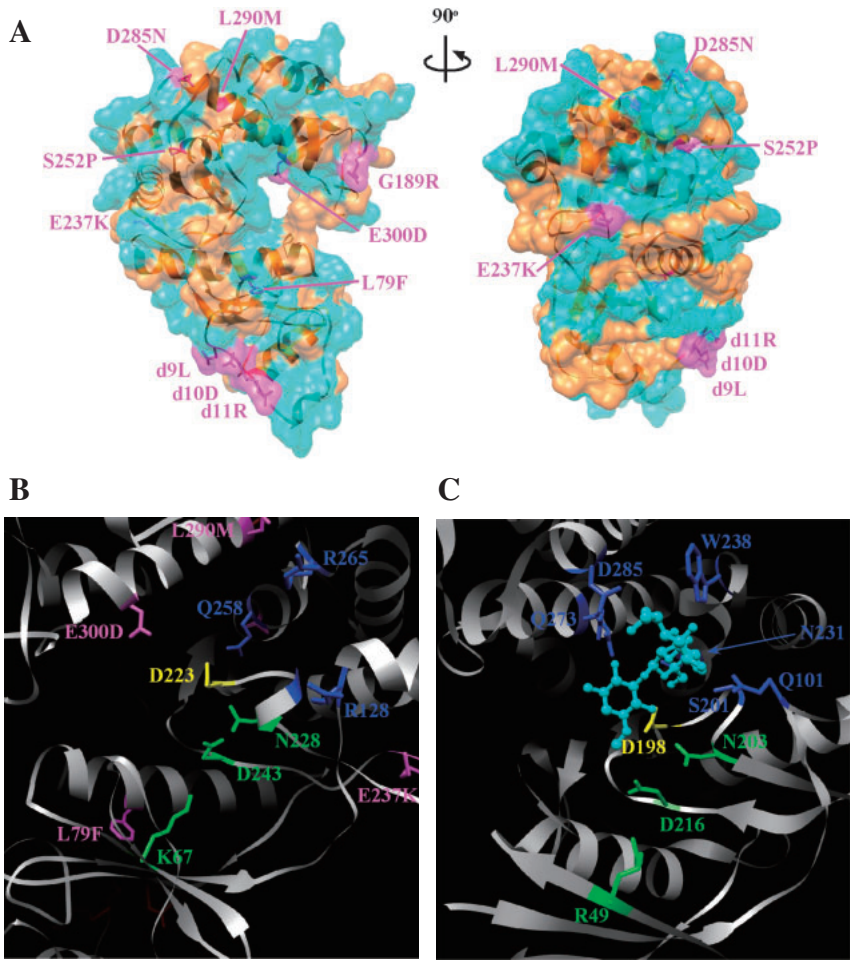

Fig. 4. Positions of Mutated Residues in the Predicted Structure of HYG10.

(A) The overall structure of HYG10 is shown as a ribbon model with a transparent surface. Hydrophobic and hydrophilic residues are shown in orange and cyan respectively, and mutated residues are shown with side chains in magenta. The duplicated residues are indicated by the prefix "d." (B) Putative catalytic center of HYG10. Putative residues for catalysis, ATP-binding, and HygB-binding, deduced from alignment with HPH, are shown in yellow, green and blue respectively. (C) The corresponding view of HPH (PDB code, 3TYK). HygB is shown as a ball and stick model in cyan.

L290M substitution on thermostabilization is currently unknown.

On the other hand, the three substitutions, G189R, E237K, and D285N, were located at the surface of the protein, particularly at the edges of the hydrophobic patches. Since the G189R and E237K substitutions gave decreased hydropathy indexes (from −0.4 and −3.5 to −4.5 and −3.9 for the G189R and E237K mutations respectively) and the D285N substitution did not alter hydrophilicity, these substitutions might cause increased hydrophilicity of HYG10. Since the G189R and E237K mutations produce positive charges, these residues might also contribute to thermostabilization by forming salt bridges with nearby acidic amino acids. In addition, the three-amino-acid duplication was located at the protein surface, including two hydrophilic amino acids (Asp11 and Arg12), and hence these mutations might also have contributed to increased hydrophilicity. Based on this, we suggest that these substitutions contribute to the thermostabilization of HYG10 by increasing hydrophilicity at the protein surface, thereby stabilizing the protein in aqueous solution. This is known as the reverse hydrophobic effect.[37] Gromiha reported that this effect is realized if the replacement of a hydrophobic by a hydrophilic amino acid is located in the coil structure at the protein surface.[38] The locations of the G189R, E237K and D285N substitutions at such positions (Fig. S6) supports this possibility.

A precise comparison of the model structure with the crystal structure of HPH showed that the putative catalytic residue, Asp223, and the conserved ATP-binding residues, Lys67, Asn228, and Asp243, were topologically conserved in HYG10 (Fig. 4BC). On the other hand, the corresponding residues for HygB binding were not conserved in the amino acid sequence or in the resulting structure. This might have been because the two enzymes are known to phosphorylate HygB at different sites,[11,12] and thus the binding mode of HygB to the enzymes may be different.

The E300D substitution was located in an α-helix (corresponding to α10 of HPH) that overlapped with the putative catalytic cleft, the side chain of which was predicted to face the putative catalytic center. Since two residues for HygB-binding in HPH, Gln273 and Asp285, are located at the corresponding positions, we postulate that this substitution affects HygB binding, thereby decreasing the K_m value at higher temperatures.

In conclusion, we obtained a thermostabilized mutant of HYG by introducing seven amino acid substitutions

and the duplication of three amino acids. The resulting mutant gene, *hyg10*, provided a HygB^r phenotype at up to 74 °C in *T. thermophilus* cells. The mutant enzyme, HYG10, showed increased thermostability and optimum temperature by 13 and 12 °C respectively compared to wild-type HYG. Biochemical and biophysical analyses and three-dimensional modeling of HYG10 suggested that thermostabilization was achieved by strengthening of the hydrophobic core and by increasing protein stability in aqueous solution.

In terms of application, the T_{max} value, the maximum selection temperature, is important. As we wrote above, determination of T_{max} apparently includes efficiency of transcription and translation in the cells as well as the thermostability of the protein, as indicated by the T_{half} and T_m values. The difference between the T_{max} and the T_{half} value of HYG10, 26 °C, was larger than that of HPH5, 14 °C.[7] Since these two genes were expressed under the same promoter, P31, in the same plasmid, the difference might have been due to efficiency of translation. In this regard, a higher G + C content of HYG (70.8%) than of HPH (58.1%) might contribute to efficient translation in *T. thermophilus*, which possesses a G + C content (69.4%) similar to that of HYG. Thus HYG10 prove useful as a selection marker gene in thermophiles with high G + C contents.

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