J. Biochem. 126, 951-956 (1999)

Directed Evolution of Thermostable Kanamycin-Resistance Gene: A Convenient Selection Marker for *Thermus thermophilus*[1]

Jun Hoseki,* Takato Yano,† Yoshinori Koyama,‡ Seiki Kuramitsu,* and Hiroyuki Kagamiyama†,2

*Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043; †Department of Biochemistry, Osaka Medical College, Takatsuki, Osaka 569-8686; and ‡National Institute of Bioscience and Human-Technology, AIST, MITI, Tsukuba Science City, Ibaraki 305-8566

Received August 27, 1999; accepted September 6, 1999

The whole-genome sequencing of an extreme thermophile, *Thermus thermophilus*, is now in progress. Like other genome projects, major concern is shifting from the sequence itself to post-sequencing research such as functional or structural genomics. Under such circumstances, the demand for convenient genetic-engineering tools is increasing. In this study we have increased the thermostability of a kanamycin-resistance gene product using strategies based on directed evolution in *T. thermophilus* to the upper limit of its growth temperature. The most thermostable mutant has 19 amino-acid substitutions, whereby the thermostability is increased by 20°C, but the enzymatic activity is not significantly changed. Most of the mutated residues are located on the surface of the protein molecule, and, interestingly, five of the 19 substitutions are those to proline residues. The evolved kanamycin-resistance gene products could be used as selection markers at the optimum growth temperature of *T. thermophilus*. The development of such a convenient genetic-engineering tool would facilitate post-sequencing research on *T. thermophilus*.

Key words: directed evolution, DNA shuffling, kanamycin-resistance gene, thermostability, *Thermus thermophilus*.

Thermophiles have attracted much attention mainly because of the biotechnological potential of their proteins. They are also useful as a source of research materials to study the structure-function relationships of enzymes because their proteins are easy to handle, stable at extreme pHs, and can be crystallized more readily than non-thermophilic proteins. Thermophilic proteins, however, sometimes do not fold into the native conformation when expressed in *Escherichia coli*. These proteins cannot be expressed in thermophiles at high temperatures because standard genetic-engineering tools are not available for most of these organisms. This situation hampers those attempts to explore the biological roles of a protein by knocking out and subsequently reintroducing the gene whose function cannot be deduced from the sequence data obtained from genome projects. *Thermus thermophilus*, a eubacterium, is particularly attractive because it can grow at the highest temperature (50-82°C) among organisms whose molecular biology has been studied (*1, 2*). The whole-genome sequencing of *T. thermophilus* is in progress in Japan (strain HB8) and Germany (strain HB27) and will soon be completed. Also, there are plans to analyze systematically the structure and biological functions of *T. thermophilus* proteins. The development of convenient genetic-engineering tools is thus urgently needed. Among the most essential tools are easy-to-use selection markers. Although *T. thermophilus* is susceptible to general antibiotics, there has been only one antibiotic-resistance marker available for *T. thermophilus*, a mutant gene of the *Staphylococcus aureus* kanamycin nucleotidyltransferase (KNT) (*3, 4*). This mutant KNT, however, is far from ideal as a selection marker because it cannot be used above 60°C. At this temperature, considerably below the optimum growth temperature (70-75°C), cell growth is very slow. So we set out to increase the thermostability of KNT.

Numerous attempts have been made to improve the thermostability of proteins with various levels of success. Most such attempts have been so-called rational designs based on an understanding of protein folding or structure formation: for example, the introduction of disulfide bonds (*5*), rearrangement of packing in the hydrophobic core (*6*), and substitutions to proline (*7-9*). The sequences of homologous proteins have been compared between those from non-thermophiles and thermophiles to find stabilizing amino-acid replacements (*10-13*). On the other hand, non-rational methods have also been used to increase the thermostability of proteins (*14-18*). In most of these studies, however, a single round of random mutagenesis and

[1] This work was supported by the Ministry of Education, Science, Sports and Culture of Japan (to T.Y.) and the Japan Society for the Promotion of Science ("Research for the Future" Program) (to H.K.).
[2] To whom correspondence should be addressed. Tel: +81-726-83-1221 (Ext. 2644), Fax: +81-726-84-6516, E-mail: med001@art.osaka-med.ac.jp

Abbreviations: KNT, kanamycin nucleotidyltransferase; HTK, a highly thermostable KNT created in this study; MES, 2-morpholinoethanesulfonic acid; PEI, polyethyleneimine; WT*, a mutant of the *Staphylococcus aureus* KNT that has two substitutions, Asp80Tyr and Thr130Lys.

© 1999 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

screening is used instead of multiple rounds of mutagenesis, selection, and amplification with increasing selection pressure, that is, Darwinian or directed evolution. Only a few studies (*16–18*) have been reported so far where thermostability was successfully enhanced by directed evolution.

In the present study, we succeeded in increasing the thermostability of KNT by directed evolution in *T. thermophilus* to the upper limit of its growth temperature. The resultant thermostable KNT provides a convenient selection marker for *T. thermophilus*.

EXPERIMENTAL PROCEDURES

Medium—T. thermophilus cells were cultured in a liquid medium containing 0.4% tryptone, 0.2% yeast extract, and 0.1% NaCl (pH 7.5). *T. thermophilus* transformants were selected on 3% agar plates supplemented with 50 μg/ml kanamycin (<70°C) or on 1.5% gellan gum plates with 500 μg/ml kanamycin (>70°C). Divalent cations, 1.5 mM $CaCl_2$ and 1.5 mM $MgCl_2$, were added to solidify the gellan gum. The concentration of kanamycin is high in gellan gum plates because these ions are antagonists of kanamycin.

Construction of the Plasmid pJHK1—Directed evolution was started from a mutant of *Staphylococcus aureus* KNT (designated WT*) with two substitutions, Asp80Tyr and Thr130Lys (*19–21*). The WT* KNT gene was amplified by PCR from pYK134 (12.3 kb) (*4*) using a pair of primers: 5′-primer, 5′-GACTGTACGggtaccCGTTGACGGCGGATATGGTA-3′ (*Kpn*I site in lower case); 3′-primer, 5′-GACTGTACGctgcagCGTAACCAACATGATTAACA-3′ (*Pst*I site in lower case). pYK134 is a derivative of pTT8, a plasmid isolated from *T. thermophilus* HB8, in which the *ori* is located around the *Bcl*I site (*4, 22*). To reduce the size of pTT8 (9.7 kb), the *Kpn*I-*Pst*I fragment (5.5 kb) spanning the *Bcl*I site was gel-purified and ligated with the amplified WT* gene. The resultant plasmid, pJHK1 (6.5 kb), was used for directed evolution experiments.

Transformation of T. thermophilus HB27—T. thermophilus cells were transformed as follows (*1*): An overnight culture was diluted 1:100 with fresh medium containing 0.4 mM $CaCl_2$ and 0.4 mM $MgCl_2$, and incubated with shaking at 70°C for 2 h. The culture (1×10^8 cells/ml) was mixed with plasmids, incubated with shaking at 70°C for 2 h, and then spread on plates containing kanamycin.

Directed Evolution of Thermostable KNT Mutants—DNA shuffling was done as described by Stemmer (*23*). The coding region of the WT* gene was amplified by PCR from pJHK1: 5′-primer, 5′-GACTGTACGgaattcGAGCTCGAGCAAATCTAAAA-3′ (*Eco*RI site in lower case); the sequence of the 3′-primer is described above. The shuffled fragment was digested with *Eco*RI and *Pst*I, gel-purified, and inserted into pJHK1 digested with the same restriction enzymes. Then, *T. thermophilus* HB27 was transformed with the pJHK1 derivative. After a library of transformants (3.2×10^5) was screened on plates containing kanamycin (64°C, 36 h), 431 transformants were picked and spotted on a kanamycin plate, and the plate was incubated at 64°C for 40 h. The cells were scraped from the plate with sterile water, and a mixture of the plasmids, pKT1 mix, was prepared. The mutant genes amplified from the pKT1 mix were shuffled, and the second round of screening was done in the same way as the first round: a library size of 4.8×10^5, incubation at 69°C for 40 h, 109 colonies selected. The pKT2 mix was prepared as described above. The pKT3 mix was prepared from 209 colonies obtained from the third round of screening: a library size of 2.4×10^5, incubation at 79°C for 20 h. After *T. thermophilus* HB27 was transformed with the pKT3 mix and incubated at 81°C for 40 h, twenty of the largest colonies were picked up and cultured, and the plasmids pKT3-1-3-20 were prepared. The coding region from each plasmid was amplified, subcloned into the *Kpn*I and *Pst*I sites of pUC18, and expressed in *E. coli*. Lysates were prepared from the *E. coli* culture and heated at 70°C for 10 min, and the residual KNT activity was compared among the twenty KT3 mutants. Ten mutants with the highest residual activities were selected, and their coding regions were sequenced.

Expression and Purification of KNTs—An expression plasmid, pUT7, was constructed by ligating the *Pvu*II-*Sca*I fragment of pUC18 containing the *ori* with the *Bgl*II-*Sca*I fragment of pET21b containing the T7 promoter. The mutant KNT genes amplified by PCR were subcloned into the *Nde*I and *Xho*I sites of pUT7. The primers were: 5′-primer, 5′-GACTGTACGcatatgAATGGACCAATAATAATGAC-3′ for WT*, or 5′-GACTGTACGcatatgAAAGGACCAATAATAATGAC-3′ for KT3-11 and HTK, (*Nde*I site in lower case); 3′-primer, 5′-GACTGTACGctcgagCGTAACCAACATGATTAACA-3′ (*Xho*I site in lower case). By this procedure, the initiation codon of the KNTs was changed from GTG to ATG. *E. coli* BL21 (DE3, pLysS) cells carrying the resultant expression plasmid were grown in medium containing 1.0% polypepton, 0.5% yeast extract, 1.0% NaCl (pH 7.0), 100 μg/ml ampicillin, and 1 mM isopropyl-1-thio-β-D-galactoside at 37°C overnight. The cells were harvested, resuspended in 20 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl and 2 mM 2-mercaptoethanol, and disrupted by sonication. All the following procedures were done at 4°C. The supernatant of the crude extract after centrifugation was applied to a DEAE-toyopearl column (Tosoh, Tokyo) equilibrated with the above buffer and eluted with a linear gradient of 50 to 250 mM NaCl. Fractions were checked by the KNT assay and SDS-PAGE. The fractions containing KNT were pooled and dialyzed overnight against 5 mM potassium phosphate buffer, pH 7.0, containing 2 mM 2-mercaptoethanol. The dialyzed solution was applied to a hydroxyapatite column equilibrated with the dialysis buffer and eluted with a linear gradient of 5 to 100 mM potassium phosphate. The fractions containing KNT were pooled and concentrated by ultrafiltration. Then, the concentrated solution was applied to a Sephacryl S-200 column equilibrated with 20 mM potassium phosphate buffer, pH 7.0, containing 0.1 M KCl. The purity of the enzyme was >90% by SDS-PAGE.

KNT Assay—The *Staphylococcus aureus* KNT catalyzes the transfer of AMP from ATP to the 4′-hydroxyl group of kanamycin (*24*). Enzyme activity was measured at 25°C in 50 mM Na-MES buffer, pH 6.0, containing 50 mM $MgCl_2$, 0.1-2 mM kanamycin, and 0.4-5.4 mM [8-^{14}C]ATP (0.4-4 mCi/mmol). The reaction was stopped by adding a half volume of 6 N HCl at various times, and the quenched solution was spotted on a PEI-cellulose TLC plate. The plate was developed in solvent (1-propanol/H_2O/acetic acid=60:39:1) for 45 min. The radioactivity of the product, kanamycin-[^{14}C]AMP, was counted using a Fujifilm phosphorimager, BAS-2000. The RF value of the product was 0.3.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

Thermal Denaturation—Thermal denaturation curves were recorded in a temperature range of 30-90°C (30-95°C for HTK) on a Jasco J-720WI spectropolarimeter equipped with a PTC-348WI thermoelectric temperature control system in a cuvette with a 5 mm path length. Protein concentrations were 0.8 μM. The buffer system was 50 mM potassium phosphate, pH 7.0, containing 0.1 M KCl. The samples were heated at a rate of 1°C/min with continuous CD monitoring at 222 nm.

RESULTS AND DISCUSSION

Directed Evolution—The first round of screening was carried out as follows: Mutations were introduced into the WT* gene by DNA shuffling (*23*); 431 positive colonies were picked from a library of 3.2×10^5 transformants screened at 64°C; and finally a mixture of plasmids, "pKT1 mix," was prepared from the positive colonies. Then, DNA shuffling was done for the mutant KNT genes amplified from the pKT1 mix to recombine the mutant genes while concomitantly introducing additional point mutations. The second round of screening was performed at 69°C (library size, 4.8×10^5; 109 colonies selected), and the third round at 79°C (library size, 2.4×10^5; 209 colonies selected). *T. thermophilus* cells transformed with the pKT3 mix formed colonies at 81°C on a plate containing kanamycin. But as the host *T. thermophilus* cannot form colonies above 81°C, the fourth round of screening could not be done.

KT3 Mutants—Twenty KT3 mutants taken from the largest colonies in the 81°C screening were examined in greater detail. The KT3 mutants, KT3-1-3-20, were expressed in *E. coli*, and the thermostability of each mutant was estimated from the residual catalytic activity after heating the lysate at 70°C for 10 min. Based on this assay, the ten most stable KT3s were selected, and their DNA sequences were determined. Each mutant has about 15 point mutations, 4-5 of which are silent. Although there is a possibility that the GC content of the KNT gene is increased after screenings at high temperature, no bias toward G or C was observed for the 39 silent mutations (data not shown). The Val75Ala substitution was found in all of ten KT3s (Table I). Other "conserved" substitutions are Glu61Gly in 7 mutants, His66Tyr in 8 mutants, Gln91Arg in 9 mutants, Ser112Pro in 7 mutants, and Ser199Pro in 7 mutants. Gln102 is replaced with basic amino acids in all KT3s examined (arginine in 7 mutants and lysine in 3 mutants). Interestingly, five of the 29 substitutions are those to proline. There are several examples of proline substitutions that increase the thermostability of proteins (discussed briefly below).

KT3-11, which was the most thermostable among the ten KT3s, was expressed in *E. coli* and purified. The purified KT3-11 is active after treatment at 70°C for 10 min, but is completely inactivated at 75°C (data not shown). pKT3-11 can, however, transform *T. thermophilus* cells at 81°C. KT3-11 might be more thermostable in the cytoplasm or expressed at quickly enough to replenish the KNT activity.

Creation of a More Thermostable Mutant—The directed evolution was stopped after only three rounds of screening and selection. If further rounds of screening were possible, more thermostable mutants should have evolved by recombinations among the mutations of KT3s. A strategy based on this assumption worked well when we altered the substrate specificity of an enzyme (*25, 26*). Therefore, the same strategy was employed to further increase the thermostability of KT3-11: Mutations conserved in more than two other KT3s and those to prolines were chosen as potential mutations. The mutations found in KT3-3 but not in KT3-11 were also chosen because KT3-3 is unique among the KT3 mutants (Table I). These mutations were added to KT3-11 independently. Each "single mutant" of KT3-11 was expressed in *E. coli*, and the thermostability of each mutant in the crude lysate was compared to that of KT3-11. The following nine substitutions increased the thermostability of KT3-11: Met57Leu, Ala62Val, Ser94Pro, Ser203Pro, Asp206Val, His207Gln, Ser220Pro, Ile234Val, and Thr238Ala. Three mutations, Asp25Asn, Glu117Gly, and Ser190Leu, which are unique to KT3-3, were found to be destabilizing, at least in the context of the sequence of KT3-11. A "highly thermostable kanamycin nucleotidyltransferase" (HTK), was constructed by incorporating all nine positive mutations into KT3-11 (Table I). HTK has 19 amino-acid substitutions compared to WT*. During the course of the construction of HTK, it was observed that the effects of these mutations on the thermostability are approximately additive (data not shown). Although the k_{cat} and K_m values of HTK for ATP (at 2 mM kanamycin) are about twice as large as the corresponding values of WT*, the catalytic properties of HTK are almost unaltered by the mutations (Table II).

Thermostability of the Three KNTs—To evaluate the thermostability of WT*, KT3-11, and HTK, the thermal denaturation of each protein was monitored by CD spectroscopy (Fig. 1A). The apparent T_m values for WT*, KT3-11,

TABLE I. **Amino acid substitutions of KNT mutants.**

Residue No.	2	17	25	57	61	62	66	75	91	94	102	112	116	117	159	188	190	196	197	198	199	203	206	207	211	220	234	238	246
WT*	N	H	D	M	E	A	H	V	Q	S	Q	S	L	E	T	S	S	V	K	Q	S	S	D	H	F	S	I	T	D
KT3-1		Y						A	R		R	P	F					L									V	A	
KT3-3			N	L	G	V		A		P	R			G			L								L				
KT3-5					G		Y	A	R	P	R										P						V		N
KT3-7	S				G		Y	A	R		R	P	F				T			L							V		
KT3-11	K				G		Y	A	R		R	P	F								P				L				
KT3-12						T	Y	A	R	P	K	T			I	G			R		P	P						A	
KT3-13					G		Y	A	R		R	P	F								P		V	Q					
KT3-15*					G		Y	A	R		R	P	F								P		V	Q					
KT3-16					G		Y	A	R	P	K	P									P		V	Q		P			
KT3-19				L			Y	A	R	P	K	P									P		V	Q					
HTK	K			L	G	V	Y	A	R	P	R	P	F								P	P	V	Q	L	P	V	A	

*KT3-15 has the same missense mutations as KT3-13. These two mutants have three silent mutations in common, but two of the silent mutations in KT3-13 and one in KT3-15 are unique to each mutant. Thus, these two mutants are thought to be different clones.

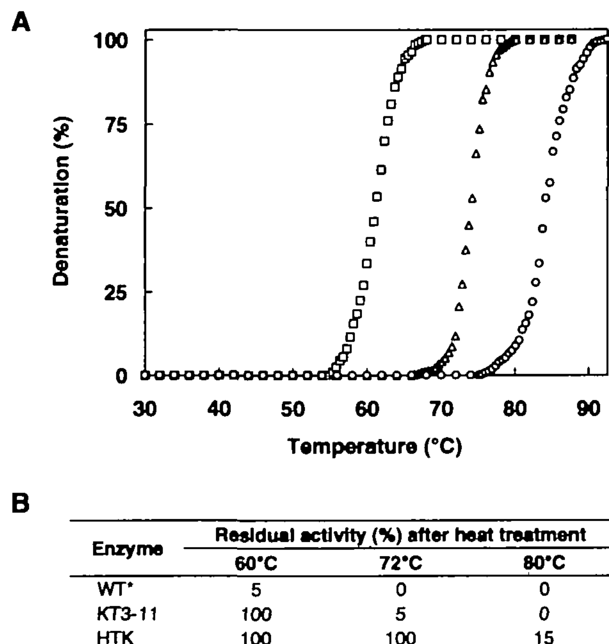
Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

and HTK are 61, 73, and 84°C, respectively. Since the denaturation of the three KNTs is irreversible and KT3-11 and HTK appear to aggregate upon denaturation, the denaturation curves shown in Fig. 1A cannot be used to calculate the $\Delta\Delta G$ values. To confirm the relative stabilities of the three KNTs, the residual activity of each KNT was measured after heat treatment at 60, 72, or 80°C for 10 min (Fig. 1B). The activity of WT* is decreased to 5% at 60°C, while KT3-11 and HTK are fully active. At 72°C, WT* is completely inactivated, the activity of KT3-11 is decreased to 5%, but HTK is still active. HTK retains 15% activity even after heat treatment at 80°C for 10 min. These results are in good accordance with the T_m values obtained from the denaturation curves. It is, therefore, reasonable to say that the thermostability of HTK is increased by at least 20°C compared to that of WT*. WT* has two mutations as mentioned above, which increase the thermostability by about 10°C. Thus, the thermostability of HTK has been increased by 30°C in total by introducing 21 substitutions to the wild-type enzyme from *Staphylococcus aureus* (*27*). To our knowledge, this example is the most successful among attempts to improve the thermostability of a protein. The thermostabilities of glucose dehydrogenase (*28, 29*) and iso-1-cytochrome (*30*) were increased by 20 and 17°C, respectively, in both cases by a single amino-acid replacement. While these two examples are interesting, we believe that our approach with KNT will prove to be more general. The natural evolution of proteins is likely to proceed through the accumulation of many mutations, each of which makes a small contribution to the total effect. Recently, we reported similar results in attempts to change the substrate specificity of aspartate aminotransferase (*25, 26*), where 17 mutations resulted in a 10^6-fold increase in the catalytic efficiency for a non-native substrate. This idea that the accumulation of small effects is important is supported by the fact that many studies, in which a few rationally-designed substitutions were introduced, have resulted in only limited success. It should be noted here that two independent studies to isolate thermostable KNT mutants by a single round of random mutagenesis and screening found only two identical stabilizing mutations (*19-21*). These findings, together with a recent report (*18*) in which nine amino-acid substitutions found by directed evolution increased the thermostability of an esterase by 14°C, show how powerful directed evolution is for searching sequence space for mutations with small but positive effects.

TABLE II. **Kinetic parameters of KNT mutants.**

Enzyme	ATP = 5.4 mM		Kan = 2.0 mM	
	K_{mKan} (mM)	k_{cat} (min^{-1})	K_{mATP} (mM)	k_{cat} (min^{-1})
WT*	0.46±0.31	13±2.6	2.7±0.52	12±1.1
HTK	0.59±0.21	18±2.3	5.9±2.6	24±6.7

Enzyme activity was measured at 25°C, pH 6.0. Kinetic parameters for one substrate were obtained at a fixed concentration of the other substrate. Kan, kanamycin.



Enzyme	Residual activity (%) after heat treatment		
	60°C	72°C	80°C
WT*	5	0	0
KT3-11	100	5	0
HTK	100	100	15

Fig. 1. **Thermostability of KNTs.** (A) Thermal denaturation of WT* (squares), KT3-11 (triangles), or HTK (circles) recorded by monitoring CD at 222 nm. Measurements were made in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1 M KCl at a protein concentration of 0.8 μM. Denaturation (%) = $(\Theta^{T}_{222}-\Theta^{N}_{222})/(\Theta^{D}_{222}-\Theta^{N}_{222})$, where Θ^{T}_{222} is the mean residue ellipticity at 222 nm at T°C, and Θ^{N}_{222} or Θ^{D}_{222} is that of the native or denatured enzyme, respectively. (B) Thermal inactivation. Enzyme solutions were heated in a water bath at the indicated temperatures for 10 min. After the sample was cooled on ice, the activity was measured at 25°C. Heat treatment was done in the same buffer as used for CD measurements at a protein concentration of 1.2 μM. Values for each enzyme represent the relative activity compared to that of non-heat-treated enzyme. Each value has a deviation of ±10%.

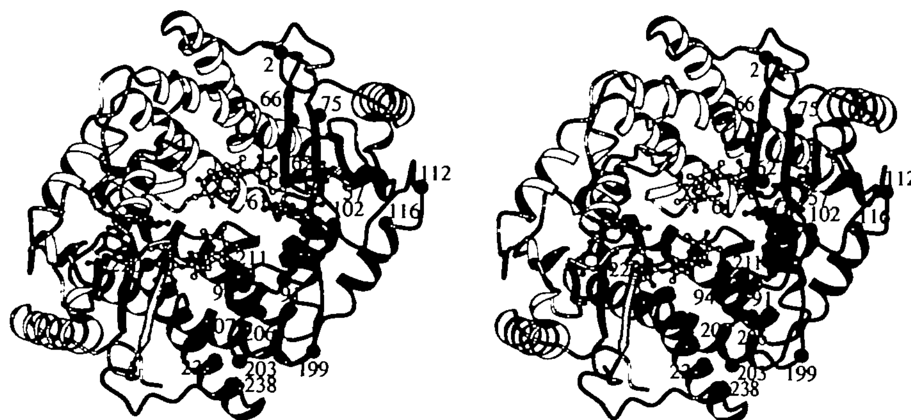


Fig. 2. **Stereo representation of the structure of KNT with an Asp80Tyr mutation (*32*).** Although KNT is a homodimer, the locations of the mutated residues and their residue numbers are shown for only one subunit (pink). The residues mutated in KT3-11 are shown in blue, the additional 9 residues mutated to construct HTK are in red, kanamycin is in yellow, and an ATP analog, adenosine 5′-α,β-methylenetriphosphate, is in green. This figure was produced using MOLSCRIPT (*33*).

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

Distribution of the Mutated Residues in the KNT Structure—Except for Val75 and Ile234, which are located in the hydrophobic core, all of the other mutations in HTK are on the surface of the molecule (Fig. 2); there are no mutated residues located at the subunit interface. The Gln91Arg substitution presumably stabilizes the helix dipole (*31*). It has been reported that the thermostability of a protein can be increased by proline substitutions (*7-9*). There are five proline substitutions in HTK: Ser94 and Ser112 are at the second site of β turns, Ser199 and Ser203 on a surface loop, and Ser220 at the N-terminal cap of an α helix. Surprisingly, even though the hydroxyl group of the Ser94 side chain is located within hydrogen-bonding distance of the 1-amide group of kanamycin (*32*), the catalytic efficiency of HTK is almost unaltered by the Ser94Pro mutation.

Creation of a Convenient Vector for T. thermophilus—pJHK3, a plasmid that incorporates both a *T. thermophilus* origin of replication and the HTK gene, provides a convenient vector for carrying out molecular biology experiments in *T. thermophilus*. Only two selection systems are available for *T. thermophilus* so far. In one system, auxotrophic host strains are rescued by the corresponding plasmid-encoded genes (*1, 2*). The other system utilizes the WT* KNT gene, which can be used only below 60°C. Auxotrophic markers are not so convenient for routine use because the preparation of the selection plates is cumbersome and because cell growth on the nutritionally restricted plate is slow even at the optimum growth temperature. Likewise, the WT* KNT gene is not a practical marker because *T. thermophilus* growth at 60°C is slower than growth at the optimum growth temperature of 70-75°C. Using pJHK3, it is possible to complete a standard procedure from transformation and colony-picking to liquid culture in two days, while the same protocol takes four days using the WT* gene. This new selection marker will greatly accelerate future research on *T. thermophilus* and other thermophilic bacteria.

We thank V.W. Cornish for careful reading of and helpful comments concerning the manuscript.

REFERENCES

1. Koyama, Y., Hoshino, T., Tomizuka, N., and Furukawa, K. (1986) Genetic transformation of the extreme thermophile *Thermus thermophilus* and of other *Thermus* spp. *J. Bacteriol.* **166**, 338-340
2. Tamakoshi, M., Yamagishi, A., and Oshima, T. (1995) Screening of stable proteins in an extreme thermophile, *Thermus thermophilus*. *Mol. Microbiol.* **16**, 1031-1036
3. Mather, M.W. and Fee, J.A. (1992) Development of plasmid cloning vectors for Thermus thermophilus HB8: expression of a heterologous, plasmid-borne kanamycin nucleotidyltransferase gene. *Appl. Environ. Microbiol.* **58**, 421-425
4. Hoshino, T., Maseda, H., and Nakahara, T. (1993) Plasmid marker rescue transformation in *Thermus thermophilus*. *J. Ferment. Bioeng.* **76**, 276-279
5. Matsumura, M., Becktel, W.J., Levitt, M., and Matthews, B.W. (1989) Stabilization of phage T4 lysozyme by engineered disulfide bonds. *Proc. Natl. Acad. Sci. USA* **86**, 6562-6566
6. Hurley, J.H., Baase, W.A., and Matthews, B.W. (1992) Design and structural analysis of alternative hydrophobic core packing arrangements in bacteriophage T4 lysozyme. *J. Mol. Biol.* **224**, 1143-1159
7. Matthews, B.W., Nicholson, H., and Becktel, W.J. (1987) Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. *Proc. Natl. Acad. Sci. USA* **84**, 6663-6667
8. Suzuki, Y., Hatagaki, K., and Oda, H. (1991) A hyperthermostable pullulanase produced by an extreme thermophile, *Bacillus flavocaldarius* KP 1228, and evidence for the proline theory of increasing protein thermostability. *Appl. Microbiol. Biotechnol.* **34**, 707-714
9. Watanabe, K., Masuda, T., Ohashi, H., Mihara, H., and Suzuki, Y. (1994) Multiple proline substitutions cumulatively thermostabilize *Bacillus cereus* ATCC7064 oligo-1,6-glucosidase. *Eur. J. Biochem.* **226**, 277-283
10. Perutz, M.F. and Raidt, H. (1975) Stereochemical basis of heat stability in bacterial ferredoxins and in haemoglobin A2. *Nature* **255**, 256-259
11. Argos, P., Rossmann, M.G., Grau, U.M., Zuber, H., Frank, G., and Tratschin, J.D. (1979) Thermal stability and protein structure. *Biochemistry* **18**, 5968-5703
12. Imanaka, T., Shibazaki, M., and Takagi, M. (1986) A new way of enhancing the thermostability of proteases. *Nature* **324**, 695-697
13. Guez-Ivanier, V., Hermann, M., Baldwin, D., and Bedouelle, H. (1993) Mapping the stability determinants of bacterial tyrosyl transfer RNA synthetases by an experimental evolutionary approach. *J. Mol. Biol.* **234**, 209-221
14. Alber, T. and Wozniak, J.A. (1985) A genetic screen for mutations that increase the thermal stability of phage T4 lysozyme. *Proc. Natl. Acad. USA* **82**, 747-750
15. Kotsuka, T., Akanuma, S., Tomuro, M., Yamagishi, A., and 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
16. Zhao, H., Giver, L., Shao, Z., Affholter, J.A., and Arnold, F.H. (1998) Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nat. Biotechnol.* **16**, 258-261
17. Buchholz, F., Angrand, P.-O., and Stewart, A.F. (1998) Improved properties of FLP recombinase evolved by cycling mutagenesis. *Nat. Biotechnol.* **16**, 657-662
18. Giver, L., Gershenson, A., Freskgard, P.-O., and Arnold, F.H. (1998) Directed evolution of a thermostable esterase. *Proc. Natl. Acad. USA* **95**, 12809-12813
19. Matsumura, M. and Aiba, S. (1985) Screening for thermostable mutant of kanamycin nucleotidyltransferase by the use of a transformation system for thermophile, *Bacillus stearothermophilus*. *J. Biol. Chem.* **260**, 15298-15303
20. Matsumura, M., Yasumura, S., and Aiba, S. (1986) Cumulative effect of intragenic amino-acid replacements on the thermostability of a protein. *Nature* **323**, 356-358
21. Liao, H., McKenzie, T., and Hageman, R. (1986) Isolation of a thermostable enzyme variant by cloning and selection in a thermophile. *Proc. Natl. Acad. USA* **83**, 576-580
22. Wayne, J. and Xu, S.-Y. (1997) Identification of a thermophilic plasmid origin and its cloning within a new *Thermus-E. coli* shuttle vector. *Gene* **195**, 321-328
23. Stemmer, W.P.C. (1994) Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature* **370**, 389-391
24. Davies, J. and Smith, D.I. (1978) Plasmid-determined resistance to antimicrobial agents. *Annu. Rev. Microbiol.* **32**, 469-518
25. Yano, T., Oue, S., and Kagamiyama, H. (1998) Directed evolution of an aspartate aminotransferase with new substrate specificities. *Proc. Natl. Acad. Sci. USA* **95**, 5511-5515
26. Oue, S., Okamoto, A., Yano, T., and Kagamiyama, H. (1998) Redesigning the substrate specificity of an enzyme by cumulative effects of the mutations of non-active site residue. *J. Biol. Chem.* **274**, 2344-2349
27. Matsumura, M., Katakura, Y., Imanaka, T., and Aiba, S. (1984) Enzymatic and nucleotide sequence studies of a kanamycin-inactivating enzyme encoded by a plasmid from thermophilic bacilli in comparison with that encoded by plasmid pUB110. *J. Bacteriol.* **160**, 413-420
28. Makino, Y., Negro, S., Urabe, I., and Okada, H. (1989) Stability-increasing mutants of glucose dehydrogenase from *Bacillus*

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

megaterium IWG3. *J. Biol. Chem.* **264**, 6381-6385
29. Nagao, T., Makino, T., Yamamoto, K., Urabe, I., and Okada, H. (1989) Stability-increasing mutants of glucose dehydrogenase. *FEBS Lett.* **253**, 113-116
30. Das, G., Hickey, D.R., McLendon, D., McLendon, G., and Sherman, F. (1989) Dramatic thermostabilization of yeast iso-1-cytochrome *c* by an asparagine→isoleucine replacement at position 57. *Proc. Natl. Acad. Sci. USA* **86**, 496-499
31. Sali, D., Bycroft, M., and Fersht, A.R. (1988) Stabilization of protein structure by interaction of α-helix dipole with a charged side chain. *Nature* **335**, 740-743
32. Pederson, L.C., Benning, M.M., and Holden, H.M. (1995) Structural investigation of the antibiotic and ATP-binding sites in kanamycin nucleotidyltransferase. *Biochemistry* **34**, 13305-13311
33. Kraulis, P.J. (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structure. *J. Appl. Crystallogr.* **24**, 946-950

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012