Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 576–580, February 1986
Biochemistry

Isolation of a thermostable enzyme variant by cloning and selection in a thermophile

(kanamycin nucleotidyltransferase/*Bacillus stearothermophilus*/protein stability)

HANS LIAO, TIM MCKENZIE, AND ROBERT HAGEMAN

Synergen, 1885 33rd Street, Boulder, CO 80301

Communicated by John Abelson, September 11, 1985

ABSTRACT **We developed a method for rapidly generating thermostable enzyme variants. Our strategy is to introduce the gene coding for a given enzyme from a mesophilic organism into a thermophile, *Bacillus stearothermophilus*. Variants that retain the enzymatic activity at the higher growth temperatures of the thermophile are then selected. This strategy was applied to kanamycin nucleotidyltransferase, which confers resistance to the antibiotic kanamycin. *B. stearothermophilus* carrying the wild-type enzyme is resistant to the antibiotic at 47°C but not at 55°C and above. Variants that were kanamycin resistant at 63°C were obtained by selection of spontaneous mutants, by passage of a shuttle plasmid through the *Escherichia coli mutD5* mutator strain and introduction into *B. stearothermophilus* by transformation, and by growing the thermophile in a chemostat. The kanamycin nucleotidyltransferases purified from these variants were all more resistant to irreversible thermal inactivation than is the wild-type enzyme, and all have the same single amino acid replacement, aspartate to tyrosine at position 80. Mutants that are even more heat stable were derived from the first variant by selecting for kanamycin resistance at 70°C, and these carry the additional change of threonine to lysine at position 130. This strategy is applicable to other enzymatic activities that are selectable in thermophiles or that can be screened for by plate assays.**

The ability of some microorganisms to grow at extreme temperatures (1) implies that their enzymes are stable and active at these temperatures. This is largely borne out when enzymes from thermophilic sources are studied *in vitro*; such enzymes are indeed more thermostable than the equivalents isolated from phylogenetically related mesophilic organisms (2). Correlations between an increase in the proportion of hydrophobic residues and the degree of thermostability have been observed (3–5). Internal electrostatic interactions (6) and disulfide linkages (7) have also been proposed as features that stabilize proteins. However, the role of individual amino acid residues in enhancing the resistance to thermal denaturation of an enzyme from a thermophile is not known.

We wish to understand the contributions of individual amino acids to the overall stability of a protein's structure. Comparisons between enzymes from mesophiles and thermophiles are complicated because these proteins, although homologous, usually differ in more than one residue. More precise inferences can be based on comparisons of temperature-sensitive mutations which encode, as a result of single amino acid changes, proteins that retain activity but are less resistant to heat denaturation than the wild-type counterpart. Thus, for example, many temperature-sensitive mutations of phage T4 lysozyme have been studied with the aim of correlating the changes in stability with changes in the protein structure (8). However, an x-ray crystallographic study of one of these mutant enzymes (9) revealed that a variant in which a histidine residue replaced an arginine in the wild type nevertheless had a three-dimensional structure identical to that of the parent protein, suggesting that the forces that govern stability are subtle.

Mutant enzymes that are more thermostable have been isolated in a few genetic investigations. For example, a mutant of the *Escherichia coli* tryptophan synthase a subunit (10, 11) and one of the phage λ repressor (12) are more heat resistant than their wild-type counterparts. These mutant enzymes were identified by enzymatic assay or by circular dichroism of the proteins purified from a large number of candidates, most of which were less thermostable than the wild type. Obviously a technique that directly identifies thermally stable mutants would reduce the number of candidates that must be examined. Recently, such mutants of T4 lysozyme were isolated by assay on lawns of infected *E. coli* after incubation at a temperature that inactivated the wild-type lysozyme (13). However, screening systems are not as powerful as a direct selection for thermostable variants.

We have developed such a selection. Our strategy consists of cloning a gene encoding an enzyme from a mesophilic organism, introducing the gene into a thermophile, and selecting for the enzymatic activity at the higher growth temperatures of the host organism. We used *Bacillus stearothermophilus* as the thermophile to test the feasibility of this approach and kanamycin nucleotidyltransferase (KNTase) as the enzyme to be modified to higher thermostability.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Plasmid pUB110 was isolated from *Bacillus subtilis* strain 1E6 from the *Bacillus* Genetic Stock Center (Ohio State University, Columbus, OH). Plasmid pBST1 was obtained from *B. stearothermophilus* 1102 from the Northern Regional Research Laboratories (Peoria, IL), as was *B. stearothermophilus* strain 1174.

Media. Media used for growth and transformation of *B. stearothermophilus* are as follows: TSY (2% Bacto-tryptone/1% NaCl/1% yeast extract, solidified with 2% agar when required) was used to grow the organism. SMM buffer was as described (14), and SMMX contained the components of TSY medium, SMM buffer, and in addition 0.5% glucose and 0.05% bovine serum albumin. Medium DM3a for the regeneration of *B. stearothermophilus* protoplasts was similar to DM3 (14) except it contained 0.15 M sodium succinate and 2% agar rather than 0.5 M and 0.8%, respectively.

Transformation of *B. stearothermophilus*. *B. stearothermophilus* were transformed by using a modification of the method used to transform *B. subtilis* protoplasts with plasmid DNA (14). Cells of *B. stearothermophilus* strain 1174, which

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: KNTase, kanamycin nucleotidyltransferase; Kan^R, kanamycin-resistant; kb, kilobase(s); Mes, 2-(*N*-morpholino)ethanesulfonate.

does not carry pBST1, were grown in TSY at 55°C to 10^8 cells per ml, harvested at room temperature by centrifugation, and resuspended in 0.1 vol of SMMX. Protoplasts were formed by treatment with lysozyme (50 μg/ml) for 15 min at 37°C, washed twice with SMMX by centrifugation at 1400 × *g* for 7 min in a clinical centrifuge at room temperature, and resuspended in the same volume of SMMX. The transformation reaction was carried out by adding 0.5 ml of protoplasts to DNA in 40–100 μl of SMM buffer, followed by the addition of 1.5 ml of 40% polyethylene glycol 8000 dissolved in SMM buffer, prewarmed to 47°C. The mixture was gently shaken at 47°C for 2 min, and the polyethylene glycol was diluted with 5 ml of SMMX. The protoplasts were recovered by centrifugation, resuspended with 0.5 ml of SMMX, and incubated with gentle shaking at 47°C for 1 hr before plating on DM3a agar. Kanamycin-resistant (Kan^R) transformants were selected by incorporating kanamycin in the regeneration agar at 25 μg/ml. The efficiency of protoplast regeneration to colony-forming units was 10%.

Other DNA Methods. Standard methods were used to transform *E. coli*, to prepare plasmid DNA from this organism, and to construct chimeric plasmids (15). Rapid screening of transformants of *E. coli* or *B. stearothermophilus* for plasmids was done by using an alkaline extraction procedure in which a phenol extraction step was incorporated (16). This procedure yielded pBST16 from either species capable of transforming the other. Large-scale (2-liter) preparation of plasmid from *B. stearothermophilus* was carried out by alkaline extraction and CsCl/ethidium bromide gradient centrifugation. Oligodeoxynucleotides were prepared by the method of Caruthers *et al.* (17), using an Applied Biosystems model 380A DNA synthesizer. The gene coding for KNTase was sequenced by the dideoxynucleotide method (18). Subclones of pBST2 were made in M13 mp8 and mp9 phage and sequences generated by extension of the universal M13 pentadecameric primer. From the sequence obtained from these subclones, a set of eight oligodeoxynucleotides, each complementary to a portion of the KNTase gene and approximately 250 bases apart, in both orientations, were synthesized to permit sequencing directly from the plasmid. This strategy obviated the need to construct sets of overlapping subclones of the wild-type gene and also allowed rapid sequencing of the thermostable mutant genes. The primers were 5′-labeled with [^{32}P]ATP and mixed in equimolar ratio with linearized plasmid carrying the KNTase gene. After denaturation by boiling, the mixture was cooled slowly and dideoxynucleotide incorporation reactions were catalyzed with reverse transcriptase (Boehringer Mannheim) at 46°C and analyzed by gel electrophoresis in the standard manner (18).

Assay for KNTase. KNTase activity was assayed by coupling the release of PP_i from ATP to the reduction of NADP. Incubation mixtures contained 50 mM sodium 2-(*N*-morpholino)ethanesulfonate (Mes) at pH 6.1, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 100 μM kanamycin, and the following coupling reagents: 0.5 unit of UDP glucose pyrophosphorylase, 1 unit of phosphoglucomutase, 0.2 unit of glucose-6-phosphate dehydrogenase, 0.2 mM UDP-glucose, 2 μM glucose 1,6-bisphosphate, and 0.2 mM NADP. The reactions were carried out at 37°C and continuously monitored at 340 nm. One unit of KNTase activity is equal to 1 nmol of PP_i released per min as measured by the production of NADPH. All chemicals and coupling enzymes were obtained from Sigma.

Purification of KNTase. A crude extract of *E. coli* harboring pBST16 was prepared by a French press, and the supernatant free of cell debris was chromatographed on DEAE-cellulose as described (19). The active fractions were precipitated with ammonium sulfate (70% saturation) and dialyzed versus 50 mM Na Mes, pH 6.1/5 mM $MgCl_2$/1 mM dithiothreitol/50 mM NaCl. The dialyzed extract was applied to a kanamycin affinity column (prepared by attaching kanamycin to epoxy-activated Sepharose 6B from Pharmacia) which was then washed with 50 mM Na Mes, pH 6.1/5 mM $MgCl_2$/1 mM dithiothreitol/200 mM NaCl, and the KNTase was eluted with a gradient of kanamycin from 0 to 10 mM in the same buffer. The enzyme was purified to homogeneity (as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis) by the use of a hydrophobic interaction column (Bio-Gel TSK phenyl-5-PW from Bio-Rad). The affinity-purified material was applied to the column in 50 mM sodium 3-(*N*-morpholino)propanesulfonate, pH 7.1/5 mM $MgCl_2$/1 mM dithiothreitol/0.5 M $(NH_4)_2SO_4$ and eluted with a reverse salt gradient to the same buffer without $(NH_4)_2SO_4$. Protein was measured by the Coomassie blue method (20), calibrated by using the extinction coefficient of the wild-type KNTase as calculated from the predicted protein sequence. Bovine serum albumin was utilized as an intermediate standard.

Thermal Denaturation. Experiments were performed after dialyzing aliquots of the enzymes vs. 10 mM sodium piperazine-*N*,*N*′-bis(2-hydroxypropanesulfonate), pH 8.0. The pH of this buffer remains above 7.0 at the temperatures used; the thermal stability of the wild-type enzyme was independent of pH above this value.

RESULTS

Cloning in *B. stearothermophilus*

The KNTase gene is carried on the *Staphylococcus aureus* plasmid pUB110 (19) and confers kanamycin resistance. This plasmid, which transforms *B. subtilis* (21), was used to develop the *B. stearothermophilus* transformation procedures. Transformants arose at a frequency of 4×10^4 per μg of plasmid DNA when selected at 47°C on protoplast regeneration plates containing kanamycin at 25 μg/ml.

Although pUB110 was maintained in *B. stearothermophilus* at 47°C, it was lost at higher temperatures and thus would not maintain cloned genes at the growth temperatures where selection for thermostable mutants would be conducted. However, we found that many strains of *B. stearothermophilus* carry large [approximately 80-kilobase (kb)] cryptic plasmids even in cells grown at 70°C. One plasmid, pBST1, was isolated from the kanamycin-sensitive strain NRRL 1102. A chimera carrying the presumably heat-stable origin of replication of pBST1 and the kan^R marker of pUB110 was constructed as follows. pUB110 was digested with *Taq* I, which inactivates its origin of replication but leaves the kan^R gene function intact (22), and pBST1 was digested with *Cla* I. The pUB110 and pBST1 fragments were ligated with T4 DNA ligase and the products of this reaction were used to transform *B. stearothermophilus*. Kan^R transformants were selected, grown on plates without the drug at 70°C, and replated on kanamycin-containing plates at 47°C. Transformants carrying only pUB110 lost this plasmid during the high-temperature growth, but a chimeric plasmid (pBST2) was obtained from a thermally stable transformant. When analyzed by restriction endonuclease mapping and Southern hybridization (23), pBST2 was found to contain a 1.4-kb DNA fragment from pBST1 as well as the three *Taq* I fragments corresponding to the complete pUB110. Restriction endonuclease mapping indicated that these *Taq* I fragments which carry the origin of replication of pUB110 were rearranged in pBST2 (Fig. 1). Furthermore, in contrast to pUB110, which is present in *B. stearothermophilus* at approximately 40 copies per cell, pBST2 is present at 1 to 3 copies per cell. We believe that the 1.4-kb fragment from

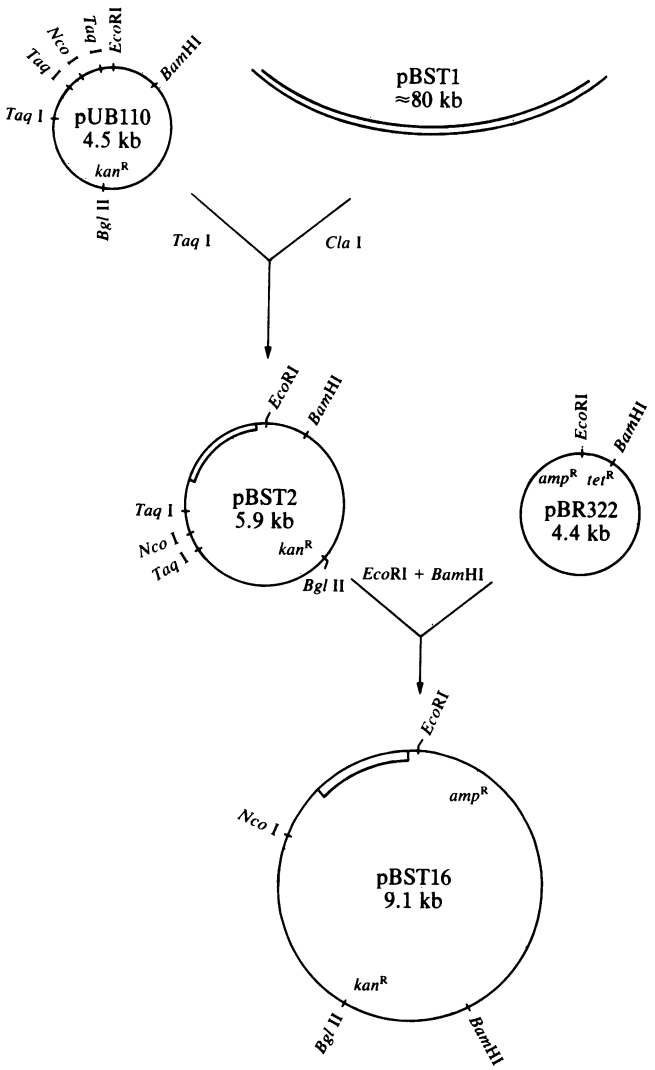

FIG. 1. Plasmid constructions. The construction of the chimeric plasmids pBST2 and pBST16 is described in the text. The pBST1 fragment cloned in pBST2 is bounded by *Taq* I sites and also contains seven *Taq* I sites, which are not marked in the figure.

pBST1 cloned in pBST2 is acting as the origin of replication of this plasmid, although other functions cannot be excluded.

The stabilities of pUB110 and pBST2 at various temperatures were measured by growing cultures for 20 generations in the absence of kanamycin, then plating in the presence of kanamycin at 25 μg/ml. As shown in Table 1, the chimeric plasmid is markedly more stable than pUB110, even though initially present at a lower copy number. The stability of pBST2 is comparable to that of naturally isolated drug-resistant plasmids found in thermophilic *Bacillus* species (24). pBST16 was constructed by joining pBST2 with the *E. coli* plasmid pBR322 to allow shuttling between the two species. This plasmid construction is shown in Fig. 1.

Table 1. Plasmid stability in *B. stearothermophilus*

Growth temp., °C	% Kan^R colonies*	
	pUB110	pBST2
47	99	100
55	36	95
59	27	95
63	1	58
67	1	46

*At 47°C, after 20 generations in the absence of kanamycin.

Generation of Thermostable Mutants

B. stearothermophilus carrying pBST2 or pBST16 is not phenotypically Kan^R at temperatures above 55°C, even though the plasmid is present. (The antibiotic itself is stable and inhibits growth of *B. stearothermophilus* up to 72°C, the highest temperature at which this organism grows.) A mutant resistant to kanamycin at 63°C was obtained by plating 10^8 cells carrying pBST2 at 63°C in the presence of kanamycin at 25 μg/ml. The plasmid was isolated from this mutant (TK1) and found to be indistinguishable by restriction analysis from the wild-type pBST2. When used to transform *B. stearothermophilus* lacking experimentally introduced plasmids, pBST2-TK1 conferred kanamycin resistance at 63°C, indicating that the high-temperature drug resistance was plasmid borne.

Mutant TK1 was isolated without the use of mutagens. To increase the frequency of mutagenesis the plasmid-borne KNTase gene was passaged through an *E. coli mutD5* mutator strain (25). The effect of the *mutD5* mutation of *E. coli*, which alters the editing function of DNA polymerase III (26), is to increase the frequency at which spontaneous mutations are generated. The shuttle plasmid pBST16 was used to transform *E. coli* LE30 (*galU strA azi^R mutD5*), and plasmid DNAs were prepared from individual colonies grown up under mutagenic conditions (27). Each of these DNAs was then separately used to transform *B. stearothermophilus* and a single transformant resistant to kanamycin at 63°C was saved from each transformation to avoid the isolation of sibling mutants. Such mutants, represented by strains TK3, TK4, and TK6, arose at frequencies of 1×10^{-4} per transformant.

To investigate the possible effect of sequential mutations, each of which may confer increased thermal stability to KNTase, mutant TK13 was generated by continuous growth in a chemostat. The continuous culture was inoculated with the wild-type strain and growth was initiated at 47°C in the presence of kanamycin. The temperature was increased slowly while the medium flow rate was adjusted to prevent washing out the stressed culture. At the indicated harvest temperature (Table 2) the culture was allowed to equilibrate for at least 50 generations before a single colony was isolated. Sequential mutations were also obtained by plating TK1 in the presence of kanamycin at 25 μg/ml at 69°C (generating TK101, which arose at a frequency of 1×10^{-7}) and by starting the continuous culture with TK1 (generating *TK108*).

Table 2 lists the mutants and the way each was obtained. All of these were plasmid borne, as shown by the ability of the respective plasmids to transform plasmid-free *B. stearothermophilus* to high-temperature kanamycin resistance. Because the chemostat-derived mutants may have changes in addition to mutations in the plasmid-borne KNTase gene, the enzymes coded for by these mutants were prepared from retransformants.

Table 2. Isolation of thermostable KNTase mutants

Mutant	Plasmid carried	Isolation method	Temp., °C	Kanamycin, μg/ml	Parent strain
TK1	pBST2	Plating	63	25	Wild type
TK3	pBST16	*mutD5*	63	25	Wild type
TK4	pBST16	*mutD5*	63	25	Wild type
TK6	pBST16	*mutD5*	63	25	Wild type
TK13	pBST2	Chemostat	63	25	Wild type
TK101	pBST2	Plating	69	25	TK1
TK108	pBST2	Chemostat	71	200	TK1

Table 3. Purification of wild-type KNTase from *E. coli*

Fraction	Total protein, mg	Total activity, units	Specific activity, units/mg
Crude extract	1008	305	0.30
DEAE-cellulose	147	1500	10.2
Affinity column	1.48	1900	1280
Hydrophobic column	0.195	488	2500

Isolation and Characterization of KNTase Mutant Proteins

Mutant enzymes were characterized after being purified to avoid possible artifacts caused by, for example, proteolytic or ATPase activity. The purification method was developed by purifying to homogeneity the enzyme from *E. coli* harboring pBST16 (Table 3). These conditions were then used to purify the enzymes from the *B. stearothermophilus* strains carrying wild-type or thermostable mutants. The enzyme preparations were homogeneous as determined by NaDodSO$_4$/polyacrylamide gel electrophoresis and were of identical mobility. Irreversible thermal denaturation kinetics for these activities were measured at several temperatures, and the points were fitted to a first-order curve. Half-lives were calculated by using a nonlinear regression least-squares program and are listed in Table 4. It is apparent from these data that the mutant enzymes are all more stable to heat denaturation than is the wild type. Furthermore, the mutants may be grouped into those with denaturation characteristics similar to those of TK1 and others with characteristics similar to those of TK101, with the latter class being more stable. This result correlates with the Kan^R phenotypes of the mutants, because TK101 is resistant at 69°C, whereas TK1 is resistant only to 63°C. There were no significant changes in the K_m, V_{max}, or k_{cat} values for the mutant enzymes assayed at 37°C.

Sequence of the KNTase Gene

The sequence we determined for the wild-type KNTase gene is identical to that reported by Matsumura *et al.* (28). The sequences of the mutant genes were obtained from the respective plasmids by using the oligodeoxynucleotide primers. The single change of aspartate to tyrosine at position 80 was found in TK1, TK3, TK4, TK6, and TK13. Mutants TK101 and TK108 had an additional change of threonine to lysine at position 130. Fig. 2 shows the regions of the gene adjacent to the positions of the mutations, as well as the nature of the base changes. Both mutations occur in regions predicted to form α-helices (29).

Table 4. Thermal denaturation properties of mutant and wild-type KNTase

KNTase	Half-life, min			
	50°C	55°C	60°C	65°C
Wild type	17	1.9	<0.3	<0.3
TK1	Stable	>60	16.5	<1
TK3	Stable	>60	17.5	<1
TK4	Stable	>60	15	<1
TK6	Stable	>60	21	<1
TK13	Stable	>60	20	<1
TK101	Stable	Stable	>60	15.2
TK108	Stable	Stable	>60	12

Stable denotes no loss of activity in 60 min.

DISCUSSION

In general, enzymes from mesophilic organisms are less thermostable than the equivalent enzymes from thermophiles. We have demonstrated that variants of an enzyme from a mesophile with increased stability towards heat denaturation can be obtained in a thermophile by introducing the gene coding for this enzyme into the thermophile and selecting for the activity at the higher growth temperatures of this organism. Furthermore, because we are selecting for enzymatic activity, we avoid the possible generation of variants in which the enhancement of stability was made at the expense of catalytic efficiency; the mutants of KNTase we obtained have specific activities at 37°C equivalent to the specific activity of the wild-type enzyme.

The isolation of a mutant enzyme with increased thermostability in *B. stearothermophilus* has been previously reported (30). In this case the activity is an endogenous restriction enzyme that fortuitously is not active *in vivo* at high growth temperatures; mutants that prevented phage infection were isolated and found to have an enzyme that is more resistant to thermal denaturation. The ability to introduce foreign DNA into *B. stearothermophilus* by our method greatly extends the range of enzymes whose thermostability can be increased by selection in this thermophile.

Only one mutation was isolated from the wild-type mesophilic parent in our KNTase test case. The repeated reisolation of the TK1 mutation (Asp-80→Tyr) by several routes may be due either to the presence of a mutagenic hotspot at that site or to the limited number and kind of changes in this protein that both preserve activity and enhance heat stability. At least one alternative mutation that confers increased thermal stability to KNTase is known; an activity from a naturally Kan^R thermophilic *Bacillus* species was described recently; this enzyme differs from the pUB110 enzyme only in the replacement of threonine by lysine at position 130 (18).

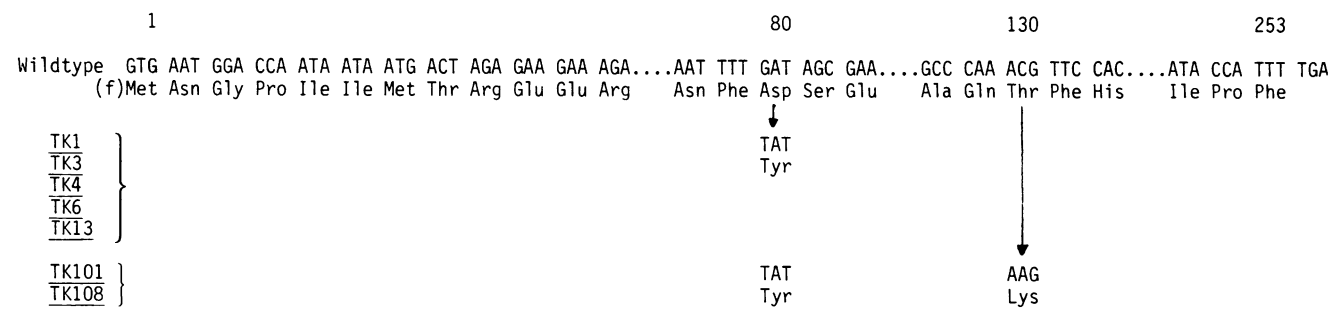

FIG. 2. Sequence of thermostable KNTase mutants. The complete sequence of the wild-type KNTase gene is given in ref. 28; thus only the portions relevant to this work are presented. The starting codon of the gene was aligned according to the sequence of the amino-terminal 12 amino acids of the pure enzyme (sequencing performed by Al Smith, University of California, Davis), and the positions and nature of the mutations that yielded thermostable variants are shown.

From the reported data we calculate that the Lys-130 mutant has a half-life of approximately 9 min at 55°C, whereas the TK1 enzyme is essentially stable at this temperature. The Lys-130 mutant may therefore be insufficiently stable to survive the selection conditions we used.

Nevertheless, it is evident that Lys-130 in combination with Thr-80 results in a variant that is more resistant to thermal denaturation than either mutation alone. This additive effect is seen in mutant TK101, which was isolated at 69°C in the presence of kanamycin from a strain already carrying the TK1 mutation but unable to grow under these conditions. We suggest that the separate changes at positions 80 and 130 act independently and additively to stabilize KNTase from thermal denaturation.

Protein stability is clearly the sum of multiple intramolecular interactions, many of which may be affected when even a single amino acid residue is altered. Thus attempts to predict enzyme variants that are more stable may be subject to unforeseen complications, such as the loss of the desired activity. In contrast, our method, based on biological selection, accounts for all variables of activity and structural stability simultaneously and requires no assumptions regarding the outcome. Furthermore, since the method does not use predictive strategies, we can isolate thermostable enzyme variants without structural information on the protein, and therefore without the time-consuming research needed to obtain this information. The sequential isolation of variants, each more thermostable than its predecessor, under laboratory conditions that are more extreme than natural ones, demonstrates that our method is able to generate enzymatic activities with properties unlikely to be found in natural sources. Lastly, even though we used an enzyme whose gene is naturally carried on a plasmid and whose activity is selectable in *B. stearothermophilus* to demonstrate the feasibility of this approach, the strategy can be readily extended to cloned genes and to screen for mutant proteins whose activities are not selectable.

Using thermostable enzymes in industrial applications offers the benefits of increased rates of reaction, higher substrate solubilities, and lowered risk of microbial contamination when reactions are carried out at higher temperatures. Thermostable enzymes also generally have longer shelf lives at normal storage temperatures. The enhancement of the heat stability of other enzymes by our method will greatly extend the repertoire of industrial tasks that these catalysts may perform.

We thank Michael McDonell for expert advice in the construction of the plasmids; Larry Forney for setting up the chemostat experiments; Amy Moore Geis for the initial M13 sequencing; Pradip Bandyopadhyay and Tom Gleason for the synthesis of oligodeoxynucleotide primers; and Patrick Shannon for helpful discussions on the properties of enzymes. The selection strategy described in this paper was first suggested to the authors by Larry Gold.

1. Brock, T. D. (1978) *Thermophilic Microorganisms and Life at High Temperatures* (Springer, New York).
2. Zuber, H. (1979) in *Strategies of Microbial Life in Extreme Environments*, ed. Shilo, M. (Verlag Chem, New York), pp. 393–415.
3. Argos, P., Rossmann, M. G., Grau, U. M., Zuber, H., Frank, G. & Tratschin, J. D. (1979) *Biochemistry* **18**, 5698–5703.
4. Ikai, A. (1980) *J. Biochem. (Tokyo)* **88**, 1895–1898.
5. Merkler, D. J., Farrington, G. K. & Wedler, F. C. (1981) *Int. J. Pept. Protein Res.* **18**, 430–442.
6. Perutz, M. (1978) *Science* **201**, 1187–1191.
7. Thornton, J. M. (1981) *J. Mol. Biol.* **151**, 261–287.
8. Schellman, J. A., Lindorfer, M., Hawkes, R. & Grutter, M. (1981) *Biopolymers* **20**, 1989–1999.
9. Grutter, M. G., Hawkes, R. B. & Matthews, B. W. (1979) *Nature (London)* **277**, 667–669.
10. Yutani, K., Ogasahara, K., Sugino, Y. & Matsushiro, A. (1977) *Nature (London)* **267**, 274–275.
11. Yutani, K., Ogasahara, K., Kimura, A. & Sugino, Y. (1982) *J. Mol. Biol.* **160**, 387–390.
12. Hecht, M. H., Sturtevant, J. M. & Sauer, R. T. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5685–5689.
13. Alber, T. & Wozniak, J. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 747–750.
14. Chang, S. & Cohen, S. N. (1979) *Molec. Gen. Genet.* **168**, 111–115.
15. Davis, R. W., Botstein, D. & Roth, J. R. (1980) *Advanced Bacterial Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
16. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1513–1523.
17. Caruthers, M. H., Beaucage, S. L., Becker, C., Efcavitch, W., Fisher, E. F., Gallupi, G., Goldman, R., deHaseth, P., Martin, F., Matteucci, M. & Stabinsky, Y. (1982) in *Genetic Engineering: Principles and Methods*, eds. Setlow, J. K. & Hollaender, A. (Plenum, New York), Vol. 4, pp. 1–17.
18. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
19. Sadaie, Y., Burtis, K. C. & Doi, R. H. (1980) *J. Bacteriol.* **141**, 1178–1182.
20. Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254.
21. Gryczan, T. J., Contente, S. & Dubnau, D. (1978) *J. Bacteriol.* **134**, 318–329.
22. Sheer-Abramowitz, J., Gryczan, T. J. & Dubnau, D. (1981) *Plasmid* **6**, 67–77.
23. Southern, E. (1975) *J. Mol. Biol.* **98**, 503–517.
24. Imanaka, T., Fujii, M., Aramori, I. & Aiba, S. (1982) *J. Bacteriol.* **149**, 824–830.
25. Degnen, G. E. & Cox, E. C. (1974) *J. Bacteriol.* **117**, 477–487.
26. Echols, H., Lu, C. & Burgers, P. M. J. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2189–2192.
27. Fowler, R. G., Degnen, G. E. & Cox, E. C. (1974) *Molec. Gen. Genet.* **133**, 179–191.
28. Matsumura, M., Katakura, Y., Imanaka, T. & Aiba, S. (1984) *J. Bacteriol.* **160**, 413–420.
29. Garnier, J., Osguthorpe, D. J. & Robson, B. (1978) *J. Mol. Biol.* **120**, 97–120.
30. Hendrix, J. D. & Welker, N. E. (1985) *J. Bacteriol.* **162**, 682–692.