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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

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The product of a kanamycin resistance gene encoded by plasmid pTB913 isolated from a thermophilic bacillus was identified as a kanamycin nucleotidyltransferase which is similar to that encoded by plasmid pUB110 from a mesophile, *Staphylococcus aureus*. The enzyme encoded by pTB913 was more thermostable than that encoded by pUB110. In view of a close resemblance of restriction endonuclease cleavage maps around the *BglII* site in the structural genes of both enzymes, ca. 1,200 base pairs were sequenced, followed by amino-terminal amino acid sequencing of the enzyme. The two nucleotide sequences were found to be identical to each other except for only one base in the midst of the structural gene. Each structural gene, initiating from a GUG codon as methionine, was composed of 759 base pairs and 253 amino acid residues (molecular weight, ca. 29,000). The sole difference was transversion from a cytosine (pUB110) to an adenine (pTB913) at a position +389, counting the first base of the initiation codon as +1. That is, a threonine at position 130 for the pUB110-coded kanamycin nucleotidyltransferase was replaced by a lysine for the pTB913-coded enzyme. The difference in thermostability between the two enzymes caused by a single amino acid replacement is discussed in light of electrostatic effects.

It is well known that enzymes from thermophilic origins are generally more stable against heat, pH, organic solvents, and chemical denaturants. The stable characteristics of thermophilic enzymes are stimulating not only for biochemical and enzymatic studies but also useful for industrial applications. Various approaches to disclose the nature of thermostable proteins have been made by many workers (2, 22), but no definite conclusion on the cause and effect of thermostability has been reached yet.

Recent studies have revealed that the thermostability of a protein could be achieved solely by a single amino acid substitution without any significant change of the three-dimensional protein structure (5, 29). This finding suggests the possibility of enhancing the thermostability of an enzyme, for example, by selectively changing one or more amino acid residues in the enzyme via genetic manipulations such as base substitutions (25) or the use of synthetic oligonucleotides (15).

A kanamycin nucleotidyltransferase is encoded by a plasmid pUB110 (24), originally isolated in England from *S. aureus*, a mesophile (13). We have isolated separately from a thermophilic bacillus another plasmid, pTB19, which conferred kanamycin and tetracycline resistance (Km^r Tc^r) on *Bacillus stearothermophilus* (7). The thermophile, once it has been transformed by pUB110, is not able to grow in L-broth containing kanamycin at temperatures above 55°C, whereas the same host cells transformed by pTB19 can grow at temperatures up to 65°C under the same culture conditions (8).

Whatever the kanamycin-inactivating enzyme encoded by pTB19 may be, the above observation gave rise to a surmise that the enzyme thermostability would differ between pTB19 and pUB110. Hence, the kanamycin-inactivating enzyme encoded by pTB913, a derivative of pTB19 (9), had to be identified at the beginnning of this work. We identified a product of the kanamycin resistance gene (*kan*) encoded by pTB913 as kanamycin nucleotidyltransferase and confirmed that the enzyme from pTB913 was more stable than that from pUB110 (see below).

Despite the widespread use of pUB110 as a cloning vector, the *kan* gene structure of pUB110 still remains to be elucidated. The purpose of this work is to determine the nucleotide sequence of the *kan* genes of both pUB110 and pTB913 and to discuss the difference of thermostability between these two enzymes.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Bacillus subtilis* MI113 (*arg-15 trpC2* r_M^- m_M^-) (7) and *B. stearothermophilus* CU21 (8) were used as the host cells of plasmids. The plasmids used were pUB110 (molecular weight; 3.0 megadaltons [Md]; Km^r) from *S. aureus* (13) and pTB913 (2.9 Md; Km^r) (9), a deletion plasmid of pTB19 (Km^r Tc^r; 17.2 Md) which is originally isolated from a thermophilic bacillus (7). Both plasmids pUB110 and pTB913 can be transferred into *B. subtilis* MI113 and *B. stearothermophilus* CU21 (8, 9), respectively, and the host cells carrying either pUB110 or pTB913 were used throughout this study.

Medium. L-broth containing 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract, and 5 g of NaCl in 1 liter of deionized water (pH 7.3) was used for cultivation of bacteria.

Enzyme assay. Nucleotidyltransferase was assayed as described by Sadaie et al. (24). The assay mixture for adenylylation contained 0.86 mM kanamycin, 0.2 mM (1 μCi) of [^{3}H]ATP, 4 mM $MgCl_2$, 10 mM mercaptoethanol, 62.5 mM Tris-maleate buffer (pH 6.25), and 8 μl of enzyme in a total volume of 20 μl. The reaction mixture was incubated at 37°C for 10 min, after which a 5-μl sample was placed onto phosphocellulose paper (0.49 cm^2; Whatman P-81), washed with large volume of distilled water, dried, and counted in a scintillation counter (Beckman LS 7500; Beckman Instru-

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ments, Inc., Fullerton, Calif.). To reduce the background, the phosphocellulose paper was pre-soaked in a solution of 20 mM ATP–10 mM sodium phosphate (pH 8.0).

Phosphotransferase and acetyltransferase were assayed by the methods of Ozanne et al. (21) and Benveniste and Davies (3), respectively. The procedure was exactly the same as mentioned above in the assay of nucleotidyltransferase, except for components of each reaction mixture and the reaction condition (3, 21).

Purification of kanamycin nucleotidyltransferase. *B. subtilis* MI113 carrying either pUB110 or pTB913 was used as the enzyme source. The purification procedure of the enzyme was essentially the same as described by Sadaie et al. (24).

The bacteria grown up to a late-log phase in 50 liters of L-broth containing 5 μg of kanamycin per ml at 37°C were harvested with a Sharples centrifuge. The cells (100 g [wet weight]), suspended in buffer N (20 mM Tris-hydrochloride [pH 7.5], 20 mM $MgCl_2$, 10% glycerol, 0.2 mM dithiothreitol, 50 mM NaCl) (24), were disrupted for 10 min by an ultrasonic oscillation (19.5 KHz), followed by centrifugation (20,000 × *g*, 30 min). The crude extract was applied to a column (6 by 40 cm) of DEAE-Cellulofine AM (Seikagaku Kogyo, Tokyo) and eluted with a 4-liter linear gradient of NaCl from 50 to 350 mM in buffer N. Active fractions were concentrated by ultrafiltration (UK10, Toyo Filter Paper Co., Tokyo) and desalted by gel filtration on Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden). The eluate was adsorbed in a column (4 by 40 cm) of DEAE-Toyopearl 650M (Toyo Soda Co., Tokyo) and eluted with a 6-liter linear gradient from 50 to 350 mM NaCl in buffer N without glycerol. Active fractions were pooled, and the protein was precipitated with 70% ammonium sulfate. The precipitate was collected by centrifugation and suspended in buffer N without glycerol, and then gel filtration was done in a column (2.15 by 60 cm) of TSKgel 3000SWG (2.15 by 60 cm; Toyo Soda Co.). The partially purified enzyme was used to study the thermostability.

For determinations of both amino acid composition and amino-terminal amino acid sequence, the enzyme was purified further by reversed-phase liquid chromatography; i.e., the enzyme was applied to a YMC-PAC AP-303 column (Yamamura Chemical Laboratory Co. Ltd., Kyoto, Japan) and eluted with a linear gradient from 0 to 80% acetonitrile-isopropanol (3:7) in 0.1% trifluoroacetate. The purified enzyme was freeze-dried and used for amino acid analyses.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (14) was used for the analysis of kanamycin nucleotidyltransferase at 12% acrylamide concentration. Molecular weight standards used were cytochrome *c* oligomers (Oriental Yeast Co., Osaka, Japan) and trypsin inhibitor (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany).

Amino acid composition and amino-terminal amino acid sequence. The amino acid composition of kanamycin nucleotidyltransferase was analyzed by hydrolysis at 110°C for 24 h with 4-*N*-methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole by the method of Simpson et al. (26). The hydrolysate was analyzed with an amino acid analyzer (835-S; Hitachi Works, Tokyo).

A partial amino-terminal, amino acid sequence of kanamycin nucleotidyltransferase was determined manually by the Edman degradation (1). Ether extracts from each cycle of the degradation were dried in a stream of nitrogen, and the phenylthiohydantoin-amino acid was determined by high-performance liquid chromatography with a Microsorb C18 column (Rainin Instrument Co., Inc., Emeryville, Calif.).

Preparation of plasmid DNA. The plasmids were prepared from *B. subtilis* MI113 carrying pUB110 or pTB913 by the cleared-lysate method of Niaudet and Ehrlich (20), followed by cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

Molecular size determination of DNA fragment. Molecular sizes of DNA fragments of pUB110 and pTB913 were determined by either 1% agarose or 5% polyacrylamide gel electrophoresis (17) with *Hin*dIII-cleaved λ phage DNA or

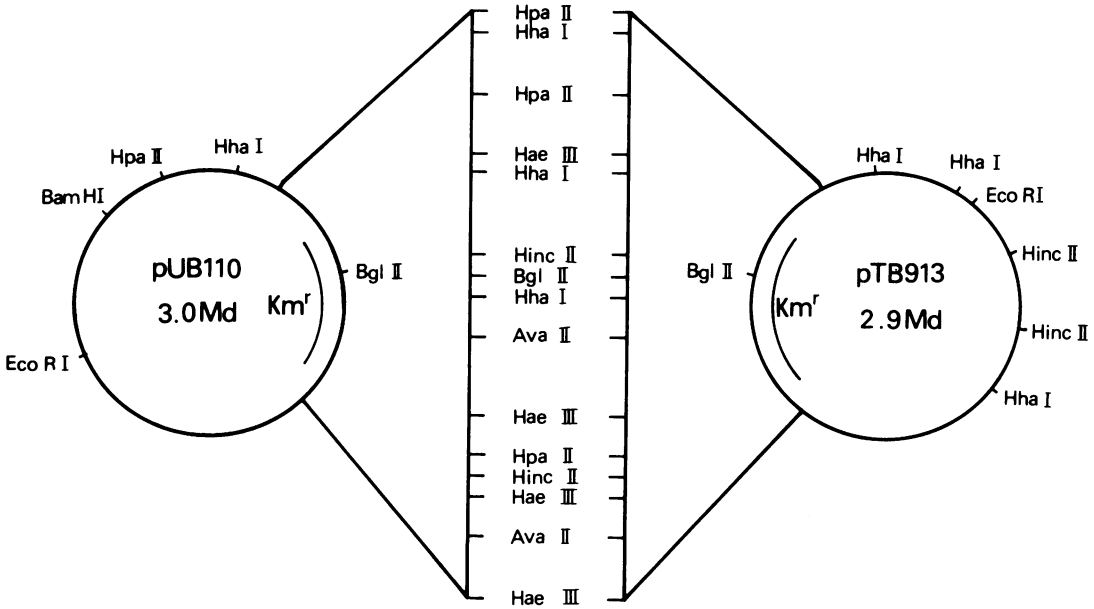

FIG. 1. Restriction endonuclease cleavage maps of pUB110 and pTB913. Detailed maps around the *Bgl* II site for both plasmids are shown vertically.

TABLE 1. Assays for kanamycin-inactivating enzymes of pTB913[a]

Enzyme assayed	Substrate used	Activity (cpm) in extract:			Activity ratio of extracts:	
		(1)	(2)	(3)	(2)/(1)	(3)/(1)
Nucleotidyltransferase	[^{3}H]ATP	130	872	592	6.7	4.6
Phosphotransferase	[γ-^{32}P]ATP	37	50	45	1.4	1.2
Acetyltransferase	[^{14}C]Acetylcoenzyme A	47	52	50	1.1	1.1

[a] Crude extracts were prepared from cells of *B. subtilis* MI113 without plasmid (extract 1) and with plasmids pUB110 (extract 2) or pTB913 (extract 3). Cells from late-log-phase cultures grown at 37°C in 1 liter of L-broth containing 5 μg of kanamycin per ml were collected by centrifugation, suspended in buffer N, and disrupted by sonic oscillation; the supernatant used for the assays was obtained after the centrifugating the crude extract at 20,000 × *g* for 30 min.

*Hin*fI-cleaved pBR322 fragments as the molecular size standards.

DNA nucleotide sequence determination. Plasmid DNA was digested with several restriction endonucleases, and the resulting fragments were labeled at the 5′ termini by polynucleotide kinase and [γ-^{32}P]ATP. DNA sequencing was performed by the method of Maxam and Gilbert (18).

Computer analysis of hydropathic character of protein. A C program to assess hydrophathy as described by Kyte and Doolittle (12) was translated to BASIC and implemented on an NEC PC-8001 computer (Nippon Electric Co., Tokyo). The BASIC program was assigned the mean hydropathy value of a moving segment of nine amino acid residues in the amino acid sequence, and proceeded from the amino to the carboxyl terminus.

Enzymes and chemicals. Restriction endonucleases, polynucleotide kinase, and bacterial alkaline phosphatase were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan) or Bethesda Research Laboratories (Rockville, Md.). The use of each enzyme was according to specifications of the manufacturer.

Kanamycin was purchased from Sigma Chemical Co. (St. Louis, Mo.). [^{3}H]ATP, [γ-^{32}P]ATP, and [^{14}C]acetyl coenzyme A were obtained from New England Nuclear Corp. (Boston, Mass.). All other chemicals used were of the reagent grade and were purchased from Wako Pure Chemical Industries (Osaka, Japan).

RESULTS

Restriction endonuclease mapping of plasmids pUB110 and pTB913. The plasmid pUB110, which confers resistance to kanamycin, has single cleavage sites for the restriction endonucleases *Eco*RI, *Bam*HI, and *Bgl*II, and this antibiotic resistance marker is subjected to an insertional inactivation at the *Bgl*II site (6). This characteristic indicates that a kanamycin inactivation enzyme, kanamycin nucleotidyltransferase (24), is encoded around the *Bgl*II site of pUB110.

Referring to the above findings and the endonuclease cleavage sites of pUB110 (10, 27), we constructed anew the cleavage maps of both pTB913 and pUB110 by either single or double endonuclease digestions, and we compared the maps. Particular attention was given to the area around the *Bgl*II site. As shown in Fig. 1, restriction endonucleases *Eco*RI and *Bgl*II were found to cleave pTB913 DNAs at a single site, but no cleavage site for *Bam*HI was found. However, the cleavage map of pTB913 was completely identical with that of pUB110 around the *Bgl*II site; the same endonuclease cleavage sites were found for *Hpa*II, *Hha*I, *Hae*III, *Hin*cIII, *Bgl*II, and *Ava*II. In addition to these six endonucleases, *Fok*I and *Sau*96I digested both plasmids at the same sites near the *Bgl*II site (data not shown here, but see Fig. 4). The close resemblance of the cleavage maps around the *Bgl*II site between the two plasmids implies that the kanamycin resistance gene in pTB913 would be located in proximity of the *Bgl*II site, suggesting also that the gene product might be kanamycin nucleotidyltransferase as in pUB110.

Identification of kanamycin-inactivating enzyme encoded by pTB913. The crude extract of *B. subtilis* MI113 carrying pTB913 was examined for activities of three kanamycin modification enzymes, i.e., nucleotidyltransferase, phosphotransferase, and acetyltransferase. As a control, the assays were also performed with an extract of *B. subtilis* MI113 carrying pUB110.

The results (Table 1) showed that only the nucleotidyltransferase activity was increased in the extract of cells carrying pTB913, whereas the phosphotransferase and acetyltransferase activities did not differ between cells with and without the plasmid. Consequently, it was concluded that kanamycin resistance gene of pTB913 codes for kanamycin nucleotidyltransferase as pUB110 does.

Comparison of thermostability of the enzymes. Both plasmids pUB110 and pTB913 can be transferred into a thermophile, *B. stearothermophilus* CU21 (8, 9). As has already been mentioned, owing to the difference in the maximum temperature for the growth of transformants either harboring pUB110 or harboring pTB19, from which pTB913 was derived, kanamycin nucleotidyltransferase encoded by pTB913 was deemed to be more thermostable than that encoded by pUB110.

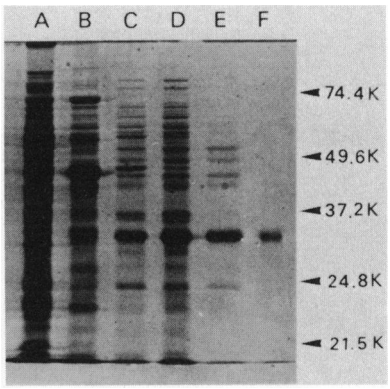

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel elctrophoresis pattern of kanamycin nucleotidyltransferase of various steps of purification. The enzyme was prepared from *B. subtilis* MI113 carrying pUB110 as described in the text. Lane A, crude extract; lane B, DEAE-Cellulofine column pooled fractions; lane C, DEAE-Toyopearl column pooled fractions; lane D, salting-out fractions by 70% ammonium sulfate; lane E, TSKgel 3000SWG column pooled fractions; lane F, YMC-PAC AP-303 column pooled fractions. Marker proteins used are trypsin inhibitor (molecular weight, 21,500) and cytochrome *c* oligomers (molecular weights 24,800 to 74,400).

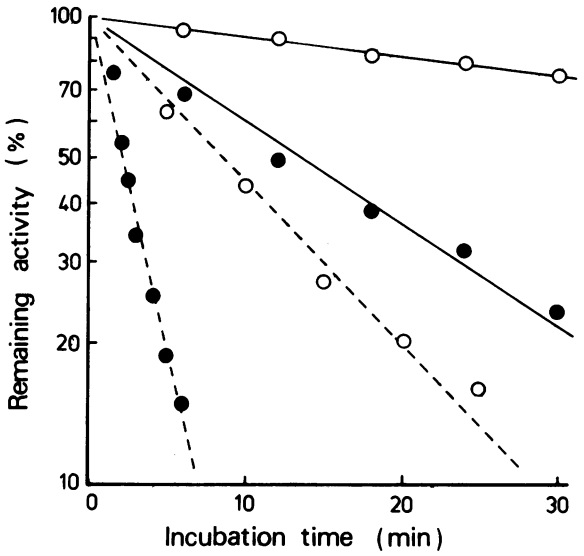

FIG. 3. Thermostability of kanamycin nucleotidyltransferase. Both enzymes from pUB110 (●) and pTB913 (○) purified up to the step of the gel filtration (Fig. 2, lane E) were used. Remaining activity after heating at 50°C (solid lines) or 55°C (broken lines) was expressed as the percent of original activity.

Before examination of the thermostability of kanamycin nucleotidyltransferase, the enzymes from both pUB110 and pTB913 were purified as described in above. Figure 2 shows the protocol of purification for the enzyme from pUB110. About 80-fold purification for the enzyme of pUB110 was attained with a yield of 20% after gel filtration (Fig. 2, lane E). Almost the same protocol was used for the enzyme from pTB913 (data not shown).

Small test tubes containing 100 μl of the enzyme solution (ca. 0.5 mg of protein per ml) in buffer N were incubated at either 50 or 55°C. At the various times (minutes) indicated, one of the test tubes was taken out and cooled quickly in ice water, followed by centrifugation (3,000 × *g*, 5 min). The remaining activity in the supernatant was assayed. The results shown in Fig. 3 justify the previous argument that kanamycin nucleotidyltransferase from pTB913 is more thermostable than that from pUB110.

Nucleotide sequence of kanamycin-resistance determinants. The strategy of the nucleotide sequencing for the *kan* gene region (ca. 1,200 base pairs [bp]) of both pUB110 and pTB913 is shown in Fig. 4. The endonuclease cleavage sites by *Sau*96I and *Fok*I are described additionally in the figure (also see Fig. 1). The sequencing was done on both strands, and all restriction sites were overlapped. The result of the nucleotide sequencing is shown in Fig. 5.

To correlate the DNA sequence data with the structure of kanamycin nucleotidyltransferase, the amino-terminal, amino acid sequence of the enzyme was determined through seven cycles of the Edman degradation procedure (1) by using the enzyme purified to homogeneity (Fig. 2, lane F for pUB110). The first seven amino acids were Met-Asn-Gly-Pro-Ile-Ile-Met for both enzymes (data not shown). The sequence of the seven amino acids completely matched that deduced from the nucleotide sequence only when GTG was taken as the initiation codon at position +1 (Fig. 5). The above result that GTG functioned as the initiation codon in this study is significant.

Starting from the GTG codon (nucleotide +1 to +3, methionine) and ending a TTT (+757 to +759, phenylalanine), the single open reading frame comprised 759 nucleotides (Fig. 5). The deduced amino acid sequence for the 253 amino acid residues of kanamycin nucleotidyltransferase from pUB110 is also given in Fig. 5. At 12 bases upstream from the GTG codon, there was a 11-base sequence AAAGGGAATGA (−16 to −6) which exhibits complementarity with 3′ end of *B. subtilis* 16S rRNA, HO-UCUUUC CUCCACUAG—(16, 19). The free energy of formation of the most stable, double-helical Shine-Dalgarno pairing (28) was calculated as −13.0 kcal/mol. A range of free energies (−11.6 to −21.0 kcal/mol) has been reported for the Shine-Dalgarno interactions present in initiation sites recognized by *B. subtilis* 16S rRNA (16). Accordingly, this 11-base sequence could serve as a ribosome binding site for the

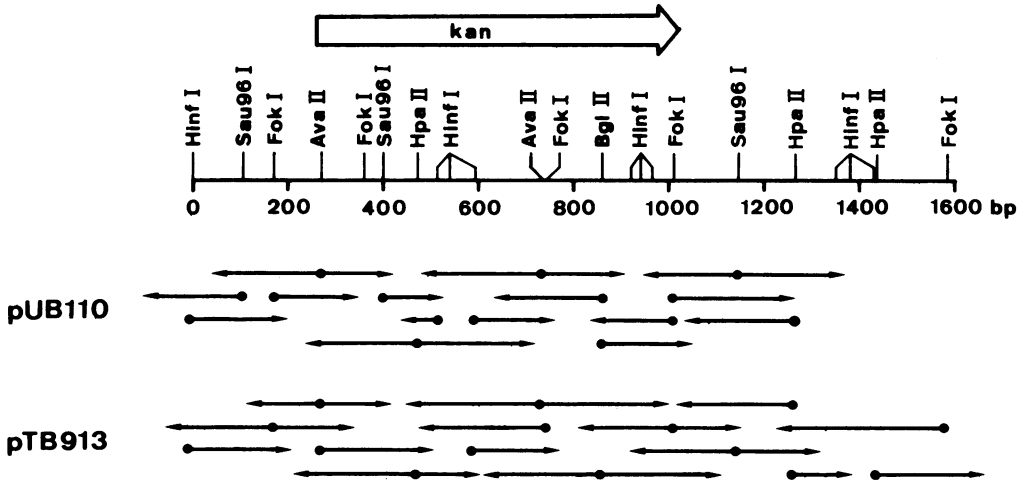

FIG. 4. Nucleotide sequencing strategy for the *kan* region of both pUB110 and pTB913. The location of the *kan* structural gene is shown by an open bar. Restriction endonuclease cleavage sites shown are for both plasmids. Sites of 5′ end labeling are indicated by closed circles, and arrows denote the direction of sequencing and correspond to length of the sequence determined. The scale is in bp as measured from the *Hin*fI recognition site on the left.

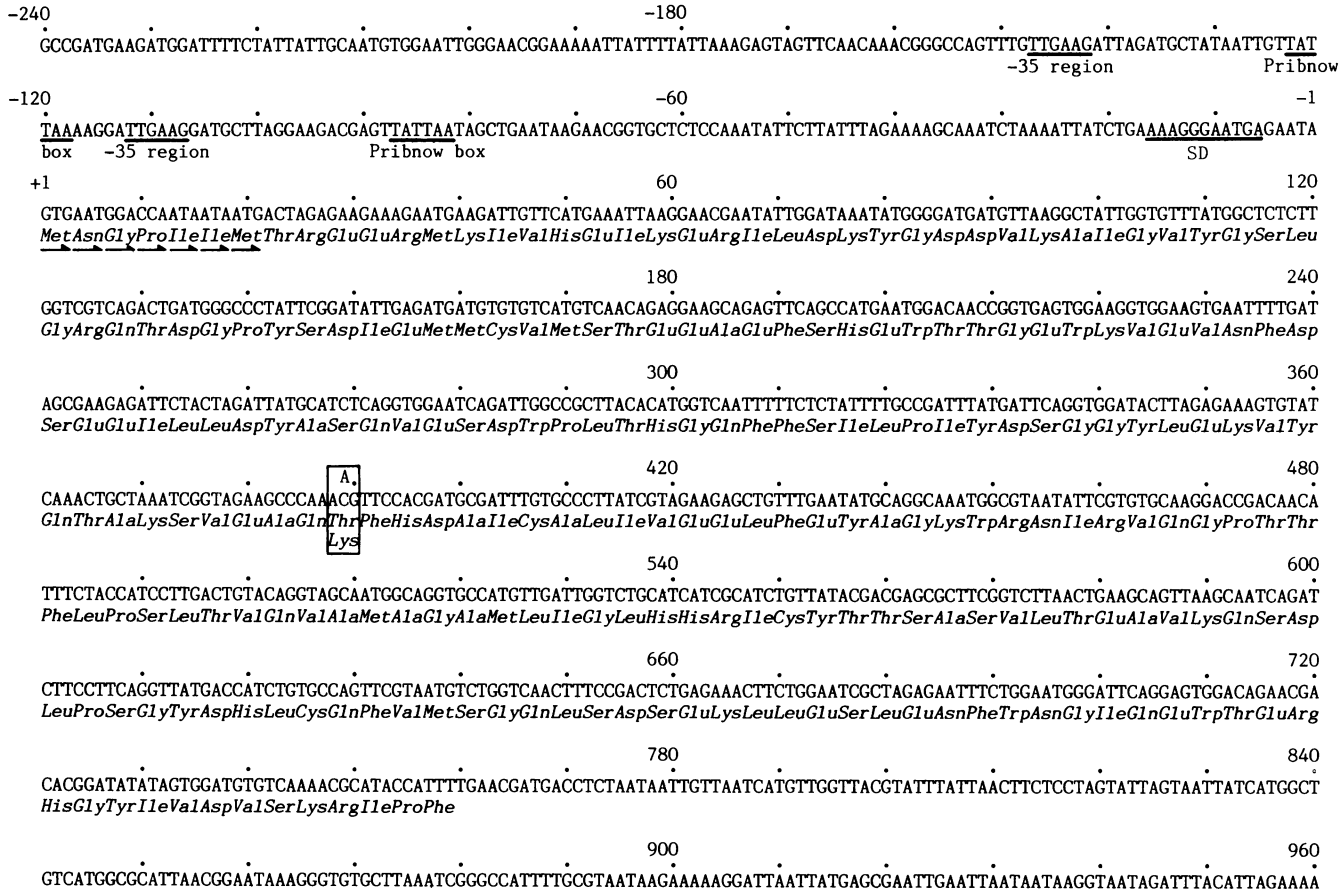

FIG. 5. Nucleotide sequence of kanamycin nucleotidyltransferase gene and flanking regions of pUB110. The nucleotide sequence shown is equivalent to the mRNA sequence except for the replacement of T with U. The nucleotide sequence is numbered from the first base of initiation codon GTG, and dots are positioned every 10 bp. Amino acid sequence is shown under the nucleotide sequence, and the amino-terminal amino acid sequence determined by the Edman method is indicated by arrows. The only difference in the bases (A, position +389), and the corresponding amino acid (Lys-130) for pTB913, is indicated by a box in the figure. A probable Shine-Dalgarno sequence (AAAGGGAATGA, −16 to −9) and two putative promoters (−35 region and Pribnow box) are underlined.

translation of kanamycin nucleotidyltransferase. Two putative promoters (−35 region and the Pribnow box) are shown in Fig. 5; one is TTGAAGATTAGATGCTATAATTGTTAT TAA (−147 to −118) and the other TTGAAGGATGCTTAG GAAGACGAGTTATTAA (−112 to −82). However, these two putative promoters were somewhat different from the consensus sequence for the TTGACA of the −35 region and the TATAAT of the Pribnow box in *B. subtilis* (19). The distance between the TTGAAG of the −35 region and the TATTAA of the Pribnow box was 18 or 19 bp; it appeared to be slightly longer than the consensus distance of 17 bp in *B. subtilis* (19).

It was confirmed that the whole nucleotide sequence (Fig. 5) was identical between both determinants of pUB110 and pTB913 except for only one base. Namely, cytosine at position +389 in the midst of the structural gene in pUB110 was replaced by adenine in pTB913 (Fig. 5), or a threonine at position 130 (Thr-130) for the pUB110-coded kanamycin nucleotidyltransferase was replaced by a lysine at the same position in the pTB913-coded enzyme. The results pointed out that a single, amino acid substitution could have caused the difference in thermostability between the two enzymes.

Amino acid analyses of enzymes. Amino acid compositions of both enzymes (Table 2) agreed with those assessed from the nucleotide sequence except for some differences in tryptophan, cystein, and isoleucine. The former two amino acids are known to be unstable during the hydrolysis, whereas the discrepancy with the latter could be explained by low recovery of the amino acid, owing to resistance against hydrolysis (26). The molecular weights of the enzymes from pUB110 and pTB913 were calculated from the nucleotide sequence as 28,796 and 28,823, respectively. These values are consistent with the molecular weight (about 30,000) estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). That the amino acid compositions and the molecular weights of the kanamycin nucleotidyltransferases agreed with those deduced from the respective nucleotide sequences may support the structure of the *kan* gene as determined in this work.

DISCUSSION

The source of plasmid pTB913 (Km^r, 2.9 Md) used in this work definitely differs from that of pUB110 (Km^r, 3.0 Md); i.e., the former is a derivative of the plasmid pTB19 (Km^r Tc^r, 17.2 Md) (9) which has been isolated from an antibiotic-resistant thermophilic bacillus in Japan (7), whereas the latter was isolated in England from a mesophile, *S. aureus*

TABLE 2. Amino acid composition of kanamycin nucleotidyltransferase from pUB110 and pTB913[a]

Amino acid	No. of residues in enzyme from:	
	pUB110	pTB913
Lys	11 (11.5)	12 (12.3)
His	8 (7.6)	8 (7.7)
Trp	6 (3.7)	6 (4.0)
Arg	9 (8.7)	9 (8.3)
Asp	14	14
Asn	5 (18.5)	5 (18.3)
Thr	15 (12.7)	14 (12.1)
Ser	20 (18.1)	20 (16.7)
Glu	25	25
Gln	11 (32.7)	11 (32.6)
Pro	8 (8.5)	8 (8.2)
Gly	18 (17.6)	18 (18.1)
Ala	13 (14.2)	13 (14.0)
Cys	4 (0.6)	4 (0.5)
Val	18 (16.6)	18 (16.3)
Met	9 (8.3)	9 (8.0)
Ile	18 (13.5)	18 (13.5)
Leu	20 (19.7)	20 (19.4)
Tyr	11 (9.8)	11 (10.0)
Phe	10 (9.4)	10 (9.7)

[a] Integers are values deduced from the nucleotide sequence, whereas decimals in parentheses are from the amino acid analysis.

(13). However, nucleotide sequences of the *kan* genes and its flanking regions (about 240 bp upstream from the initiation codon and about 200 bp downstream from the termination codon) of pUB110 and pTB913 were completely identical to each other except for only one base in the midst of the structural gene. This surprisingly identical nucleotide sequence of the two plasmids of different origins may stimulate further studies not only on the transfer of plasmids but also on the artificial enhancement of thermostability of an enzyme in question.

The nucleotide sequencing revealed that GUG was the initiation codon for the kanamycin nucleotidyltransferase encoded by both plasmids pUB110 and pTB913 for gram-positive bacteria. According to the recent review by Gold et al. (4) on translational initiation in procaryotes, 119 of 123 initiation sequences in *Escherichia coli* have AUG as the initiation codon, whereas the other 4 are GUG. In gram-positive bacteria, *spoVG* and *0.3 Kb* genes of *Bacillus* spp. (19), subtilisin genes of *Bacillus amyloliquefaciens* (30) and *B. subtilis* (31), and the *tox-228* gene of *Corynebacterium diphtheriae* (11) have GUG as the initiation codon. The finding that GUG functioned as the initiation codon of the *kan* gene in both pUB110 and pTB913 may be of significance for studying further the role of GUG, especially in gram-positive bacteria, because data on the initiation codon in procaryotes referred to earlier demonstrated a striking bias in favor of AUG despite nearly the same functionings of both AUG and GUG in binding experiments with fMet-tRNA in vitro (4).

A distinct enhancement of the thermostability of kanamycin nucleotidyltransferase was ascribed here to the replacement of Thr-130 by lysine among the 253 amino acid residues of the enzyme. Indeed, the observation that the subtle change of a single amino acid residue stabilized the enzyme deserved particular attention, even though the enzymes studied in this work were isolated from natural environments and were not subjected to any artificial mutagenesis. Conversely, if subjected to mutagenesis such that a single replacement in the amino acid sequence of a given enzyme occurs, it may be possible to create more thermostable enzymes (29).

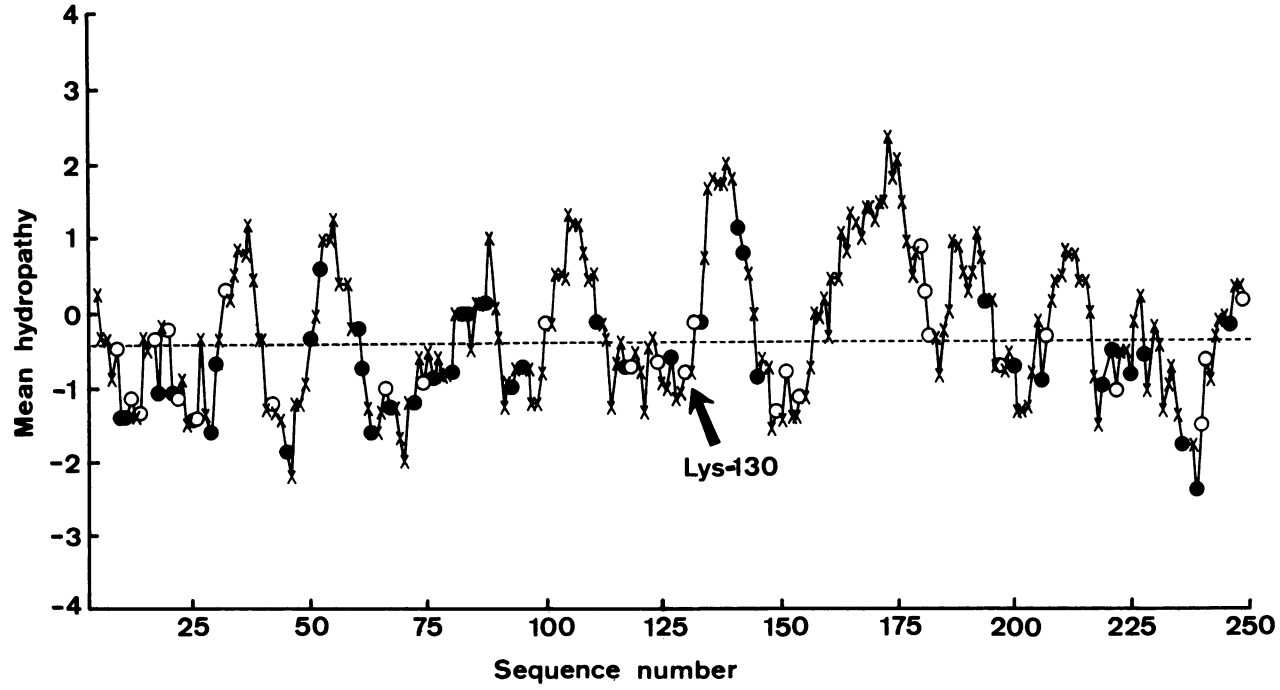

FIG. 6. Hydrophathy profile of kanamycin nucleotidyltransferase of pTB913. The mean hydropathy values of a moving segment of nine amino acid residues are plotted at the midpoint of each segment: X, uncharged amino acid; ○, positively charged amino acid; ●, negatively charged amino acid. The broken line parallel to the abscissa represents the grand average of hydropathy of the soluble proteins (12). The location of the substituted amino acid from Thr-130 (pUB110) to Lys-130 (pTB913) is shown by an arrow.

Since the net free energy of stabilization is small for a protein which rests on a delicate balance between stabilizing and destabilizing forces (5, 23), it may be inferred that thermostability of a protein can be affected even by a slight change in hydrophobic interactions, hydrogen bonds, or electrostatic interactions, etc., among the amino acid residues that constitute the protein. Although we have no concrete evidence to judge what kind of forces have contributed to the stabilization of this enzyme, we tried to interpret the enhanced thermostability solely on the basis of the change of net charge, since the lysine of pTB913 is a positively charged amino acid, whereas the threonine of pUB110 is an uncharged amino acid.

Figure 6 shows the hydropathic character of kanamycin nucleotidyltransferase from pTB913 as assessed by the method of Kyte and Doolitle (12), wherein the hydrophobicity and hydrophilicity of the enzyme are evaluated by a moving segment of nine amino acid residues along the sequence. The jutting portions above the broken line in the figure denote strongly hydrophobic segments and interior regions of the enzyme, whereas by contrast, those projected below the line designate strongly hydrophilic segments and the exterior. Lysine at position 130 (Lys-130) (pTB913) in Fig. 6 was located between the exterior and the interior regions (the protein surface). This means that the Lys-130 on the protein surface could acquire an additional and effective electrostatic interaction (salt bridge) with any neighboring negatively charged amino acid, such as glutamic acid and aspartic acid, without any significant change of three-dimensional protein structure. If the lysine were located at the exterior region of the protein, this positively charged amino acid would not form an effective salt bridge with another negatively charged one because of the existence of a large amount of water surrounding the amino acid.

It has been pointed out that enzymes of thermophilic bacteria often owe their extra stability to additional salt bridges (22, 23). For instance, the thermostability of *B. stearothermophilus* glyceraldehyde phosphate dehydrogenase was attributed to the salt bridges between the four subunits which the enzyme from the mesophile lacked (23). Further, the increased stabilities of ferredoxins from thermophilic bacteria such as *Clostridium thermosaccharolyticum* are reported to have arisen from additional salt bridges gained by replacements of a few amino acid residues (22). Hence, the effective salt bridge that would be formed by the Lys-130 (pTB913) in place of the Thr-130 (pUB110) on the protein surface would account for the difference in thermostability between these two kanamycin nucleotidyltransferases.

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