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Nucleotide Sequence of the Uricase Gene from *Bacillus* sp. TB-90[1]

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The nucleotide sequence of the uricase gene from the thermophilic bacterium *Bacillus* sp. TB-90 was determined. The primary structure of the uricase deduced from the nucleotide sequence comprised 332 amino acids, with a total molecular mass of 37,994 Da. The molecular mass of the subunit of the uricase produced by the transformant of *Escherichia coli* agreed well with this value. However, the molecular mass of a subunit of the uricase produced by *Bacillus* sp. TB-90 was found to be 34,000 Da by SDS-PAGE. The difference between these molecular masses was attributed to processing of the C-terminal 13 amino acid residue in *Bacillus* sp. TB-90. Comparison of the enzymatic properties of both uricases showed that the thermostability of the uricase produced by the transformant was enhanced by about 10°C in comparison to that produced by *Bacillus* sp. TB-90.

Key words: *Bacillus* sp., nucleotide sequence, processing C-terminus, thermostability, uricase.

Uricase (urate oxidase, EC 1.7.3.3) is an enzyme involved in the purine degradation pathway, where it catalyzes the oxidation of urate to allantoin, which exists in various living organisms such as animals, plants, fungi, yeasts, and bacteria. In primates, however, uricase would be present but not catalytically active, so that urate is secreted into the blood as the final degradation product of purine. Uric acid and its salts are relatively insoluble in water, easily precipitated, and the abnormal accumulation of uric acid is a causative factor of gout in humans. Determination of the concentration of uric acid in blood and urine is effective in the diagnosis of gout, and enzymatic determination by uricase is commonly used (*1-3*). Though several uricases from microorganisms are used in diagnostic reagents for determination of uric acid, these enzymes have low thermostability and a narrow range of pH in which they are active (*4, 5*). We thus investigated uricase activity among the thermophilic bacteria and *Bacillus* sp. TB-90 was found to produce uricase having good thermostability and high activity across the wide range of pH 6-9 (*6*).

We have already reported cloning and expression in *Escherichia coli* of the uricase gene from *Bacillus* sp. TB-90 (defined as *uao* gene), and development of a stable liquid reagent for determination of uric acid using recombinant enzyme (*7*). The present report describes the nucleotide sequence and deduced amino acid sequence of the *uao* gene. We also compare the enzymatic properties of uricase produced by *Bacillus* sp. TB-90 with that produced by a transformant of *E. coli*. This is the first report describing the nucleotide sequence of a procaryotic uricase.

[1] The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D49974.

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MATERIALS AND METHODS

Enzymes and Chemicals—Restriction endonucleases and other enzymes for DNA manipulation were provided by Toyobo (Osaka). [α-^{32}P]dCTP (3,000 Ci/mmol) was obtained from NEN research Products (USA). Carboxypeptidase A and B were obtained from Sigma (USA) and P from Takara Shuzo (Kyoto). All other chemicals used were of analytical grade.

Strains, Plasmid, and Culture Conditions—*Bacillus* sp. TB-90 and *E. coli* JM109 were used as donor strain of the gene and a host strain for cloning, respectively. Plasmid pUC18 was used as subcloning and expression vector. *E. coli* cells, which harbored the recombinant plasmid containing the *uao* gene, were grown in L-broth (1% polypeptone, 0.5% yeast extract, 1% NaCl, pH 7.4) containing 50 μg/ml of ampicillin and 0.2 mM IPTG (isopropyl-β-D-thiogalactoside) at 37°C for 24 h with shaking. *Bacillus* sp. TB-90 cells were grown in a urate medium (3% glucose, 1% yeast extract, 0.5% polypeptone, 4% uric acid, 1% potassium dihydrogenphosphate, 0.5% magnesium sulfate, and 0.5% soybean oil) at 55°C for 13 h with shaking.

Nucleotide Sequence of the uao Gene—Cloning of the *uao* gene was reported in a previous paper (*7*). The expression plasmid pUO6 was constructed by insertion of a 1.4-kb *Eco*RI-*Hin*cII DNA fragment including the *uao* gene into plasmid pUC18. A series of deletion mutants were prepared using an Exo/Mung Deletion Kit (Stratagene). Each DNA fragment was sequenced by the dideoxy-chain termination method using T7 DNA polymerase (7-deaza sequencing kit, Toyobo) (*8*). The sequences obtained were analyzed with the GENETYX program (Software Development, Tokyo).

Purification of Uricase—*Bacillus* sp. TB-90 cells grown in urate medium and *E. coli* JM109 harboring pUO6 cells

grown in L-broth containing 50 μg/ml ampicillin and 0.2 mM IPTG were used for the purification of uricase. All purification steps were done according to the previous paper (*7*).

Determination of N- and C-Terminal Amino Acid Sequences—The N- and C-terminal amino acid sequences of purified uricase produced by *Bacillus* sp. TB-90 and *E. coli* JM109 harboring pUO6 were analyzed. The N-terminal amino acid sequence was determined by Edman degradation using a gas-phase sequencer 470A (Applied Biosystems, USA). The C-terminal amino acid sequence was determined by degrading uricase protein in 0.1 M pyridine-collidine-acetate (pH 8.2) and 0.1 M pyridine-acetate (pH 5.5) using carboxypeptidase A, B, or P. The degradation product was sampled in time sequence and analyzed with an amino acid analyzer (Beckman system 6300, USA).

Amino Acid and Metal Analysis—A purified uricase produced by *Bacillus* sp. TB-90 was hydrolyzed using a 2 : 1 (v/v) mixture of hydrochloric acid/trifluoroacetic acid, containing 0.005% (v/v) phenol in an evacuated and sealed tube at 166°C for 25 or 50 min (*9*). The hydrolyzate was then analyzed with a Beckman system 6300 amino acid analyzer. Copper content of uricase produced by *Bacillus* sp. TB-90 and *E. coli* JM109 harboring pUO6 was measured using a Shimazu AA-640-12 atomic absorption spectrophotometer.

Uricase Assay—Uricase was assayed by following the disappearance of uric acid, detected by a decrease in absorbance at 290 nm in the presence of enzyme. The assay

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   1 GAATTCATCATGGGTGGCCGAACGAGCAGAACCCAATCGCCCATACTCTTCCTTTCAATC   60
  61 TCTATATAATAAAATGGTGGAGATTGTCGAAACCGCTTCGGATAACGAACAATTAAAATT  120
 121 AATTCAAATGCATCCGCATCTAGGCACCAACGTCAAAATAACCGATTTCTCTCAAGAGGA  180
 181 ACAGAAACATGCCGGATTAAATGAATTAACAAAAGATGAACAGAATCATCTCATTTTACT  240
 241 CAACCAAAAATACAAGGATAAATTTGGATTTCCATTTGTAATGGCCGTTCGAGGAAAAAT  300
 301 CAAACAAGAAATTTTTAGAACGATCAAAGAACGATTACAAAATAATCATCAAACGGAGTT  360
 361 TAAACAAGCATTGGAAGAAATTAAAAAAATCGCAATGTTTCGTTTGCAAGAAATATTTCG  420
 421 TGAAGGAGAGAATAATTCGATGACCAAACACAAAGAAAGAGTGATGTATTATGGAAAAGG  480
   1                   M  T  K  H  K  E  R  V  M  Y  Y  G  K  G   14
 481 TGACGTATTTGCTTATCGCACCTATTTAAAACCACTTACTGGAGTTAGAACGATTCCTGA  540
  15  D  V  F  A  Y  R  T  Y  L  K  P  L  T  G  V  R  T  I  P  E   34
 541 ATCTCCATTTTCCGGTCGAGATCATATTCTTTTTGGAGTAAATGTAAAAATCTCAGTAGG  600
  35  S  P  F  S  G  R  D  H  I  L  F  G  V  N  V  K  I  S  V  G   54
 601 AGGAACAAAATTGCTGACCTCCTTTACGAAAGGGGATAACAGCTTAGTCGTTGCAACAGA  660
  55  G  T  K  L  L  T  S  F  T  K  G  D  N  S  L  V  V  A  T  D   74
 661 CTCGATGAAAAACTTTATACAAAAACATTTAGCTAGTTATACAGGAACAACGATAGAAGG  720
  75  S  M  K  N  F  I  Q  K  H  L  A  S  Y  T  G  T  T  I  E  G   94
 721 TTTTTTAGAATATGTAGCTACTTCTTTTTTGAAGAAATATTCTCATATTGAAAAGATTTC  780
  95  F  L  E  Y  V  A  T  S  F  L  K  K  Y  S  H  I  E  K  I  S  114
 781 GTTGATAGGAGAGGAAATTCCCTTTGAAACAACTTTTGCAGTAAAGAATGGAAATAGAGC  840
 115  L  I  G  E  E  I  P  F  E  T  T  F  A  V  K  N  G  N  R  A  134
 841 AGCTAGTGAGCTAGTATTTAAAAAATCACGAAATGAATATGCCACCGCTTATTTGAATAT  900
 135  A  S  E  L  V  F  K  K  S  R  N  E  Y  A  T  A  Y  L  N  M  154
 901 GGTTCGTAATGAAGATAACACCCTAAACATTACTGAACAACAAAGCGGACTTGCTGGTCT  960
 155  V  R  N  E  D  N  T  L  N  I  T  E  Q  Q  S  G  L  A  G  L  174
 961 TCAATTAATAAAAGTCAGCGGAAATTCCTTTGTCGGTTTTATTCGTGACGAATACACAAC 1020
 175  Q  L  I  K  V  S  G  N  S  F  V  G  F  I  R  D  E  Y  T  T  194
1021 TCTTCCAGAGGATTCAAACCGCCCTCTATTTGTTTACTTAAACATCAAATGGAAGTACAA 1080
 195  L  P  E  D  S  N  R  P  L  F  V  Y  L  N  I  K  W  K  Y  K  214
1081 AAACACGGAAGACTCATTTGGAACGAATCCAGAAAATTATGTTGCAGCTGAACAAATTCG 1140
 215  N  T  E  D  S  F  G  T  N  P  E  N  Y  V  A  A  E  Q  I  R  234
1141 CGACATCGCCACTTCCGTATTTCATGAAACCGAGACGCTTTCCATCCAACATTTAATTTA 1200
 235  D  I  A  T  S  V  F  H  E  T  E  T  L  S  I  Q  H  L  I  Y  254
1201 TTTAATCGGCCGCAGAATATTAGAAAGATTCCCTCAACTTCAAGAAGTTTACTTCGAATC 1260
 255  L  I  G  R  R  I  L  E  R  F  P  Q  L  Q  E  V  Y  F  E  S  274
1261 TCAAAATCATACATGGGATAAAATAGTGGAGGAAATTCCTGAATCAGAAGGGAAAGTATA 1320
 275  Q  N  H  T  W  D  K  I  V  E  E  I  P  E  S  E  G  K  V  Y  294
1321 TACAGAACCGCGACCGCCATATGGATTTCAATGCTTTACTGTCACCCAAGAAGACTTGCC 1380
 295  T  E  P  R  P  P  Y  G  F  Q  C  F  T  V  T  Q  E  D  L  P  314
1381 ACACGAAAACATTCTTATGTTCTCTGATGAACCCGATCATAAAGGAGCACTTAAATGATC 1440
 315  H  E  N  I  L  M  F  S  D  E  P  D  H  K  G  A  L  K        332
1441 GGGTTAAC                                                     1448
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Fig. 1. **Nucleotide sequence and the deduced amino acid sequence of the 1.4-kb region containing *uao* gene.** The putative Shine-Dalgarno sequence is boxed. The proposed promoter consensus sequences are doubly underlined. The N-terminal sequence determined by Edman degradation of uricase from *Bacillus* sp. TB-90 and *E. coli* transformant are underlined with a thin line. The C-terminal sequence determined by the digestion of uricase from *Bacillus* sp. TB-90 and *E. coli* transformant with carboxypeptidases are indicated with a dashed line and double dashed line, respectively.

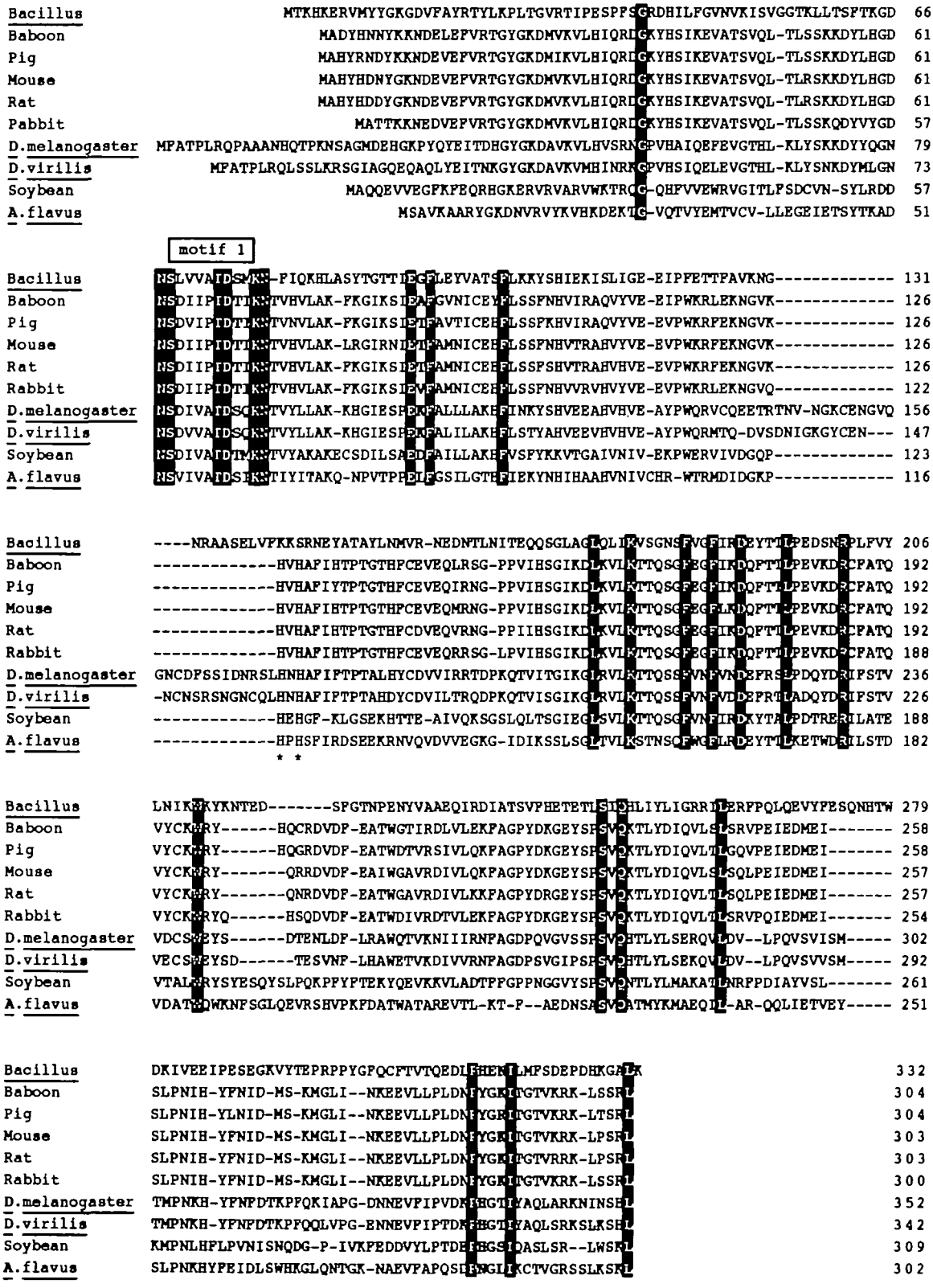

Fig. 2. **Comparison of deduced amino acid sequence of *Bacillus* sp. TB-90 uricase with sequences of other uricases.** The deduced amino acid sequences of uricase from *Bacillus* (this paper), baboon, pig, mouse (*14*), rat (*15*), rabbit (*16*), *Drosophila melanogaster* (*18*), *Drosophila virilis* (EMBL Accession X57114), soybean (*17*), and *Aspergillus flavus* (*19*) are aligned. Dashes indicate gaps introduced into the sequences so that the maximum homology may be obtained. Identical amino acid in all enzymes are shown with white-on-black letters. Motif 1 indicates a highly conserved region between procaryotic and eucaryotic enzymes. Asterisks indicate copper-binding histidine residues of eucaryotic enzymes.

mixture contained 0.5 ml of enzyme solution (50 mM borate buffer containing 0.001% Triton X-100 and 1.0 mM EDTA, pH 8.0) and 40 μM uric acid in a final volume of 3.0 ml. One unit is defined as the amount of enzyme which transforms 1 μmol of uric acid into allantoin in 1 min at 37°C and pH 8.0. Protein was estimated by the method of Lowry *et al.* (*10*) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Nucleotide Sequence of the uao Gene—The nucleotide sequence of the 1.4-kb *Eco*RI-*Hin*cII DNA fragment including the *uao* gene was identified. The determined nucleotide sequence and deduced amino acid sequence are shown in Fig. 1. An open reading frame comprised of 996 bases encoding 332 amino acids was identified. The six-base sequence AAGGAG was found at a site 11 bp upstream from the ATG initiation codon of the *uao*, which was complementary to the HO-UCUUUCCUCCACUAG sequence of 16S rRNA from *Bacillus subtilis*. This six-base sequence was considered to be the ribosome-binding site of the *uao* gene. Possible promoter sequences (TTTAAA in −35 region and TAAAAA in −10 region) are shown in Fig. 1. These promoter sequences are similar to various promoter sequences obtained from *Bacillus stearothermophilus*, and especially resemble the promoter sequence in the tetracycline-resistant gene (*11*). The 17 bp between the −35 and −10 regions is in accordance with the concensus distance between −35 and −10 in *B. subtilis* (*12*). However, these sequences differ from concensus sequences recognized by the σ^A-RNA polymerase of *B. subtilis* (*13*). It is considered that the different form of the promoter sequence was utilized poorly by σ^A-RNA polymerase and caused low expression of uricase in *Bacillus* sp. TB-90.

Comparison of Amino Acid Sequences of Several Uricases—The amino acid sequence of uricase from *Bacillus* sp. TB-90 is compared with those of uricases from other sources in Fig. 2. Uricase from *Bacillus* sp. TB-90 shares 24-28% identical residues with mammalian enzymes (*14-16*), 24% with the soybean uricase (*17*), 29% with the *Drosophila* enzymes (*18*), and 26% with the *Aspergillus* uricase (*19*). Bairoch and Legoux identified consensus sequences of uricase from eucaryotes (*19, 20*). The sequences Val-Leu-Lys/Thr-Thr-Asn/Gln-Ser and Ser-Pro/Ala-Ser-Val-Gln-*X*-Thr-Leu/Met-Tyr are found in all uricase sequences published so far. In uricase from *Bacillus* sp. TB-90, the Leu-Ile-Lys-Val-Ser-Gly-Asn (176-182 in Fig. 2) sequence and the Thr-Leu-Ser-Ile-Gln-His-Leu-Ile-Tyr (246-254 in Fig. 2) sequence correspond to this conserved sequence, but high homology was not recognized. Moreover uricase is known as a copper-binding enzyme, and a copper-binding site (Fig. 2) is found in every uricase of eucaryotic origin (*14*). However, the His-*X*-His copper-binding sequence is absent in the amino acid sequence of uricase from *Bacillus* sp. TB-90. In fact, copper was not detected in metal analyses of uricases produced by *Bacillus* sp. TB-90 and *E. coli* JM109 harboring pUO6. These results indicate that the uricase from *Bacillus* sp. TB-90 differs structurally from eucaryotic uricases. On the other hand, comparison of the amino acid sequence of uricase from *Bacillus* sp. TB-90 with that from eucaryotes revealed the sequence Asn-Ser-*X*-Val/Ile-Val/Ile-Ala/Pro-Thr-Asp-Ser/Thr-*X*-Lys-Asn (motif 1 in Fig. 2) to be a highly conserved region. This sequence is the commonly conserved sequence in both procaryotes and eucaryotes, and is considered to be concerned with the expression of uricase activity.

The Primary Structure of Uricase—The N- and C-terminal amino acid sequences produced by *Bacillus* sp. TB-90 and *E. coli* JM109 harboring pUO6 were analyzed. N-terminal amino acid sequences of both uricases were entirely identical to that deduced from the nucleotide sequence from threonine, position 2, to valine, position 29. N-terminal methionines in both uricases were eliminated. The C-terminal amino acid sequence of uricase produced by the transformant was determined to be -Gly-Ala-Leu-Lys. This four-amino-acid sequence was identical to the C-terminal amino acid sequence deduced from the nucleotide sequence. The C-terminal amino acid sequence of uricase produced by *Bacillus* sp. TB-90, however, was determined to be -Glu-Asn-Ile-Leu. This four-amino-acid sequence was identical to positions 316-319 of the deduced amino acid sequence. So, the C-terminal 13 amino acids must have been cleaved from the mature enzyme produced by *Bacillus* sp. TB-90. Consequently, the molecular masses of the subunit produced by the transformant and *Bacillus* sp. TB-90 were calculated to be 37,863 and 36,406 Da, respectively. These values agrees well with the molecular weight determined by SDS-PAGE as reported previously (7). The result of amino acid composition analysis of the uricase produced by *Bacillus* sp. TB-90 and the amino acid composition deduced from the nucleotide sequence are shown in Table I. The amino acid composition of 318 residues estimated from the result of C-terminal analysis of uricase produced by *Bacillus* sp. TB-90 agreed well with the results of amino acid composition analysis. These results strongly suggest that in uricase produced by *Bacillus* sp. TB-90, a 332-residue polypeptide, is translated according to the nucleotide sequence followed by processing of N-terminal

TABLE I. **Amino acid composition of uricase from *Bacillus* sp. TB-90.**

Amino acid	Observed[a]	Deduced
Asp	30 (Asx)	13[b] (11)[c]
Asn		18 (18)
Thr	25	28 (28)
Ser	20	22 (21)
Glu	42 (Glx)	30 (29)
Gln		11 (11)
Gly	23	21 (20)
Ala	15	14 (13)
Val	21	21 (21)
Cys	3	1 (1)
Met	3	4 (3)
Ile	20	22 (22)
Leu	25	27 (26)
Tyr	14	17 (17)
Phe	18	20 (19)
Lys	20	23 (21)
His	7	9 (8)
Arg	14	14 (14)
Pro	15	14 (13)
Trp	—	2 (2)
Total	315	331 (318)

[a]Uricase produced by *Bacillus* sp. TB-90 was analyzed. Observed values were recalculated with the subunit molecular weight (34,000). [b]Values were deduced from the nucleotide sequence. [c]Values were derived from the cleavage of 13 residues from the C-terminus.

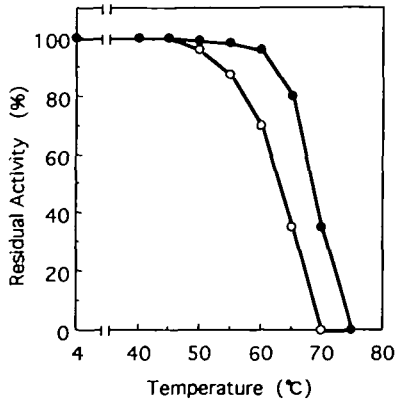

Fig. 3. **Thermostabilities of the uricase purified from *E. coli* transformant (●) and *Bacillus* sp. TB-90 (○).** The enzyme (about 5 U of uricase activity per ml in 50 mM borate buffer, pH 8.0) was treated for 10 min at various temperatures, then the residual activity was determined. The residual activity at 4°C was defined as 100%.

methionine and the C-terminal 13-residue sequence.

Comparison of Enzymatic Properties—Enzymatic properties of uricases produced by *Bacillus* sp. TB-90 and *E. coli* JM109 harboring pUO6 were compared. Several enzymatic properties (Michaelis constant, optimum pH, optimum temperature, pH stability) of uricase produced by the transformant were identical with those of the enzyme produced by *Bacillus* sp. TB-90. However, the thermostability of uricase produced by the transformant was promoted by about 10°C over that produced by *Bacillus* sp. TB-90 (Fig. 3). This result is attributed to the fact that uricase produced by the transformant was not subject to processing of the C-terminal 13 amino acids, which are responsible for the thermostability of uricase. Yashigi *et al.* described that a β-amylase lacking the C-terminus had decreased thermostability compared to the original enzyme (*21*). Their report may support our hypothesis that the change in thermostability depends on processing of C-terminal amino acids. Furthermore, it is of interest to know whether this C-terminal 13 amino acid sequence would increase the thermostability of another enzyme. Further research on the addition of the C-terminal 13 amino acids to another enzyme is in progress.

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