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Cloning, sequence analysis and expression in *Escherichia coli* of the gene encoding a uricase from the yeast-like symbiont of the brown planthopper, *Nilaparvata lugens*

Yuichi Hongoh *, Tetsuhiko Sasaki, Hajime Ishikawa

Department of Biological Sciences, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

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Abstract

A urate oxidase (uricase; EC 1.7.3.3) gene of the yeast-like fungal endosymbiont of the brown planthopper, *Nilaparvata lugens*, was cloned, and sequenced together with its flanking regions. The gene comprised a open reading frame of 987 bp, that was split into two parts by a single 96 bp intron. The encoded uricase was 296 amino acids with 62% sequence identity with that of *Aspergillus flavus*. The molecular weight deduced was 32,882, and the predicted isoelectric point was 6.06. The symbiont's uricase conserved all the known consensus motifs, except the C-terminal PTS-1, Ser–basic–Leu. The leucine at the third position of PTS-1 was replaced by serine in the C-terminus of the symbiont's uricase. The symbiont's uricase gene was successfully expressed in *Escherichia coli*, and the product, tagged with histidine residues, was purified. The symbiont's uricase, thus produced, was as active as those from plants and animals, but less active than those from other fungi.

Keywords: *Nilaparvata lugens*; Planthopper; Yeast-like symbiont; Fungi; Symbiosis; Uric acid; Urate oxidase; Uricase; PTS

1. Introduction

Most organisms produce uric acid in the process of purine breakdown. Microorganisms such as bacteria and yeasts further catalyze the oxidation of uric acid to allantoin and, successively, to allantoic acid, urea and ammonia. In animals, however, the chain of reactions became shorter progressively in the course of evolution, losing some uricolytic enzymes. Birds, reptiles and hominoids including humans are devoid of uricase (urate oxidase; EC 1.7.3.3), and excrete uric acid as the final product of purine degradation (Keilin, 1959). Many insects also lack uricase and excrete uric acid, while some species, such as *Drosophila*, exhibit uricolytic activity in certain periods of their life cycles and excrete allantoin, allantoic acid, urea or ammonia (Bursell, 1967; Friedman and Johnson, 1977; Wallrath and Friedman, 1991). Homopterans do not excrete a detectable amount of uric acid or other uricolytic products, but various kinds of amino acids (Bursell, 1967; Klingauf, 1987; Sasaki et al. 1990, 1996). In aphids, no uricolytic products were detected in honeydew, their excreta or in the whole body (Mittler, 1958; Lamb, 1959; Sasaki et al., 1990). In the brown planthopper, *Nilaparvata lugens*, a considerable amount of uric acid was detected in the whole body, but not in honeydew (Sasaki et al., 1996; Hongoh and Ishikawa, 1997).

Planthoppers normally harbor yeast-like, intracellular symbionts (Buchner, 1965). Our previous studies suggested that these symbionts play a pivotal role in nitrogen metabolism in the brown planthopper (Sasaki et al., 1996; Hongoh and Ishikawa, 1997). Planthoppers feeding on a synthetic diet showed an accumulation of uric acid, without its excretion, in proportion to the concentration of amino acids in the diet. The accumulated uric acid was mobilized by the symbiotic planthoppers, but not by the heat-treated or aposymbiotic insects. When the amino acid concentration in the diet was lowered, the symbiotic insects retained their growth rate to some degree and exhibited a sharp decline of their uric acid content. In contrast, in the aposymbiotic insects, no decline of the uric acid content was observed when the

* Corresponding author. Fax: +81-3-5841-4448.
E-mail address: ss67243@hongo.ecc.u-tokyo.ac.jp (Y. Hongoh)

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amino acid concentration was lowered. These insects never reached adulthood regardless of the amino acid concentration in the diet (Hongoh and Ishikawa, 1997). In addition, uricase activity was detected in the symbiotic insects and the isolated symbionts, but not in the aposymbiotic insects (Sasaki et al., 1996). All these results together suggest that planthoppers themselves are devoid of uricase, and that the uricase of the symbionts plays a key role in the host's utilization of the stored uric acid and is essential for the host to grow normally.

The intracellular symbionts of planthoppers were shown to belong to the class Pyrenomycetes in the subphylum Ascomycotina by molecular phylogenetic analysis based on partial 18S rDNA sequences (Noda et al., 1995). It has been reported that many fungi assimilate uric acid and that some species, including *Neurospora crassa* and many yeasts, can grow even in media containing uric acid as a sole nitrogen source (LaRue and Spencer, 1968; Reinert and Marzluf, 1975; Watanabe et al., 1973; Middelhoven et al. 1983, 1985; Mahmoud and Fallar, 1996). It is probable that one of these uricolytic fungi had become symbiotic with an ancestor of planthoppers and supplied them with an ability to recycle uric acid as a storable nitrogen source in the long evolutionary history. These intracellular symbionts now appear to be transmitted only vertically through ovaries and proliferate only by asexual budding, lacking teleomorph (Buchner, 1965; Noda, 1977; Chen et al., 1981). It is interesting to characterize the uricase of these symbionts because it not only provides basic information to elucide the uric-acid-mediated nitrogen metabolism in the planthopper at a molecular level, but also information concerning evolutionary changes of the fungal uricase caused by the symbiosis with insects.

We here report the genic and cDNA sequences of the uricase gene of the yeast-like symbiont of the brown planthopper and its successful expression in *Escherichia coli*.

2. Materials and methods

2.1. Insects and yeast-like symbionts

Brown planthoppers, *N. lugens*, were maintained on rice seedlings at 25°C under a 16 h light:8 h dark photoperiodic regime.

The yeast-like symbionts were isolated from adult insects according to the method we described before (Sasaki et al., 1996). The insects were homogenized in 50 mM Tris–HCl buffer (pH 7.5) containing 250 mM sucrose. The homogenate was filtered through nylon mesh with a pore size of 90 μm. Percoll (Pharmacia) was added to the filtrate to give a final concentration of 30%, and the filtrate centrifuged at 1000***g*** for 20 min. The pellet was suspended in 50 mM Tris–HCl buffer (pH 7.5) containing 250 mM sucrose and 75% Percoll, and centrifuged at 100,000***g*** for 20 min. The symbionts were collected from the 65–85% region of Percoll gradient.

2.2. DNA preparation

DNA extraction from the yeast-like symbionts was carried out based on the method of DNA extraction from yeast cells (Woolford et al., 1979). The isolated symbionts were rinsed in 50 mM Tris–HCl buffer (pH 7.5), and suspended in 700 μl of 0.1 M potassium phosphate buffer (pH 6.8) containing 1 M sorbitol and 0.1 M ethylene diamine triacetic acid (EDTA). 1.5 μl 2-Mercaptoethanol and 20 μl of 2 mg/ml Zymolyase-100T (Seikagaku) were added, and the mixture was incubated at 37°C for 90 min. The resulting spheroplasts were collected by centrifugation. The pellet was suspended in 500 μl of 50 mM Tris–HCl buffer (pH 7.5) containing 20 mM EDTA, 0.1% sodium dodecyl sulfate (SDS) and 100 μg/ml proteinase K (Merck), and incubated at 55°C for 2 h. The lysate was processed according to the standard protocol for DNA purification including phenol extraction, phenol/chloroform extraction and precipitation in isopropyl alcohol. After treating with 5 μg/ml RNase A at 37°C for 1 h, the ethanol-precipitated DNA was suspended in TE buffer and stored at −20°C.

2.3. RNA preparation

The isolated symbionts were suspended in 400 μl of TRIZOL solution (Gibco). The suspension was dropped into aqueous nitrogen and ground vigorously into fine powder. The powder was processed according to the TRIZOL manual. The purified total RNA was stored at −80°C.

2.4. Cloning of the uricase gene

Purified DNA of the symbionts was digested completely with one of the restriction enzymes, *Bam*HI, *Hin*dIII, *Xba*I and *Xho*I (Takara). The digested DNA was subjected to electrophoresis on 0.7% GTG–Agarose (FMC), and transferred on to Hybond N^+ (Amersham). The transferred DNA was hybridized with a polymerase chain reaction (PCR) product from the uricase gene of *Aspergillus flavus* (IAM culture collection; IAM13835) as a probe. The PCR for producing the probe was performed with the primers AF1 and AF2 (Table 1). The template DNA was prepared from mycelia of *A. flavus* according to the DNA extracting method described by Biel and Parrish (1986). The 629 bp region flanked by two introns was amplified by PCR, and the products were labeled with ^{32}P-deoxycytidine 5′-triphosphate (dCTP), using the Bca-BEST DNA Labelling Kit (Takara). The hybridization was performed according to Church and Gilbert (1984). After hybridization overnight

Table 1
The primers used in this study for PCR and reverse transcriptase reaction

AF1[a]	5′-TACACCAAGGCCGACAACAGC-3′
AF2[a]	5′-GTTAGGCAACGAGTACTCGAC-3′
R1	5′-AGATGACCATAAGCTGCCTCAT-3′
R2	5′-GCTCAAAATTGTGCTTGTTAGGCA-3′
AP	5′-GGCCACGCGTCGACTAGTAC-3′
UO55	5′-GTCGAGCAGAGCGCTGCTAAG-3′
3R2	5′-TCGCTGTATTACTCTCGAGCTG-3′
UOPRT	5′-CCGACAATGGAACT-3′
5R2a	5′-GGCGACGTGAATATGGCTGTA-3′
5R2b	5′-CGCATGGACGTTGACGGGAA-3′
5R3a	5′-TGATGATGGAAGCGAAGAGCTC-3′
5R3b	5′-CCCACAGCTTCATCAAAGACG-3′
UOint	5′-GCTGTAGCCATATGCCGTACGTCTCTGTCG-3′
UOterm	5′-GCTGTAGCCATATGCTAAGAGCGAGATACTTCGCA-3′

[a] The sequence of *A. flavus* uricase was obtained from Legoux et al. (1992).

at 60°C, the membrane was rinsed with 40 mM sodium phosphate buffer (pH 7.2) containing 1% SDS at 65°C for 20 min. Signals were detected and analyzed by a MacBAS-2500 image analyzer (Fuji Film).

The 7–9 kb *Hind*III fragments corresponding to positive signals were cut out from the agarose gel, and ligated to *Hind*III-digested pBluescript II SK+ with the DNA ligation Kit version 2 (Takara), to transform *E. coli*, DH5α. About 1000 clones were plated and transferred on to Hybond N (Amersham). The transferred DNA was hybridized with the AF1–AF2 probe, and positive clones were isolated.

2.5. Cloning of the uricase cDNA

The reverse transcriptase reaction was performed with an oligo(dT)-polylinked adapter primer and SUPERSCRIPT II (Gibco), according to the manufacturer's instructions. An aliquot of the reaction mixture was subjected to PCR with the specific primers R1 and R2. The products were sequenced directly with R1 and R2 primers.

For analysis of the 3′ end region of cDNA, nested PCR was performed with the adapter primer AP and the specific primers UO55 and 3R2. The products were purified with agarose gel electrophoresis and GENECLEAN III (BIO 101), and cloned into pCR2.1 of the Original TA Cloning Kit (Invitrogen). The clones were sequenced.

The 5′-Full RACE Core Set (Takara) was used for analysis of the 5′ end region of cDNA, according to its protocol. The reverse transcriptase reaction was performed with the 5′-phosphorylated primer UOPRT, and the products were circularized or concatemerized with T4 RNA ligase. These products were used as templates for nested PCR with the two specific primer sets, 5R2a and 5R2b, and 5R3a and 5R3b. The products were purified and sequenced as above.

All the PCRs in this study were performed with *EX-Taq* polymerase (Takara). The sequences of the primers and their location on the uricase gene are shown in Table 1Fig. 1, respectively.

2.6. DNA sequencing and analysis

The clones were sequenced with an ABI PRISM 310 auto-sequencer. Samples were prepared with the DNA Cycle Sequencing Kit using Big-Dye Terminator (Perkin Elmer). The subcloned plasmids as templates were prepared in an Automatic DNA Isolation System (Kurabo). For direct sequencing, PCR products were purified with the Spin Column S-300HR (Pharmacia) for templates.

DNASIS-MAC V3.7 (Hitachi) was used for analysis of sequences except multi-alignment of amino acid sequences, which was performed by GENETYX-MAC V8 (Software Development).

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number, AB027293.

2.7. Detection of the uricase expression in the symbionts

Total RNA purified from the isolated symbionts was electrophoresed on 1.0% GTG–Agarose (FMC) under the denaturing conditions, and transferred on to Hybond N^+ (Amersham). The transferred RNA was hybridized with a probe prepared from a PCR product from the uricase gene of the symbionts. PCR was performed with

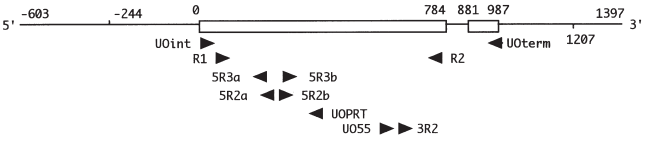

Fig. 1. Location of the primers used in this study. Open boxes represent the two exons comprising the uricase gene of the symbiont.

the primers 5R2b and R2 (Table 1 and Fig. 1). The resulting 461 bp products were labeled with ^{32}P-dCTP as above. Hybridization and analysis were also performed as above.

2.8. Expression of the uricase gene in E. coli

PET system (Novagen) was used to express the uricase gene of the symbiont. The entire coding region of the uricase cDNA was amplified by PCR with the primers UOint and UOterm that contained the *Nde*I site (Table 1). The PCR products were digested with *Nde*I and inserted into the *Nde*I site of the expression vector pET16b, which inserts 10 histidine residues to the amino terminus of a target protein, to construct pETUO1 (Fig. 2). The protease-deficient *E. coli* strain pLysS, which expresses T7 lysozyme, was transformed with pETUO1. The transformation and the induction of expression of the His-tagged uricase by adding isopropylthio-β-D-galactoside (IPTG) were carried out basically according to the PET system manual (Novagen). The expression was done at 25°C for 8 h, and the cells collected were stored at −80°C.

2.9. Purification of the uricase expressed in E. coli

Cells from 50 ml culture, stored at −80°C, were thawed at room temperature for 10 min. The cells, lyzed by the intragenic lysozyme, were suspended in 1.2 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 15 mM imidazole, 15 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1% Triton X-100 and 10% glycerol, and sonicated on ice for 1 min, at output level '1' and 0.5 s interval (Branson, SONIFIER 250). The lysate was centrifuged at 15,000***g*** at 4°C for 30 min, and the supernatant was collected for purification. The supernatant was loaded on to two Ni-NTA Spin Columns (Qiagen), and purification was performed based on the manufacturer's manual. Columns were washed with 50 mM sodium phosphate buffers (pH 8.0) containing 300 mM NaCl, 10 mM 2-mercaptoethanol, 10% glycerol and imidazole whose concentration was increased from 40 to 80 mM, and next to 100 mM. The His-tagged uricase was eluted with total 500 μl of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 10% glycerol and 1 M imidazole. The buffer of the eluate was changed to 0.1 M Tris–HCl buffer (pH 9.5) containing 1 mM dithiothreitol (DTT), 1 mM EDTA and 10% glycerol, using a MOLCUT L (5000 NMWL; Millipore). The final concentration of protein was 150–200 μg/ml, and that of imidazole was below 15 mM.

2.10. Uricase assay

A reaction mixture comprised 0.9 ml of air-saturated 0.1 M Tris–HCl buffer (pH 9.5) containing 1 mM EDTA and 0.1 mM sodium urate, and 5–10 μl of enzyme solution. The mixture was incubated at 25°C for 5 min, and the reaction was stopped by addition of 0.1 ml of 20% KOH (Koyama et al., 1996). The uricase activity was

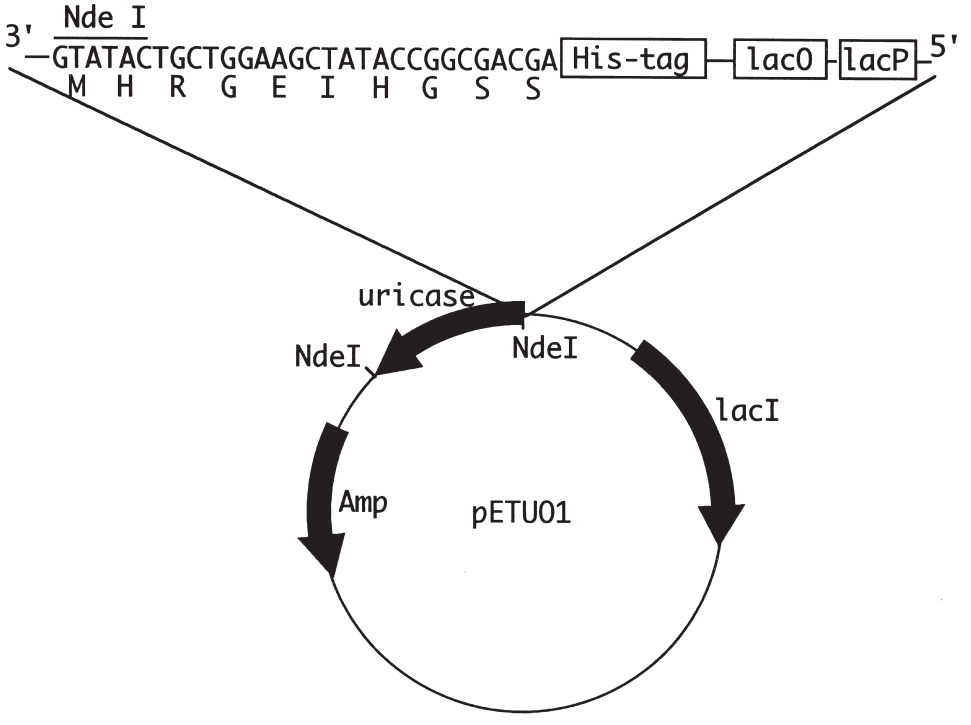

Fig. 2. Structure of the uricase expression vector, pETUO1. The coding region of the uricase cDNA of the symbiont was amplified by PCR, and the products were inserted into the *Nde*I site of the expression vector, pET16b.

determined by the decrease in absorbance at 292 nm. One unit was defined as the amount of enzyme necessary to convert 1 μmol of uric acid into allantoin in 1 min at 25°C and pH 9.5.

2.11. Protein analysis

Protein concentration was estimated by the Bio-Rad Protein Assay (Bradford, 1976), using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using Laemmli's buffer system (Laemmli, 1970).

3. Results

3.1. Cloning and sequencing of the uricase gene and its cDNA

The genomic DNA of the yeast-like symbiont gave a single signal when digested with any of four restriction enzymes and hybridized with the AF1–AF2 probe, the PCR product from the uricase gene of *A. flavus* (Fig. 3). The 7.2 kb *Hind*III fragment with positive signal was inserted into pBluescript II SK+, and the cloned plasmid was double-digested with *Sac*I and *Hind*III. When the 5.0, 0.55 and 1.7 kb were hybridized with the AF1–AF2 probe, the latter two gave positive signals. The two fragments were subcloned into pBluescript II SK+ and sequenced.

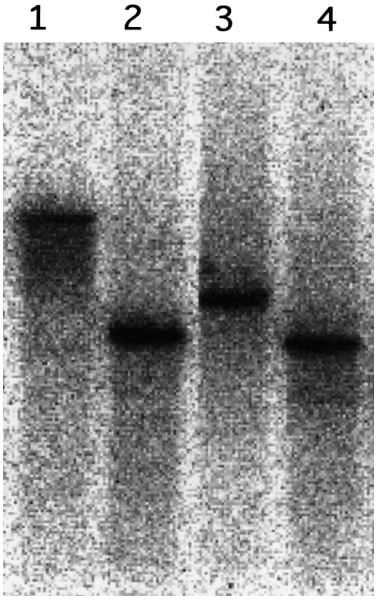

Fig. 3. Southern hybridization analysis of the genomic DNA of the symbiont. The genomic DNA was completely digested with one of the four restriction enzymes, *Bam*HI, *Hind*III, *Xba*I and *Xho*I. A PCR product from the uricase gene of *A. flavus* was used as a probe, which was labeled with ^{32}P-dCTP. Signals were detected by a MacBAS-2500 image analyzer. Lane 1, *Bam*HI; lane 2, *Hind*III; lane 3, *Xba*I; lane 4, *Xho*I.

In sequencing 2000 bp, we identified an open reading frame of 987 bp. In comparison with the cDNA sequence, it was revealed that the gene contained a single 96 bp intron at the homologous position of the second intron of the uricase gene of *A. flavus*, and encoded an uricase of 296 amino acids that shared 62% identical residues with the *A. flavus* uricase (Fig. 4) (Legoux et al., 1992). The deduced molecular weight was 32,882, and the predicted isoelectric point was 6.06.

The initiation site of transcription was determined at the position −244 by the 5′RACE method. The termination sites of transcription determined by the 3′RACE method were located at at least six points whose positions were 987, 1042, 1066, 1157, 1165 and 1206, as shown in Fig. 5.

3.2. Detection of the uricase expression in the symbionts

Expression of the uricase gene in the symbionts was detected by the Northern blot analysis (Fig. 6). A signal was detected at the position below 18S rRNA (1.9 kb). The RNA length coincided with that predicted by sequencing of the cDNA.

3.3. Expression and purification of the uricase in E. coli

E. coli strain pLysS cells, which were transformed with the recombinant expression vector pETUO1, were cultured in luria broth (LB) medium containing 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol at 37°C, and expression of the His-tagged uricase was induced by adding 1 mM IPTG at optical density (OD)=0.8–1.0. Although the His-tagged uricase was expressed most efficiently by inducing at 37°C for 3 h, about 90% of the expressed proteins were trapped into inclusion bodies (data not shown). Induction at 25°C for 8 h increased the proportion of the His-tagged uricase found in the soluble fraction up to about 70%, retaining a high expression level (Fig. 7).

The His-tagged uricase in the soluble fraction was purified in a single step, using Ni^{2+} affinity columns, to almost complete purity (Fig. 7). The purified His-tagged uricase in eluate was unstable, aggregating and precipitating in several hours at 4°C. When stored in 0.1 M Tris–HCl buffer (pH 9.5), the protein remained soluble and retained 90% of the enzyme activity after 5 days at 4°C. The activity of the His-tagged uricase was 5.2 U/mg protein.

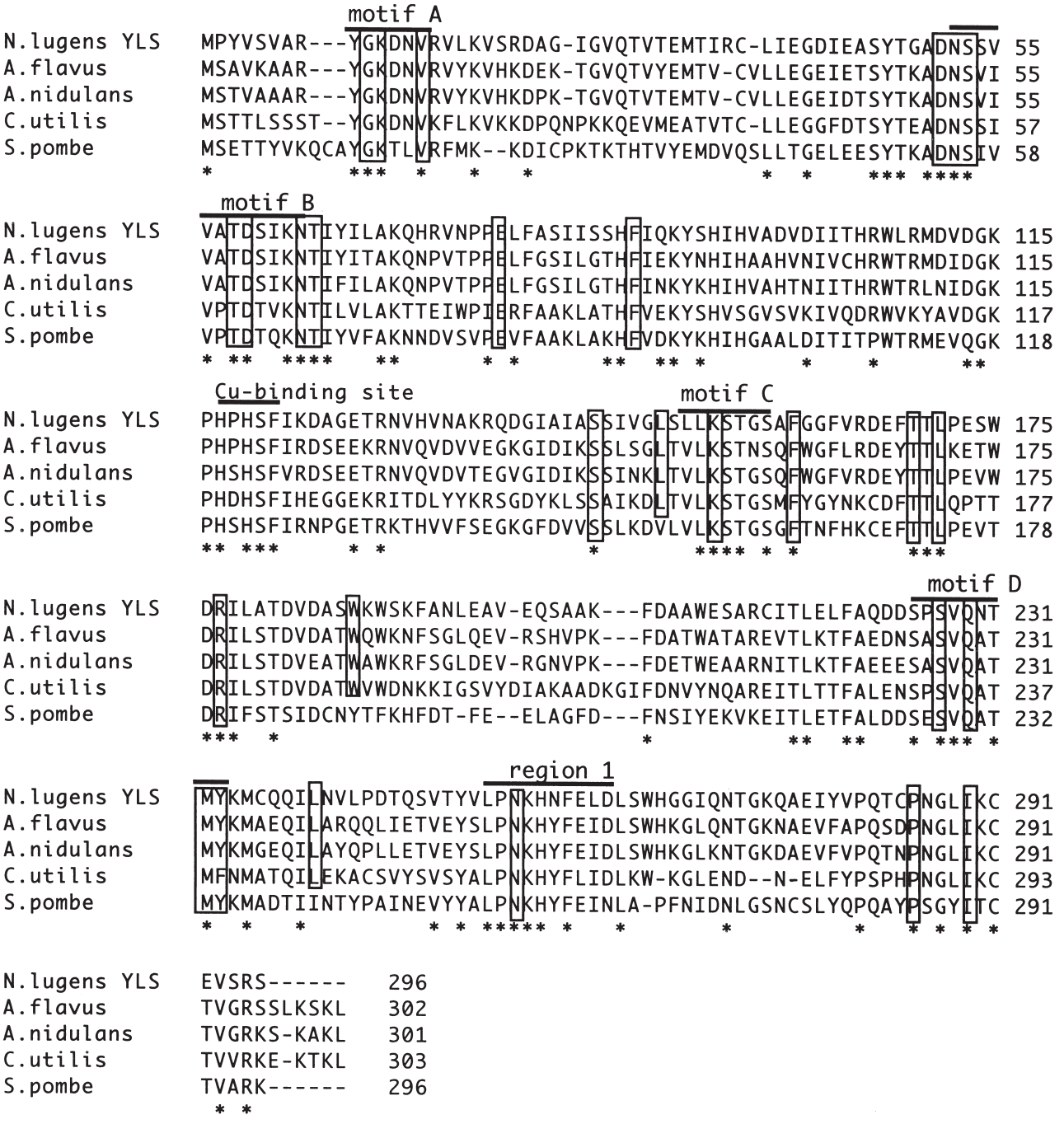

Fig. 4. Comparison of the deduced amino acid sequence of the symbiont's uricase with those of other fungal uricases. Uricases of the symbiont, *A. flavus* (Q00511), *Aspergillus nidulans* (Oestreicher and Scazzocchio, 1993), *Candida utilis* (D32043) and *Shizosaccaromyces pombe* (AL031579-9) are aligned. The sequence of the C-terminal region of *S. pombe* uricase may be incomplete. The consensus motifs (motif A–D and region 1) and the putative copper-binding site are indicated by bars. The conserved amino acid residues identified by Koyama et al. (1996) are boxed.

4. Discussion

The uricase gene identified in this work was thought to be a sole and single copy gene of the yeast-like symbiont, judging from the result of the genomic Southern hybridization (Fig. 3). The putative amino acid sequence of the uricase showed 62%, 47%, 45%, 38%, 36%, 35% and 32% identity with those of uricases from *A. flavus* (Q00511), *C. utilis* (D32043), *S. pombe* (ALO31579-p), *Rattus rattus* (X13098), *Drosophila virilis* (P23194), *Glycine max* (M63743), and *Bacillus* sp. TB-90 (D49974), respectively.

Two consensus regions in both eukaryotic and prokaryotic uricases have been identified (Yamamoto et al., 1996; Koyama et al., 1996). These sequences, motif A (Tyr/His–Gly–Lys–X–X–Val) and motif B (Asn–Ser–

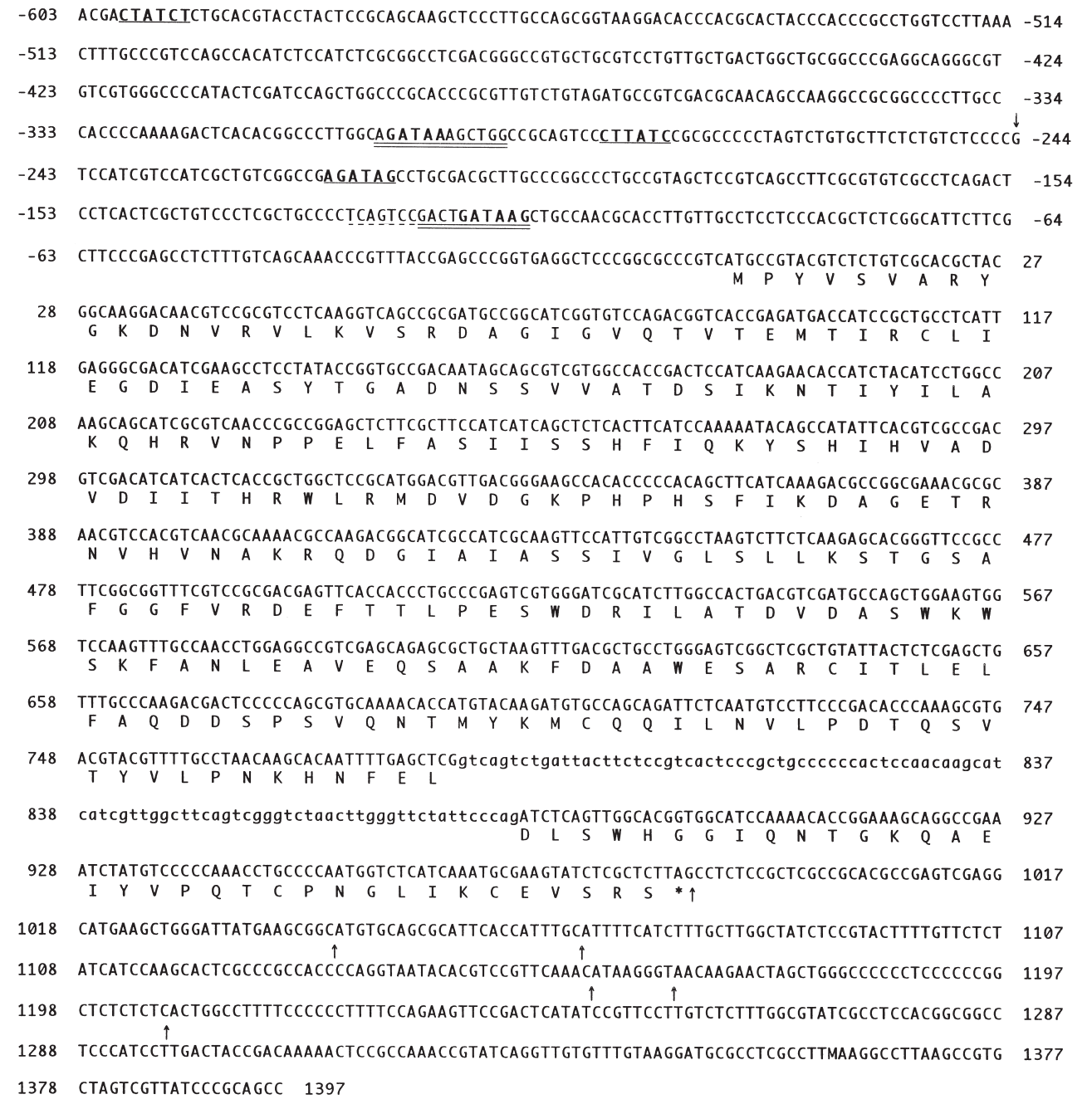

Fig. 5. DNA sequence of the uricase gene of the symbiont and its flanking region. The amino acid sequence deduced is shown under the nucleotide sequence. The intron sequence is depicted in small letters. The positions of the 5′ and 3′ end of the cDNA are indicated by arrows. The double underlined sequences were identical to those found in the 5′ flanking region of the uricase gene of *A. nidulans* that was determined by Oestreicher and Scazzocchio (1993). The dotted underline shows the sequence to form a palindrome with the following double underlined sequence. The single and the double underlined sequences were the putative GATA sites.

X–Val/Ile–Val/Ile–Ala/Pro–Thr–Asp–Ser/Thr–X–Lys–Asn), were completely conserved in the sequence of the symbiont's uricase at 9–14 and 52–63, respectively (Fig. 4). The sequence of the symbiont's uricase also shared the conserved amino acid residues identified by Koyama et al. (1996), although there were differences at some positions in the recently reported sequence of a eubacterial uricase from *Arthrobacter globiformis* (E13255). In the uricase, the conserved Ser residues at 53, 145 and 227 in Fig. 4 were all replaced by Ala, and Leu at 240 by Ile. Before identification of these regions, Bairoch (1991) proposed consensus patterns for eukaryotic uricases, which are motif C (Val–Leu–Lys–Thr–Thr–Gln–Ser) and motif D (Ser–Pro–Ser–Val–Gln–Ala–Thr–Met–Phe). In the sequence of the symbiont's uricase these were conserved with slight modifications at 152–158 and

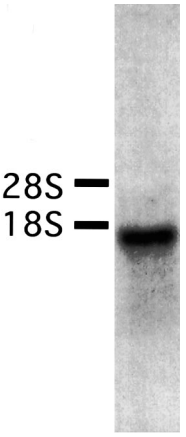

Fig. 6. Northern blot analysis of the total RNA of the symbiont. 15 μg of the total RNA was subjected to electrophoresis on 1.0% GTG–Agarose under the denaturing conditions in 20 mM 3-monopholino propanesulfonic acid (MOPS) buffer (pH 7.0) containing 5 mM sodium acetate and 1 mM EDTA. A PCR product from the uricase gene of the symbionts was used as a probe, which was labeled with ^{32}P-dCTP. The signal was detected by a MacBAS-2500 image analyzer.

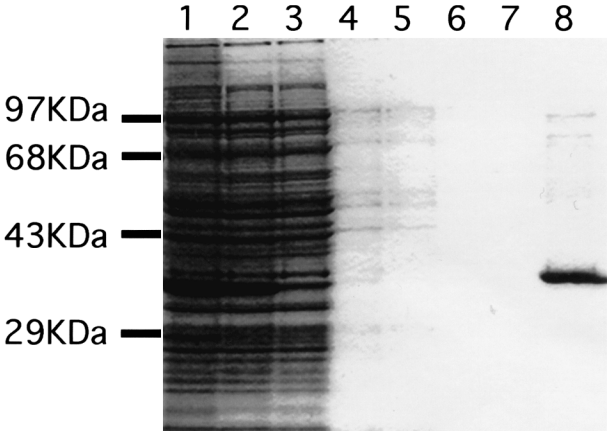

Fig. 7. SDS-PAGE analysis of the proteins produced in the transformed *E. coli*, and of the proteins purified with Ni^{2+} affinity spin columns. Lane 1, crude extract; lane 2, soluble fraction; lane 3, flow-through; lanes 4–7, washes; lane 8, eluate. The samples were loaded onto 10% polyacrylamide gel. The His-tagged uricase produced in *E. coli* was shown at 35 kDa, which corresponded to the deduced molecular weight for the His-tagged uricase.

225–233, respectively (Fig. 4). In addition to these four motifs, another conserved sequence, region 1 (253–262 in Fig. 4; LPNKHFELD), was identified. The sequence, Leu/Met–Pro–Asn–Lys/Leu/Ile–His–Phe/Tyr–Phe/Leu–Asn/Pro/Glu–Ile/Phe/Val–Asp/Asn, was shared by all the uricase sequences published so far, including that of *A. globiformis*, but not that of *Bacillus* sp. T-90.

Eukaryotic uricases are known to be cuproproteins, and the putative copper-binding site has been identified to be His–X–His–X–Phe (Wu et al., 1989; Chu et al., 1996). All the uricases sequenced so far, except again that of *Bacillus* sp. T-90, possess this motif. Although the symbiont's uricase conserves this motif, the essential role of copper in its activity is dubious because the presence of EDTA up to 10 mM in the reaction mixture had no effect on the uricase activity (data not shown). Since it has been indicated that the uricases of *A. flavus*, *Streptomyces cyanogenus* and *C. utilis* do not contain copper (Conley and Priest, 1980; Ohe and Watanabe, 1981; Nishimura et al., 1982), it is possible that copper is not essential for the oxidative activity of some fungal uricases.

As peroxisomal proteins, all the eukaryotic uricases sequenced so far have a necessary and sufficient consensus peroxisomal targeting signal (PTS), Ser/Ala–basic–Leu, known as PTS-1, at their carboxyl extremity (Terlecky et al., 1996). In the symbont's uricase, the consensus C-terminal region was deleted, and its carboxyl extremity comprised Ser–Arg–Ser. Whereas the leucine at the third position of PTS-1 is reported to be essential for proteins to be targeted to peroxisomes in mammalian cells and in vitro experiments (Gould et al., 1989; Miura et al., 1994), exceptions have been found in yeast peroxisomal proteins. The extreme C-terminus of methanol oxidase of *Hansenula polymorpha* and that of hydratase–dehydrogenase–epimerase of *Candida tropicalis* comprise Ala–Arg–Phe and Ala–Lys–Ile, respectively, which were evidenced to be functional PTSs (Aitchson et al., 1991; Hansen et al., 1992). Thus, it is possible that the tripeptide Ser–Arg–Ser at the carboxyl extremity of the symbiont's uricase functions as PTS as well.

The uricase gene of the symbiont possessed a TATA box-like sequence, –ATAAA–, at 58 bp upstream from the initiation point of transcription, and the 12 bp region containing this sequence coincided completely with a sequence found in the 5′ flanking region of the uricase gene of *A. nidulans* (Oestreicher and Scazzocchio, 1993). In spite of low identity between the whole 5′ flanking regions of the two uricase genes, in addition to the 12 bp region, a 10 bp sequence of the 5′ untranscribed region, −122 to −113 in Fig. 5, was also identical to the sequence found in that of the uricase gene of *A. nidulans*. Both the two shared sequences contained GATA sequence. The GATA sequence motif is known to be the recognition site by transcription factors of the GATA family. Because GATA-family factors in fungi are known to control transcription of genes related to nitrogen metabolism in particular (Peters and Caddick, 1994; Hoe et al., 1998), it seems likely that these sequences upstream of the uricase gene of the symbiont act as transcriptional *cis*-elements. No polyadenylation site corresponding to the eukaryotic consensus was found in the 3′ downstream region of the uricase gene of the symbiont (Proudfoot and Brownlee, 1976). The biological meaning of the multiple termination points of this gene, suggested by the 3′RACE method, is totally unknown.

Expression of the uricase gene of the symbiont in *E. coli* was successfully performed, using the His-tag system, which facilitated purification of the gene product. The activity of the symbiont's His-tagged uricase produced in *E. coli* was 5.2 U/mg protein, which was only about one-fifth of those of *C. utilis* and *A. flavus* (Conley and Priest, 1980; Nishimura et al., 1982) but was comparable to those of beans, fish and a camel (Kinsella et al., 1985; Osman et al., 1989; Suzuki and Verma, 1990). It was reported that expression of the uricase genes from *A. flavus*, *C. utilis* and soybean in *E. coli* produced recombinant uricases that were as active as their natural counterparts (Suzuki and Verma, 1990; Legoux et al., 1992; Koyama et al., 1996). Thus, the activity of the symbiont's uricase expressed in *E. coli*., although not compared in this study, is probably comparable to that of its natural counterpart, unless the His-tagging decreases the uricase activity significantly. It is possible that the activity of the symbiotic uricase has been lowered by mildly deleterious mutations that are liable to accumulate in genes of asexual organisms of a small population size, such as intracellelar symbionts (Moran, 1996).

A utilization of uric acid and the symbiont's role in it have been reported for some species of cockroaches and termites that are phylogenetically distant from planthoppers (Vogels and Van Der Drifft, 1976; Potrikus and Breznak, 1981; Cochran, 1985). Unlike the yeast-like, intracellular symbionts of planthoppers, endosymbionts of these insects are not eukaryotes, but bacteria (Buchner, 1965). Yet these symbionts, like those of planthoppers, contribute to their hosts by mobilization of the stored uric acid, though their uricases are yet to be identified (Potrikus and Breznak, 1981; Cochran, 1985). While the diet of the planthopper is different from those of cockroaches and termites, it is similarly poor in nitrogenous compounds (Mittler, 1958; Potrikus and Breznak, 1981; Sasaki et al., 1990). It is likely that insects under similar nutritional conditions acquired different microorganisms with similar ability as symbionts evolutionarily independently of each other.

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