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Isolation and characterization of a 1,4-β-endoxylanase gene of *A. awamori*

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Abstract. An enzyme with a particular 1,4-β-xylanase activity was identified and purified from wheat-bran culture medium of an *Aspergillus awamori* strain. With oligonucleotides based on the N-terminal amino-acid sequence of the enzyme, the *exlA* gene of *A. awamori*, encoding 1,4-β-xylanase A, has been cloned. Based on the deduced amino-acid sequence, 1,4-β-xylanase A is produced as a 211 amino-acid-residue-long precursor, which is converted post-translationally into a 184-aa-residue-long mature protein. Transformation of the original *A. awamori* strain with multiple copies of the *exlA* gene resulted in a 40-fold overproduction of 1,4-β-xylanase A. The overproduced enzyme has the same biochemical and enzymological properties as the wild-type enzyme.

Key words: *Aspergillus awamori* – Enzyme (1,4-β-D-xylanohydrolase E.C. 3.2.1.8) purification – *exlA* gene isolation – Multicopy transformant – Overexpression

Introduction

The filamentous fungus *Aspergillus niger* is widely used in industry for the production of fermented foods, organic acids, and enzymes (Barbesgaard 1977; Bennett 1985). Based on its capacity to secrete large amounts of protein (up to 30 g/l) in the culture medium this fungus is an attractive organism for the overproduction and secretion of homologous or heterologous proteins, with the aid of recombinant DNA techniques. During the last decade genetic modification techniques have been developed for this organism (van den Hondel et al. 1991). Enzymes which receive much attention in industrial application are polysaccharide-degrading enzymes such as xylanases. These enzymes are able to hydrolyse D-xylans consisting of 1,4-β-linked polymers of D-xylose as a backbone. Xylanases from filamentous fungi such as *Aspergillus* and *Trichoderma* species (Wong et al. 1988; Ito et al. 1992b) have been especially well studied and many xylanases from these species have been purified and characterized. Commercially, fungal-enzyme preparations with xylanolytic activity are used in bakery. The application of these preparations, however, is hampered because a wide variety of other enzymes is also present and the type of enzymes, as well as their respective quantities, vary from one preparation to another. In a study to improve control of the effect of the enzymes on bakery performance, commercial fungal enzyme preparations were analyzed. A specific 1,4-β-xylanase of *Aspergillus awamori* was identified as a very important contributor in bakery improvement (van Gorcom et al. 1991). In this paper we describe the isolation and characterization of the *exlA* gene, encoding 1,4-β-xylanase A (EXLA). Furthermore, enhancement of EXLA production level by *A. awamori* strains comprising multiple copies of the EXLA-encoding expression cassette is described.

Materials and methods

Strains, plasmids, media and transformation procedures. A. awamori ATCC11358 (CBS 115.52) was employed for the isolation of 1,4-β-xylanases from culture fluid, for preparing a gene library, and as a recipient for transformation experiments. *Escherichia coli* JM109 was used for the propagation of plasmids (Yanish-Perron et al. 1985). *E. coli* NM538 (*supF, hsdR*) and NM539 [*supF hsdR* (P2cox3)] were used for the construction and amplification of the *A. awamori* gene bank (Frischauf et al. 1983). *E. coli* transformations were carried out as described by Hananan (1983). *A. awamori* transformations were carried out as described by Punt et al. (1987). $AmdS^+$ transformants were selected after 6–10 days at 25 °C on plates with acetamide as sole nitrogen source. Culturing of *A. awamori* strains was performed in xylan medium [1% oat-spelt xylan (Sigma, Fluka), 0.67% yeast extract without amino acids (Difco), 0.1% cas amino acids] or medium with wheat bran [4% wheat bran, 0.5% $(NH_4)_2SO_4$, 0.15% KH_2PO_4, 0.025% $MgSO_4$ and 0.025% KCl pH 5.0] or minimal growth medium (Bennett and Lasure 1991) supplemented with 1% glucose, 0.2% trypticase (BBL), 0.5% yeast extract, 0.1% cas amino acids. Media were inoculated with 2×10^5 spores/ml. Incubation was performed at 25 °C (300 rpm).

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Protein analysis and characterization of 1,4-β-xylanase A (EXLA). After growth on wheat bran, EXLA was isolated from an *A. awamori* culture filtrate by chromatography on DEAE-Sepharose. The buffer used was 0.05 M acetate buffer, pH 5.5, and elution was performed with a stepwise NaCl gradient. The purification of EXLA was completed by gel filtration of the 0.15-M NaCl fraction on Ultrogel AcA-54. The purity of EXLA was tested by IEF. Determination of the N-terminal amino-acid (aa) sequence by automated Edman degradation revealed S A G I N Y V Q N Y N G N L G D F. SDS-PAGE and Western-blot analysis were performed using the Phast™ system (Pharmacia). Antisera against semi-purified EXLA were raised in rabbits.

Enzyme assay. Total xylanase activity was quantitatively determined by measuring the production of newly-liberated reducing groups using the DNS (3,5 dinitro-salicylic acid) method (Amado et al. 1985). Appropriate amounts of medium sample were incubated with a 2% xylan suspension in 0.5 M sodium acetate (pH 5.0) at 40 °C for 30 min. After reaction with the DNS reagent the optical density at 543 nm was measured. One unit of xylanase activity is defined as the amount of enzyme which, per min, releases an amount of reducing groups from xylan equivalent to 1 mg of xylose.

DNA and RNA manipulations. The λ EMBL3 genomic library was constructed essentially as described by Van Hartingsveldt et al. (1987). Sixty-five-thousand plaques, representing about 32 times the genome, were screened with a degenerate oligonucleotide probe [Xyl06: 3′CG (G/C) CC (G/C) TA (G/C) TTG ATG CA (G/C) GT (C/T) TTG ATG TTG CC (G/C) TTG GA (G/C) CC (G/C) CTG AA 5′)] based on the N-terminal aa sequence (residues 2 to 17) of EXLA. Total RNA was isolated as described by Kolar et al. (1988). Poly $(A)^+$ mRNA was obtained using the oligo-dT mRNA purification kit of Pharmacia. S1-nuclease analysis was performed as described by Punt et al. (1988). For cloning of the *exlA* gene, sequence analysis and primer extension, standard recombination DNA techniques were used (Sambrook et al. 1989).

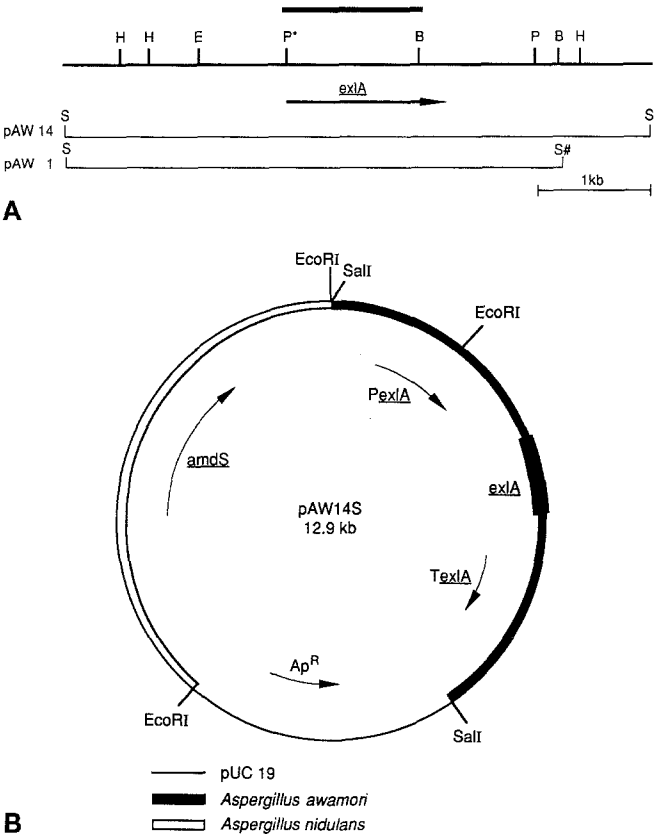

Fig. 1. **A** Restriction map of the genomic DNA region of *A. awamori*, comprising the cloned *exlA* gene. Restriction sites are given by: *S*, *Sal*I; *E*, *Eco*RI; *H*, *Hin*dIII; *P*, *Pst*I; *B*, *Bam*HI; *S#*, *Sal*I-site present in the λ-EMBL3 polylinker. The direction of transcription is indicated by the arrow, the solid block represents a 1.2-kb *Pst*I*-*Bam*HI fragment hybridising with the Xyl06 probe. **B** Plasmid used for the expression of the *exlA* gene of *A. awamori*

Results and discussion

Isolation, localization and DNA sequence of the exlA gene

1,4-β-xylanase (EXLA), with an isoelectric point of 3.7, was purified to homogeneity from the *A. awamori* culture medium. The apparent molecular weight of this protein as determined by SDS-PAGE was 30 000. Amino-acid sequence analysis of the N-terminus of EXLA yielded a sequence of 17 residues. Southern-blot analysis (hybridisation at 62 °C, washing in 5× and 3×SSC) of chromosomal *A. awamori* DNA digested with *Eco*RI, *Sal*I and *Bam*HI revealed single hybridizing bands of 4.4, 5.3 and 9.5 kb, respectively, when Xyl06 – based on the N-terminus of EXLA – was used as a probe.

Screening of a lambda EMBL3 genomic DNA library of *A. awamori* with this Xyl06 probe resulted in the isolation of three λ-positive clones which contain the putative *exlA* gene. A restriction map of the chromosomal DNA present in two overlapping λ phages is given in Fig. 1 A. Subcloning in pUC19 of a 4.6-kb and a 5.3-kb *Sal*I fragment – hybridizing with the Xyl06 probe – resulted respectively in pAW1 and pAW14 (Fig. 1 A). The putative *exlA* gene was further localized by digestion with restriction enzymes followed by Southern analysis with the Xyl06 probe. A 1.2-kb *Pst*I-*Bam*HI fragment hybridizes strongly with Xyl06 (see Fig. 1 A). This fragment and an adjacent 1.0-kb *Bam*HI-*Pst*I fragment were subcloned in M13 to determine the direction of transcription and the DNA sequence of the putative *exlA* gene. By spot-blot hybridization of single-stranded M13 DNA subclones with Xyl06 – based on the non-coding strand – the direction of transcription was determined (Fig. 1 A).

The DNA sequence comprising the pre(pro)xylanase gene (*exlA*) is shown in Fig. 2. This nucleotide sequence reveals an open reading frame (ORF) which includes the DNA sequence coding for the N-terminal aa sequence of the purified EXLA. The DNA-deduced protein sequence reveals that mature EXLA is preceded by a putative leader peptide of 27 amino acids. The first 16 residues fulfil all the requirements of a canonical signal sequence: i.e., charged N-terminus, hydrophobic core, cleavage downstream from small residues (Ala^{-3}, Ala^{-1}) (von Heijne 1983; von Heijne and Abrahmsen 1989). Its length corresponds well to that of other fungal signal sequences (17–23 amino acids). The cleavage of the bond between Arg 27 and Ser 28 may take place by a KEX2-like processing protease. However, whereas in most cases, two basic residues are located at the C-terminus of the leader peptide, only one basic residue is present at the C-terminus of the putative *exlA* leader peptide. The ORF is interrupted by a

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.      -100          .          -80          .    ↓↑ ↓↑↓
CGCAGAATATAAATAGAGGTAGAGTGGGCTCGCAGCAATATTGACCAGCACAGTGCTTCT

.      -40           . ↑        -20          .          1          .
GTTCCAGTTGCATAAATCCATTCACCAGCATTTAGCTTTCTTCAATCATCATGAAGGTCA
                                                      M  K  V  T

       20            .          40           .          60         .
CTGCGGCTTTTGCAGGTCTTTTGGTCACGGCATTCGCCGCTCCTGTGCCGGAAGCTGTTC
  A  A  F  A  G  L  L  V  T  A  F  A  A  P  V  P  E  P  V  L

       80            .          100          Xyl19  120
TGGTGTCGGGAAGTGCTGGTATTAACTACGTGCAAAACTACAACGGCAACCTTGGTGATT
  V  S  R  S  A  G  I  N  Y  V  Q  N  Y  N  G  N  L  G  D  F

       140           .          160          .          180        .
TCACCTATGACGAGAGTGCCGGAACATTTTCCATGTACTGGGAAGATGGAGTGAGCTCCG
  T  Y  D  E  S  A  G  T  F  S  M  Y  W  E  D  G  V  S  S  D

      200            .          220          .          240        .
ACTTTGTCGTTGGTCTGGGCTGGACCACTGGTTCTTCTAACTGAGTGACTGTATTCTTTA
  F  V  V  G  L  G  W  T  T  G  S  S  N

       260           .          280          .          300        .
ACCAAAGTCTAGGATCTAACGTTTTCTAGCGGCTATCACCTACTCTGCCGAATAGAGTGCT
                                A  I  T  Y  S  A  E  Y  S  A

       320           .          340          .          360        .
TCTGGCTCCTCTTCCTACCTCGCTGTGTACGGCTGGGTCAACTATCCTCAGGCTGAATAC
S  G  S  S  S  Y  L  A  V  Y  G  W  V  N  Y  P  Q  A  E  Y

       380     Xyl25 400          .          420        .
TACATCGTCGAGGATTACGGTGATTACAACCCTTGCAGCTCGGCCACAAGCCTTGGTACC
Y  I  V  E  D  Y  G  D  Y  N  P  C  S  S  A  T  S  L  G  T

       440           .          460          .          480        .
GTGTACTCTGATGGAAGCACCTACCAAGTCTGCACCGACACTCGAACTAACGAACCGTCC
V  Y  S  D  G  S  T  Y  Q  V  C  T  D  T  R  T  N  E  P  S

       500           .          520          Xyl13      .
ATCAGGGGAACAAGCAGGTTCACGCAGTACTTCTCCGTTCGAGAGAGCAGGCGCAGATCT
I  T  G  T  S  T  F  T  Q  Y  F  S  V  R  E  S  T  R  T  S

       560           .          580          .          600        .
GGAACGGTGACTGTTGCCAACGCATTTCAACTTCTGGGCGCAGCATGGGTTCGCAAATAGC
G  T  V  T  V  A  N  H  F  N  F  W  A  Q  H  G  F  G  N  S

       620           .          640          .          660        .
GACTTCAATTATCAGGTCATGGCAGTGGAAGCATGGAGCGGTGCTGGCAGCGCCAGTGTC
D  F  N  Y  Q  V  M  A  V  E  A  W  S  G  A  G  S  A  S  V

       680           .          700          .          720        .
ACGATCTCCTCTTAAGGGATAAGTGCCTTGGTAGTCGGAAGATGTCAACGCGGAACTTTG
T  I  S  S  *

       740           .          760          .          780        .
TTCTCAGCTGGTGTGATGATCGGATCCGGCCTCTGGTGGTTACATTGAGGCTGTATAAGT
       800           .          820          .          840        .
TATTCTGGGGCCGAGCTGTCAGCGGCTGCGTTTCCAATTTGCACAGATAATCAACTTTCG
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Fig. 2. Nucleotide and deduced amino-acid sequence of the genomic region of *A. awamori* comprising the *exlA* gene. The putative translation start, the translation stop codon, and the putative intron acceptor site are *shaded*. The 49-bp intron is *double underlined*; the major transcription start points (*tsp*) are indicated by *arrows* (↑ primer extension; ↓ S1); the N-terminal amino-acid sequence of the mature protein is *shadowed*. The complementary sequence of the primers Xyl19, Xyl25 and Xyl13 used for S1 and primer extension experiments are *underlined*

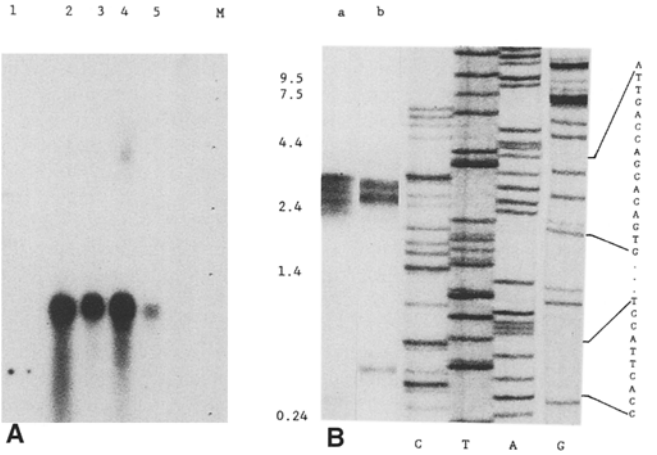

Fig. 3. A Northern-blot analysis of *A. awamori* total RNA after hybridisation with probe Xyl106. *Lane 1*, uninduced RNA. *Lanes 2 and 3*, wheat-bran induced RNA after culturing for 6 and 3 days, respectively. *Lanes 4 and 5*, xylan-induced RNA after 11 and 6 days culturing, respectively. *M*, RNA markers. **B** Mapping of *tsp* (*arrows*) by S1 nuclease analysis (*lane a*) and primer extension (*lane b*) with Xyl19 (Fig. 2) and the nucleotide sequence obtained with the same primer

putative intron of 49 or 76 bp, predicted on the basis of the presence of sequences corresponding to "donor" and "acceptor" sites of introns (Gurr et al. 1988). From the results of primer-extension experiments with primers located at the 5′ (Xyl19) and 3′ (Xyl13 and Xyl25) end of the putative intron (Fig. 2) the presence of an intron of 49 bp was deduced. Northern analysis with two oligonucleotides, comprising both the 3′ end of the first putative exon and the 5′ end of the second putative exon starting either at position 280 (intron 49 bp) or at 307 (intron 76 bp), revealed only hybridisation of the former oligonucleotide, supporting the presence of a 49-bp intron.

The cloned *exlA* gene encodes a pre(pro)protein of 211 amino acids. The predicted mature EXLA, comprising 184 aa, has a calculated molecular weight of 19.9 kDa and a pI of 3.7. The former corresponds well with that estimated by SDS-PAGE under non-reducing conditions (20 kDa) although a much higher molecular weight (MW, 30 kDa) was found under reducing conditions. The calculated pI of the mature polypeptide corresponds with that of the purified protein (3.7). The discrepancy between the MW estimation from SDS PAGE under reducing conditions and the calculated MW (based on the DNA sequence) is probably not due to glycosylation since for a very similar xylanase (XYNC) from *A. kawachii* with Ito et al. (1992a) determined a carbohydrate content of only 8.7%.

The N-terminal aa sequence of purified EXLA produced by a transformant with multiple *exlA* copies (see below) was identical to that of wild-type EXLA. Subsequent sequence determination of the peptides generated by *Staphylococcus aureus* protease digestion (at C-terminal Glu-residues) of this EXLA, confirmed the DNA-deduced aa sequence of several parts of the protein. One peptide with the N-terminal sequence YSASG (from position 302 in Fig. 2) gave definite proof for the presence of a 49-bp intron. Another peptide with the sequence AWSGAGSASVTISS (from 640, Fig. 2) confirmed the position of the deduced translation stop codon of the gene.

Determination of transcription start point

Northern-blot analysis was carried out to determine the size of the *exlA* RNA messenger and to analyse the regulation of the expression of the *exlA* gene. After hybridization of induced (xylan or wheat-bran medium) or uninduced (supplemented MM medium) total RNA with the Xyl06 probe (Fig. 3A) and the 5.3-kb *Sal*I fragment of pAW14 (data not shown) a clear band of about 1 kb was found only in RNA obtained from mycelium cultivated with xylan or wheat bran. S1-nuclease mapping and primer extension were used to determine the *exlA* transcription

Table 1. Xylanase activity level of AW14S transformants after different culturing periods in xylan medium at 25 °C. Xylanase activities are expressed in kilo-units per ml of medium. Standard deviations are less than 10%

Strain	No. of days			
	3	7	10	13
# 1 A	28	55	58	53
# 2	21	58	56	44
# 3	20	32	31	17
# 4	25	55	58	49
# 5	8	13	22	
A. awamori	5	11	13	13

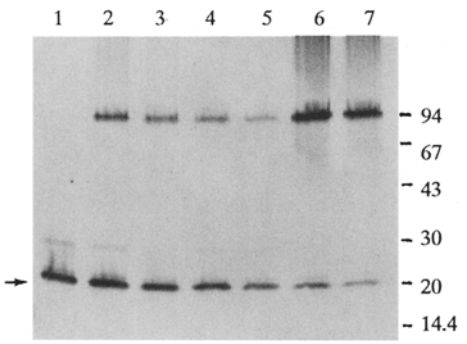

Fig. 4. Western-blot analysis of medium samples of *A. awamori* and transformant # 1 A developed with anti-EXLA serum. *Lane 1*, EXLA; *lanes 2 to 5*, medium samples of # 1 A diluted 10×, 20×, 30× and 40×; *lanes 6 and 7*, medium samples of wild-type *A. awamori* undiluted and diluted twice. The 94-kDa signal is due to the reaction of the antiserum with another secreted protein as the signal was also found in medium of an *A. awamori* strain in which the *exlA* gene was deleted (R. Gouka, unpublished results)

starting points (*tsp*). For S1 mapping a radio-labelled ssDNA fragment was generated using Xyl19 (Fig. 2) and a ssM13 vector containing the *PstI*-BamHI* fragment indicated in Fig. 1 A. After S1 treatment fragments of about 185 basepairs were obtained (Fig. 3 B). Fragments of similar size, as well as a smaller fragment of about 150 bp (Fig. 3 B), were found after primer extension with Xyl19 and with poly $(A)^+$ RNA. These results locate the major *tsp*s between positions –65 and –61 relative to the putative translation initiation codon (together with a minor *tsp* at –28). Based on these results the position of the translation start (ATG indicated in Fig. 2) downstream from the transcription start site, is confirmed.

Functional analysis of the exlA gene

Evidence for the isolation of a functional copy of the *exlA* gene was obtained by the introduction of additional copies of the gene into *A. awamori* and an analysis of the resulting transformants. Transformants were obtained after transformation of the wild-type strain with pAW14S (Fig. 1 B). This vector was produced by the insertion of the *A. nidulans amdS* gene, located on a 5.0-kb *Eco*RI fragment of pGW325 (Wernars 1986), into the *Eco*RI site of the polylinker in pAW14.

Four out of the five $amdS^+$ transformants obtained grew well on acrylamide as sole nitrogen source, suggesting that multiple copies of the *amdS* gene and, as a consequence of the *exlA* gene, are integrated into the genome (Verdoes et al. 1993). The extracellular production level of xylanase by the multicopy *exlA* transformants was determined after cultivation in medium containing xylan as an inducer. As given in Table 1, one of the transformants (# 1 A) showed a maximal production level of 58 kU of xylanase activity after 8–10 days of cultivation whereas the parent strain produces only 13 kU. Under the same conditions with two other transformants, # 2 and # 4, similar expression levels were observed, whereas in transformants # 3 and # 5 lower xylanase activities were found. As no decrease in activity is observed even after relatively-long incubation periods (up to 14 days) we conclude that the enzyme produced is very stable.

The increase in xylanase activity indicates the cloning of a functional *exlA* gene. Further proof that the increased xylanase activity originates from enhanced extracellular production of EXLA was obtained by protein analysis of the culture medium of strain # 1 A. After IEF and staining with Coomassie, a protein with a pI of 3.7 was identified in greatly-enhanced quantities compared to the amount in the wild-type strain (data not shown). Based on Western-blot analysis (Fig. 4), the amount of EXLA in the culture medium of # 1 A is about 40-fold higher than in the wild-type strain. The fact that the total xylanase activity of # 1 A is only four-fold the activity of the wild-type strain may be explained by the presence of other proteins with xylanase activity in the wild-type strain, as was indicated by zymogen analysis according to the method of Biely et al. (1985; data not shown). Also in *A. kawachii* (Ito et al. 1992 b) other xylanases were found besides the EXLA-related XYNC. If all the EXLA produced in # 1 A is present in an active form the discrepancy between the activity and the Western-blot result indicates that EXLA makes only a minor contribution to the total xylanase activity in the wild-type strain.

The EXLA production levels observed in shake-flask cultures can be further increased upon fermentation optimisation (results to be published). Based on these results we infer that the *exlA* promoter is useful for the high-level, regulated expression of genes in *Aspergillus*.

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Note added in proof. The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence. Databases under the accession number X78115 AA14BEXS.

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