Full Paper

Cloning, nucleotide sequencing, and expression of the β -galactosidase-encoding gene (*lacA*) from *Aspergillus oryzae*

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lacA coding for β -galactosidase (β -gal) was cloned from the genomic DNA of *Aspergillus oryzae* RIB40. There were 9 exons in *lacA* and the coding region of 3,015 bp encoded a protein of 1,005 aa with a deduced molecular mass of 109,898. *A. oryzae lacA* was highly homologous to fungal β -gals, with the highest aa identity of 70.7% to *A. niger lacA*, and also showed significant identity to acid β -gals belonging to family 35 glycosyl hydrolases. Approximately 10 copies of *lacA* under control of *A. oryzae glaA* promoter were integrated into the chromosome of *A. oryzae* M-2-3. The recombinant strain expressed more than 700-fold of the β -gal activity as compared to the wild type strain under induction by maltose.

Key words—*Aspergillus oryzae*; β -galactosidase; gene cloning; genomic gene; *lacA*

Introduction

Aspergillus oryzae is a filamentous fungus which has been used for centuries in fermentation of several traditional Japanese and Asian foodstuffs such as *sake* (rice wine), *shoyu* (soy sauce), and *miso* (soybean paste). *A. oryzae* is known to produce and secrete several glycosidases including α -amylase, glucoamylase, and α -glucosidase, which play an important role in those fermentations. *A. oryzae* also excretes β -galactosidase (β -gal; EC 3.2.1.23.) (Akasaka et al., 1976; Tanaka et al., 1975), which hydrolyzes β -

galactoside bond and is alternatively called lactase since lactose in milk is one of the natural substrates of the enzyme. The *A. oryzae* β -gal is commercially available from several suppliers and is used in the treatment of milk and whey in order to make dairy products sweeter and more affordable to those who suffer lactose-intolerance. The enzyme has also been used in the synthesis of galactose-containing oligosaccharides via transgalactosylation (Binder et al., 1994; Reuter et al., 1999; Toba et al., 1985) or reversed hydrolysis (Ajisaka et al., 1988).

In spite of its usefulness, there have been a few studies focused on fungal β -gal genes. Cloning and expression in *Saccharomyces cerevisiae* was reported for cDNA of the β -gal gene (*lacA*) from *Aspergillus niger* (Kumar et al., 1992). Partial genomic sequence was reported for *Penicillium canescens lacA* (Nikolaev

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et al., 1992). But so far no genetic study of *A. oryzae* β -gal gene has been published. Though conditions for induction of the wild-type *A. oryzae* β -gal expression have not been reported, *A. niger lacA* was reported to be induced by arabinose, xylose, xylan, and pectin, but not by galactose-containing sugars such as lactose, and regulation via XlnR was suggested (de Vries et al., 1999).

This study is the first report of cloning and characterization of a full-length genomic gene of a fungal β -gal, *lacA* from *Aspergillus oryzae*. Sequencing of the gene showed the exon-intron structure as well as the upstream region, which may contain expression controlling machinery. The sequence should help the assembly process of the on-going genome sequencing project of the species. Over-expression of the β -gal enzyme by a recombinant technique was also carried out.

Materials and Methods

Strains and plasmids. *A. oryzae* RIB40 is a laboratory strain from the National Research Institute of Brewing. *A. oryzae* M-2-3 (Gomi et al., 1987) is an arginine-auxotrophic (Arg⁻) strain and was used as the host for transformation. *Aspergillus niger* IFO4066, the source of hybridization probes, was obtained from the culture collection of the Institution for Fermentation Osaka (Osaka, Japan). These fungi were cultivated in dextrin-peptone (DP) media (Minetoki et al., 1995) with vigorous aeration at 30°C. *Escherichia coli* DH5 (Sambrook et al., 1989) and TG1 (Amersham, Buckinghamshire, UK) were used as the hosts for genetic manipulation. pUC118 and pUC119 were purchased from TaKaRa Shuzo (Kyoto, Japan). pMARG (Tsuchiya et al., 1993) is an *Aspergillus* expression vector equipped with the *A. oryzae glaA* promoter for inducible transcription and *Aspergillus nidulans argB* as a selection marker.

Transformation. Protoplast of *A. oryzae* M-2-3 was prepared and transformed according to the method of Gomi et al. (1987) and transformants were selected on Czapek-Dox (CD) minimum agar plates (O'Donnell and Peterson, 1992). Transformation of *E. coli* was carried out as described in the literature (Sambrook et al., 1989).

DNA manipulation. DNA manipulation was performed according to the literature (Sambrook et al., 1989) or to instructions of the suppliers. PCR was

done with a *Taq* polymerase (TaKaRa Shuzo) using a GeneAmp® PCR System 2400 (Applied Biosystems, Foster City, CA, USA).

Cloning of plain style β -gal gene. Genomic DNA of *A. oryzae* RIB40 and *A. niger* IFO4066 were prepared from mycelia as described by Minetoki et al. (1995). A DNA library was constructed in a λ EMBL3 phage vector (Stratagene, La Jolla, CA, USA) with partial *Sau3AI* digest of the genomic DNA of *A. oryzae* RIB40 as described previously (Tada et al., 1989). Two genomic DNA fragments were amplified from the *A. niger* IFO4066 chromosome DNA by PCR using primers based on the *lacA* cDNA sequence (Kumar et al., 1992) and were used as hybridization probes to select clones containing *lacA* out of the genomic library of *A. oryzae* RIB40. Plaque hybridization was performed according to the literature (Sambrook et al., 1989) at 50°C in the absence of formamide.

Nucleotide sequencing. Two neighboring *EcoRI* fragments, together containing *A. oryzae lacA*, were digested completely with *Sau3AI* or *AluI* and subcloned into *Bam*HI or *Sma*I site of pUC118/119. Shotgun sequencing of the subclones was done by using a Sequenase version 2.0 7-deaza sequencing kit (United States Biochemical, Cleveland, OH, USA). Assembly and analysis of the sequences were performed by using the GENETYX ver 8.0 program (Software Development, Tokyo, Japan).

Assay of β -gal activity. The β -gal activity was measured according to Nevalainen (Nevalainen, 1981) using ONPG (*o*-nitrophenyl- β -galactopyranoside) as a substrate in 75 mM sodium acetate buffer (pH 4.5). One unit was defined as the amount of the enzyme needed to degrade 1 μ mol ONPG per minute at 45°C.

Results and Discussion

Cloning of the plain style β -gal gene (plain style)

Since *A. oryzae* genes cloned so far are more than 70% identical to *A. niger* equivalents, we used *A. niger lacA* as hybridization probes to screen the *A. oryzae* gene. A 0.7-kb fragment was amplified from the *A. niger* IFO4066 chromosome DNA by PCR using primers TCCTACGATGATTCTCTCTGG (corresponding to nt. 2075–2095; Kumar et al., 1992) and TGT-TGAGGAACAGTGGGACAT (nt. 2743–2723), and a 1.1-kb fragment was amplified by using GCCATTAT-GAACCTCTACATG (nt. 1019–1039) and TTTCTAGA-AGAGTGTCCACGTACTTCCA (TTTCTA+nt. 2060–

2039). These fragments were sequenced and it was confirmed they represented parts of *A. niger lacA* (data not shown). The two *A. niger lacA* fragments were used as probes to screen homologous clones out of the genomic library of *A. oryzae* RIB40. Two independent plaques were positive under a low-stringent plaque hybridization. One clone (clone #1-6; Fig. 1) which hybridized to the both probes was further characterized.

The nucleotide sequences of two *EcoRI* fragments (Fig. 1) were determined. The total sequence (5,319 bp) is shown in Fig. 2 and available from the GenBank (Accession No. E12173). The coding region consisting of nine exons is 3,015-bp long and highly homologous (71.2% identity) to the *A. niger lacA* cDNA. It was considered to be the *A. oryzae* β -gal gene and was designated *lacA* according to the *A. niger* counterpart. The region encodes a polypeptide of 1,005-amino acid (aa) with a deduced molecular mass of 109,898. The deduced value was in a good accordance with an experimentally determined molecular weight of 105,000 (Tanaka et al., 1975). Eight introns of 48 to 72 bp were located (Fig. 2) by comparing the genomic sequence with the cDNA sequence of the *A. niger* β -gal (Kumar et al., 1992) or with a cDNA sequence cloned by RT-PCR of mRNA from an overexpressing strain of the *A. oryzae* β -gal described below (data not shown). All of the introns started from GT and ended in AG as common introns. A putative polyA-signal (AATGAAA; nt. 4523–4529) and a polyA-attaching sequence (GATAAA; nt. 4543–4548) were found downstream of the coding region of *A. oryzae lacA*.

The exon-intron organization of *A. oryzae lacA* was compared to that of the partial genomic β -gal gene from *P. canescens* (Nikolaev et al., 1992), the only genomic gene of fungal β -gal available so far. The first three exons of the two genes were identical in length (135, 77, and 303 bp), while lengths of the two introns were different from each other (72 and 46 bp for *A. oryzae* and 64 and 51 bp for *P. canescens*, respectively). The introns of the two genes are less conserved (56.0%/50 bp and 65.4%/26 bp) than the three exons (69.4%/134 bp, 72.6%/73 bp, and 72.4%/301 bp). A TATA box-like sequence (TATATT; nt. 911–916) resides 71-bp upstream from the initiation codon (nt. 988–990) of *A. oryzae lacA*, whereas in the *P. canescens* gene a TATA box is found 120-bp upstream from its initiation codon. Two CAAT-like sequences are found (nt. 583–586 and nt. 901–904). Transcription of

the *P. canescens* gene starts from an adenine residue at nt. 345 (underlined) in the sequence TGCAAGCT (Nikolaev et al., 1992). A similar sequence (TGTAAGCT; nt. 950–957) found on the *A. oryzae* gene may indicate the transcription start site of *A. oryzae lacA*.

Amino acid sequence homology

The aa sequence of *A. oryzae lacA* was searched for homologies to the GenBank database by using the tfasta (Devereux et al., 1984) program (Table 1). It showed the highest identities to the aa sequences of the two known fungal β -gals; 70.7% to *A. niger lacA* (Kumar et al., 1992) and 77.2% to the N-terminal 171 aa of *P. canescens lacA* (Nikolaev et al., 1992). The amino acid sequences of the three fungal β -gals are aligned in Fig. 3. The first 19 aa residues of *A. niger lacA* are deduced to be the secretion signal sequence (Kumar et al., 1992). The N-terminal 20 residues of *A. oryzae lacA* is 80% identical to the *A. niger* sequence and the processing site is conserved (Fig. 3). Whether *A. oryzae lacA* would be processed at the same site awaits determination of the N-terminal sequence of the mature enzyme. *A. oryzae* and *A. niger lacA* share an overall similarity but a block of significant dissimilarity is seen near the C-termini of the two enzymes (Fig. 3). The nucleotide sequences of the regions are homologous but frame-shifts at three positions cause the difference between the aa sequences (Fig. 2). There are 11 possible N-glycosylation sites (Fig. 2), of which nine are shared with *A. niger lacA* (Fig. 3). *A. oryzae* β -gal should actually be glycosylated at more than one of the sites because the native *A. oryzae* β -gal was reported to contain N-linked sugar chain(s) which can be liberated by endo- β -N-acetylglucosaminidase H from *Streptomyces griseus* (Nakao et al., 1987).

The N-terminal aa sequence of *A. oryzae lacA* also showed significant similarities to those of acid β -gals of plants and mammals and of a recently identified group of bacterial β -gals from *Streptomyces coelicolor* (Accession No. AL356992), *Bacillus circulans* (Ito and Sasaki, 1997), *Arthrobacter* (Gutshall et al., 1995), and *Xanthomonas manihotis* (Taron et al., 1995) (Table 1). These β -gals are classified in family 35 glycosyl hydrolases (Henrissat and Bairoch, 1993). Mammalian β -gal is known to be a lysosomal enzyme which cleaves the terminal galactose from gangliosides, glycoproteins, and glycosaminoglycans. Regions conserved in the β -gals are also found in the aa sequence of *A. oryzae*

Exons and introns are shown by uppercase and lowercase letters, respectively. The putative CAAT sequence, TATA-box, and poly-A signal are indicated. Boxed are three positions where the reading frame of *A. oryzae lacA* shifts from that of *A. niger lacA*.

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AO  MKLLSVAVALLAQAAGASIKHRLNGFTILEHPDPAKRDLQDIVTWDDKSLFINGERIMLFSGEVHPFRLPVPSLWLD
    *** * . *****
AN  MKLSSACAIALLAAQAAGASIKHRINGFTLTHESDPAKRELLQKYVTWDDKSLFINGERIMIFSGEFHPFRLPVKELQLD
    ***** . * . *****
PC  MKLLSSWVVAALAAQAAGAAISHKLDGFTIREHADPAKRALLQKYVTWDEHSIFVNGERLMIFSGEVHPYRLPVASLYID

AO  IFHKIRALGFNCVSFYIDWALLEGKPGDYRAEGIFALEPFFDAAKEAGIYLIARPGSYINAEVSGGGFGWLQRVNGTLR
    ** * . *****
AN  IFQKVKALGFNCVSFYVDWALVEGKPGEYRADGIFDLEPFFDAAKEAGIYLLARPGPYINAESSGGGFGWLQRVNGTLR
    ** * . *****
PC  IFEKVKALGFNCVSFYVDWALLEGNPGHYSAEGIFDLQPFDDAAKEAGIYLLARPGPYINAEVSGGGFGWLQRVNGTLR

AO  SSDEPFLKATDNYIANAAAVAKAQITNGGPVILYQPENESGGCCGVKYPDADYMQYVMDQARKADIVVPFISNDASPS
    *** . * . *****
AN  SSDKAYLDATDNYVSHVAATIAKYQITNGGPILYQPENEYTSGCSGVEFPDPVYMQYVEDQARNAGVVIPLINNDASAS
    . *** . *****
PC  TSDEAYLKATD

AO  GHNAPGSGTSAVDIYGHDSYPLGFDGANPSVWPEGKLPDNFRTLHLEQSPSTPYSLLEFQAGAFDPWGGPGFEKCYALVN
    * . *****
AN  GNNAPGTGKGAVDIYGHDSYPLGFDGANPTVWPSGDLPTNFRTLHLEQSPSTPYAIVEFQGGSYDPWGGPGFAACSELLN

AO  HEFSRVFYRNDLSFGVSTFNLVMTFGGTNWGNLGHGGYTSYDYGSPITETRNVTREKYSIDIKLLANFVKASPSYLTATP
    . * . *****
AN  NEFERVIFYKNDFSFQIAIMNLYMIFGGTNWGNLGYPNGYTSYDYGSAVTESRNI TREKYSSELKLLGNFAKVSPGYLTASP

AO  RNLTTGVYTDTSDLAVTPLIGDSPGSFFVVRHTDYSSQESTSYKLLPTSAGNLTPQLEGTLNLNDRDSKIHVVVDYNS
    ***** . * . *****
AN  GNLTTSYADTTDLTVTPFVGNSTGSFFVVRHSDYSSEESTSYKLLPTSAGSVTIPQLGGTLTLNDRDSKIHVVDHNVN

AO  GTNIIYSTAEVFTWKKFDGNKVLVLYGGPKHEHLAIAKSNVTIIEGSDSGIVSTRKGSSVIGWVDSSTRRIIVQVGD
    *****
AN  GTNIIYSTAEVFTWKKFADGKVLVLYGGAGDDHELAISTKSNVTIIEGSESGISSKQTSVVVWGVVDSTTRRIIVQVGD

AO  RVFLLDNRNAYNYWVPELPTGTS PGFSTSKTASSIIVKAGYLLRGALHDGADLHLTADFNATTPIEVIGAPTGAKNLF
    *****
AN  KILLLDNRNAYNYWVPQLATDGTSPGFSTPEKVASSIIVKAGYLVRTAYLKSGSLYLTADFNATTSVEVIGVPSTAKNLF

AO  VNGEKASHTVDKNGIWSSEVKYAAPEIKLPLKDLWDKYLDTLPEIKSSYDDSAWVSADLPKTKNTHRPLDTPTSLYSSD
    * . *****
AN  INGDKTSHTVDKNGIWSATVDYNAPDISLPSLKDLDWKYVDTLPEIQSSYDDSLWPAADLKQTKNTRLSTPTPTSLYSSD

AO  YGFHTGYLIYRGHVFVANGKESEFFIRTQGGSAFGSSVWLNETYLGSWTGADYAMDGNSTYKLSQLESGKNYVITVVIDNL
    *****
AN  YGFHTGYLLYRGHFTATGNESTFAIDTQGGSAFGSSVWLNETYLGSWTGLYANSYDNATYNLPQLQAGKTYVITVVIDNM

AO  GLDENWTVGEETMKNPRGILSYKLSGQDASAITWKL TGNLGGEDYQDKVRGPLNEGGLYAERQGFHQPPSESWESGSP
    ** . *****
AN  GLEENWTVGEDLMKSPRGISTSCLPDGAAPISWKL TGNLGGEDYEDKVRGPLNEGGLYAERQGFHQPEPPSNQWSSSP

AO  LEGLSKPGIGFYTAQFDLDPKGDVPLYFNFGNNT-QAARQLYVNGYQYG-KFTGNVGPQTSFPVPEGILNYRGTYNV
    *****
AN  LEGLSEAGIGFYASFDLDPKD-GMHSSTSVTALRHPRTACRSTSTDIVCEIHKQHRTSDQLPCPRGNPELSRNLV

AO  ALSLWALESDGAKLGSFELS YTPVLTGYGNVESPEQPKYEQRKGAY
    * . *****
AN  GGDPAVLD SAGGKLESLELS YTPVLTALGEVESVDQPKYKRRKGAY

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Fig. 3. Alignment of amino acid sequences of fungal β -galactosidases.

Amino acid sequences of β -gals of *A. oryzae* (AO), *A. niger* (AN; Kumar et al., 1992), and *P. canescence* (PC; Nikolaev et al., 1992) were aligned. Asterisks and dots indicate identical and similar residues, respectively. An arrow indicates the signal processing site known for the *A. niger* β -gal (Kumar et al., 1992). Asparagine residues of possible N-glycosylation sites are underlined. A box indicates the region of dissimilarity between *A. oryzae* and *A. niger lacA*.

lacA. GGPVILYQPENYEY (aa 189–201) in *A. oryzae* LacA stands for the best conserved motifs (G-G-P-[LIVM](2)-x(2)-Q-x-E-N-E-[FY]), where the second glutamic acid residue is thought to be a proton donor from similarities with other families of glycosyl hydrolases (Henrissat et al., 1995). *A. oryzae lacA* is a new member of the glycosyl hydrolase family 35.

Construction of a β -gal over-expressing strain BGH26-4

The clone #1-6 DNA was partially digested with *Pst*I and a 6.0 kb fragment containing the entire coding sequence of *A. oryzae lacA* (Fig. 4A) was isolated. The fragment was subcloned downstream of the *A. oryzae*

glaA promoter of an *Aspergillus* expression vector pMARG (Tsuchiya et al., 1993) to construct pAOBG26 (Fig. 4A). pAOBG26 was introduced to *A. oryzae* M-2-3 (Arg⁻) strain by the protoplast-transformation method and Arg⁺-transformants were selected on CD minimal agar plates. One of the transformants obtained was designated BGH26-4 and further analyzed. Southern hybridization showed that approximately 10 copies of pAOBG26 were integrated tandemly into the chromosomal DNA of BGH26-4 (data not shown). Expression of the β -galactosidase was demonstrated for BGH26-4 and the parental strain M-2-3 in liquid CD medium containing 3% glucose or maltose as a sole carbon source (Fig. 4B). In the media for M-2-3, 1 mM arginine was added in order to permit growth of the Arg⁻ strain. When cultivated with maltose, an inducer of the *glaA* promoter (Hata et al., 1992), BGH26-4 secreted more than 700-fold higher β -galactosidase activity into the culture medium than M-2-3. A northern hybridization experiment showed that mRNA of *lacA* in BGH26-4 was more than 10⁴ abundant than in M-2-3 (data not shown). The overproduction of β -gal is thought to be due to the strong transcription activity of the *glaA* promoter under induction and the gene-dosage effect.

Table 1. Amino acid sequence homology of the *A. oryzae* β -galactosidase to other β -galactosidases.

β -Galactosidase from	aa identity	Acc. No.
<i>Aspergillus niger</i>	70.7%/1,006 aa	A00968
<i>Penicillium canescens</i>	77.2%/171 aa	X70408
<i>Lycopersicon esculentum</i>	21.7%/568 aa	AF154424
<i>Nicotiana tabacum</i>	23.6%/394 aa	AJ250431
<i>Streptomyces coelicolor</i>	39.2%/166 aa	AL356992
<i>Bacillus circulans</i>	30.0%/370 aa	D88750
<i>Arthrobacter</i> species	25.8%/349 aa	ASU78028
<i>Xanthomonas manihotis</i>	34.1%/179 aa	L35444
<i>Felis catus</i>	27.0%/367 aa	AF029974
<i>Canis familiaris</i>	27.0%/367 aa	AF056084

Fig. 4. Over-expression of the *A. oryzae* β -galactosidase.

A. pAOBG26 plasmid. B. The β -gal activity of culture supernatants. *Aspergillus oryzae* strains M-2-3 and BGH26-4 were cultivated in CD medium containing 3% glucose for 138 h. Cells were harvested, resuspended in CD medium supplemented with 3% glucose or maltose, and cultivated at 30°C. The β -gal activity of culture supernatants are indicated by ○ (M-2-3 with glucose), ● (M-2-3 with maltose), □ (BGH26-4 with glucose), and ■ (BGH26-4 with maltose).

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