Direct cloning of a xylanase gene from the mixed genomic DNA of rumen fungi and its expression in intestinal *Lactobacillus reuteri*

Je-Ruei Liu a, Bi Yu b, Shiou-Hua Lin c, Kuo- Joan Cheng d, Yo-Chia Chen c,*

a Department of Biotechnology, National Formosa University, Yunlin, Taiwan, ROC
b Department of Animal Science, National Chung-Hsing University, Taichung, Taiwan, ROC
c Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung, Taiwan, ROC
d Institute of BioAgricultural Sciences, Academia Sinica, Taipei, Taiwan, ROC

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Abstract

A relatively newly defined xylanase gene, *xynR8*, was obtained directly from a mixed DNA sample prepared from unpurified rumen fungal cultures by PCR amplification. The DNA sequence of *xynR8* revealed that the gene was 884 bp in size and encoded amino acid sequences with a molecular weight of 27.9 kDa. XynR8 belonged to glycosyl hydrolase family 11, and the catalytic site residues were also found in its amino acid sequence. The main hydrolysis products of XynR8 were xylobiose, xylotriose and xylotetrose, which indicated that it belonged to the endoxylanases. The *xynR8* gene was constructed so as to express and secrete under the control of the *Lactococcus lactis lac A* promoter and its secretion signal, and was transformed into *L. reuteri* Pg4, a strain isolated from the gastrointestinal tract of broiler chickens. The *L. reuteri* transformants harboring *xynR8* not only acquired the capacity to break down xylan, but also maintained their high adhesion efficiency to mucin and mucus and their resistance to bile salts and acid.

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1. Introduction

Cereals are a major component of diets fed to mono-gastric animals, the endosperm cell wall of cereals containing complex sugars known as non-starch polysaccharide (NSP), which include β-glucans in barley and wheat, and arabinoxylans in rye and oats [1]. The animals do not synthesize the enzymes xylanase and β-glucanase, which are capable of degrading these structural polysaccharides, and as a result, these undigested NSP can often be problematic for mono-gastric animals being fed such a diet, causing intestinal disturbances, typified by sticky droppings and poor growth in young animals. It has been demonstrated previously that the anti-nutritive effects of NSP are related to their propensity to form high molecular-weight viscous aggregates in the gastrointestinal tract [2]. Such a scenario is suggested to be responsible for a reduction in the rate of food passage through the gastrointestinal tract, for reducing diffusion of digestive enzymes, and for stimulating bacterial prolif-
2. Materials and methods

2.1. Preparation of rumen fungal biomass and DNA extraction

Rumen fluid from a water buffalo was sampled through a cannula, squeezed through 2 layers of cheesecloth, following which 0.5 ml of filtrate was syringed into a Hungate tube (125 x 16 mm, Bellco Glass) containing 5 ml enrichment medium. The enrichment method as reported by Chen et al. [10] was followed throughout. The tubes containing medium and rumen fluid were incubated at 39 °C for 1 day, and the biomass was collected by centrifuge (4 °C, 6000 rpm, 30 min). All of the samples used for DNA extraction had been frozen in liquid nitrogen, ground to a fine powder with a mortar and pestle, and then stored at −20 °C. The protocol for DNA extraction was based upon phenol–chloroform extraction [11]. The extracted genomic DNA samples were stored at −20 °C prior to use.

2.2. Amplification of the xylanase gene from rumen fungal genomic DNA

The PCR reaction adopted herein was used for the amplification of xylanase genes obtained from the mixed genomic DNA samples extracted from unpurified rumen fungal cultures [7]. Two primers, xynF4 (5'-ACT-GTTGCTAAAGGCCAATGG-3') and xynR2 (5'-CCCCATTACCCTCAGTCAATG-3' ), were designed based upon the rumen fungal xylanase sequences [7]. The diluted PCR product so produced was subsequently amplified again using BamHI-xynF4 (5'-CGGATCCGTTAAGTCTCTTGCTAAAGGCCCATG-3') and NotI-xynR2 primers (5'-ATTTGCGGGCGCTTTACCCCATCCTCGTCA-3') and an appropriate PCR process. BamHI and NotI restriction sites were incorporated into xynF4 and xynR2, respectively, in order to facilitate the cloning of the xylanase gene to the pGEX4T-1 (Amersham-Pharmacia, Piscatway, NJ) expression vector for subsequent screening purposes. The xylanase gene enriched library was constructed by ligating the BamHI- and NotI-digested (New England Biolabs, Beverly, MA) PCR products into the pGEX4T-1 vector. The ligation mixture was used to transform E. coli DH5α (Invitrogen, Carlsbad, CA) by electroporation [12]. The electrooporated cells were spread on Luria–Bertani (LB) agar (Difco, Detroit, MI) containing 0.2% xylan (Oat spelt, Sigma, St. Louis, MO). Subsequent to overnight incubation at 37 °C, the transformants were transferred to another LB plates and screened by Congo-red staining [13]. Those colonies surrounded by yellow halo indicated a level of xylanase activity of the clones. The resultant plasmids (pGEX4T-1R8) were purified and the sequence of the xylanase...
gene (xynR8) inserts was determined by automatic sequencing (MDBio Inc. Taipei). The nucleotide sequence of xynR8 has been deposited with GenBank (Accession No. AY941119). The computer program Bioedit was used to analyze and align the xylanase sequences [14].

2.3. Cloning of xynR8 into L. reuteri Pg4

The primers, 32F (5'-GCACCTCGAGCCGTT-AACTGTTG-3') and 32R (5'-TCTGCAATCAGTAC-GATGCCGCGGCG-3'), were designed to amplify xyn R8 from pGEX4T-1R8. These two primers were designed so as to insert, respectively, a XhoI site at the 5' end and a PstI site at the 3' end of the PCR product. The PCR fragments encoding xynR8 were digested with XhoI and PstI, and ligated with SalI–PstI digested pNZ3004 [15] to generate pNZXYNR8 (SalI and XhoI produce compatible ends), which was sequenced to ensure that there were no errors introduced by PCR. The competent E. coli cells were prepared and transformed by means of techniques described above. The transformants were selected on LB agar plates containing erythromycin (200 μg ml⁻¹; Sigma). Plasmids expressing xylanase activity in E. coli were transformed into L. reuteri Pg4 as described by Serror et al. [16]. Subsequent to electroporation, the L. reuteri transformants were incubated in MRS (Difco) broth containing MgCl₂ (10 mM) at 37 °C for 3 h, following which the transformants were spread on MRS agar plates containing erythromycin (50 μg ml⁻¹) and incubated at 37 °C until such time as the appearance of transformants were also confirmed by Congo-red staining [13].

2.4. Enzyme assay and analysis of xylan degradation products

The xylanase activity of each L. reuteri cell culture was estimated by dividing cell culture into two fractions, one being an extracellular supernatant and the other an intracellular extract prepared as described by Cho et al. [6]. Xylanase activity was determined by estimating the quantity of reducing sugar that resulted from the enzymatic reaction. One unit of xylanase activity was defined as 1 μmol of reducing sugar equivalents released from xylan per minute under the assay conditions [7].

For the analysis of the hydrolysis products of xylan, 2.0% (w/v) solutions of xylan in 50 mM citrate buffer (pH 6.0) were incubated with appropriately diluted enzyme at 50 °C for 10 min. Hydrolysis products were identified by thin-layer chromatography (TLC) using TLC plastic sheets (Merck, Darmstadt, Germany), the specific TLC technique adopted being the method described by Carmona et al. [17].

2.5. In vitro adhesion assay

Lactobacillus was biotinylated according to the method of Fallgren et al. [18]. Partially purified porcine gastric mucin (Sigma) was purified by the method of Glenister et al. [19]. Mucus was isolated from the small intestinal wall of 35-day-old chickens according to the method reported by Gusils et al. [20]. Mucin, mucus, and BSA were dissolved in 0.1 M sodium carbonate buffer (pH 9.6) and immobilized in 96-well microtiter plates (Nunc, Roskilde, Denmark) by incubation at 37 °C for 3 h. The wells were then washed three times in PBS supplemented with 0.05% Tween 20 (PBST) and refilled with 250 μl of blocking buffer (PBS containing 1% BSA). Subsequent to further incubation for 2 h, the mucin- and mucus-coated microtiter plates were washed three times with PBST, and then filled with 200 μl of biotinylated bacterial cell suspension in PBS (5 × 10⁸ CFU ml⁻¹), and incubated for 1 h at 37 °C. Following a further three washes in PBST, a solution of avidin–horseradish peroxidase conjugate (Sigma) was added to the plates and incubated for one hour. Following a final three washes with PBST, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) solution was added to each well, and incubated at room temperature for 5 min, color development being halted by the addition of 100 μl of 1 N HCl. The absorbance of each well at a wavelength of 450 nm was determined using a microplate reader (Multiskan Ascent, Labsystems, Helsinki, Finland).

2.6. Lactobacillus tolerance to bile salts and acidic pH

The bile salts and acid tolerance of Lactobacillus strains was determined according to the method of Strompfova et al. [21]. In order to determine Lactobacillus survival at low pH, a 1 ml aliquot of the overnight cultures of Lactobacillus cells were harvested by centrifugation at 5000g for 10 min at 4 °C. The pellets were then washed twice in sterile PBS (pH 7.4), resuspended in 50 mM phosphate buffer (pH 3.0), and held at 37 °C for, variously, 0–4 h. Aliquots of 0.1 ml of suspension were removed after 0, 2, and 4 h and added onto MRS agar plates for the determination of the presence of colony forming units. In order to test the Lactobacillus resistance to bile salts, overnight cultures were inoculated (1%) into MRS broth with oxgall (0.5%) and incubated at 37 °C. The number of viable bacterial cells present was determined at 0 h and after 24 h by plating onto MRS agar plates.

2.7. Statistical analysis

All results were analyzed using the general linear model procedure available from the Statistical Analysis System (version 8.1; SAS Institute Inc, Cary, NC).
Duncan’s multiple range test [22] was used to detect differences between treatment means. Each experiment was conducted in triplicate and repeated 3 times.

3. Results and discussion

3.1. The nucleotide and deduced amino acid sequences of xynR8

The nucleotide sequences of xynR8 are illustrated in Fig. 1. The total length of the xylanase insert (xynR8) was 884 bp, and it contained a putative coding region encoding a polypeptide of amino acids with a molecular mass of 27,929 Da. According to sequence analysis, xynR8 exhibited an amino acid sequence highly similar to that of xynA of Orpinomyces sp. PC-2 (Accession No. U57819), xynw1-4 of N. patriciarum (Accession No. AY133992) and xynsk1-15 of N. frontalis (Accession No. AY134032). The xyn R8 gene revealed amino acid identities of 95.9%, 89.1% and 88.8% when compared, respectively, with xynA, xynw1-4 and xynsk1-15. According to the sequence-based glycosyl hydrolase (GH) classification, a putative conserved domain of GH family 11 was detected at position 106–660 of xynR8 (Fig. 1). Certain regions, which would appear to be conserved amongst the GH family 11 xylanases, including YGW, EYY, and SVR residues, were also found in the deduced amino acid sequence of xynR8 (Fig. 1), therefore, xynR8 was placed into this family. These deduced amino acid residues proved to be rich in Gly (12.93%) and Thr (11.22%). A particular region of xynR8, position 744–831, is typically rich in threonine and contains the reiterated sequence RTTT (Fig. 1). These characteristic features have previously been identified as regions of linkers separating the different domains of the proteins for several fibrolytic enzymes. The linker enables the two domains to act independently and may provide a greater chance of substrate catalysis [23]. The Dockerin domain was also observed at the C-terminal of xynR8, (Fig. 2) the domain

![Fig. 1. Nucleotide and deduced amino acid sequence of xynR8 cloned from the mixed genomic DNA of rumen fungi. The forward and reverse primers for PCR amplification are underlined. The putative region and conserved residues of glycosyl hydrolase family 11 are showed in bold type and double underline, respectively. The reiterated sequence RTTT is boxed.](https://academic.oup.com/femsle/article-abstract/251/2/233/601709)
belonging to the type I subfamily within rumen fungal dockerin domains (noncatalytic docking domains, NCDD). The specific role of NCDD is thought to be involved with the function of a particular high molecular-weight polypeptide associated with cellulases [7,24].

3.2. Cloning of xynR8 in L. reuteri Pg4

The xylanase xynR8 gene was cloned into a Lactobacillus expression vector pNZ3004 to generate pNZXYNR8. L. reuteri Pg4 harboring pNZXYNR8 efficiently expressed and secreted xylanase under the control of lac A promoter and its secretion signal. The enzyme activity levels for xylanase in the extracellular and intracellular fractions were determined to be, respectively, 2.25 ± 0.20 and 1.90 ± 0.11 U ml⁻¹. More than 54% of the total xylanase activity was demonstrated to present in the extracellular fraction.

To the best of our knowledge, and following a thorough review of the relevant literature, the expression of the xylanase gene in intestinal lactobacilli would not appear to have been reported significantly previously. Further, following our investigations of the literature, only a few studies have focused on the expression of bacterial β-glucanase genes in strains of intestinal lactobacilli. Heng et al. [25] reported the cloning and expression of a Bacillus macerans β-glucanase gene in L. gasseriand L. johnsonii, and reported that the β-glucanase activity of the Lactobacillus transformed strains was 0.73–0.79 U ml⁻¹. Again to the best of our knowledge, most other related studies would appear to have focused mainly on the description of genetic manipulation in the silage starter bacteria L. plantarum, although, the level of the heterologous enzyme genes expressed in the genetically modified L. plantarum would appear to typically be rather low [6,26–28].

Fig. 2. Alignment of the deduced amino acid sequence of xynR8 with other fungal xylanase genes. Amino acid residues with an identical match (*) and those with different degrees of conservation (: or .) are indicated. The reiterated sequences (RTTT) of linker are showed in bold type. Dockerin domains (partial) of xylanase are boxed. Gaps (dashes) were introduced to maximize the regions of sequence alignment. The reference sequences shown in this figure are Orpinomyces sp. PC-2 xylanase A (xynA_PC2, GenBank No. U57819), Neocallimastix patriciarum W-1 xylanase W1-4 (xynw1-4NP, GenBank No. AY133992) and Neocallimastix frontalis SK xylanase sk1-15 (xynskl-15NF, GenBank No. AY134032).
3.3. Xylan hydrolysis by the recombinant xylanase XynR8

The hydrolysis products released from oat spelt xylan by XynR8 were analyzed using TLC, the results being presented in Fig. 3. The principal products of xylan hydrolysis of oat spelt xylan were xylobiose and xylotriose, which indicated that the recombinant xylanase was an endoxylanase, and the pattern of such hydrolysis classified the xylanase R8 as being endoenzyme β-1,4 xylan xylanohydrolase (EC 3.2.1.8) [7,29].

Prebiotics, which are suggested to elicit a beneficial effect through their selective metabolism in the intestinal microflora of individuals consuming such dietary supplements, consist mainly of oligosaccharides, sugar molecules of three to six chains and soluble fiber. Endoxylanase is able to hydrolyze the xylan of certain cereals and produce xylooligosaccharides that serve profitable factors on growth of probiotics. There would appear to be abundant evidence to show that probiotics are effective in enhancing the immune system, thus encouraging body-weight gain, reducing diarrhea, and improving feed-conversion efficiency [30,31]. Therefore, Lactobacillus transformed with the xylanase gene can not only used as a probiotic supplement but can also be added dietarily in order to produce oligosaccarides to maintain a healthy balance of intestinal flora.

3.4. Adhesion of L. reuteri Pg4 transformed strains to mucus and mucin

The colonization of different portions of the intestinal tract by beneficial microorganisms constitutes the first defensive barrier against the invasion of pathogenic microorganisms or toxic substances. The ability of various probiotic strains to adhere to the intestinal epithelium and to compete with other microorganisms seems to be crucial for a probiotic strain to effectively colonize...
the gastrointestinal tract [21]. As illustrated by the results presented in Fig. 4, *L. casei* ATCC 393 reveals a rather low ability to adhere to immobilized mucin and mucus, such an observation being quite consistent with the results of Edelman et al. [32]. On the other hand, our study has revealed that all *L. reuteri* Pg4 strains adhered efficiently to both mucin and mucus, and there appeared to be no significant difference between the adherence ability of *L. reuteri* Pg4 transformed strains and that of the *L. reuteri* Pg4 wild-type strain (p > 0.05; Fig. 4). *L. reuteri* is one of the major species of this genus found in both human and animal intestines. When present in sufficient numbers within gastrointestinal tract, *L. reuteri* is believed to be able to create a healthy equilibrium between beneficial and potentially harmful microflora in the gut [31,32]. In the current study, we have not only

![Graph](https://example.com/graph.png)  
Fig. 5. Survival of *Lactobacillus reuteri* Pg4 strains during incubation at pH 3.0 (a) or in presence of 0.5% bile salts (b).
demonstrated that chicken *L. reuteri* Pg4 was able to adhere to the small intestinal mucus, but we have also revealed that the presence of the heterologous xylanase gene in *L. reuteri* Pg4 did not affect its adherence ability. Further investigation is required to verify the colonization and the xylanase secretion of the *L. reuteri* pNZXYNR8 in vivo.

3.5. Tolerance of *L. reuteri* Pg4 strains to low pH and bile salts

All the *L. reuteri* Pg4 strains tested herein survived after 4 h incubation at pH 3.0 (over 23%) (Fig. 5(a)). Further, we also noted that they also survived an incubation period of 24 h in MRS broth containing 0.5% bile salts under the rate over 16% (Fig. 5(b)). Our results have revealed that both acid- and bile salts tolerance of transformed *L. reuteri* Pg4 strains did not differ significantly from those of the wild-type strain of *L. reuteri* Pg4 ($p > 0.05$).

*Lactobacillus* used as a dietary probiotic adjunct is commonly delivered in a food and/or feed system. Therefore, probiotic bacteria need to be able to tolerate acid and bile salts, in order to attach to gut epithelium, and proliferate in the lower intestinal tract before they can start to provide any real health benefits [33]. All of the *L. reuteri* Pg4 strains tested in this study exhibited resistance to acidic conditions and contact with bile salts. Such a result indicated that *L. reuteri* Pg4 strains retain the potential to survive transit through the stomach and possess the ability to survive in the intestine for a protracted period of time, and thus could be important to the microbial ecology of the intestinal environment.

In conclusion, we have successfully obtained a xylanase gene, *xynR8*, directly from the mixed DNA sample prepared from unpurified rumen fungal cultures by PCR amplification. We have also cloned *xynR8* in *L. reuteri* Pg4 and demonstrated that the introduction of this heterologous gene into certain bacterial cells did not appear to affect probiotic properties of such bacterial cells. Further studies attempting to evaluate the ability of the transformed *L. reuteri* Pg4 strain to colonize the gastrointestinal epithelium and secret xylanase into the gastrointestinal tract of chicken are now in progress.

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References


