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# Coexpression of rumen microbial β-glucanase and xylanase genes in *Lactobacillus reuteri*

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**Abstract** The aim of this study was to clone and coexpress two rumen fibrolytic enzyme genes in *Lactobacillus reuteri*. The ability of the genetically modified strain to degrade β-glucan and xylan was evaluated. The *Fibrobacter succinogenes* β-glucanase (1,3–1,4-β-D-glucan 4-glucanohydrolase [EC 3.2.1.73]) gene and the *Neocallimastix patriciarum* xylanase gene, *xynCDBFV*, were constructed to coexpress and secrete under control of the *Lactococcus lactis lac*A promoter and its secretion signal and then transformed into *L. reuteri* Pg4, a strain isolated from the gastrointestinal tract of broiler chickens. The transformed *L. reuteri* strain acquired the capacity to break down soluble β-glucan and xylan. The introduction of the recombinant plasmids and production of β-glucanase and xylanase did not affect cell growth. To the best of our knowledge, this is the first report of coexpression of rumen microbial fibrolytic enzyme genes in *L. reuteri*.

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**Keywords** Rumen microbe · β-Glucanase · Xylanase · *Lactobacillus reuteri*

## Introduction

Although in many areas of the world, the majority of poultry diets are corn–soybean based, barley, wheat, and rye remain the major feed grains in certain regions of the USA and in European countries. Water-soluble fractions of nonstarch polysaccharides (NSP), including rye and wheat arabinoxylan and barley β-glucan, are known to exert adverse effects on performance and nutrient digestibility in broilers (Englyst et al. 1989). In general, NSP have antinutritional effects related to their low digestibility and viscousness, which reduce the rate of passage, decrease diffusion of digestive enzymes, promote endogenous losses, and stimulate bacterial proliferation (Bedford and Schulze 1998; Choct and Annison 1992). The antinutritive effects of NSP can be attenuated by adding specific enzymes such as xylanase or β-glucanase into wheat- or barley-based diets for nonruminant animals (Mathlouthi et al. 2003). However, enzyme supplementation substantially increases feed cost. An alternative and less expensive strategy might be to develop probiotics with the capacity to digest plant cell wall structural carbohydrates by introduction of heterologous genes encoding fibrolytic enzymes (Cho et al. 2000).

Probiotic, which means "for life" in Greek (Gibson and Fuller 2000), has been defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller 1989). There is sufficient evidence to show that probiotics are effective at improving growth rate, feed efficiency, and disease resistance and reducing gut shedding of enteropathogenic bacteria (Patterson and Burkholder 2003; Reid and Friendship 2002).

Therefore, addition of probiotics to feed may be a potential replacement for antibiotics. Current probiotics have been mostly selected from native gut microflora, with the selection of optimal strains often largely empirical. In recent years, there has been interest in the development of methods for the expression of heterologous proteins in probiotics to improve their efficiency. Examples of such potential applications of genetically modified probiotics include strains that produce antibodies for use as recombinant live vaccines and enzymes for detoxification and fiber degradation (Steidler 2003).

To date, several studies have investigated the expression of fibrolytic enzyme genes in lactobacilli. Most of these reports describe the genetic manipulation of β-glucanase in *L. plantarum*, which is the primary bacterium used in silage fermentation (Rossi et al. 2001; Scheirlinck et al. 1990a, b). Only a few these investigations have focused on expression of β-glucanase genes in intestinal probiotic strains (Cho et al. 2000; Irvin and Teather 1988). In our previous study, the rumen microbial cellulase, β-glucanase, and xylanase genes were, respectively, cloned and expressed into a probiotic *Lactobacillus reuteri* strain (Liu et al. 2005). It was also demonstrated that the *L. reuteri* Pg4-transformed strains not only acquired the capacity to break down carboxymethyl cellulose, β-glucan, or xylan but also that there was no difference from the parent strain in terms of the adhesion efficiency to mucin and mucus and its resistance to bile salts and acid (Liu et al. 2005). To the best of our knowledge, however, coexpression of different fibrolytic genes in intestinal lactobacilli has not been reported previously.

In the present study, we have described the cloning, coexpression, and secretion of a β-glucanase (1,3–1,4-β-D-glucan 4-glucanohydrolase [EC 3.2.1.73]) gene from the ruminal bacterium *F. succinogenes* and a xylanase gene *xynCDBFV* from the ruminal fungus *N. patriciarum* in a strain of *L. reuteri* isolated from the gastrointestinal tract of healthy broilers. We also examined the growth characteristics, heterologous enzyme production, and plasmid stability of this genetically modified *L. reuteri* strain.

## Materials and methods

### Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in Table 1. *L. reuteri* Pg4 and *Escherichia coli* were grown at 37°C in de Man–Rogosa–Sharpe (MRS) broth and Luria–Bertani (LB) broth (Difco Laboratories, Detroit, MI), respectively. Agar plates were prepared by adding agar (1.5% w/v; Difco) to each broth.

### DNA isolation and manipulation

Plasmid deoxyribonucleic acid (DNA) was isolated from *E. coli* using the alkali lysis method (Birnboim and Doly 1979). *Lactobacillus* plasmid DNA was isolated according to the method described by O'Sullivan and Klaenhammer (1993). Restriction enzymes and T4 DNA ligase (New England BioLabs, Beverly, MA) were used according to the manufacturer's instructions. All other DNA manipulations were performed using established procedures (Sambrook and Russell 2001).

### Construction of xylanase and β-glucanase coexpression plasmids

The DNA sequences encoding xylanase and β-glucanase genes were amplified by polymerase chain reaction (PCR).

**Table 1** Bacterial strains and plasmids used in this study

| Strains or plasmid | Relevant features | Reference or source |
|---|---|---|
| Strains | | |
| *E. coli* DH5α | Cloning host; $F^-$ φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17($rk^-$, $mk^+$) phoA supE44 thi-1 gyrA96 relA1 $\lambda^-$ | Invitrogen, Carlsbad, CA |
| *L. reuteri* Pg4 | *L. reuteri* isolate from the gastrointestinal tract of broilers | Yu et al. 2007 |
| *L. reuteri*pNZ3004 | *L. reuteri* Pg4 carrying pNZ3004 | Liu et al. 2005 |
| *L. reuteri* pNZJ021(xyn) | *L. reuteri* Pg4 carrying pNZJ021 | Liu et al. 2005 |
| *L. reuteri* pNZJ023(glu) | *L. reuteri* Pg4 carrying pNZJ023 | Liu et al. 2005 |
| *L. reuteri* pNZ-xyn/glu | *L. reuteri* Pg4 carrying pNZ-xyn/glu | This study |
| Plasmids | | |
| pNZ3004 | $Cm^r$, $Em^r$; *E. coli–L. reuteri* shuttle vector, 5.0 kb | van Rooijen et al. 1992 |
| pNZJ021(xyn) | pNZ3004 containing *N. patriciarum* xylanase gene (*xynCDBFV*), 5.7 kb | Liu et al. 2005 |
| pNZJ023(glu) | pNZ3004 containing *F. succinogenes* β-glucanase gene (*glu*), 5.8 kb | Liu et al. 2005 |
| pNZ-xyn | $Cm^r$, $Em^r$; 0.68-kb xylanase gene *xynCDBFV* PCR amplicon from pNZJ021 cloned into pNZ3004, 5.7 kb | This study |
| pNZ-xyn/glu | $Cm^r$, $Em^r$; pNZ3004 containing *N. patriciarum* xylanase gene (*xynCDBFV*) and *F. succinogenes* β-glucanase gene (*glu*), 6.4 kb | This study |

The DNA sequence encoding xylanase xynCDBFV (Chen et al. 2001), a thermostable and alkalophilic mutant of the catalytic domain of *N. patriciarum* xylanase xynC (GenBank accession number AF123252), was amplified by PCR from pNZJ021 (Liu et al. 2005) using the oligonucleotide forward primer, xynF (5′-GCA*GTCGAC*CCAAAGTTT CTGTAGTTCAG-3′), and the reverse primer, xynR (5′-TTC***CTGCAG***ATGCATCACCAATGTAAAC-3′). These two primers were designed to insert a *Sal*I site at the 5′ end and *Nsi*I and *Pst*I sites at the 3′ end of the PCR product, respectively. The PCR fragments encoding *xynCDBFV* were digested with *Sal*I and *Pst*I and ligated with *Sal*I–*Pst*I-digested pNZ3004 (van Rooijen et al. 1992) to generate pNZ-xyn. The DNA sequence encoding the signal peptide derived from *Lactococcus lactis lac*A fusing to the catalytic domain of *F. succinogenes* β-glucanase glu (GenBank accession number M33676) was amplified by PCR from pNZJ023 (Liu et al. 2005) using the primers gluF (5′-GCA*ATGCAT*GATGGCTATTGTTGTTG-3′) and gluR (5′-TTC*CTGCAG*TCACGATTGCGGAG-3′). These two primers were designed to place a *Nsi*I site at the 5′ end and a *Pst*I site at the 3′ end of the PCR product, respectively. The PCR fragments encoding *glu* were digested with *Nsi*I and *Pst*I and ligated with *Nsi*I–*Pst*I-digested pNZ-xyn to generate the coexpression plasmid pNZ-xyn/glu, which was sequenced to ensure that there were no errors introduced by PCR.

## Transformation of plasmid DNA

Competent *E. coli* cells were prepared and transformed by standard techniques (Sambrook and Russell 2001). The transformants were selected on LB agar plates containing erythromycin (100 μg $ml^{-1}$; Sigma Chemical, St. Louis, MO). Plasmids expressing the desired enzyme activity in *E. coli* were electroporated into *L. reuteri* Pg4 as described by Serror et al. (2002). Subsequent to electroporation, the *L. reuteri* Pg4 transformants were incubated in MRS broth containing $MgCl_2$ (10 mM) at 37°C for 3 h, with the transformants subsequently spread on MRS agar plates containing erythromycin (10 μg $ml^{-1}$) and incubated at 37°C until the appearance of transformants. The transformants were not only confirmed by direct colony PCR using the two primer pairs, xynF/xynR and gluF/gluR, but also transferred to another MRS plates and screened using Congo red staining (Teather and Wood 1982). Those colonies surrounded by a yellow halo were selected for further analysis.

## Growth characteristics and enzyme production by *L. reuteri* Pg4 transformants

For analysis of growth characteristics, 10 ml of MRS broth (Difco) was inoculated at 1% with an overnight culture of *Lactobacillus* and was incubated statically at 37°C for 48 h. During the incubation period, samples were taken every 4 h for counting the cell numbers by using the standard agar plate method and measuring turbidity at 600 nm ($OD_{600}$).

For estimation of enzyme activity, each transformant screened above was transferred to the MRS broth containing erythromycin (10 μg $ml^{-1}$) and incubated at 37°C for 24 h. The xylanase and β-glucanase activities of each *L. reuteri* cell culture were estimated by dividing cell culture into two fractions, one being an extracellular supernatant and the other being an intracellular extract, both prepared as described by Cho et al. (2000). Xylanase and β-glucanase activities were determined by measuring the amounts of reducing sugar liberated by fractions incubated with 0.5% (w/v) oat spelt xylan (Sigma) or barley β-glucan (Megazyme International, Wicklow, Ireland) in 50 mM sodium citrate buffer (pH 5.0), respectively. After incubation for 20 min at 40°C, the reaction mixture was stopped by boiling for 10 min. The reducing sugar produced was estimated by the dinitrosalicyclic acid reagent (Konig et al. 2002). One unit of fibrolytic enzyme activity was defined as 1 μmol of reducing sugar equivalents released from the respective substrate per minute under the assay conditions.

## Gel electrophoresis and zymography

A sensitive zymogram technique modified from that of Beguin (1983) was routinely used to identify β-glucanase and xylanase using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). In brief, intracellular extracts of each *L. reuteri* cell culture were electrophoretically separated on a 15% SDS-PAGE containing 0.1% glucan or 0.1% xylan. After electrophoresis, the gels were washed three times for 30 min at 4°C in 100 mM acetate buffer (pH 5.0) containing 25% isopropanol for the first two washes to remove SDS and then incubated in the same buffer for 20–40 min at 40°C. The gels were stained in 0.1% Congo red solution for 15 min at room temperature, then washed with 1 M NaCl before 0.5% acetic acid to enable visualization of regions of enzyme activity within the gel.

## Plasmid stability assay

The stability of the plasmids harbored by *L. reuteri* Pg4 was investigated using a modification of the described method (Gurakan et al. 1998). *L. reuteri* Pg4 harboring pNZ3004, pNZJ021(xyn), pNZJ023(glu), and pNZ-xyn/glu were, respectively, grown in MRS broth without antibiotics and maintained in mid-log phase throughout 50 generations by serial dilution. At appropriate times, bacteria were plated onto MRS agar to determine the total number of viable cells and all the resulting colonies were transferred to erythromycin-containing MRS agar plates to screen for erythromy-

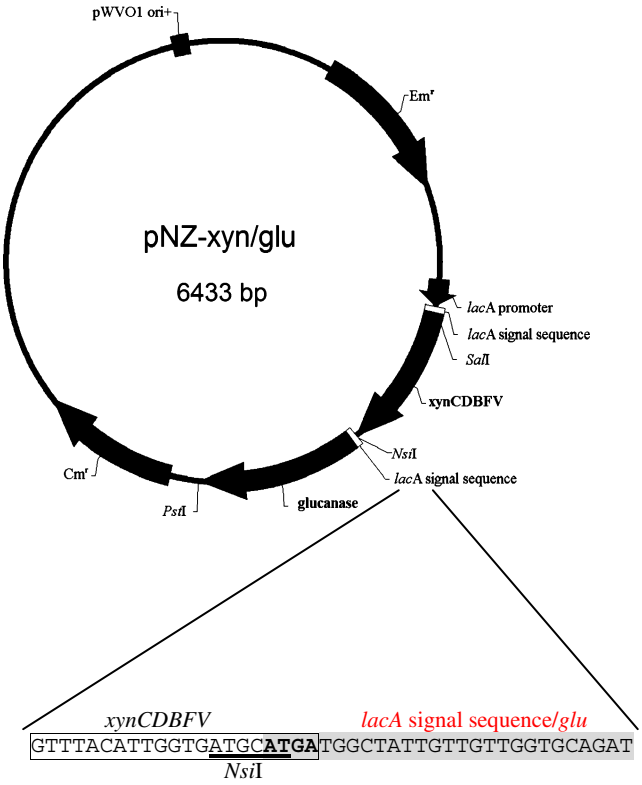


**Fig. 1** *Lactobacillus* expression plasmid harboring the *Fibrobacter succinogenes* β-glucanase gene, *glu*, and the *Neocallimastix patriciarum* xylanase gene, *xynCDBFV*. The *xynCDBFV* termination codon overlaps the *glu* initiation codon in an overlapping stop-start tetranucleotide 5′-AUGA-3′

cin-resistant ($Em^r$) cells. The number of colonies growing on a MRS agar plate but not on an erythromycin-containing MRS agar plate represented the percentage segregational instability. The colonies growing under erythromycin selection underwent the Congo red dye assay to detect the presence of fibrolytic enzyme activity. The structural stability was assumed to be complete when all the $Em^r$ colonies were xylanase and β-glucanase positive as well.

### Statistical analysis

All results were analyzed using the general linear model procedure available with Statistical Analysis System software (version 8.1; Statistical Analysis System Institute 1998). The Duncan's multiple range test (Montgomery 1999) was used to detect differences between treatment means. Each experiment was conducted in triplicate and repeated three times.

## Results

### Construction of glucanase–xylanase-coexpressing plasmids

To coexpress the β-glucanase and xylanase genes in *L. reuteri* Pg4, the recombinant plasmid was constructed with *E. coli* DH5α as a host. The β-glucanase gene *glu* of *Fibrobacter succinogenes* (0.75-kb *Nsi*I–*Pst*I fragment) and the xylanase gene *xynCDBFV* of *Neocallimastix patriciarum* (0.68-kb *Sal*I–*Nsi*I fragment) were cloned into the *Lactobacillus* expression vector pNZ3004, generating pNZ-xyn/glu (Fig. 1). The *xynCDBFV* termination codon overlaps the *glu* initiation codon in an overlapping stop–start tetranucleotide 5′-AUGA-3′. Therefore, termination of *xynCDBFV* translation triggers reinitiation on the *glu* AUG and hence coordinate synthesis of the two enzymes.

### Transformation of plasmid DNA

The expression plasmid pNZ-xyn/glu was electroporated to *L. reuteri* Pg4 with efficiency similar to that of pNZ3004 ($5–10\times10^2$ transformants $\mu g^{-1}$ of DNA). The presence of the *glu* and/or *xynCDBFV* genes in the *L. reuteri* transformants was verified by direct-colony PCR. In the *L. reuteri* pNZJ021(xyn), primers xynF and xynR amplified a 0.68-kb fragment representing *xynCDBFV* (Fig. 2, lane 3), while in the *L. reuteri* pNZJ023(glu), primers gluF and gluR amplified a 0.76-kb fragment representing *glu* (Fig. 2, lane 4). In the *L. reuteri* pNZ-xyn/glu, the checking primers not only amplified the 0.68-kb *xynCDBFV* fragment and the 0.76-kb *glu* fragment but also a 1.44-kb fragment representing the tandem arrangement of *xynCDBFV* and *glu* genes (Fig. 2, lane 5).

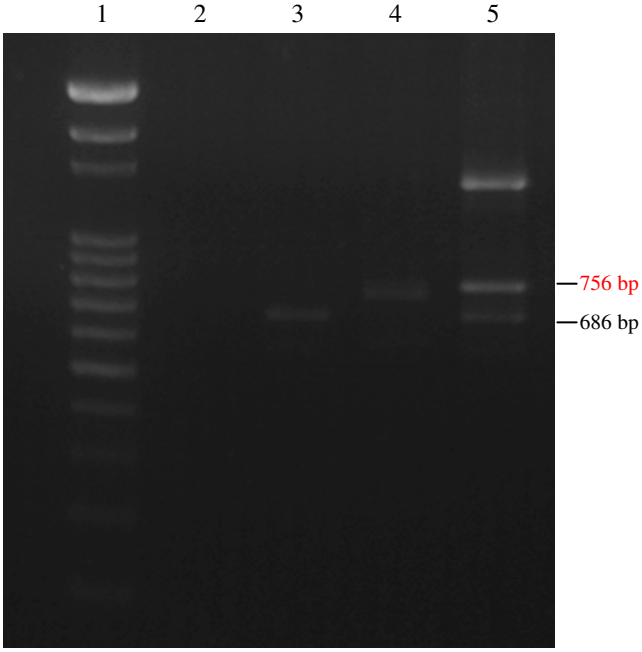


**Fig. 2** PCR confirmation of glu and/or xynCDBFV in the transformants. *Lane 1*, 100-bp ladder; *lane 2*, *L. reuteri* pNZ3004; *lane 3*, *L. reuteri* pNZJ021(xyn); *lane 4*, *L. reuteri* pNZJ023(glu); *lane 5*, *L. reuteri* pNZ-xyn/glu

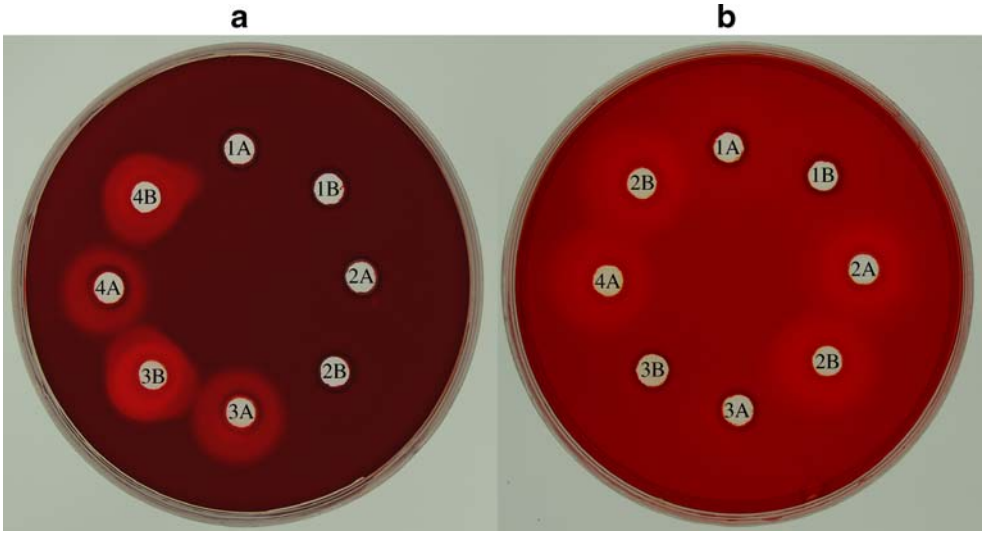


**Fig. 3** Plate test for fibrolytic enzyme activity of *Lactobacillus reuteri* transformants. Extracellular supernatant (*A*) and intracellular extract (*B*) of growing cells were pipetted into wells of glucan-containing (**a**) and xylan-containing (**b**) plates, allowed to incubate, and stained with Congo red. *Well 1*, *L. reuteri* pNZ3004; *well 2*, *L. reuteri* pNZJ021(xyn); *well 3*, *L. reuteri* pNZJ023(glu); *well 4*, *L. reuteri* pNZ-xyn/glu

## Coexpression of β-glucanase and xylanase in *L. reuteri* Pg4

A Congo red plate assay was used to confirm the fibrolytic enzymes activity of the transformants. The *L. reuteri* pNZ3004-transformed strain was devoid of glucanase and xylanase activity (Fig. 3). The culture supernatant and cell lysate of the *L. reuteri* pNZJ023(glu) produced yellow halo zones on the glucan-containing plate but not on the xylan-containing plate (Fig. 3a), while the analogue of the *L. reuteri* pNZJ021(xyn) produced degradation zones on the xylan-containing plate but not on the glucan-containing plate (Fig. 3b). The *L. reuteri* pNZ-xyn/glu, which showed clear halos on the glucan-containing plates, also produced clear halos on the xylan-containing plates, indicating that glucanase and xylanase were functionally coexpressed by *L. reuteri* pNZ-xyn/glu transformants (Fig. 3).

The enzymatic activities of the *L. reuteri*-transformed strains were further confirmed by the zymogram assay. In the zymographic analysis of glucan-containing SDS-PAGE, the intracellular extracts from *L. reuteri* pNZJ023(glu) and *L. reuteri* pNZ-xyn/glu transformants revealed a glucanase activity band of about 29-kDa but not those of *L. reuteri* pNZ3004 and *L. reuteri* pNZJ021(xyn) (Fig. 4a). Zymographic analysis of xylan-containing SDS-PAGE revealed a xylanase activity band of about 26-kDa in the intracellular extracts from *L. reuteri* pNZJ021(xyn) and *L. reuteri* pNZ-xyn/glu transformants but not *L. reuteri* pNZ3004 and *L. reuteri* pNZJ023(glu) (Fig. 4b). This demonstrates that the rumen microbial β-glucanase and xylanase can be functionally coexpressed by *L. reuteri* pNZ-xyn/glu and did not form a fusion protein.

## Growth characteristics and enzyme production of *L. reuteri*-transformed strains

When cultured in the MRS medium at 37°C, *L. reuteri* Pg4 reached stationary growth after 16 h of fermentation, with a concentration of 9.76±0.21 log colony-forming unit (CFU) $ml^{-1}$ ($OD_{600}$ of 1.18±0.26). The growth pattern of *L. reuteri* pNZ-xyn/glu was similar to that observed for *L. reuteri* Pg4. The cell counts did not differ significantly between *L. reuteri* pNZ-xyn/glu and *L. reuteri* Pg4 throughout the fermentation period (data not shown).

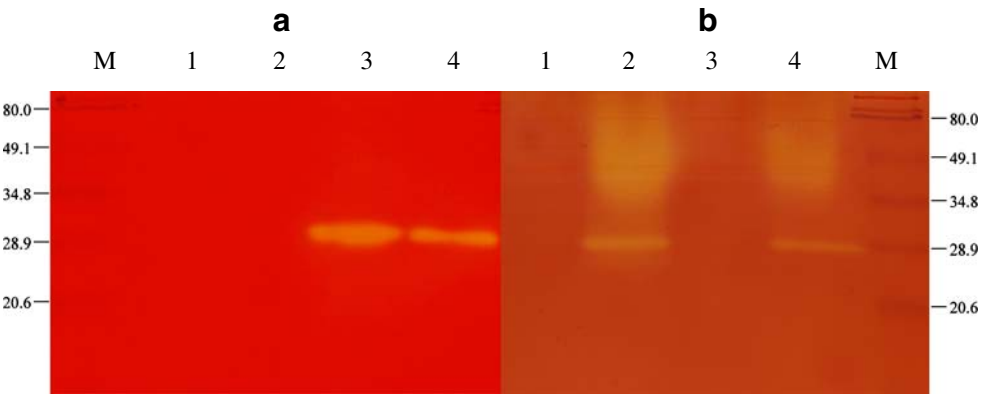


**Fig. 4** Zymogram of glucanase and xylanase in SDS gel electrophoresis. The intracellular extracts of *Lactobacillus reuteri* transformants were analyzed by glucan-containing (**a**) and xylan-containing (**b**) SDS-PAGE, renaturated, then zymography was performed. *Lane M*, molecular weight marker; *lane 1*, *L. reuteri* pNZ3004; *lane 2*, *L. reuteri* pNZJ021(xyn); *lane 3*, *L. reuteri* pNZJ023(glu); *lane 4*, *L. reuteri* pNZ-xyn/glu

**Table 2** The activity of fibrolytic enzymes of *L. reuteri* Pg4 transformants

| Strain | Enzyme activity (U ml$^{-1}$)[a] | | | |
|---|---|---|---|---|
| | β-Glucanase | | Xylanase | |
| | Intracellular | Extracellular | Intracellular | Extracellular |
| *L. reuteri* pNZ3004 | nd[b] | nd | nd | nd |
| *L. reuteri* pNZJ021 (xyn) | nd | nd | 1.54±0.24 | 2.37±0.19 |
| *L. reuteri* pNZJ023 (glu) | 1.06±0.09 | 1.69±0.20 | nd | nd |
| *L. reuteri* pNZ-xyn/glu | 0.72±0.09 | 0.86±0.09 | 0.98±0.14 | 1.50±0.15 |

[a] Enzyme activity was defined as that releasing 1 μmol of reducing sugar equivalents per minute from the respective substrate

[b] Not detected

In the *L. reuteri* pNZJ023(glu) culture, the β-glucanase activity in the extracellular fraction was 1.6-fold that of the intracellular fraction. Approximately 62% of β-glucanase activity was detected in the extracellular fraction (Table 2). The xylanase activity in the extracellular fraction of the *L. reuteri* pNZJ021(xyn) culture was 1.5-fold that of the intracellular fraction, with more than 60% of total xylanase activity detected in the extracellular fraction (Table 2). These results were consistent with the previous findings (Liu et al. 2005). Both β-glucanase and xylanase activities were observed for *L. reuteri* pNZ-xyn/glu. The enzyme activity levels for glucanase in the extracellular and intracellular fractions was 0.72±0.09 and 0.86±0.09 U ml$^{-1}$, respectively, while the related xylanase activity was 0.98±0.14 and 1.50±0.15 U ml$^{-1}$, respectively. More than 50% of the total β-glucanase and xylanase activity of the *L. reuteri* pNZ-xyn/glu culture was present in the extracellular fraction (Table 2). Furthermore, the specific enzyme activity levels for glucanase and xylanase in the intracellular fractions of *L. reuteri* pNZ-xyn/glu was 1.01±0.24 and 1.37±0.27 U mg$^{-1}$, respectively.

Plasmid stability in *L. reuteri*-transformed strains

In industrial fermentations, antibiotic selection is not economically feasible for the production of recombinant proteins. Therefore, the stability of the recombinant plasmids in *L. reuteri* was assessed in batch culture in the absence of antibiotics. As shown in Table 3, even after 50 generations, more than 55% of the CFU were resistant to erythromycin, and there was no significant difference between *L. reuteri* harboring pNZ3004, pNZJ021(xyn), pNZJ023(glu), and pNZ-xyn/glu ($p>0.05$). However, Em$^r$ and fibrolytic enzyme-producing cells were much lower in number than erythromycin-resistant cells during growth in the antibiotic-free broth. After 50 generations, approximately 70% of the Em$^r$ cells of *L. reuteri* pNZ-xyn/glu had lost their fibrolytic enzyme-producing phenotype (Table 3). Further, Em$^r$ *L. reuteri* pNZ-xyn/glu, which had lost the β-glucanase-producing phenotype, also lost its xylanase-producing phenotype. Thus, the plasmids used in this study showed modest segregational instability and much higher levels of structural instability under the conditions examined.

## Discussion

Probiotics, which contain viable organisms and exert a beneficial effect on animal performance through modification of gastrointestinal microflora, offer great potential both as feed additives and as replacements for antibiotics (Fuller 1989). Using probiotics that secrete specific enzymes can provide additional benefits by reducing the cost of enzyme supplementation in that these enzyme genes could be replicated and expressed by the probiotics within the digestive tract. In addition, probiotics could directly deliver the enzyme to the target site, typically the intestine, where most of the feed digestion and nutrient absorption occurs. To this end, we successfully cloned two rumen microbial fibrolytic enzyme genes into a probiotic *L. reuteri* strain and demonstrated that these *L. reuteri*-transformed strains not only acquired the capacity to break down soluble β-glucan or xylan but also showed high adhesion efficiency to mucin and mucus and resistance to bile salt and acid (Liu et al. 2005). We also demonstrated that the supplementation of barley-based poultry diets with *L. reuteri* pNZJ023(glu) could decrease digesta viscosity and improve body weight gain and feed-conversion efficiency in broilers 0–6 weeks of age (Yu et al. 2007). By contrast, however, supplemen-

**Table 3** Stability of plasmids in *L. reuteri* pNZ-xyn/glu under nonselective growth conditions

| Strain | $n$[a] | Em$^r$ (%) | Em$^r$, β-glucanase$^+$ (%) | Em$^r$, xylanase$^+$ (%) |
|---|---|---|---|---|
| *L. reuteri* pNZ-xyn/glu | 0 | 100 | 100 | 100 |
| | 25 | 75.1±2.9 | 41.9±0.1 | 41.9±0.1 |
| | 50 | 57.2±2.3 | 15.9±0.1 | 15.9±0.1 |

[a] $n$ Number of generations

tation of *L. reuteri* pNZJ021(xyn) in wheat-based poultry diets improved body weight gain in the broilers at only 0–3 weeks but not at 4–6 weeks (Liu et al. 2007). In certain regions of the USA and in European countries, wheat is the major feed grain used in poultry diets. Although arabinoxylans are the main wheat polysaccharides, significant amounts of β-glucan and cellulose are also present (Steenfeldt et al. 1995). Therefore, the combinations of fibrolytic enzymes with xylanase, β-glucanase, and cellulase are more effective than supplementation with solely xylanases at improving nutrient utilization in broilers fed wheat-based diets (Meng et al. 2005). In this study, we successfully cloned and coexpressed rumen microbial β-glucanase and xylanase genes in *L. reuteri*. To the best of our knowledge, and following a thorough review of the relevant literature, this is the first report of successful coexpression of β-glucanase and xylanase in intestinal lactobacilli. Although previous studies have demonstrated that *L. reuteri* pNZJ021(xyn) and *L. reuteri* pNZJ023(glu) could survive and secrete xylanase and glucanase in the gastrointestinal track of broilers (Liu et al. 2007; Yu et al. 2007), future research should be directed at evaluation of the *L. reuteri* glucanase–xylanase-coexpressing strain as a probiotic in poultry diets.

The development of *Lactobacillus* strains expressing heterologous proteins at sufficient levels for application in industry has been hindered by the lack of detailed knowledge of gene expression control in these organisms. They are also generally considered to be poor protein secretors (Kerovuo and Tynkkynen 2000). Low-level expression and secretion of heterologous proteins are described in most publications on *Lactobacillus*; however, efficient expression and secretion have only recently been reported (Kerovuo and Tynkkynen 2000; Bates et al. 1989). In this study, *L. lactis lac*A promoter and its secretion signal were used to constitutively express and secrete fibrolytic enzymes in *L. reuteri*. Further, it was demonstrated that the *L. reuteri* pNZ-xyn/glu-transformed strains were able to secrete fibrolytic enzymes into the medium and that the capacity to break down soluble β-glucan and xylan was acquired. Future research on utilization of different promoters may further increase expression of the fibrolytic enzyme in *Lactobacillus*.

Plasmid instability constitutes a major problem for industrial utilization of many recombinant microorganisms. The instability of a recombinant plasmid in a microbial culture may reduce the overall levels of the desired product in the bioprocess. This leads to lower specific activities and increases production costs as growth substrates are consumed by nonproductive cells that may have a significant growth rate advantage over cells harboring the intact recombinant plasmids (Nayak and Vyas 1999). Two types of plasmid instability, segregational and structural, occur frequently. Segregational instability is defined as the loss of the entire plasmid from the cells because of defective partitioning, whereas structural instability results in a plasmid population carrying structural rearrangements or deletions (Nugent et al. 1983). Studies have shown that plasmid stability is determined by many factors, such as plasmid load and copy number, replication pattern, substrate type, medium composition, host inheritable background, culture conditions and temperature, and expression protein toxicity (Nayak and Vyas 1999; Xu et al. 2006). The plasmids used in this study showed modest segregational instability and much higher levels of structural instability under the conditions examined. Cordes et al. (1996) indicated that both divergent transcription and the export of plasmid-specified proteins interfere with the structural stability of a pWVO1-derived plasmid. The explanation for these phenomena is that altered local DNA supercoiling is an important cause of plasmid deletion formation. Transcription, in particular the divergent form, induces positively and negatively supercoiled domains in plasmids (Drlica 1992). Expression of plasmid-specified membrane and exported proteins also has a drastic effect on local supercoiling through anchoring of the transcription ensemble to the membrane (Lynch and Wang 1993). Local hypersupercoiling may induce topoisomerase-dependent nicking activity if the plasmid becomes anchored to the membrane. Through uncoupling of the nicking and closing activities of DNA topoisomerase I, free 3′-OH ends may become available for processing by exonucleases, producing single-stranded DNA gaps and, finally, deletions (Cordes et al. 1996). The pNZ3004 series plasmids used in this study are derived from pWVO1. Two of the phenomena mention above applied to plasmid pNZ3004: (1) divergent transcription of truncated *lac*I and recombinant protein genes and (2) directed export of the recombinant proteins by the *lac*A signal sequence. We constructed a pNZJ021(xyn) derivative that lacks the entire *lac*A signal sequence and found that xylanase xynCDBFV was mainly present in the intracellular extract of the *L. reuteri* transformants (data not shown). Furthermore, lower levels of structural plasmid instability were demonstrated for the pNZJ021(xyn) variant, with a deletion in the *lac*A signal sequence relative to the parental pNZJ021(xyn) (data not shown). Further investigation is required to improve the plasmid DNA stability in the transformed *L. reuteri* cells under an antibiotic-free culture condition.

In conclusion, we have successfully cloned the rumen microbial xylanase and β-glucanase genes in a probiotic *L. reuteri* strain, and we have demonstrated that these heterologous fibrolytic enzyme genes are functionally co-expressed by the transformed *L. reuteri* strain. New studies for evaluating the *L. reuteri* glucanase–xylanase-coexpressing strain as a probiotic in poultry diets are now in progress.

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