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Expression of Rumen Microbial Fibrolytic Enzyme Genes in Probiotic *Lactobacillus reuteri*

Je-Ruei Liu,[1,3] Bi Yu,[2] Fu-Hwa Liu,[1] Kuo-Joan Cheng,[1] and Xin Zhao[3]*

Institute of BioAgricultural Sciences, Academia Sinica, Taipei, Taiwan[1]; Department of Animal Science, College of Agriculture and Natural Resources, National Chung-Hsing University, Taichung, Taiwan[2]; and Department of Animal Science, Faculty of Agricultural and Environmental Sciences, McGill University, Ste-Anne-de-Bellevue, Quebec, Canada[3]

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This study was aimed at evaluating the cloning and expression of three rumen microbial fibrolytic enzyme genes in a strain of *Lactobacillus reuteri* and investigating the probiotic characteristics of these genetically modified lactobacilli. The *Neocallimastix patriciarum* xylanase gene *xynCDBFV*, the *Fibrobacter succinogenes* β-glucanase (1,3-1,4-β-D-glucan 4-glucanohydrolase [EC 3.2.1.73]) gene, and the *Piromyces rhizinflata* cellulase gene *eglA* were cloned in a strain of *L. reuteri* isolated from the gastrointestinal tract of broilers. The enzymes were expressed and secreted under the control of the *Lactococcus lactis lacA* promoter and its secretion signal. The *L. reuteri* transformed strains not only acquired the capacity to break down soluble carboxymethyl cellulose, β-glucan, or xylan but also showed high adhesion efficiency to mucin and mucus and resistance to bile salt and acid.

Animal agriculture relies heavily on antibiotics, both for treatment of diseases and for growth promotion. With increasing public concerns associated with antibiotic resistance, the ban on subtherapeutic antibiotic usage in Europe and the potential for a ban in other regions of the world, there is increasing pressure to reduce the use of antibiotics in feed. Addition of probiotics to feed is one of the alternatives to be used as a replacement for antibiotics. There is sufficient evidence to show that probiotics are effective in enhancing the immune system, increasing body weight gain, reducing diarrhea, and improving feed conversion efficiency (27, 28). Research on animal probiotics is mainly focused on calves, pigs, and poultry. The currently used or researched probiotics are mostly selected from native gut microflora, and the selection of optimal strains has often been empirical. It has been speculated that more efficacious probiotics can be developed through genetic modification. Examples of such genetically modified probiotics include strains that produced antibodies, enzymes for detoxification, and cytokines for immune intervention (38).

Cereals, such as barley, wheat, rye, and oats, are major feed components for monogastric animals. The cell walls of cereals are primarily composed of carbohydrate complexes referred to as nonstarch polysaccharides (NSP), which include β-glucans in barley and wheat and arabinoxylans in rye and oats (11). It has been demonstrated that the antinutritive effect of β-glucans and arabinoxylans is related to their low digestibility and their propensity to form high-molecular-weight viscous aggregates in the gastrointestinal tract (8). This reduces the rate of passage, decreases diffusion of digestive enzymes, promotes endogenous losses, and stimulates bacterial proliferation (3). Therefore, it is not surprising that addition of specific enzymes such as xylanase or β-glucanase into wheat- or barley-based diets for nonruminant animals decreases viscosity and consequently reduces the antinutritional effect of NSP, leading to better production performance (23). However, enzyme supplementation substantially increases the cost of feed and is only used for a short-term solution in enhancing digestion of cereals. 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 (7).

The rumen microbial population represents a rich and underutilized source of novel enzymes with tremendous potential for industrial application. The enzyme activities confirmed to exist in the rumen are diverse and include plant cell wall polymer-degrading enzymes (e.g., cellulase, β-glucanase, xylanase, and pectinase), amylase, protease, phytase, and specific plant toxin-degrading enzymes (e.g., tannases) (6). At least 100 different genes, the majority of which encode enzymes involved in fiber degradation, have been cloned from ruminal microbes. Most of these have been isolated from a small group of bacterial species, including *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes*, *Prevotella ruminicola*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Actinomyces* (6). Researchers have only recently turned to study the genetics of anaerobic fungi and protozoa from rumen. In a previous study, we reported isolation of a cellulase gene, *eglA*, from the ruminal fungus *Piromyces rhizinflata.* The cellulase gene *eglA* showed carboxymethyl cellulase (CMCase), β-glucanase, and xylanase activity amounting to 345, 576, and 106 units per mg of protein, respectively (22). We also reported isolation of a xylanase gene from the ruminal fungus *Neocallimastix patriciarum* strain 27 and used site-directed mutagenesis to produce a thermostable and alkalophilic mutant xylanase gene (*xynCDBFV*). The xylanase gene *xynCDBFV* showed remarkably high activities, amounting to 10,128 units per mg of protein (5, 21).

To date, several previous studies have investigated the expression of bacterial β-glucanase genes in lactobacilli. Most of

* Corresponding author. Mailing address: Department of Animal Science, McGill University, 21111 Lakeshore Rd., Ste-Anne-de-Bellevue, Quebec, Canada H9X3V9. Phone: (514) 398-7975. Fax: (514) 398-7964. E-mail: Xin.Zhao@mcgill.ca.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant feature(s)	Source or reference
Strains		
E. coli DH5α	Cloning host; F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(rk^- mk^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen, Carlsbad, CA
L. reuteri Pg4	*L. reuteri* isolate from the gastrointestinal tract of broilers	B. Yu, unpublished data
L. reuteri pNZ3004	*L. reuteri* Pg4 carrying pNZ3004	This study
L. reuteri pNZJ021(xynCDBFV)	*L. reuteri* Pg4 carrying pNZJ021	This study
L. reuteri pNZJ023(glu)	*L. reuteri* Pg4 carrying pNZJ023	This study
L. reuteri pNZJ068(eglA)	*L. reuteri* Pg4 carrying pNZJ063	This study
Plasmids		
pxyn-CDBFV	Amp^r; source of *Neocallimastix patriciarum* xylanase gene (*xynCDBFV*); 5.6 kb	5
pJI10	Amp^r; source of *Fibrobacter succinogenes* β-glucanase gene; 4.1 kb	16
pGEX-EGLA	Amp^r; source of *Piromyces rhizinflata* cellulase gene (*eglA*); 5.9 kb	22
pNZ3004	Cm^r Em^r; *E. coli-L. reuteri* shuttle vector; 5.0 kb	41
pNZJ021(xynCDBFV)	Cm^r Em^r; pNZ3004 containing *N. patriciarum* xylanase gene (*xynCDBFV*); 5.7 kb	This study
pNZJ023(glu)	Cm^r Em^r; pNZ3004 containing *F. succinogenes* β-glucanase gene; 5.8 kb	This study
pNZJ068(eglA)	Cm^r Em^r; pNZ3004 containing *P. rhizinflata* cellulase gene (*eglA*); 6.0 kb	This study

these studies described the genetic manipulation in *Lactobacillus plantarum*, which is the primary bacterium used in silage fermentation. However, the level of the heterologous β-glucanase gene expressed in *L. plantarum* was usually low (31, 33, 34). Only a few studies were focused on expression of bacterial β-glucanase genes in intestinal lactobacilli strains (7, 15). To the best of our knowledge, however, expression of a xylanase gene in intestinal lactobacilli has not been reported previously.

In the present study, we describe the cloning, expression, and secretion of the xylanase gene *xynCDBFV* from the ruminal fungus *N. patriciarum*, the β-glucanase (1,3-1,4-β-D-glucan 4-glucanohydrolase [EC 3.2.1.73]) gene from the ruminal bacterium *F. succinogenes*, and the cellulase gene *eglA* from the ruminal fungus *P. rhizinflata* in a strain of *Lactobacillus reuteri* isolated from the gastrointestinal tract of healthy broilers. We also examined the probiotic characteristics such as adherence capability, acid tolerance, and bile salt tolerance of these genetically modified *L. reuteri* strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Lactobacillus reuteri* Pg4 and *Escherichia coli* were grown at 37°C in MRS broth and Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.), respectively. Agar plates were prepared by the addition of agar (1.5% [wt/vol]; Difco) to each broth.

DNA isolation and manipulation. Plasmid DNA was isolated from *E. coli* by the alkali lysis method (4). *Lactobacillus* plasmid DNA was isolated according to the method described by O'Sullivan and Klaenhammer (26). Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs, Inc. (Beverly, Mass), and were used according to the manufacturer's instructions. All other DNA manipulations were performed by established procedures (32).

Construction of xylanase, β-glucanase, and cellulase expression plasmids. The DNA sequences encoding xylanase, β-glucanase, and cellulase genes were amplified by PCR. The oligonucleotide forward primer 21F (5′ GCAGTCGAC CCAAAGTTTCTGTAGTTCAG 3′) and the reverse primer 21R (5′ TTCCTG CAGTTAATCACCAATGTAAACCTTTG 3′) were designed to amplify the *N. patriciarum* xylanase gene *xynCDBFV* (GenBank accession number AF123252) in pxyn-CDBFV (5). The primers 23F (5′ GCAGTCGACCGTTAG CGCAAAGG 3′) and 23R (5′ TTCCTGCAGTCACGATTGCGGAG 3′) were used to amplify the *F. succinogenes* β-glucanase gene (GenBank accession number M33676) in pJI10 (16). The primers 68F (5′ GCAGTCGACATGATCCGT GATATTT 3′) and 68R (5′ TTCCTGCAGTTATTCCTCTGTTTCTT 3′) were employed to amplify the *P. rhizinflata* cellulase gene *eglA* (GenBank accession number AF094757) in pGEX-EGLA (22). These primers were designed to place a SalI site (underlined) at the 5′ end and a PstI site (underlined) at the 3′ end of the PCR product, respectively. The PCR fragments encoding xylanase, β-glucanase, and cellulase were digested with SalI and PstI and ligated with SalI-PstI-digested pNZ3004 (41) to generate pNZJ021(xynCDBFV), pNZJ023(glu), and pNZJ068(eglA), respectively.

Transformation of plasmid DNA. Competent *E. coli* cells were prepared and transformed by standard techniques (32). Transformants were selected on LB agar plates containing erythromycin (100 μg/ml) (Sigma Chemical Co., St. Louis, MO). Plasmids expressing the desired enzyme activity in *E. coli* were electroporated into *L. reuteri* Pg4 as described by Serror et al. (36). After electroporation, the *L. reuteri* Pg4 transformants were incubated in MRS broth containing 10 mM $MgCl_2$ at 37°C for 3 h. Then, they were spread on MRS agar plates containing erythromycin (10 μg/ml) and incubated at 37°C until the appearance of transformants, usually 24 to 48 h.

Screening of xylanase-, β-glucanase-, and CMCase-positive *Lactobacillus reuteri* Pg4 transformants on agar plates. *Lactobacillus reuteri* Pg4 transformants were detected on replica plates by making an overlay of MRS agar containing 0.01% oat-spelled xylan (Sigma), 0.1% barley β-glucan (Megazyme, Wicklow, Ireland), or 0.1% carboxymethyl cellulose (CMC; Sigma), respectively. After overnight incubation, the plate was stained with a 1% (wt/vol) Congo red (Sigma) for 1 h and destained with 1 M NaCl solution for 2 h (40). Colonies forming yellow halos were selected for further analyses.

Xylanase, β-glucanase, and CMCase production by *Lactobacillus reuteri* Pg4 transformed strains. For estimation of the intra- and extracellular enzymes levels, each transformant screened above was transferred to MRS broth containing erythromycin (10 μg/ml) and incubated at 37°C for 24 h. The xylanase, β-glucanase, and CMCase activities of each *L. reuteri* cell culture were estimated for culture supernatant and whole-cell extract. The culture was centrifuged at 5,000 × *g* for 20 min at 4°C. The supernatant was collected. The cell pellet was resuspended in 50 mM sodium citrate buffer (pH 5.0) and sonicated for 10 min with an ultrasonicator (model XL; Misonix, Farmingdale, NY). Cellular debris was subsequently removed by centrifugation at 5,000 × *g* for 20 min, and the clarified extract was retained for subsequent enzyme analyses. Xylanase, β-glucanase, and CMCase activities were determined by measuring the amounts of reducing sugar liberated by fractions incubated with 0.5% (wt/vol) xylan, β-glucan, and CMC in 50 mM sodium citrate buffer (pH 5.0), respectively. After incubation for 20 min at 40°C, the reaction was stopped by being boiled for 10 min. The reducing sugar produced was estimated by the dinitrosalicyclic acid reagent (20). One unit of the fibrolytic enzyme activity was defined as that releasing 1 μmol of reducing sugar equivalents per min from the respective substrate under the assay conditions.

Plasmid stability assay. The stability of plasmids harbored by *L. reuteri* Pg4 was investigated following the modified method of Gurakan et al. (13). *L. reuteri* Pg4 strains harboring pNZ3004, pNZJ021(xynCDBFV), pNZJ023(glu), and

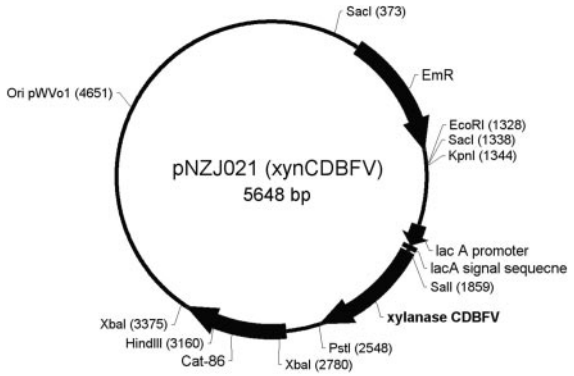

FIG. 1. *Lactobacillus* expression plasmid harboring the *Neocallimastix patriciarum* xylanese gene *xynCBDFV*.

pNZJ068(eglA) were 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 erythromycin-resistant (Em^r) cells. The colonies growing under erythromycin selection underwent the Congo red dye assay to detect the presence of fibrolytic enzyme activity.

In vitro adhesion assay. Partially purified porcine gastric mucin (Sigma) was purified by the method of Glenister et al. (12). Mucus was isolated from the small intestinal wall of 35-day-old chickens by the method of Gusils et al. (14). Both small intestinal mucus and gastric mucin were conjugated with horseradish peroxidase (HRP) by the method of Rojas and Conway (29). The HRP-conjugated mucus and gastric mucin were dialyzed against phosphate-buffered saline (PBS; 0.01 M, pH 7.4), mixed with equal volumes of 80% glycerol, and stored at −20°C until being used. For preparation of formaldehyde-killed bacteria, *Lactobacillus* cells were washed once in PBS and diluted to an optical density at 600 nm (OD_{600}) of 0.5 in PBS containing 0.5% formaldehyde. After incubation overnight at 4°C, the bacteria were washed and diluted to an OD_{600} of 0.3 in sterile PBS. Ninety-six-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 200 μl of formaldehyde-killed *Lactobacillus* in PBS and incubated for 3 h at 37°C. After 2 days at 4°C, plates were washed three times in PBS supplemented with 0.05% Tween 20 (PBST) and refilled with 250 μl of blocking buffer (PBS containing 1% bovine serum albumin). After incubation at 37°C for 2 h, the microtiter plates, coated with lactobacilli, were washed three times with PBST and incubated at 37°C for 1 h with 200 μl of HRP-labeled mucin and mucus solution (1 μg/μl of blocking buffer). Following another three washes with PBST, 100 μl of 3,3′,5,5′-tetramethylbenzidine (Sigma) solution was added to each well. After incubation at room temperature for 5 min, color development was stopped by the addition of 100 μl of 1 N HCl. The absorbance of each well at a wavelength of 450 nm was measured using a Multiskan Ascent ELISA reader (Labsystems, Helsinki, Finland).

Tolerance to acidic pH. Acid tolerance of *L. reuteri* Pg4 strains was determined according to the method of Ehrmann et al. (10). A 1-ml aliquot of the *Lactobacillus* cell cultures was centrifuged at 5,000 × *g* for 10 min at 4°C, and the pellets were washed twice in sterile PBS and resuspended in the same buffer. Each strain was diluted 1/100 in pH-adjusted MRS broth (pH 2.0 and 3.0) and incubated at 37°C. Aliquots of 0.1 ml (each) were removed after 0, 30, 60, and 180 min for determination of total viable count in MRS agar plates.

Tolerance of bile salt. Bile salt tolerance of *L. reuteri* Pg4 strains was determined following the modified method of Ehrmann et al. (10). A 1-ml aliquot of the *Lactobacillus* cell cultures was inoculated into MRS broth containing 0.3 or 0.5% ox gall (Sigma) and incubated at 37°C. The OD_{600} values were monitored for 10 h.

Statistical analysis. All results were analyzed using the general linear-model procedure available from the Statistical Analysis System software package, version 8.1 (37). The Duncan multiple-range test (24) was used to detect differences among treatment means. Each experiment was conducted in triplicate and repeated three times.

RESULTS

Cloning of rumen microbial xylanase, β-glucanase, and cellulase genes in *L. reuteri* Pg4. To express the xylanase, β-glucanase, and cellulase genes in *L. reuteri* Pg4, three recombinant plasmids were constructed with *E. coli* DH5α as a host. The xylanase gene *xynCDBFV* of *Neocallimastix patriciarum* was cloned as a 0.7-kb SalI-PstI fragment into *Lactobacillus* expression vector pNZ3004, generating pNZJ021(xynCDBFV) (Fig. 1). The β-glucanase gene of *Fibrobacter succinogenes* was cloned as a 0.75-kb SalI-PstI fragment into pNZ3004, generating pNZJ023(glu). The cellulase gene *eglA* of *Piromyces rhizinflata* was cloned as a 1.0-kb SalI-PstI fragment into pNZ3004, generating pNZJ068(eglA).

The expression plasmids pNZJ021(xynCDBFV), pNZJ023(glu), and pNZJ068(eglA) were electroporated to *L. reuteri* Pg4 with efficiencies similar to that of pNZ3004 (5×10^2 to 10×10^2 transformants per μg of DNA). The Congo red plate assay was applied to confirm the fibrolytic enzyme activities of transformants. Xylanase, β-glucanase, and CMCase activities were observed for *L. reuteri* pNZJ021(xynCDBFV), *L. reuteri* pNZJ023(glu), and *L. reuteri* pNZJ068(eglA), respectively.

In the culture of *L. reuteri* pNZJ021(xynCDBFV), the xylanase activity in the extracellular fraction was 1.5-fold higher than that in the intracellular fraction (Table 2). Similarly, β-glucanase activity in the extracellular fraction from the culture of *L. reuteri* pNZJ023(glu) was 1.6-fold higher than that in the intracellular fraction (Table 2). Both β-glucanase and CMCase activities were observed for *L. reuteri* pNZJ068(eglA). More than 50% of the total β-glucanase and CMCase activities of the culture of *L. reuteri* pNZJ068(eglA) were present in the extracellular fraction (Table 2). No xylanase activity was detected from *L. reuteri* pNZJ068(eglA) culture.

Plasmid stability in *L. reuteri* Pg4. In industrial fermentations, antibiotic selection is not economically feasible for production of recombinant proteins. Therefore, stability of recom-

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

Strain of *L. reuteri*	Enzyme activity (U/ml)[a]					
	Xylanase		β-Glucanase		CMCase	
	Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular
pNZ3004	ND[b]	ND	ND	ND	ND	ND
pNZJ021(xynCDBFV)	1.48 ± 0.42	2.33 ± 0.24	ND	ND	ND	ND
pNZJ023(glu)	ND	ND	1.03 ± 0.07	1.66 ± 0.23	ND	ND
pNZJ068(eglA)	ND	ND	3.14 ± 0.75	3.15 ± 0.11	1.13 ± 0.16	1.36 ± 0.10

[a] Enzyme activity was defined as that releasing 1 μmol of reducing sugar equivalents per min from the respective substrate.
[b] ND, not detectable.

TABLE 3. Stability of plasmids in *L. reuteri* Pg4 under nonselective growth conditions

Strain	n^a	Em^r (%)	Em^r, fibrolytic enzyme positive (%)
L. reuteri pNZ3004	0	100	0
	25	76.0 ± 2.0	0
	50	56.3 ± 2.0	0
L. reuteri pNZJ021(xynCDBFV)	0	100	100
	25	76.5 ± 1.3	48.7 ± 1.3
	50	56.9 ± 1.3	16.2 ± 0.8
L. reuteri pNZJ023(glu)	0	100	100
	25	77.6 ± 1.0	40.8 ± 2.4
	50	55.9 ± 4.7	14.1 ± 0.9
L. reuteri pNZJ068(eglA)	0	100	100
	25	71.9 ± 1.5	41.2 ± 0.8
	50	57.7 ± 3.1	16.1 ± 0.3

[a] *n*, number of generations.

binant plasmids in *L. reu*teri was assessed in batch culture in the absence of antibiotic. As shown in Table 3, >55% of the CFU was resistant to erythromycin, even after 50 generations. There were no significant differences among *L. reuteri* harboring pNZ3004, pNZJ021(xynCDBFV), pNZJ023(glu), and pNZJ068(eglA) ($P > 0.05$). However, erythromycin-resistant and fibrolytic enzyme-producing cells were much lower in number than erythromycin-resistant cells during the growth in antibiotic-free broth. After 50 generations, approximately 70% of the erythromycin-resistant cells lost their fibrolytic enzyme-producing phenotype (Table 3). Thus, the plasmids used in this study showed modest segregational instability and much higher levels of structural instability under the conditions examined.

Adhesion of *L. reuteri* Pg4 transformed strains to small intestinal mucus and gastric mucin. Association of *Lactobacillus* with an epithelial or mucosal surface is undoubtedly an important determinant in its colonization of the gastrointestinal tract. As shown in Fig. 2, the adhesion of *L. casei* ATCC 393 to immobilized pig gastric mucin and chicken small intestinal mucus was very low. On the other hand, all *L. reuteri* Pg4 strains adhered efficiently to both pig gastric mucin and chicken small intestinal mucus. There was no significant difference between the adherence ability of *L. reuteri* Pg4 transformed strains and that of the *L. reuteri* Pg4 wild type ($P > 0.05$). This result indicated that the introduction of a heterologous fibrolytic enzyme gene into *L. reuteri* Pg4 did not alter its adherence ability.

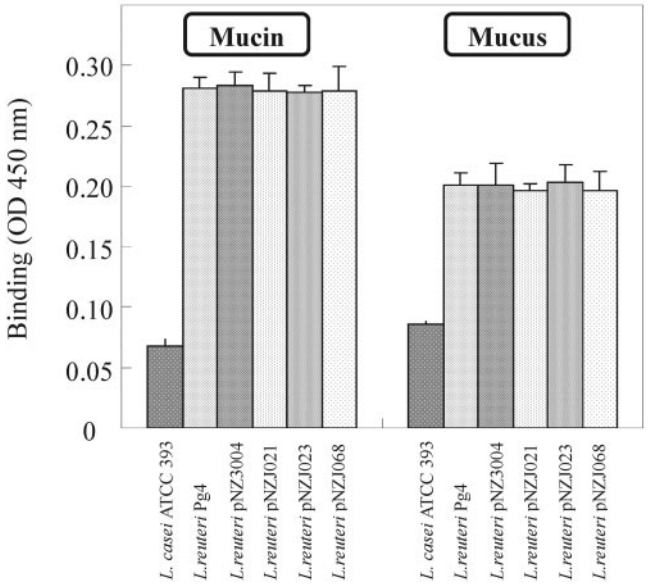

FIG. 2. Adhesion of *L. reuteri* Pg4 strains to immobilized porcine gastric mucin or chicken small intestinal mucus.

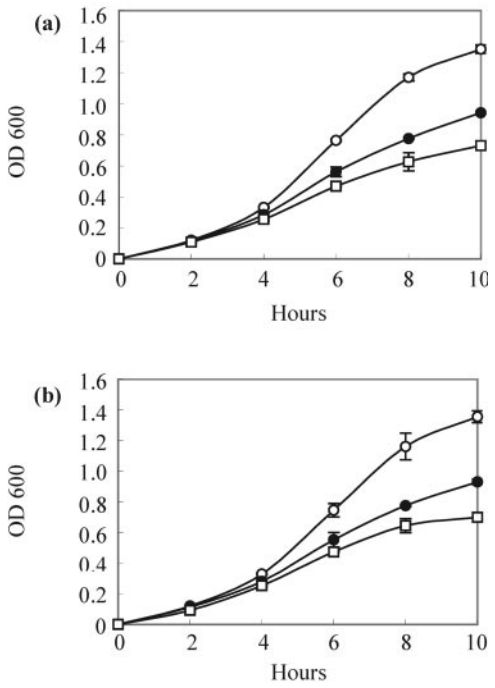

FIG. 3. Growth of lactobacilli cultured in MRS medium (○), MRS containing 0.3% oxgall (●), and MRS containing oxgall (□). *L. reuteri* Pg4 (a); *L. reuteri* pNZ3004 (b).

Tolerance of *L. reuteri* Pg4 transformed strains to different pH values and growth with bile salt. The results for acid tolerance showed that approximately 35% or 50% of all the *L. reuteri* Pg4 strains tested survived after a 180-min incubation at pH 2.0 and 3.0, respectively. There was no significant difference between the acid tolerance of *L. reuteri* Pg4 transformed strains and that of the *L. reuteri* Pg4 wild type ($P > 0.05$) (Table 4).

Resistance to bile salt is important for *Lactobacillus* to survive and grow in the intestinal tract. The growth curves of *L. reuteri* Pg4 and *L. reuteri* pNZ3004 in the presence of 0.3% and 0.5% ox gall are shown in Fig. 3. There was no significant difference ($P > 0.05$) between the viable counts of *L. reuteri* Pg4 and that of *L. reuteri* pNZ3004 after culture in MRS broth containing 0.5% ox gall for 10 h. The bile salt resistance of the other *L. reuteri* Pg4 transformed strains [*L. reutrei* pNZJ021(xynCDBFV), *L. reutrei* pNZJ023(glu), and *L. reutrei* pNZJ068(eglA)] was also similar to that of the *L. reuteri* Pg4 wild type (data not shown). All of the *L. reuteri* Pg4 strains tested in this study survived after an incubation period of 10 h in MRS broth containing 0.5% ox gall.

TABLE 4. Survival of *L. reuteri* Pg4 strains after incubation at various pH values

Strain	Survival (%) after incubation at:					
	pH 2.0			pH 3.0		
	30 min	60 min	180 min	30 min	60 min	180 min
L. reuteri Pg4	83.2 ± 0.6	53.6 ± 6.2	34.1 ± 0.5	87.0 ± 1.7	73.3 ± 1.3	53.5 ± 1.7
L. reuteri pNZ3004	83.9 ± 1.4	57.0 ± 3.3	35.4 ± 1.6	86.8 ± 3.8	74.9 ± 3.5	55.8 ± 3.1
L. reuteri pNZJ021(xynCDBFV)	81.8 ± 2.3	55.4 ± 3.4	34.6 ± 3.8	86.4 ± 2.9	73.3 ± 0.8	54.3 ± 3.2
L. reuteri pNZJ023(glu)	82.6 ± 2.5	53.5 ± 0.5	35.8 ± 1.9	86.3 ± 1.6	72.9 ± 2.3	53.1 ± 1.9
L. reuteri pNZJ068(eglA)	81.9 ± 2.9	55.5 ± 1.3	35.7 ± 2.4	86.6 ± 0.9	71.2 ± 1.8	52.1 ± 2.5

DISCUSSION

The potential of enzyme supplementation for improving the efficiency of feed utilization by nonruminant livestock is increasingly recognized. Enzymes have changed the way nutritionists select ingredients for a nutritionally balanced, least-cost diet. Using feed enzymes can also alleviate the problem of environmental pollution and control certain diseases (3). Other than phytase to release available phosphorus from phytate hydrolysis, enzymes in the feed industry have mostly been used for poultry and pigs to neutralize the effects of the viscous nonstarch polysaccharides in cereals such as barley and wheat (3). These antinutritive carbohydrates are undesirable, as they reduce digestion and absorption of all nutrients in the diet, especially fat and protein (8). It has been demonstrated that addition of specific enzymes such as xylanase or β-glucanase into wheat- or barley-based diets reduces the antinutritional effect of NSP and leads to better performance of nonruminants (23).

Probiotics are now being considered as one of the alternatives to antibiotics for livestock production. Using probiotics with high activities of specific enzymes can provide additional benefits in reducing the cost of enzyme supplementation. The rumen microbial population presents a rich source of novel enzymes with tremendous potential for industrial applications. It is reported that cellulases and xylanases produced by ruminal fungi are among the most active fibrolytic enzymes described to date (35). To this end, we have successfully cloned and expressed three rumen microbial fibrolytic enzyme genes into a strain of *Lactobacillus reuteri* in this study. Further, we have demonstrated that the transformed *L. reuteri* strains not only acquired the capacity to break down soluble CMC, β-glucan, or xylan, but also showed no difference from the parent strain in terms of the efficiency of adhesion to mucin and mucus and resistance to bile salt and acid.

To the best of our knowledge, this is the first report of successful expression of xylanase in intestinal lactobacilli. In addition, both the rumen fungal cellulase gene *eglA* and the rumen bacterial β-glucanase gene were expressed in a strain of *L. reuteri* isolated from the gastrointestinal tract of broilers. Interestingly, the extracellular β-glucanase activity of *L. reuteri* pNZJ068(eglA) was much higher than that of *L. reuteri* pNZJ023(glu) or those in previous studies in which the bacterial β-glucanase gene was transformed into *Lactobacillus*. Liu et al. (22) reported that the cellulase gene *eglA* also had xylanase activity. However, the xylanase activity of *L. reuteri* pNZJ068(eglA) was not detectable in this study. The exact reason for this discrepancy is not clear.

To date, only a few studies were focused on the expression of gram-positive bacterial β-glucanase genes in intestinal *Lactobacillus* strains (7, 15). Earlier studies described genetic manipulation of silage starter bacteria of *L. plantarum*. However, the expression level of heterologous glucanase genes was usually low (0.04 to 0.2 μmol of glucose equivalent per ml of supernatant per min) (31, 33, 34). The only studies to express heterologous fibrolytic enyzme genes in intestinal lactobacilli strains were reported by Heng et al. (15) and Cho et al. (7). Heng et al. (15) reported cloning and expression of a β-glucanase gene from *Bacillus macerans* in *L. reuteri*, but found that the enzyme activity was <0.5 nmol of glucose equivalent per ml of supernatant per min. Cho et al. (7) described cloning of a *Clostridium thermocellum* β-glucanase gene in *Lactobacillus gasseri* and *Lactobacillus johnsonii* and found efficient expression of β-glucanase in these lactobacilli strains with activity of 0.73 to 0.79 μmol of glucose equivalent per ml of supernatant per min.

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 secretors of protein (19). Low-level expression and secretion of heterologous proteins were described in most publications on *Lactobacillus*, but recently efficient expression and secretion have been reported (2, 19). Various inducible gene expression systems have been developed for lactic acid bacteria. These systems allow expression of homologous or heterologous genes based on promoters controlled by sugar, salt, temperature upshift, pH decrease, phage infection, or antibiotics (1). In this study, the *Lactococcus lactis lacA* promoter and its secretion signal were used to overexpress and secrete fibrolytic enzymes by *L. reuteri*. The *L. reuteri* transformed strains were able to secrete fibrolytic enzymes into the medium and acquired the capacity to break down soluble CMC, β-glucan, or xylan. Future research on using different promoters and/or secretion signal elements may further increase expression and secretion of the fibrolytic enzyme genes in *Lactobacillus*.

Plasmid instability constitutes a major problem for industrial utilization of many recombinant microorganisms. The scaling up of recombinant fermentation processes depends largely on the ability to control genetic parameters and to maintain a stable recombinant organism. Two types of plasmid instability occur frequently, segregational and structural (25). Segregational instability, which can be defined as the loss of the entire plasmid from the cells due to defective partitioning, is affected by multiple factors including host and plasmid genotype and

culture conditions such as temperature, pH, aeration, composition of the growth medium, and dilution rate. However, structural instability involves the rearrangement, loss, or insertion of plasmid DNA sequences, associated typically with transposition or recombination (25). Gurakan et al. (13) estimated the stability of a recombinant plasmid carrying α-amylase gene and found that the higher rate of retention of the antibiotic resistance gene than the α-amylase gene was due to the position of the antibiotic resistance gene closer to the origin of replication. The plasmids used in this study showed modest segregational instability and much higher levels of structural instability under the conditions examined. It could be suggested that under nonselective growth conditions the retention of the erythromycin resistance gene, rather than the fibrolytic enzymes gene, is higher because of its location close to the origin of replication.

The epithelial cells of the intestine are covered by a protective layer of mucus, which is a complex mixture of glycoproteins and glycolipids with the large glycoprotein mucin being the main component. Ability of probiotic bacteria to adhere to the intestinal mucus is considered important for transient colonization, antagonism against pathogens, modulation of the immune system, and enhanced healing of damaged gastric mucosa (39). *Lactobacillus casei* ATCC 393 had a low ability to adhere to immobilized pig gastric mucin and chicken small intestinal mucus in this study. The result is consistent with the report of Edelman et al. (9). *Lactobacillus reuteri* frequently occurs in the intestinal microflora of various mammals and has been widely regarded as a potential probiotic. Strains of this species have been shown to possess several properties believed to be important for its capacity to colonize. These are adherence to epithelial cells, production of an autoaggregation-promoting protein, and production of the antimicrobial substance reuterin (30). It has been reported that many strains of *L. reuteri* adhere to components in mucus (18). In this study, we not only cloned the rumen microbial fibrolytic enzymes genes in *L. reuteri* but also demonstrated that these heterologous genes did not affect the bacterial adherence ability. Further investigation is required to verify the colonization and the fibrolytic enzymes secretion of these *L. reuteri* Pg4 transformed strains in vivo.

Prior to reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach, where secretion of gastric acid represents a primary defense mechanism against the majority of ingested microorganisms (39). All of the *L. reuteri* Pg4 strains tested in this study survived after an incubation period of 180 min at pH 2.0 and 3.0. This indicated that *L. reuteri* Pg4 strains would have the potential to survive transit through the stomach and might possess the ability to reach the intestinal environment in where they can effectively work.

Once the bacteria reach the intestinal tract, their ability to survive depends on their resistance to bile. Bile entering the duodenal section of the small intestine has been found to reduce survival of bacteria. This is probably due to the fact that all bacteria have cell membranes consisting of lipids and fatty acids that are very susceptible to destruction by bile salts (17). Hence, tolerance of bile salts is a prerequisite for colonization and metabolic activity of probiotic bacteria in the small intestine of the host. All of the *L. reuteri* Pg4 strains tested in this study survived after an incubation period of 10 h in MRS broth containing 0.5% ox gall. Therefore, it is likely that *L. reuteri* Pg4 strains are able to survive in the intestine.

In conclusion, we not only cloned the rumen microbial xylanase, β-glucanase, and cellulase genes in *L. reuteri* Pg4 but also demonstrated that introduction of these heterologous fibrolytic enzyme genes into cells did not affect their adherence to mucin and mucus or tolerance of acid and bile salts. New studies for evaluating the ability of these *L. reuteri* Pg4 transformed strains to colonize the gastrointestinal epithelium and secrete the fibrolytic enzymes into gastrointestinal tract of chickens are now in progress.

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