Expression of *Clostridium thermocellum* Endoglucanase Gene in *Lactobacillus bulgaricus* and *Lactobacillus plantarum* and *in vitro* Survival Characteristics of the Transformed *Lactobacilli*

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*Lactobacillus bulgaricus*와 *Lactobacillus plantarum* 균주에서 *Clostridium thermocellum* 유래 endoglucanase의 발현과 발현 유산균의 *in vitro* 생존 특성

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ABSTRACT

Endoglucanase A from *Clostridium thermocellum* which is resistant to pancreatic proteinase was selected out of numbers cellulases then were expressed in *lactobacilli*. Recombinant *lactobacilli* expression vector, pSD1, harboring the endoglucanase gene from *C. thermocellum* under the control of its own promoter, was constructed. Both *L. bulgaricus* and *L. plantarum* were electrotransformed with pSD1. The endoglucanase activities of 0.120 and 0.144 U/ml were found in culture media of *L. bulgaricus* and *L. plantarum* containing pSD1, respectively. *In vitro* survival characteristics of the transformed lactobacilli were tested. Both *L. bulgaricus* and *L. plantarum* showed a similar resistance to low pH 3. Moreover, *L. plantarum* was bile-salt resistant in the presence of 0.3 and 1% oxgall. *L. bulgaricus* and *L. plantarum* showed a rather homogenous resistant pattern against the tested antibiotics. Both of the strains were resistant to amikacin, gentamicin, streptomycin, kanamycin, and colistin.
(**Key words** : Endoglucanase activity; *Clostridium thermocellum*; Survival of *Lactobacilli*)

Ⅰ. INTRODUCTION

Lactobacillus species are normal inhabitants of the gastrointestinal tracts of the mammalians and are main constituents of probiotics which are fed to newborn livestock with the aim of enhancing

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the immune system, increasing body weight gain, and improving feed conversion rates (Abe *et al*., 1995). Recently, it was suggested that more efficacious probiotics could be developed through the genetic modification of gastrointestinal strains of *lactobacilli* (Tannock, 1992). The modified strains would colonize in the digestive tract when the animal hosts consume and synthesize a novel product (Heng *et al*., 1997). But, so far, the development of genetically modified *lactobacilli* has been proved difficult, largely because suitable plasmid expression cassettes with strong promoters and signal sequences of *Lactobacillus* origin have not yet been established.

In mono-gastric animal, the major plant cell wall components of cereals, primarily β-glucans and arabinoxylans, form gel-like structures in the small intestines, trap nutrients, and therefore hinder enzymatic hydrolysis and absorption(Hall *et al*., 1993). The viscous polysaccharides can also cause severe gastrointestinal disorders (Hall *et al*., 1993). To improve the performances of non-ruminant animals, barley-based diets are often supplemented with endoglucanases and endoxylanases, particularly in poultry. However, enzyme supplementation substantially increases the cost of feed and thus is only used as a short-term solution in enhancing digestion of cereals. An alternative and less expensive strategy should be designed to develop *lactobacilli* with the capacity to digest plant structural carbohydrates by the introduction of heterologous genes encoded by polysaccharide-degrading enzymes.

The expression of cellulases and xylanases genes in *lactobacilli* is mainly described in silage starter and the probiotic bacteria, *L. plantarum*, while little information is known about the other probiotic bacteria, *L. bulgaricus* (Bates *et al*., 1989; Scheirlinck *et al*., 1990).

Clostridium thermocellum, a gram-positive thermophilic anaerobe, secretes a highly potent and thermostable cellulase complex (Cornet *et al*., 1983). The *cel* A gene codes for a previously purified endoglucanase (1,4-β-D-glucan glucanohydrolase EC 3.2.1.4.), endoglucanase A, which has a MW of 56,000 and is one of the major endoglucanases secreted by *C. thermocellum* (Petre *et al*., 1981).

In this study, we expressed the *C. thermocellum* endoglucanase gene in *L. plantarum* and *L. bulgaricus* and also examined probiotic characteristics such as acid and bile-salt tolerances and antibiotic susceptibility using these genetically modified *lactobacillus* strains.

Ⅱ. MATERIALS AND METHODS

1. Bacterial strains, plasmids, and media

Escherichia coli MC 1061 (Casadaban and Cohen, 1980) was used as a host for the construction of recombinant expression vectors. *L. bulgaricus* KCTC 3188 and *L. plantarum* KCTC 1048 were used as hosts for endoglucanase expression. Plasmids pCT104 (Beguin *et al*., 1985) and pNZ123 (de Vos *et al*., 1992) were used for the construction of recombinant expression vector. *Lactobacilli* were cultivated in MRS media (Difco) at 37℃. *Lactobacillus* transformants were selected on MRS plates with 0.5% CMC (carboxymethyl cellulose) solidified with 1.5% agar. Antibiotics were amended with 10 μg/ml chloramphenicol (Cm) were amended to select of *lactobacilli* transformant and the maintenance of plasmid.

2. Isolation of plasmid DNA and DNA manipulation

Standard methods, as described by Maniatis *et al*. (1982) were used for recombinant DNA work. Isolation of plasmid DNA from *Lactobacilli* was carried out according to the method of O'Sullivan and Klaenhammer (1993).

3. Transformation of *Lactobacilli* and *E. coli*

Transformations of *L. bulgaricus* and *L. plantarum* were carried out by electroporation according to the method of Bates *et al*. (1989). Transformation of *E. coli* was also done through electroporation or the $CaCl_2$ treatment method (Maniatis *et al*., 1982).

4. Preparation of total *lactobacilli* cell extracts

The endoglucanase activity of each *lactobacillus* cell culture was estimated by dividing into two fractions of culture supernatant and whole-cell extract. The 16-h culture broth was centrifuged at 8,000g for 20min at 4℃. The Harvested cells were washed twice with 50 mM sodium citrate buffer (pH 6.0), suspended in the buffer, and sonicated for 20 min with the Fisher scientific sonic dismembrator Model 550. Cell debris was removed from the extract by centrifugation for 15 min at 9,000 g.

5. Determination of endoglucanase activity

For the qualitative detection of endoglucanase synthesis, congo red dye method was used (Teather and Wood, 1982). Enzyme assays were carried out in 50 mM sodium citrate buffer (pH 6.0) containing 0.5% CMC. After incubation for 30 min at 60℃, boiling for 10 min stopped the reaction mixture. The reducing sugar produced was measured by the DNS (dinitrosalicyclic acid) reagent (Miller et al., 1960). One unit (U) of enzyme activity was defined as that forming 1μmole of glucose equivalent of reducing sugar per minute under the given conditions. Specific activity was defined as units per milligram of protein. Protein concentration was determined by the Bradford method (1976).

6. Proteinases inactivation test of cellulases

The selection of small intestinal proteinase-resistant cellulase was carried out using the method of Hall *et al*.(1993).

7. Determination of acid tolerance

MRS broth was adjusted to pH 2 and 3 by the addition of sterile 0.1 N HCl. Overnight *lactobacillus* cells were collected by low speed centrifugation at 5,000 g for 5 min, rinsed once with phosphate buffered saline (PBS: pH 7.2) and 0.9% NaCl, and suspended in 2 mL PBS. Test tubes containing pH-adjusted MRS broth at pH 2 and 3 were inoculated with the bacterial suspensions to achieve a final cell concentration of 10^8 CFU/mL. All tubes were incubated at 37℃ for 2 h. Populations were determined at 20 min intervals using MRS agar pour plates during 48 h incubation at 37℃.

8. Determination of bile-salt tolerance

Lactobacilli MRS broth was prepared with 0.3 and 1% oxgall (Difco) dispensed in 30 ml volumes and sterilized by autoclaving at 121℃ for 15 min. For each culture tested, one tube of each medium was inoculated with 0.3 ml of freshly prepared overnight *lactobacilli* culture. The inoculated media were incubated in a 37℃ water bath for 6 h. Growth was determined in terms of the increases of OD_{600} with a spectrophotometer.

9. Antibiotic susceptibility test

Antibiotic susceptibility patterns of *Lactobacilli* were assayed using the disc diffusion method (Charteris *et al*., 1998a) as described previously using eleven antibiotic-impregnated paper discs

(Difco). The results were expressed in terms of resistance, moderate susceptibility or susceptibility according to the interpretative standards (Charteris *et al*., 1998a).

Ⅲ. RESULTS AND DISCUSSION

To develop recombinant *Lactobacillus* strains having capacity to hydrolyze cellulose in the small intestine, the secreted cellulase must not be inactivated by proteinases of pancreatic origin. Hall *et al*. (1993) previously reported that endoglucanase E from *C. thermocellum* was completely resistant to small intestinal proteinases. Similar results were also obtained in our study. Endoglucanase A from *C. thermocellum* was also most resistant to inactivation by small intestinal proteinases such as elastase, trypsin, and pancreatin (Table 1).

For the expression of endoglucanase gene in *L. bulgaricus* and *L. plantarum*, recombinant plasmid was constructed using *E. coli* MC1061 as a host. A 3.2-kb *Hind* III fragment containing full-length *cel*A gene from pCT104 was introduced into a promoterless *Lactobacillus-E. coli* shuttle vector pNZ123, resulting in pSD1 (Fig 1).

Table 1. Resistance of cellulases to proteolytic inactivation

Enzymes[a]	Half life of enzyme incubated with proteinase (min)		
	Pancreatin	Trypsin	Elastase
Endoglucanase A	〉120	〉110	〉60
Endoglucanase II	30	20	15
Endoglucanase B	60	60	60
Mixed-glucanase	10	8	8
CMC-xylanase	5	6	5

[a] Endoglucanase A (Beguin *et al*., 1985), II (Min *et al*., 1994), and B (Fugino and Ohmiya, 1991) were derived from *Clostridium thermocellum*, *Actinomyces* KNG 40, and *Clostridium josui*, respectively. Mixed-glucanase (Teather and Erfle, 1990) and CMC-xylanase (Woo, 1995) were derived from *Fibrobacter succinogenes*.

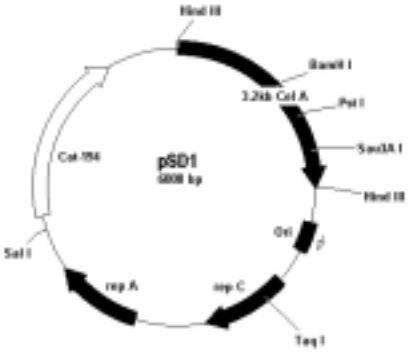


Fig 1. *Lactobacillus* expression plasmid harboring the *C. thermocellum* cel A gene.

The recombinant plasmid pSD1 was transformed into *E. coli* MC 1061 through the $CaCl_2$ competent cell method, and transformants showing endoglucanase activity were detected with Congo Red dye (data not shown). pSD1 was then electrotransformed into *L. bulgaricus* and *L. plantarum.* The electroporation efficiencies of *L. bulgaricus* and *L. plantarum* were 6.4×10^1 and 2.4×10^2 CFU/㎍ of pSD1 plasmid DNA, respectively (Table 2).

To investigate whether *L. bulgaricus* and *L. plantarum* harboring the recombinant plasmid pSD1 showed endoglucanase activity, the cells were cultured in MRS plate containing CMC and chloramphenicol. When the colonies on the plate were treated with Congo Red, halo zones indicating endoglucanase activity were formed (Fig 2). The specific enzyme activities of

Table 2. Electroporation efficiencies of plasmids transformed into *L. bulgaricus* and *L. plantarum*

Strains or plasmids	Efficiency[a](CFU/㎍)
L. bulgaricus (pNZ123)	1.2×10^2
L. bulgaricus (pSD1)	6.4×10^1
L. plantarum (pNZ123)	8.0×10^2
L. plantarum (pSD1)	2.4×10^2

[a] Average of two experiments.

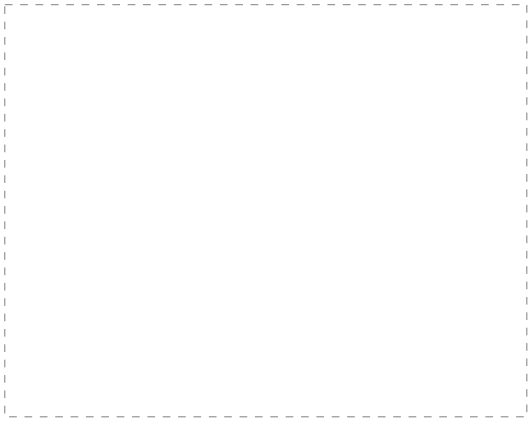


Fig 2. Congo-red test of *Lactobacillus* transformants indicating endoglucanase activity.
A : *L. bulgaricus* (pSD1)
B : *L. plantarum* (pSD1)

transformed *E. coli*, *L. bulgaricus*, and *L. plantarum* were 1.20, 4.28, and 6.36 U/mg of protein, respectively. The levels of *cel A* expression in *L. bulgaricus* and *L. plantarum* compared to those of cellulases expressed by other *Lactobacillus* species were relatively high. Micromole quantities of glucose equivalents were released by hydrolysis of CMC compared to nanomole quantities of *L. reuteri* reported proviously. Also, most endoglucanases were secreted into extracellular fraction, both in *L. bulgaricus* and *L. plantarum* (Table 3). This result provides further evidence for the universal nature of signal peptide recognition by gram-positive bacteria (Bates et al., 1989), suggesting that the endoglucanase signal peptide of *C. thermocellum* is efficiently recognized by the protein transport machinery of *L. bulgaricus* and *L. plantarum.*

Both *L. bulgaricus* and *L. plantarum* were evaluated to be acid-tolerant. Viable counts of both of the strains were maintained at pH 3 for at least 120 min (Fig 3). Acid-tolerant strains are thought to be more capable to survive at low pH conditions in the stomach (even at pH 2.0 in extreme cases), where hydrochloric and gastric acids are secreted. Toit *et al*.(1998) reported that *L. reuteri* BFE 1058 and *L. johnsonii* BFE 1061 isolated from pig faeces were able to grow at pH 3 and 4 and may thus be regarded as acid-tolerant. Generally, intrinsic acid resistances of *L. delbruelkii* supsp. *bulgaricus* and *S. thermophilus* are poor, whereas *L. acidophilus* and *bifidobacteria* have been reported to be more resistant, although great differences exist between strains (Charteris *et al*., 1998b; Conway *et al*., 1987; Klein *et al*., 1998).

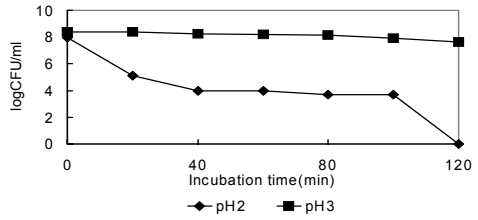
The growth curves of *L. bulgaricus* and *L. plantarum* in the presence of 0.3 and 1% oxgall were presented in Fig 4. At 3h incubation, *L. bulgaricus* was greatly inhibited in MRS broth with oxgall, but *L. plantarum* was relatively bile-salt resistant. Bile salt tolerance is important for *Lactobacillus* strains to grow and survive in the upper small intestine; non-intestinal bacteria such as *L. bulgaricus* and *L. lactis* were very sensitive to bile concentrations lower than 0.05% (Gillinand and Speck, 1977; Toit *et al*., 1998).

Probiotics have been considered as substitutes of feed antibiotics, but, in some cases, it may be

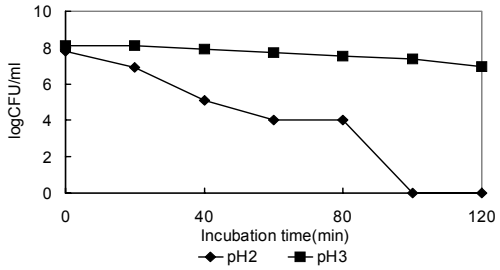
Table 3. The activity of endoglucanase in transformants of *L. bulgaricus* and *L. plantarum*

Strain (Plasmid)	Secretion Rate (%)	Endoglucanase activity[a](U/ml)		
		Total	Supernatant	Whole cell extract
L. bulgaricus(pSD1)	94.5	0.127	0.120	0.007
L. plantarum(pSD1)	91.7	0.157	0.144	0.013

[a] One unit (U) of enzyme activity was defined as that forming 1μmole of glucose equivalent of reducing sugar per minute under the given conditions.

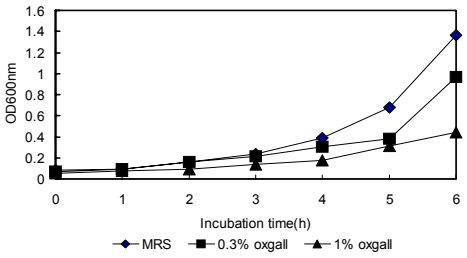


(A)

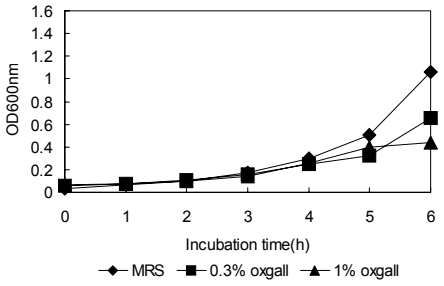


(B)

Fig 3. Survival of *L. bulgaricus KCTC 3188* and *L. plantarum KCTC 1048* in MRS broth adjusted to pH 2 and 3 with 0.1N HCl.
A : *L. bulgaricus*
B : *L. plantarum*



(A)



(B)

Fig 4. Survival of *L. bulgaricus KCTC 3188* and *L. plantarum KCTC 1048* in MRS broth with 0.3 and 1% oxgall.
A : *L. bulgaricus*
B : *L. plantarum*

feasible to combine the probiotic and antibiotic treatments to obtain extra advantages (Nemcova *et al*., 1997). *L. bulgaricus* and *L. plantarum* showed a rather homogenous resistant pattern to antibiotics (Table 4). Both strains were resistant to amikacin, gentamicin, streptomycin, kanamycin, and colistin, while susceptible to penicillin G, amphicillin, tetracyclin, oxytetracyclin, and erythromycin. Dutta and Devriese (1981) investigated the minimal inhibitory concentrations of some commonly used feed antimicrobial agents against *lactobacilli* isolated from pigs, cattle, and poultry. The pencentages of resistant strains varied in pigs, cattle, and poultry ranging from 2, 10, and 8 to 70, 95, and 83, respectively, according to the type of antibiotics, this suggest a potential for combined treatment of antibiotics and *lactobacilli* probiotics (Dutta and Devriese, 1981).

Table 4. Responses of *L. bulgaricus* and *L. plantarum* to antibiotics

Antibiotics	Concentration	*L. bulgaricus*	*L. plantarum*
Penicillin G	10 unit	[a]MS	MS
Ampicillin	10 mcg	[b]S	MS
Kanamycin	30 mcg	[c]R	R
Streptomycin	10 mcg	R	R
Erythromycin	15 mcg	S	S
Amikacin	30 mcg	R	R
Tetracycline	30 unit	S	S
Bacitracin	10 unit	MS	R
Colistin	10 mcg	R	R
Oxytetracycline	30 mcg	S	S
Gentamycin	10 mcg	R	R

[a] MS : Moderately susceptible.
[b] S : Susceptible.
[c] R : Resistant.

Morever, the occurrence of bacitracin-resistant *L. acidophilus* strains from cattle and poultry was related to the bacitracin used as a growth promoter in these animals (Charteris *et al*., 1998).

In conclusion, this study provides important findings concerning a probiotic species of *lactobacilli*. First, the expression of an endoglucanase gene of heterologous origin was demonstrated for the first time in *L. bulgaricus*. Second, the probiotic properties of *L. bulgaricus* and *L. plantarum* were described (such as, acid tolerance, bile-salt tolerance, and antibiotic susceptibility). These results may provide useful basic information to employ lactobacilli in genetic modification for biotechnological application.

Ⅳ. 적 요

다양한 미생물들로부터 유래한 cellulase 중에서, 특히 장내 단백질 가수분해효소에 안정한 *Clostridium thermocellum* 균주 유래의 endoglucanase를 선별하였다. 그 후 그 유전자의 자체 프로모터에 의해 발현되는 재조합 *Lactobacillus* 용 발현벡터를 구축하였고, 그 발현벡터를 pSD1이라 명명하였다. 이 발현벡터를 *L. bulgaricus*와 *L. plantarum* 균주에 각각 전기천공법을 이용하여 형질전환시키는데 성공하였으며 그 재조합 균주들로부터 endoglucanase 효소역가를 조사한 결과 각각 배지 상층액에서 0.12, 0.144 U/ml로 조사되었다.

한편 이들 균주들의 생균제로 갖추어야할 특성인 내산성, 내담즙성 및 항생제내성 여부를 조사한 결과, 이들 균주들은 모두 pH3과 같은 산성 조건하에서도 안정하였으며, 내담즙성에 있어서는 특히 *L. plantarum* 균주의 경우 0.3, 1% 의 oxgall에서도 안정하였다. 또한 항생제 내성을 조사한 결과 두 균주 모두 amikacin, gentamicin, kanamycin, colistin에 저항성이 높은 것으로 나타났다.

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