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Transformation and Expression of β-glucanase Gene in *Lactobacillus* Strains Isolated from Gastrointestinal Tract of Chickens

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Abstract: Ten *Lactobacillus* strains (*L. crispatus* I12, *L. brevis* I23, I211, I218 and C10C, *L. fermentum* I25, I24C and C17C and *L. acidophilus* I16C and I26C) which originated from the gastrointestinal tract of chickens were transformed with plasmids pSA3b3 and pSA3b6. These plasmids harbored a *bgl*A gene, cloned in opposite orientation, which encoded for β-glucanase enzyme. Enzyme expression was only detected in pSA3b6 transformants. The plasmid stability in transformants *L. crispatus* I12pSA3b6, *L. brevis* I23pSA3b6, I211pSA3b6, I218 pSA3b6 and C10CpSA3b6 and *L. fermentum* I25pSA3b6, were comparatively higher than the other strains. Approximately 30-48% of the cells retained the plasmid after 21-36 generations of growth. All the strains, except for *L. brevis* C10CpSA3b6, produced 22.8-25.1 U mL^{-1} of β-glucanase during late exponential growth phase (14 h old cultures). In *L. brevis* C10CpSA3b6, 37.2 U mL^{-1} of β-glucanase was detected during early exponential phase (6 h old culture).

Key words: Expression, β-Glucanase, *Lactobacillus*, Transformation

Introduction

Many gram positive bacteria are well known for their important contributions to medical, agricultural and food biotechnology and also as production organisms for industrial enzymes (de Vos Willem *et al*., 1997). Of these, the low GC gram positive bacteria belonging to the genera *Bacillus*, *Clostridium*, *Lactococcus*, *Lactobacillus*, *Staphylococcus* and *Streptococcus* have established biotechnological applications. In fact, the food-grade lactic acid bacteria that include *Lactococcus*, *Lactobacillus* and *Streptococcus* species have been regarded as potentially useful organisms for production of commercially important proteins (Billman-Jacobe, 1996).

The lactic acid bacteria are widely used in various food and agricultural fermentation processes and health-promoting products for humans and animals, which made this group of microorganisms the target for genetic exploitation. These microorganisms are considered either as suitable vehicles for genetic modification or as cell factories for the synthesis of novel proteins (Thompson *et al*., 1999). Much interest has also been generated to improve the characteristics of these strains through DNA recombinant technology, so as to allow new applications in the health, food and feed industries (Gaeng *et al*., 2000). By far, the best studied lactic acid bacteria are the lactococci, specifically *L. lactis*, an important starter in cheese production (Leenhouts *et al*., 1998). Due to their non-pathogenic and non-toxic nature, food-fermenting lactobacilli also attract attention as potential expression systems for heterologous gene products.

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The genetic modification of lactobacilli only became practical after the first report of successful electroporation in *L. casei* (Chassy and Flickinger, 1987). The development of convenient cloning and controlled expression system, which would expand the metabolic capabilities and widen the scope of applications for *Lactobacillus*, is certainly promising. Genetic modification of lactobacilli has usually been achieved by the introduction of plasmids, either derived from other naturally occurring *Lactobacillus* plasmids or by using broad host range plasmid replicons from other gram positive bacteria (Rush *et al*., 1994). Various vectors have been constructed and gene expression systems have been developed for *Lactobacillus*. A large number of different strains isolated from a variety of hosts are now transformable, including species that can colonize the gastrointestinal tract. However, the successful expression of introduced heterologous gene is the ultimate concern in the transformation procedure, especially when practical applications are desired. Thus, the aims of the present study were to transform several probiotic *Lactobacillus* strains with plasmid vectors carrying a heterologous gene (β-glucanase gene from *Bacillus amyloliquefaciens*) and to study the expression of the novel heterologous gene product. The segregational stability of the plasmid in the host and the yield of enzyme produced were also investigated to determine the feasibility of using these *Lactobacillus* strains as an alternative host to deliver heterologous gene into chickens to enhance feed degradation.

Materials and Methods

Lactobacillus Strains, Plasmids and Media

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain JM109 was the host for the plasmids used in the present study and was routinely grown with shaking in Luria-Bertani medium (Difco Laboratories, Detroit, USA) containing 12.5 μg mL^{-1} tetracycline. Ten *Lactobacillus* strains were used as hosts for studying β-glucanase expression. They were cultured under anaerobic conditions at 39°C, with every 48 h transfer into fresh Man Rogosa Sharpe (MRS) broth (Oxoid, Unipath Ltd., Basingstoke, UK). During cultivation of transformed *Lactobacillus*, 50 μg mL^{-1} erythromycin was added to MRS broth for plasmid maintenance. The plasmid vectors used were pSA3, pSA3b3 and pSA3b6. Plasmid vector pSA3 is a 10.2 kb *Streptococcus-E. coli* shuttle vector, donated by J.J. Ferretti (University of Oklahoma, USA) (Fig. 1) (Dao and Ferretti, 1985). Plasmids pSA3b3 and pSA3b6 were gifts from K. Thompson (University of Belfast, Northern Ireland, UK) (Fig. 2). These plasmids carry a *bgl*A gene from *Bacillus amyloliquefaciens* cloned in both orientations at the *Eco*RI site of pSA3. Both plasmids are about 13.8 kb in size (Thompson and Collins, 1991). The *bgl*A gene encoded for β-glucanase (E.C. 3.2.1.73; 1,3-1,4-β-glucan-4-glucanohydrolase) which specifically hydrolyses glucans containing a mixture of β-1,3 and β-1,4 linkages, such as lichenan and barley β-glucan (Borris *et al*., 1985).

Transformation of Lactobacillus Strains

Late exponential phase (OD_{600}) cultures were used for transformation. *Lactobacillus brevis* I23, I211, I218 and C10C, *L. acidophilus* I16C and I26C and *L. crispatus* I12 were grown in MRS broth containing 6% (w/v) glycine and 0.3 M sucrose while *L. fermentum* I24, I25, C17, I24C and C17 were grown in similar broth but contained 8% (w/v) glycine. The cultures were harvested and washed with appropriate buffer. *Lactobacillus crispatus* I12 and *L. acidophilus* I16C and I26C were washed and resuspended in 1/10 of their original volume in 3.5x SMEB buffer (272 mM sucrose, 1mM $MgCl_2$, pH 7.4). For *L. brevis* I23, I211, I218 and C10C and *L. fermentum* I24, I25, C17, I24C and C17C, 2.5x SMEB buffer was used. The bacterial suspension was kept on ice before plasmid DNA (1.0 μg mL^{-1} dissolved in 10 mM Tris HCl, 1 mM EDTA, pH 8.0) was mixed into 80 μL of the ice-cold bacterial suspension. The suspension was transferred into a pre-cooled Gene Pulser™ disposable cuvette (inter-electrode distance 0.2 cm; Bio-Rad Laboratories, Richmond, CA). The cell suspensions were then exposed to electrical pulse delivered by a gene pulser apparatus (Bio-Rad) at different field

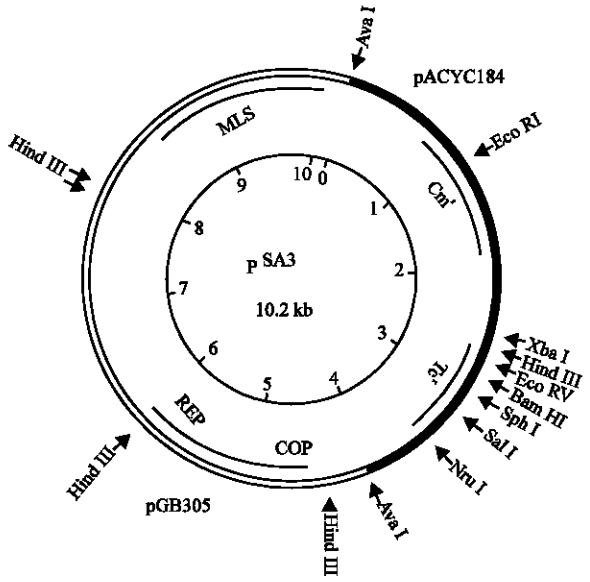

Fig. 1: Physical map of plasmid pSA3. ■ = DNA segment derived from pACYC184; □ = DNA segment derived from pGB305; MLS = Marcolide Linocosamide Streptogramin B esistance; Cm^r = chloromphenicol resistance; Tc^r = Tetracycline resistance; REP = replication region; COP = Copy Control Region (Dao and Ferretti, 1985)

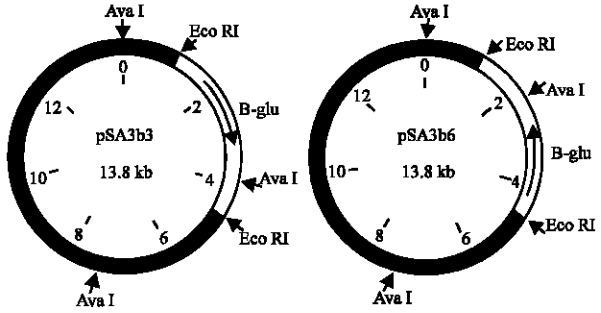

Fig. 2: Diagrammatic representation of the structure of plasmids pSA3b3 and pSA3b6 showing the relative position of the *Eco*RI and *AvaI* sites and the position if the β-glucanase determinant (*bglA*) (Thompson and Collins, 1991)

strengths. *Lactobacillus crispatus* I12 and *L. acidophilus* I16C and I26C were electroporated at 12.5 kV cm^{-1}, while *L. brevis* I23, I211, I218 and C10C and *L. fermentum* I24, I25, C17, I24C and C17C were exposed to field strength of 7.5 and 10 kV cm^{-1}, respectively. During the pulse, the pulse controller was set to 200 ohm and the capacitance to 25 μF. After the pulse, the cells were allowed to recover in MRS broth supplemented with 0.3 M sucrose for phenotypic expression. Selection of transformants was then carried out on MRS agar plates containing 50 μg mL^{-1} erythromycin and scored after 48 h incubation at 39°C under anaerobic condition. The plasmid profiles of the parental strains and transformants were then analyzed on 0.8% agarose gel.

Isolation of Plasmid DNA

The plasmid DNA from *E. coli* was prepared using the Wizard® DNA Purification Systems (Promega, Madison, USA). The plasmid DNA from *Lactobacillus* strains were extracted according to the method described by O'Sullivan and Klaenhammer (1993) with slight modifications. Instead of using overnight cultures, 10 mL^{-1} of 6-8 h cultures were used for plasmid extraction. The cells were harvested by centrifugation at 3,000 x g for 10 min at 4°C and washed twice in 2 mL^{-1} of 10 mM Tris-HCl buffer, pH 8.0. After the final wash, the cells were collected by centrifugation and 200 μL of freshly prepared lysozyme solution (6 mg mL^{-1} in 10 mM Tris HCl, pH 8.0) was added to the cell pellet. The cells were resuspended gently in the lysozyme solution and incubated at 39°C for 25 min. Then 400 μL of alkaline SDS solution (3% SDS in 0.2 N NaOH) was added to the tube and mixed immediately. The mixture was incubated at room temperature for 7 min, after which 300 μL of ice-cold 3 M sodium acetate pH 4.8 was added and mixed gently. The mixture was centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was transferred to a new tube and 10 μL of RNase (10 mg mL^{-1}) was added. The mixture was gently inverted a few times and incubated at 39°C for 1 h, after which 650 μL of absolute isopropanol was added. The mixture was centrifuged at 15,000 x g for 15 min at 4°C. The pellet was resuspended gently in 320 μL of sterilized distilled water, 300 μL of 7.5 M ammonium acetate containing 0.5 mg mL^{-1} ethidium bromide and 350 μL phenol/chloroform solution. The mixture was mixed gently and centrifuged at 15,000 x g for 15 min at 4°C. The upper phase was transferred into a new tube and 1 mL of absolute ethanol was added. The mixture was left overnight at -20°C and then centrifuged at 15,000 x g for 15 min at 4°C. The pellet was resuspended in 70% ethanol and centrifuged again. The pellet obtained, which contained the plasmids, was vacuum dried before it was resuspended in 50 μL of TE buffer pH 7.5.

Qualitative Study on Expression of β-glucanase Gene by Agar Plate Assay

The transformed *Lactobacillus* strains expressing β-glucanase activity were identified by agar plate assay. Wells of 4 mm in diameter were made in PHB agar (per L, 12 g of Todd-Hewitt broth, 3 g of MRS medium, 15 g of agar) supplemented with 0.1% (v/v) Tween 80 (Sigma Chemical Co., USA) and 0.05% (w/v) lichenan (Sigma Chemical Co., USA). Then, 40 μL of supernatant obtained from an overnight culture of transformed *Lactobacillus* strain or parental strain (centrifuged at 8,000 x g for 20 min at 4°C) was placed into the wells. After overnight incubation, the surface of the plates was flooded with 0.1% (w/v) Congo red solution [dissolved in 20% (v/v) ethanol]. β-Glucanase activity was shown by a clear zone surrounding the wells (Teather and Wood, 1982).

Plasmid Stability

An overnight culture of transformed *Lactobacillus* strain grown in MRS medium containing antibiotic was inoculated at 1% (v/v) into antibiotic-free MRS broth and incubated at 39°C for 12 h. A series of sequential propagations in antibiotic-free broth was performed every 12 h. Plasmid stability was observed throughout 5 sequential transfers. Samples were taken from each culture of each transfer, serially diluted and plated onto MRS agar with or without erythromycin. The β-glucanase activity of erythromycin resistant colonies was determined by agar plate assay. Plasmid stability was determined by the ratio of antibiotic-resistant β-glucanase-producing colonies to total colonies on non-selective plates.

Quantitative Analysis of Expressed β-glucanase in Transformed Lactobacillus Strains

A 14 h culture of transformed *Lactobacillus* strain was used as inoculum. The culture was inoculated at 1% (v/v) into 3 mL of fresh MRS broth containing 50 μg mL^{-1} erythromycin and incubated at 39°C for 12 h. The culture was then centrifuged at 8,000 x g for 20 min at 4°C and the

β-glucanase activity in the supernatant was determined. *Lactobacillus* strains carrying plasmid vector pSA3 was used as negative controls. For the enzyme and substrate controls, both components were replaced by 0.1 M sodium phosphate buffer (pH 6.5). All tests were repeated three times, each in triplicate.

Determination of β-glucanase Activity

The β-glucanase activity in crude enzyme preparations was determined by using the method described by Waffenschmidt and Jaenicke (1987). Sample for assay was prepared by centrifuging 1 mL culture at 8,000 x g for 20 min at 4°C, after which 250 μL of the supernatant was mixed with an equal volume of the substrate [0.05 % (w/v) lichenan in 0.1 M sodium phosphate buffer (pH 6.5)] (Heng *et al*., 1997). The mixture was incubated at 39°C for 1 h. β-Glucanase activity was determined by measuring the amount of reducing sugars (as glucose equivalents) liberated. At the end of the incubation period, 100 μL of the mixture was added to 9.9 mL of 0.1 M sodium phosphate buffer (pH 6.5). Then, 500 μL of sample was mixed with an equal volume of fresh reagent and incubated for 15 min at 98°C. The reagent was prepared by mixing equal volumes of solution A (5 mM disodium 2,2'-bicinchoninate, 0.5 M $Na_2CO_3H_2O$ and 0.3 M $NaHCO_3$) and solution B (5 mM $CuSO_4.5H_2O$ and 12 mM L-serine). After incubation, the tubes were cooled to room temperature for 20 min and optical density determined at 560 nm in a spectrophotometer, against a blank without sugar. One unit of β-glucanase activity was defined as the amount of enzyme required to liberate 1 μmol of glucose equivalent of reducing sugar per h under the given assay conditions.

Statistical Analysis

Data obtained were analyzed using the General Linear Model (GLM) procedure for analysis of variance (SAS Institute, 1997). Significant differences among the means were separated by the Duncan's New Multiple Range Test (Duncan, 1955) at 5% level of probability.

Results

Transformation of Lactobacillus Strains

The *Lactobacillus* strains were successfully transformed with plasmids pSA3, pSA3b3 and pSA3b6 with the electroporation efficiency ranging from 10^3-10^4 cfu μg^{-1} plasmid DNA (Table 1 and 2). The plasmid profiles of the parental strains and those transformed by plasmids pSA3, pSA3b3 and pSA3b6 were also verified (a representative gel shown in Fig. 3). In addition to the indigenous plasmids, additional plasmid with the expected size (10.6 kb for pSA3 and 13.8 kb for pSA3b3 and pSA3b6) was observed in the respective transformants.

Table 1: Bacterial strains and plasmids

Strain or plasmid	Relevant feature	Origin or references
Strain		
E. coli JM109		
L. crispatus I12		Jin *et al.* (1998)
L. brevis I23		Jin *et al.* (1998)
L. fermentum I25		Jin *et al.* (1998)
L. brevis I211		Jin *et al.* (1998)
L. brevis I218		Jin *et al.* (1998)
L. acidophilus I16C		Sieo *et al.* (2005)
L. acidophilus I26C		Sieo *et al.* (2005)
L. fermentum I24C		Sieo *et al.* (2005)
L. brevis C10C		Sieo *et al.* (2005)
L. fermentum C17C		Sieo *et al.* (2005)
Plasmid		
pSA3	Em^r, Cm^r, Tet^{ra}	Dao and Ferretti (1985)
pSA3b3	Em^r, *bgl*A+	Thompson and Collins (1991)
pSA3b6	Em^r, *bgl*A+	Thompson and Collins (1991)

Em^r = resistant to erythromycin; Cm^r = resistant to chloromphenicol; Tet^{ra} = tetracycline resistance expressed in *E. coli*; *bgl*A+ = gene encoding for β-glucanase from *Bacillus amyloliquefaciens*

Fig. 3: Agarose gel of plasmid preparation of *L. brevis* 1218 transformed with plasmid pSA3 and pSA3b6. Lane 1, molecular weight marker; Lane 2, parental strain; Lane 3, pSA3 transformant; Lane 4, pSA3b3 transformant; Lane 5, pSA3b6 transformant; Lane 6, plasmid pSA3; Lane 7, plasmid pSA3b3, Lane 8, plasmid pSA3b6.↘ indicates introduced plasmid

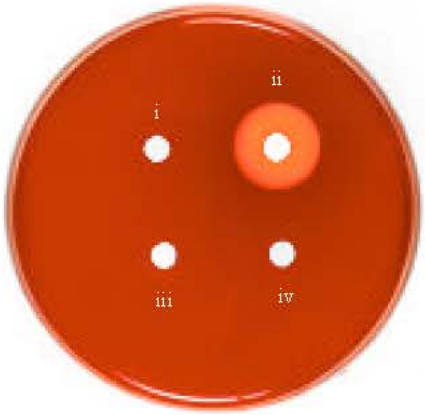

Fig. 4: Agar plate assay for detection of β-glucanase activity in *L. crispatus* I12. I = parental strain; ii = pSA3b6 transformant; iii = pSA3 transformant; iv = pSA3b3 transformant

Qualitative Study on Expression of β-glucanase Gene

The β-glucanase activities of the parental strains and transformants containing plasmid pSA3, pSA3b3 or pSA3b6 examined on PHB agar containing 0.05% (w/v) lichenan as substrate are shown in Fig. 4. The presence of β-glucanase activity was indicated by the formation of clear zones. β-Glucanase activity was detected in pSA3b6 transformants but not in the parental strains, pSA3 transformants or pSA3b3 transformants of all the *Lactobacillus* strains. Thus, subsequent studies on the expression of the β-glucanase gene were focused on pSA3b6 transformants only.

Plasmid Stability

Figure 5 shows the stability of plasmid pSA3b6 in *Lactobacillus* strains growing under non-selective growth condition (antibiotic-free MRS broth). Under this condition, the plasmid stability of six *Lactobacillus* strains transformed with pSA3b6, namely *L. crispatus* I12pSA3b6, *L. brevis* I23pSA3b6, I211pSA3b6, I218pSA3b6 and C10CpSA3b6 and *L. fermentum* I25pSA3b6, were comparatively higher than those of the other strains. After the first and second transfers,

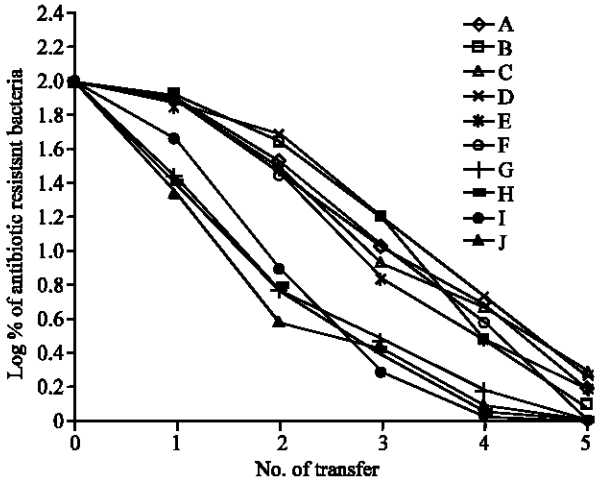

Fig. 5: Stability of pSA3b6 in *Lactobacillus* strains grown under non-selective condition. A = *L. crispatus* I12pSA3b6; B = *L. brevis* I23pSA3b6; C = *L. fermentum* I25pSA3b6; D = *L. brevis* I211pSA3b6; E = *L. brevis* I218pSA3b6; F = *L. acidophilus* I16CpSA3b6; G = *L. acidophilus* I26CpSA3b6; H = *L. fermentum* I24CpSA3b6; I = *L. brevis* C10CpSA3b6; J = *L. fermentum* C17CpSA3b6

Table 2: Transformation efficiencies of *Lactobacillus* strains

Strain	Plasmid	Transformation efficiency (cfu μg^{-1} plasmid DNA)
L. crispatus I12	pSA3	5.29×10^{4}
	pSA3b3	5.65×10^{4}
	pSA3b6	5.02×10^{4}
L. brevis I23	pSA3	2.73×10^{4}
	pSA3b3	2.20×10^{4}
	pSA3b6	2.31×10^{4}
L. fermentum I25	pSA3	1.30×10^{4}
	pSA3b3	1.22×10^{4}
	pSA3b6	1.39×10^{4}
L. brevis I211	pSA3	2.95×10^{3}
	pSA3b3	3.29×10^{3}
	pSA3b6	3.08×10^{3}
L. brevis I218	pSA3	6.44×10^{3}
	pSA3b3	6.02×10^{3}
	pSA3b6	6.00×10^{3}
L. acidophilus I16C	pSA3	3.99×10^{4}
	pSA3b3	3.73×10^{4}
	pSA3b6	3.86×10^{4}
L. acidophilus I26C	pSA3	2.74×10^{4}
	pSA3b3	2.59×10^{4}
	pSA3b6	2.38×10^{4}
L. fermentum I24C	pSA3	3.95×10^{4}
	pSA3b3	3.47×10^{4}
	pSA3b6	3.60×10^{4}
L. brevis C10C	pSA3	2.85×10^{3}
	pSA3b3	3.38×10^{3}
	pSA3b6	3.18×10^{3}
L. fermentum C17C	pSA3	5.48×10^{4}
	pSA3b3	5.86×10^{4}
	pSA3b6	5.74×10^{4}

Table 3: β-Glucanase activities of *Lactobacillus* transformants

Strain	β-Glucanase activity * (U mL^{-1})
L. brevis C10CpSA3b6	2.28±0.06^{a}
L. crispatus I12pSA3b6	25.10±0.50^{b}
L. brevis I23pSA3b6	24.30±0.81b^{c}
L. fermentum I25pSA3b6	23.07±0.66^{c}
L. brevis I211pSA3b6	24.39±0.53^{bc}
L. brevis I218pSA3b6	22.75±0.70^{c}

* Values represent means±SE (n = 9). $^{a-c}$Means with different superscripts in the same column are significantly different ($p<0.05$)

approximately 72-81 and 30-48% of the cells of these strains retained the erythromycin resistance property (as conferred by the plasmids transformed), respectively. As for *L. acidophilus* I16CpSA3b6 and I26CpSA3b6 and *L. fermentum* I24CpSA3b6 and C17CpSA3b6), only 21-46% of the cells retained the erythromycin resistance characteristic after the first transfer and the number of erythromycin resistant cells was reduced to 3-7% after the second transfer. Due to the instability of the plasmid, no further studies were carried out on *L. acidophilus* I16CpSA3b6, *L. acidophilus* I26CpSA3b6, *L. fermentum* I24CpSA3b6 and *L. fermentum* C17CpSA3b6.

Quantitation of β-glucanase Produced by Transformed Lactobacillus Strains

The β-glucanase activities of the *Lactobacillus* strains transformed with plasmid pSA3b6 are shown in Table 3. All the strains, except for *L. brevis* C10CpSA3b6, produced approximately 22.75-25.10 U mL^{-1} of enzyme under the conditions provided.

Discussion

The possibility to present proteins, enzymes and antigenic epitopes in selected intestinal locations *via* a bacterial delivery and expression system is fast becoming a reality. Despite substantial progress made in this field over the past decade, many barriers remained to be overcome before their genetically manipulated functional roles are considered important (Kullen and Klaenhammer, 1999). The main concern in all cases of genetic manipulation is the expression of the cloned gene in heterologous hosts (de Vos Willem, 1987). In the present study, the susceptibility of 10 *Lactobacillus* strains to transformation and expression of a heterologous gene were investigated.

The β-glucanase gene (*bgl*A) which originated from *B. amyloliquefaciens* was expressed in all the 10 strains of *Lactobacillus* transformed with plasmid pSA3b6. When the *bgl*A gene was cloned into plasmid pSA3 in the opposite direction, as in pSA3b3, β-glucanase activity was not detected although the pSA3b3 transformants possessed a band of approximately the size of the introduced plasmid (13.8 kb), as determined by electrophoretic examination (Fig. 3). In an attempt to alter the proteolytic characteristics of *Streptococcus thermophilus,* O'Sullivan and Fitzgerald (1999) also observed that the transformants remained inactive in proteolytic assays even though the introduced plasmid was detected electrophoretically and the heterologous proteinase gene was retained as assessed by Polymerase Chain Reaction (PCR) in the transformants. They suggested that this may indicate that the heterologous gene was not expressed or was expressed in an inactive form. In some cases, expression from a gene cloned in both orientations could be detected. Baik and Pack (1990) reported the expression of an endoglucanase gene isolated from *B. subtilis* cloned in both orientations in a plasmid in *L. acidophilus* transformants. Similar observations have been reported in *L. plantarum* in which an α-amylase gene is expressed in both orientations (Scheirlinck *et al*., 1989). In these cases, the expression would be by using the gene's own promoter (Baik and Pack, 1990). Although two putative promoters are located in the *bgl*A gene (Hofemeister *et al*., 1986), the promoters are somehow not functional when the *bgl*A gene is cloned in the opposite direction. Altered expression of *Bacillus* genes cloned on *E. coli* vector DNA, that is dependent upon the orientation of insert, has also been observed by Fouet *et al*. (1982) and Panbangred *et al*. (1983).

Further analyses of pSA3b6 in *Lactobacillus* transformants showed that the stability of the plasmid in several strains such as *L. acidophilus* I16CpSA3b6, *L. acidophilus* I26CpSA3b6, *L. fermentum* I24CpSA3b6 and *L. fermentum* C17CpSA3b6 was lower when compared to the other strains. Instability of introduced plasmid is common in *Lactobacillus*. A plasmid, which is found to be stable in a species, may not be so in another species. For instance, plasmid pGK12 has been reported to be stable in *L. plantarum* (Bates *et al*., 1989) but disappeared at a rate of 20% per generation in *L. acidophilus* (Baik and Pack, 1990). In a similar study, Baik and Pack (1990) found that several plasmids (pUB110, pAM610, pACM1 and pACM2) were stably maintained, with a loss of only 0.5% per generation, whereas other plasmids, pCK98 and pBSKTAU, were lost at a rate of 4-6% per generation in *L. acidophilus*. The decrease in stability of plasmid could be due to lower plasmid copy numbers in these hosts (O'Sullivan and Fitzgerald, 1999). Bhowmik and Steele (1993) observed differences in stability in different isolates produced from a single transformation and suggested that it might be due to differences in the extent of amplification of the plasmid in the host. Alternatively, it could be explained by the lack of tolerance of the strain to a foreign DNA or by a plasmid re-organization resulting in deletion of tolerance either at the antibiotic resistant gene or at the plasmid replication level (Changnaud *et al*., 1992). Incompatibility of native plasmids with the introduced plasmid could also contribute to the instability of the plasmid (Posno *et al*., 1991). As none of the *Lactobacillus* strains used in the present study was plasmid-free, this may be one of the factors which led to plasmid loss.

Plasmid pSA3b6, however, was maintained fairly well in *L. crispatus* I12pSA3b6, *L. brevis* I23pSA3b6, I211pSA3b6, I218pSA3b6 and C10CpSA3b6 and *L. fermentum* I25pSA3b6. Approximately 30-48% of the cells retained the erythromycin resistance after 21-36 generations of non-selective growth. Bates *et al*. (1989) found that 10% of *L. plantarum* transformed with pSA3 carrying a *cel*E gene from *Clostridium thermocellum* retained the erythromycin resistant marker after 45 generations. In another study, Scheirlinck *et al*. (1989) observed that only 10% of *L. plantarum* retained the recombinant pSA3 plasmid carrying a *cel*A gene from *C. thermocellum* after 26 generations. The plasmid pSA3::pVA797 co-integrates constructed by Thompson and Collins (1989) were stable during sub-culture without selection for 20 generations only. With plasmid pGK12, Zink *et al*. (1991) reported that only 26.8 % chloromphenicol resistant transformants were detected after growth for 20 generations without selective pressure.

The successful functional expression of the *bgl*A gene cloned in pSA3b6 indicated that the expression signals and secretion signals of *B. amyloliquefaciens* were recognized by the *Lactobacillus* strains in the present study. As suggested by Bates *et al*. (1989), the signal peptide recognition may be universal in gram positive bacteria. Thus, the signal peptide of *B. amyloliquefaciens* could be efficiently recognized by the protein transport machinery of the *Lactobacillus* strains and, hence, facilitated the expression and secretion of β-glucanase. Successful expression of heterologous genes illustrated the genetic compatibility of regulatory signals from gram positive bacteria in *Lactobacillus* species (Scheirlinck *et al*., 1989). A *bgl*M gene from *Bacillus macerans* which encodes for endo-1,3-1,4-β-glucanase has also been expressed in *L. reuteri* under the control of its own expression and secretion signals (Heng *et al*., 1997).

All the strains, except for *L. brevis* C10CpSA3b6, produced 22.8-25.1 U mL^{-1} of β-glucanase during late exponential growth phase, as detected in 14 h old cultures. In *L. brevis* C10CpSA3b6, higher enzyme activity of β-glucanase was detected during early exponential phase. Quantitative study on the production of β-glucanase showed that 37.2 U mL^{-1} of catalytically active β-glucanase was secreted in 6 h cultures (data not shown). The efficiency of the expression of the gene depends greatly on the choice of the vector plasmid on which the gene is to be inserted or specifically, on the strength of the promoter (McCracken and Timms, 1999) for the particular strain. Hols *et al*. (1994) found that the expression of an amylase gene from *B. licheniformis* in *L. plantarum* was significantly improved by including a promoter isolated from the chromosome of the same strain.

In conclusion, the ability of the *Lactobacillus* strains, especially *L. crispatus* I12pSA3b6, *L. brevis* I23pSA3b6, I211pSA3b6, I218pSA3b6 and C10CpSA3b6 and *L. fermentum* I25pSA3b6, to express the cloned enzyme indicated that these strains could be potential vector for delivering enzyme to heterologous hosts. This is particularly important for monogastric animals such as poultry and swine, in which they required enzyme supplementation to enhance utilization of feed. The advantages of using *Lactobacillus* as a delivery vehicle as compared to direct enzyme supplementation in the feed is that the enzyme could be protected from the harsh environment (low pH and proteolytic activity) in the gastrointestinal tract and the bacteria could be further manipulated for site-targeted delivery.

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