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Expression of *Bacillus licheniformis* α-amylase Gene in *Lactobacillus casei* Strains

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As a first step for developing *Lactobacillus* strains capable of fermenting starch directly, the α-amylase gene (*amyL*) from *Bacillus licheniformis* (Kim *et al.*, 1988. *Kor. J. Appl. Microbiol. Bioeng.* 16: 369-373) was introduced into *Lactobacillus casei* strains and the level of α-amylase expression in transformants was examined. 3 kb *Eco*RI fragments encompassing *amyL* were subcloned into the suitable lactococcal cloning vectors (pSA3, pMG36e, and pIL2530) and then recombinant plasmids were introduced into *E. coli* and *L. casei* strains by electroporation. Only one recombinant plasmid, pIL2530α was able to transform few *L. casei* strains tested at low efficiencies. The transformation efficiencies with the plasmid into *L. casei* YIT 9018 and *L. casei* ATCC 4646 were less than 10^2/μg pIL2530α. The level of amylase activities in *L. casei* was five to ten-fold lower than that in *E. coli* cells. pIL2530α was stably maintained in *Lactobacillus* strains in the presence of Em (5 μg/ml) but without antibiotic selection, it was unstable so more than 95% of cells lost plasmids after a week of daily subculturing.

Lactic acid, the major end product of lactic acid fermentation, is a commercially important organic acid used as a food preservative, acidulant, medicinal ingredient, and recently as a raw material for biodegradable plastic. Natural lactic acid is produced by lactic acid bacteria, traditionally by *Lactobacillus delbrueckii*. Refined sucrose is one of the most commonly used substrates (1). Less expensive starch can not be used directly since most of the lactic acid bacteria lack starch degrading enzymes. Therefore, in commercial lactic fermentation where starch is employed as a substrate, the starch is first treated with α-amylase and glucoamylase to produce glucose and oligosaccharides upon which lactic starter can nourish. If lactic acid bacteria could produce lactic acid directly from starch, they would be ideal industrial strains for lactic acid production since employment of such strains would be very economical in liquefaction and saccharification processes (5). Recombinant DNA Technique seems to be an attractive approach to develop such microorgnisms for introduction and operation of a few related genes at the same time in a specific host cell. As a first step to develop the starch-utilizing strains, α-amylase gene (*amyL*) from *Bacillus licheniformis* was introduced into L(+)-lactic acid producing *Lactobacillus casei* strains which are commercial yogurt starters and the level of expression was examined.

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Key words: heterologous gene expression, *Lactobacillus casei*, α-amylase, *Bacillus licheniformis*

MATERIALS AND METHODS

Bacterial Cultures and Media

Bacterial strains and plasmids used in this study are described in Table 1.

Lactobacillus strains were grown in MRS broth (Difco Laboratories, Detroit, Mich. USA) without agitation or on MRS plates (1.5% agar) at 37°C (7). *Lactococcus* strains were grown in M17 broth (19) without shaking at 30°C and *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C with vigorous agitation.

Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μg/ml; tetracycline (Tc), 10 μg/ml; erythromycin (Em), 200 μg/ml for *E. coli*, 5 μg/ml for lactic acid bacteria.

DNA Isolation and Manipulation

Plasmid DNA from lactic acid bacteria was isolated as described by O'sullivan and Klaenhammer (15). Plasmid DNA from *E. coli* was isolated by the alkaline lysis method of Birnboim and Doly (2). For electroporation experiments, plasmids were further purified by CsCl-ethidium bromide density gradient ultracentrifugation (16). Restriction enzyme digestions

Table 1. Bacterial strains and plasmids.

Bacterial strain or plasmid	Description	Reference
E. coli		
DH5α	ϕ80*dlacZ*ΔM15, *recA1*, *endA1*, *gyrA96*, *thi*-1, *hdR17* (r_k^-, m_k^+), *supE44*, *relA1*, *deoR*, Δ(*lacZYA-argF*)U169	BRL
MC1061	*araD139*, *lacX74*, *galU*, *galK*, *hsr*, *hsm*$^+$, *strA*	3
Lactococcus lactis subsp. *lactis*		
MG1363	plasmid-free derivative of NCDO 712, Lac$^-$	9
Lactobacillus casei		
YIT 9018 ATCC 4646		
plasmids		
pTA322	pBR322 containing a 3 kb *Eco*RI fragment encompassing the *amyL* gene of *Bacillus licheniformis*	13
pIL253	Em^r, 4.9 kb, derived from pAMβ1	18
pIL2530	7.1 kb, a shuttle vector derived from pIL253, containing the 2.2 kb *Eco*RI-*Sal*I fragment of pACYC184	This study
pIL2530α	pIL2530 containing the 3 kb *amyL* fragment from pTA322 in *Eco*RI site	This study
pSA3	Em^r, Tc^r, Cm^r, 10.2 kb; *E. coli-L. lactis* shuttle vector, constructed by ligation of pACYC184 and pGB305	6
pSA3α	pSA3 containing the 3 kb *amyL* fragment at *Eco*RI site	This study
pMG36e	Em^r, 3.6 kb; expression vector carrying the origin of *L. lactis* ssp. *cremoris* Wg2 cryptic plasmid, pWV01	20
pMG36eα	pMG36e containing the 3 kb *amyL* fragment at *Eco*RI site	This study

were performed in accordance with the supplier's instructions (Promega, Boehringer Mannheim Biochemical). Agarose gel electrophoresis was conducted with Tris-Acetate-EDTA buffer (pH 8.0) at 4 V/cm. DNA ligations were conducted with T4 DNA ligase (Promega) at 16°C. Isolated restriction fragments for subcloning were obtained from agarose gels with GeneClean II kit (Bio 101, Inc., LaJolla, CA, USA).

Electroporation

Introduction of plasmids into lactic acid bacteria and *E. coli* strains was done by the electroporation method. Frozen competent *Lactobacillus* and *Lactococcus* cells were prepared as described by Holo and Nes (11). MRS broth containing 20 mM DL-threonine was used for cultivation of *Lactobacillus* strains, and M17 broth with DL-threonine for *Lactococcus* strains. After growth to an optical density 0.5 to 0.7 at 660 nm, the cells were harvested by centrifugation at 4°C at 5,000×g. Following two washes in ice-cold sterile water and two washes in 0.5 M sucrose containing 10% glycerol, the cells were resuspended in 1/200 original culture volume of washing solution and then stored in aliquots of 40 μl at -76°C until use. 40 μl of the frozen competent cells was added to 1 μg of plasmid DNA (resuspended in 2 μl of TE buffer) and mixed by drawing the mixture up and down with a micropipette. The mixture was added to the cold electroporation cuvette (0.2 cm), and a single pulse was applied (25 μF capacitance, 200 Ω resistance, and a field strength of 10.0 kv/cm) with Gene Pulser Apparatus (BioRad Laboratories, Richmond, CA, USA). The pulsed mixture was immediately diluted with 1 ml of appropriate broth (MRS for lactobacilli, M17 for lactococci), transferred to a 1.5 ml eppendorf tube, incubated for 2 hours at 37°C (lactobacilli) or 30°C (lactococci), and then plated onto MRS-0.5 M sucrose Em (5 μg/ml) or onto M17G-0.5 M sucrose Em (5 μg/ml) plate. Transformants were usually visible after 48 hrs of incubation. Frozen *E. coli* competent cell preparation and electroporation procedures were followed by the method of Dower *et al.* (8).

Enzyme Assay

Qualitative α-amylase assay was done by staining plates containing soluble starch with iodine and examining the presence and size of clear zones (halo) around colonies with α-amylase plasmids. For the quantitative enzyme assay, the method described by Kawaguchi *et al.* was employed with some modifications (12). *Lactobacillus* strains were cultivated in MRS-lactose (1%) broth until the early stationary phase. The optical density of the culture was measured at 660 nm and then cells were separated from the supernatant by centrifugation. 0.5 ml of supernatant was mixed with 1 ml of 2% soluble starch solution (in 100 mM Tris-HCl; pH 7.0) and incubated at 50°C for 30 min. Then 2.5 ml of stop solution (0.5 N acetic acid to 0.5 N HCl; 5:1) was added to stop the reaction. 0.5 ml of final reaction mixture was added to 5 ml of 0.01% I_2/0.1% KI solution and incubated for 20 min at room temp., and then the absorbance at 660 nm was measured. One enzyme unit was defined as the amount of enzyme which could reduce the absorbance by 1.0 at 660 nm within 30 min when cells were grown to A_{660} 1.0. For determining enzyme activities remaining in the cell, cell pellet was resuspended in 100 mM Tris-HCl buffer, and then lysed by ultrasonication. Insoluble materials were removed by centrifugation, and enzyme activities in the soluble fraction were determined as mentioned above.

Plasmid Stability

Stability of pIL2530α in *L. casei* strains was examined as follows. Actively growing *L. casei* cells harboring pIL 2530α in MRS Em (5 μg/ml) broth were inoculated into fresh MRS broth (1%) without antibiotic, incubated for 24 hr at 37°C. Then grown cells were again inoculated into fresh medium without Em. Continued subculturing in fresh MRS broth at daily intervals was repeated up to a month. Every week, aliquots of culture were taken and serially diluted with 1/10 MRS broth. 0.1 ml of appropriately diluted sample was spread onto MRS Em and MRS plates, respectively, incubated at 37°C for 48 hrs. The percentage of cells still harboring pIL2530α was calculated as follows.

$$\text{Percentage of cells keeping pIL2503}\alpha = \frac{\text{number of cells on MRS Em}}{\text{number of cells on MRS plate}} \times 100$$

RESULTS AND DISCUSSION

Construction of pIL2530α and Expression of α-amylase Gene in *Escherichia coli*

As vehicles for introduction and expression of the α-amylase gene from *B. licheniformis* in lactic acid bacteria, three different lactococcal cloning vectors; pIL253, pSA3 and pMG36e (Table 1) were employed. Among them, pIL253 (4.9 kb, Em, 18) turned out to be the most efficient in terms of α-amylase production (see below). Since pIL253 cannot replicate in *E. coli*, a derivative shuttle plasmid, pIL2530 (7.1 kb, Em), was constructed for this work. As shown in Fig. 1, pIL2530 was obtained by ligating the 2.2 kb *Eco*RI-*Sal*I fragment containing Gram - origin from pACYC 184 to pIL253, previously cut with the same enzymes. Since the 2.2 kb fragment contains Gram - origin, pIL 2530 can replicate in both Gram + and Gram - hosts. pIL2530 was stable in both *E. coli* and lactic acid bacteria in the presence of Em (5 μg/ml in lactic acid bacteria, 200 μg/ml in *E. coli*) after repeated subculturing (results not shown). 3 kb *Eco*RI fragment encompassing *amyL* from *B. licheniformis* was obtained by *Eco*RI digestion of pTA322 (13, see Fig. 1), and the GeneClean purified fragment was introduced into the unique *Eco*RI site in MCS of pIL2530, thus generating pIL2530α. Likewise, pSA3α and pMG36eα were obtained by ligating the 3 kb fragment into the unique *Eco*RI site of pSA3 and pMG36e, respectively. The

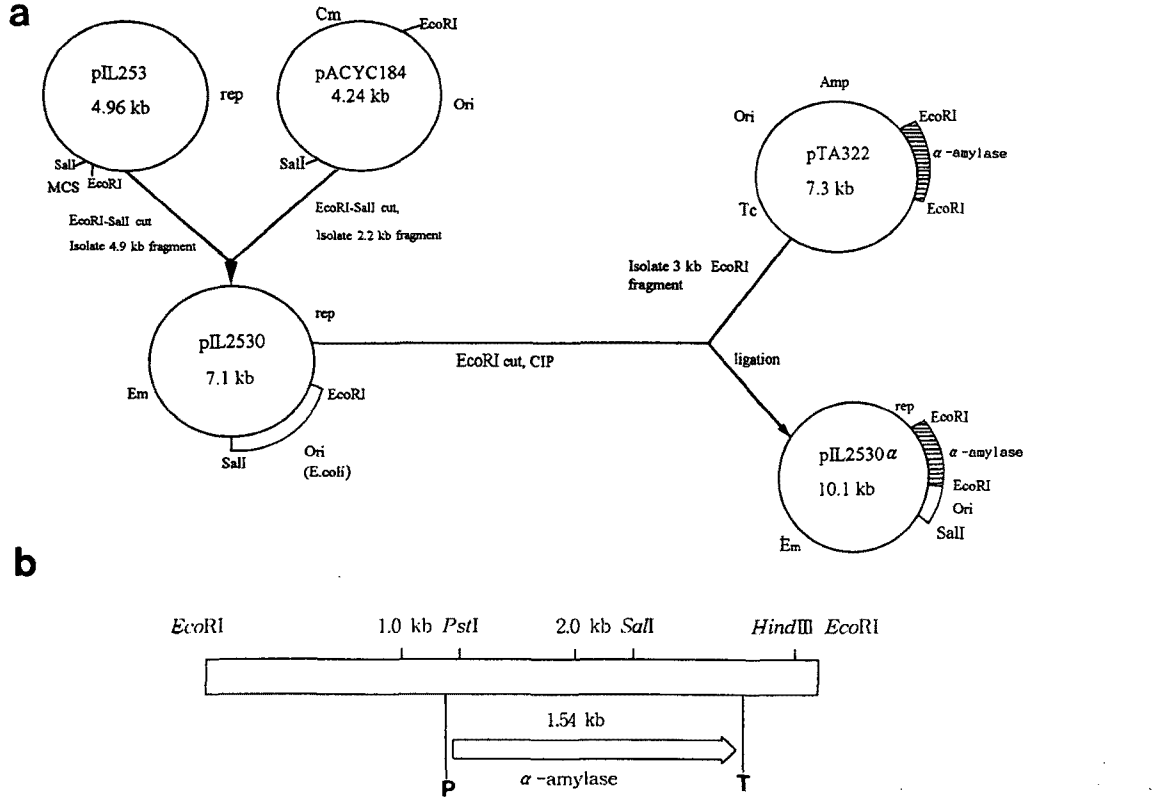

Fig. 1. a, Construction of pIL2530α for expression of *Bacillus licheniformis* α-amylase gene in *Lactobacillus* strains. b, Restriction map of 3 kb *Eco*RI fragment encompassing the α-amylase gene (13).
P denotes promoter sequences and T denotes transcription terminator.

complete nucleotide sequences of *amyL* were determined and analyzed by Kim (14). The *amyL* gene within the 3 kb fragment is 1,536 bp in size, capable of encoding a polypeptide of 512 amino acids. Promoter sequences with features observed among the major *E. coli* promoters (σ^{70} recognized) and *B. subtilis* vegetative promoters (σ^{43} recognized) locate upstream of the start codon (ATG), and a transcription terminator locates immediate downstream of the last codon.

In *E. coli*, cells harboring pIL2530α showed the highest enzyme activities whereas cells with pSA3α showed the lowest. Table 2 shows the results of α-amylase assays for *E. coli* and lactic acid bacteria harboring various plasmids and Fig. 2 shows the formation of clearing zones on LB soluble starch (1%, w/v) plate by *E. coli* cells harboring various α-amylase plasmids. As mentioned above, pIL2530α was the most efficient vector for α-amylase expression and then pMG36eα, pSA3α followed in decreasing order when extracellular α-amylase activities were considered. The differences in the level of expression might be resulted from the differences in plasmid copy numbers in *E. coli* cells, the vector sizes, and the adjacent vector sequences. Obviously, the *amyL* promoter was functional in *Lactobacillus* strains, suggestive of the similarity in transcriptional control mechanisms between *Bacillus* and *Lactobacillus*. About half of the enzyme activity in each case was detected inside the cell, indicating the signal sequence of *amyL* was not so effective as in the original host or in other Gram + cells. Kim *et al.* reported that the proportion of the secreted α-amylase was about 10% of total enzyme produced when *amyL* was cloned in pBR322 (13). Thus, the proportion of enzyme secreted outside the cell observed in this work was 5 times higher than that of Kim *et al*'s. Separate experiments consistently gave similar results and the values shown in Table 2 are average values of at least three measurements.

Table 2. α-amylase activities of *E. coli* and lactic acid bacteria harboring various α-amylase plasmids.

Strains	Enzyme activity (unit)	
	culture supernatant	cell
E. coli		
DH5α (pIL2530α)	7.78	7.42
DH5α (pSA3α)	4.77	9.07
MC1061 (pMG36eα)	5.20	5.53
MC1061 (pIL2530α)*	6.18	2.05
L. casei YIT 9018 (pIL2530α)	0.92	0.23
L. casei YIT 9018 (control)	0.00	0.00
L. casei ATCC 4646 (pIL2530α)	0.59	0.06
L. casei ATCC 4646 (control)	0.00	0.00
Lac. lactis MG 1363 (pIL2530α)	0.85	nd

nd, not determined; *, MC 1061 transformed with pIL2530α DNA derived from *L. casei* ATCC 4646 (pIL2530α). In this transformant, size change in pIL2530α was not detected.

Introduction of pIL2530α into *Lactobacillus* Strains

Among pIL2530α, pSA3α and pMG36eα, only pIL 2530α was able to transform *Lactobacillus* strains tested in this work. With pSA3α and pMG36eα, no transformants were obtained under the electroporation conditions described in the Materials and Methods section. For some *Lactobacillus* strains, numerous tiny colonies appeared on the selective medium in 48 hrs but these turned out to be pseudotransformants since they could not grow in MRS broth containing Em (5 μg/ml) and did not contain plasmids. Chassy *et al.* reported that pSA3 transformed *L. casei* ATCC 393 at efficiency of 1.1×10^4 transformants/μg DNA (4). Since no true transformants with pSA3α were obtained after repeated trials, it was suspected that pSA3 might have replication problems in *Lactobacillus* strains and the data of Chassy *et al.* might be due to the appearance of pseudotransformants. No other data confirming the presence of pSA3 in transformants were presented in their paper. The possibility, however, that the conditions employed for this experiment were not optimal for pSA3 transformation cannot be ruled out. Both pSA 3α and pMG36eα were successfully introduced into *Lactococcus lactis* strains and production of α-amylase was confirmed (results not shown). Only for *L. casei* ATCC 4646 and *L. casei* YIT 9018 strains, transformants with pIL2530α were obtained. But the efficiencies were quite low; less than 1×10^2 trans-

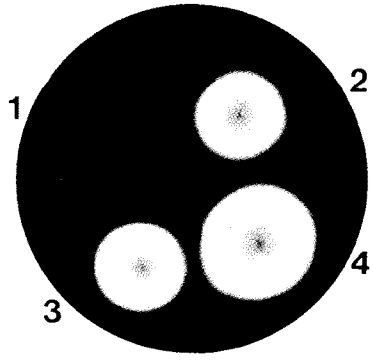

Fig. 2. Formation of clearing zones on LB soluble starch medium resulting from α-amylase activity of *E. coli* strains. 1, DH5α (pIL2530); 2, MC1061 (pMG36eα); 3, DH5α (pSA3α); 4, DH5α (pIL2530α).

formants/μg DNA. Although no extensive efforts to improve transformation efficiency were done, these results clearly indicate that one of the most serious obstacles for genetic manipulation of *Lactobacillus* strains is still the lack of efficient gene transfer methods. *L. casei* ATCC 4646 (pIL2530α) and *L. casei* YIT 9018 (pIL2530α) cells grew fast in MRS broth containing Em (5 μg/ml) and showed significant enzyme activities when assayed (Table 2 and Fig. 3). But the pIL2530α (10.1 kb) was difficult to locate when plasmid preparations were analyzed by agarose gel electrophoresis. To confirm the presence of pIL2530α in *L. casei* transformants, plasmid DNA was prepared from *L. casei* transformants and used to transform competent *E. coli* MC1061 cells. Transformants which could grow on LB Em (200 μg/ml) plates were obtained and they showed clear zones when spotted on LB plates containing soluble starch. Furthermore, when plasmid DNA from *E. coli* transformants were prepared, digested with *Eco*RI and examined by agarose gel electrophoresis, two bands corresponding to the 7.1 kb pIL2530 and the 3 kb α-amylase containing fragment, respectively clearly appeared (Fig. 4). These results confirm that true transformants were obtained for *L. casei* ATCC 4646 and *L. casei* YIT 9018 strains. It is not clear why pIL 2530α was difficult to locate from the plasmid preps of *Lactobacillus* transformants. It might be due to the low copy number of pIL2530α in *L. casei* strains tested or the interference by resident plasmids since both ATCC 4646 and YIT 9018 have few cryptic plasmids. Another observation was that when plasmids were prepared from MC1061 transformants, two differently sized plasmids were detected. In addition to the expected 10.1 kb (pIL2530α) size plasmid, a larger plasmid (11 kb) was recovered from some transformants. In figure 4, 4-1, 4-5 and 6-3 plasmids are larger than 6-1 and 6-4 by approximately 0.9 kb. By *Eco*RI digestion, these larger plasmids produced a 3 kb α-amylase band and an 8 kb band. No significant differences in α-amylase activities in *E. coli* were observed between two plasmids. Obviously, some structural changes in pIL2530α occurred during the transformation of *Lactobacillus* strains but the exact cause is unknown. The increase in vector size might be due to incorporation of an insertion sequence as reported by Walker *et al.* (21) but the possible involvement of an insertion sequence was not examined.

α-amylase Gene Expression in Lactic Acid Bacteria

L. casei cells harboring pIL2530α showed significant α-amylase activities and Table 2 shows the results of α-amylase assays. The overall levels of activities were five to ten-fold lower than those of *E. coli* transformants. But most enzyme activities were detected in the culture supernatant, indicating the proper working of *Bacillus* signal sequences in *Lactobacillus* hosts. Further work of purifying α-amylase from the culture supernatant and determining its N-terminal amino acid sequence will confirm the exact processing of *Bacillus* α-amylase during secretion in *Lactobacillus* cells. Although there were some attempts to express α-amylase gene from various *Bacillus* species in *Lactobacillus* strains including *Lactobacillus plantarum* (17) and *Lactobacillus helveticus* (10), no detailed results were presented regarding the efficiency of expression. Therefore, it was difficult to compare the efficiency of expression ob-

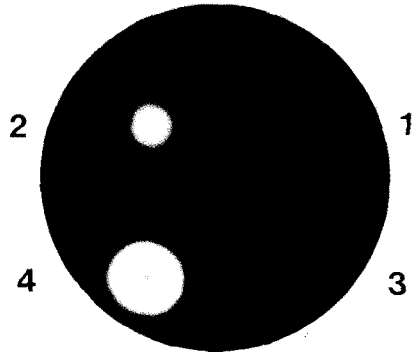

Fig. 3. *Formation of clearing zones on MRS soluble starch* medium resulting from α-amylase activity of *Lactobacillus strains.*

1, *L. casei* ATCC 4646; 2, *L. casei* ATCC 4646 (pIL2530α); 3, *L. casei YIT 9018;* 4, *L. casei* YIT 9018 (pIL2530α).

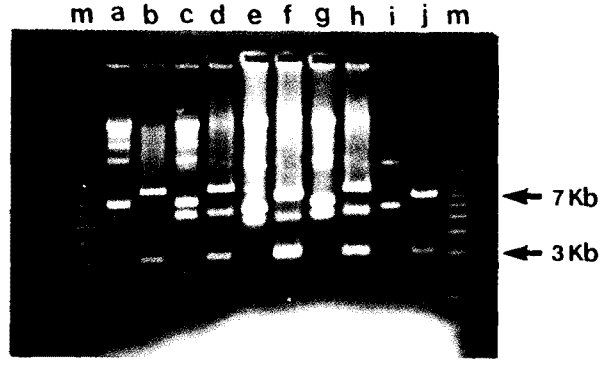

Fig. 4. Agarose gel electrophoresis of plasmids prepared from MC1061 strains transformed with pIL2530α derived from *L. casei* ATCC 4646 (designated 4-1, 4-5) and *L. casei* YIT 9018 (designated 6-1, 6-3, 6-4) transformants.

m, 1 kb ladder size marker (BRL); a, 4-1; b, *Eco*RI digested 4-1; c, 4-5; d, *Eco*RI digested 4-5; e, 6-1; f, *Eco*RI cut 6-1; g, 6-3; h, *Eco*RI cut 6-3; i, 6-4; j, *Eco*RI cut 6-4 DNA.

Table 3. Stability of pIL2530α in *L. casei* strains.

Strain	Incubation Time (days)				
	0	5	10	15	21
YIT 9018	100%	5.2	0.53	0.28	0.01
ATCC 4646	100%	3.8	0.70	0.88	0.10

tained through this study with those of previous reports. Another interesting observation was that expression of α-amylase in *L. casei* was affected by "catabolite repression phenomena" as in *Bacillus*. Cells grown on MRS-Glucose (0.05%-0.2%, w/v)-soluble starch plates (1%) showed obvious clear zones while cells on the same plate, except the glucose concentration was increased to 0.5%, hardly showed clear zones although the colony size increased. It will be interesting to investigate whether α-amylase gene expression in the presence of glucose is repressed by the common mechanisms in both genera.

Stability of pIL2530α in *L. casei* Strains

The results of stability tests for pIL2530α in *L. casei* strains are shown in Table 3. As shown in Table 3, pIL2530α was not quite stable without antibiotic selection, a common problem in plasmid-based expression systems. In the absence of erythromycin, both strains lost pIL2530α quickly. After 10 days of daily subculturing in MRS media, less than 1% of cells maintained plasmids. After 3 weeks, only 0.1% of cells still maintained pIL2530α. These results indicate that measures to improve plasmid stability without use of antibiotics are necessary. Plasmid stability, or more precisely the maintenance of a specific gene, is an important issue especially for the development of so-called food-grade vectors or cloning systems where use of antibiotics is undesirable. Currently, attempts to integrate the whole pIL2530α plasmid and only the α-amylase gene into the chromosome of *Lactobacillus* strains are in progress and will be reported, elsewhere.

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