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Cloning of the Entire Gene Encoding the 140-kDa α-Amylase of *Lactobacillus amylovorus* and Expression in *Escherichia coli* and *Lactococcus lactis*

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A 4.6-kb *Hin*dIII fragment encompassing the complete 140-kDa α-amylase gene of *Lactobacillus amylovorus* B 4540 was cloned into pBR322 by the shot gun method. Southern blotting and restriction mapping for the insert were performed. The recombinant 9.0-kb plasmid, pFML1, conferred α-amylase activity to *E. coli* and *Lactococcus lactis* hosts when introduced by electroporation. SDS-PAGE and zymography confirmed the production of 140-kDa α-amylase and its proteolytic degradation products with enzyme activity in transformants. Total α-amylase activity of *E. coli* DH5α cells harboring pFML1 was 1.8 units and most activity was detected from cell pellets. Total enzyme activity of *L. lactis* subsp. *lactis* MG1363 transformant was five to ten-fold lower than that of *E. coli* cell but more than half of the activity was detected in the culture supernatant.

Lactobacillus amylovorus first isolated from cattle waste-corn fermentations by Nakamura, possesses amylolytic activities and thus is able to utilize starch directly to form lactic acid (15). Burgess-Cassler *et al.* (4) reported that *L. amylovorus* produced three different amylases; 160-kDa amylase, 140-kDa α-amylase, and 65-kDa α-amylase. They also partially purified the 140-kD α-amylase, a major amylase of *L. amylovorus*, from the culture supernatant and showed that the optimum pH and temperature for the reaction were 5.5 and 60~65°C, respectively. Since *L. amylovorus* can produce lactic acid directly from starch without additional liquefication/saccharification processes, it can be employed as a starter for developing an economical process for lactic acid production from inexpensive waste materials containing starch. Cheng *et al.* (6) investigated the potential of *L. amylovorus* as a starter for a novel lactic acid production process from corn starch. The final level of lactic acid obtained under optimized conditions was similar to that obtained with *L. delbrueckii* B-445, a traditional starter for lactic acid fermentation, using glucose as the substrate. Fitzsimons *et al.* (9) attempted to clone α-amylase genes from *L. amylovorus* for the purpose of constructing amylolytic *L. plantarum* silage strains but they only succeeded in cloning part of an α-amylase gene corresponding to N-terminal domain of 140-kD α-amylase. We have tried to clone α-amylase genes from *L. amylovorus* for the purpose of constructing recombinant *Lactobacillus* strains capable of performing lactic acid fermentation from starch. Here, we report the cloning of the entire gene encoding the 140-kDa α-amylase of *L. amylovorus* and the expression of the cloned gene in heterologous hosts such as *Escherichia coli* and *Lactococcus lactis* cells.

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MATERIALS AND METHODS

Bacterial Cultures and Media

Bacterial strains and plasmids used in this study are described in Table 1. *L. amylovorus* B4540 was obtained from Dr. Burgess-Cassler at USDA (Peoria, IL, U.S.A.) and was grown in MRS broth (Difco Laboratories, Detroit, MI, U.S.A.) without agitation or on MRS plates (1.5% agar) at 37°C (7). *Lactococcus lactis* subsp. *lactis* MG 1363 was grown in M17G (glucose, 1%) broth (18) 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), 12.5 μg/ml; erythromycin (Em), 200 μg/ml for *E. coli*, 5 μg/ml for *Lactococcus lactis*.

DNA Isolation and Genomic Library Construction

Table 1. Bacterial strains and plasmids.

Bacterial strain or plasmid	Description	Reference
E. coli		
DH5α	ϕ80*dlacZ*ΔM15 *recA1 endA1 gyrA96 thi*-1 *hsdR17* (r_k^-, m_k^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)U169	BRL
MC 1061	*araD139 lacX74 galU galK hsr hsm*$^+$ *strA*	5
Lactobacillus amylovorus B 4540		
	amylolytic strain	15
Lactococcus lactis subsp. *lactis* MG 1363		
	plasmid-free derivative of NCDO 712, Lac$^-$	10
plasmids		
pBR322	4.3 kb, Ap^r, Tc^r, general cloning vector	3
pUC19	2.7 kb, Ap^r, general cloning vector	20
pMG36e	Em^r, 3.6 kb; expression vector carrying origin of cryptic plasmid, pWV01	19
pFML1	9.0 kb, pBR322 containing 4.6-kb *Hin*dIII fragment encompassing 140-kDa α-amylase gene	This study
pFML1d	6.1 kb, a shortened derivative of pFML1, 2.9-kb *Bam*HI fragment was deleted.	This study
pFML2	8.2 kb, pMG36e containing 4.6-kb *Hin*dIII fragment encompassing 140-kDa α-amylase gene	This study
pFU3	6.6 kb, pUC19 containing 3.9-kb *Hin*dIII fragment derived from 4.6-kb insert of pFML1	This study
pFML5	a shortened derivative of pFML1, 1.3-kb 3' region of 4.6-kb insert was deleted by *Acc*I digestion.	This study

Total DNA from *L. amylovorus* B4540 was prepared by the method of Luchansky *et al.* (14) with some modifications. Instead of mutanolysin, the amount of lysozyme used for cell-wall disruption was increased from 20 to 30 μg/ml and the incubation time at 37°C was extended to one hour. Plasmid DNA from *Lactococcus lactis* was isolated by the method of O'Sullivan and Klaenhammer (16). Plasmid DNA from *E. coli* was isolated by the alkaline lysis method of Birnboim and Doly (2). For electroporation experiments, plasmid DNA was prepared using a Qiagen plasmid midikit (Qiagen, Hilden, Germany). A genomic library of *L. amylovorus* B4540 was constructed in *E. coli* DH5α using pBR322. 50 μg of total DNA was digested with *Hin*dIII. DNA fragments of 2 to 10 kilobase pairs (kb) in length were isolated by agarose gel electrophoresis followed by electroelution. Then 1.0 μg of the isolated fragments and 0.2 μg of pBR322 cleaved with *Hin*dIII and dephosphorylated with CIP (Calf Intestinal Phosphatase) were joined with T4 DNA ligase at 16°C for 16 h. *E. coli* DH5α cells were transformed with the ligation mix, plated on LB agar containing ampicillin (100 μg/ml). Transformants were replicated onto LB agar plates supplemented with 0.3% soluble starch. After growth at 37°C for 48 h, α-amylase positive clones were detected by staining with 10 mM I_2-KI solution.

DNA Manipulations and Southern Blotting

Restriction enzyme digestions 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). Restriction fragments for subcloning were isolated from agarose gels by using GeneClean II kit (Bio 101, LaJolla, CA, U.S.A.). Southern blotting was performed as described by Sambrook *et al.* (17). DNA probes were labeled with α-^{32}P-dATP by using multiprime™ DNA labelling kits (RPN 1600Y) supplied by Amersham Corp. and were hybridized to Southern blots as directed in the kits.

Electroporation

Introduction of plasmids into competent *E. coli* and *L. lactis* cells was achieved by the electroporation method. Frozen competent *L. lactis* MG1363 cells were prepared as described by Holo and Nes (11). M17G broth containing 20 mM DL-threonine was used for cultivation. After growth to an optical density at 600 nm of 0.5 to 0.7, cells were harvested by centrifugation at 4°C. Following two washes in ice-cold sterile water and two washes in 0.5 M sucrose containing 10% glycerol, cells were resuspended in 1/200 of original culture volume of washing solution and then stored in aliquots of 40 μl at -76°C. Frozen competent cells were added to the ligation mixture (resuspended in 2 μl TE buffer) and the mixture was transferred to a cold electroporation cuvette (0.2 cm). A single pulse was applied (25 μF capacitance, 200 Ω resistance, and a field strength of 12.5 kv/cm) with Gene Pulser Apparatus (BioRad, Richmond, CA, U.S.A.). The pulsed mixture was immediately diluted with 1 ml of M 17G, incubated for 2 h at 30°C, and then spread onto M 17G plates containing 0.5 M of sucrose and Em (5 μg/ml). Transformants were usually visible after 48 h of incubation at 30°C. Frozen *E. coli* competent cell preparation and electroporation procedures were followed by the method of Dower *et al.* (8).

Enzyme Assay

Qualitative α-amylase activity measurement was performed by staining plates containing soluble starch (0.3%) with 10 mM I_2-KI solution and examining the size of clear zones arround colonies with α-amylase plasmids. For quantitative enzyme assay, total reducing sugars released from soluble starch were measured according to the method of Bernfeld (1). Cell extracts were prepared by washing cell pellets twice in half of the original culture volume of 0.2 M sodium acetate buffer (pH 5.5) and resuspending the cells in 1/10 the original culture volume of 0.2 M sodium acetate buffer (pH 5.5). The cell suspension was placed on ice and subjected to sonication with a Bandelin Sonopuls (model HD60) ultrasonic homogenizer. The sonicated cells were centrifuged at 5,000×g for 10 min at 4°C to remove cell debris, and the supernatants were used for activity measurements. Culture supernatants for measuring the activity of secreted amylase were prepared by centrifugation at 12,000×g for 10 min at 4°C. The reaction mixture consisted of 2 ml of 0.02 M acetate buffer (pH 5.4)-5 ml of 0.5% soluble starch solution-1 ml of 1% NaCl solution-0.5 ml of distilled water-0.5 ml of enzyme solution. After incubation at 65°C for 30 min, the reaction was stopped by cooling on ice. The released reducing sugars were measured by dinitrosalicylic acid. 1 enzyme unit was defined as the amount of enzyme which release 1 μmol of maltose per min.

SDS-PAGE and Zymogram

SDS-PAGE was carried out according to the method of Laemmli (13). Stacking (4%) and separating (8%) gels contained 0.25% soluble starch. Enzyme samples were mixed with an equal volume of 2× sample buffer (2.25% glycerol, 0.25% β-mercaptoethanol, 2.3% SDS, 0.001% bromphenol blue, and 62 mM Tris HCl, pH 6.8) and heated for 5 min at 100°C prior to electrophoresis. After electrophoresis, one-half of the gel was stained with Coomassie brilliant blue G250. For the zymogram, the other half of the gel was washed (four times for 30 min) in 10 mM Tris HCl (pH 6.8) plus 0.25% starch to remove SDS and to allow renaturation of the proteins for the zymogram. Subsequently, the gel was incubated overnight at 37°C in the same buffer. α-amylase activity was visualized as clear zones after the gel was stained with 10 mM I_2-KI solution. Culture supernatants were used as enzyme samples for zymography after concentration (more than 10 fold) using Centriprep-10 (Amicon, Beverly, MA, U.S.A.).

RESULTS AND DISCUSSION

Cloning of a 140-kDa α-Amylase Gene from *L. amylovorus*

Among about 10,000 transformants obtained, only one clone showed a large, clear halo around the colony when the plates were stained with 10 mM KI-I_2 solution. The Amy^+ transformant contained a 9.0-kb plasmid and when the plasmid was digested with *Hin*dIII, two fragments of 4.4 and 4.6 kb were generated. In addition, the Amy^+ phenotype was transferable to other *E. coli* cells by electrophoration with purified 9-kb plasmid DNA. All these results indicated that a 4.6-kb *Hin*dIII fragment containing an α-amylase gene was cloned into the *Hin*dIII site of pBR322. The 9-kb recombinant plasmid was designated pFML1. To prove that the 4.6-kb *Hin*dIII fragment really originated from the *L. amylovorus* chromosome, Southern blot analysis was performed using the 4.6-kb fragment as a probe. The hybridization results are shown in Fig. 1. As shown in Fig. 1, the probe strongly hybridized to the ca. 4.6-kb *Hin*dIII fragment and 15-kb *Eco*RI fragment, respectively. Other signals might be due to partial digestion of *L. amylovorus* chromosomal DNA. Therefore, Southern blot results confirmed that the 4.6-kb fragment originated from the *L. amylovorus* chromosome. The restriction map of the 4.6-kb fragment was obtained and shown in Fig. 2. For restriction enzyme *Acc*I, *Bam*HI, *Pst*I, *Pvu*II and *Sph*I, a single site was located within the insert but sites for *Ava*I, *Bcl*I, *Bgl*II, *Eco*RI, *Eco*RV, *Kpn*I, *Sac*I, *Sal*I, *Sma*I, and *Xho*I were not detected. From inspection of the restriction map, it became clear that the cloned 4.6-kb fragment encompassed the major α-amylase gene, encoding the 140-kDa enzyme, of *L. amylovorus* since the restriction map of the first 1-kb region from the left-junction of the fragment (left-junction corresponds to the *Hin*dIII site near the +1 position in pBR322 sequence) exactly matched

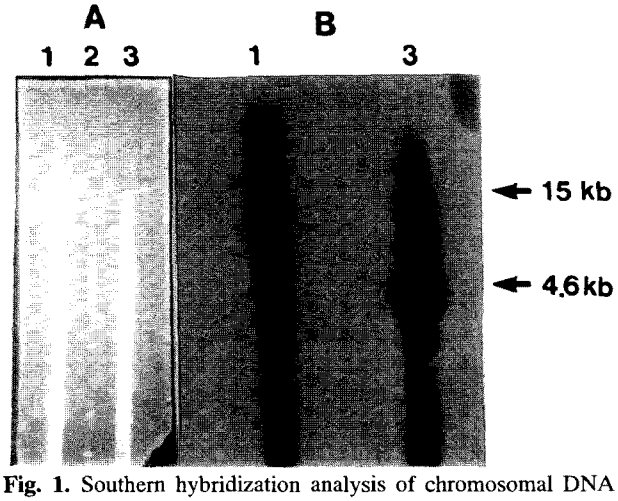

Fig. 1. Southern hybridization analysis of chromosomal DNA from *L. amylovorus*.

A. Ethidium bromide stained agarose gel. Lanes 1 and 3, *L. amylovorus* chromosomal DNA cleaved with *Eco*RI and *Hin*dIII, respectively. Lane 2, 1 kb size ladder (BRL). B. Autoradiogram of gel A. Arrows indicate the major hybridization signals and their sizes.

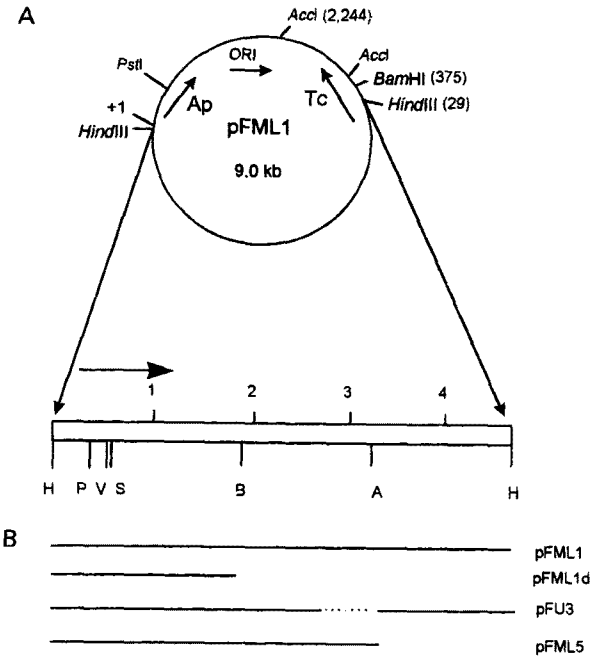

Fig. 2. A: Restriction map of the cloned 4.6-kb *Hin*dIII fragment encompassing the entire gene for the 140-kDa α-amylase of *L. amylovorus* B4540.
Abbreviations: A, *Acc*I; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; V, *Pvu*II; S, *Sph*I. Arrow indicates the direction of transcription. The numbers in parentheses refer to the corresponding positions in pBR322 sequences. B: the size and position of insert DNA in pFML1, pFML1d, pFU3 and pFML5.

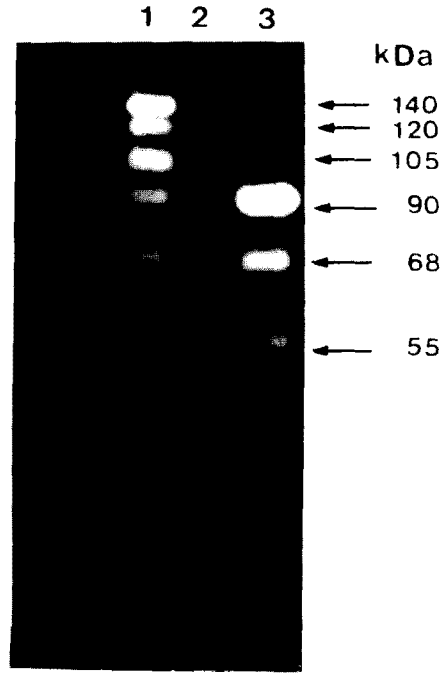

Fig. 3. Zymogram of SDS-PAGE gel.
Lane 1, Culture supernatant of *E. coli* DH5α (pFML1); lane 2, supernatant of DH5α (pFML1d); lane 3, supernatant of DH5α (pFU3). Arrows indicate the location and size of each band with α-amylase activity.

with that of the 2.0-kb *Mbo*I fragment cloned by Fitzsimons *et al.* (9). Since Burgess-Cassler *et al.* (4) reported that by partial proteolytic digestion of 140-kDa α-amylase with trypsin, proteins in the 48- to 105-kDa size range with amylolytic activity were generated, Fitzsimons *et al.* concluded that the 2.0-kb *Mbo*I fragment carried the 5' region of the 140-kDa α-amylase gene, corresponding to the N-terminal domain responsible for enzyme activity. To confirm that the 4.6-kb fragment overlapped with the 2.0-kb *Mbo*I fragment of Fitzsimons *et al.*, Southern blot analysis was repeated using the 2.0-kb *Mbo*I fragment (from pYYZ2110, provided by Professor Delcour) as a probe. The same hybridization signals as shown in Fig. 1 were obtained (results not shown). The size of our cloned fragment is large enough to encode the entire 140-kDa protein (the left-junction of the 4.6-kb fragment corresponds to the immediate upstream regulatory region of the α-amylase gene) and the production of 140-kDa enzyme was confirmed in transformants harboring pFML1 by SDS-PAGE and subsequent zymography (see Fig. 3). Therefore, we concluded that the 4.6-kb *Hin*dIII fragment encompassed the entire gene encoding the 140-kDa α-amylase of *L. amylovorus*.

Expression of the Cloned α-Amylase Gene in *E. coli*

Expression of the cloned α-amylase gene in *E. coli* was investigated using pFML1 and its derivatives. In the 4.6-kb insert of pFML1, a single *Bam*HI site locates 1.8 kb downstream from the left-junction, and another *Bam*HI site locates approximately 350 bp downstream from the right-junction of the insert (in pBR322 sequence). When pFML1 was digested with *Bam*HI and self-ligated, a shortened derivative with the 3.3-kb *Bam*HI fragment deleted was obtained and designated pFML1d (6.7 kb). pFML1d contained only 1.8-kb 5' region of the insert (see Fig. 1) but still maintained α-amylase activity (see Table 2), confirming the observations of Fitzsimons *et al.* (9). When *Hin*dIII-digested pFML1 DNA was ligated with *Hin*dIII-cut pUC19 DNA, a 6.6 kb Amy$^+$ plasmid where 3.9-kb *Hin*dIII fragment was inserted into the *Hin*dIII site of pUC19 was obtained and named as pFU3. In pFU3, a 0.7-kb region between the *Bam*HI site and the *Acc*I site on the 4.6-kb insert of pFML1 was missing (see Fig. 2) and the direction of the α-amylase promoter was opposite to that of the *lac* promoter in pUC19. When pFU3 was first obtained, we suspected that the 4.6-kb insert contained an additional *Hin*dIII site located 0.7 kb upstream from the right-junction. Later, it was found that a 0.7-kb internal region between the *Bam*HI site and the *Acc*I site of the insert

Table 2. α-Amylase activities of *E. coli* and *L. lactis* strains harboring α-amylase plasmids.

Strains	Enzyme activity (unit)*	
	Culture supernatant	Cell pellet
E. coli		
DH5α (pFML1)	0.23	1.58
DH5α (pFML1d)	0.26	0.82
DH5α (pFU3)	0.20	1.25
MC1061 (pFML2)	0.26	0.25
DH5α (pBR322)	0.08	0.07
L. lactis MG1363 (pFML2)	0.22	0.17
L. lactis MG1363	0.09	0.07

*1 emzyme unit is the amount of enzyme which produce 1 μmol reducing sugars (maltose) from soluble starch at 65°C per min.

was deleted in pFU3. The presence of an internal *Hin*dIII site near the right-junction of the insert was not confirmed after extensive *Hin*dIII digestion of either pFML1 or a Gene Clean isolated 4.6-kb insert and the sizes of restriction fragments derived from 3.9-kb insert of pFU3 were identical with those from 4.6-kb insert of pFML1 except those of *Bam*HI-*Acc*I fragments (data not shown). Additional evidence indicating internal deletion in pFU3 was the size of the α-amylase bands on the zymogram. If deletion occurred near the right-junction, the resulting 3.9-kb fragment should direct synthesis of at least 120-kDa protein. But the largest protein directed by pFU3 was only 90 kDa in size as shown in Fig. 3. Therefore, we concluded that somehow the 0.7-kb internal region (probably near the *Acc*I site, judged from the size of the largest protein directed by pFU3) of insert was deleted in pFU3. The exact site and cause of deletion are currently under investigation. When pFML1 was digested with *Acc*I and then self-ligated, a 5.5 kb shortened derivative, pFML5, was obtained. In pFML5, a 1.3-kb downstream region from *Acc*I site in the insert was deleted together with pBR322 sequences up to the second *Acc*I site (at 2,244 position in pBR322 sequence). *E. coli* DH5α (pFML5) directed synthesis of 120- and 105-kDa α-amylase proteins in addition to smaller-sized proteins when zymography was performed (data not shown). Results obtained from pFML5 again support the internal region deletion in pFU3. α-amylase activities of *E. coli* strains harboring plasmid constructs were examined and represented in Table 2. *E. coli* DH5α cells harboring pFML1 showed the highest enzyme activity and most activity was detected in the cell pellet (87% of total activity), indicating poor secretion of *L. amylovorus* α-amylase in *E. coli* cell. DH5α cells harboring either pFML1d or pFU3 sustained ca. 60% or 80%, respectively, of total activity of cells with pFML1. *E. coli* MC 1061 cells harboring pFML2 (see Table 1 for description) showed the lowest enzyme activity and this might be due to a low copy number of pMG36e in *E. coli* cells. The origin of pMG36e was derived from a cryptic, broad-host plasmid, pWV01, of *Lactococcus lactis* subsp. *cremoris* Wg2 (19).

SDS-PAGE and Zymogram

SDS-PAGE and Zymography of enzyme samples were performed as described in Materials and Methods and a zymogram is shown in Fig. 3. As shown in Fig. 3, pFML1 directs synthesis of ca. 140-, 120-, 105-, 90-, 68- and 55-kDa proteins (Lane 1). Among them, the larger three bands were missing in the enzyme sample from *E. coli* DH5α (pFU3) (Lane 3) and all bands except the smallest one (55 kDa) were missing in the enzyme sample from *E. coli* DH5α (pFML1d) (Lane 2). These results indicate that all five bands smaller than 140 kDa were generated by partial proteolytic degradation of the 140-kDa enzyme and the minimum size required for enzymatically active fragments is ca. 55 kDa. These results again agree well with the observations of Burgess-Cassler *et al.* (4) except some discrepancies in the estimated size of fragments. The discrepancies might be due to differences in SDS-PAGE conditions such as the acrylamide content of the gel. Considering the size of the upstream regulatory region, the 1.8-kb insert of pFML1d might correspond to the minimally required gene fragment capable of encoding a protein with α-amylase activity. Protein bands showing α-amylase activity were apparent on zymograms but the corresponding bands were not clearly distinguished from other *E. coli* protein bands on SDS-PAGE gels stained with Coomassie Blue. Obviously, *E. coli* cells produce a lot of proteins of similar sizes, thus obscuring proteins directed by pFML1 or its derivatives. Although 140-kDa and smaller protein bands corresponding to the active bands on zymograms were visible on SDS-PAGE gels, those bands were not clearly distinguished when photographed (data not shown). Overproduction of the α-amylase using efficient expression vector systems might alleviate the masking problem.

α-Amylase Gene Expression in *L. lactis* MG1363

L. lactis MG1363 transformants harboring pFML2 produced halos around colonies on M17G(0.2%) plates containing soluble starch(0.3%). The results of enzyme assays are represented in Table 2. The overall level of total enzyme activity was five to ten-fold lower than that of *E. coli* cells harboring either pFML1, pFML1d or pFU3. The low level of α-amylase activity in *L. lactis* might be due to the low copy number of pMG36e as shown in *E. coli* cells harboring pFML2. It will be interesting to investigate whether the level of α-amylase gene expression is increased by the use of other lactococcal expression vectors. Although the total enzyme activity in *L. lactis* was lower than that of *E. coli* cell, more than half of the activity was detected in the culture supernatant rather than in the cell pellet, indicating the

proper working of *Lactobacillus* signal sequences in the *Lactococcus* host. The same results were obtained for the expression of *Bacillus licheniformis* α-amylase gene (*amyL*) in lactic acid bacteria (12). When the expression levels of the α-amylase gene of *L. amylovorus* were compared with those of the *amyL* of *B. licheniformis*, the activities of *Bacillus* enzyme were higher both in *E. coli* and in *L. lactis* (data not shown). But the α-amylase gene of *L. amylovorus* has one important advantage over other α-amylase genes; it is derived from a safe, food-grade-microorganism, a desirable property for various food fermentations. Obviously, more studies are required on the characteristics of the *L. amylovorus* α-amylase before the potential of this enzyme for commercial applications is fully appreciated.

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