APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2006, p. 269–275 Vol. 72, No. 1
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.1.269–275.2006

Display of α-Amylase on the Surface of *Lactobacillus casei* Cells by Use of the PgsA Anchor Protein, and Production of Lactic Acid from Starch

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Received 29 May 2005/Accepted 28 September 2005

We developed a new cell surface engineering system based on the PgsA anchor protein from *Bacillus subtilis*. In this system, the N terminus of the target protein was fused to the PgsA protein and the resulting fusion protein was expressed on the cell surface. Using this new system, we constructed a novel starch-degrading strain of *Lactobacillus casei* by genetically displaying α-amylase from the *Streptococcus bovis* strain 148 with a FLAG peptide tag (AmyAF). Localization of the PgsA-AmyA-FLAG fusion protein on the cell surface was confirmed by immunofluorescence microscopy and flow cytometric analysis. The lactic acid bacteria which displayed AmyAF showed significantly elevated hydrolytic activity toward soluble starch. By fermentation using AmyAF-displaying *L. casei* cells, 50 g/liter of soluble starch was reduced to 13.7 g/liter, and 21.8 g/liter of lactic acid was produced within about 24 h. The yield in terms of grams of lactic acid produced per gram of carbohydrate utilized was 0.60 g per g of carbohydrate consumed at 24 h. Since AmyA was immobilized on the cells, cells were recovered after fermentation and used repeatedly. During repeated utilization of cells, the lactic acid yield was improved to 0.81 g per g of carbohydrate consumed at 72 h. These results indicate that efficient simultaneous saccharification and fermentation from soluble starch to lactic acid were carried out by recombinant *L. casei* cells with cell surface display of AmyA.

The anchoring of proteins to the cell surfaces of bacteria is potentially important in several areas of biotechnological application, including the construction of oral live vaccines and the production of recombinant proteins. Many related studies have reported the cell surface display of peptides and enzymatically active heterologous proteins in *Escherichia coli* (15, 32), *Lactobacillus*, and *Lactococcus*. Research into the cell surface display of lactic acid bacteria is aimed mainly at its use as an oral vaccine vehicle (3, 17, 20, 29). Most of the reported cell surface display systems have made use of the C-terminal cell wall-anchoring LPXTG motif (26, 31). This is because many cell surface proteins of gram-positive bacteria have an anchoring region that consists of an LPXTG motif followed by a hydrophobic domain and a charged tail at the predicted C terminus (23). The proteins with the LPXTG motif are cleaved after translocation of the plasma membrane and are amide linked to a free amino group of the peptide cross-bridge in the cell wall by a postulated sortase (18). Other reported types of display system have made use of S-layer subunits (3, 13), BspA anchor protein (12, 29), and AcmA anchor protein (4, 25, 30). S-layer subunits and BspA are anchored via charge interactions and can be extracted from the cell surface with charge-occupying agents such as lithium chloride. The AcmA anchor protein binds specifically to peptidoglycan (25).

In most of the previously reported cell surface display systems mentioned above, the target proteins are displayed by N-terminal fusion to the anchoring motif. In the present study, we developed a new system of cell surface display in lactic acid bacteria by using as an anchor protein *Bacillus subtilis* subsp. *chungkookjang* PgsA, which is able to fuse the target protein to its C terminus. PgsA is a transmembrane protein and is one of the poly-γ-glutamate synthetase complexes (1). According to Ashiuchi et al. (2), PgsA functions to stabilize the complex by anchoring in the cell membrane and may function as a poly-γ-glutamate transporter. The PgsA protein is expected to be located between the cell surface and membrane and contains only a transmembrane region consisting of amino acids 25 to 44 as analyzed by the SOSUI (11) system. The PgsA anchor protein is classified as an A1 type anchor (transmembrane anchor) in the system proposed by Leenhouts et al. (16). Since the transmembrane region is located at the N terminus of PgsA, it is predicted that the C terminus of PgsA is exposed outside the cell wall. This is the first report on a cell surface display system of the *Lactobacillus* genus that uses a transmembrane protein.

For the heterologous target protein, we selected the α-amylase (AmyA) (EC 3.2.1.1) of the *Streptococcus bovis* 148 strain, which has a strong ability to hydrolyze and be adsorbed onto corn starch (19, 22). AmyA has been reported to maintain its activity by fusing its N terminus to an anchor protein (24). For the host strain, we selected *Lactobacillus casei* BLSJ 03135, because it produces 0.8 g of L-lactic acid per g of glucose consumed. We propose to develop a process for direct lactic acid fermentation from starch in which the recombinant lactic acid bacteria with cell surface display of AmyA makes it possible to saccharify starch near the cell surface and utilize the resulting sugar to produce lactic acid. Furthermore, we repeat-

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TABLE 1. Plasmids and oligonucleotide primers used

Plasmid or oligonucleotide primer	Phenotype or sequence[a]	Reference
Plasmids		
pSECE1	Wide-host-range vector; erythromycin resistance marker	21
pHCE1LB-pgsBCA-HB168	Vector for display of HB168 using PgsBCA anchor motif; ampicillin and chloramphenicol resistance marker	27
pL3U	Vector under ldhUTLS promoter control; erythromycin resistance marker	Present study
pL3UA	Cell surface display vector containing *pgsA* gene under ldhUTLS promoter control; erythromycin resistance marker	Present study
pL3UAαAF	Vector for display of α-amylase with FLAG tag; derivative of pL3UA	Present study
Primers		
pSH_F	5′-CCCAAGCTTGGTACCGCTGTTTTGGCGGATGAGAGAAG-3′	
pSH_R	5′-CCCAAGCTTAGATCTGCACAAAAAGAAAAACGAAATGATACACC-3′	
Pldh_F	5′-CGCGGATCCAGATCTCCATGGCAAAATTATGAAAAAGTCTGTCAATTTTGTTTCG-3′	
Pldh_R	5′-CGCGGATCCGCATGCGTACAGGCAACACGCCTGAAGCACGTCCTATTACGTGAGTAT AACACATTATCAATTCGCCG-3′	
UTLS_F	5′-CGCGGATCCGCATGCTGATTCTTCAGCAAGACTACTACCTCATGAGAG-3′	
UTLS_R	5′-CGCGGATCCGATATCCATATGGTCTTTTCCTCCTTGAAATAT-3′	
pgsA_F	5′-CCCAAGCTTCAGCTGATGAAAAAAGAACTGAGCTTTCATGAAAAGC-3′	
pgsA_R	5′-TCTGGATCCTTTAGATTTTAGTTTGTCACTATGATCAATATCAAACGTC-3′	
amyA_F	5′-CGCGGATCCGATGAACAAGTGTCAATGAAAGATGGTACG-3′	
amyA_R	5′-CCCAAGCTTACTTGTCATCGTCATCCTTGTAGTCTTTTAGCCCATCTTTATTATAGT TTCCAGATTTTACAAGG-3′	

[a] Restriction enzyme sites are underlined; a FLAG sequence is double underlined.

edly used AmyA-displaying lactic acid bacteria for fermentation. Since recovered cells could hydrolyze starch immediately by use of the displayed AmyA, the efficiency of lactic acid fermentation is expected to be high during repeated utilization.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* NovaBlue {*endA1 hsdR17* [r_{K12}^- m_{K12}^+] *supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB*$^+$ *lacI*^{q}*Z*ΔM15::Tn*10* (Tet^r)]} (Novagen, Inc., Madison, Wis.) was used for construction of vectors. It was cultivated in Luria-Bertani medium or on Luria-Bertani agar plates and grown at 37°C. *L. casei* BLSJ 03135 was grown at 37°C in MRS broth (Difco Laboratories, Detroit, Mich.). Where appropriate, antibiotics were added (250 μg/ml of erythromycin for *E. coli* and 20 μg/ml of erythromycin for *L. casei*).

Construction of plasmids for cell surface display. All PCRs were carried out using KOD-Plus polymerase (Toyobo Co. Ltd., Osaka, Japan), and the oligonucleotides used to construct the plasmids are shown in Table 1. The basic plasmid pL3U has the replication gene of a wide range of hosts from pSECE1 (21) and an ldhUTLS promoter, which consists of the *ldh* core promoter from the lactate dehydrogenase gene of *Lactobacillus casei* (14) and an untranslated leader sequence (UTLS) from the S-layer gene of *Lactobacillus acidophilus* (5). The details of this promoter system will be described elsewhere. pL3U was constructed as follows. First, the plasmid pSH was constructed by inserting an *rrnBT1T2* terminator from pHCE1LB-pgsBCA-HB168 (27) at the SacI site of pSECE1. The pSH plasmid fragment containing a replication gene, an erythromycin resistance gene, and an *rrnBT1T2* terminator was amplified from the plasmid pSH with pSH_F and pSH_R primers. The core promoter of *ldh* was synthesized by PCR with Pldh_F and Pldh_R primers, and the UTLS fragment was amplified from *L. acidophilus* JCM 1132 with UTLS_F and UTLS_R primers. The pSH plasmid fragment, *ldh* promoter fragment, and UTLS fragment were digested with BglII-HindIII, BglII-SphI, and SphI-HindIII, respectively, and then these three fragments were ligated. The resulting plasmid was designated pL3U. To produce the basic plasmid for cell surface display, pL3UA derived from pL3U, the *pgsA* gene was PCR amplified directly from pHCE1LB-pgsBCA-HB168 by using the oligonucleotides pgsA_F and pgsA_R, and the PCR fragment was inserted into pL3U with the EcoRV and BamHI sites. To construct the plasmid for cell surface display of α-amylase, the mature α-amylase gene (*amyA*) fused with the peptide FLAG tag (DYKDDDDK) gene at the C terminus was prepared by PCR from pQE31::*amyA* (24) with amyA_F and amyA_R primers. The amplified fragment was digested with BamHI and HindIII and introduced into the BamHI and HindIII sites of plasmid pL3UA. The resulting plasmid, in which the fusion protein of the PgsA-AmyA-FLAG was expressed, was designated pL3UAαAF (Fig. 1).

Transformation of *L. casei* BLSJ 03135 was performed by electroporation. The sample was subjected to a 1.25-kV, 200-Ω, 25-μF electric pulse in a 0.2-cm cuvette, using a Gene Pulser (Bio-Rad, Richmond, Calif.).

Western blot analysis. Membrane and cell wall fractions were isolated from a 5-ml culture of *L. casei* grown at 37°C for 18 h using the following method. The cultures were diluted to an optical density of 0.8 at 600 nm with 20 mM Tris-HCl (pH 8.0) buffer. Samples of 1 ml were centrifuged at 4,000 × *g* for 5 min at 4°C and then resuspended in 20 mM Tris-HCl (pH 8.0) buffer, after which the cells were disrupted by sonication (UD-201; Tomy Seiko, Tokyo, Japan) (output, 1; duty, continue; time, 20 s five times on ice) and undisrupted cells removed by repeated centrifugation at 1,500 × *g* for 5 min at 4°C. The resulting supernatant was centrifuged for 30 min at 21,800 × *g* at 4°C and the membrane and cell wall

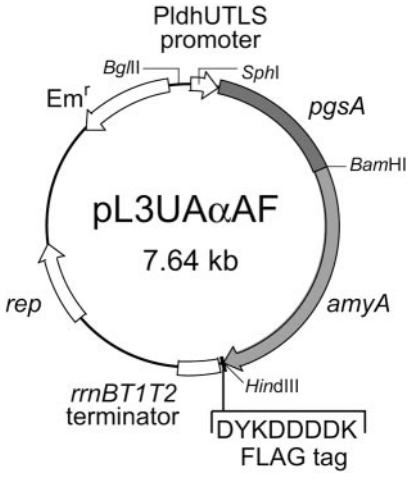

FIG. 1. Expression plasmid (pL3UAαAF) for display of α-amylase on the *Lactobacillus casei* BLSJ 03135 cell surface. Details of the construction are given in Materials and Methods. PldhUTLS, high-level expression promoter consisting of the *ldh* core promoter and an untranslated leader sequence; Em^r, erythromycin resistance gene from pSECE1; *rep*, replication gene from pSECE1.

fraction recovered as the pellet, which was resuspended in 20 mM Tris-HCl buffer and mixed with sample buffer. All samples were then boiled for 5 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 8% gel. As the molecular size marker, dual-color prestained Precision Plus protein standards (Bio-Rad Laboratories, Inc. Richmond, Calif.) were used. The samples were subsequently electroblotted on a polyvinylidene difluoride membrane (Millipore Corp., Boston, Mass.) and allowed to react with primary mouse anti-FLAG M2 antibody (Sigma Chemical Co., St. Louis, Mo.) at a dilution of 1:5,000 and secondary goat anti-mouse immunoglobulin G (IgG) alkaline phosphatase-conjugated antibody (Promega Co., Madison, Wis.) at a dilution of 1:5,000. The membrane was then stained with nitroblue tetrazolium (Promega Co.) and 5-bromo-4-chloro-3-indolylphosphate (Promega Co.) according to the protocol specified by the supplier. The staining solution was prepared by adding 95 μl of nitroblue tetrazolium and 50 μl of 5-bromo-4-chloro-3-indolylphosphate sequentially to 15 ml of alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.0] containing 150 mM NaCl and 1 mM $MgCl_2$).

Immunofluorescence microscopy. Immunostaining was performed as follows. *L. casei* cells were cultivated in MRS medium at 37°C for 24 h, collected by centrifugation at 3,500 × *g* for 5 min at 4°C, and washed with phosphate-buffered saline (PBS) (pH 7.2). After resuspension in PBS containing 10 g/liter bovine serum albumin (optical density at 600 nm = 1.0) and incubation for 30 min at room temperature, the cells and the primary antibody were incubated in PBS containing 10 g/liter of bovine serum albumin for 1.5 h at room temperature; the primary antibody was mouse IgG against FLAG used at a dilution of 1:500. The cells were then incubated for 1 h at room temperature with the secondary antibody, a 1:300 dilution of goat anti-mouse IgG (heavy plus light chains) antibody conjugated with Alexa Fluor 488 (Molecular Probes, Inc., Eugene, Oreg.); washed with PBS; and finally observed by microscopy. Immunofluorescence microscopy analysis was performed using a BZ-8000 inverted fluorescence and phase-contrast microscope (Keyence Co., Osaka, Japan) equipped with a 12-V, 100-W halogen lamp for transmitted light illumination; a 120-W mercury arc lamp for fluorescence illumination; a green fluorescent protein filter set (excitation wavelength, 480 to 530 nm; absorption wavelength, 510 nm); a Nikon PlanApo VC60×H NA1.40 oil objective lens; and an cooled charge-coupled-device camera. Data were analyzed with the BZ analyzer program (version 2.0; Keyence Co.).

Flow cytometric analysis. Flow cytometric analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) equipped with a 15-mW, 488-nm, air-cooled argon ion laser and a cell-sorting catcher tube. The cells were cultivated in MRS medium at 37°C for 24 h, collected by centrifugation at 3,500 × *g* for 5 min at 4°C, and washed with PBS (pH 7.2). Cells were then immunostained as described above. Immunostained cell samples were diluted to approximately 10^6 cells per ml and delivered at a low flow rate, corresponding to 150 to 300 cells per second. A band pass filter of 530 nm (515 to 545 nm) was used to collect the green fluorescence (FL1). Data were analyzed with the CELLQuest program (version 3.3; Becton Dickinson Immunocytometry Systems), with 10,000 counts analyzed in each experiment. Counts were made in triplicate for each procedure. The performance of the instrument was monitored by using CaliBRITE Beads (Becton Dickinson Immunocytometry Systems).

Measurement of α-amylase activity. The α-amylase activity on the *L. casei* cell surface was measured with an α-amylase measurement kit (Kikkoman Co., Tokyo, Japan), using 2-chloro-4-nitrophenyl 6^5-azido-6^5-deoxy-β-maltopentaosideas substrate. The assay mixture contained 400 μl of reaction solution and 40 μl of sample solution. For preparation of the sample solution, *L. casei* cells harvested from the culture broth were washed and resuspended in 20 mM HEPES buffer (pH 7.0) under vigorous agitation. The suspension was diluted with 20 mM HEPES buffer to an appropriate concentration for measurement and then used for α-amylase activity assay. The assay mixture was incubated at 37°C for 10 min and the enzyme reaction terminated by addition of 800 μl of reaction stop solution. The activity was determined according to the supplier's instruction by measuring the absorbance of liberated 2-chloro-4-nitrophenol at 400 nm. One unit of activity was defined as the amount of enzyme needed to release 1 μmol of 2-chloro-4-nitrophenol from 2-chloro-4-nitrophenyl 6^5-azido-6^5-deoxy-β-maltopentaoside per min at 37°C.

Fermentation experiment. Lactic acid fermentation by recombinant *L. casei* was performed in a 2-liter fermentor with a 1.0-liter working volume. The autoclave sterilization (121°C, 15 min) of the fermentor with MRS medium (without glucose) and the preparation of the soluble starch solution were carried out separately. After autoclaving, the soluble starch solution was added to the fermentor to a final soluble starch concentration of 50 g/liter. This fermentation medium was defined as SMRS. The fermentor was then inoculated at 1% (vol/vol) with 15-h seed cultures, and fermentation was carried out at 37°C. To maintain the pH at 6.0, 5 N NaOH was added automatically to SMRS and was gently stirred (100 rpm) to maintain homogeneity.

In the repeated utilization of cells, the first batch fermentation was carried out for 24 h, and then the fermentation broth was recovered and centrifuged at 3,000 × *g* for 10 min. The supernatant was separated, and the recovered cells were resuspended in 1.0 liter fresh SMRS medium and added to the fermentor to start the next cycle. At the starting point of each cycle, approximately 1.0×10^{10} cells/ml were retained in the fermentor. Repeated utilization was continued four times. In each cycle, fermentation was carried out for 12 h.

Viable cells of *L. casei* during fermentation were counted by the pour plate method using bromocresol purple plate count agar (Nissui Pharmaceutical Co., Tokyo, Japan). The plate was incubated anaerobically for 72 h at 37°C. The concentration of lactic acid was determined by using an organic acid analysis system (Shimadzu Co., Kyoto, Japan; solvent delivery system, LC-10ADvp; column, Shim-pack SPR-H; column temperature, 40°C; detector, CDD-10A). A 5 mM concentration of *p*-toluenesulfonic acid was used as the mobile phase, and 20 mM bis-Tris containing 5 mM *p*-toluenesulfonic acid and 100 μM EDTA was mixed just before detection to enhance sensitivity. The colorimetric method based on the phenol-sulfuric acid reaction described by Dubois et al. was used to determine the amount of total sugars corresponding to starch and starch hydrolysis products (7). The concentration of glucose was determined using a Wako Glucose CII-Test kit (Wako Pure Chemical Industries, Osaka, Japan).

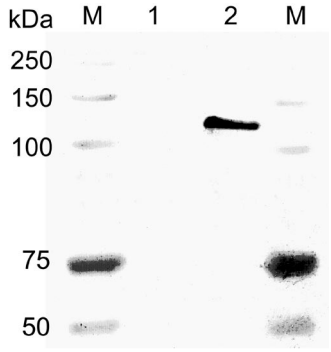

FIG. 2. Western blot analysis of PgsA-AmyA-FLAG fusion protein. Lanes M, marker proteins with the sizes shown; lanes 1 and 2, membrane and cell wall fraction of *L. casei* BLSJ 03135 harboring pL3UA and pL3UAαAF, respectively. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an 8% gel and stained with primary mouse anti-FLAG M2, followed by secondary goat anti-mouse IgG conjugated with alkaline phosphatase.

RESULTS

Expression of PgsA-AmyA fusion protein on the cell surface. To ferment starch to lactic acid, the α-amylase (AmyA)-displaying plasmid pL3UAαAF for expression of the PgsA-AmyA-FLAG (PgsA-AmyAF) fusion protein on the cell surface (Fig. 1) was constructed and transformed into *L. casei* BLSJ 03135 by electroporation. Since the PgsA-AmyAF fusion protein has a FLAG tag at the C terminus of AmyA, the expressed fusion protein was analyzed by Western blotting with mouse anti-FLAG M2 antibody. Figure 2 shows the Western blot analysis of the cell wall fractions of *L. casei* cells harboring the plasmid pL3UAαAF. The respective molecular sizes of the PgsA and AmyA-FLAG (AmyAF) proteins were approximately 43 and 78 kDa, and that of the PgsA-AmyAF fusion protein was therefore approximately 121 kDa. The clear band of the fusion protein is observed at the estimated molecular size, indicating the successful expression of the fusion protein in the membrane and cell wall fraction.

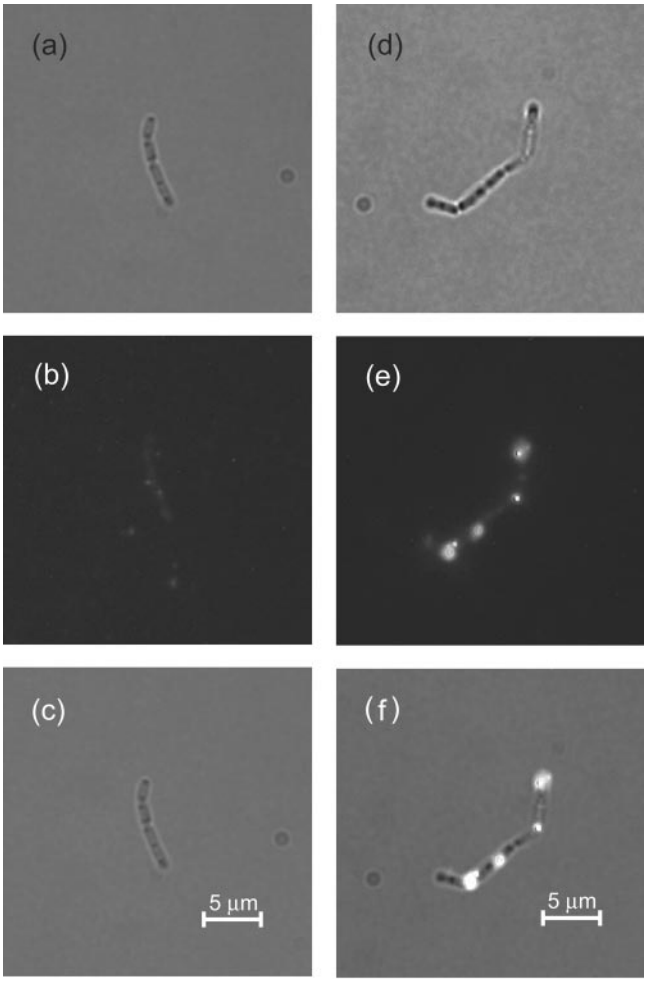

FIG. 3. Immunofluorescence labeling of *L. casei* harboring pL3UA and pL3UAαAF. (a and d) Nomarski differential interference micrographs of *L. casei* harboring pL3UA (a) and pL3UAαAF (d). (b and e) Immunofluorescence micrographs of *L. casei* harboring pL3UA (b) and pL3UAαAF (e). (c and f) Merged images of Nomarski differential interference and immunofluorescence micrographs of *L. casei* harboring pL3UA (c) and pL3UAαAF (f). Cells were labeled with mouse anti-FLAG M2, followed by goat anti-mouse IgG conjugated with Alexa Fluor 488.

Immunofluorescence microscopy and flow cytometric analysis. Immunofluorescence labeling of the cells was performed using mouse anti-FLAG M2 antibody as the primary antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG as the secondary antibody. As shown in Fig. 3, the green fluorescence of the immunostained PgsA-AmyAF fusion protein was observed in *L. casei* cells harboring the plasmid pL3UAαAF, whereas cells harboring the control plasmid pL3UA were not immunostained, indicating that AmyAF was displayed on the cell surface of the former set of *L. casei* cells. In most of cells, green fluorescence was localized around the septa of cells (Fig. 3). Flow cytometry was used to quantitatively analyze the cell surface display of AmyAF (Fig. 4). The cell surface-displayed AmyAF was stained with the first and second antibodies, and *L. casei* cells harboring the plasmid pL3UA were used as a control for flow cytometry. Cells displaying AmyAF showed a

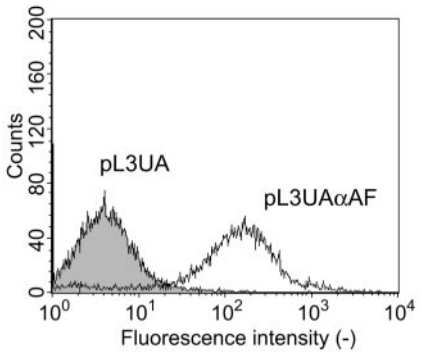

FIG. 4. Flow cytometric analysis of *L. casei* harboring pL3UA and pL3UAαAF. Cells were labeled with mouse anti-FLAG M2, followed by goat anti-mouse IgG conjugated with Alexa Fluor 488. For each experiment 10,000 cells were analyzed.

significantly greater intensity of fluorescence signals than the control cells. This result is consistent with the data shown in Fig. 3. To further confirm the cell surface localization of displayed AmyAF, trypsin digestion of whole cells was carried out (28, 33). When *L. casei* cells harboring the plasmid pL3UAαAF were treated with 20 mg/liter of trypsin (incubated at 37°C for 30 min), their immunofluorescence micrograph did not show clear green fluorescence (data not shown), and their mean fluorescence intensity analyzed by flow cytometry decreased to 25.2% of that of untreated cells. These results confirm the successful cell surface display of AmyAF.

Lactic acid fermentation using AmyAF-displaying *L. casei* BLSJ 03135 cells. The *L. casei* strain displaying AmyAF was used for direct simultaneous saccharification and fermentation for production of lactic acid from soluble starch (Fig. 5). The AmyAF-displaying *L. casei* cells were fermented in MRS medium containing 50 g/liter of soluble starch and with no glucose as a carbon source. To maintain α-amylase activity and the viable cell count, the pH of the fermentation medium was maintained at 6.0 by automatic addition of 5.0 N NaOH.

Figure 5a shows the α-amylase activities and the viable cell counts of the AmyAF-displaying and the control *L. casei* cells in batch fermentation. The α-amylase activity indicates the activity of AmyAF displayed on the cell surface per milliliter of culture broth. *L. casei* harboring pL3UAαAF showed a high level of α-amylase activity (0.93 U/ml) at 36 h, while no activity was detected in *L. casei* harboring the control plasmid pL3UA. The α-amylase activity was detected in supernatants during prolonged fermentation, probably due to cell lysis or cell wall turnover. The activity of cell surface-displayed AmyAF was nearly equal to that of cell surface-displayed AmyA (data not shown). The active AmyAF-displaying cells grew to a higher density than the control cells by utilizing soluble starch as a carbon source. The control cells grew a little using glucose (0.68 g/liter of medium before inoculation of cells) produced by the thermal decomposition of soluble starch.

Figure 5b shows the time courses of lactic acid production and starch consumption by AmyAF-displaying and control *L. casei* cells in batch fermentation. The production of lactic acid was almost completed within 24 h of fermentation, and the lactic acid concentration reached around 21.8 g/liter. Since the lactic acid concentration increased as the total sugar concentration de-

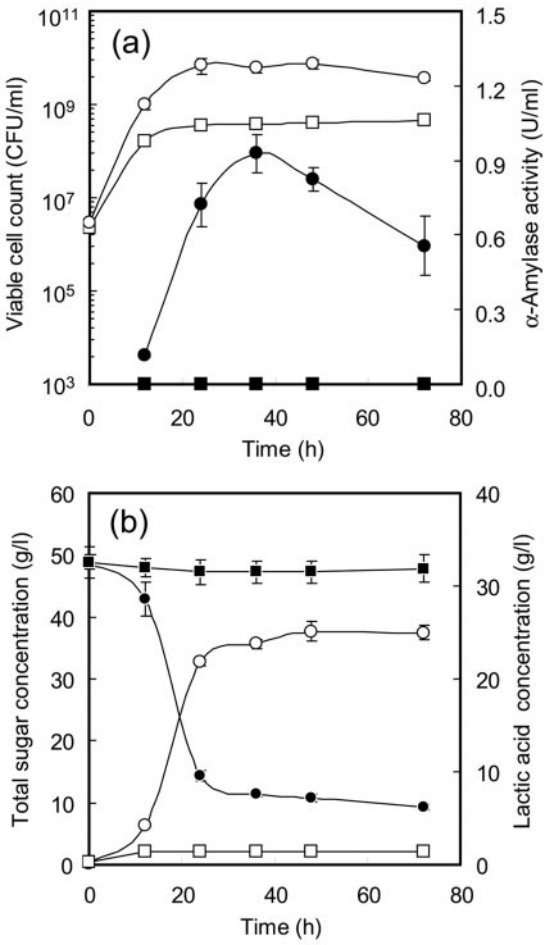

FIG. 5. Lactic acid fermentation from soluble starch as the sole carbon source, using *L. casei* harboring pL3UA (squares) and pL3UAαAF (circles). (a) Time course of viable cell count (open symbols) and α-amylase activity (closed symbols) on the cell surface during lactic acid fermentation. (b) Time course of lactic acid (open symbols) and total sugar (closed symbols) concentrations. The data points represent the averages and standard deviations from three independent experiments.

creased, it seems clear that the lactic acid was produced by simultaneous saccharification and fermentation of soluble starch by the AmyAF-displaying *L. casei*. The lactic acid productivity was 1.50 g/liter/h, and the yield of lactic acid produced was 0.6 g per g of carbohydrate consumed at 24 h. Glucose was not detected after inoculation of the AmyAF-displaying *L. casei* (data not shown). The cells probably consumed the glucose immediately.

Figure 6 shows the repeated utilization of AmyAF-displaying *L. casei* for lactic acid fermentation. The time courses of lactic acid production and starch consumption by AmyAF-displaying *L. casei* are shown. During repeated utilization, cells showed high lactic acid production rates without a time lag. The lactic acid productivity was 1.71 g/liter/h, and the yield of lactic acid produced was 0.81 g per g of carbohydrate consumed at 72 h.

DISCUSSION

Study of cell surface display in lactic acid bacteria has been carried out using various anchor proteins. Among them, the

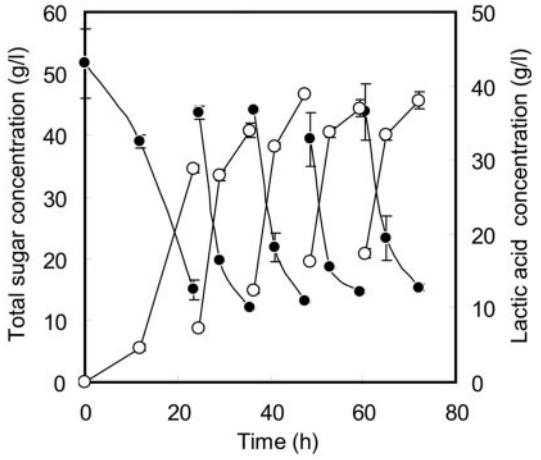

FIG. 6. Repeated utilization of *L. casei* harboring pL3UAαAF for lactic acid fermentation from soluble starch as the sole carbon source. Time courses of lactic acid (open symbols) and total sugar (closed symbols) concentrations are shown. The data points represent the averages and standard deviations from three independent experiments.

C-terminal cell wall-anchoring LPXTG motif has been most commonly used (26, 31). After their translocation across the plasma membrane, the proteins with the LPXTG motif are apparently cleaved between the threonine and glycine of the conserved LPXTG motif by a postulated sortase (18). Subsequently the carboxyl group of the threonine is amide linked to a free amino group of the peptide cross-bridge in the cell wall by sortase. Therefore, the difference in sortase activity between strains causes a problem in using the LPXTG motif for cell surface display. The limiting sortase in the cell causes an insufficient display of target protein using the LPXTG motif (6). We also performed cell surface display using proteinase P (PrtP) (17) as an LPXTG motif anchor. However, the display of proteins on the surface of *L. casei* BLSJ 03135 was not successful (data not shown). This might be caused by he insufficient activity of sortase in anchoring PrtP in our strain.

In the present study, we have developed a novel system of cell surface display in lactic acid bacteria by using a PgsA anchor system. Since the PgsA protein is classified as an A1-type anchor (transmembrane anchor) (16), PgsA can be anchored on the cell surface without sortase. As shown in Fig. 3 and 4, the AmyAF displayed on the cell surface by the PgsA anchor protein was successfully immunostained, but the cells treated with trypsin were not, demonstrating that AmyAF was successfully displayed on the cell surface by the PgsA anchor. Strong green fluorescence was observed around the septa of cells (Fig. 3). The septum is a place where the cell wall is synthesized in the early stage of cell division. Since a smaller number of cell wall components (e.g., lipoteichoic acid) exist around the septum (25), this part of the cell might be more suitable to accumulate large proteins such as AmyA. Since A1-type anchor proteins pass through the membrane and are anchored, it is thought that the target protein is more strongly anchored on the cell surface than with the S-layer subunit or BspA. The PgsA-AmyAF fusion protein could not in fact be removed from the cell by a 5 M LiCl wash (data not shown).

In studies of cell surface display, various peptides, antigens, and receptors have been displayed in lactic acid bacteria for use as oral live vaccines (3, 17, 20, 29). However, bioconversion using lactic acid bacteria displaying large proteins has not been studied. In the present study, we constructed a starch-degrading *L. casei* strain with active AmyAF display by using the PgsA anchor protein. We thus propose a new approach which directly produces lactic acid from biomass by using lactic acid bacteria displaying a hydrolytic enzyme. Many studies have been carried out on direct lactic acid production from starch by using an amylase-secreting lactic acid bacterium (8, 9, 10, 19). We thought that efficient lactic acid fermentation from starch could be performed by lactic acid bacteria displaying α-amylase on their cell surface, and we developed a relevant novel process. In order to hydrolyze starch, the displayed AmyAF must maintain activity. As shown in Fig. 5a, AmyAF was successfully displayed on the *L. casei* cell surface in an active form, and its activity reached 0.93 U/ml at 36 h. The fusion position of the target protein is a very important factor for activity retention. In the yeast cell surface display system, fusion of the N terminus of AmyA with the C terminus of the Flo1 anchor protein resulted in high α-amylase activity (24), and the present results are consistent with this observation. Immobilization on the cell surface at the N terminus by using PgsA allows AmyA to be efficiently bound and to degrade starch.

In order to confirm simultaneous saccharification and fermentation by the AmyAF-displaying *L. casei*, lactic acid fermentation from soluble starch as a carbon source was carried out. The α-amylase from *S. bovis* 148 which was displayed on the *L. casei* cell surface was able to efficiently hydrolyze corn starch. As shown in Fig. 5b, the total sugar concentration decreased immediately, and lactic acid was produced. Since glucose was not detected, the saccharification step might be a rate limiting. The yield of lactic acid was significantly improved by repeated utilization of cells and reached 0.81 g per g of carbohydrate consumed at 72 h (Fig. 6). As we expected, recovered cells could hydrolyze starch immediately by displayed AmyA, and hence the efficiency of lactic acid fermentation was high during repeated utilization. This yield is similar to that obtained by using *S. bovis*, which is one of the most powerful α-amylase-secreting natural strains (19). The AmyAF-displaying *L. casei* is more advantageous, because *L. casei* has showed higher cell viability than *S. bovis* during lactic acid fermentation, and it is possible to produce lactic acid for a longer time by repeated utilization of cells. On the other hand, the yields of lactic acid in the other α-amylase-secreting lactic acid bacteria, *Lactobacillus manihorivorans* LMG 18010T (10) and *Lactobacillus plantarum* A6 (9), have been reported to be 0.67 and 0.84 g per g of carbohydrate consumed, respectively. Although *L. plantarum* A6 showed a better yield than α-amylase-displaying *L. casei*, this strain produces DL-lactic acids.

Although AmyAF-displaying *L. casei* directly produced L-lactic acid from starch, starch is not completely consumed during fermentation by α-amylase-displaying *L. casei*. This is probably because α-1,6-glycosidic bonds cannot be completely cleaved by α-amylase. The yield would be further improved by the coexpression of glucoamylase.

This is the first report of the successful application of lactic acid bacteria displaying enzymes to the field of bioconversion. As described above, we demonstrated direct and simultaneous saccharification and lactic acid fermentation from soluble starch by *L. casei* displaying α-amylase.

ACKNOWLEDGMENTS

The present study was financed by the 2003 regional innovative consortium project of the Ministry of Economy, Trade and Industry, Japan.

We thank Meiji Dairies Corporation for supplying the pSECE1 plasmid.

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