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Yong Jun Goh
North Carolina University, Raleigh, NC

Jong-Hwa Lee
School of Bioresource Sciences, Andong National University, Andong, South Korea

Robert W. Hutkins
University of Nebraska-Lincoln, rhutkins1@unl.edu

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Functional analysis of the fructooligosaccharide utilization operon in *Lactobacillus paracasei* 1195

Yong Jun Goh[1], Jong-Hwa Lee[2] and Robert W. Hutkins*

University of Nebraska
Department of Food Science and Technology
Lincoln, NE 68583-0919

[1]Current address: North Carolina State University, Department of Food Science, Raleigh, NC 27695-7624

[2]Current address: School of Bioresource Sciences, Andong National University, Andong, South Korea

*Corresponding author. Mailing address: University of Nebraska, Department of Food Science and Technology, 338 FIC, Lincoln, NE 68583-0919. Phone: 402-472-2820. Fax: 402-472-1693. E-mail: rhutkins1@unl.edu

ABSTRACT

The *fosABCDXE* operon encodes components of a putative fructose/mannose phosphoenolpyruvate-dependent phosphotransferase system (PTS) and a β-fructosidase precursor (FosE) that are involved in the fructooligosaccharide (FOS) utilization pathway of *Lactobacillus paracasei* 1195. The presence of an N-terminal signal peptide sequence and a LPQAG cell wall anchor motif at the C-terminal region of the deduced FosE precursor amino acid sequence predicted that the enzyme is cell wall-associated, indicating that FOS may be hydrolyzed extracellularly. In this study, cell fractionation experiments demonstrated that the FOS hydrolysis activity was contained exclusively in the cell wall extract of *L. paracasei* previously grown on FOS. In contrast, no measurable FOS hydrolysis activity was detected in the cell wall extract from the isogenic *fosE* mutant. Induction of β-fructosidase activity was observed when cells were grown on FOS, inulin, sucrose, or fructose, but not glucose. A diauxic growth pattern was observed when cells were grown on FOS in the presence of limiting glucose (0.1%). Analysis of the culture supernatant revealed that glucose was consumed first, followed by the longer chain FOS species. Transcription analysis further showed that the *fos* operon was expressed only after glucose was depleted in the medium. Expression of *fosE* in a non-FOS-fermenting strain, *Lactobacillus rhamnosus* GG, enabled the recombinant strain to metabolize FOS, inulin, sucrose, and levan.

INTRODUCTION

The consumption of fermented food products or dietary supplements containing probiotic species of *Lactobacillus* and *Bifidobacteria* has been suggested to promote gastrointestinal (GI) health in humans and other animals by increasing the population of these microorganisms in the GI tract (10, 40). However, the beneficial effects of these bacteria may be transient due to colonization resistance by the commensal microbiota, which restricts the ability of probiotic bacteria to become well established in the intestinal environment (3, 15). An approach to overcome this limitation is to include prebiotics in the host diet. Prebiotics are specific nondigestible dietary sugars that are selectively metabolized by certain probiotic bacteria and that enhance their survival and colonization in the GI tract (12). Such an approach would also enrich the population of indigenous bifidobacteria and lactobacilli, allowing them to occupy a more dominant position in the gut ecosystem.

Fructooligosaccharides (FOS) are among the prebiotic substances that have been shown to selectively stimulate the growth and activity of certain strains of *Lactobacillus* and *Bifidobacterium* (4, 11, 13, 16, 47). Two types of FOS, that differ based on their methods of preparation, are commercially available and are widely used in food. One type, referred to as the GFn-type of FOS, is enzymatically produced from sucrose, and consists of a glucose monomer (G) linked α-1,2 to two or more β-2,1-linked fructose units (F), forming a mixture of GF_2, GF_3, and GF_4 (16, 17). The other type of commercial FOS is produced by partial enzymatic hydrolysis of the fructan polymer, inulin. The resulting product consist of a mixture of linear fructose oligomers, in the FFn form, also linked β-2,1, and having a degree of polymerization varying from 2

to 10. Due to the presence of a terminal glucose on the inulin molecule, the latter products also contain oligosaccharide species in the GFn form (6).

Despite considerable commercial and research interests on the beneficial effects of FOS, the molecular basis for FOS metabolism by probiotic bacteria and specific members of the intestinal microflora has only recently been examined. It now appears, however, that utilization of FOS occurs via one of two metabolic routes. Either the substrate is transported intact and is hydrolyzed in the cytoplasm, or it is hydrolyzed by extracellular enzymes, followed by subsequent accumulation of the hydrolysis products. In *Lactobacillus acidophilus* NCFM, for example, the FOS metabolic pathway is encoded by a multiple sugar metabolism (*msm*) operon that resembles the *msm* operon of *Streptococcus mutans* and the raffinose (*raf*) operon of *Streptococcus pneumoniae* (1). The *msm* operon encodes an ATP-dependent binding cassette (ABC)-type transport system and a cytoplasmic β-fructosidase that mediate FOS uptake and intracellular hydrolysis. Expression of the operon was inducible by sucrose and FOS, but not glucose or fructose. Similarly, cytoplasmic β-fructofuranosidases from *Bifidobacterium adolescentis*, *B. infantis*, and *B. lactis* have also been reported to hydrolyze FOS (9, 19, 20, 34-36, 46). Although FOS transport in bifidobacteria has not been reported, the presence of at least seven gene loci encoding oligosaccharide transport and metabolism in the genome sequence of *B. longum* (44) suggests that uptake of FOS may also be mediated by specific oligosaccharide transporters. In contrast, extracellular enzymes that hydrolyze FOS have also been reported for non-intestinal bacteria, including a fructan β-fructosidase from *Lactobacillus pentosus*, and

levanbiohydrolases from *Streptomyces exfoliatus* and *Microbacterium laevaniformans* (39, 41, 45).

Recently, microarray expression analyses of *Lactobacillus paracasei* 1195 grown on FOS led to the identification of a putative *fos* operon that plays a major role in the FOS utilization pathway (14). The *fosABCDXE* operon encodes a putative fructose/mannose PTS (FosABCDX) and a β-fructosidase precursor (FosE) that has high sequence identity with the putative levanase (*lev*) operons of *Lactobacillus casei* strains ATCC 334 and BL23. Inactivation of the *fosE* gene led to the inability of the mutant strain to grow on FOS and other β-fructose-linked sugars. The deduced amino acid sequence of FosE contains an N-terminal signal peptide sequence and a LPQAG cell wall anchor motif at the C-terminal region, suggesting that FOS may be hydrolyzed extracellularly by FosE, with the subsequent uptake of the hydrolysis products mediated by the FosABCDX PTS. Microarray analyses also indicated that expression of the FOS-induced genes was subject to catabolite regulation by glucose (14). Hence, the objectives of this study were to establish the location of the FOS hydrolysis activity in *L. paracasei* 1195 and to examine the effect of glucose on FOS utilization by *L. paracasei*. Additionally, we established the functional role of the *fos* operon by expressing the *fosE* gene in *Lactobacillus rhamnosus* GG, a widely used probiotic strain that has a limited ability to metabolize FOS (21) and other β-fructose-linked carbohydrates.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. Parental strains of *L. paracasei* 1195 and *L. rhamnosus* GG were

routinely grown in MRS broth (Difco, Inc., Ann Arbor, MI) at 37°C in ambient atmosphere under static condition, and recombinant strains were grown in MRS medium containing 5 μg/ml of erythromycin (Erm). For growth and enzyme experiments, cells were grown in modified MRS (mMRS) basal medium (14), supplemented with filter-sterilized solutions of FOS of the GFn (GTC Nutrition, Westminster, CO) or the FFn type (Orafti North America, Malvern, PA), glucose (Sigma-Aldrich, St. Louis, MO), fructose (Sigma), or sucrose (Sigma), at the indicated concentrations. Inulin- or levan-containing mMRS was prepared by addition of inulin (Sigma) or levan (Sigma) into mMRS prior to heat sterilization of the culture medium. For diauxie experiments, *L. paracasei* 1195 were grown in semi-defined medium (SDM) (23) containing (per liter): 10 g Bacto casitone (Difco), 5 g yeast nitrogen base (Difco), 1 g polysorbate 80 (Fisher Chemicals, Fairlawn, NJ), 2 g ammonium citrate (Sigma), 5 g sodium acetate (Sigma), 0.1 g magnesium sulfate (Sigma), 0.05 g manganese sulfate (Sigma), 2 g dipotassium phosphate (MCB Manufacturing Chemists, Norwood, OH), and supplemented with 0.1% (wt/vol) glucose, 0.35% (wt/vol) FOS (GFn form), or 0.1% glucose plus 0.35% FOS. *Escherichia coli* DH5α, used as host for routine cloning procedures, was grown in Luria-Bertani (LB) medium or Brain Heart Infusion (BHI) medium at 37°C with aeration at 200 rpm. When necessary, Erm was added at final concentrations of 250 μg/ml and 450 μg/ml for BHI and LB media, respectively.

DNA isolation and manipulations. Isolation of genomic DNA from *L. rhamnosus* GG was performed as previously described for *L. paracasei* (14). Plasmid DNA from *E. coli* was isolated using Zyppy Plasmid Miniprep I Kit (Zymo Research Corp., Orange, CA) according to the manufacturer's recommendations. Restriction enzymes (New England

Biolabs Inc., Ipswich, MA, and Takara Mirus Bio Inc., Madison, WI) were used as recommended by manufacturers. DNA ligation was performed using Fast-Link DNA Ligation Kit (Epicentre Biotechnologies, Madison WI) according to supplied instructions. PCR amplicons were generated using Easy-A High Fidelity PCR cloning enzyme or PfuTurbo DNA polymerase (Stratagene Corp., La Jolla, CA) in an Amplitron II Thermolyne thermocycler (Barnstead/Thermolyne Corp., Dubuque, IA). Primers were synthesized by Sigma-Genosys (The Woodlands, TX). The PCR products were electrophoresed in 0.8% agarose gel, and DNA fragments were purified using Zymoclean Gel DNA Recovery Kit (Zymo Research) prior to downstream applications. DNA sequencing was performed by the Genome Core Research Facility (University of Nebraska, Lincoln, NE).

For electroporation, *E. coli* cells were prepared according to protocols of the Wolf Laboratory (http://www.research.umbc.edu/%7Ejwolf/m7.htm). Electroporation was performed in pre-chilled 0.2-cm electroporation cuvettes (Boca Scientific Inc., Boca Raton, FL) using a Gene Pulser electroporation system (Bio-Rad Laboratories, Inc., Hercules, CA) set at 12.5 kV cm^{-1}, 200 Ω, and 25 μF. Electrotransformation of *L. rhamnosus* GG were performed as previously described (14). Briefly, stationary-phase cells were used to inoculate 100 ml of MRS broth (2% inoculum) and grown for 3 hrs (optical density at 625 nm ~ 0.1 to 0.2). Then, freshly prepared filter-sterilized penicillin G solution was added to a final concentration of 10 μg/ml, and the culture was grown for additional 1.5 to 2.0 hrs. Cells were harvested at 5,500 x *g* for 15 min at 4°C, washed with 10 ml and 50 ml of ice-cold filter-sterilized 1X PEB buffer (per liter: 272 mM sucrose, 1 mM $MgCl_2$, 7mM potassium phosphate [KPO_4], pH 7.4) sequentially, followed by a

third wash with 10 ml of ice-cold 10% (vol/vol) glycerol, and finally resuspended in 1 ml of 10% glycerol. For electroporation, ~ 0.1 μg of DNA was added to 50 μl of the cells, and the mixture was transferred into a pre-chilled 0.2-cm electroporation cuvette, and incubated on ice for 2 min. Cells were electroporated at 12.5 kV cm^{-1}, 400 Ω, and 25 μF and placed on ice immediately. Transformed cells were supplemented with 950 μl of MRS broth and recovered for 3 to 4 hrs at 37°C. Cells were then plated onto MRS agar containing 2.5 to 5.0 μg/ml of Erm and incubated at 37°C for 48 to 72 hrs under ambient atmospheric condition.

Purification of *L. paracasei* total RNA. Total RNA was isolated as previously described (14) using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). The RNA samples were subsequently treated with DNase I using Turbo DNA*free* kit (Ambion Inc., Austin, TX). The quality and integrity of RNA samples were assessed spectrophotometrically (A_{260}/A_{280} 1.6 to 1.9) and gel electrophoresis, as described previously (14).

FOS hydrolysis assay. *Lactobacillus paracasei* 1195 and the BHe mutant strain were grown in mMRS broth containing 1% FOS (GFn form), and harvested by centrifugation at 3,000 *x g* for 15 min at room temperature when the $OD_{625\ nm}$ reached 0.60 and 0.35, respectively. Culture supernatants were filter-sterilized through 0.45 μm filters and concentrated to 1/20 of the initial volume using Amicon Ultra-4 centrifugal filter units (30,000 MWCO; Millipore Corp., Bedford, MA). Cell pellets were washed twice in 0.1 M potassium phosphate buffer (pH 6.6) and resuspended in 1 ml of the same buffer. The cell suspension was transferred into 1.5 ml conical tubes (BioSpec Products, Inc., Bartlesville, OK) containing 400 mg of 0.1 mm diameter glass beads (BioSpec

Products), and cells were disrupted by homogenization using a Mini-Beadbeater (BioSpec Products) at 4,200 rpm for 6 cycles of 1 min, with 1 min on ice between each interval. Cell lysates were transferred into fresh tubes, and the fraction containing cell wall fragments was separated from the cytoplasmic extract by centrifugation at 13,800 *x g* for 10 min at room temperature. This cell wall fraction was resuspended in 1 ml of phosphate buffer, whereas the cytoplasmic extract was concentrated to 1/5 of initial volume using Amicon Ultra-4, as described above.

For induction experiments, *L. paracasei* 1195 was sub-cultured twice in mMRS containing 1% FOS (separately, in both GFn and FFn types), sucrose, inulin, fructose, glucose, or 0.5% levan. The cultures were subsequently used to inoculate (2% inoculum) 30 ml of mMRS containing the respective sugars at the same concentrations. When the $OD_{625\ nm}$ reached 0.6 to 0.7, the cells were collected by centrifugation at 3,000 *x g* for 20 min at 4°C. Cell fractionation was performed as described above.

For all β-fructosidase assays, 10 μl of the concentrated culture supernatant, cell wall fraction, or cytoplasmic extract was added to 190 μl of 1% (wt/vol) FOS (GFn or FFn type), sucrose, or inulin solution. Reaction mixtures were incubated at 37°C for 3 hrs and inactivated by boiling for 2 min, and activities were reported as the amount of fructose released per minute per mg of protein. Fructose concentrations were determined by using a Fructose Assay Kit (Sigma), according to the manufacturer's instructions or by HPLC using an Aminex HPX-42C column (Bio-Rad Laboratories) and a RI 410 reflective index detector. The internal and external temperatures of the column were maintained at 40°C and 85°C, respectively, with a column heater. Water was used as mobile phase with a flow rate of 0.6 ml/min. Protein concentrations were

determined by the Bradford Reagent (Sigma), based on the manufacturer's specifications. All experiments were done in duplicate.

Lactate dehydrogenase (LDH) assay. Samples of cell-free culture supernatant, cell wall extract and cytoplasmic extract were assayed for LDH activity as previously described (18). Briefly, the reaction mixtures contained 1 ml of 0.1 M triethanolamine-hydrochloride (pH 7.5), 80 μl of 0.1 M sodium pyruvate, 40 μl of 30 mM fructose-1,6-diphosphate, 40 μl of freshly prepared 4 mM NADH, and 40 μl of each cell fraction or the culture supernatant. The decrease in absorbance at 340 nm was recorded over 6 minutes and used to calculate LDH activity.

Catabolite repression studies. Overnight cultures of *L. paracasei,* grown in SDM containing 1% FOS (GFn), were used to inoculate 1.2 liter of SDM containing 0.1% glucose, 0.35% FOS (GFn), or 0.1% glucose plus 0.35% FOS (GFn). Cultures were incubated at 37°C in ambient atmosphere under static condition. At various times, the cell densities were recorded, and portions of cultures grown were centrifuged and cell supernatants saved for analysis. In addition, cells grown on SDM-0.1% glucose + 0.35% FOS were centrifuged at 3,000 *x g* for 10 min at room temperature for isolation of total RNA. To prepare RNA samples for gel electrophoresis on formaldehyde gel, 30 μg of each sample in 10 μl was mixed with 2.5 μl of 10X MOPS (0.2 M MOPS, 80 mM sodium acetate, 10 mM EDTA; pH 7.0), 3 μl of formaldehyde solution (Fisher [37% vol/vol]), 12.5 μl of formamide, and 1 μl of 1 mg/ml ethidium bromide. The mixtures were incubated at 65°C for 10 min, chilled on ice for 2 to 3 min, followed by electrophoresis on a formaldehyde gel (1% agarose, 0.66 M formaldehyde, 1X MOPS).

The RNA was subsequently transferred onto Zeta-Probe blotting membrane (Bio-Rad Laboratories) using standard procedures (42). The membrane was then soaked in 2X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min, and RNA was subsequently immobilized on the wet membrane by UV-crosslinking twice at 120,000 μJoules in a Stratalinker Crosslinker (Stratagene). The internal region of *fosE* gene (981 bp) used for synthesis of hybridization probe was amplified from *L. paracasei* 1195 genomic DNA using fosE-for1 and fosE-rev1 primers (Table 1) in a 50 μl reaction containing 1 μl of 10 mM dNTP mix, 0.5 μg of genomic DNA, 2.5 U of Taq DNA polymerase, and 25 pmol of each primer in 1X Taq DNA polymerase buffer (Stratagene). PCR amplification was carried out in the following condition: 1 cycle at 95°C for 3 min, 30 cycles of 30 sec at 95°C, 30 sec at 55°C, 1 min at 72°C, and a final cycle at 72°C for 10 min. Synthesis of DIG-labeled *fosE* probe with the *fosE* PCR product, hybridization, and detection of hybridized signals were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics Corp., Indianapolis, IN) as described by manufacturer. Hybridization signals were exposed onto X-Omat Blue XB-1 imaging films (Gold Biotechnology, Inc., St. Louis, MO) in multiple exposure times (2 to 8 min) to obtain optimum signal strength.

Sugar analyses. Glucose concentration in culture supernatants was measured using a YSI 2700 SELECT Biochemistry Analyzer (YSI Incorp., Yellow Springs, OH) equipped with glucose membranes (YSI 2365). To determine the concentration of each FOS fraction in the culture supernatants, the samples along with FOS standards (0.05%, 0.1%, 0.2%, and 0.4%) were spotted onto 20 x 20 cm thin layer chromatography (TLC) silica gel plates (Whatman Ltd., Kent, UK). The plates were developed twice in acetic

acid-chloroform-water (7:5:1) solvent. Spots were visualized by spraying plates with ethanolic 50% sulfuric acid, and heated at 115 °C for 5 min. The TLC plates were subsequently scanned on a Epson Perfection 1660 Photo scanner (Epson America, Inc., Long Beach, CA), and density of spots on the scanned image was analyzed using the Scion Image for Windows software (http://www.scioncorp.com/frames/fr_download_now.htm).

Expression of the *L. paracasei* β-fructosidase gene in *L. rhamnosus* GG. To introduce the *fosE* gene into *L. rhamnosus* GG, a fragment containing the *fosE* gene with its native ribosomal binding sequence (RBS) and a promoter sequence isolated from *L. rhamnosus* GG, P-GL1 (33), were sequentially cloned into the pTRKH2 shuttle vector (38). Briefly, the 4,131-bp *fosE* gene was PCR-amplified from the genomic DNA of *L. paracasei* 1195 using fosE-for2 and fosE-rev2 primers (Table 1). The *fosE* amplicon was digested with *Xho*I and *Pst*I, ligated into pTRKH2 with compatible ends, and transformed into *E. coli* DH5α. The recombinant plasmid, designated as pRH5, was verified by restriction digest and sequencing. Next, the P-GL1 promoter and the RBS for the *fosE* gene were cloned upstream of the *fosE* gene in the pRH5 plasmid. The P-GL1 promoter region was PCR-amplified from *L. rhamnosus* GG genomic DNA using PGL1-for and PGL1-rev primers (Table 1), with the *fosE* RBS incorporated into the latter primer. The 103-bp PCR amplicon was restricted with *EcoR*V and *Xho*I, and ligated into similarly digested pRH5. The ligation products were purified using DNA Clean & Concentrator-5 kit (Zymo Research) and transformed into *E. coli* DH5α. Ligation of the PGL-1 promoter and the *fosE* RBS upstream of *fosE* gene in the recombinant plasmid, designated as pYG582, was confirmed by DNA sequencing. The

recombinant plasmid pYG582 was subsequently electroporated into *L. rhamnosus* GG, and transformants were recovered on MRS plates containing 2 to 5 μg/ml of Erm after incubation at 37°C in ambient atmosphere for 48 to 72 hrs. The *L. rhamnosus* GG transformants harboring pYG582 were streaked on mMRS-1% FOS agar containing 5 μg/ml Erm and 100 mg/L bromcresol purple to determine their ability to ferment FOS. One recombinant isolate that formed a yellow zone as a result of acid production from fermentation of FOS was selected and designated as *L. rhamnosus* GGE582. The presence of pYG582 in the GGE582 strain was verified by direct cell PCR method essentially as described previously (5). For phenotypic analysis, strains of GG and GGE582 were grown in mMRS and mMRS containing 5 μg/ml of Erm, respectively, and supplemented with 1% of glucose, fructose, sucrose, FOS (both types), inulin, or 0.5% levan.

RESULTS

Location of β-fructosidase activities in *L. paracasei* 1195. To identify the location of the β-fructosidase activity in *L. paracasei* 1195, cells grown in mMRS broth containing 1% FOS (GFn type) were harvested, and three fractions, representing the concentrated culture supernatant, crude cell wall extract, and cytoplasmic extract, were prepared as described above. The same fractions were also obtained from the mutant strain, BHe. Using FOS (GFn type) as the substrate, the β-fructosidase activity of the wild type strain was detected almost exclusively in the cell wall extract (Table 2). In contrast, FOS hydrolysis activity in the culture supernatant or the cytoplasmic extracts was negligible, relative to that in the cell wall extract. No FOS hydrolysis activity was detected in the

BHe strain. To confirm that the cell fractionation procedure had adequately separated the different fractions, all cell fractions and supernatants were assayed for LDH, a cytoplasmic marker enzyme. As expected, LDH activity was detected only in the cytoplasmic extract (data not shown).

Induction of β-fructosidase activity during growth on various sugars. The influence of various carbohydrate growth substrates on the induction of β-fructosidases and their substrate specificities was examined (Fig. 1). Regardless of the carbohydrate source in the media, β-fructosidase activities were only present in the cell wall extracts. Cells grown on inulin resulted in the highest enzyme activities, followed by cells grown in both types of FOS. The two FOS products (GFn and FFn) and inulin also served as the preferred substrates. In contrast, sucrose- and fructose-grown cells had the lowest activities, and only when FOS was the substrate. Sucrose was the least preferred substrate, even for sucrose-grown cells. No β-fructosidase activity was detected from the cell wall extract of glucose-grown cells, indicating that the enzyme was either not induced or repressed in the presence of glucose. Analysis of the FOS (GFn) hydrolysis products by HPLC showed that fructose and sucrose were the major products from FOS hydrolysis. Inulin hydrolysis generated primarily fructose with no oligomeric intermediate released. These observations suggested that the β-fructosidases hydrolyzed the substrates in an exo-type fashion.

Catabolite repression of FOS utilization by glucose. Previous microarray expression analyses suggested that the expression of FOS-induced genes in *L. paracasei* 1195 was subject to catabolite repression by glucose (14). To further assess the effect of glucose on FOS utilization, growth of cells in SDM containing both glucose

and FOS (0.1% and 0.35%, respectively) was compared to cells grown in SDM supplemented with either 0.1% glucose or 0.35% FOS. A typical diauxic growth pattern was observed during growth on glucose plus FOS (Fig. 2). The diauxic lag was likely caused by the depletion of glucose, since cessation of growth was also observed at a similar time and cell density for cells grown separately on the same amount of glucose. After the diauxic lag phase, cells resumed growth and entered a second growth phase using FOS as the carbon source, with the culture ultimately reaching approximately similar cell density that was achieved for cells grown on 0.35% FOS alone (i.e., about 1.5). Sugar analyses of the culture supernatants revealed that FOS was utilized only after glucose was consumed, confirming that glucose was metabolized preferentially (Fig. 3A). When cells entered the second growth phase, GF_4 and GF_3 were rapidly hydrolyzed, resulting in a transient increase in the GF_2 concentration. Subsequently, the GF_2 concentration gradually decreased to an undetectable level, with a simultaneous increase in the concentrations of glucose and sucrose (data not shown) from the hydrolysis of GF_2.

To examine the kinetics of transcription of the *fos* operon during the diauxic shift, northern blot analysis, using a *fosE* probe, was performed on RNA samples obtained from cells grown on 0.1% glucose plus 0.35% FOS. As expected, no hybridization signal for the *fos* genes was detected during the first growth phase when glucose was utilized as the preferred carbon source (Fig. 3B and 3C). Shortly after the onset of the diauxic lag phase, the signal intensity associated with *fosE* gradually increased, with maximum transcript levels observed during the period when GF_4 and GF_3 were actively hydrolyzed (Fig. 3A). This was followed by a dramatic reduction in the *fosABCDXE*

mRNA level as the GF_4 and GF_3 were depleted, along with a slight increase in the glucose concentration. A second induction of the *fos* mRNA transcript was then observed (Fig. 3C, lane 14), coinciding with a decrease in the GF_2 concentration. During the next few hours, the signal intensity of the *fos* operon decreased to an undetectable level (Fig. 3C, lanes 15 to 17). This time frame is associated with the depletion of GF_2 and an increase in glucose and sucrose levels in the culture supernatant.

Expression of the *fosE* gene in *L. rhamnosus* GG. In a previous study it was reported that *L. rhamnosus* GG, a widely used probiotic strain, was unable to utilize FOS as an energy source (21). However, this strain is able to ferment fructose, indicating the presence of at least one fructose transport system. Thus, only the *fosE* gene from the *fos* operon was introduced into the GG strain. To construct a recombinant GG strain capable of metabolizing FOS, the *fosE* gene, along with its RBS, and the P-GL1 promoter sequence from *L. rhamnosus* GG (33) were cloned into the pTRKH2 shuttle vector (see Materials and Methods). The resulting construct, pYG582, was transformed into the GG strain. Unlike the parent strain, the recombinant GGE582 strain harboring the pYG582 was able to utilize FOS for growth (Fig. 4). In addition, the GGE582 strain gained the ability to grow in mMRS medium containing sucrose, inulin, and levan. None of these sugars supported the growth of the parent strain.

DISCUSSION

Recent microarray transcriptome analyses of *L. paracasei* revealed the presence of an FOS metabolic pathway, encoded by the *fosABCDXE* operon, that was comprised

of a putative cell wall-associated β-fructosidase and a fructose/mannose PTS (14). Expression of the *fos* genes was induced by FOS and repressed in the presence of glucose. Previous studies of FOS metabolism in *L. paracasei* 1195, however, had suggested that FOS uptake and hydrolysis were mediated by an ABC transport system and a cytoplasmic β-fructofuranosidase, respectively (22). The cytoplasmic location of the FOS hydrolyzing enzyme, was based, in part, on the absence of activity in the supernatant, and also on the presence of activity associated with the crude cytoplasmic fraction. In this report, the intracellular as well as the cell wall fractions were both examined, and β-fructosidase assays showed that the FOS hydrolysis activity was present primarily in the cell wall extract. This fraction had very high activity and had not previously been assayed for β-fructosidase activity. No cytoplasmic-specific LDH activity was detected in the culture supernatant or in the cell wall fraction. These results indicate that cell lysis was minimal when the cultures were harvested prior to cell fractionation and also that the location of the β-fructosidase activity was distinct from the LDH activity. These data provide evidence that FosE is a cell wall-associated β-fructosidase, that, like other enzymes possessing LPXTG anchor motifs, faces the extracellular side of the cell wall and therefore, catalyzes FOS hydrolysis extracellularly (2, 25). The anchoring of the FosE to the cell wall is likely mediated by the action of a sortase that cleaves between the alanyl and glycyl residues of the LPQAG motif, and subsequently catalyzes the formation of amide-linkage of the alanyl residue to the peptide crossbridge in the peptidoglycan layer (37). The resulting 1,303-amino acid residue of the mature anchored β-fructosidase thus has an estimated molecular weight of 139 kDa.

The essential role of FosE in the FOS utilization pathway was demonstrated previously, when it was reported that insertional inactivation of *fosE* gene severely impaired the ability of the *L. paracasei* BHe mutant to grow on FOS (14). In the present study, no β-fructosidase activity was detected from the cell wall extract of the BHe mutant. In addition, the mutation prevented the utilization of FOS (FFn type), inulin, levan, and sucrose as sole carbon source, indicating that the *fos* operon is essential for metabolism of not only FOS and but also other fructose-containing carbohydrates.

Expression of the β-fructosidase was induced during growth on FOS, inulin, and to a lesser extent, sucrose and fructose, but not on glucose. Similarly, the preferred substrates were FOS of the FFn and GFn form, followed by inulin, with minor activity towards sucrose. These results indicate that this enzyme may have preference for oligosaccharides having β-2,1-linkages. The FFn form of FOS is composed of ca. 75% of fructose oligomers with a degree of polymerization of 2 to 10 and which do not contain a terminal glucose molecule. Thus, most of the FOS chains have more fructosyl units per oligomer as substrates for successive exo-hydrolysis by β-fructosidase compared to the GFn form of FOS. The low activity against the α-1,2 glucose-fructose bond in GFn, as indicated by the near absence of free glucose in reaction mixtures, would also explain why sucrose was not hydrolyzed. Furthermore, the lower activities observed for inulin also indicate a preference for intermediate short chain length oligosaccharides. The exo-hydrolysis activity of the β-fructosidase is supported by the observation that hydrolysis of the GF_4 and GF_3 fractions in FOS occurred first, producing GF_2, sucrose, and fructose. The latter two then accumulated gradually as the concentration of GF_2 decreased. Finally, that no growth was observed on raffinose, a

trisaccharide composed of galactose, glucose, and fructose, implies that raffinose is not a substrate for the β-fructosidase (data not shown).

The diauxic growth pattern exhibited by *L. paracasei* 1195 grown on FOS in the presence of limiting glucose demonstrated that FOS utilization is subject to catabolite repression by glucose. This observation was consistent with the results from transcriptome experiments showing that glucose repressed the transcription of FOS-induced genes (14). During growth on limiting glucose plus FOS, glucose was consumed first, although the cells had been sub-cultured in medium containing FOS. After the diauxic lag period, FOS was utilized in the order of GF_4, GF_3, and GF_2, presumably due to the substrate preferences of FosE. Interestingly, Northern hybridization analysis revealed that the expression of the *fos* genes was not constant during the post-diauxie secondary growth phase. Rather, repression of the *fos* operon also occurred during the second growth phase. While a small amount of glucose was generated from the hydrolysis of FOS, which may have contributed to the decreased transcript level of the *fos* mRNA, it also appears that the repression effect was not sufficient to cause a second diauxic lag.

Although the molecular basis of regulation of the *fos* operon expression was not examined in detail during the present study, given the similarity in operon structure, the transcription of *fos* in *L. paracasei* 1195 is likely controlled by similar regulatory mechanisms as described for the *lev* operons in *L. casei* BL23 and *Bacillus subtilis* (27-32). However, unlike the *lev* operon of *B. subtilis*, transcriptional activation of the *lev*-PTS in *L. casei* BL23 and the *fos* operon by LevR and FosR, respectively, are independent of a σ^{54}-like sigma factor, since no -12, -24 promoter sequence

(CTGGCACN$_5$TTGCA) was found in regions preceding both the BL23 *lev* operon and the *fos* operon (7, 8, 32). In BL23, the activity of LevR is regulated by dual PTS-catalyzed phosphorylation at conserved histidine residues in the EIIA and PRD2 domains by P~His-HPr and P~His-EIIB^{Lev}, respectively (32). In the presence of substrate for Lev-PTS, P~His-EIIB^{Lev} preferably donates its phosphoryl group to the transported sugar, leading to dephosphorylation of LevR at His-776 by P~His-EIIB^{Lev} and LevR activation, and thereby induction of the *lev*-PTS. On the other hand, when metabolically preferred PTS sugars are present, such as glucose, the phosphoryl group of P~His-HPr is used for sugar phosphorylation. Poor phosphorylation at His-488 by P~His-HPr renders LevR less active and down regulates expression of *lev*-PTS. Therefore, the *lev* operon is subject to carbon catabolite repression (CCR) by P~His-HPr dephosphorylation via LevR. The presence of a putative *cre* sequence overlapping the transcriptional start site of the *lev* operon of BL23 (32) and the *fos* operon indicated that the expression of both operons are also controlled by CCR via binding of catabolite control protein CcpA to the *cre* site (14, 32). In *B. subtilis*, accumulation of glycolytic intermediates, such as fructose-1,6-bisphosphophosphate (FBP) from uptake of rapidly metabolizable sugars was proposed to stimulate the phosphorylation of HPr by HPr kinase (HprK) at Ser-46 (29). P~Ser-HPr acts as a co-repressor by interacting with CcpA, enabling CcpA to bind to *cre* and prevents transcription of the *lev* operon.

Although certain strains of *Lactobacillus* are widely used as probiotics due to their various desirable traits (24), their ability to utilize prebiotic oligosaccharides, such as FOS, may be limited (21). We have shown that the introduction of the *fosE* gene into the non-FOS-fermenting *L. rhamnosus* GG not only conferred on the recombinant

GGE582 strain the ability to utilize both forms of FOS efficiently, but also other prebiotics such as inulin and levan. Although β-fructosidase activity was not measured in the FOS-fermenting transformant, this strain appeared to grow on these fructans as well as on glucose and fructose. This demonstrates the feasibility of developing novel probiotic strains having enhanced metabolic functionality.

In contrast to our findings that *L. paracasei* 1195 could grow on both forms of FOS, Saulnier et al. (43) recently reported that *L. plantarum* WCF1 was unable to grow on the FFn form. Although *L. plantarum* WCF1 also possesses a putative β-fructofuranosidase, this enzyme is apparently intracellular and is part of a sucrose transport and metabolic system. The authors suggest that the small GFn oligosaccharides are transported via this sucrose system in *L. plantarum* WCF1. This strain also had preference for GF_2 and GF_3, with relatively little consumption of GF_4. Although *L. paracasei* 1195 was originally reported to have a similar substrate preference (21), the current data indicates that all of the FOS fractions, including GF_4 were metabolized by this strain.

Another related strain, *Lactobacillus paracasei* subsp. *paracasei* 8700:2 was also reported to use short and long chain fractions of FFn FOS, simultaneously, although when grown on inulin and FOS, the FFn chains were preferred (26). Fructose, as well as sucrose and various FFn and GFn oligosaccharides, were also formed during growth on FOS and inulin, indicating that an enzyme capable of extracellular hydrolysis is present in this organism.

Overall, results from this study and previous mutational analysis of the *fosE* gene (14) have provided evidence that the *fos* operon encodes key components for the

utilization of FOS and other structurally similar carbohydrates by *L. paracasei* 1195. While the cell wall-anchored FosE of the *fos* system may provide versatility in the utilization of larger prebiotic substrates without dependence on dedicated transporters for uptake of the substrates, it may also promote cross-feeding by providing access of the hydrolysis products to other intestinal microorganisms that do not possess a FOS metabolic pathway. In addition, the results show that glucose, generated from hydrolysis of FOS or other glucose-containing polysaccharides, may catabolite repress, at least transiently, FOS metabolism in the GI environment. Collectively, these results emphasize that understanding the mechanisms and regulation of prebiotic sugar utilization by probiotic bacteria and targeted commensals is necessary for rational selection and development of effective probiotics and prebiotics.

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FIGURE LEGENDS

Figure 1. Induction and substrate specificities of β-fructosidases in cell wall extracts of *L. paracasei* 1195.

Figure 2. Growth of *L. paracasei* 1195 in SDM supplemented with 0.1% glucose (□), 0.35% FOS (Δ), or 0.1% glucose plus 0.35% FOS (•).

Figure 3. Sugar utilization and *fos* operon expression during diauxic growth of *L. paracasei* 1195. Cells were grown in SDM (A) containing 0.1% glucose plus 0.35% FOS. Cell densities (•) and the concentrations of glucose (o), GF_4 (Δ), GF_3 (X), and GF_2 (□) present in the culture supernatant were determined. In a parallel experiment,

cells were grown in the same medium (B), and Northern analysis of the *fosABCDXE* mRNA transcript levels (C), relative to the cell density (•) and glucose concentration (o) in the culture supernatant were determined. Numbers labeled on the growth curves correspond to the lane numbers on the Northern blot indicating the time points at which cells were collected.

Figure 4. Growth of *L. rhamnosus* GG wild type (A) and GGE582 recombinant strain (B) in mMRS only (no CHO), or mMRS supplemented with 1% sugars or 0.5% levan, with 5 μg/ml of Erm added into each growth medium for GGE582 strain. All cultures were inoculated to an initial OD_{625nm} of ~ 0.02 to 0.05 and grown at 37°C in ambient atmosphere under static conditions of growth.

TABLE 1. Bacterial strains, plasmids, and primers used in this study

	Genotype or characteristics	Source or reference
Strains		
L. paracasei		
1195	Parent strain, FOS-fermenter	UNL collection[a]
BHe	1195 isogenic strain with *fosE* gene disrupted by insertion inactivation	14
L. rhamnosus		
GG	Parent strain, non-FOS-fermenter	ConAgra[b]
GGE582	GG harboring pYG582	This study
E. coli		
DH5α	F^- ϕ80d*lac*ZΔM15 Δ(*lacZYA-argF*)U169 *deoR endA1 recA1 hsdR17*(r_K^- m_K^+) *phoA supE*44 λ^- *thi*-1 *gyrA*96 *relA*1	Gibco-BRL[c]
Plasmids		
pTRKH2	High copy number shuttle cloning vector, P15A *ori*, pAMβ1 *ori*, Erm^r, *lacZ'*	38
pRH5	pTRKH2 with *fosE* gene cloned into *Xho*I/*Pst*I sites	This study
pYG582	pRH5 with P-GL1 promoter and *fosE* RBS cloned upstream of *fosE*	This study
Primers	Sequence (5' to 3')[d]	This study
fosE-for1	TGGCTTAGGAAAAGACGCCA	
fosE-rev1	TGATCATCAGATACTCGCAA	
fosE-for2	CGGACCTCGAGTTGGAAATGGATGAAAAGAAAC	
fosE-rev2	ATTATCTGCAGTTAGACTCGCTTCACCCGCCTC	
PGL1-for	ATCAATGATATCACGGTTTTAAAATGAGCGTTG	
PGL1-rev	GCTACCTCGAG**tcatcctcc**AACTTATTATGTTAATAA	

[a]University of Nebraska Department of Food Science and Technology Culture Collection, Lincoln, NE.

[b]ConAgra Foods Inc., Omaha, NE.

[c]Gibco-BRL, Rockville, MD.

[d]restriction enzyme sites, underlined; ribosomal binding site, lower case in bold.

TABLE 2. FOS hydrolysis activities from culture supernatants and cell extracts of *L. paracasei* 1195 wild type and BHe mutant strain previously grown on mMRS containing 1% FOS.

	Fructose released (nmoles/min/mg protein)
L. paracasei 1195	
culture supernatant	4
cell wall extract	3,400
cytoplasmic extract	13
L. paracasei BHe	
culture supernatant	0.2
cell wall extract	nd[a]
cytoplasmic extract	nd

[a] nd , none detected

Figure 1. Goh et al.

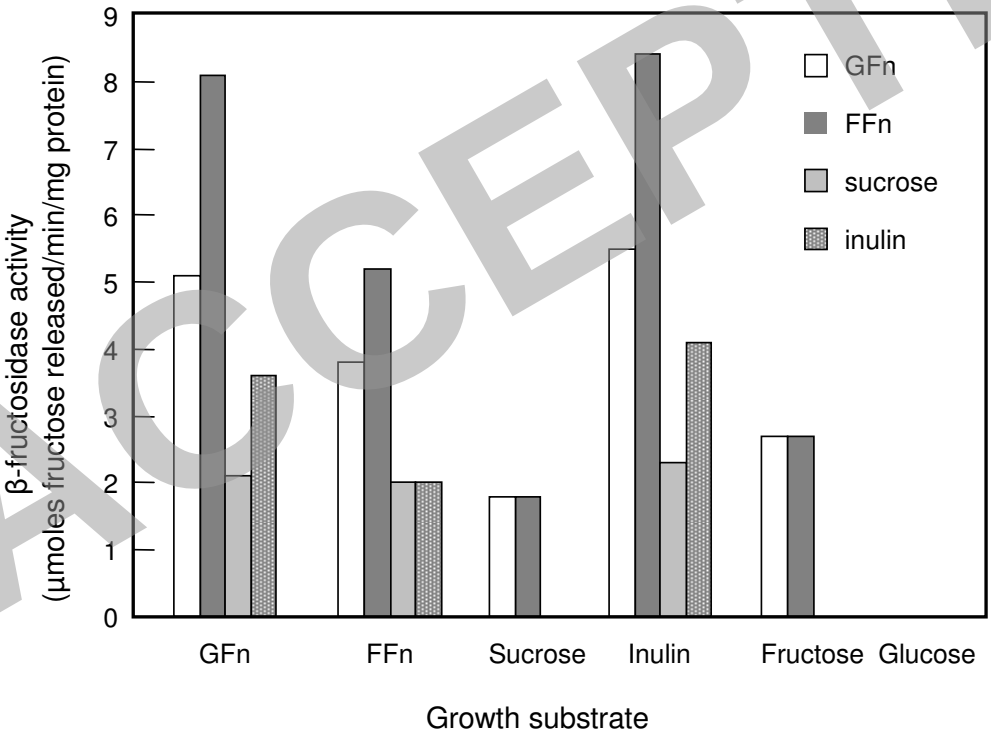

Figure 2. Goh et al.

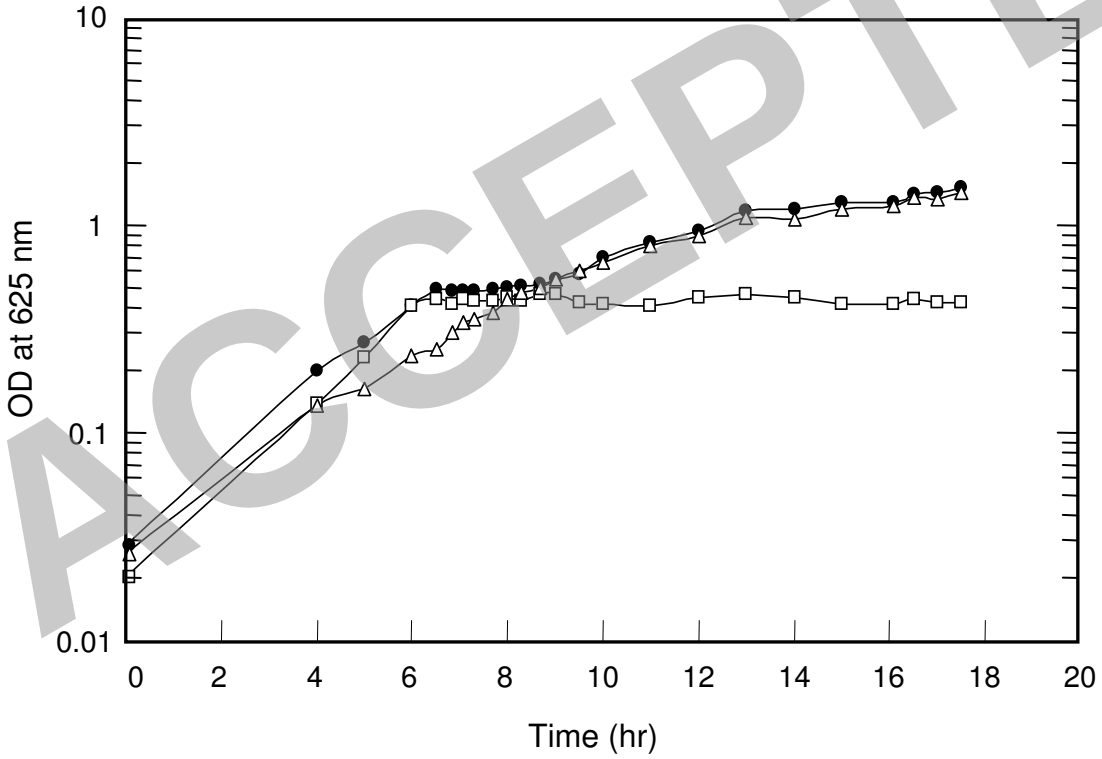

Figure 3. Goh et al.

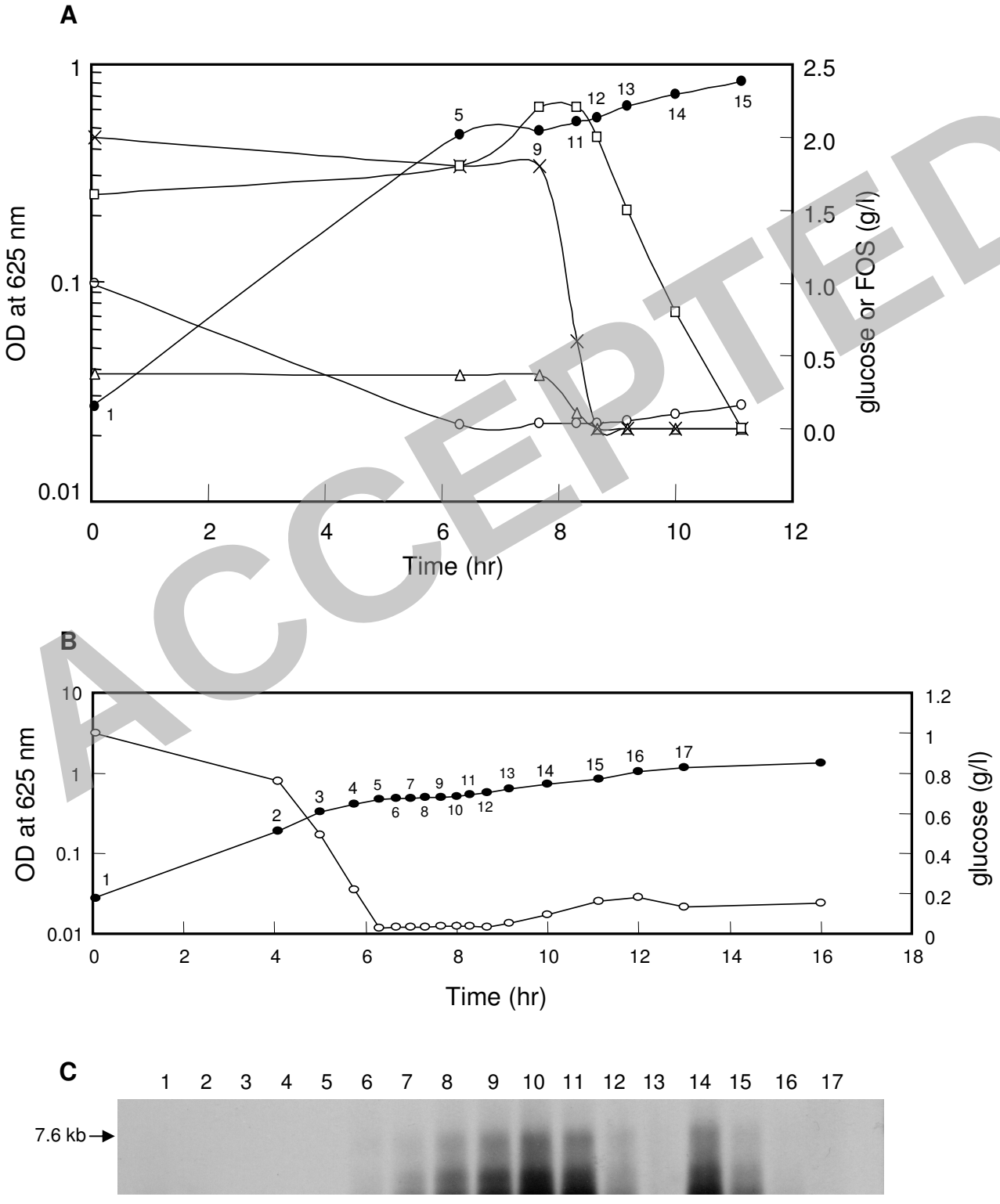

Figure 4. Goh et al.