



# An Inducible Operon Is Involved in Inulin Utilization in *Lactobacillus plantarum* Strains, as Revealed by Comparative Proteogenomics and Metabolic Profiling

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**ABSTRACT** The draft genomes of *Lactobacillus plantarum* strains isolated from Asian fermented foods, infant feces, and shrimp intestines were sequenced and compared to those of well-studied strains. Among 28 strains of *L. plantarum*, variations in the genomic features involved in ecological adaptation were elucidated. The genome sizes ranged from approximately 3.1 to 3.5 Mb, of which about 2,932 to 3,345 protein-coding sequences (CDS) were predicted. The food-derived isolates contained a higher number of carbohydrate metabolism-associated genes than those from infant feces. This observation correlated to their phenotypic carbohydrate metabolic profile, indicating their ability to metabolize the largest range of sugars. Surprisingly, two strains (P14 and P76) isolated from fermented fish utilized inulin.  $\beta$ -Fructosidase, the inulin-degrading enzyme, was detected in the supernatants and cell wall extracts of both strains. No activity was observed in the cytoplasmic fraction, indicating that this key enzyme was either membrane-bound or extracellularly secreted. From genomic mining analysis, a predicted inulin operon of *fosRABCDXE*, which encodes  $\beta$ -fructosidase and many fructose transporting proteins, was found within the genomes of strains P14 and P76. Moreover, *pts1BCA* genes, encoding sucrose-specific IBCA components involved in sucrose transport, were also identified. The proteomic analysis revealed the mechanism and functional characteristic of the *fosRABCDXE* operon involved in the inulin utilization of *L. plantarum*. The expression levels of the *fos* operon and *pst* genes were upregulated at mid-log phase. FosE and the LPXTG-motif cell wall anchored  $\beta$ -fructosidase were induced to a high abundance when inulin was present as a carbon source.

**IMPORTANCE** Inulin is a long-chain carbohydrate that may act as a prebiotic, which provides many health benefits to the host by selectively stimulating the growth and activity of beneficial bacteria in the colon. While certain lactobacilli can catabolize inulin, this has not yet been described for *Lactobacillus plantarum*, and an associated putative inulin operon has not been reported in this species. By using comparative and functional genomics, we showed that two *L. plantarum* strains utilized inulin and identified functional inulin operons in their genomes. The proteogenomic data revealed that inulin degradation and uptake routes, which related to the *fosRABCDXE* operon and *pstBCA* genes, were widely expressed among *L. plantarum* strains. The present work provides a novel understanding of gene regulation and mechanisms of

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inulin utilization in probiotic *L. plantarum* generating opportunities for synbiotic product development.

**KEYWORDS** lactic acid bacteria, genomes, prebiotic, *fos* operon,  $\beta$ -fructosidase

Inulin-type fructans are among the prebiotic substances found in several fruits and vegetables, such as artichokes, chicory, bananas, garlic, and asparagus (1). Inulin chains can be found in many lengths, with a degree of polymerization (DP) varying from 2 to 60, or even more (2). Inulin and its partially hydrolyzed fructooligosaccharides (FOS) are linear D-fructose polymers linked by a  $\beta$  (2-1) glycosidic bond, with a terminal glucose or fructose unit (3). The  $\beta$  (2-1) linkages protect these fructans from digestion in the upper gastrointestinal tract of humans. However, these prebiotics can be hydrolyzed by  $\beta$ -fructosidase-producing bacteria residing in the colon (3). Inulin-type fructans are readily degraded by the majority of bifidobacteria (4). On the other hand, inulin degradation is quite rare among lactobacilli, and is mainly limited to some strains of *L. paracasei*, *L. casei*, *L. acidophilus*, and *L. delbrueckii* (5–8).

The ability of lactobacilli to metabolize prebiotics, especially long-chain FOS of the GF<sub>n</sub> and FF<sub>n</sub> types, has been documented. Several pathways, including the *fos* (9, 10), *msm* (8), and *pts1BCA* (11) operons, are reportedly associated with FOS (fructooligosaccharide) metabolism in *Lactobacillus* species. In *L. plantarum* WCSF1, proteins encoded by the *pts1BCA* genes involved in the sucrose phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) can transport short-chain FOS (scFOS) into the cytosol, which are then further digested by intracellular  $\beta$ -fructofuranosidase, encoded by *sacA* (11). The *fosRABCDXE* operon is involved in the FOS utilization pathway of *L. paracasei* strain 1195 and encodes components of a putative fructose/mannose PEP-dependent PTS and a  $\beta$ -fructosidase precursor (FosE). FosE contains an N-terminal signal peptide sequence and an LPQAG cell wall anchor motif in the C-terminal region, indicating its location outside the cell (9, 10). Another system for FOS utilization that has been genetically characterized in *L. acidophilus* NCFM is the *msm* operon. The *msm* operon encodes an ABC transport system (*msmEFGK*) and a putative intracellular  $\beta$ -fructosidase (*bfrA*), which were found to hydrolyze sucrose, inulin-type fructans, and inulin (8).

Although many strains of *L. plantarum* have been found to exhibit a FOS-degrading ability, none can degrade the long-chain inulin (12). Thus far, only *L. plantarum* no. 14 has been reported to grow in the presence of inulin with a DP range of 5 to 30 (5). In this study, we aimed to compare the genomic features and metabolic properties of 28 strains of *L. plantarum* isolated from Asian fermented foods, infant feces, and shrimp intestines. Apart from the insights in the core and pan genomes of the *L. plantarum*, this approach enabled the identification and characterization of a new inducible operon involved in efficient utilization of inulin, a natural fructose polymer with high industrial relevance that, to the best of our knowledge, has not been described as being degradable by *L. plantarum*.

## RESULTS AND DISCUSSION

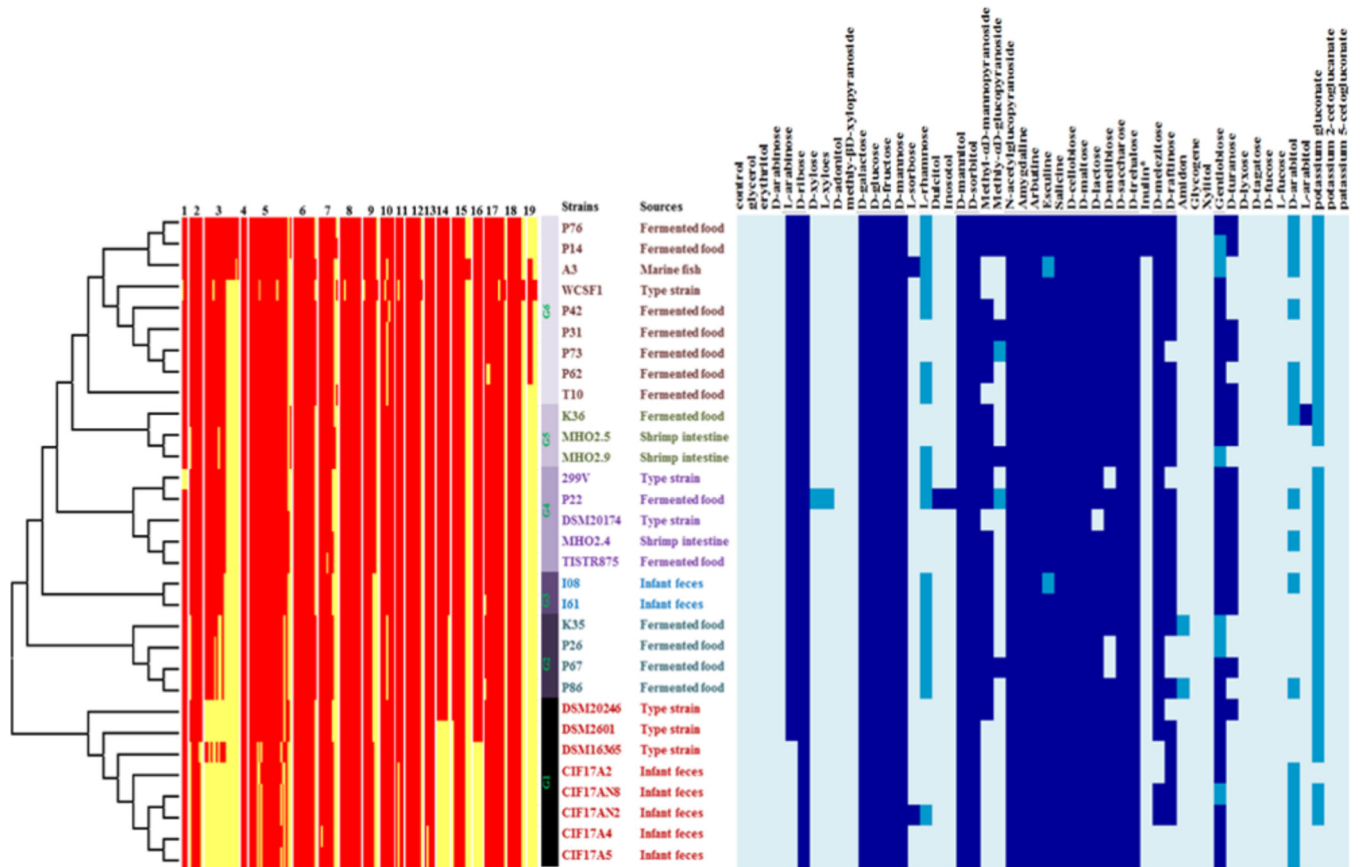
The genomic diversity of *Lactobacillus* spp. has been shown to be unusually high and indicates that *L. plantarum* and its two subspecies are part of a deeply rooted group (13). It is known that *L. plantarum* strains are quite versatile, but there have been no comparative next-generation genomics studies with functional analyses that can shed light on novel functions. In this study, the genomic and phenotypic characterizations were determined and compared among 28 strains of *L. plantarum*, the earlier analyzed *L. plantarum* strain WCSF1 isolated from the oral cavity, the type strain *L. plantarum* DSM20246, *L. plantarum* subsp. *plantarum* DSM20174, and *L. plantarum* subsp. *argenteratensis* DSM16365 (see Table S1 in the supplemental material). The collection of 28 strains included 13 strains newly isolated from fermented rice and fish from Thailand, since comparative hybridization analysis has shown that *L. plantarum* strains from Asian fermented foods are quite divergent from those found in other

habitats (14). Moreover, this collection also contained several *L. plantarum* isolates from marine fish, shrimp intestine, and other foods, as well as isolates of human intestinal origin, including strains from Asian infants of various ages, isolated under aerobic and anaerobic conditions (see Table S1). All strains were compared for their metabolic and genomic properties under identical circumstances and using the same technology to reveal new functionalities, as described below.

**General genomic features of *L. plantarum* and its predicted core and pan genomes.** All Asian strains of *L. plantarum* showed variation in their genome size ranging from approximately 3.1 to 3.5 Mb, with an overall GC content of 44.1 to 45.1%. The predicted numbers of protein-coding sequences (CDS) ranged from 2,932 to 3,345 (see Table S1). No plasmids were detected in any of the newly sequenced genomes, as opposed to *L. plantarum* WCSF1, which harbors three plasmids (15). The total gene pool of the *L. plantarum* species was calculated based on the genome comparison between all Asian *L. plantarum* strains with the best quality genome sequence of *L. plantarum* WCSF1 (16) (see Fig. S1). The pan genome was estimated to include 4,408 orthologous genes, of which 2,280 were common to the core genomes of all analyzed strains. The number of genes shared between the 28 *L. plantarum* strains and strain WCSF1 ranged from 2,445/2,841 (86.1%) to 2,630/2,841 (92.6%), with a median number of 2,555/2,841 (89.9%). None of the Asian strains shared the complete set of 2,841 genes present in *L. plantarum* WCSF1.

**Genomic clustering and sugar utilization capacity.** Since the genomic properties of the *L. plantarum* strains should translate into specific functions, the sugar utilization capacities of all strains were determined and clustered into 6 genomic groups (G1 to G6) based on the presence and absence of genes (Fig. 1). Of note, these genome-based clusters partly correlated to the order seen in the sugar utilization profile. Moreover, the genomic groups also correlated with the origins of the *L. plantarum* strains, where the strains from infant feces were enriched in G1 and G3, strains from fermented foods were enriched in G2 and G6, and strains from shrimp intestine were enriched in G5 (Fig. 1). The carbohydrate utilization profile further revealed that most *L. plantarum* strains use a broad range of simple and complex carbohydrates, and all strains grew on a group of 15 sugars, including glucose, galactose, mannose, sucrose, and fructose. The distinct carbohydrate metabolism may reflect their genomic diversity and evolution and adaptation to various environmental niches by gene decay, mutation (17, 18), gain, or loss (19, 20).

The capability to utilize carbohydrates depends on the presence of a functional transport system and of intracellular metabolic pathways. In this study, we detected genes encoding a PTS system and essential enzymes, which were originally annotated with functions involved in the uptake and metabolism of D-ribose, D-galactose, D-fructose, D-mannose, D-mannitol, D-sorbitol, D-cellobiose, D-maltose, D-lactose, D-saccharose, and D-trehalose (see Fig. S2). Strains isolated from fermented food and shrimp intestine (in G2, G3, G4, G5, and G6) had higher numbers of sugar utilization genes than the infant isolates (in G1). This was also evident from the summary of total genes predicted to be involved in sugar utilization as obtained from the Rapid Annotations using Subsystems Technology (RAST) server (see Fig. S3). Further comparison of genes involved in carbohydrate utilization revealed that *L. plantarum* isolated from fermented food and shrimp intestine comprised a high variety of genes, while strains isolated from infant feces lost genes involved in the utilization of fructooligosaccharides (FOS), raffinose, L-rhamnose, and L-arabinose (see Fig. S2 and S3). Therefore, the source of isolation displayed a remarkable degree of phenotypic and genotypic variety. There were large differences in carbohydrate availability in the various habitats from which the *L. plantarum* strains were recovered. The plant-associated niches were abundant in sucrose, trehalose, maltose, cellobiose, raffinose, starch, and inulin, whereas the mammalian intestinal tract was found to contain a variety of diet-derived carbohydrates and substantial quantities of host-derived carbohydrates (21, 22).

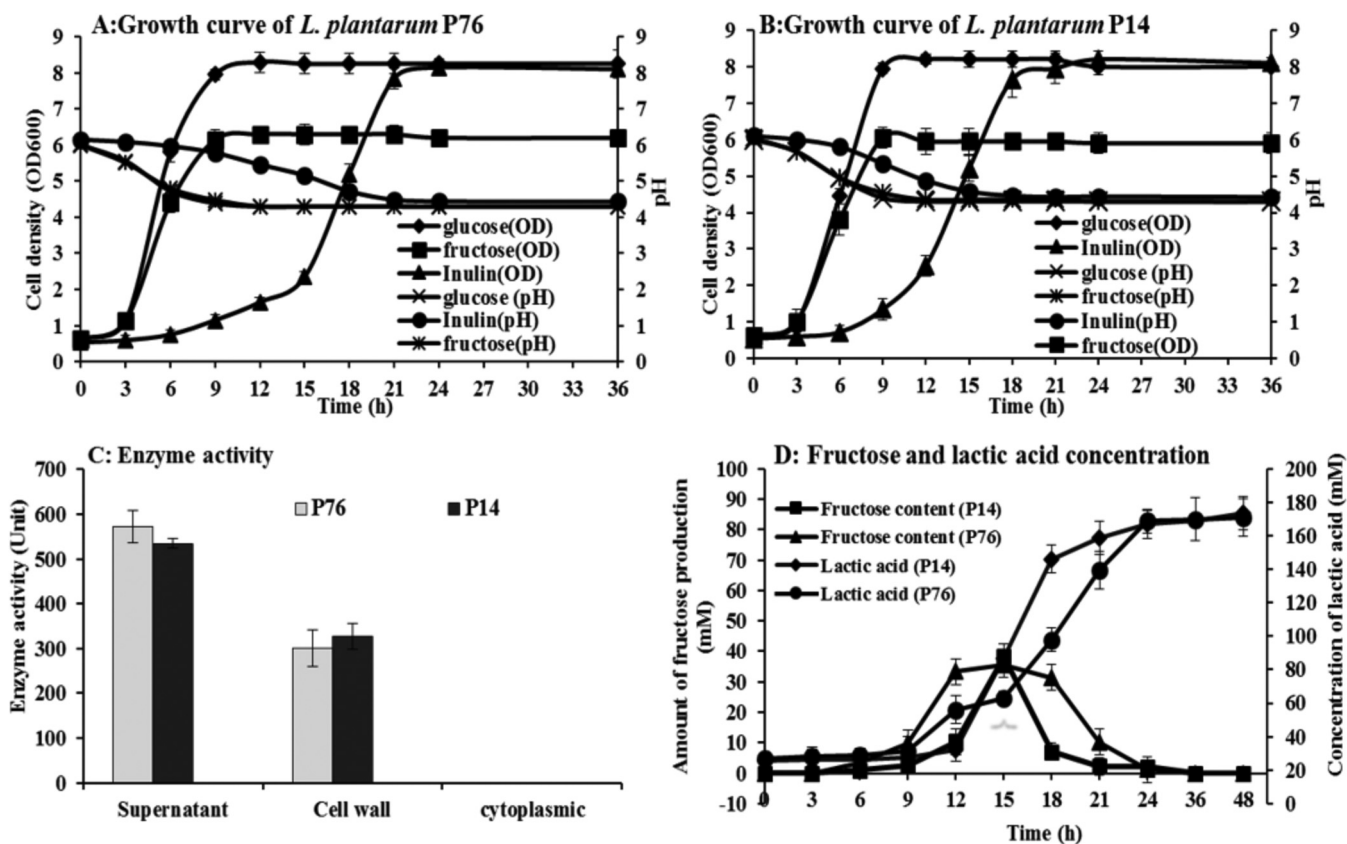


**FIG 1** Correlation genome diversity based on carbohydrate gene presence (red) and absence (yellow) (left side) in *L. plantarum* isolated from various sources by using the Mega 6 program (unweighted pair group method using average linkages [UPGMA] bootstrap value of 1,000, P-distance model) with the phenotypic data (API 50 CH) (right side); dark blue, completely fermented; lighter blue, partially fermented; lightest blue, nonfermented. The numbers at the top of the left figure refer to the PTS system and other transport and metabolic genes involved in sugar metabolism: 1, trehalose biosynthesis; 2, sucrose utilization; 3, fructooligosaccharide (FOS) and raffinose utilization; 4, lactose utilization; 5, maltose and maltodextrin utilization; 6, beta-glucoside metabolism; 7, trehalose uptake and utilization; 8, lactose and galactose uptake and utilization; 9, mannose metabolism; 10, D-tagatose and galactitol utilization; 11, D-gluconate and ketogluconate metabolism; 12, fructose utilization; 13, D-ribose utilization; 14, L-rhamnose utilization; 15, D-sorbitol (D-glucitol) and L-sorbosose utilization; 16, L-arabinose utilization; 17, glycerol and glycerol-3-phosphate uptake and utilization; 18, mannitol utilization; and 19, inositol catabolism.

**Adaptation to early life.** The genomes of the five *L. plantarum* strains (CIF17A2, CIF17A4, CIF17A5, CIF17AN2, and CIF17AN8) originating from the 5-month-old infant’s intestine had slightly lower GC contents and were considerably smaller than those of *L. plantarum* strains from other sources, including those from older infants and adults (see Table S1). Since infants at this young age are predominantly breast-fed, this implies a specific adaptation of these *L. plantarum* strains to the mother’s milk diet and that the strains in the baby’s intestine may be associated with gene loss events, well-known in lactic acid bacteria and often mediated by insertion sequences (13, 23).

The young infant’s *L. plantarum* strains lacked the capacity to utilize arabinose, a typical sugar found only in plants, notably in rice and banana plants that are provided to Thai infants after weaning. This is in line with the observation that the *L. plantarum* strains from the older and weaned infants (I08 and I61, respectively) and adults (299V) utilize arabinose. In contrast, the *L. plantarum* strains from the young infants used arabinol, a reduced sugar formed in the human body from arabinose and found in blood, urine, and likely also in the mother’s milk. Similarly, the arabinose-utilizing human strains consumed D-turanose, while the young infant’s strains did not utilize this plant signaling sugar for growth.

**Analysis of inulin-utilizing strains.** Two strains (P14 and P76) showed vigorous growth in the presence of inulin as a sole carbon source, indicating the presence of genes involved in inulin metabolism (Fig. 2). There are some reports of genes encoding

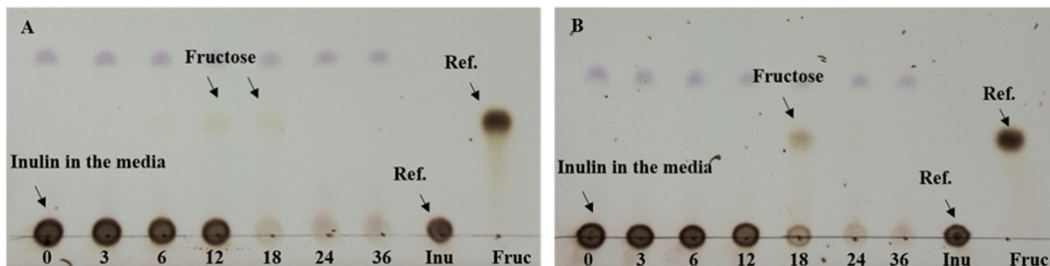


**FIG 2** Growth,  $\beta$ -fructosidase enzyme activity, and products of inulin-grown *L. plantarum* strains. Growth kinetics on inulin in comparison with other sugars for *L. plantarum* strains P76 (A) and P14 (B).  $\beta$ -Fructosidase enzyme activities in the various fractions (C). Amounts of lactic acid and fructose produced when grown on inulin (D).

enzymes belonging to the  $\beta$ -fructofuranosidase and  $\beta$ -fructosidase family in the genomes of *L. plantarum* isolates (11, 24). However, the consumption of FOS, inulin-type fructan, or inulin has rarely been reported (11, 25). Hence, we further focused on the conversion of this natural fructose polymer. Two inulin-utilizing strains (P14 and P76) were isolated from a fermented fish product (Pla Pang Dang), which includes sticky rice, salt, and sugar, as well as garlic that may serve as a source of inulin (26).

The capacity of *L. plantarum* strains P14 and P76 to ferment inulin was confirmed by a batch growth experiment in modified MRS-inulin in comparison to growth in the glucose- and fructose-containing media (Fig. 2A and B). Both strains grew faster in glucose (specific growth rates of 0.50 and 0.54 h<sup>-1</sup>, respectively) than in fructose (specific growth rates of 0.44 and 0.45 h<sup>-1</sup>, respectively). The stationary phase was reached in approximately 9 h on glucose or fructose compared to 18 to 21 h on inulin. *L. plantarum* P14 grew faster on inulin than *L. plantarum* P76. It reached the stationary phase several hours earlier than the P76 strain (after approximately 18 h and 21 h, respectively). In addition, the initial log phases of the P14 and P76 strains were 9 and 15 h, respectively, and their specific growth rates were 0.24 and 0.27 h<sup>-1</sup>, respectively, at mid-log phase. When grown on inulin, both *L. plantarum* strains reached optical densities at 600 nm (OD<sub>600</sub>) of about 8.0, similar that of strains grown on glucose. Lactic acid was the main metabolic end product of the P14 and P76 strains, with concentrations of 166.85  $\pm$  5.25 and 168  $\pm$  7.50 mM, respectively, after incubating for 24 h (Fig. 2D). Inulin utilization analysis showed that strains P14 and P76 consumed 86.28%  $\pm$  5.60% and 86.22%  $\pm$  4.55% of inulin, respectively, after incubating for 24 h. Thin-layer chromatographic (TLC) analysis of the culture supernatant revealed the presence of fructose after 12-h (P14) and 18-h (P76) incubations (Fig. 3).

Inulin degradation was confirmed by high-pressure liquid chromatography (HPLC), which showed that 38.38  $\pm$  5.50 and 35.69  $\pm$  4.50 mM fructose was generated by



**FIG 3** Sugar analysis of culture supernatants from inulin-grown *L. plantarum* strains. Culture supernatants obtained following the growth of *L. plantarum* P14 (A) and P76 (B) strains on inulin (time in hours is indicated) were spotted onto thin-layer chromatography silica gel plates and run in an acetic acid-chloroform-water (7:5:1) solvent. Sugars were visualized by spraying the plates with ethanolic 50% sulfuric acid and heating them at 115°C for 5 min. Inulin and fructose were used as a reference (Ref.).

strains P14 and P76 after 6 h and 15 h, respectively, which was then completely metabolized at 24 h (Fig. 2D). This observation reflected the presence of an active  $\beta$ -fructosidase. To further substantiate this, the activity of this particular enzyme was evaluated in three fractions of cell culture using inulin as a substrate, i.e., cell-free supernatant, cell wall extract, and cytoplasmic fractions. The  $\beta$ -fructosidase activity was detected in the cell-free supernatant and cell wall extract fractions, but not in the cytoplasmic one. The highest  $\beta$ -fructosidase enzyme activities from the cell-free supernatants of strains P14 and P76 were observed at 18 h ( $649.24 \pm 47.14$  and  $572.62 \pm 35.92 \mu\text{mol/ml/min}$ , respectively), and the highest enzyme activities in the cell wall fractions were found at 15 h ( $367.06 \pm 47.58$  and  $387.70 \pm 28.62 \mu\text{mol/ml/min}$ , respectively) (Fig. 2C). These data provide evidence indicating that the  $\beta$ -fructosidase enzyme was not only secreted extracellularly but was also anchored to the cell wall, like other enzymes possessing an LPXTG anchor motif (27, 28). Results from a previous mutational study of  $\beta$ -fructosidase encoded by the *fosE* gene provided evidence that the *fos* operon encodes key components for the utilization of fructooligosaccharide and other, structurally similar carbohydrates in *L. paracasei* 1195 (9). In addition, the cell wall-anchored FosE was encoded by the last gene of the *fosRABCDXE* operon, which may encode for the degradation of inulin, as discussed below.

**Proteogenomic analysis reveals an inducible inulin operon.** Three pathways, encoded by the *fos*, *msm*, and *pts1BCA* operons, are reportedly involved in FOS and inulin metabolism in *Lactobacillus* species (8–11). Microarray and other functional analyses revealed that the *pts1BCA* operon encodes a sucrose PTS transport system involved in metabolizing scFOS in *L. plantarum* WCSF1 in conjunction with an intracellular  $\beta$ -fructofuranosidase encoded by *sacA* (11). The *pts1BCA* gene complex, found in the draft genome sequences of strains P14 and P76, is also predicted to encode a sucrose PTS EIIBCA. It also shares complete identity (100% sequence similarity) with the gene of strain WCSF1. In addition, two other pathways have been described for metabolizing FOS and long-chain carbohydrates (inulin) in *Lactobacillus* species, but they have not yet been reported in *L. plantarum*. The *fosRABCDXE* operons present in the genomes of strains P14 and P76 are predicted to encode components of a fructose/mannose PEP-dependent PTS and a  $\beta$ -fructosidase precursor (the product of the *fosE* gene; see above) that are involved in the FOS utilization pathway of *L. paracasei* 1195 (9, 10). The presence of an N-terminal signal peptide sequence and an LPQAG cell wall anchor motif in the C-terminal regions of the deduced FosE precursor amino acid sequences predicts that the enzymes are cell wall associated, indicating that FOS may be extracellularly hydrolyzed, and the subsequent uptake of the hydrolysis products may be mediated by the FosABCDX PTS (9, 10). Another system for FOS utilization was previously described for *L. acidophilus* NCFM, where genes encoding the ABC transport system (*msmEFGK*) and a putative intracellular  $\beta$ -fructosidase (*bfrA*) were located in a multiple sugar metabolism (*msm*) operon. The *bfrA* gene product hydrolyzes sucrose, inulin-type fructan, and inulin (8). Therefore, in this study, the *fosRABCDXE* operon is

**TABLE 1** Proteome analysis of inulin-grown *L. plantarum* strains

Gene	Protein annotation for indicated <i>L. plantarum</i> strain <sup>a</sup>			Fold change <sup>b</sup>	
	P14	P76	Annotation	P14	P76
<i>fosR</i>	LBP14_156	— <sup>c</sup>	Transcription regulator related to NorR	6.31	—
<i>fosA</i>	LBP14_155	—	PTS system, fructose/mannose-inducible, IIA component	104.71	—
<i>fosB</i>	LBP14_154	LBP76_3114	PTS system, fructose/mannose-inducible, IIB component	60.26	58.88
<i>fosC</i>	LBP14_153	—	PTS system, fructose/mannose-inducible, IIC component	537.03	—
<i>fosD</i>	LBP14_152	LBP76_3112	PTS system, fructose/mannose-inducible, IID component	213.80	64.57
<i>fosE</i>	LBP14_150	LBP76_3198	Sucrose-6-phosphate hydrolase (beta-fructosidase)	26.92	11.75
<i>sacK1</i>	LBP14_1639	LBP76_917	Fructokinase	165.96	245.47
<i>pts1BCA</i>	LBP14_1640	LBP76_918	PTS system, sucrose-specific, IIB, IIC, and IIA components	1258.93	72.44
<i>sacC</i>	LBP14_1641	LBP76_919	Sucrose-6-phosphate hydrolase (EC 3.2.1.26)	288.40	9.77
<i>fruK</i>	LBP14_1067	LBP76_2192	1-Phosphofructokinase	21.38	20.89
—	LBP14_1804	LBP76_1082	Aerobic glycerol-3-phosphate dehydrogenase	151.36	630.96
<i>araT2</i>	LBP14_2218	LBP76_1662	<i>N</i> -Acetyl-L,L-diaminopimelate aminotransferase	177.83	109.65
—	LBP14_1423	LBP76_705	Maltose-6-phosphate glucosidase	54.95	380.19
—	LBP14_2003	LBP76_999	PTS system, cellobiose-specific, IIC component	33.88	154.88
<i>pts6C</i>	LBP14_1721	LBP76_1344	PTS system, cellobiose-specific, IIC component	20.89	22.39
<i>pts33BCA</i>	LBP14_1357	—	PTS system, beta-glucoside-specific, IIB, IIC, and IIA components	354.81	—
<i>pbg10</i>	LBP14_1356	—	6-Phospho-beta-glucoside	125.89	—
—	LBP14_566	—	LSU ribosomal protein L35p	630.96	—
<i>ntpT</i>	LBP14_3012	—	Di-/tripeptide permease (DtpT)	194.98	—
<i>mvaS</i>	LBP14_1040	—	Hydroxymethylglutaryl-CoA synthase	151.36	—
<i>pps</i>	LBP14_909	—	Phosphoenolpyruvate synthase	125.89	—
<i>mleR1</i>	LBP14_2541	—	Malolactic regulator	107.15	—
<i>nox5</i>	—	LBP76_584	NADH peroxidase (Npx)	—	269.15
<i>glpK</i>	—	LBP76_1081	Glycerol kinase	—	234.42
<i>minC</i>	—	LBP76_2308	Septum site-determining protein MinC	—	83.18
—	—	LBP76_427	ATP/GTP-binding protein	—	83.18
—	—	LBP76_1793	Erk/YbiS/YcfS/YnhG family protein, putative	—	81.28
<i>elaC</i>	—	LBP76_2187	RNase Z	—	72.44
<i>coaA</i>	—	LBP76_1274	Pantothenate kinase	—	67.61
<i>ppx3</i>	—	LBP76_1220	Exopolyphosphatase	—	64.57
<i>pheS</i>	—	LBP76_54	Phenylalanyl-tRNA synthetase alpha chain	—	60.26
—	—	LBP76_1033	Hypothetical protein	—	53.70
<i>dnal</i>	—	LBP76_19	Helicase loader (Dnal)	—	47.86
<i>mecA</i>	—	LBP76_2882	Negative regulator of genetic competence (MecA)	—	42.66
<i>pox1</i>	—	LBP76_1225	Pyruvate oxidase	—	39.81
—	—	LBP76_2377	Fumarate reductase, flavoprotein subunit precursor	—	39.81
<i>pflA</i>	—	LBP76_489	Pyruvate formate-lyase activating enzyme	—	36.31
<i>pps</i>	—	LBP76_365	Phosphoenolpyruvate synthase	—	35.48
—	—	LBP76_659	Galactitol-1-phosphate 5-dehydrogenase	—	32.36

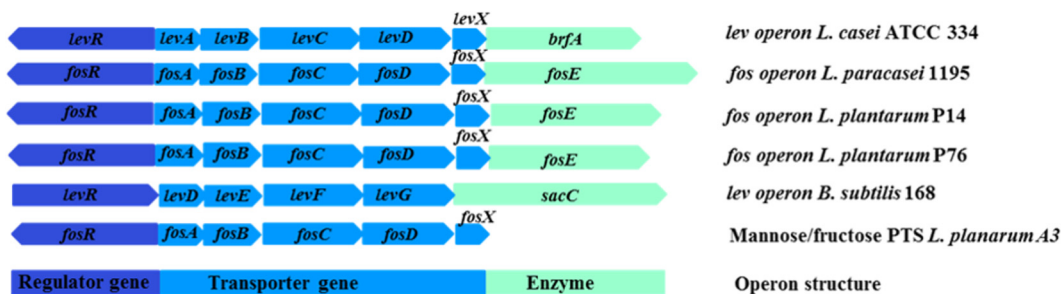
<sup>a</sup>P14 and P76 were grown on inulin and on glucose.

<sup>b</sup>Quantitative proteome analysis was performed on cell extracts. Inulin-induced proteins with a >5-fold change versus glucose are listed.

<sup>c</sup>—, not available.

likely involved in inulin utilization by *L. plantarum* strains P14 and P76. This operon has not yet been observed in other *L. plantarum* strains.

To substantiate the expression of the *fosRABCDXE* operon in *L. plantarum* P14 and P76, these strains were grown on inulin and the proteins produced were analyzed by shotgun proteomics. The results revealed an increased abundance of  $\beta$ -fructosidase, FosE, and other products of this operon (Table 1).  $\beta$ -Fructosidase (FosE) is a key enzyme in inulin degradation and hydrolyzes the termini of nonreducing 2,1- and 2,6-linked beta-D-fructofuranose residues in fructans. In the presence of inulin, 10- and 25-fold increased abundances of FosE were measured in strains P76 and P14, respectively (LBP14\_150 and LBP76\_3198, respectively) (Table 1). The 2,900-bp and 2,386-bp *fosE* genes of P14 and P76, respectively, were located next to *fosX* and coded in the same direction (Fig. 4).  $\beta$ -Fructosidase belongs to glycosyl hydrolase family 32 and contains conserved amino acid residues essential for its catalytic activity (29). However, the Gram-positive anchor was found in the FosE amino acid sequences from strains P14 and P76. The alignment of these amino sequences with those of fructan hydrolases of *L.*



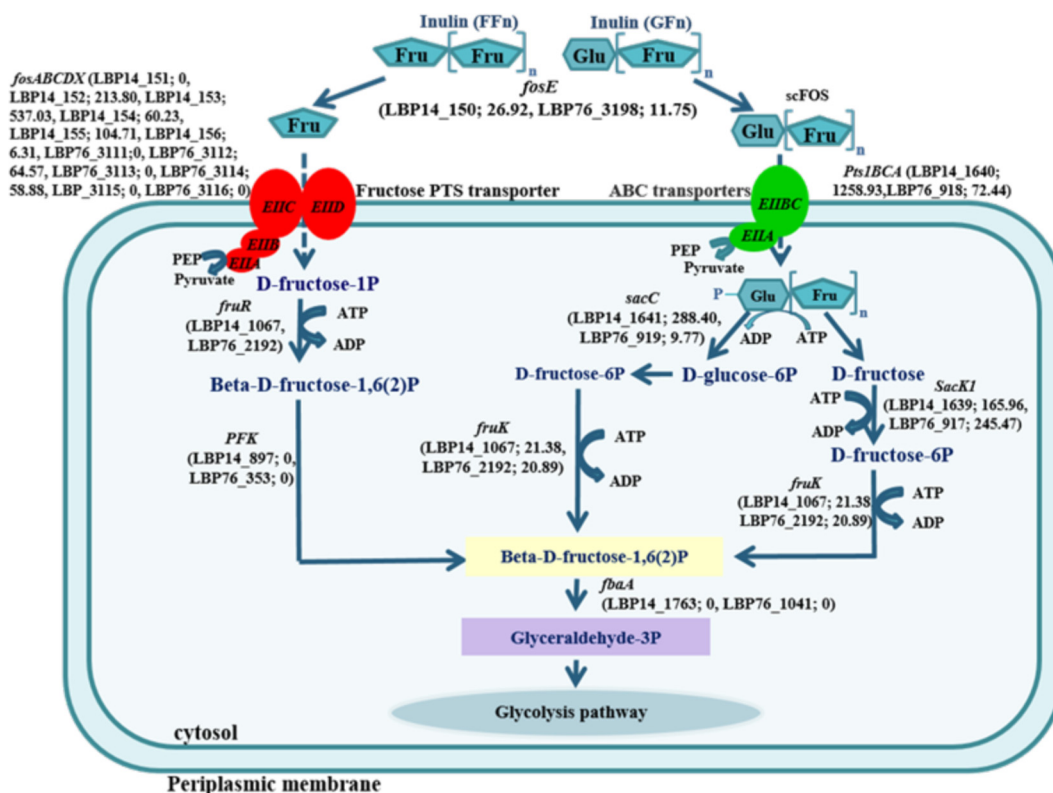
**FIG 4** Comparative gene organization of the *L. plantarum* inulin utilization operon. The predicted inulin degradation genes of *L. plantarum* strains P76 and P14 are shown in comparison with those from the *fos* operon of *L. paracasei* 1195 and the *lev* operon of *L. casei* ATCC 334. The fructose/mannose PTS system of *L. plantarum* A3 is also depicted. Dark blue, regulator gene; blue, PTS genes involved in sugar transport; light green, beta-fructosidase gene.

*casei* ATCC 334 (BfrA), *L. paracasei* 1195 (FosE), and *Bacillus subtilis* 168 (SacC) revealed that these enzymes were closely related to those encoded by *bfrA* and *fosE*, with sequence identities of 96 and 89.8%, respectively. On the other hand, the sequence shared only moderate sequence identity (38.6%) with levanase of *B. subtilis* 168. A dendrogram based on the deduced amino acid sequences can be seen in Fig. S4 in the supplemental material. These proteins were similar to each other (100% identity) and to  $\beta$ -fructosidase sequences of *L. casei* and *L. paracasei*.

The proteomics analysis of strains P14 and P76 also provided evidence for the inducible expression of the sugar PTS genes in the presence of inulin (Table 1). Increases of 60- and 540-fold were observed for the FosABCD transporter in strains P14 and P76, respectively. FosE and FosABCDX are encoded by the *fosRABCDXE* operon, which was previously documented in a strain of *L. paracasei* (9, 10). The identified putative FosABCD transporter located in the genomes of *L. plantarum* P14 and P76 comprises genes encoding a transcriptional regulator (FosR) and the EIIA, IIB, IIC, IID, and EII components of a fructose/mannose-specific PTS. The structural organizations and gene sequences of these operons were extremely similar to those of the putative *fos* operon of *L. paracasei* 1195 and the putative levanase operon of *L. casei* ATCC 334 (Fig. 4). The products of *fosR* genes (LBP14\_156 and LBP76\_3116) exhibited 69% and 47% amino acid sequence identity with the *fosR* transcriptional regulator of *L. paracasei* 1195 (GenBank accession no. ABD57313.1) and a putative levanase operon transcriptional regulator in *L. casei* ATCC 334 (GenBank accession no. ABJ69406.1), respectively. The structural genes consist of four genes (FosABCD) that encode the EIIABCD components of the fructose/mannose PTS. FosA and FosB are presumably responsible for phosphorylating substrates, while FosC and FosD are likely specific for fructose and/or mannose (30). The nucleotide sequences of FosB, FosC, and FosD shared 85.6, 91.5, and 69.1% identities, respectively, with the structural genes of *L. paracasei* 1195 and *L. casei* ATCC 334, whereas FosA is similar (49% identity) to *L. paracasei* 1195 and *L. casei* ATCC 334. The *fosX* gene encodes a 109-residue putative EII component that shares 69% identity with a hypothetical protein in *L. paracasei* 1195 (GenBank accession no. ABD57318.1) and *L. casei* ATCC 334 (GenBank accession no. ABJ69401.1).

In *L. acidophilus* and *Bifidobacterium breve*, ABC transporters were observed to be involved in FOS uptake (8, 31), but these systems were not found in the inulin-utilizing P14 and P76 strains of *L. plantarum* described here. However, these strains were found to contain an additional sucrose PTS, encoded by *pts1BCA*, which was upregulated during inulin fermentation in strains P14 and P76, resulting in 1,259-fold (LBP14\_1640) and 72-fold (LBP76\_918) increased abundances, respectively. Moreover, expression of sucrose-6-phosphate hydrolase (*sacC*) was increased 288-fold (LBP14\_1641) and 9.7-fold (LBP76\_919) while expression of fructokinase (*sacK1*) was increased 165- and 245-fold in strains P14 and P76, respectively, when grown on inulin (Table 1). The upregulated sucrose-6-phosphate hydrolase in strains P14 and P76 revealed a 99% similarity to beta-fructofuranosidase in other *L. plantarum* strains (accession no.





**FIG 5** Proposed model for inulin degradation by *L. plantarum* strains P14 and P76 based on the proteogenomic analysis. The locus tags and fold induction of protein based on proteomic data are indicated for *L. plantarum* strains P76 and P14. *fosE*, beta-fructofuranosidase; *fosABCDX*, the putative *fos* operon; *sacK1*, fructokinase; *fruR*, 1-phosphofructokinase; PFK, 6-phosphofructokinase; *fbaA*, fructose-bisphosphate aldolase class II.

AGE\_37932.1 and WP\_02400235.1), which hydrolyzed sucrose and scFOS. In addition, *pts33BCA* may be involved in metabolizing glucose residues present as terminal sugars of certain types of inulin (32, 33). The proposed pathway of inulin utilization is depicted in Fig. 5.

**Conclusion.** Twenty-eight Asian isolates of *L. plantarum* were investigated at the genomic and phenotypic levels. Obviously, genome size correlated with the isolation sites. The strains isolated from foods typically harbored more genes than those isolated from humans. Although information related to their niches might parallel their ecological fitness and adaptation, each strain harbors sets of strain-specific genes. To further illustrate the association between genotype and phenotype, we reported and examined genes involved in carbohydrate utilization among *L. plantarum* strains in this study. Specifically, strains P14 and P76 harbored the operon *fosRABCDXE* and *pts1BCA* in their genomes, enabling these two strains to hydrolyze inulin. We further demonstrated the expression and the functionality of the inulin metabolic pathway using a proteomic approach.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Twenty-eight strains of *L. plantarum* were isolated and selected from various sources, including infant feces, marine fish, shrimp intestines, fermented fish, and fermented rice noodle products from Thailand (see Table S1 in the supplemental material). All strains were classified as *L. plantarum* according to their 16S rRNA nucleotide sequences and sugar utilization profiles (using the API 50 CH kit; see below for further details). Additionally, these strains were differentiated from the closest species, *L. pentosus* and *L. paraplantarum*, based on the nucleotide sequences of *recA* and *dnaK* genes. Seven infant strains (CIF17A2, CIF17A4, CIF17A5, CIF17AN2, CIF17AN8, I08, and I61) (34, 35) as well as the marine fish and shrimp intestine strains (A3, MHO2.4, MHO2.5, and MHO2.9) (36, 37) were previously described. The 13 fermented-food isolates were cultured from Thai fermented rice noodles and fish essentially as described previously (38) (kindly provided by A. Nuyler and K. Kanjanasmith). *L. plantarum* TISTR 875 (39) was isolated from a Thai fermented food

(pickled cabbage) donated by the Thailand Institute of Scientific and Technological Research (Bangkok, Thailand). The *L. plantarum* type strain DSM20246, *L. plantarum* subsp. *plantarum* DSM20174, and *L. plantarum* subsp. *argenteratensis* DSM16365 and DSM2601 (previously described as *L. arizonensis*; see reference 40) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany), *L. plantarum* strain 299V was isolated from a fermented ProViva drink produced with strain 299v (41), and *L. plantarum* strain WCSF1 was obtained from the Laboratory of Microbiology at Wageningen University (The Netherlands). All *L. plantarum* strains/isolates were cultivated in MRS broth at 37°C for 24 h.

**Sugar fermentation and other metabolic assays.** Carbohydrate fermentation profiles were determined using the API 50 CH kit (bioMérieux, France) according to the manufacturer's instructions. The inulin-utilizing strains (P14 and P76) were cultured in modified MRS media supplemented with 1% inulin (Sigma, USA) as a main carbon source and incubated at 37°C for 48 h (glucose and fructose were used as controls). Bacterial growth was determined every 3 h for up to 48 h by measuring the optical density at 600 nm, and the pH of the cell-free supernatants was also monitored.

**Analysis of sugar and other inulin-degraded metabolites.** To determine the concentrations of glucose and fructose residues in the bacterial cultures, cells were removed by centrifugation at  $9,500 \times g$  for 10 min at 4°C. The supernatants were transferred to HPLC vials. Twenty microliters of these supernatants was injected by an autosampler into the HPLC system (Spectra system AS3000) equipped with a Metacarb 67H column (300 mm  $\times$  6.5 mm). The sugar molecules were eluted with 0.01 N H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.8 ml/min. Standard curves for glucose and fructose (0, 2.5, 5, 10, and 20 mM) were prepared. Thin-layer chromatography (TLC) was used to monitor the extent of inulin degradation by spotting the supernatants onto a silica gel plate (Merck, Germany) using 1% inulin and fructose as a control. The plates were developed in a solvent mixture of acetic acid-chloroform-water (7:5:1) as a mobile phase. The sugar spots were visualized by spraying the plates with ethanolic 50% sulfuric acid (ethanol:sulfuric acid, ratio 1:1) and heating them at 115°C for 5 min. In addition, a general phenol-sulfuric acid assay was performed to determine the total carbohydrate contents after incubating for 24 h (42).

**$\beta$ -Fructosidase assays.** Enzyme activity was investigated as previously described (8) with some modifications. Briefly, cells were harvested by centrifugation at  $9,500 \times g$  for 10 min at 4°C, and 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 homogenized with a Branson sonicator equipped with a 3-mm tip using six pulses of 30 s with a 1-min incubation on ice in between each pulse. Cell lysates were transferred into new microtubes and cell walls were separated from the cytoplasmic fraction by centrifuging at  $9,500 \times g$  for 10 min at 4°C, essentially as described previously (9). The cell wall fraction was then resuspended in 1 ml 0.1 M potassium phosphate buffer (pH 6.6).

Ten microliters of each fraction (culture supernatant, cell wall fraction, or cytoplasmic extract) was added to 190  $\mu$ l of a 1% (wt/vol) inulin solution. The reaction mixtures were incubated at 37°C for 3 h and heat inactivated at 95°C for 5 min. The  $\beta$ -fructosidase activity was measured by determining the amount of inulin released as fructose, and fructose residue concentrations were determined using a fructose assay kit (EnzyChrom; BioAssay Systems, USA) according to the manufacturer's instructions. All experiments were performed in duplicate. One unit of enzyme activity was defined as the enzyme necessary to release 1  $\mu$ mol of fructose per min at the specified conditions.

**Protein extraction and proteomic analysis.** Bacterial proteins were extracted and then fractionated using SDS-PAGE. Bacterial cells at mid-log phase grown (separately twice) in the presence and absence of inulin were centrifuged and washed three times with phosphate-buffered saline (PBS; pH 6.8). The pellet was resuspended in 0.5 ml 100 mM Tris-HCl (pH 7.5) with 4% SDS and 0.1 M dithiothreitol and transferred into 2-ml low-binding microcentrifuge tubes (Eppendorf, The Netherlands). The cell pellets were lysed using a sonicator as described above. Proteins were denatured by heating at 95°C for 20 min, followed by centrifugation at  $9,500 \times g$  for 10 min. Protein concentrations were determined as described previously (43). The protein concentrations were equalized, and each of the samples containing 25  $\mu$ g of protein mixed with 5  $\mu$ l of loading buffer (250 mM Tris-HCl [pH 6.8], 0.5 M dithiothreitol, 10% SDS, 0.02% bromophenol blue, and 30% glycerol) was subjected to SDS-PAGE using a 10% acrylamide gel (MiniProtein III system; Bio-Rad, The Netherlands). Gels were stained with Coomassie brilliant blue R250.

Protein extractions, separations, and tryptic digestions were performed as described previously (44). The trypsin-digested peptides were analyzed by nano-liquid chromatography coupled with mass spectrometry (nLC-MS/MS) using a Proxeon nLC and a LTQ-Orbitrap mass spectrometer (44, 45), and all spectral data obtained were analyzed with MaxQuant v.1.3.0.5 (45, 46) using a contaminants database and a protein database generated from *L. plantarum* P14 and *L. plantarum* P76 after genomic sequencing and proteomic data were obtained (as described below).

The "label-free quantification" (LFQ) and "match between runs" (set to 2 min) options were enabled. Deamidated peptides were used for protein quantification, and all other quantification settings were kept at the default. Filtering and further bioinformatic analysis of the MaxQuant/Andromeda workflow output and the analysis of the abundances of the identified proteins were performed with the Perseus 1.3.0.4 module (47). Peptides and proteins with a false discovery rate (FDR) of less than 1% were accepted, as were proteins with at least 2 identified peptides, of which at least one was unique and at least one was unmodified. Reversed hits were deleted from the MaxQuant result table. Zero LFQ intensity values were replaced by a value of 15,848 (just below the lowest value measured) to permit sensible ratio calculations. Relative protein quantitation of inulin-grown samples to glucose-grown controls was calculated as the ratio of the average inulin-grown LFQ intensities to the average glucose-grown LFQ intensities.

**Genome sequencing and annotation.** For sequencing, genomic DNA from all *L. plantarum* strains (see Table S1) was extracted from 1.5-ml volume overnight cultures using a commercial kit (MasterPure; Epicentre, Madison, USA). Bacterial genomic DNA was then sequenced on a paired-end (R1 = 326 bp, R2 = 286 bp) Illumina MiSeq run. Adapter sequences and low-quality bases (Q <30) at the ends of reads were trimmed out using cutadapt (version 1.6) (48). Overlapping paired-end reads were merged and extended as single reads by FLASh (Fast Length Adjustment of Short reads) (version 1.2.6) (49). *De novo* genome assemblies were performed from the extended paired-end and orphan reads using SPAdes assembler (v. 3.1.1) (50). DNA sequencing was performed at the DNA Sequencing and Genomics Laboratory, Institute of Biotechnology, University of Helsinki. Gene prediction and annotation were conducted using the RAST server (51). The presence of plasmid sequences was evaluated by using PlasmidFinder version 1.3 (52). The pan and core genomes were evaluated using eggNOG v4.0 (53). The signal peptide prediction was performed using SignalP 4.1 (54), and the transmembrane helices were predicted by the TMHMM server (V.2.0) (55).

**Accession number(s).** Genome sequences of the 28 strains were determined, analyzed, and deposited into the GenBank database. The new whole-genome shotgun sequences reported here are deposited at DDBJ/EMBL/GenBank under the accession numbers LEAV000000000 (for *L. plantarum* 299v), LEAW000000000 (for *L. plantarum* TISTR875), LEAX000000000 (for *L. plantarum* DSM 20246), LEAY000000000 (for *L. plantarum* DSM 2601), LEAZ000000000 (for *L. plantarum* C1F17AN8), LEBA000000000 (for *L. plantarum* C1F17AN2), LEBB000000000 (for *L. plantarum* C1F17A5), LEB000000000 (for *L. plantarum* C1F17A4), LEBD000000000 (for *L. plantarum* C1F17A2), LEBE000000000 (for *L. plantarum* I08), LEBF000000000 (for *L. plantarum* A3), LEBG000000000 (for *L. plantarum* MHO2.9), LEBH000000000 (for *L. plantarum* MHO2.5), LEBI000000000 (for *L. plantarum* MHO2.4), LEBJ000000000 (for *L. plantarum* P86), LEBK000000000 (for *L. plantarum* P76), LEBL000000000 (for *L. plantarum* P73), LEBM000000000 (for *L. plantarum* P67), LEBN000000000 (for *L. plantarum* P62), LEB000000000 (for *L. plantarum* P42), LEBP000000000 (for *L. plantarum* P31), LEBQ000000000 (for *L. plantarum* P26), LEBR000000000 (for *L. plantarum* P22), LEB000000000 (for *L. plantarum* T10), LEBT000000000 (for *L. plantarum* K35), LEBU000000000 (for *L. plantarum* I61), LEBV000000000 (for *L. plantarum* P14), and LEBW000000000 (for *L. plantarum* K36) (see Fig. S1 in the supplemental material). Sequences were compared with those of the previously reported strains WCFS1 (14, 56), *L. plantarum* subsp. *plantarum* DSM20174, and *L. plantarum* subsp. *argenteratensis* DSM16365 (13).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02402-16>.

**TEXT S1**, PDF file, 0.8 MB.

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