Pseudofructophilic *Leuconostoc citreum* Strain F192-5, Isolated from Satsuma Mandarin Peel

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ABSTRACT Fructophilic lactic acid bacteria (FLAB), composed of *Fructobacillus* spp., *Lactobacillus kunkeei*, and *Lactobacillus apinorum*, are unique in that they prefer D-fructose over D-glucose as a carbon source. Strain F192-5, isolated from the peel of a satsuma mandarin and identified as *Leuconostoc citreum*, grows well on D-fructose but poorly on D-glucose and produces mainly lactate and acetate, with trace amounts of ethanol, from the metabolism of D-glucose. These characteristics are identical to those of obligate FLAB. However, strain F192-5 ferments a greater variety of carbohydrates than known FLAB. Comparative analyses of the genomes of strain F192-5 and reference strains of *L. citreum* revealed no signs of specific gene reductions, especially genes involved in carbohydrate transport and metabolism, in the genome of F192-5. The bifunctional alcohol/acetaldehyde dehydrogenase gene (*adhE*) is conserved in strain F192-5 but is not transcribed. This is most likely due to a deletion in the promoter region upstream of the *adhE* gene. Strain F192-5 did, however, ferment D-glucose when transformed with a plasmid containing the allochthonous *adhE* gene. *L. citreum* F192-5 is an example of a pseudo-FLAB strain with a deficiency in D-glucose metabolism. This unique phenotypic characteristic appears to be strain specific within the species *L. citreum*. This might be one of the strategies lactic acid bacteria use to adapt to diverse environmental conditions.

IMPORTANCE Obligate fructophilic lactic acid bacteria (FLAB) lack the metabolic pathways used in the metabolism of most carbohydrates and differ from other lactic acid bacteria in that they prefer to ferment D-fructose instead of D-glucose. These characteristics are well conserved at the genus or species level. *Leuconostoc citreum* F192-5 shows similar growth characteristics. However, the strain is metabolically and genomically different from obligate FLAB. This is an example of a strain that evolved a pseudo-FLAB phenotype to adapt to a fructose-rich environment.

KEYWORDS *Leuconostoc citreum*, *adhE*, comparative genomics, fructophilic lactic acid bacteria, pseudofructophilic

Lactic acid bacteria (LAB) are found in a variety of environments, including dairy products, fermented food, silage, and the gastrointestinal tracts of humans and animals, and are known to adapt to habitats at the genomic level (1). Fructophilic lactic acid bacteria (FLAB) differ from LAB in that they prefer fructose over glucose as a growth substrate (2, 3). External electron acceptors such as pyruvate, oxygen, and fructose dramatically enhance glucose metabolism in FLAB. Species thus far described have been isolated from D-fructose-rich niches such as flowers, fruits, fermented food

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made from fruits, and the gastrointestinal tracts of insects such as bumblebees, honeybees, tropical fruit flies, and *Camponotus* ants that feed on D-fructose-rich diets (3). FLAB are divided into two taxonomic groups, i.e., the *Fructobacillus* group and the *Lactobacillus kunkei*-*Lactobacillus apinorum* group (2). Species from both groups lack the bifunctional alcohol/acetaldehyde dehydrogenase (ADH/ALDH) gene (*adhE*) (4–6), resulting in an imbalance of NAD/NADH during glucose metabolism (7). No other species have been reported as typical obligate FLAB.

The genus *Leuconostoc* is the type genus of the family *Leuconostocaceae* which contains the genus *Fructobacillus*. *Fructobacillus* spp. were originally classified into the genus *Leuconostoc* but later reclassified as members of a novel genus (8). The two genera are phylogenetically related but are clearly separated based on genetic, biochemical, and morphological characteristics (4). *Leuconostoc* spp. have diverse physiological and biochemical characteristics and have been isolated from different niches, mainly, fermented plant materials and the gastrointestinal tracts of omnivores, including human infants (9–11). Fructophilic characteristics have, however, never been reported for *Leuconostoc* spp.

During an ecological study of FLAB, a unique strain (F192-5) was isolated from the peel of a satsuma mandarin. The strain shares similar growth characteristics with typical FLAB but is phylogenetically related to *Leuconostoc citreum*. Comparative genomic analyses and characterization of the *adhE* gene distinguished strain F192-5 from other strains of *L. citreum*. Here, we report strain-specific pseudofructophilic evolution.

RESULTS

Isolation, identification, and metabolic characteristics. Strain F192-5 grew actively in fructose-yeast extract-peptone (FYP) but poorly in glucose-yeast extract-peptone (GYP) broth and was regarded a candidate of FLAB. BLAST analysis of nearly complete 16S rRNA gene sequences (1,542 bp) showed 99.7% similarity to *L. citreum* ATCC 49370^T, and strain F192-5 was identified as *L. citreum*. Strain F192-5 grew well in the presence of D-fructose (FYP) and D-glucose, but only when D-glucose was supplemented with pyruvate (GYP-P). Good growth of strain F192-5 was recorded in GYP under aerobic conditions but not in GYP without electron acceptors. The type strain of *L. citreum* (NRIC 1776^T), on the other hand, grew well under all conditions tested (Fig. 1a and b). Growth characteristics of *L. citreum* NRIC 1772, NRIC 1579, and NRIC 1581 were similar to that of strain NRIC 1776^T, and the strains reached optical density at 660 nm (OD₆₆₀) values of 1.5 to 2.0 after 24 h in GYP broth (data not shown). Strain F192-5 produced pinpoint colonies (0.1 to 0.2 mm in diameter) on GYP agar when anaerobically incubated for 48 h (Fig. 1c). Reference strains of *L. citreum* produced large colonies (2 to 3 mm in diameter) under these conditions. Strain F192-5 produced lactate and acetate, with trace amounts of ethanol, from the fermentation of D-glucose (Table 1). Reference strains of *L. citreum* produced lactate and ethanol as main end products from the fermentation of D-glucose. Strain F192-5 fermented D-fructose within 1 day but took 2 days to ferment D-glucose. The unique growth and metabolic properties recorded for strain F192-5 are consistent with those of typical FLAB (2, 3). Strain F192-5 does, however, differ from typical FLAB by fermenting more carbohydrates, including L-arabinose, D-xylose, D-mannose, mannitol, methyl- α -D-glucopyranoside, N-acetylglucosamine, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, and turanose, after 1 to 5 days of incubation (3). Reference strains of *L. citreum* showed similar carbohydrate fermentation patterns to those of strain F192-5 but fermented D-glucose within 1 day.

Genomic analysis. *L. citreum* F192-5 has a slightly larger genome (2.06 Mbp) and more coding DNA sequences (CDSs; 2,051) than other *L. citreum* strains (average $\pm$ standard deviation [SD], 1.84 ± 0.08 Mbp and $1,835 \pm 90$ CDSs), as shown in Table 2. Strain F192-5 and all reference strains, including the type strain of *L. citreum* (NRIC 1776^T), had average nucleotide identity (ANI) values of 0.990 to 0.998, indicating that they belong to a single taxon. *L. citreum* strains have 1,357 core genes, which accounts for 66.2% of the total CDSs in the F192-5 genome. The pan-genome size is 2,893.

FIG 1 Growth characteristics of *L. citreum* NRIC 1776^T (a) and F192-5 (b) in GYP broth (○), FYP broth (Δ), GYP-P broth (◇), and GYP broth under aerobic conditions (□) and on GYP agar under anaerobic conditions (c). In panels a and b, lines and error bars indicate averages and standard deviations from triplicates, respectively.

Functional classification of CDSs predicted in each strain showed large gene repertoires of classes for [J] translation, [G] carbohydrate transport and metabolism, [K] transcription, and [E] amino acid metabolism and transport. Few differences were found between strain F192-5 and other *L. citreum* strains (see Table S1 in the supplemental material). Contents of genes involved in metabolic/biosynthesis pathways, as determined by ortholog assignment and pathway mapping using the KEGG Automatic Annotation Server (KAAS), showed that strain F192-5 has gene repertoires for metabolism of carbohydrates and amino acids similar to those for other *L. citreum* strains (data not shown). Strain F192-5 has 14 genes involved in the phosphotransferase system (PTS), encoding an estimated six complete PTS transporters. Similar results were obtained for other *L. citreum* strains.

Strain-specific genes in each strain were initially screened by orthologous clustering. The number of strain-specific genes ranged from 16 to 175 (average $\pm$ SD, 81.6 ± 51.7) (Table 3). Specificity of the genes was further studied based on standard protein BLAST (BLASTP) analysis against other *L. citreum* strains using the NCBI BLAST web server. The analysis dramatically reduced the number of strain-specific genes, ranging from 1 to 68

TABLE 1 End products from D-glucose

Strain	Consumed D-glucose (mM)	End products (mM) ^a			Molar ratio of L:E:A	Carbon recovery (%)
		L	E	A		
NRIC 1776 ^T	51.3	56.3	47.8	1.9	1:0.85:0.03	87.2
NRIC 1772	51.4	52.4	45.5	3.2	1:0.87:0.06	82.6
NRIC 1579	51.3	51.8	44.2	1.9	1:0.85:0.04	80.4
NRIC 1581	51.4	53.3	45.7	3.3	1:0.86:0.06	83.6
F192-5	13.9	14.6	0.2	14.1	1:0.02:0.97	86.7
31-11	53.9	58.3	71.8	1.2	1:1.23:0.02	99.3
2-1	9.6	10.3	0.3	12.5	1:0.03:1.21	98.2

^aL, lactate; E, ethanol; A, acetate.

TABLE 2 General genomic characteristics of *L. citreum* strains used for genomic analysis

Strain	Source	Genome size (Mbp)	No. of CDSs	Genome status ^a	Contamination (%)	Completeness (%)	Accession no.
F192-5	Peel of satsuma mandarin	2.06	2,051	D	4.02	98.67	BJJW01000000.1
NRIC 1776 ^T	Honey dew of rye ear	1.81	1,781	D	1.49	99.65	BJJX01000000.1
1301_LGAS	Intensive care units	1.82	1,784	D	0.56	97.69	NZ_JVUU00000000.1
NRRL B-1299	Unknown	1.75	1,717	D	0.23	98.63	NZ_CCNH00000000.1
NRRL B-742	Unknown	1.75	1,749	D	0.21	97.50	NZ_CCNG00000000.1
DmW_11	Wild <i>Drosophila</i>	1.83	1,847	D	2.67	98.67	NZ_NDXG00000000.1
KM20	Kimchi	1.90	1,915	C	0.03	99.65	NC_010471.1
LBAE_C10	Sourdough	1.93	1,939	D	1.18	99.59	NZ_CAGE00000000.1
LBAE_C11	Sourdough	1.97	1,976	D	0.52	99.65	NZ_CAGF00000000.1
LBAE_E16	Sourdough	1.80	1,806	D	0.03	99.65	NZ_CAGG00000000.1

^aD, draft genome sequence; C, complete genome sequence.

(average $\pm$ SD, 27.5 $\pm$ 24.4) (Table 3). Strain F192-5 possessed the largest number of specific genes (68 genes). The number of genes missing specifically in F192-5 but conserved in the other nine strains was one, which was the smallest in this analysis (ranging from 1 to 39 in the tested strains). BLASTP analysis has shown that 7 of the 68 specific genes in strain F192-5 were conserved in certain strains of *Fructobacillus* spp. The seven genes encode (i) abortive infection gene product AbiN, (ii) DNA replication initiation protein, (iii) type II toxin-antitoxin system antitoxin, (iv) RelB/DinJ family, (v) zinc-binding alcohol dehydrogenase family protein, (vi) phage portal protein, and (vii) hypothetical protein. The zinc-binding alcohol dehydrogenase gene was sandwiched between genes encoding the MarR family transcriptional regulator and a hypothetical protein in the F192-5 genome. This gene organization is identical to that observed in *Fructobacillus fructosus* NRIC 1058^T (GCA_001047095.2). Similarities of the three proteins between strains F192-5 and *F. fructosus* NRIC 1058^T ranged from 66% to 75%.

The bifunctional alcohol/acetaldehyde dehydrogenase gene (*adhE*) consisting of 2,703 bp was conserved in the genomes of strain F192-5 and the other strains of *L. citreum*. The *adhE*/AdhE of F192-5 and NRIC 1776^T shared sequence similarities of 99.7% at the DNA level and 99.8% at the amino acid level. The *adhE* gene in all strains studied had no stop codon.

Characterization of *adhE* gene and relevant enzyme activities. As described above, the *adhE* gene was found in the genome of strain F192-5. The gene is sandwiched between genes encoding glycerophosphoryl diester phosphodiesterase and an uncharacterized protein (Fig. 2). This gene organization was identical with two of the nine reference strains (NRIC 1776^T and DmW_11). In seven of the reference strains, the gene encoding the uncharacterized protein was replaced with a gene encoding a membrane protein. In the genome of strain F192-5, only 62 bp separated the *adhE* gene and the gene encoding glycerophosphoryl diester phosphodiesterase (Fig. 2). This area in the genomes of the nine reference strains was much longer (229

TABLE 3 Strain-specific genes in *L. citreum* strains

Strain	No. of strain-specific genes		No. of genes shared with <i>Fructobacillus</i> spp. ^a
	Orthologous clustering	BLASTP analysis	
F192-5	123	68	7
NRIC 1776 ^T	16	5	1
1301_LGAS	70	23	0
NRRL B-1299	43	10	1
NRRL B-742	45	1	1
DmW_11	58	37	4
KM20	47	7	0
LBAE_C10	149	68	0
LBAE_C11	175	24	0
LBAE_E16	90	32	4

^aAs determined by BLASTP analysis.

FIG 2 Organization of *adhE* and its upstream and downstream genes in *L. citreum* NRIC 1776^T and F192-5. White, black, and slashed arrows indicate genes encoding glycerophosphoryl diester phosphodiesterase, *AdhE*, and uncharacterized protein, respectively. Single-elbow arrows indicate promoters predicted.

or 230 bp) (Fig. 2). Alignment of the sequences has shown a 167- to 168-bp gap in the genome of F192-5 (see Fig. S1). A putative promoter was found between the *adhE* and glycerophosphoryl diester phosphodiesterase genes of the reference strains but not for F192-5 (Fig. 2).

Reverse-transcriptase PCR revealed that *L. citreum* NRIC 1776^T expressed its *adhE* (*adhE-citreum*) gene, but F192-5 did not (Fig. 3). The expression of reference genes, i.e., *glk* and the 16S rRNA gene, was recovered in both strains. Strong ADH activities and relatively weak ALDH activities were recorded in NRIC 1776^T but not in strain F192-5 (Table 4).

Transformation of strain F192-5 and characterization of the derivatives. *Leuconostoc citreum* F192-5 was successfully transformed with pSJE-*adhEpro*, and a recombinant strain (strain 31-11) was obtained. Sequencing analysis of the inserted promoter region (*PslpA*) in pSJE-*adhEpro* of the recombinant strain revealed single nucleotide mutation compared with the original *PslpA* sequence in pIGM1. This mutation is located in the upstream –35 region of the dual tandem –35 and –10 regions (Fig. 4b; see also Fig. S2). The mutated region was not recognized as a promoter by promoter prediction. Sequences of *adhE-mesenteroides* in strain 31-11 were identical to those of *adhE-mesenteroides* in *Leuconostoc mesenteroides* (data not shown). Strain 2-1 was also obtained by transformation of *L. citreum* F192-5 with pSJE and used as a control strain.

Expression of *adhE-mesenteroides* was recorded from strain 31-11 but not from strains F192-5 and 2-1 (Fig. 5). All strains studied expressed reference genes *glk* and 16S rRNA. None of the strains expressed the *adhE-citreum* gene. Strong ADH activities and relatively weak ALDH activities were recorded in strain 31-11 but not in strains F192-5 and 2-1 (Table 4).

The *adhE-mesenteroides*-expressing strain 31-11 grew rapidly on D-glucose and reached stationary phase after 1 day (Fig. 6a), whereas strain 2-1 grew poorly on D-glucose. Strain 31-11 produced large colonies (1 to 2 mm in diameter) after 48 h on GYP agar under anaerobic conditions, but strain 2-1 produced pinpoint colonies (approximately 0.1 to 0.2 mm in diameter) (Fig. 6b). Strain 31-11 produced lactate and

FIG 3 Reverse transcriptase PCR (RT-PCR) detection of expression of *adhE-citreum*, *glk*, and 16S rRNA gene in *L. citreum* NRIC 1776^T and F192-5. Lanes: M, Hyper ladder 1 kb (Biolone, London, UK); 1, genomic DNA of NRIC 1776^T; 2, cDNA of NRIC 1776^T; 3, genomic DNA of F192-5; 4, cDNA of F192-5; N, negative control.

TABLE 4 Enzyme activities of ADH, ALDH, and NADH oxidase

Strain	Activity (mU/mg protein)		
	ADH	ALDH	NADH oxidase
NRIC 1776 ^T	214	4	60
F192-5	0	0	8,137
31-11	468	3	496
2-1	0	0	1,285

ethanol at almost equimolar ratios, but strain 2-1 mainly produced lactate and acetate with trace ethanol (Table 1).

DISCUSSION

LAB are known to adapt to their habitats. Adaptation on a genomic level usually occurs at a species level or a niche-associated group level within species (12–14). Previous studies have shown that niche-specific fructophilic evolutions are common at the genus level, e.g., the genus *Fructobacillus*, or at species level, e.g., *Lactobacillus kunkeei* and *Lactobacillus apinorum* (4–6).

Strain F192-5 grows poorly on D-glucose but well on D-fructose. Acetate is mainly produced with lactate from D-glucose metabolism, but not ethanol. Supplementation of pyruvate or aerobic culturing on D-glucose dramatically enhanced the growth of strain F192-5. These characteristics differentiated the strain from the reference strains of *L. citreum* but were consistent with the characteristics of obligate FLAB (2, 8, 15, 16). On the other hand, strain F192-5 metabolized 15 carbohydrates, separating it from obligate FLAB that usually metabolize two to four carbohydrates (2, 16). Obligate FLAB species are known to have poor gene repertoires for class of carbohydrate transport and metabolism (ranging from 61 to 79 genes) compared to their phylogenetic relatives (4, 6). The gene class of FLAB ranks 8th or 9th in the 21 Cluster of Orthologous Groups (COG) functional classes (3). Strain F192-5 has 171 genes in the class, and the class ranks 3rd in the 21 classes (see Table S1 in the supplemental material). FLAB have poor carbohydrate transport systems, and they especially lack a complete PTS (3). Strain F192-5, on the other hand, has six complete PTS transporters encoded by its genome. Specific reduction of genomic size and total CDSs, previously reported in FLAB (4, 6), was not seen in the genome of F192-5. These characteristics indicate that strain F192-5 is a phenotypically FLAB-like strain but is genetically distinct from other FLAB.

Orthologous clustering is frequently used for the identification of specific genes in specific bacterial species or strains (17, 18). We found 16 to 175 strain-specific genes in

FIG 4 Construction of a vector (a) and features of *PslpA* (b). The arrow in panel b indicates a mutation point in pSJE-adhEpro.

FIG 5 RT-PCR detection of expression of *adhE-mesenteroides*, *adhE-citreum*, *glk*, and 16S rRNA gene in *L. citreum* F192-5, 31-11, and 2-1. Lanes: M, Hyper ladder 1 kb; 1, genomic DNA of NRIC 1776^T; 2, cDNA of F192-5; 3, cDNA of 31-11; 4, cDNA of 2-1; N, negative control.

L. citreum strains, which accounted for 1% to 9% of total CDSs in each strain. The ratio of specific genes seemed to be too large within a single species. In fact, the numbers of strain-specific genes dramatically reduced after manual BLASTP analyses (Table 3). This discrepancy may be due to more stringent criteria of get-homologues, which depends on bidirectional BLAST hits as evidence for orthologues. Strain F192-5 has the highest number of strain-specific genes of all strains included in the present study. Seven of the strain-specific genes are conserved in *Fructobacillus* spp. *L. citreum* LBAE_C10, originating from sourdough, has the same number of specific genes as strain F192-5 (Table 3), but none of the genes were shared with *Fructobacillus* spp. Concluded

FIG 6 Growth characteristics of *L. citreum* 31-11 (○) and 2-1 (Δ) in GYP broth (a) and on GYP agar under anaerobic conditions (b). In panel a, lines and error bars indicate averages and standard deviations from triplicates, respectively.

from these findings, the same set of genes was stored for survival in fructose-rich environments in *Fructobacillus* spp. and strain F192-5.

The gene encoding bifunctional ADH/ALDH (*adhE*) plays a key role in the phosphoketolase pathway of heterofermentative LAB. This gene is either absent in obligate FLAB or is partially present (4–6, 19). Transformation of the *adhE* gene into *F. fructosus* NRIC 1058^T resulted in a remarkable change of metabolic properties, i.e., there was no requirement for the external electron acceptors during the metabolism of D-glucose (7). Furthermore, ethanol instead of acetate was produced from D-glucose (7). The key gene, i.e., *adhE*, was conserved in all *L. citreum* strains tested, including strain F192-5. However, strain F192-5 did not grow on D-glucose without electron acceptors. A detailed study of the genome sequence revealed a 167- to 168-bp gap upstream of the *adhE* gene on the genome of F192-5 compared with reference strains of *L. citreum*. A single promoter was predicted upstream of the *adhE* gene of the reference strains but not in the region of F192-5. This would explain why strain F192-5 did not express its *adhE*. Introduction of the *adhE-mesenteroides* gene led to significant phenotypic changes, and strain 31-11, transformed with the *adhE* gene, did not require the electron acceptors for D-glucose metabolism. These results clearly indicated that deletion of the promoter region of *adhE* is the reason for the fructophile-like metabolic characteristics of strain F192-5.

Toxicity of AdhE was reported in *Escherichia coli* (20, 21). In the former study, the terminator region of pSJE-*adhE*pro² was not practically removed in *F. fructosus* for transformation of *adhE*, and a leaky promoter activity assisted the cells in producing acceptable levels of AdhE (7). Interestingly, the recombinant strain 31-11 contained a single nucleotide mutation in one of the dual tandem –35 and –10 regions of the inserted promoter sequences (*PslpA*), and the mutated region was not predicted as a promoter. This would attenuate promoter activity. This attenuation would repress excess production of AdhE, resulting in a successful production of the recombinant.

Conclusion. A novel unique *L. citreum* strain, F192-5, was isolated from the peel of a satsuma mandarin. The strain shows fructophile-like growth characteristics. Unlike other FLAB, strain F192-5 possesses phenotypically and genetically rich carbohydrate metabolic systems, and the genome of strain F192-5 remained large; the size is comparable to the genomes of nonfructophilic *L. citreum* strains. Furthermore, unlike obligate FLAB, the *adhE* gene of strain F192-5 is intact. However, a large gap upstream of the *adhE* gene with no promoter was observed in the genome of strain F192-5, explaining the inability to ferment D-glucose. These results do not classify the strain as obligate FLAB, and the strain could be described as pseudo-FLAB. D-Glucose metabolism is a key for energy production in most organisms, including LAB, and a strain-specific deficiency of glucose metabolism is unique in LAB. This strain-specific unique phenotype might be one of the strategies to survive in diverse environments of LAB. All heterofermentative LAB have a possibility to have a pseudofructophilic phenotype by inactivating the *adhE* during adaptation to fructose-rich niches. This adaptation seems to be strain specific.

MATERIALS AND METHODS

Isolation. A satsuma mandarin was purchased at a supermarket in Stellenbosch, South Africa. Fruit peels were collected in sterilized plastic bags and crushed. Five milliliters of FYP broth was added to the crushed samples and incubated at 30°C for 24 h. FYP broth was composed of (per liter) 10 g D-fructose, 10 g yeast extract, 5 g polypeptone, 2 g sodium acetate, 0.5 g Tween 80, 0.2 g MgSO₄·7H₂O, 0.01 g MnSO₄·4H₂O, 0.01 g FeSO₄·7H₂O, 0.01 g NaCl, 0.05 g cycloheximide, and 0.05 g sodium azide (pH 6.8). After incubation, a loop full of sample was inoculated in 30% FYP broth and incubated aerobically on an orbital shaker (120 rpm) at 30°C until growth was visible. The 30% FYP broth was FYP broth supplemented with 300 g liter^{−1} D-fructose and was used for selective isolation of FLAB (2). The culture was streaked onto FYP agar containing (per liter) 5 g CaCO₃ and 12 g agar. Plates were incubated at 30°C under aerobic conditions. After 48 h of incubation, ten colonies were selected from the plates based on the sizes of the colony and clear zone surrounding the colonies and transferred to GYP and FYP broth, respectively. Incubation was conducted for 24 h at 30°C without aeration. GYP broth differed from FYP broth by containing 10 g liter^{−1} of D-glucose instead of D-fructose. The isolate was stocked in MRS broth containing 20% (vol/vol) glycerol at −80°C.

TABLE 5 Bacterial strains and plasmids used in the present study

Bacterial strain or plasmid	Description	Source or reference
Strains		
<i>Escherichia coli</i> DH5 α -pSJE- <i>adhEpro</i> ²	Donor of pSJE- <i>adhEpro</i> ²	7
<i>Leuconostoc citreum</i> NRIC 1776 ^T	Reference strain	NRIC ^a
<i>Leuconostoc citreum</i> NRIC 1772	Reference strain	NRIC
<i>Leuconostoc citreum</i> NRIC 1579	Reference strain	NRIC
<i>Leuconostoc citreum</i> NRIC 1581	Reference strain	NRIC
<i>Leuconostoc citreum</i> F192-5	Wild type (fructophile-like isolate)	This study
<i>Leuconostoc citreum</i> 31-11	Derivative of F192-5 possessing <i>adhE-mesenteroides</i>	This study
<i>Leuconostoc citreum</i> 2-1	Derivative of F192-5 containing pSJE	This study
Plasmids		
pSJE	Em ^{trb} ; <i>E. coli</i> - <i>Leuconostoc</i> shuttle vector	29
pSJE- <i>adhEpro</i> ²	pSJE harboring <i>adhE-mesenteroides</i> and promoter region of pLGM ^t (removed pSJE-originated NotI site)	7
pSJE- <i>adhEpro</i>	pSJE- <i>adhEpro</i> ² derivative with terminator removed	This study

^aNODAI Research Institute Culture Collection, Tokyo, Japan.^bEm^r, erythromycin resistant.

Reference strains. *Leuconostoc citreum* NRIC 1776^T, NRIC 1772, NRIC 1579, and NRIC 1581 were obtained from NODAI Culture Collection Center, Tokyo University of Agriculture, and used as reference strains for biochemical characterization (Table 5). The strains were cultured in MRS broth at 30°C for 24 h.

Identification and biochemical characterization. Identification of the isolate was performed by phylogenetic analysis based on the 16S rRNA gene sequence. Almost full-length 16S rRNA gene sequences (1,542 bp) were determined by a method described previously (22). BLAST analysis was performed for species identification.

Carbohydrate fermentation reactions were recorded using API CHL galleries (bioMérieux, Marcy-l'Etoile, France), according to the manufacturer's instructions. Readings were taken for 7 days at 30°C. Growth characteristics on D-glucose and D-fructose and the requirement of external electron acceptors for D-glucose dissimilation were determined in GYP broth, FYP broth, and GYP broth supplemented with 10 g liter⁻¹ pyruvate (GYP-P). Oxygen utilization as an electron acceptor was determined in GYP broth under aerobic conditions on a shaker (170 rpm). Incubation was at 30°C for 5 days. Growth was monitored at 660 nm with a spectrophotometer (model U-2800A; Hitachi, Tokyo, Japan). Anaerobic growth on D-glucose was determined by streaking cultures onto GYP agar incubated in an anaerobic workstation (10% H₂, 10% CO₂, and 80% N₂, Bugbox chamber; Baker Ruskinn, Sanford, ME, USA). Production of lactate, acetate, and ethanol from D-glucose metabolism was determined after 2 days of incubation at 30°C in GYP broth using the F-kit (Roche Diagnostics, Mannheim, Germany). All experiments were performed in triplicates, unless indicated otherwise.

Draft genome sequencing and de novo assembly. Whole-genome sequencing was conducted by Illumina MiSeq, with insert lengths of approximately 500 bp. A total of 5,502,480 and 6,743,618 reads with average lengths of 294 and 275 bp were obtained from *L. citreum* F192-5 and NRIC 1776^T, respectively. *De novo* assembly, using the Velvet Assembler for short reads with parameters optimized by the VelvetOptimiser (version 1.2.10), resulted in 42 contigs (length, 2,061,777 bp; *N*₅₀, 161,012 bp) and 8 contigs (length, 1,806,779; *N*₅₀, 1,253,651 bp) for strains F192-5 and NRIC 1776^T, respectively. Sequences shorter than 300 bp were eliminated. The genome was annotated using the DDBJ Fast Annotation and Submission Tool (DFAST; <https://dfast.nig.ac.jp>) (23), which is based on the annotation pipeline Prokka (24) and the curated reference database tailored for lactic acid bacteria. The sequence coverages of *L. citreum* F192-5 and NRIC 1776^T were 785- and 1,028-fold, respectively.

Genomic data of other *L. citreum* strains and genome analysis. The draft genome and complete genome sequences of eight strains of *L. citreum*, which were all genomic data of *L. citreum* strains available in the GenBank or RefSeq database at the time of analysis (May 2017), were obtained and were included in the comparative genomic study (Table 2). The completeness and contamination of the genomic data were assessed by CheckM (version 1.0.4) (25), by which the existence of gene markers specific to *Leuconostoc* spp. was inspected. Genomic data of *L. citreum* 1300_LCIT (NZ_JVUV00000000.1), which were available at the GenBank database, were excluded in the analysis, since the average nucleotide identity (ANI) analysis clearly separated this strain from *L. citreum* and classified it as *Leuconostoc lactis*.

To estimate the size of conserved genes in *L. citreum*, all protein sequences were grouped into orthologous clusters by GET_HOMOLOGUES software (version 1.3) based on the all-against-all bidirectional BLAST alignment and the MCL graph-based algorithm. Conserved genes were defined as gene clusters present in all analyzed genomes (26). This analysis was also used to identify strain-specific genes (nonorthologs). For functional comparison of the gene contents among *L. citreum* strains, coding sequences (CDSs) predicted in each strain were assigned to Cluster of Orthologous Groups (COG) functional classification using the COGNITOR software (27). Prediction of promoter sequences was conducted by using Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/promoter.html) (28).

TABLE 6 Primers used in the present study

Primer	Sequence (5'→3')	Purpose
pSJE- <i>adhE</i> -BamHI-F2	AAAGGATCCGTGCACTAATCTAAATATGTG	Amplification of <i>adhE-mesenteroides</i> without promoter
pSJE- <i>XbaI</i> - <i>adhE</i> SD-R	AAATCTAGATTAATACTGATCTTTCAACAATTGACG	
<i>adhE</i> -R100	ATCAATACCTCAGTCATCTTC	Sequencing of <i>PslpA</i>
<i>adhE</i> -F350	CAAGGTAAGGTAGATTAGC	Sequencing of <i>adhE-mesenteroides</i>
<i>adhE</i> -R500	ATAAACTGATTCTGAGGCGA	
<i>adhE</i> -F1100	TGCATACCTTGTTCCTAAGA	Sequencing of <i>PslpA</i>
<i>adhE</i> -R1250	TAATTCGAACAAAAGTACAT	
<i>adhE</i> -F1700	CTTACTTACAAGACTTGCTT	
<i>adhE</i> -R1800	AATCGCCTCGTCATCTTCTA	
<i>adhE</i> -F2000	GTTGCTATTGTTGATCCAGA	
<i>adhE</i> -F2440	GCACGCACAAAGATGCACTA	
pLPD4-1t	ATTACAAAGGGTTTAAGCAG	
pLPD4-2t	GAACAACCGGGCCTCGAC	
F192-5- <i>adhE</i> -EXP-F	TCACAGAACGCCAAGAAGCA	
F192-5- <i>adhE</i> -EXP-R	CATGATCCGGTACCAAGCGT	
Lcit_ <i>glk</i> _F	TTAGACAACGATGCGAATGC	Amplification of <i>glk</i>
Lcit_ <i>glk</i> _R	TCTTGTGCCAAGCGTACAAC	
8F	AGAGTTTGATCATGGCTCAG	Amplification of 16S rRNA gene
15R	AAGGAGGTGATCCAACCGCA	

DNA manipulation, recombination, and transformation. *Escherichia coli* strain DH5 α possessing the pSJE-*adhEpro*² plasmid was cultured at 37°C in brain heart infusion broth (Oxoid, Thermo Fisher Scientific, Tokyo, Japan), supplemented with 200 μ g/ml erythromycin. The pSJE-*adhEpro*² (Table 5) was produced from the promoterless *E. coli*-*Leuconostoc* shuttle vector pSJE (29) by insertion of *adhE* of *Leuconostoc mesenteroides* (here, *adhE-mesenteroides*) and the modified *slpA* promoter of *Lactobacillus brevis* (Fig. 4) used in a previous study (7). The modified promoter contains dual tandem –35 and –10 regions. The pSJE-*adhEpro*² plasmid was digested with NotI and ligated to itself to restore the promoter activity by removing the terminator (pSJE-*adhEpro*). *L. citreum* F192-5 was transformed with the plasmid. A Qiagen Plasmid MAXI kit (Qiagen, Hilden, Germany), restriction endonucleases (TaKaRa-bio), and a Ligation-Convenience kit (Nippon Gene, Tokyo, Japan) were used as specified by the manufacturers.

Preparation of competent cells and electrotransformation of *L. citreum* F192-5 were conducted based on the method used for *Fructobacillus fructosus* (7), with slight modifications. Concentrations of sucrose, glycine, and penicillin G in culture broth were changed to 100 mM, 1% (wt/vol), and 0.375 μ g/ml, respectively. After electrotransformation, cells were immediately resuspended in 1 ml freshly prepared MRS broth containing 0.5 M sucrose, 1% (wt/vol) sodium pyruvate, and 0.1 M magnesium chloride and incubated statically for 1 h at 30°C. The transformed cells were plated onto MRS agar supplemented with 2 μ g/ml erythromycin and incubated aerobically at 30°C until colonies developed.

Gene expression of *adhE* and enzyme assays. Gene expression of autochthonous *adhE* (*adhE-citreum*) and/or *adhE-mesenteroides* was confirmed from 20-h-old cultured cells of *Leuconostoc citreum* NRIC 1776^T, F192-5, and derivatives (strains 31-11 and 2-1) by reverse transcription of total RNA combined with a PCR with gene-specific primers, as described previously (6). Genes encoding 16S rRNA and glucokinase (*glk*) were used as references. Primers used are listed in Table 6.

ADH, ALDH, and NADH oxidase activities were determined in cells of *L. citreum* NRIC 1776^T, F192-5, and the derivatives (31-11 and 2-1). Culturing of the cells, preparation of cell extracts, and enzyme assays were performed as described previously (7).

Accession number(s). Newly determined sequence data have been deposited in the GenBank database under accession numbers [BJJW01000000.1](https://doi.org/10.1128/AEM.01077-19) and [BJJX01000000.1](https://doi.org/10.1128/AEM.01077-19).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01077-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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