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# *Lactococcus lactis* Metabolism and Gene Expression during Growth on Plant Tissues

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**Lactic acid bacteria have been isolated from living, harvested, and fermented plant materials; however, the adaptations these bacteria possess for growth on plant tissues are largely unknown. In this study, we investigated plant habitat-specific traits of *Lactococcus lactis* during growth in an *Arabidopsis thaliana* leaf tissue lysate (ATL). *L. lactis* KF147, a strain originally isolated from plants, exhibited a higher growth rate and reached 7.9-fold-greater cell densities during growth in ATL than the dairy-associated strain *L. lactis* IL1403. Transcriptome profiling (RNA-seq) of KF147 identified 853 induced and 264 repressed genes during growth in ATL compared to that in GM17 laboratory culture medium. Genes induced in ATL included those involved in the arginine deiminase pathway and a total of 140 carbohydrate transport and metabolism genes, many of which are involved in xylose, arabinose, cellobiose, and hemicellulose metabolism. The induction of those genes corresponded with *L. lactis* KF147 nutrient consumption and production of metabolic end products in ATL as measured by gas chromatography-time of flight mass spectrometry (GC-TOF/MS) untargeted metabolomic profiling. To assess the importance of specific plant-inducible genes for *L. lactis* growth in ATL, xylose metabolism was targeted for gene knockout mutagenesis. Wild-type *L. lactis* strain KF147 but not an *xylA* deletion mutant was able to grow using xylose as the sole carbon source. However, both strains grew to similarly high levels in ATL, indicating redundancy in *L. lactis* carbohydrate metabolism on plant tissues. These findings show that certain strains of *L. lactis* are well adapted for growth on plants and possess specific traits relevant for plant-based food, fuel, and feed fermentations.**

Lactic acid bacteria (LAB) found on plants are essential for the production of a wide variety of plant-derived fermented foods with desirable organoleptic properties, improved nutritional attributes, and extended shelf life (1). LAB are a diverse group of bacterial species in the *Firmicutes* phylum that are characterized by the production of lactic acid as the main end product of carbohydrate metabolism. Some of the most commonly recognized genera among the LAB include *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Oenococcus*. LAB-fermented plant-based food products include sauerkraut, sourdough, and olives, as well as diverse foods unique to different ethnic groups (e.g., pulque, nukadoko, or gundruk [2, 3]). LAB are responsible for the conversion of plant tissues such as alfalfa into silage for animal feed with enhanced nutritional content and stability (4). LAB are also used to ferment plant-based substrates into industrial-grade lactic acid for the manufacture of polylactide bioplastics (5). Conversely, LAB are frequent contaminants of bioethanol fermentations (6).

In contrast to commercial dairy fermentations that utilize starter cultures, initiation of plant-based fermentations typically relies on the LAB that are located on or associated with plant tissues. LAB are frequent residents of the phyllosphere (the aboveground surfaces of a plant) and can be detected by both culture-dependent (3) and -independent (7–9) methods. Although LAB are more commonly found in relatively low cell numbers ($10^2$ to $10^4$ CFU/g) (3), significant seasonal differences exist, and LAB were among the most abundant bacteria found on Romaine lettuce grown in an agricultural field in the early summer over multiple years and sampling dates (7).

Plant-associated LAB grow rapidly and reach high cell densities following the harvest of plant leaves and fruits and exposure to humid conditions with reduced oxygen concentrations (3). However, because of variation in LAB numbers and species on living plants at the time of harvest, plant fermentations are sometimes prone to quality defects and product losses due to spoilage (10, 11). A better understanding of LAB adaptations for growth on plant materials should result in improved control over plant-based fermentations as well as guide the selection of LAB strains with desired functional traits for use as starter cultures.

Although the LAB species *Lactococcus lactis* is best studied for its role in dairy fermentations (12), this species is often associated with plants. Plants were originally regarded as the natural habitat of *L. lactis* (previously termed *Streptococcus lactis*) (13). Comparative genomics studies indicate that the *L. lactis* species originated in association with plants, and through anthropogenic forces, members of this species adapted to the dairy niche (14, 15). Compared to their dairy-isolated counterparts, *L. lactis* isolates collected from plants have a greater genetic potential as reflected by traits such as the capacity to consume a broader array of carbohydrates, especially those derived from plants, increased biosynthetic capacity for nonribosomal peptides and polyketides, and fewer amino acid auxotrophies (15, 16). The transition to the

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dairy environment is not unique to *L. lactis*, and other LAB species have gone through analogous domestication processes, including *Lactobacillus bulgaricus* (17) and *Lactobacillus casei* (18). Because *L. lactis* is a well-characterized LAB species, it serves as an attractive model to elucidate the contributions of specific genetic traits toward fitness in plant-based fermentations.

Using a combination of transcriptomics, metabolomics, and targeted deletion mutagenesis, this study aimed to identify genetic adaptations that contribute to *L. lactis* fitness during growth in plant fermentations. Two *L. lactis* subsp. *lactis* strains, KF147 and IL1403, from different origins were compared for their capacity to grow in a lysate, ATL, derived from leaves of *Arabidopsis thaliana*. *L. lactis* KF147 was originally isolated from mung bean sprouts (19) and is the first plant-associated strain of this species for which the complete genome sequence has been published (20). *L. lactis* IL1403, the prototypical dairy isolate and first of its species to undergo complete genome sequencing, is well suited for growth in milk (21). *L. lactis* KF147 was subjected to transcriptome sequencing (RNA-seq) and untargeted gas chromatography-time of flight mass spectrometry (GC-TOF/MS) metabolome profiling during growth in the leaf lysate. Based on gene expression levels and metabolic profiles, we targeted *L. lactis* xylose metabolism to understand how individual genetic adaptations influence plant-based fermentations.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *Lactococcus lactis* subsp. *lactis* strains KF147 (19) and IL1403 (22) were provided by NIZO food research (Ede, The Netherlands) and were maintained as frozen glycerol stocks at −80°C. *L. lactis* was routinely grown at 30°C without agitation on M17 broth (BD, Franklin Lakes, NJ) or 1.5% (wt/vol) agar plates with either 0.5% (wt/vol) D-glucose (GM17) or 0.5% (wt/vol) D-xylose (XM17) (MP Biomedicals, Santa Ana, CA) as the sole carbon source. *Escherichia coli* DH5α was grown at 37°C on LB (Fisher Scientific, Waltham, MA). When necessary, the growth medium was supplemented with erythromycin at 150 μg/ml for *E. coli* and 5 μg/ml for *L. lactis*. Bacterial growth rates in M17 were measured using overnight cultures of *L. lactis* cells that were collected by centrifugation at 10,000 × *g* for 3 min, washed twice in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$ [pH 7]), and then inoculated into microtiter plates at a concentration of $10^5$ CFU/ml in 200 μl M17 broth. The optical density at 600 nm was measured using a Synergy 2 microplate reader (BioTek, Winooski, VT). Bacterial growth rates in *A. thaliana* lysate (ATL) were measured by dilution plating on GM17 agar. Bacterial cells were washed and inoculated in ATL in the same manner as for M17 cultures.

**ATL preparation.** *A. thaliana* Col-0 plants were maintained at the University of California—Davis Controlled Environment Facility (UC Davis, Davis, CA) in a GR48 walk-in growth chamber (Conviron, Pembina, ND) with T8 fluorescent lighting. Prior to planting, seeds were incubated in 0.1% (wt/vol) agarose for 48 h at 4°C in the dark to synchronize germination time. Seeds were sewn in moist Sunshine Mix 1 (Sun Gro Horticulture, Agawam, MA) and bottom watered as necessary. Plants were kept under a 16-h photoperiod and a constant temperature of 20°C and rH of 60%. After 4 weeks of growth, leaves were picked for lysate preparation. Leaves were first macerated in 1 mM sodium phosphate buffer (0.39 mM $NaH_2PO_4$, 0.61 mM $Na_2HPO_4$ [pH 7]) at a ratio of 1:4 (g leaves/ml buffer) in a Whirl-Pak filter bag (Nasco, Modesto, CA) using a Homex-6 homogenizer (Bioreba AG, Reinach, Switzerland). The resulting lysate was then centrifuged at 10,000 × *g* for 10 min to remove insoluble debris. Finally, the lysate was filter sterilized and stored at 4°C in the dark.

**RNA sequencing sample collection and RNA extraction.** *L. lactis* KF147 was washed and inoculated in the same manner as for growth experiments in triplicate into 5 ml of either GM17 or ATL at a cell density of $10^5$ CFU/ml. After 8 h, the cells were collected by centrifugation at 10,000 × *g* for 2 min at 4°C, snap-frozen in liquid nitrogen, and then stored at −80°C. For RNA extraction, cell pellets were thawed in 500 μl of cold acidic phenol-chloroform-isoamyl alcohol (125:24:1) (Ambion, Carlsbad, CA) and then transferred to a 2-ml screw-cap tube containing 500 μl TE (10 mM Tris-HCl, 1 mM EDTA), 30 μl 3 M sodium acetate (pH 5.2), 30 μl 10% SDS, and 300 mg 0.1-mm-diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK). Cells were mechanically lysed in a Fastprep-24 bead beater (MP Biomedicals, Santa Ana, CA) by homogenization twice at 6 m/s for 40 s with 1 min on ice between runs. The cell lysate was then centrifuged at 10,000 × *g* for 10 min, and the upper RNA-containing aqueous phase was washed twice in two volumes of chloroform-isoamyl alcohol (24:1) and then concentrated by ethanol precipitation. The RNA was suspended in 50 μl nuclease-free water and quantified spectrophotometrically using a NanoDrop 2000c (Thermo Scientific, Waltham, MA).

DNase treatment was performed on 5 μg total RNA using the DNA-free kit (Ambion, Carlsbad, CA). RNA quality was assessed using a Bioanalyzer RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA), and all samples were found to have 23S/16S rRNA ratios of 1.9 to 2.0 (data not shown). A lack of contaminating DNA was confirmed by quantitative-real-time-PCR using primers K and L for *rpoA* (see Table S1 in the supplemental material).

**RNA-seq library construction and transcriptome analysis.** rRNA was first depleted from the total RNA using a Ribo-Zero rRNA removal kit for Gram-positive bacteria (Epicentre, Madison, WI) according to the manufacturer's instructions. mRNA was then purified using RNA Clean & Concentrator 5 spin columns (Zymo Research, Irvine, CA) and eluted with 10 μl nuclease-free water. Purified mRNA was examined using the Bioanalyzer RNA 6000 Pico kit to confirm the absence of any remaining detectable quantity of rRNA. Strand-specific library construction was performed using a ScriptSeq v2 RNA-Seq library preparation kit (Epicentre, Madison, WI) according to the manufacturer's instructions (http://www.epibio.com). Terminal-tagged cDNA was purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, Indianapolis, IN). Unique Illumina-compatible barcode primers (Epicentre, Madison, WI) were added to each library prior to PCR amplification of the cDNA. Amplified barcoded cDNA libraries were purified and size selected for 250-bp inserts using Agencourt AMPure XP magnetic beads. The library insert size distribution was confirmed using a Bioanalyzer high-sensitivity DNA kit. Libraries were pooled in equimolar amounts onto a single lane of a HiSeq 2000 (Illumina, San Diego, CA), and 50-bp single-read sequencing was performed. Sequencing reactions were carried out at the UC Davis DNA Technologies Core (http://dnatech.genomecenter.ucdavis.edu/).

Reads were aligned to the *L. lactis* KF147 chromosome and plasmid (20) using Bowtie2 (23) in the "[–sensitive]" mode. Differential gene expression in ATL was determined by calculating the number of fragments per kilobase per million reads (FPKM) for each gene using Cuffdiff (24). Reads that aligned to either rRNA or tRNA sequences were removed prior to performing FPKM calculations. Genes were tested for differential expression by comparing FPKM values from ATL cultures to those from the GM17 reference cultures using Cuffdiff (24). Differential expression was considered significant if the false discovery rate (FDR)-adjusted *P* value was less than 0.05 and the fold change in gene expression was greater than 2-fold compared with the reference condition. Genes were grouped into Clusters of Orthologous Groups (COGs) categories for analysis (25).

**GC-TOF/MS metabolomic analysis.** Metabolomic analysis was performed on uninoculated ATL and on cell-free supernatants of *L. lactis* KF147 during growth in ATL after 8 and 24 h ($n = 3$ for each sample type). Samples were prepared by centrifugation of 5 ml of culture at 10,000 × *g* for 3 min at room temperature. The resulting supernatant was then heated (80°C for 15 min), filtered (0.22 μm), and subsequently stored at −20°C until gas chromatography-time of flight mass spectrometry (GC-TOF/MS) analysis at the West Coast Metabolomics Center (UC Davis, Davis,

CA). One hundred-microliter-sample aliquots were dried in a vacuum concentrator and subjected to trimethylsilyl (TMS) derivatization for GC-TOF/MS analysis using previously established methods (26) followed by raw data processing for compound annotation using BinBase (27). Raw chromatogram peak heights for each metabolite within each sample were normalized to the sum of the chromatogram peak heights for fatty acid methyl ester (FAME) internal standards for the respective sample. Individual normalized peak heights were further scaled by the average of the sum of FAME peak heights for all samples to assist in data visualization. Statistically significant differences were determined by one-way analysis of variance followed by pairwise comparisons using Tukey's honest significant difference test ($P < 0.05$). A complete list of detectable metabolites using this methodology can be found at http://metabolomics.ucdavis.edu/core-services/metabolites.

**Construction of an *L. lactis* KF147 Δ*xylA* deletion mutant.** Standard molecular biology techniques were performed by previously described methods (28). A markerless deletion of *L. lactis* KF147 *xylA* was performed using homologous recombination according to the selection/counterselection system described by Solem et al. (29). All primers used for mutant construction and validation are described in Table S1 in the supplemental material. The upstream flanking region of *xylA* was PCR amplified using primers A and B and the downstream flanking region of *xylA* was PCR amplified using primers C and D. Primers B and C contain 5′ reverse complementary linker sequences (30) to be used during splicing by overlap extension (SOEing) PCR. The flanking upstream and downstream PCR products were pooled and used as a template for SOEing PCR using primers A and D to link the two original products. The linked PCR product was double digested with XbaI and XhoI and subsequently cloned into pCS1966 (29) to yield pXYLA-KO. The plasmid pXYLA-KO was transformed into *E. coli* DH5α.

*L. lactis* KF147 electrocompetent cells were prepared by growing cells in the presence of 2.0% glycine (wt/vol) as previously described (31). Cells were transformed with pXYLA-KO (250 ng) in 2-mm electroporation cuvettes using a GenePulser Xcell (Bio-Rad, Hercules, CA) according the parameters of Holo and Nes (31). The integration location of the plasmid was checked using primers A and J and primers D and I. A single band was found using the primer combination A and J, indicating that the plasmid had integrated at the downstream flanking region of *xylA* (data not shown). To screen for cells that had lost the plasmid via a second homologous recombination event, bacteria were plated on yeast synthetic complete (SC) medium supplemented with 5-fluoroorotate (10 μg/ml). SC medium was prepared at a 2× concentration with yeast nitrogen base (catalog no. 239210; BD, Franklin Lakes, NJ), yeast synthetic dropout mixture without uracil (catalog no. Y1501; Sigma-Aldrich, St. Louis, MO), and 2% (wt/vol) glucose. We observed that the 5-fluoroorotate counterselection agent was not lethal to the single-crossover *L. lactis* KF147 integrant (on the SC medium) and thus instead plated the cells on GM17 followed by replica plating onto GM17 supplemented with erythromycin to screen for colonies that had lost the plasmid. Erythromycin-sensitive colonies were screened via PCR using primers E and F and primers I and J to identify colonies in which *xylA* had been deleted.

**RNA-seq data accession number.** All RNA-seq data are freely available in the NCBI Sequence Read Archive (SRA) and can be accessed under BioProject accession no. PRJNA235954.

## RESULTS

**Growth characteristics of *L. lactis* in ATL.** *L. lactis* KF147 originating from plants exhibited a significantly higher growth rate (1.06 $h^{-1}$) than the dairy-associated strain *L. lactis* IL1403 (0.63 $h^{-1}$) ($P < 0.05$, Student's *t* test) (Fig. 1). Strain KF147 also reached significantly higher cell densities ($3.3 \times 10^8$ CFU/ml) than IL1403 ($4.9 \times 10^7$ CFU/ml), equivalent to 7.9-fold more CFU/ml (Fig. 1). In comparison, both strains exhibited similar growth rates and reached nearly identical cell densities when grown in GM17, a standard laboratory culture medium for the species *Lactococcus* (see Fig. S1 in the supplemental material).

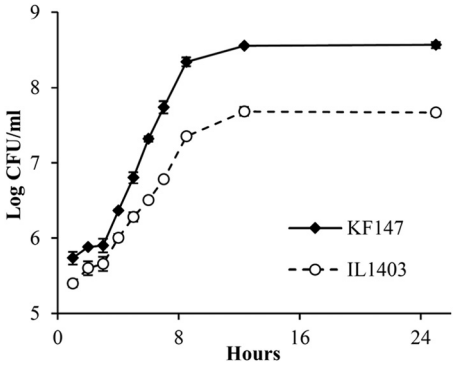


FIG 1 Growth of *L. lactis* in ATL. *L. lactis* KF147 and *L. lactis* IL1403 were inoculated into ATL, and growth was measured over 25 h. Growth curves were analyzed in triplicate, and data points represent the average ± standard error (SE).

**Transcriptome analysis of *L. lactis* KF147 in ATL.** To identify *L. lactis* genes specifically expressed during growth on plant leaf tissues, whole-genome transcriptome profiling using RNA-seq was performed on rRNA-depleted total RNA extracted from triplicate cultures of exponential-phase *L. lactis* KF147 cells grown in either ATL or GM17 (as a reference condition). An average of 31,190,418 DNA reads per culture was collected, and among those reads, 91.5% and 97.7% mapped to the *L. lactis* KF147 genome for the ATL- and GM17-grown cells, respectively. rRNA and tRNA constituted 1.2% and 20.3% of the nucleotide sequences from *L. lactis* KF147 grown in ATL and 0.7% and 10% of those from this strain grown in GM17. Excluding rRNA and tRNA sequences, the rates of genome coverage were, on average, 428- and 533-fold for KF147 cells in ATL and GM17, respectively.

Expression levels of a total of 853 *L. lactis* KF147 genes (33.1% of the genome) were significantly higher, and 264 genes (10.3% of the genome) were significantly lower in ATL compared with GM17 (see Table S2 in the supplemental material). These genes were distributed among 19 COGs and numerous cellular pathways (Fig. 2). COGs with high numbers of upregulated genes included the carbohydrate transport and metabolism COG (140 genes total, 71.1% within the COG), as well as COGs for secondary metabolite biosynthesis, transport, and catabolism (10 genes, 52.6% within the COG), defense mechanisms (20 genes, 46.5% within the COG), and those lacking functional assignments (229 genes, 39.3% within the COG). A total of 21 out of 38 annotated genes carried on the plasmid pKF147A harbored by this strain were induced, while none were repressed in ATL. Downregulated chromosomal genes were overrepresented in the translation, ribosomal structure, and biogenesis COG (73 genes, 49.7% of genes within that COG). For example, large subunit ribosomal proteins and small subunit ribosomal proteins were repressed on average 7.6- and 6.2-fold, respectively. Translation initiation factor genes (*infA*, *infB*, and *infC*) and elongation factor genes (*fusA*, *tsf*, *tuf*, and *efp*) were also repressed. Genes with the highest downregulation in expression in ATL were in the nucleotide transport and metabolism COG. *L. lactis* genes coding for carbamoyl-phosphate synthetase, catalyzing the first step in pyrimidine and arginine synthesis, were highly (27-fold) downregulated in ATL (see Table S2).

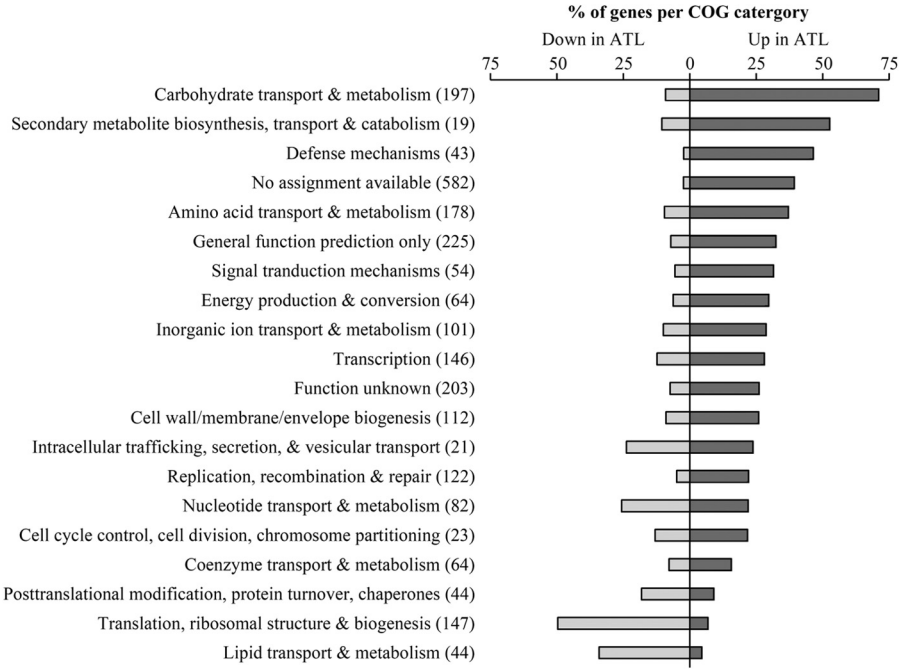


**FIG 2** Percentage of KF147 genes in COG categories that were differentially expressed in ATL compared to GM17. The number of genes in each COG category is indicated in parentheses. Genes were considered to be differentially expressed if there was at least a 2-fold change in expression in ATL compared to GM17 and an FDR-adjusted *P* value of $<0.05$.

**Expression of carbohydrate metabolism genes by *L. lactis* KF147 in ATL.** *L. lactis* KF147 genes required for the consumption of diverse plant-associated carbohydrates were induced in ATL (Fig. 2; see also Table S2 in the supplemental material). Genes for sucrose metabolism were significantly induced in ATL compared to GM17, including the gene coding for a sucrose phosphorylase (*sucP*) and an ATP-binding cassette transporter locus (llkf_2259 to -2262). Genes coding for the uptake and degradation of fructose and mannitol (a reduced form of fructose) were also induced and encompassed a mannitol-1-phosphate dehydrogenase gene (*mtlD*), an α-mannosidase/fructosidase gene (*amn*), mannitol-specific phosphotransferase system (PTS) genes (*mtlA* and *mtlF*), and mannitol/fructose PTS genes (*fptABC*) (see Table S2). The *malEFG* ABC transporter for maltose uptake was also induced (upregulated 8.6-fold) in ATL compared with GM17, as well as associated genes responsible for the degradation of starch, such as an α-amylase gene (*amyY*) and neopullulanase gene (*dexC*). Finally, glycerol utilization genes encoding a glycerol uptake facilitator protein (*glpF*), glycerol kinase (*glpK*), and α-glycerophosphate oxidase (*glpD*) were highly upregulated (39.6-fold on average) (see Table S2).

Genes for metabolism of the pentoses xylose, arabinose, and ribose were induced during *L. lactis* KF147 growth in ATL. The xylose degradation pathway included several of the most highly induced and coordinately expressed genes by *L. lactis* KF147 in ATL (Fig. 3A; see also Table S2 in the supplemental material). For example, the gene for xylose isomerase (*xylA*), responsible for the conversion of xylose to xylulose, was the second most highly induced gene within the genome (90-fold induced). Also upregulated were *xylR*, encoding a putative positive transcriptional regulator (induced 72-fold), and *xylB*, encoding xylulose kinase, responsible for the phosphorylation of xylulose to xylulose-5-P (induced 19-fold) (Fig. 3A; see also Table S2 in the supplemental material). The expression of other genes likely important for xylose/xylan consumption was also highly upregulated and encompassed genes coding for a β-xyloside transporter (*xynT* [18-fold]), a β-1,4-xylosidase (*xynB* [36-fold]), a xylose/proton symporter (*xylT* [9-fold]), and an epimerase (*xylM* [24-fold]) (Fig. 3A; see also Table S2). Genes for arabinose degradation were induced on average 7.6-fold during KF147 growth in ATL compared to GM17 (see Table S2). Notably, the capacity for arabinose metabolism is not found in dairy lactococci, including strain IL1403 (15). Finally, ribose uptake (*rbsABC*) and metabolism (*rbsD* and *rbsK*) genes were also induced 2.7-fold on average (see Table S2).

Xylose and arabinose are both components of hemicellulose, an important structural polysaccharide in plants. Genes in the locus llkf_1368 to -1374 involved in hemicellulose breakdown were induced, including genes encoding an α-glucuronidase (*algA*), a polysaccharide deacetylase (*pda*), an endoxylanase/endoglucanase (llkf_1370), and an acetylesterase (llkf_1374) (see Tables S2 and S3 in the supplemental material). Also upregulated was the locus llkf_1440 to -1447, which is putatively associated with hemicellulose breakdown (Fig. 3D; see also Table S2). This locus contains genes for a β-mannanase (llkf_1446) and an acetylxylan esterase (*axe* [llkf_1447]), as well as a gene encoding a putative substrate-binding protein for oligosaccharides (llkf_1445). The latter was the most highly induced gene in the genome in ATL (107-fold upregulated) (Fig. 3D; see also Table S2).

Genes involved in the consumption of the constituents of pectin (D-galacturonate and D-glucuronate) were also induced in *L. lactis* KF147 in ATL compared to GM17 (Fig. 3B and C; see also Table S2 in the supplemental material). D-Galacturonate is the primary component of pectin (32, 33), whereas D-glucuronate is a component of both pectin and xylan (32, 34). D-Galacturonate

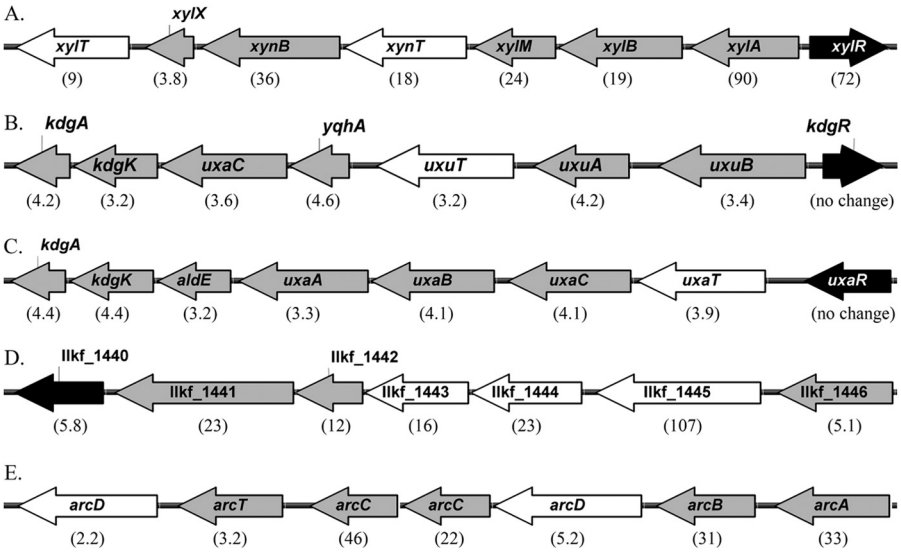


**FIG 3** Organization and fold changes in expression of selected induced genes during *L. lactis* KF147 growth in ATL. (A) Genes involved in xylose/xylan metabolism (llkf_1623 to -1630); (B) genes involved in D-glucuronate metabolism (llkf_1783 to -1790); (C) genes involved in D-galacturonate metabolism (llkf_1705 to -1712); (D) genes involved in transport and metabolism of unknown sugars (llkf_1440 to -1446); (E) genes in the arginine deiminase (ADI) pathway (llkf_2217 to -2223). The fold change is indicated in parentheses below each gene. Predicted regulatory genes are in black, transporter genes are in white, and enzyme genes are in gray. See the text and Table S2 in the supplemental material for a complete description of each gene.

is metabolized to 2-dehydro-3-deoxy-D-gluconate (KDG) through the action of UxaT (D-galacturonate transporter), UxaC (llkf_1710 [uronate isomerase]), UxaB (altronate oxidoreductase), and UxaA (altronate hydrolase). The corresponding genes, *uxaTCBA*, were upregulated on average 3.8-fold during growth in ATL (Fig. 3C; see also Table S2). Similarly, D-glucuronate is metabolized to KDG through UxuT (D-glucuronate transporter), UxaC (llkf_1785 [uronate isomerase]), UxuB (fructuronate reductase), and UxuA (mannonate dehydrogenase). The genes coding for those proteins were induced on average 3.6-fold in KF147 during growth in ATL (Fig. 3B; see also Table S2). Moreover, the *kdgK* and *kdgA* genes responsible for the further breakdown of KDG to pyruvate and glyceraldehyde-3-phosphate were also induced (Fig. 3B and C; see also Table S2).

The breadth of *L. lactis* KF147 responses to growth on plant tissues was demonstrated by the induction of multiple glycosyl hydrolases (GHs) as well as PTS and ABC transport systems. Genes coding for a total of 41 out of 51 annotated GH family enzymes were induced in *L. lactis* KF147 during growth in ATL compared to GM17 (see Table S3 in the supplemental material). Comparisons of the *L. lactis* KF147 genome to the CAZy carbohydrate-active enzyme database (35) revealed that this strain contains 51 glycoside hydrolases (GH) (data not shown). This is this highest number of glycoside hydrolases among all *L. lactis* strains for which genome sequences are available. Among those induced genes were genes for nine GH family 1 enzymes (β-glucosidases and β-galactosidases), eight GH family 13 enzymes (α-glucosidases), and three GH family 43 enzymes (α-L-arabinofuranosidases, endo-α-L-arabinases, and β-D-xylosidases) (see Table S3). Similarly, 23 out of 28 known PTS genes were also induced in ATL, including those specific for cellobiose, other β-glucosides, and unknown sugars (see Table S2 in the supplemental material). Notably, genes for five ABC transporters for which the transported sugar has yet to be determined were also upregulated (see Table S2).

**Fermentative metabolism during *L. lactis* KF147 growth in ATL.** Expression levels of two out of three annotated lactate dehydrogenases were downregulated by *L. lactis* KF147 in ATL compared to GM17 during exponential-phase growth (see Table S2 in the supplemental material). Genes leading to the production of acetoin (*butA*, encoding acetoin dehydrogenase) and 2,3-butanediol (*butB*, encoding butanediol dehydrogenase) were highly induced (89-fold and 21-fold, respectively) (see Table S2). Other enzymes involved in the production of fermentative end products other than lactic acid were upregulated, including those coding for pyruvate oxidase (*poxL*), pyruvate formate-lyase activating enzyme (*pflA*), alcohol dehydrogenase 1 (*adhA*), and acetolactate synthase (*ilvBH*) (see Table S2).

**Environmental stress- and defense-associated responses of *L. lactis* KF147 in ATL.** One response of *L. lactis* to environmental stress is the conversion of arginine to ornithine through the arginine deiminase (ADI) pathway and release of $NH_4$ and $CO_2$. Genes coding for the ADI pathway (*arcABC* and *arcD*) were significantly upregulated from 2.2- to 46-fold in ATL compared to GM17 (Fig. 3E; see also Table S2 in the supplemental material). Also upregulated were both copies of the *ymgGHIJ* genes (llkf_0277 to -0280 and llkf_2281 to -2284), which were previously correlated with oxidative stress tolerance (36) (see Table S2). However, *L. lactis* did not appear to be exposed to high levels of environmental stress in ATL, as indicated by the finding that other stress-responsive genes were downregulated (*tig* [encoding a trigger factor, repressed 10.1-fold], *dnaK* [repressed 3-fold], *grpE* [repressed 2.6-fold], and *groEL* [repressed 2.4-fold]) compared to cells grown in GM17 (see Table S2). In this regard, the cells did not appear to be under acid stress in ATL, most likely because the pH decreased only 0.2 pH unit from pH 6.6 to 6.4 during the 8 h of incubation.

Genes involved in secondary metabolite and antimicrobial production were induced in *L. lactis* in ATL. A putative hybrid nonribosomal peptide synthetase/polyketide synthase (NRPS/

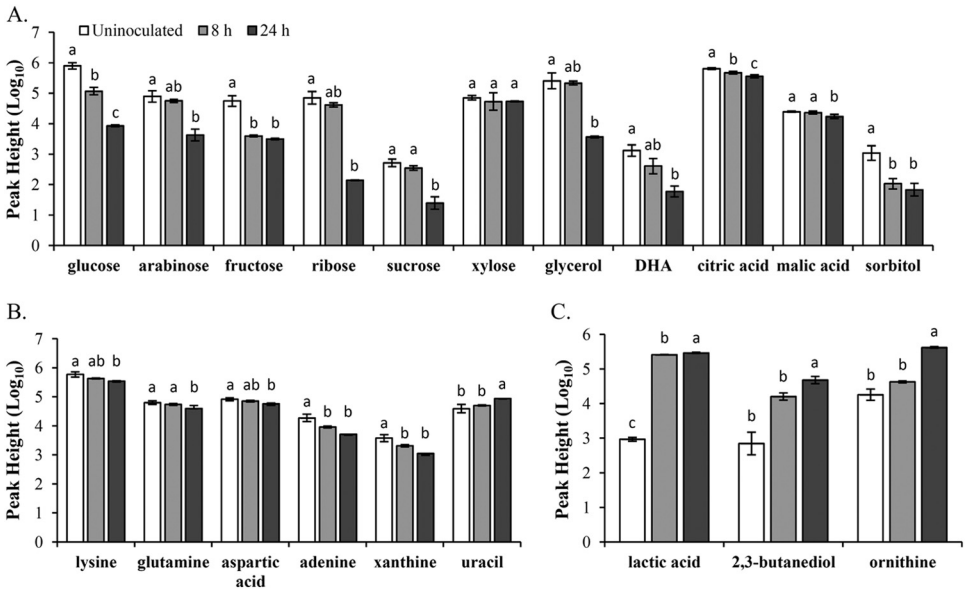


**FIG 4** Selected metabolites identified during *L. lactis* KF147 fermentation of ATL. (A) Sugars, sugar alcohols, and organic acids; (B) amino acids and nucleobases; (C) fermentation end products. *L. lactis* KF147 was inoculated into ATL, and cell-free supernatants were subjected to GC-TOF/MS metabolomic analysis after 8 and 24 h. Uninoculated ATL was included as a reference control. Analysis was performed in triplicate, and results are shown as the average ± standard deviation (SD), with statistical groupings represented by letters above each bar column.

PKS) locus (llkf_1211 to -1222) was induced 3.6-fold on average during growth in ATL compared with GM17 (see Table S2 in the supplemental material). The gene coding for the putative bacteriocin lactococcin 972, *yujF*, was also induced 3.4-fold (see Table S2). The *L. lactis* KF147 *nisBC* nisin biosynthesis genes have an internal deletion that renders them nonfunctional (37); however, these pseudogenes were induced as well as *nisP* (also a pseudogene), the immunity gene, *nisI*, and the regulatory and transport genes for nisin biosynthesis (see Table S2).

Genes responsible for natural competence in other bacteria were previously found to be induced in *L. lactis* under glucose starvation conditions (38, 39). Similarly, the *L. lactis* KF147 gene coding for the alternative sigma factor ComX, which putatively controls the expression of late competence genes, was induced during growth in ATL, as were several of the late competence genes associated with DNA uptake and processing (see Table S2 in the supplemental material).

**Cell envelope gene expression of *L. lactis* KF147 in ATL.** Many cell surface-associated genes were upregulated during growth in ATL. Pilus biosynthesis was recently characterized in *L. lactis* (40), and the genes *yhgD* (for pilus shaft oligomers), *yhgE* (pilus cap gene), and *yhhB* (for basal pilin), and *srtC* (encoding sortase C) were induced 3.4-fold, on average, compared to those in cells grown in GM17 (see Table S2 in the supplemental material). Genes encoding LPXTG motif-containing proteins were induced, including llkf_0311, *lpxAB*, *ynfH*, and *ywfG* (see Table S2). The LPXTG motif is a recognition signal for sortase enzymes that localize those proteins to the cell wall (41). Finally, both of the KF147 *csc* cell surface protein gene clusters with putative roles in the degradation of plant-derived polysaccharides (42) were induced (see Table S2).

***L. lactis* KF147 metabolite profiles during growth in ATL.** Untargeted GC-TOF/MS metabolomic analysis was performed on ATL and cell-free supernatants of *L. lactis* KF147 during the exponential (8 h of incubation) and stationary (24 h of incubation) phases of growth in ATL. Metabolomic analysis successfully annotated 125 compounds, and another 14 compounds were assigned to a specific chemical class (e.g., carbohydrates) (see Table S4 in the supplemental material). After 8 h of KF147 growth in ATL, 37 metabolites were significantly different from the ATL medium alone, and after 24 h, 53 metabolites were significantly changed (see Table S4).

Glucose was the most abundant sugar identified in ATL. The large amount of glucose observed in the ATL could have been due to endogenous *A. thaliana* myrosinase activity, which hydrolyzed glucosinolates into glucose and thiol-containing compounds upon plant cell lysis (43). Within 8 h of exponential-phase growth, *L. lactis* KF147 had consumed 85.2% of the glucose, equivalent to a 0.8-$\log_{10}$ reduction in total amounts of glucose. At 24 h, glucose concentrations were reduced another 1.1 $\log_{10}$, such that nearly all of the glucose was consumed (Fig. 4A; see also Table S4 in the supplemental material). Arabinose, fructose, ribose, sucrose, and sorbitol were also highly consumed by *L. lactis* KF147 during growth in ATL, to the extent that 94 to 99% of these compounds were removed from the medium within 24 h of incubation (Fig. 4A; see also Table S4). Notably, the fold reductions in glucose, fructose, and sorbitol were similar during the first 8 h of growth (Fig. 4A; see also Table S4). Despite the fact that genes coding for xylose degradation were highly induced in *L. lactis* KF147 in ATL, xylose consumption was variable and only moderately (25% [0.1 $\log_{10}$]) reduced after 24 h, and this difference was not significant (see Table S4). Moreover, the three-carbon compounds glycerol and dihydroxyacetone were also both metabolized by *L. lactis* to the extent that there was approximately a 2-$\log_{10}$ reduction in the amounts of those compounds in ATL after 24 h of incubation (Fig. 4A; see also Table S4). Other sugar alcohols were also detected in ATL; however, only sorbitol was found to decrease following KF147 growth (94% [1.2 $\log_{10}$]) (Fig. 4A; see also Table S4).

Growth of *L. lactis* also resulted in reductions of organic acids in ATL with large decreases in fumaric acid (91%) and gluconic acid (99%) and intermediate decreases in ribonic acid (50.2%), citric acid (44.2%), saccharic acid (41.5%), mucic acid (26%), malic acid (29.7%), and shikimic acid (42.8%) (Fig. 4A; see also Table S4). Within 24 h, *L lactis* also consumed at least 80% of certain nitrogenous compounds in ATL, including urea, citrulline, *N*-acetylgalactosamine, and *N*-acetyl-D-mannosamine (Fig. 4A; see also Table S4 in the supplemental material). Lastly, carbohydrates with limited structural annotation were also reduced up to 2.6 $\log_{10}$ (99.7% consumed) lower levels after KF147 incubation (see Table S4).

Out of a total of 18 amino acids detected in ATL, alanine was the most abundant (see Table S4 in the supplemental material). A total of three amino acids were consumed in significant quantities by *L. lactis* KF147, resulting in reductions in the amounts of lysine (0.2 $\log_{10}$), glutamine, (0.2 $\log_{10}$), and aspartic acid (0.2 $\log_{10}$) (Fig. 4B; see also Table S4). The levels of adenine and xanthine, an intermediate in purine metabolism, were also highly reduced in ATL within 24 h of incubation with *L. lactis* KF147 (Fig. 4B). Thymine was detected in very small amounts and did not change with the presence of *L. lactis* (see Table S4). In contrast, the amounts of uracil increased more than 2-fold after 24 h of *L. lactis* growth in ATL (Fig. 4B).

Polyamines are small, cationic, organic molecules that are essential for cell growth and contribute to multiple cellular functions, including maintenance of nucleic acid structure and scavenging of reactive oxygen species (44). During KF147 growth in ATL, the polyamines spermidine and spermine were both consumed 73.4% (0.6 $\log_{10}$) and 52% (0.3 $\log_{10}$), respectively, after 24 h (see Table S4 in the supplemental material). Although putrescine levels decreased significantly within 8 h of incubation of KF147, significantly higher quantities of this compound were found at 24 h when the cells were in the stationary phase (see Table S4).

Fermentation end products were also detected after incubation of *L. lactis* KF147 in ATL (see Table S4 in the supplemental material). By 24 h, the amounts of lactic acid and 2,3-butanediol had increased 314-fold (2.5 $\log_{10}$) and 57-fold (1.8 $\log_{10}$), respectively (Fig. 4C). Also notable was a 22-fold increase in ornithine in stationary-phase *L. lactis* ATL cultures (Fig. 4C).

***xylA* is essential for xylose metabolism.** *xylA* coding for xylose isomerase was the second most highly induced gene in *L. lactis* KF147 during growth in ATL; however, only modest (1.3-fold) reductions in xylose levels were found in those cultures. Those findings led us to further investigate the importance of *xylA* for *L. lactis* growth in association with plants. In lactococci, XylA isomerizes xylose to xylulose and is essential for the metabolism of xylose (45). Therefore, we constructed an *L. lactis* KF147 *xylA* deletion mutant and examined the growth of the wild-type and mutant strains in M17 containing xylose as the sole carbon source (XM17). Whereas *L. lactis* KF147 was able to multiply to high cell densities in XM17, the isogenic Δ*xylA* mutant was unable to grow in XM17 (Fig. 5). *L. lactis* KF147 Δ*xylA* reached the same low numbers as cells incubated in M17 lacking any carbon source (Fig. 5). In comparison, the KF147 Δ*xylA* mutant grew equally well as the wild-type strain in ATL (see Fig. S2 in the supplemental material). The mutant strain did not have an impaired growth rate in ATL nor did it exhibit a reduced competitive fitness when cocultured with *L. lactis* KF147 in ATL (data not shown).

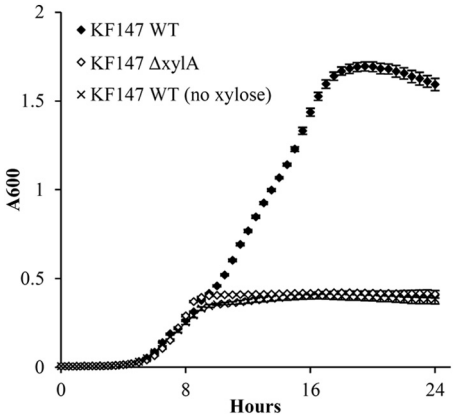


**FIG 5** Growth of *L. lactis* KF147 in XM17. The *L. lactis* KF147 WT strain and Δ*xylA* mutant were inoculated into XM17. As a control, the KF147 WT strain was incubated in M17 with no sugar added. Bacteria were incubated in 96-well microtiter plates and incubated at 30°C under static conditions. Growth was assessed by measurement of absorbance at 600 nm ($A_{600}$) for 24 h. Eight replicate wells were used for each strain; data points represent the average ± SE.

## DISCUSSION

Although *Lactococcus* is frequently isolated from plant environments, the molecular adaptations of these bacteria for growth and survival on plants are not well understood. Here, we have shown that a plant-derived strain of *L. lactis* adapts to and grows on plant tissues in predictable ways that are distinct from those of dairy-associated *L. lactis*. Gene expression and metabolic responses of *L. lactis* support the functional diversification of this species indicated by genome content.

The growth rate of *L. lactis* KF147 in ATL was significantly greater and higher cell densities were achieved than those in *L. lactis* IL1403. These findings are in agreement with the concept that LAB autochthonous to the plant environment are better suited for plant-based fermentations than allochthonous strains (3). Strain-specific differences in the capacity to consume nutrients found in plant tissues are likely the major determinant for growth and survival in those habitats (46). Conversely, strain KF147 grows poorly in milk compared to IL1403 (47). This phenotype was partially reversed upon culturing of KF147 successively in milk for 1,000 generations (47). Following domestication to the dairy niche, the milk-adapted KF147 mutants exhibited increased biomass accumulation and acidification rates in milk concomitant with gene losses and mutations that were apparently beneficial for growth in the dairy environment (47). The transcriptomes of the milk-adapted KF147 mutants also converged toward that of *L. lactis* IL594 (the parent strain for IL1403) when grown in milk (47). Although such "experimental evolution" experiments were not the purpose of the study here, it is intriguing to consider the possible genetic and physiological outcomes of growth of the dairy-originating *L. lactis* strains on plant tissues over many generations. It seems unlikely that significant increases in growth rate and yield would be achieved by dairy-associated strains because of the variety of unique genes found among plant-associated *L. lactis* strains that are relevant for adaptation and growth on plant tissues (15). This possibility is supported by the current understanding that *L. lactis* originated on plants and was domesticated for growth in milk through reductive evolution and horizontal gene transfer (15).

Over 40% of the protein-encoding genes in *L. lactis* KF147 were differentially expressed in ATL compared with standard (complete) laboratory culture medium. Carbohydrate metabolism pathway genes constituted 16% of all genes that were induced in strain KF147 in ATL, whereas genes coding for translation and ribosomal structures accounted for 28% of all repressed genes. These findings resemble the transcriptome of *L. lactis* IL1403 under glucose-limiting conditions (38). In that study, IL1403 genes required for consumption of alternate carbon sources as well as genes in the ADI pathway were induced, and genes related to cell growth were downregulated (38). The ADI pathway is responsible for the conversion of arginine to ornithine and the subsequent generation of 1 mol ATP per mol arginine (48). This pathway was induced in *L. lactis* KF147 in ATL as well as in other other lactococci during glucose starvation (38, 49) and was repressed by CcpA in the presence of glucose (12, 50). Evidence of glucose limitation during *L. lactis* KF147 growth in ATL is also supported in the *L. lactis* metabolomes, which showed that even though glucose was the most abundant sugar in ATL, it was nearly entirely consumed by strain KF147 within 8 h and hence not available to sustain *L. lactis* over longer periods of time. By comparison, genes in the ADI pathway were induced during growth in ATL, and this response corresponded with the 22-fold increase in ornithine with the 24-h incubation period.

The quantities of arabinose, fructose, ribose, and sucrose were significantly reduced in ATL after KF147 growth. The reductions in those sugars corresponded with the induction of *L. lactis* genes in the corresponding metabolic pathways. The metabolome and transcriptome profiles were also in agreement with the depletion of glycerol and dihydroxyacetone from the ATL medium. Interestingly, strain KF147 was previously reported as being unable to metabolize glycerol (37). Other LAB were shown to degrade glycerol (51, 52), and therefore the capacity to consume this compound might depend on the environmental context and other nutrients to which the cells are exposed.

The *A. thaliana* cell wall consists of hemicellulosic, pectic, and cellulosic polysaccharides as well as structural proteins (53). The transcriptomes and metabolomes of KF147 showed that this strain consumes breakdown products of these polysaccharides for growth. Up to 20% of the *A. thaliana* cell wall contains the hemicellulose xyloglucan, consisting of a 1,4-linked β-D-glucosyl residue backbone with substituted α-D-xylosyl residues (53). This corresponds with the detection of xylose in the ATL metabolome and the finding that genes responsible for xylose degradation were among the most highly expressed genes in KF147 during growth in ATL. By comparison, genes for xylan/xylose utilization are present but apparently nonfunctional in IL1403 (37). Pectin can account for up 42% of the cell wall polysaccharides in *A. thaliana* (53). Pectin is a heterogeneous, branching, structural polysaccharide composed primarily of a polygalacturonic acid backbone interspersed with rhamnose residues and other carbohydrates, including xylose, arabinose, or D-glucuronate (32, 33). Although the KF147 genome lacks genes for pectate lyases or pectinases necessary for initial steps in pectin hydrolysis, the strain can metabolize the sugar substituents of that polysaccharide which may become available due to the activity of endogenous *A. thaliana* enzymes that are released upon tissue lysis. In this regard, genes coding for arabinose, D-galacturonate, and D-glucuronate transporters and degradative enzymes were induced during *L. lactis* KF147 growth in ATL. KF147 is the only sequenced *Lactococcus* strain to possess genes annotated for D-galacturonate catabolism, although other plant-isolated lactococci have also been demonstrated to consume this compound (15). Similarly strain KF147, and not the dairy-associated strains *L. lactis* subsp. *cremoris* SK11 and IL1403, was able to consume D-glucuronate (37).

Out of the 11 complete genome sequences available for *L. lactis*, our comparisons to the CAZy database showed that strain KF147 encodes the most glycoside hydrolases (51 total). Similarly, *L. lactis* subsp. *cremoris* KW2, which is also a plant isolate, has 48 glycoside hydrolases (data not shown). In contrast, dairy isolates, such as *L. lactis* IL1403 and MG1363 subsp. *cremoris*, possess fewer glycoside hydrolases (32 and 37, respectively) (35). Notably, the specific sugar substrates hydrolyzed by the *L.* lactis KF147 GH family enzymes are largely unknown. Many of these genes were induced in ATL, suggesting a role for these enzymes in plant fermentations.

*L. lactis* is generally considered to be a strictly homofermentative LAB species. However, this species also possesses the *ptk* gene coding for phosphoketolase, an enzyme required for heterofermentative metabolism and involved in the degradation of pentoses (54). Consistent with glucose depletion and transition to a heterofermentation (55), *L. lactis* KF147 *ptk* was induced in ATL. Other genes involved in mixed-acid fermentation were also induced, including *butAB*, responsible for the production of acetoin and 2,3-butanediol. The expression of those genes corresponded with the detection of 2,3-butanediol after *L. lactis* growth in ATL. Intriguingly, 2,3-butanediol (and acetoin) is known to act as a plant growth-promoting signaling molecule, which raises the possibility that *L. lactis* communicates with its plant host (56). The *butAB* genes are known to be induced under acidic conditions (57); however, ATL was not acidic (pH 6.6), and after 8 h of incubation, the pH was lowered to only pH 6.4, thereby indicating that other plant-associated factors contributed to the induction of those genes.

LAB might encounter a multitude of stresses in plant fermentations, such as acidic pH, various temperatures, oxidative stress, osmotic stress, competing microorganisms, nutrient limitations, and antimicrobial compounds. These stresses result in the expression of a variety of genes regulating protein folding and turnover, cell membrane modification, defense responses, reactive oxygen detoxification, and ion transport (58). Remarkably, growth of *L. lactis* KF147 in ATL did not result in the induction of many of those genes. Instead, the transcriptional responses of *L. lactis* KF147 in ATL were similar to the downregulation of general stress response genes found during glucose starvation (38). Notably, the locus llkf_1440 to -1446, which contained the most highly induced gene in our study (llkf_1445), was previously negatively correlated with tolerance against heat stress (36). However, those genes are annotated to have roles in sugar metabolism, and their specific functions in thermal sensitivity have yet to be determined.

The presence of NRPS/PKS systems in *L. lactis* is limited to plant-isolated strains (15). Although the function of the NRPS/PKS system in KF147 is unknown, these proteins are highly similar (67 to 72% amino acid identity) to an NRPS/PKS system in *Streptococcus mutans*. In *S. mutans* UA140, the NRPS/PKS locus shares the same gene arrangement as KF147 and was recently demonstrated to produce a metabolite that contributes to oxidative stress tolerance and biofilm formation (59). Our finding that KF147 expresses this locus during growth in association with plants indicates that the end metabolite might confer protection against re-

active oxygen species encountered in plant fermentations as well as on living plant surfaces (60).

Finally, genes required for pilus production were induced in *L. lactis* KF147 during growth in ATL. Pili were only recently found for members of the *Lactococcus* genus; however, pilus production is not active during growth in standard laboratory culture medium (40). Pili were also shown to facilitate *L. lactis* attachment to intestinal epithelial Caco-2 cells (61), and this capacity might also be important for binding to plant tissues. One of the pilus-associated proteins possessing a lectin domain (open reading frame 4 [ORF4]) was putatively designated to be responsible for epithelial cell binding (61). In KF147, the ATL-inducible gene *ywfG* (llkf_2426) encodes a putative LPXTG-anchored mucus binding protein with a lectin domain that might provide a similar function for *L. lactis* on plants.

To test the relevance of a specific metabolic pathway for *L. lactis* KF147 growth on plant tissues, we constructed a xylose isomerase gene deletion mutant. The *L. lactis* Δ*xylA* mutant was unable to use xylose as the sole carbon source for growth, thereby confirming the role of this gene in the metabolism of this abundant plant-associated sugar. However, the *L. lactis* Δ*xylA* strain grew similarly to wild-type strain KF147 in ATL. This outcome supports the concept of functional redundancy of carbohydrate degradative pathways in plant tissue environments containing a variety of carbohydrates. However, it is notable that the *xyl* and *xyn* genes were the most highly induced in *L. lactis* during growth in ATL. It is possible that those genes were highly expressed because the enzymatic capacity for xylose/xylan metabolism is important for long-term persistence and cell maintenance of *L. lactis* on plant materials after glucose is consumed. Alternatively, *xynT*, which is putatively responsible for xyloside uptake, was induced in ATL, and this finding might indicate that xylosides contribute more to growth in ATL than xylose. Another explanation for this finding is that *L. lactis* KF147 XylR is different from XylR transcriptional regulators found in other *Firmicutes* (62). *L. lactis* KF147 XylR is a member of the AraC family of transcriptional regulators, whereas XylR proteins from related bacteria are classified as ROK (repressor, open reading frame, kinase) family regulators (62, 63). In *E. coli*, expression of the *araBAD* operon is highly induced upon exposure to arabinose through a "light switch"-type mechanism of AraC (64). It is therefore possible that a small amount of xylose present in ATL drives a large fortuitous increase in gene expression through a similar mechanism in *L. lactis*. Further studies are necessary to determine how XylR regulates expression of the *xyl* operon in this species and the relevance of *L. lactis* allocation of cellular resources to the production of xylose isomerase and other carbohydrate-degrading enzymes in glucose-containing, plant tissue environments.

Our results support the conclusion that certain *L. lactis* strains are well adapted for plant environments. This species exhibits high levels of environmental niche specificity that support growth and survival in different animal- and plant-associated habitats. The model fermentation system applied here using the genetically tractable *A. thaliana* species will be useful for elucidating the specific nutrients provided by plant tissues that set the course for LAB growth and succession. This information can be applied to improve our understanding of how LAB transform raw starting materials into economically valuable food, feed, and industrial products.

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