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Uncovering the *Lactobacillus plantarum* WCFS1 Gallate Decarboxylase Involved in Tannin Degradation

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Lactobacillus plantarum **is a lactic acid bacterium able to degrade tannins by the subsequent action of tannase and gallate decarboxylase enzymes. The gene encoding tannase had previously been identified, whereas the gene encoding gallate decarboxylase is unknown. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of gallic-acid induced *L. plantarum* extracts showed a 54-kDa protein which was absent in the uninduced cells. This protein was identified as Lp_2945, putatively annotated UbiD. Homology searches identified *ubiD*-like genes located within three-gene operons which encoded the three subunits of nonoxidative aromatic acid decarboxylases. *L. plantarum* is the only bacterium in which the *lpdC* (*lp_2945*) gene and the *lpdB* and *lpdD* (*lp_0271* and *lp_0272*) genes are separated in the chromosome. Combination of extracts from recombinant *Escherichia coli* cells expressing the *lpdB*, *lpdC*, and *lpdC* genes demonstrated that LpdC is the only protein required to yield gallate decarboxylase activity. However, the disruption of these genes in *L. plantarum* revealed that the *lpdB* and *lpdC* gene products are essential for gallate decarboxylase activity. Similar to *L. plantarum* tannase, which exhibited activity only in esters derived from gallic and protocatechuic acids, purified His6-LpdC protein from *E. coli* showed decarboxylase activity against gallic and protocatechuic acids. In contrast to the tannase activity, gallate decarboxylase activity is widely present among lactic acid bacteria. This study constitutes the first genetic characterization of a gallate decarboxylase enzyme and provides new insights into the role of the different subunits of bacterial nonoxidative aromatic acid decarboxylases.**

Vegetable tannins are present in a variety of plants utilized as food and feed. High tannin concentrations are found in nearly every part of the plant, such as the bark, wood, leaf, fruit, root, and seed. Tannins also widely occur in common foodstuffs, such as pomegranate, banana, strawberry, grape, cashew nut, and hazelnut. Drinks like wine and tea also contain these phenolic compounds (1). Tannins have been described to exhibit opposing health effects (2). They are beneficial to health due to their chemopreventive activities against carcinogenesis and mutagenesis. However, tannins are considered nutritionally undesirable because of their ability to bind to proteins to form indigestible complexes and to chelate heavy metals, and they may be involved in cancer formation and hepatotoxicity (2).

Tannase (tannin acyl hydrolase, EC 3.1.1.20) catalyzes the hydrolysis of ester linkages in hydrolyzable tannins. The products of hydrolysis are glucose and gallic acid. In addition to esters, gallic acid can be found in plants in the free state or in the form of ethers (e.g., syringic acid and other lignins constituents), being a major pollutant present in the wastewater generated in processes involving the boiling of cork and in food manufacturing industries. Gallic acid and its derivatives are used in industry as antioxidants (3).

Although gallic acid is widely distributed in nature, it is easily oxidized at neutral or alkaline pH, at which point it becomes a product difficult for bacteria to use as a carbon source for growth. In fact, only bacteria of the genus *Pseudomonas* have been reported to be able to utilize free gallic acid as the sole carbon and energy source under aerobic conditions (4). The aerobic metabolism of gallic acid usually starts with a direct ring-cleavage reaction and formation of the central intermediate 4-oxalomesaconic acid, which then undergoes hydration to 4-carboxy-4-hydroxy-2-oxoadipic acid and aldol cleavage to oxaloacetic and pyruvic acids (5). In addition to microorganisms that use gallic acid as the sole carbon and energy source, there are also microorganisms that nonoxidatively decarboxylate gallic acid but do not possess appropriate mechanisms to further degrade the pyrogallol produced by this dead-end pathway. Strains of the species *Pantoea agglomerans* (6), *Enterococcus faecalis* (7), *Klebsiella pneumoniae* (7), *Streptococcus gallolyticus* (8), and *Lactobacillus plantarum* (9–11) were described to decarboxylate gallic acid to pyrogallol, without further metabolism. Even though several gallate decarboxylases, mainly from anaerobic sources, have been described, most of these enzymes have not been purified due to their instability. Gallate decarboxylases from *E. faecalis* and *P. agglomerans* are inducible enzymes which, due to their oxygen sensitivity, were extremely unstable when they were purified (6, 7). So far, none of these gallate decarboxylase enzymes has been genetically identified or characterized.

L. plantarum is a lactic acid bacterial species that is most frequently encountered in the fermentation of plant materials where tannins are abundant. These plant fermentations include several food and feed products, e.g., olives, grape must, and a variety of vegetable fermentation products. Among food lactic acid bacteria, strains from the *L. plantarum* group are the only ones which possess tannase activity (12–14). The proposed biochemical pathway for the degradation of tannins by *L. plantarum* implies the action of a tannase and a gallate decarboxylase to decarboxylate the gallic acid formed by tannase action (9, 10). Of the genes involved in tannin degradation in *L. plantarum*, only the gene encoding tan-

Received 14 March 2013 **Accepted** 29 April 2013
Published ahead of print 3 May 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00840-13.
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doi:10.1128/AEM.00840-13

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description/relevant marker(s)[a]	Source or reference[b]
Strains		
Escherichia coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacIZYA-argF*) *recA1 gyrA endA1 relA1 supE44 hsdR17*	Clontech
BL21(DE3)	*E. coli* B F^- *dcm ompT hsdS*$_B$(r_B^- m_B^-), *gal* λ (DE3)	Novagen
JW2308-4 (CGSC 9853)	Δ(*araD-araB)567* Δ*lacZ4787* (::*rrnB-3*) *rph-1* λ^- Δ(*rhaD-rhaB*)*568* Δ*ubiX732*(*del*)::*kan*	31
Lactobacillus plantarum		
ATCC 14917^T	Wild-type strain	CECT
WCFS1	Wild-type strain	M. Kleerebezem
WCFS1ΔlpdB	WCFS1 derivative, Δ*lpdB*	This study
WCFS1ΔlpdC	WCFS1 derivative, Δ*lpdC*	This study
WCFS1ΔlpdD	WCFS1 derivative, Δ*lpdD*	This study
Other		
Enterococcus faecium CECT 410^T	Wild-type strain	CECT
Enterococcus faecium CECT 4102	Wild-type strain	CECT
Lactobacillus brevis CECT 4121^T	Wild-type strain	CECT
Lactobacillus brevis CECT 5354	Wild-type strain	CECT
Lactobacillus hilgardii RM62	Wild-type strain	42
Lactobacillus hilgardii RM63	Wild-type strain	42
Lactobacillus pentosus DSM 20314^T	Wild-type strain	DSMZ
Lactobacillus sakei DSM 15831^T	Wild-type strain	DSMZ
Leuconostoc mesenteroides CECT 219^T	Wild-type strain	CECT
Oenococcus oeni CECT 4100^T	Wild-type strain	CECT
Oenococcus oeni RM1	Wild-type strain	42
Pediococcus pentosaceus CECT 4695^T	Wild-type strain	CECT
Streptococcus gallolyticus UCN34	Wild-type strain	P. Glaser
Plasmids		
pIN-III-A3	Expression vector for producing proteins in *E. coli*	30
pURI3	Expression vector for producing His-tagged proteins in *E. coli*; pT7-7 derivative, Amp^r	42
pIN-lpdB	pIN-III-A3 carrying *lpdB*	This study
pIN-lpdC	pIN-III-A3 carrying *lpdC*	This study
pIN-lpdD	pIN-III-A3 carrying *lpdD*	This study
pURI3-lpdB	pURI3-carrying *lpdB*	This study
pURI3-lpdC	pURI3-carrying *lpdC*	This study
pUCE191	*L. plantarum* integrative vector, pUC19 derivative, Amp^r Em^r Lm^r	18
pUCE191-lpdB	pUCE191 carrying an internal fragment of *lpdB*	This study
pUCE191-lpdC	pUCE191 carrying an internal fragment of *lpdC*	This study
pUCE191-lpdD	pUCE191 carrying an internal fragment of *lpdD*	This study

[a] Amp^r, ampicillin resistance; Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance.
[b] CECT, Spanish Type Culture Collection; DSMZ, German Collection of Microorganisms and Cell Cultures.

nase has been identified so far (15), and the gene encoding the gallate decarboxylase enzyme involved in this degradation remains unknown. In this work, the genes involved in *L. plantarum* gallate decarboxylation have been identified. For the first time, a gallate decarboxylase enzyme has been molecularly identified and characterized. In addition, our results provide new important insights into bacterial nonoxidative aromatic acid decarboxylases.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. plantarum* WCFS1, used throughout this study, was kindly provided by Michiel Kleerebezem (NIZO Food Research, The Netherlands). This strain is a colony isolate of *L. plantarum* NCIMB 8826, which was isolated from human saliva. *L. plantarum* WCFS1 derivative strains, lactic acid bacteria, and the *Escherichia coli* strains used in this study are described in Table 1. *Escherichia coli* JW2308-4 (CGSC 9853), which has a deletion in the *ubiX* gene, was generously provided by the *E. coli* Genetic Stock Center.

Lactic acid bacteria were routinely grown on MRS broth. When gallate activity was assayed and in order to avoid the presence of phenolic compounds included in nondefined medium, bacteria were cultivated in a modified basal medium, RPM, described previously (16). The sterilized modified basal medium was supplemented with filter-sterilized gallic or protocatechuic acid at a 3 mM final concentration. Where appropriate, erythromycin was added to the culture medium at 10 μg/ml. The inoculated media were incubated at 30°C in the dark, without shaking, for 7 to 10 days. Incubated media with cells and without phenolic compound were used as controls. The phenolic compounds present in the supernatants were extracted by a standard protocol involving two extraction steps with one-third of the reaction mixture volume of ethyl acetate.

E. coli cells were routinely grown in LB medium (17) at 37°C with agitation. The *E. coli* JW2308-4 strain was grown in medium containing kanamycin at 30 μg/ml. *E. coli* transformant cells harboring recombinant plasmids were selected on LB medium plates supplemented with 100 μg of ampicillin or 200 μg of erythromycin per ml.

Molecular biology techniques. Standard molecular biology techniques were performed as previously described (17). Plasmid DNA was extracted by a High Pure plasmid isolation kit (Roche). PCR products were purified with a QIAquick gel extraction kit (Qiagen). All cloned

inserts and DNA fragments were confirmed by DNA sequencing with fluorescently labeled dideoxynucleotide terminators and AmpliTaq FS DNA polymerase (Applied Biosystems) in an ABI Prism 377 automated DNA sequencer (Applied Biosystems). Transformation of *E. coli* cells was carried out by using the RbCl method (17). Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany) (see Table S1 in the supplemental material). Proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Coomassie stained according to standard protocols (17). The protein concentration was determined by using a Bio-Rad protein assay.

***L. plantarum* cell extract preparations.** To identify the protein involved in gallate decarboxylation, cell extracts containing all soluble proteins were prepared. *L. plantarum* WCFS1 was grown in MRS medium at 30°C until exponential phase (optical density at 600 nm [OD_{600}], 0.6). The culture was induced by adding 3 mM gallic acid and incubated for 1 h at 30°C. Uninduced culture was prepared as a control. In the experiments with *L. plantarum lpd* knockout mutants, the mutants were grown in MRS medium containing 10 μg/ml erythromycin until the OD_{600} was 0.6. In all experiments, after incubation the cells were harvested by centrifugation, washed three times with phosphate buffer (50 mM, pH 6.5), and subsequently resuspended in the same buffer for cell rupture. Bacterial cells were disintegrated three times by using a French press at a 1,100-lb/in^2 pressure (Amicon French pressure cell; SLM Instruments). The cell disruption steps were carried out on ice to ensure the low-temperature conditions required for most enzymes. The disintegrated cell suspension was centrifuged at 12,000 × *g* for 20 min at 4°C in order to sediment the cell debris. The supernatant containing the soluble proteins was filtered aseptically using sterile filters with a pore size of 0.2 μm (Sarstedt, Germany).

Gallate decarboxylase assay. The gallate decarboxylase activity of the bacterial cultures was assayed by growing the strains in RPM or LB medium supplemented with 3 mM gallic acid in the dark for several days. The phenolic compounds present in the supernatant were extracted with ethyl acetate as described above. In *E. coli* cultures expressing recombinant *L. plantarum lpd* proteins, gallate decarboxylase activity was assayed in cell extracts incubated at 37°C for 1 h in the presence of 3 mM gallic acid. Similarly, cell extracts were used to assay the gallate decarboxylase activity of *L. plantarum lpd* knockout mutants incubated for 18 h in 3 mM gallic acid. Recombinant His-tagged proteins purified in 50 mM phosphate buffer, pH 6.5, containing 300 mM NaCl, 150 mM imidazole, 1 mM dithiothreitol (DTT), and 50 mM $Na_2S_2O_5$ were assayed for gallate decarboxylase activity (6). Eluted purified proteins (100 μg) were incubated at 37°C in the presence of 3 mM gallic acid and 50 mM L-ascorbate for 1 h.

The reaction products were extracted with ethyl acetate and analyzed by high-pressure liquid chromatography (HPLC) with a diode array detector. A Thermo chromatograph (Thermo Electron Corporation, Waltham, MA) equipped with a P400 SpectraSystem pump, an AS3000 autosampler, and a UV6000LP photodiode array detector was used. A gradient of solvent A (water and acetic acid, 98:2, vol/vol) and solvent B (water, acetonitrile, and acetic acid, 78:20:2, vol/vol/vol) was applied to a reversed-phase Nova-pack C_{18} cartridge (25 cm by 4.0 mm [inner diameter]; particle size, 4.6 μm) at room temperature as follows: 0 to 55 min, 0 to 80% solvent B, linear, 1.1 ml/min; 55 to 57 min, 80 to 90% solvent B, linear, 1.2 ml/min; 57 to 70 min, 90 to 95% solvent B, isocratic, 1.2 ml/min; 70 to 80 min, 95 to 100% solvent B, linear, 1.2 ml/min; 80 to 90 min, 100% linear, 1.2 ml/min; 100 to 120 min, washing with methanol 1.0 ml/min; and reequilibration of the column under initial gradient conditions. Detection was performed by scanning from 220 to 380 nm. Samples were injected onto the cartridge in duplicate, after being filtered through a 0.45-μm-pore-size polyvinylidene difluoride filter. The identification of degradation compounds was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers.

Construction of *L. plantarum lpd* knockout mutant strains. To ascertain the participation of particular *lpd* genes in gallate decarboxylase activity, insertion-duplication mutagenesis was employed. Internal fragments from the *lpdB* (*lp_0271*), *lpdC* (*lp_2945*), and *lpdD* (*lp_0272*) genes were cloned into the pUCE191 suicide vector. Plasmid pUCE191 is a pUCE19 derivative that does not replicate in *L. plantarum* but expresses lincomycin resistance upon integration into the lactobacillus genome. Plasmid pUCE191 was constructed by introducing the LnR gene from plasmid pFB9 into pUC19 (18). When pUCE191 and its derivatives were used as donor DNA, *L. plantarum* transformants were selected by plating with 10 μg/ml erythromycin and *E. coli* transformants were selected by plating with ampicillin at 100 μg/ml. Plasmids pUCE191-lpdB, pUCE191-lpdC, and pUCE191-lpdD (Table 1), constructed in *E. coli*, were used to transform *L. plantarum* WCFS1 competent cells by electroporation (19). Knockout mutants were selected by plating in MRS medium plates containing erythromycin. The correct insertion of the donor pUCE191 derivative plasmid into the *L. plantarum* WCFS1 chromosome was checked by PCR analysis using primers flanking the target region combined with vector-specific primers (primers 1131 and 1233 for *lpdB*, 388 and 1224 for *lpdC*, and 1109 and 1224 for *lpdD*) (see Table S1 in the supplemental material).

Expression of the *lpd* genes in *E. coli* and purification of the His$_6$-tagged *lpdB* and *lpdC* recombinant enzymes. The *lpdB*, *lpdC*, and *lpdD* genes from *L. plantarum* WCFS1 were PCR amplified by using HS Prime Start DNA polymerase (TaKaRa) and primer pairs 455 and 390 (*lpdB*), 454 and 388 (*lpdC*), and 1141 and 1142 (*lpdD*). The purified PCR products were inserted into the pIN-III-A3 vector following the restriction enzyme- and ligation-free cloning strategy described previously (20, 21). The procedure used to clone *lpdB* and *lpdC* containing an amino-terminal His$_6$ tag into the pURI3 vector was essentially the same as that described above for the native protein, but primers 389 and 390 and primers 387 and 388, respectively, were used. The pURI3 vector is a pT7-7 derivative that was used to overproduce recombinant proteins containing a six-histidine tag at their N termini (20).

Cells carrying the recombinant plasmids were grown at 37°C in LB medium containing ampicillin (100 μg/ml), until they reached an optical density at 600 nm of 0.4, and induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside; final concentration, 0.4 mM). After induction, the cells were grown at 22°C for 20 h and collected by centrifugation. Cells were resuspended in phosphate buffer (50 mM, pH 6.5). Crude extracts were prepared by French press lysis of the cell suspension (three times at 1,100 lb/in^2). The insoluble fraction of the lysate was removed by centrifugation at 47,000 × *g* for 30 min at 4°C, and the supernatant was filtered through a 0.2-μm-pore-size filter.

For purification of the recombinant His-tagged LpdB and LpdC proteins, the cultures were similarly prepared but the cells were resuspended in 50 mM phosphate buffer, pH 6.0, containing 30 mg/ml $FeSO_4$, 1 mM DTT, 1 mM L-ascorbate, and 50 mM $Na_2S_2O_5$ (6). The supernatants were applied to a Talon Superflow resin (Clontech) equilibrated with the buffer described above containing 0.3 M NaCl and 10 mM imidazole to improve the interaction specificity in the affinity chromatography step. The bound enzymes were eluted using 150 mM imidazole in the same buffer. The purity of the enzymes was determined by SDS-PAGE in Tris-glycine buffer. Fractions containing the His$_6$-tagged protein were pooled and analyzed for gallate decarboxylase activity. Eluted purified LpdB and LpdC proteins (100 μg) were incubated at 37°C in the presence of 3 mM gallic acid for 1 h.

***In vitro* protein-protein cross-linking experiments.** The LpdB-LpdC interaction was assayed by glutaraldehyde cross-linking. For glutaraldehyde treatment, LpdB and LpdC proteins, at concentrations ranging from 2 and 10 μM, in 50 mM sodium phosphate, 300 mM NaCl, and 150 mM imidazole buffer (pH 7) were treated with glutaraldehyde solution (0.1, 0.2, and 0.5 μM) for 20 min at room temperature. As a control, a similar glutaraldehyde treatment was applied to monomeric lysozyme. The reactions were stopped by adding Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, β-mercaptoethanol, 10% glycerol) containing 0.005% bromophenol blue. Samples were separated by SDS-PAGE and revealed by Coomassie blue staining.

PCR detection of gallate decarboxylase. Genes encoding LpdB and LpdC were amplified by PCR using chromosomal DNA from several lactic acid bacterial strains. Amplifications were performed by using degenerate primers 450 and 451 and degenerate primers 448 and 449 to amplify *lpdB* and *lpdC*, respectively. These degenerate primers were based on the well-conserved domains of the B and C proteins. The reactions were performed in a Personal thermocycler (Eppendorf), using 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 30 s. The expected sizes of the amplicons were 158 bp (subunit B) and 300 bp (subunit C). Amplified fragments were resolved on 2% agarose gels.

Protein identification via MS. The protein band was excised manually and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany). The protocol described by Shevchenko et al. (22) was used for trypsin digestion. The digestion was analyzed in an Ultraflex time-of-flight mass spectrometer (MS; Bruker-Daltonics) equipped with a LIFT-MS/MS device. Spectra were acquired in the positive-ion mode at a 50-Hz laser frequency, and 100 to 1000 individual spectra were averaged. Automated analysis of mass data was performed using flexAnalysis software (Bruker-Daltonics). Matrix-assisted laser desorption ionization MS and MS/MS data were combined through the BioTools program (Bruker-Daltonics) to search a nonredundant protein database (NCBInr Swiss-Prot) using Mascot software (Matrix Science, London, United Kingdom).

Sequence data analyses. A homology search with finished and unfinished microbial genome databases was performed with the TBLAST algorithm at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Multiple alignments were made using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) on the EBI site, after retrieval of sequences from the GenBank and Swiss-Prot databases.

RESULTS

Identification of the enzyme responsible for gallate decarboxylation in *L. plantarum* WCFS1. In order to know if gallate decarboxylase is an inducible enzyme, mid-exponential-phase *L. plantarum* WCFS1 cultures were incubated in MRS medium containing glucose as the carbon source, with or without the addition of 3 mM gallic acid for 1 h at 30°C. Cell extracts prepared from these cultures were tested for activity on gallic acid. The extract from the control culture (grown in the absence of gallic acid) did not show decarboxylase activity. However, the extract from the culture grown in the presence of gallic acid for 1 h was able to decarboxylate the gallic acid present in the reaction mixture (data not shown). Similar to the result for *p*-coumaric acid decarboxylase previously described in *L. plantarum* (23, 24), this result might indicate that gallate decarboxylase is an inducible enzyme.

Cell extracts were resolved by SDS-PAGE in order to find proteins overproduced from the induced culture (Fig. 1). The only difference clearly detected was in the gallate-induced culture and consisted of a protein band of approximately 50 kDa which was absent in the uninduced sample. The overproduced protein was excised from the gel, and its identification was done by in-gel trypsin and chymotrypsin digestion and subsequent mass spectrometry analyses. The result obtained unambiguously identified the protein as 3-octaprenyl-4-hydroxybenzoate carboxy-lyase or UbiD (NP_786283), encoded by the *lp_2945* locus.

In *E. coli*, the UbiD protein is involved in the biosynthesis of ubiquinone (or coenzyme Q) (25). Ubiquinone plays an essential role in aerobic respiratory electron transfer for energy generation. The biosynthesis of ubiquinone involved at least nine reactions. In one of these reactions, the 3-octaprenyl-4-hydroxybenzoate is decarboxylated to 2-octaprenylphenol by the enzyme 3-octaprenyl-4-hydroxybenzoate decarboxylase. There are two isofunctional enzymes in *E. coli* K-12, UbiD and UbiX, which catalyze this reaction (26). Their amino acid sequences share no similarity. UbiX, a 21-kDa protein, may require a flavin nucleotide as a cofactor, whereas UbiD is a 55-kDa protein requiring divalent metal for activity. Of the two enzymes, UbiD accounts for almost 80% of the total activity (27). However, it has been described that *L. plantarum* does not produce mena- or ubiquinones, as it needs the exogenous addition of at least menaquinones for heme-assisted respiration (28). Therefore, it is unlikely that UbiD is involved in the self-production of ubiquinones by *L. plantarum*. The precise biochemical function of the *L. plantarum* protein annotated UbiD (Lp_2945) is likely to be a gallate decarboxylase.

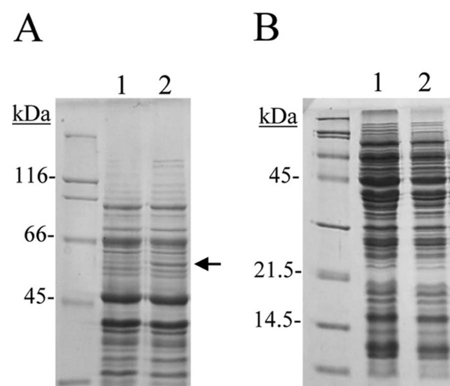

FIG 1 SDS-PAGE analysis of protein extracts from *L. plantarum* WCFS1 grown in the presence of 3 mM gallic acid. Lanes 1, uninduced cell extracts; lanes 2, extracts induced with gallic acid for 1 h. The arrow indicates the induced protein. The 8% (A) and 15% (B) gels were stained with Coomassie blue. Molecular mass markers are located on the left (SDS-PAGE standards; Bio-Rad).

Genes encoding *L. plantarum* gallate decarboxylase. Recent studies indicated that *ubiD*-like genes in many prokaryotes are located within operons that encode partner proteins, including proteins homologous to UbiX, which are required to decarboxylate a variety of hydroxyarylic or aromatic acids. Bacterial nonoxidative, reversible multisubunit hydroxyarylic acid decarboxylases/phenol carboxylases are encoded by three clustered genes (genes encoding the B, C, and D subunits) (29). The corresponding genes from *Sedimentibacter hydroxybenzoicus*, *Bacillus subtilis*, *Streptomyces* sp. strain D7, *E. coli* O157:H7, *K. pneumoniae*, and *Salmonella enterica* serovar Typhimurium were cloned and expressed in *E. coli* and shown to code for proteins exhibiting nonoxidative aromatic acid decarboxylase activity (29). Database searches revealed the existence of different gene organizations among these decarboxylases: the BCD gene arrangement (such as in *Enterobacter cloacae*, *Pantoea ananatis*, *B. subtilis*, and a *Streptomyces* sp.), CDB (*S. hydroxybenzoicus*, *Clostridium* sp. strain D5, and *Olsenella uli* DSM 7084), CDB in several lactic acid bacteria (*Oenococcus oeni*, *Lactobacillus brevis*, and *Enterococcus faecium*), and DBC in *S. gallolyticus* (Fig. 2). Surprisingly, the genes that putatively encoded gallate decarboxylase, the *lpd* (from Lactobacillus plantarum decarboxylase, to be consistent with the existing nomenclature of genes encoding aromatic acid decarboxylases) genes, are not close only in the genome of *L. plantarum*. The gene encoding the C subunit, *lpdC* or *lp_2945*, which was overproduced in the presence of gallate, is located close to the gene encoding tannase (*tanLp1* or *lp_2956*). However, the genes encoding the B (*lp_0271*) and D

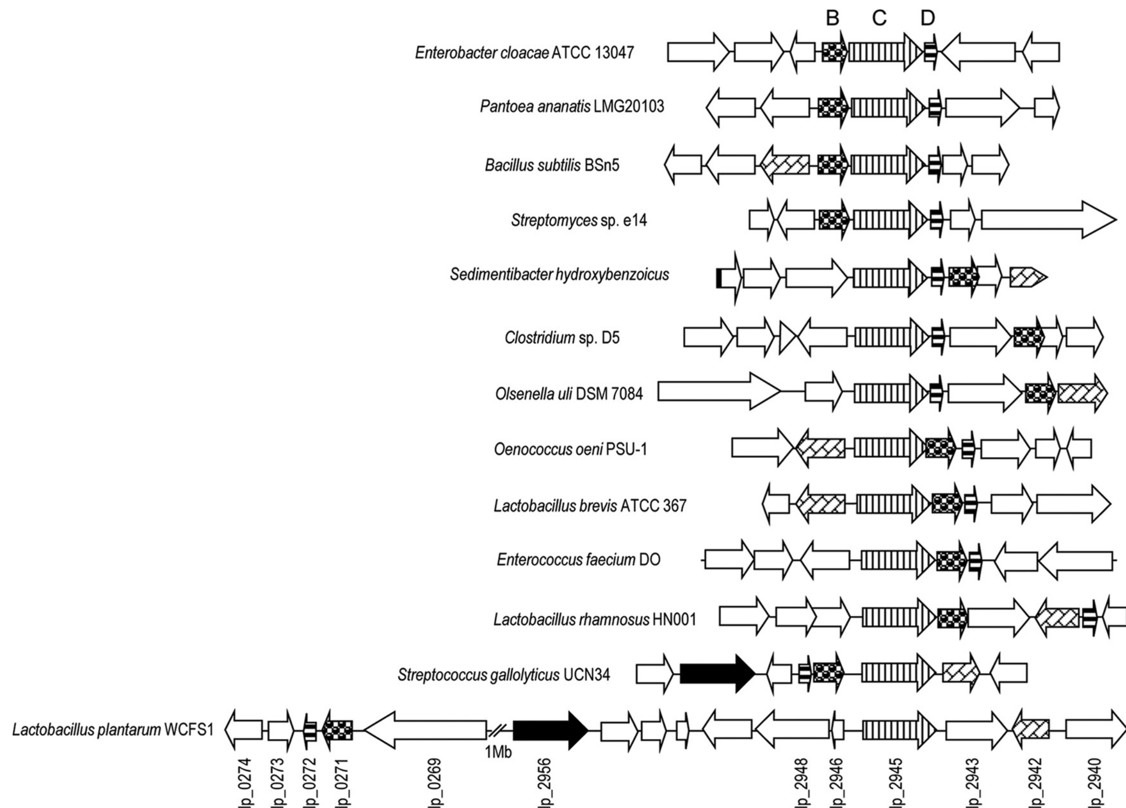

FIG 2 Genetic organization of the *L. plantarum* WCFS1 chromosomal region containing the genes encoding gallate decarboxylase (GenBank accession no. NC_004567, positions 243093 to 252815 and 2618290 to 2635122). The genetic organization from several bacterial nonoxidative aromatic acid decarboxylases (B, C, and D subunits) is also represented, such as those of *Enterobacter cloacae* subsp. *cloacae* ATCC 13047 (GenBank accession no. NC_014121, positions c/4181733 to 4189439 [where "c/" indicates positions on a complementary strand]), *Pantoea ananatis* LMG20103 (GenBank accession no. NC_013956, positions 1814209 to 1820127), *Bacillus subtilis* BSn5 (GenBank accession no. NC_014976, positions 2624421 to 2630650), *Streptomyces* sp. strain e14 (GenBank accession no. NZ_ACUR00000000, positions c/7010912 to 7018020), *Sedimentibacter hydroxybenzoicus* (GenBank accession no. AF128880, positions 1 to 5796), *Clostridium* sp. D5 (GenBank accession no. NZ_ADBG00000000, positions 5032836 to 5040967), *Olsenella uli* DSM 7084 (GenBank accession no. NC_014363, positions c/1673164 to 1680578), *Oenococcus oeni* PSU-1 (GenBank accession no. NC_008528, positions c/1104544 to 1114618), *Lactobacillus brevis* ATCC 367 (GenBank accession no. NC_008497, positions c/1942529 to 1951428), *Enterococcus faecium* DO (GenBank accession no. NC_017960, positions 1147858 to 1155656), *Lactobacillus rhamnosus* HN001 (GenBank accession no. NZ_ABWJ00000000, positions 1905984 to 1914300), and *Streptococcus gallolyticus* UCN34 (GenBank accession no. NC_013798, positions 1699210 to 1708634). Arrows indicate genes. Genes having putative identical functions are represented by identical shading. The genes having brick-like shading encode putative LysR-type transcriptional regulators. Genes coding for putative tannase proteins are represented by black arrows.

(*lp_0272*) subunits are located more than 1 Mb apart in the *L. plantarum* genome. This unusual gene organization could indicate a different catalytic mechanism of *L. plantarum* gallate decarboxylase.

Pyrogallol production by recombinant *E. coli* cells harboring *lpdB*, *lpdC*, and *lpdD* genes. In order to know the catalytic subunits involved in gallate decarboxylation, the *lpd* genes were individually cloned into the expression vector pIN-III-A3 under the control of the lpp^{P}-5 and lac^{PO} promoters, which can be induced to high levels with IPTG (30). The correct sequence of the recombinant plasmids pIN-lpdB, pIN-lpdC, and pIN-lpdD was verified by DNA sequencing.

Cell extracts were prepared from *E. coli* DH5α cells harboring the recombinant plasmids. The extracts were used to detect the presence of hyperproduced proteins. Control cells containing the pIN-III-A3 vector plasmid alone did not show expression over the time course analyzed (overnight), whereas expression of additional 54-, 21-, and 15-kDa proteins was apparent in DH5α cells harboring pIN-lpdC, pIN-lpdB, and pIN-lpdD, respectively (Fig. 3A and B). These molecular masses are in good agreement with the relative molecular masses deduced from the nucleotide sequences of the *lpdC*, *lpdB*, and *lpdD* genes.

Extracts from *E. coli* cells carrying the pIN-III-A3, pIN-lpdC, pIN-lpdB, and pIN-lpdD plasmids adjusted to the same protein concentration were assayed for gallate decarboxylase activity. Reactions with mixtures containing the same total protein concentration were done by mixing these extracts in all possible combinations, e.g., in mixtures containing the B, C, or D subunit individually; mixtures containing subunits B and C, B and D, and C and D; and finally, mixtures simultaneously containing the three different *lpd* subunits. Reactions were carried out for 1 h, and after that, the phenolic compounds present in the reaction mixtures were extracted by ethyl acetate and analyzed by HPLC.

Figure 3C shows that all the reactions with mixtures containing LpdC were able to partially decarboxylate gallic acid to a similar extent. In contrast, in reactions in which LpdC was absent from the reaction mixture, gallic acid was not metabolized. These results indicate the involvement of LpdC in the catalysis of decar-

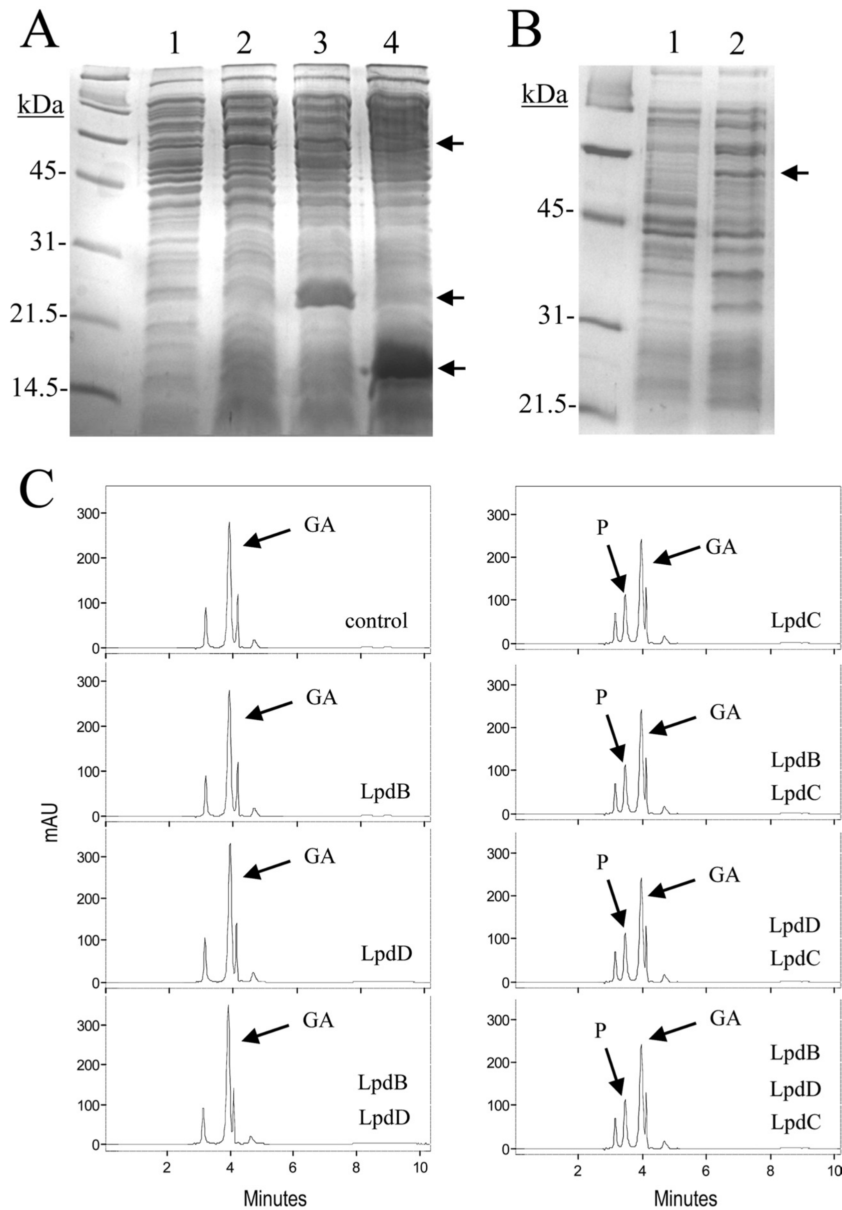

FIG 3 Pyrogallol production by cell extracts from recombinant *E. coli* cells harboring *lpdB*, *lpdC*, and *lpdD* genes. (A and B) SDS-PAGE analysis of cell extracts of IPTG-induced cultures of *E. coli* DH5α bearing recombinant pIN-III-A3 plasmids for LpdB, LpdC, and LpdD protein production. (A) LpdC, LpdB, and LpdD production (15% gel). Lane 1, *E. coli* DH5α(pIN-III-A3); lane 2, *E. coli* DH5α(pIN-lpdC); lane 3, *E. coli* DH5α(pIN-lpdB); lane 4, *E. coli* DH5α(pIN-lpdD). (B) LpdC production (12% gel). Lane 1, *E. coli* DH5α(pIN-III-A3); lane 2, *E. coli* DH5α(pIN-lpdC). The arrows indicate the overproduced proteins. The gels were stained with Coomassie blue. Molecular mass markers are located on the left. (C) Gallate decarboxylase activity of *E. coli* DH5α cell extracts harboring *lpdB*, *lpdC*, and *lpdD* genes. HPLC chromatograms of *E. coli* cell extracts, at the same total protein concentration, incubated in 3 mM gallic acid for 1 h: pIN-III-A3 (control), pIN-III-A3 plus pIN-lpdB (LpdB), pIN-III-A3 plus pIN-lpdD (LpdD), pIN-III-A3 plus pIN-lpdB and pIN-lpdD (LpdB LpdD), pIN-III-A3 plus pIN-lpdC (LpdC), pIN-III-A3 plus pIN-lpdB and pIN-lpdC (LpdB LpdC), pIN-III-A3 plus pIN-lpdC and pIN-lpdD (LpdC LpdD), and pIN-lpdB plus pIN-lpdC and pIN-lpdD (LpdB LpdC LpdD). The gallic acid (GA) and pyrogallol (P) detected are indicated. Chromatograms were recorded at 280 nm. mAU, milli-absorbance units.

boxylation, and in addition, they suggest that LpdC is the only subunit required to yield gallate decarboxylase activity.

From the results obtained using *E. coli* extracts, the possibility that in *E. coli* the missing subunits can be replaced by other *E. coli* proteins, e.g., UbiX, to yield enzyme activity could not be excluded. As explained before, UbiX is involved in ubiquinone biosynthesis and catalyzes the reaction of 3-octaprenyl-4-hydroxybenzoate to 2-octaprenylphenol. In order to avoid the presence of a functional *E. coli* UbiX protein in the extracts, plasmid pIN-lpdC was introduced into *E. coli* JW2308-4 (CGSC 9853), a UbiX-defective mutant (31). Gallate decarboxylase activity was assayed in cell extracts prepared from *E. coli* JW2308-4 harboring pIN-lpdC. The results indicated that, similar to *E. coli* DH5α extracts, pyrogallol was produced from gallic acid in the presence of LpdC even in the absence of a functional *E. coli* UbiX protein (see Fig. S1 in the supplemental material). Therefore, the possibility of the in-

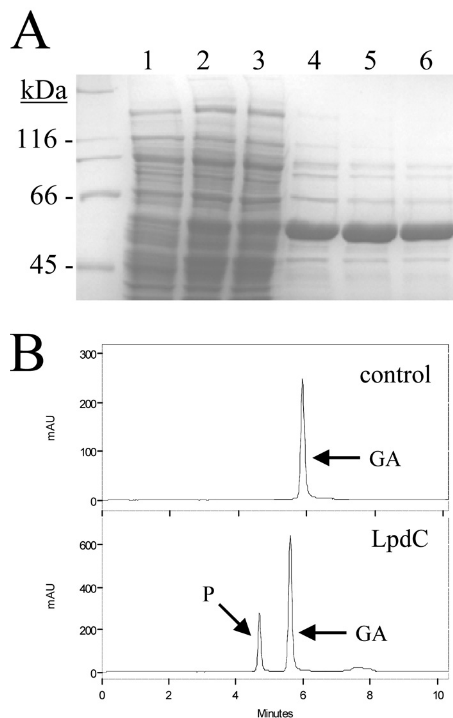

FIG 4 Purification and enzymatic activity of recombinant *L. plantarum* LpdC protein. (A) SDS-PAGE analysis of the expression and purification of the His_6-tagged LpdC. Results of analysis of soluble cell extracts of IPTG-induced *E. coli* BL21(DE3)(pURI3) (lane 1) or *E. coli* BL21(DE3)(pURI3-lpdC) (lane 2), flowthrough from the affinity resin (lane 3), or fractions eluted after His affinity resin (lanes 4 to 6) are shown. The 8% gel was stained with Coomassie blue. Molecular mass markers are located on the left (SDS-PAGE standards; Bio-Rad). (B) Gallate decarboxylase activity of purified His_6-LpdC protein. An HPLC chromatogram of LpdC (100 μg) incubated in 3 mM gallic acid for 1 h (LpdC) is shown. A chromatogram without protein (control) is also shown. The gallic acid (GA) and pyrogallol (P) detected are indicated. Chromatograms were recorded at 280 nm.

volvement of *E. coli* UbiX in the gallate decarboxylase activity observed in *E. coli* extracts could be discarded.

Enzymatic activity of purified of His_6-LpdC. To further investigate the decarboxylase activity of LpdC and the possible involvement of *lpdB* or *lpdB*-like genes, His-tagged proteins LpdB and LpdC were constructed for expression and purification from *E. coli*. His_6-tagged proteins were cloned into *E. coli* BL21(DE3), overproduced, and purified by a one-step affinity procedure as described in Materials and Methods. Only the His_6-LpdC protein showed gallate decarboxylase activity. However, even though His_6-LpdC was produced at a high yield, it presented low gallate decarboxylase activity, as only degradation similar to that of the cell extracts was observed (Fig. 4).

In order to know the involvement of LpdB on activity, reactions were performed by using both purified His_6-LpdB and His_6-LpdC proteins. Decarboxylase activity did not increase due to the presence of the LpdB subunit (see Fig. S2 in the supplemental material). Moreover, *in vitro* protein-protein cross-linking experiments using glutaraldehyde did not show physical interaction between the two proteins (see Fig. S3 in the supplemental material).

Effects of disruption of *lpdB*, *lpdC*, and *lpdD* on gallate decarboxylation by *L. plantarum*. To corroborate previous results, insertion-duplication mutagenesis was employed to construct *L. plantarum* mutants with knockouts in the *lpdB*, *lpdC*, and *lpdD* genes. The correct insertion of the donor plasmids into the *L. plantarum* WCFS1 chromosome was verified by PCR. Unexpectedly, when these mutants were grown in the presence of gallic acid, the *lpdB* and *lpdC* mutants were unable to decarboxylate it to pyrogallol (Fig. 5), suggesting the participation of both proteins, LpdB and LpdC, in the decarboxylation of this hydroxybenzoic acid. Taking into account the probable operonic organization of the *lpdBD* genes (Fig. 2), the *lpdB* mutant could, in fact, be a double-knockout mutant. The *lpdD* mutant was the only mutant able to decarboxylate gallic acid. Similar to gallic acid, protocatechuic acid was decarboxylated in the wild type and disrupted *L. plantarum* cells, except cells in which the *lpdB* and *lpdC* genes were interrupted (Fig. 5). The results obtained from the *L. plantarum* knockout mutants indicate that the B and C subunits of the decarboxylase seem to be essential for gallate and protocatechuate decarboxylase activity in *L. plantarum* WCFS1, whereas the D subunit is not involved.

To ascertain the participation of *lpd* genes in gallate decarboxylase activity, cell extracts were prepared from *L. plantarum* wild type and knockout mutants. The extracts were adjusted to the same protein concentration, and, similar to the reactions with the *E. coli* extracts described above, the reactions were done with mixtures in which these extracts were mixed in all possible combinations in the presence of gallic acid. Reactions were carried out for 18 h, and after that, the phenolic compounds present in the reaction mixtures were extracted with ethyl acetate and analyzed by HPLC.

Figure 6 shows that only the reaction with a mixture which contained a functional copy of the *lpdB* and *lpdC* genes from the same strain was able to decarboxylate gallic acid. Surprisingly, gallate decarboxylase activity was not observed when functional LpdB and LpdC proteins came from different extracts. The only explanation for this result could be that LpdB has a possible role during the maturation (e.g., folding) or activation of LpdC, the main catalytic subunit.

Gallate decarboxylase activity in lactic acid bacteria. Once the direct involvement of *lpd* genes in gallate/protocatechuate decarboxylation was demonstrated, as shown in Fig. 2, it seems probable that other species of lactic acid bacteria could also decarboxylate these aromatic acids. The sequences of LpdB and LpdC proteins from nine lactic acid bacteria were aligned. The degree of identity among these LpdB proteins ranged from 56 to 80% (see Fig. S4 in the supplemental material). The identity shown among the LpdC proteins was higher, ranging from 73 to 90% (see Fig. S5 in the supplemental material). In both cases, proteins from *L. plantarum* and *Lactobacillus pentosus* presented a 98% identity. These alignments allowed us to identify conserved amino acid domains to design degenerate oligonucleotides to detect the presence of both genes by PCR. Oligonucleotides 450 and 451 amplify a 158-bp internal fragment of the subunit B gene in lactic acid bacteria; similarly, oligonucleotides 448 and 449 amplify a 300-bp fragment of the gene encoding subunit C.

In order to associate the presence of the *lpdB* and *lpdC* genes and the ability to degrade gallic and protocatechuic acids, selected strains of lactic acid bacteria (Table 1) were grown in culture medium containing these hydroxybenzoic acids, and their supernatants were analyzed for the production of pyrogallol or catechol. In addition, their DNAs were used as the templates in PCRs using oligonucleotides 450 and 451 and oligonucleotides 448 and 449 to detect the presence of the genes encoding subunits B and C, respectively.

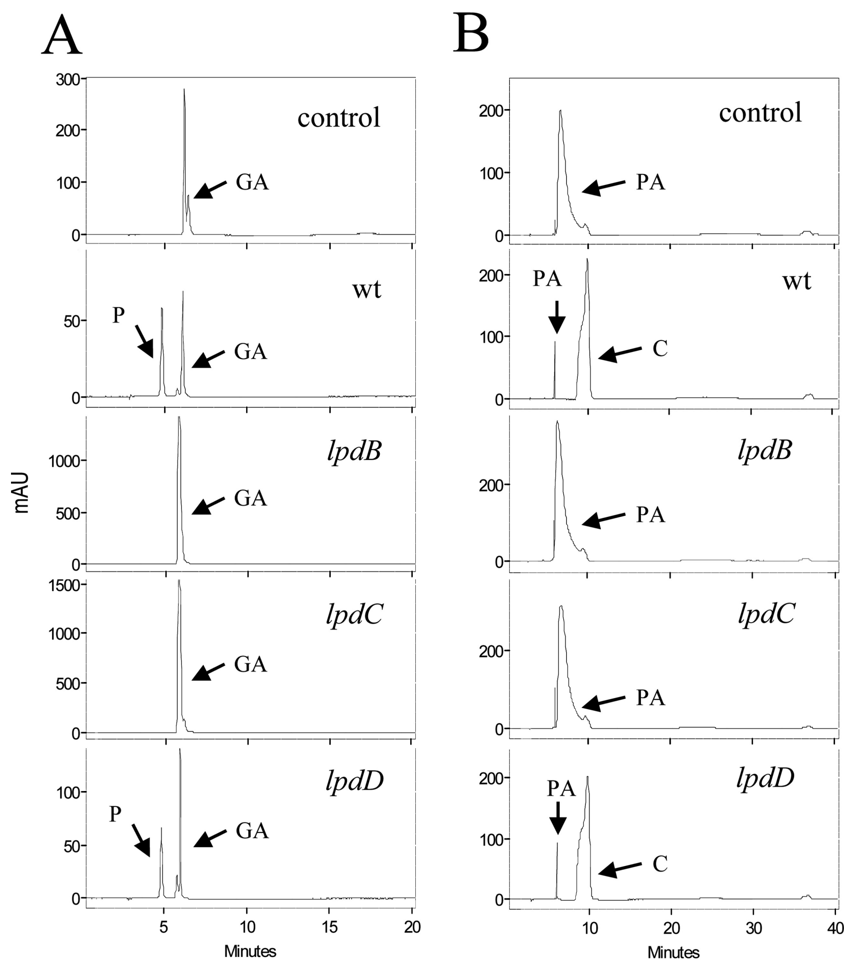

FIG 5 Effects of disruption of *lpdB*, *lpdC*, and *lpdD* on gallate and protocatechuate decarboxylation in *L. plantarum* WCFS1. HPLC chromatograms of *L. plantarum* cultures incubated in 3 mM gallic acid (A) or protocatechuic acid (B) are shown for *L. plantarum* WCFS1 (wild type [wt]), *L. plantarum* WCFS1(pUCE191-lpdB) (*lpdB* mutant), *L. plantarum* WCFS1(pUCE191-lpdC) (*lpdC* mutant), and *L. plantarum* WCFS1(pUCE191-lpdD) (*lpdD* mutant). Results for uninoculated medium are also shown (control). The gallic acid (GA), protocatechuic acid (PA), pyrogallol (P), and catechol (C) detected are indicated. Chromatograms were recorded at 280 nm.

Strains belonging to the species *E. faecium*, *L. brevis*, *L. pentosus*, *L. plantarum*, *O. oeni*, and *S. gallolyticus* amplified fragments from both genes (Fig. 7A and B); *Lactobacillus sakei* DSM 15831^T amplified only the gene encoding subunit C; and finally, strains of the species *Lactobacillus hilgardii*, *Leuconostoc mesenteroides*, and *Pediococcus pentosaceus* did not amplify either of the two genes. These results are mostly in agreement with the information obtained from the complete genome sequences of representative strains of these lactic acid bacterial species. However, unexpected results were obtained with *L. sakei* and *P. pentosaceus*. The genome sequence of *L. sakei* subsp. *sakei* 23K revealed the presence of a copy of the gene encoding subunit B which was absent from the *L. sakei* subsp. *carnosus* DSM 15831^T strain used in this study. Similarly, the genome sequence of *P. pentosaceus* ATCC 25745 revealed the presence of both genes; however, these genes were absent from *Pediococcus claussenii* ATCC BAA-344, as revealed from its sequenced genome, and from *P. pentosaceus* CECT 4695, used in this study. These results might indicate that, at least in these species, the ability to decarboxylate gallic and protocatechuic acids is strain specific.

HPLC analysis of the supernatants from cultures of these bacteria in the presence of gallic or protocatechuic acid indicated that only the bacteria which possess the genes encoding subunits B and C are able to decarboxylate gallic acid to pyrogallol and protocatechuic acid to catechol (Fig. 7C). Strains from the species *E. faecium*, *L. brevis*, *L. pentosus*, *L. plantarum*, and *O. oeni* were able to decarboxylate gallic and protocatechuic acids; however, *L. hilgardii*, *L. mesenteroides*, *L. sakei*, and *P. pentosaceus* strains were not. Therefore, the results obtained seem to indicate that the ability to decarboxylate some hydroxybenzoic acids (gallic and protocatechuic acids) is widely extended among lactic acid bacterial strains. Moreover, the ability to decarboxylate these acids is related to the presence of the B and C subunits of a putative aromatic acid decarboxylase found in these bacteria.

DISCUSSION

Bacterial nonoxidative, reversible multisubunit aromatic acid decarboxylases are encoded by three clustered genes, B, C, and D. The functions of these proteins remain unknown, and the question that arises is, which genes encode the catalytic protein? Initially, when

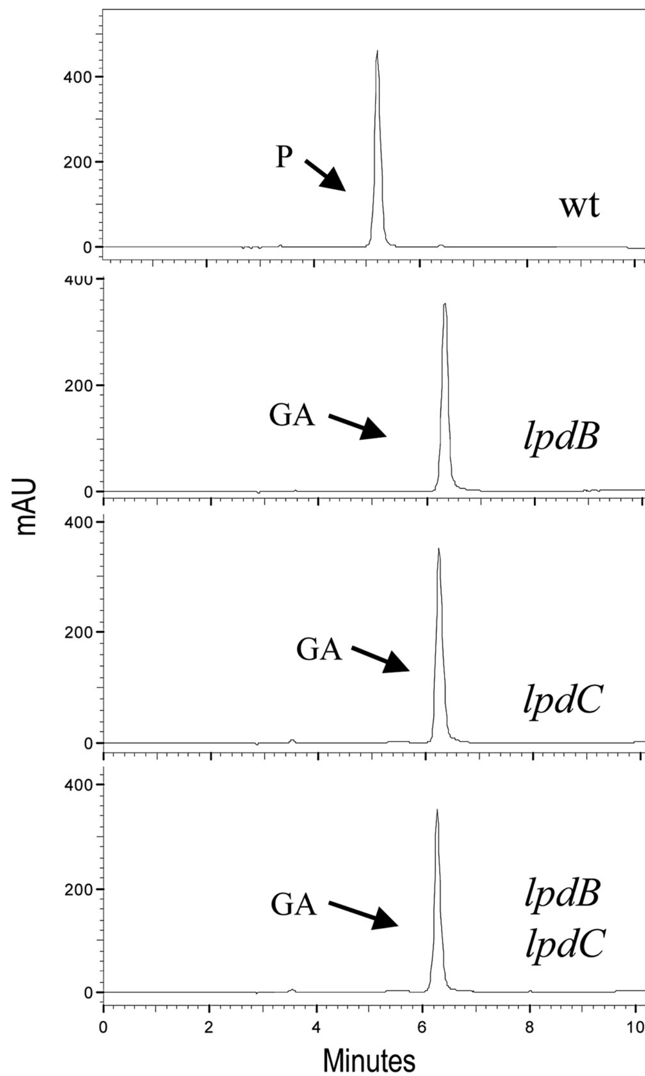

FIG 6 Pyrogallol production by cell extracts from *L. plantarum* knockout mutants. HPLC chromatograms of *L. plantarum* cell extracts, at the same total protein concentration, incubated in 3 mM gallic acid for 18 h are shown for *L. plantarum* WCFS1 (wild type), *L. plantarum* WCFS1(pUCE191-lpdB) (*lpdB* mutant), *L. plantarum* WCFS1(pUCE191-lpdC) (*lpdC* mutant), and *L. plantarum* WCFS1(pUCE191-lpdB) plus *L. plantarum* WCFS1 (pUCE191-lpdC) (*lpdB* and *lpdC* mutants). The gallic acid (GA) and pyrogallol (P) detected are indicated. Chromatograms were recorded at 280 nm.

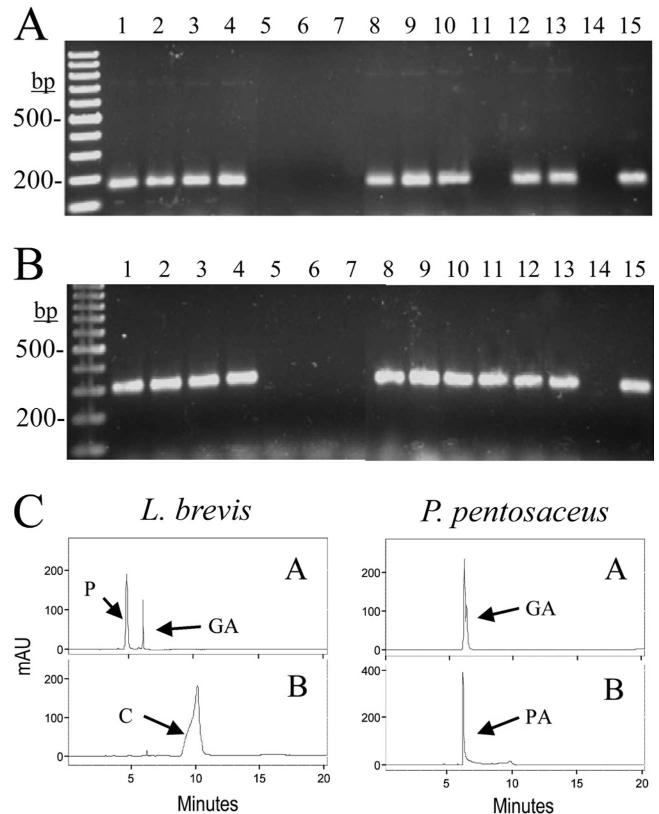

FIG 7 Gallate and protocatechuate decarboxylase activity in lactic acid bacteria. (A and B) PCR amplification of the B and C subunits of putative gallate decarboxylases. Chromosomal DNA from the following strains was used for PCR amplification with oligonucleotides 450 and 451 (A) or oligonucleotides 448 and 449 (B) to amplify 158 bp or 300 bp of the B or C subunit, respectively: *E. faecium* CECT 410^T (lane 1), *E. faecium* CECT 4102 (lane 2), *L. brevis* CECT 4121^T (lane 3), *L. brevis* CECT 5354 (lane 4), *L. hilgardii* RM62 (lane 5), *L. hilgardii* RM63 (lane 6), *L. mesenteroides* CECT 219^T (lane 7), *L. pentosus* DSM 20314^T (lane 8), *L. plantarum* ATCC 14917^T (lane 9), *L. plantarum* WCFS1 (lane 10), *L. sakei* DSM 15831^T (lane 11), *O. oeni* CECT 4100^T (lane 12), *O. oeni* RM1 (lane 13), *P. pentosaceus* CECT 4695^T (lane 14), and *S. gallolyticus* UCN34 (lane 15). PCR products were subjected to gel electrophoresis and stained with Gel Red. Left lane, 100-bp molecular size ladder. Numbers indicate some of the molecular sizes. (C) HPLC chromatograms of supernatants from lactic acid bacteria. The *L. brevis* CECT 5354 and *P. pentosaceus* CECT 4695^T strains were grown in RMP medium containing 3 mM gallic acid (A) or protocatechuic acid (B) for 10 days. The gallic acid (GA), protocatechuic acid (PA), pyrogallol (P), and catechol (C) detected are indicated. Chromatograms were recorded at 280 nm.

these decarboxylases were purified from cell extracts in an active form, the results indicated that only one multimeric protein, composed of identical subunits, was involved. Purified 4-hydroxybenzoate decarboxylase from *S. hydroxybenzoicus* showed a single band on both native gradient PAGE and denatured SDS-PAGE, having an apparent molecular mass of 350 kDa and consisted of six identical subunits of 57 kDa (32). Similarly, in *P. agglomerans*, SDS-PAGE analysis indicated that the purified gallate decarboxylase was homogeneous and consisted of six identical subunits of 57 kDa in molecular mass (6). In addition, purified 4-hydroxybenzoate decarboxylase from *E. cloacae* was a homohexamer of identical 60-kDa subunits (33). The molecular masses of these proteins are in accordance with the masses of their respective C subunits deduced from *in silico* sequence analysis.

However, when the genetic organization of these proteins was known, results involving the activity of the additional B and D subunits were obtained. Contradictory results about the involvement of the B, C, and D subunits in the activity of these decarboxylases were obtained (29, 32, 34, 35). It seems that purification from cell extracts indicated that, on the basis of the size of the purified protein, only the C subunit is involved in enzymatic activity; however, experiments of heterologous expression indicated that the three genes, or at least subunits C and D, are needed for activity (29, 36). It was also speculated that during heterologous expression in *E. coli*, the B subunit can be at least partially replaced by another gene product from *E. coli*, such as UbiX (36).

As shown in Fig. 2, genes encoding nonoxidative decarboxylase are clustered in different organizations in bacteria. However, *L. plantarum* is the only bacterial species in which the genes are sep-

arated in the chromosome by more than 1 Mb. This unusual configuration could indicate a different enzymatic organization. The first result suggesting the involvement of LpdC in gallate decarboxylase activity in *L. plantarum* was that, in induced cell extracts, only this protein was significantly overproduced, as observed in the 8 and 15% SDS-PAGE analysis (Fig. 1). In addition, only the LpdC protein was overproduced (more than 7-fold) in *L. plantarum* when it was exposed to a tannic acid challenge, as revealed by a proteomic analysis (37). Similarly, only the proteins equivalent to LpdC were overproduced in response to other phenolic acids, such as protein 3717 (VdcC) in *Streptomyces* sp. D7 upon exposure to vanillic acid (34) and BsdC in *B. subtilis* in response to salicylic acid (38). However, in *Streptomyces* sp. D7 as well as in *B. subtilis*, it has been described that at least subunits C and D are required to confer decarboxylase activity (29, 36).

The only involvement of LpdC in *L. plantarum* decarboxylation also arose from *E. coli* extracts producing LpdB, LpdC, and LpdD proteins. The three proteins were independently overproduced in *E. coli*, and the expression of *lpdC* was enough to confer gallate decarboxylase activity to *E. coli*, even in an *E. coli* UbiX-defective mutant. Finally, to ascertain the exclusive role of LpdC in gallate decarboxylase activity, the recombinant LpdC protein was purified and gallate decarboxylase activity was demonstrated *in vitro*. However, even though LpdC was produced in a high yield, it presented low gallate decarboxylase activity. It is noteworthy that addition of pure LpdB protein did not increase the activity of LpdC. It could be argued that the presence of a His tag could result in differences in activity. More likely, the low activity observed could be because these enzymes are extremely unstable in usual buffer solutions due to their oxygen sensitivity (6, 7). In addition, the batch purification protocol followed for protein purification and the presence of immobilized metal ions (cobalt) in the resin could contribute to the inactivation of the enzyme.

Unexpected results were obtained by the use of *L. plantarum* mutants with knockouts in the three *lpd* genes, as it was demonstrated that the disruption of subunit B and subunit C avoids gallate decarboxylase activity in *L. plantarum*. Decarboxylase activity was restored only when extracts containing functional B and C proteins were present. These results could be compatible with those obtained from *E. coli* extracts only if a protein from *E. coli* could assume the function of LpdB. In spite of the similarity of UbiX and LpdB, the expression of *lpdC* in a *ubiX*-negative *E. coli* mutant (*E. coli* JW2308-4) indicates that UbiX is not assuming the LpdB function. An unknown *E. coli* protein different from UbiX could be involved. The involvement of the B subunit in the decarboxylation reaction has also been clearly demonstrated in *B. subtilis* since antisense mRNA inactivation of the B subunit highly reduces the enzyme activity to below 2% of that of the wild type (29, 36).

The biochemical activities of the three different protein subunits have not been assigned. So far, it has not been possible to unequivocally correlate genes coding for aromatic acid decarboxylase and their function. In this study, interesting results came from the use of *L. plantarum* knockout mutants. In *L. plantarum*, the LpdD protein did not seem to be necessary for gallate decarboxylase activity, while the LpdC protein seemed to be the main catalytic subunit. However, the function of LpdB is unknown. To achieve gallate decarboxylase activity fully, it is not enough to have functional copies of the LpdB and LpdC proteins, since it seems that both proteins need to be synthesized in the same strain. Both mature proteins do not seem to interact, as revealed by the *in vitro* cross-linking experiments. It is tempting to speculate that LpdB could have a possible role during the maturation (e.g., folding) or activation of LpdC, and therefore, LpdB and LpdC need to be synthesized simultaneously in the same host. The mechanism of decarboxylation followed by these aromatic acid decarboxylases is a paradigm for a new type of biological decarboxylation reaction. As far as we know, this study in *L. plantarum* constitutes the first description of the involvement of only subunits B and C in the nonoxidative decarboxylation of an aromatic acid.

Apart from *E. faecalis*, among lactic acid bacteria, decarboxylation of aromatic acids has been described only in *L. plantarum* (11), *L. brevis* (39), and *S. gallolyticus* (8, 40). These bacteria decarboxylate the same hydroxybenzoic acids, gallic acid and protocatechuic acid, and all possess genes similar to the gallate decarboxylase genes described in this work (Fig. 2). Such decarboxylase activity has never been described in *E. faecium* and *O. oeni* species; however, strains from these species also decarboxylate both acids and possess both genes (Fig. 2 and 7). From the data obtained in this study, it could be assumed that, at least in some bacterial species, the ability to decarboxylate gallic and protocatechuic acids might be strain dependent, similar to the ability of some specific *E. coli* strains (e.g., strains EDL933 and VT2-Sakai from *E. coli* O157:H7) to decarboxylate 4-hydroxybenzoate (29).

The identification of the *L. plantarum* gallate decarboxylase involved in tannin degradation completes the analysis of the first route of degradation of a phenolic compound in lactic acid bacteria. The proposed biochemical pathway for the degradation of tannins by *L. plantarum* implies that tannins are hydrolyzed to gallic acid and glucose by a tannase action, and the gallic acid formed is decarboxylated to pyrogallol by the action of a gallate decarboxylase (9, 10). When purified *L. plantarum* tannase was assayed against 18 phenolic acid esters, only esters derived from gallic and protocatechuic acids were hydrolyzed (41), with these esters apparently sharing the same substrate specificity as the decarboxylase enzyme. This substrate specificity suggests a concomitant activity of tannase and gallate decarboxylase on specific phenolic substrates. This is more obvious when the chromosomal location of these genes is considered. The genes encoding gallate decarboxylase (*lp_2945*) and tannase (*lp_2956*) are only 6.5 kb apart on the *L. plantarum* WCFS1 chromosome. Interestingly, in *S. gallolyticus*, another tannin-degrading lactic acid bacterium, the gene encoding tannase (GALLO_1609) is separated by only one open reading frame from the genes encoding decarboxylase (GALLO_1611, GALLO_1612, and GALLO_1613) (Fig. 2). More interestingly, *S. gallolyticus* strains showed metabolism of these phenolic compounds identical to that of *L. plantarum*, as they hydrolyzed hydrolyzable tannins to release gallic acid, which was subsequently decarboxylated to pyrogallol, and protocatechuic acid, which was decarboxylated to catechol (8, 40). Neither the *S. gallolyticus* nor *L. plantarum* bacterial species possesses appropriate mechanisms to further degrade the compounds produced by these dead-end pathways. The physiological relevance of these reactions is unknown, but in natural ecosystems, it could be imagined that other organisms in a consortium mineralize and remove these dead-end metabolites. These enzymatic activities have ecological advantages for *L. plantarum*, as it is often associated with fermentation of plant materials. Therefore, *L. plantarum* plays an important role when tannins are present in food and the intestine, having the capability of degrading and detoxifying harmful and antinutritional constituents into simpler and innocuous compounds.

ACKNOWLEDGMENTS

This work was supported by grants AGL2008-01052, AGL2011-22745, Consolider INGENIO 2010 CSD2007-0063 FUN-C-FOOD (Comisión Interministerial de Ciencia y Tecnología), S2009/AGR-1469 (ALIBIRD; Comunidad de Madrid), and RM2008-00002 (Instituto Nacional de Investigación Agraria y Alimentaria). N. Jiménez is the recipient of an FPI fellowship from MINECO.

We are grateful to J. L. Ruíz-Barba for his help with the *L. plantarum* electroporation experiments and to M. V. Santamaría and J. M. Barcenilla for their assistance.

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