Tannin degradation by a novel tannase enzyme present in some *Lactobacillus plantarum* strains

Natalia Jiménez a, María Esteban-Torres a, José Miguel Mancheño b, Blanca de las Rivas a, Rosario Muñoz a,*,

a Laboratorio de Biotecnología Bacteriana, Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN-CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

b Grupo de Cristalografía y Biología Estructural, Instituto de Química Física “Rocasolano” (IQFR-CSIC), Serrano 119, 28006 Madrid, Spain

Running Title

NOVEL *LACTOBACILLUS PLANTARUM* TANNASE

Address correspondence to Rosario Muñoz, r.munoz@csic.es
*Lactobacillus plantarum* is frequently isolated from the fermentation of plant material where tannins are abundant. *L. plantarum* strains possess tannase activity to degrade plant tannins. A *L. plantarum* tannase (TanLp1) has been previously identified and biochemically characterized. In this study, we report the identification and characterization of a novel tannase (TanLp2). While all the 29 *L. plantarum* strains analyzed in this study possess the tanLp1 gene, the gene tanLp2 was only present in four strains. Upon methyl gallate exposure, the expression of tanLp1 was induced, whereas tanLp2 expression was not affected. TanLp2 showed only 27% sequence identity to TanLp1 but the residues involved in tannase activity are conserved. Optimum activity for TanLp2 was observed at 30 °C and pH 6 in presence of Ca$^{2+}$ ions. TanLp2 was able to hydrolyze gallate and protocatechuate esters having a short aliphatic alcohol substituent. Moreover, TanLp2 was able to fully hydrolyze complex gallotannins such as tannic acid. The presence of the extracellular TanLp2 tannase in some *L. plantarum* strains will provide them an advantage for the initial degradation of complex tannins present in plant environments.
INTRODUCTION

Tannins are present in a variety of plants which are utilized as food and feed (1). Tannins seem to be a double-edged sword since they are beneficial to health due to their chemopreventive activities against carcinogenesis and mutagenesis, but simultaneously they may be involved in cancer formation, hepatotoxicity or antinutritional activity (2). The molar mass of tannin molecules affects the tannin characteristics directly. It has been found that the higher the molar mass of tannin molecules, the stronger the antinutritional effects and the lower the biological activities (2). Small molecule tannins are suggested to have less anti-nutritional effects and can be more readily absorbed.

Based on the molecular structure, origin of currently known tannins, and their roles in plant life, tannins are defined as polyphenolic secondary metabolites of higher plants, and they are either galloyl esters or their derivatives, in which galloyl moieties or their derivatives are attached to a variety of polyol-, catechin-, and triterpenoid cores, or they are oligomeric and polymeric proanthocyanidins that can possess different interflavanyl coupling and substitution pattern (3). Gallotannins are those in which galloyl units or their meta-depsidic derivatives are bound to diverse polyol-, catechin-, or triterpenoid units. Upon hydrolysis by acids, bases or certain enzymes, gallotannins yield glucose and gallic acid (4).

Though tannins have toxic effects on various organisms, some microorganisms are resistant to tannins and have the ability to degrade them into oligomeric tannins and other useful derivatives such as gallic acid or pyrogallol. Gallotannins are degraded by some bacteria, fungi and yeasts, which can only hydrolyze the galloyl residues of galloyl esters of tannins. Tannin acyl hydrolase (EC. 3.1.1.20), commonly known as tannase, catalyzes the hydrolysis of the galloyl ester bond of tannins. Tannase belongs to the superfamily of esterases. Since its discovery, tannase has found wide applications
in the food, feed, beverage, pharmaceutical, and chemical industries (5). Despite the
extensive interest and long history of the study on tannase, there is surprisingly little
knowledge about the enzyme at the molecular level, which has become one of the
critical factors that limit the large-scale application of tannase. To our knowledge, the
only bacterial tannases that have been analyzed genetically are those from
Staphylococcus lugdunensis (6), Lactobacillus plantarum (7, 8), and Enterobacter sp.
(9). In addition, L. plantarum tannase has been biochemically and structurally
characterized (7-8, 10).

*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 possess tannase activity (11-13). The biochemical pathway
for the degradation of tannins by *L. plantarum* involved the action of a tannase and a
gallate decarboxylase to decarboxylate the gallic acid formed by tannase action (14-16).
The *L. plantarum* genes encoding tannase (*tanLp1*) (7) and gallate decarboxylase
(*lpdBCD*) (16) involved in tannin degradation have been identified. However, an
additional putative *L. plantarum* tannase sequence has been annotated in the genome of
a *L. plantarum* strain. In this work, we have characterized the biochemical properties of
this novel tannase. The presence of this tannase has been analyzed among *L. plantarum*
strains. Finally, the relative expression of both tannase genes under methyl gallate
exposure has been studied.

**MATERIALS AND METHODS**
Strains and growth conditions. A total of 29 strains of *L. plantarum* were used in this study. *L. plantarum* WCFS1, NC8, and LPT 57/1 strains were kindly provided by M. Kleerebenzem (NIZO Food Research, The Netherlands), L. Axelsson (Norwegian Institute of Food, Fisheries and Aquaculture Research, Norway), and J. L. Ruiz-Barba (Instituto de la Grasa, CSIC; Spain), respectively. Eight strains were provided by the Spanish Type Culture Collection (CECT): *L. plantarum* CECT 220 (ATCC 8014), CECT 221 (ATCC 14431), CECT 223, CECT 224, CECT 749 (ATCC 10241), CECT 4645 (NCFB 1193), and the type strain *L. plantarum* subsp. *plantarum* CECT 748<sup>T</sup> (ATCC 14917, DSMZ 20174). Seven strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ): *L. plantarum* DSM 1055, DSM 2648, DSM 10492, DSM 12028, DSM 13273, DSM 20246, and the type strain of *L. plantarum* subsp. *argentoratensis* DSM 16365<sup>T</sup>. Eleven strains were isolated from must grape or wine of different wine-producing areas of Spain over the period from 1998 to 2001: *L. plantarum* RM28, RM31, RM34, RM35, RM38, RM39, RM40, RM41, RM71, RM72, and RM73) (17). *L. plantarum* strains were cultivated in a modified basal and defined medium described previously for *L. plantarum* (18). This basal media was modified by the replacement of glucose by galactose. This defined medium was used to avoid the presence of phenolic compounds included in nondefined media. The sterilized modified basal media was supplemented with tannic acid (1 mM) filter sterilized. The *L. plantarum*-inoculated media was incubated in darkness without shaking, at 30 °C for 10 days. Incubated media with cells and without phenolic compound and incubated media without cells and with phenolic compounds were used as controls. The phenolic products were extracted from the supernatants twice with ethyl acetate (one third of the reaction volume).
Escherichia coli DH10B was used for all DNA manipulations. E. coli BL21(DE3) was used for expression in the pURI3-TEV vector (19). E. coli strains were cultured in Luria-Bertani (LB) medium at 37 °C and 140 rpm. When required, ampicillin was added to the medium at a concentration of 100 μg/ml.

**PCR detection of tannase encoding genes.** Genes encoding *L. plantarum* tannases (*tanLp1* and *tanLp2*) were amplified by PCR using chromosomal DNA from several lactic acid bacterial strains. The *tanLp1* gene (1.4 kb) was amplified by using primers 951 (5´-TGATGCTGACTGGCTGGTGC) and 952 (5´-GCACAAGCCATCAATCCAGG). Oligonucleotides 953 (5´-CCTGATGAGTGGTTTGTTAG) and 954 (5´-CTTGCGTTCTGCTTCGGTATG) were used to amplify *tanLp2* gene (1.8 kb). 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. Amplified fragments were resolved in agarose gels.

**RNA isolation, RT-PCR and qPCR.** For RNA isolation, *L. plantarum* MRS cultures were grown up to an OD 600nm of 1 and then supplemented with methyl gallate at 15 mM final concentration. As control, RNA was also isolated from cultures not supplemented with methyl gallate. After 10 min incubation the cultures were immediately processed for RNA extraction as previously described (20). After DNaseI treatment, the absence of DNA from the RNA samples was verified by PCR. The DNA-free RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer instructions. From the DNA obtained, quantitative gene expression was analyzed in an AbiPrism 7500 Fast Real Time PCR system (Applied Biosystems). Specific primer pairs were designed with the Primer Express 3.0 program to amplify internal regions of tannase.
genes. Oligonucleotides 1168 (5´- TGCGCTACCGTGGGATATTC) and 1169 (5´- AATCCAGGAAAATAATCGCTAA) were used to amplify 64 bp of tanLp1, and primers 1286 (5´- AAAGCAAGCTACGCCAAAGC) and 1287 (5´- CCCTGGGCATCCGTCTTC) a 56 bp fragment of tanLp2. The expression level of the endogenous control gene (ldh) was assayed by primers 918 (5´- AACCGCGACAATGTTTTGATT) and 919 (5´- TTGTGAACGCGCAGTTTCAGTGT). Amplifications were performed in triplicate. All qPCR assays amplified a single product as determined by melting curve analysis and by electrophoresis. A standard curve was plotted with cycle threshold (Ct) values obtained from amplification of known quantities of cDNA and used to determine the efficiency (E) as E=10^{-1/slope}. In order to measure L. plantarum gene expression, amplification of the endogenous control gene was performed simultaneously and its relative expression compared with that of the target gene. Relative expression levels were calculated with the 7500 Fast System relative quantification software using L. plantarum ldh gene as endogenous gene and the growth in the absence of methyl gallate as growth condition calibrator.

Expression and purification of TanLp2 from L. plantarum ATCC 14917T. As a peptide signal was predicted in the TanLp2 sequence, the gene tanLp2 in the locus HPREF0531_11477 from L. plantarum ATCC 14917T was PCR amplified but lacking the 22-amino acid peptide signal. The gene was amplified by Prime Star HS DNA polymerase (TaKaRa) by using the primers 805 (5´- GGTGAAAACCTGTATTTCAGGGCgcttgccgagctcagaaacgaag) and 637 (5´- ATCGATAAGCTTAGTTAGCTATtacctcagcttgctgacccactta) (the nucleotides pairing with the expression vector sequence are in italics, and the nucleotides pairing with the tanLp2 gene sequence are in lowercase letters). The gene was cloned into the pURI3-TEV vector which encodes expression of a leader sequence containing a six-histidine
affinity tag. The purified PCR product was then inserted into the pURI3-TEV vector by using a restriction enzyme- and ligation-free cloning strategy (19). *E. coli* DH10B cells were transformed, and the recombinant plasmids were isolated. Those containing the correct insert were used for transformation of *E. coli* BL21(DE3) cells.

*E. coli* cells carrying the recombinant pURI3-TEV-TanLp2 plasmid were grown at 37 °C in LB medium containing ampicillin (100 μg/ml) and induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, the cells were grown at 22 °C for 20 h and harvested by centrifugation (7,500 x g for 15 min at 4 °C). The cells were resuspended in 50 mM sodium phosphate buffer, pH 7.0, containing 300 mM NaCl. Crude extracts were prepared by French press lysis of cell suspensions (three cycles at 1,100 lb/in2). The lysates were centrifuged at 17,400 x g for 40 min at 4 °C. The supernatant obtained was filtrated through 0.22 μm filter (Millipore) and gently mixed for 20 min at room temperature with 1 ml Talon resin (Clontech). The resin was washed with 50 mM sodium phosphate buffer, pH 7.0, containing 300 mM ClNa and 10 mM imidazole. The recombinant His6-tagged protein was eluted with 50 mM sodium phosphate, pH 7.0, containing 300 mM NaCl and 150 mM imidazole. The eluted His6-tagged-TanLp2 was dialyzed overnight at 4 °C against 50 mM sodium phosphate buffer, pH 7.0, containing 300 mM NaCl. The purity of the enzyme was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-Glycine buffer.

**Tannase activity assay.** Since tannase catalyzes the hydrolysis of the galloyl ester linkage liberating gallic acid, the activity of tannase could be measured by estimating the gallic acid formed due to enzyme action (21). A method specific for the detection of gallic acid could be used for a reliable quantification of tannase activity.

Inoue and Hagerman described a rhodanine assay for determining free gallic acid (22).
Rhodanine reacts only with gallic acid and not with galloyl esters of other phenolics. Rhodanine reacts with the vicinal hydroxyl groups of gallic acid to give a red complex with a maximum absorbance at 520 nm. Since the rhodanine assay using commercial tannic acid as substrate gives high absorbance values due to small amounts of free gallic acid present in the preparation, methyl gallate was used as substrate.

Gallic acid analysis in reactions was determined in triplicate by using the following assay. TanLp2 (10 μg) in 700 μl of 50 mM phosphate buffer pH 6.5 was incubated with 40 μl of 25 mM methyl gallate (1 mM final concentration) during 5 min at 37 ºC. After incubation, 150 μl of a methanolic rhodanine solution (0.667% rhodanine in 100% methanol) was added to the mixture. After 5 min incubation at 30 ºC, 100 μl of 0.5 M KOH was added. The absorbance at 520 nm was measured on a spectrophotometer. A standard curve using gallic acid concentration ranging from 0.125 to 1 mM was prepared. One unit of tannase activity was defined as the amount of enzyme required to release 1 μmol of gallic acid per minute under standard reaction conditions.

**Biochemical characterization of TanLp2.** Activities of TanLp2 from *L. plantarum* ATCC 14917T were measured at 4, 20, 30, 37, 45, 55, and 65 ºC to determine the optimal temperature for enzymatic activity. The optimum pH value for tannase activity was determined by studying its pH-dependence within the pH range between 3 and 10. Acetic acid-sodium acetate buffer was used for pH 3-5, citric acid-sodium citrate buffer for pH 6, sodium phosphate buffer for pH 7, Tris-HCl buffer for pH 8, glycine-NaOH buffer for pH 9, and sodium carbonate-bicarbonate for pH 10. A 100 mM concentration was used in all the buffers. The rhodanine assay was used for the optimal pH characterization of TanLp2. Since the rhodanine-gallic acid complex forms only in basic conditions, after the enzymatic degradation of methyl gallate, KOH was added.
added to the reaction mixture to ensure that the same pH value (pH 11) was achieved in all samples assayed.

For temperature stability measurements, TanLp2 was incubated in 50 mM phosphate buffer pH 6.5 at 22, 30, 37, 45, 55, and 65 °C for 15 min, 30 min, and 1, 2, 5, and 18 h. After incubation, the residual activity was measured as described above.

To test the effect of metals and ions on the activity of TanLp2, the enzymatic activity was measured in the presence of different additives at a final concentration of 1 mM. The additives analyzed were MgCl₂, KCl, CaCl₂, HgCl₂, ZnCl₂, Triton-X-100, urea, Tween 80, EDTA, DMSO, and β-mercaptoethanol. All the determinations were done in triplicate.

**TanLp2 substrate specificity analysis by HPLC.** The activity of TanLp2 against 26 potential substrates has been analyzed. The substrates assayed were gallic esters (methyl gallate, ethyl gallate, propyl gallate, and lauryl gallate), benzoic esters (methyl benzoate, and ethyl benzoate), hydroxybenzoic esters (methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, and butyl 4-hydroxybenzoate), vanillic ester (methyl vanillate), dyhydroxybenzoic esters (methyl 2,4-dihydroxybenzoate, ethyl 3,4-dihydroxybenzoate or protocatechuic acid ethyl ester, and ethyl 3,5-dihydroxybenzoate), gentisic ester (methyl gentisate), salicylic ester (methyl salicylate), and ferulic esters (ferulic methyl ester and ferulic ethyl ester).

Tannic acid and epigallocatechin gallate were also assayed as potential substrates.

Tannase (50 μg), in phosphate buffer pH 6.0 (50 mM), CaCl₂ (1 mM) was incubated at 37 °C in presence of the substrate (1 mM). As controls, phosphate buffer containing the reagents but the enzyme were incubated in the same conditions. The reaction products were extracted twice with ethyl acetate; the solvent fractions were filtered through a 0.45 μm PVDF filter and analyzed by HPLC-DAD. A Thermo (Thermo Electron
Corporation, Waltham, Massachusetts, USA) chromatograph equipped with a P4000 SpectraSystem pump, and AS3000 autosampler, and a UV6000LP photodiode array detector were used. A gradient of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied to a reversed-phase Nova-pack C\textsubscript{18} cartridge (25 cm x 4.0 mm i.d., 4.6 \textmu m particle size) at room temperature as follows: 0-55 min, 80\% B linear, 1.1 ml/min; 55-57 min, 90\% B linear, 1.2 ml/min; 57-70 min, 90\% B isocratic, 1.2 ml/min; 70-80 min, 95\% B linear, 1.2 ml/min; 80-90 min, 100\% linear, 1.2 ml/min; 100-120 min, washing 1.0 ml/min, and reequilibration of the column under initial gradient conditions. Samples were injected onto the cartridge after being filtered through a 0.45 \textmu m PVDF filter. Detection of the substrates and the degradation compounds was performed spectrophotometrically by scanning from 220 to 380 nm. 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 or by LC-DAD/ESI-MS.

**Sequence data analysis.** A homology search with finished and unfinished microbial genome databases was performed with the BLAST 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 W2 Program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) on the EBI site, after retrieval of sequences from the GenBank and Swiss-Prot databases. Computer promoter predictions carried out at the Internet site (http://www.fruitfly.org/seq_tools/promoter.html). \text{pI} and MW were analysed on EXPASY (http://web.expasy.org/compute_pi/), signal peptide cleavage sites were analyzed on SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) site, and
predicted transcription terminators at ARNold site (http://rna.igmors.u-
psud.fr/toolbox/arnold/index.php#Results).

RESULTS

Presence of a putative novel tannase-encoding gene in several L. plantarum strains.

Homology searches in genome databases allowed finding the locus HMPREF0531_11477 in the L. plantarum ATCC 14917^T genome, which encodes a 626 amino acid residue protein annotated as “tannase”, herein defined as TanLp2.

Interestingly, multiple amino acid sequence alignments of TanLp2 from L. plantarum ATCC 14917^T with TanA tannase from S. lugdunensis, and TanLp1 tannase from L. plantarum revealed high sequence identity to TanA (50%) and much lower to TanLp1 (27%) (Fig. S1). Moreover, TanLp2 shares additional features to TanA from S. lugdunensis. Both proteins are 67 kDa proteins, have alkaline isoelectric points (9.54 for TanA, and 9.94 for TanLp2), and they present predicted signal peptides. Contrarily, TanLp1 is a 50 kDa protein, having an isoelectric point of 6.06, and it does not possess a signal peptide.

Based on the recently described crystal structure from TanLp1 (10), the conserved sequence motif Gly-X-Ser-X-Gly typical of serine hydrolases could be easily identified in TanLp2 from L. plantarum ATCC 14917^T (Gly-215 to Gly-219 in TanLp2). From the catalytic triad identified in the structure (Ser-163, His-451, and Asp-419, in TanLp1), only serine and histidine residues are conserved in TanLp2 as well as in TanA (Fig. S1), with Asp-419 being substituted by a Gln residue in both proteins. Contrarily, the residues which make contacts with the three hydroxyl groups of gallic
acid (Asp-421, Lys-343, and Glu-357, in TanLp1) are conserved in both TanLp2 and TanA (Fig. S1).

The genomes of ten *L. plantarum* strains are currently available. Analyses of these genomes revealed that a copy of the *tanLp2* gene is present only in *L. plantarum* ATCC 14917\(^T\) and *L. plantarum* NC8, not being found in the rest (*L. plantarum* WCFS1, JDM1, ST-III, 16, P-8, IPLA 88, UCMA 3037, and ZJ316). On ATCC 14917\(^T\) and NC8 strains, *tanLp2* is located between the genes *nox3* (encoding a NADH oxidase) and *dapE1* (encoding succinyl-diaminopimelate desuccinylase) (Fig. 1). The intergenic region between these latter genes is 121 bp long in the *L. plantarum* strains devoid of *tanLp2*; however, in the strains possessing *tanLp2*, this region is 2515 or 2516 bp long in NC8 or ATCC 14917\(^T\), respectively. In *L. plantarum* ATCC 14917\(^T\), this region encodes a 626 amino acid residues protein (TanLp2), which is preceded by a putative promoter as revealed by sequence analysis. In turn, a putative transcription terminator site follows the stop codon, where a possible stem-loop structure is predicted, which would start 45 nucleotides downstream from the TAA stop codon and would have a 19-base stem and a 22-base loop. This structure may serve as a terminator for transcription (Fig. 1). *L. plantarum* NC8 sequence is identical to ATCC 14917\(^T\) with the exception of a G deletion, which produce a frameshift from Gln-529 to the end of TanLp2 (Fig. S2).

In order to know the extend of the tannase genes among *L. plantarum* strains, the presence of the *tanLp1* and *tanLp2* genes was studied in 29 of *L. plantarum* strains isolated from different sources. To determine the presence of both genes, chromosomal DNA was extracted and PCR amplified. DNA fragments of 1.4 or 1.8-kb from *tanLp1* or *tanLp2*, respectively, were PCR amplified using oligonucleotides designed on the basis of the *L. plantarum* ATCC 14917\(^T\) sequence. All the *L. plantarum* strains analyzed gave the corresponding *tanLp1* amplicon (data not shown), which indicates that TanLp1
is generally present among *L. plantarum* strains, as described previously (7). Apart from ATCC 14917^T^ and NC8, two additional strains also possessed a copy of the *tanLp2* gene, namely, *L. plantarum* CECT 749 and RM35 (data not shown). Since the NC8 *tanLp2* copy is truncated, the complete sequences of the CECT 749 and RM35 strains were determined. Alignment of the resulting TanLp2 amino acid sequences revealed one substitution in the CECT 749 protein (Ala-107 to Asp-107), and two in the RM35 strain (Arg-563 to Lys-563, and Arg-565 to Gln-565) (Fig. S2). It is noteworthy to mention that these two mutated arginine residues are part of a motif (WRIR) conserved in all tannases (Fig. S1).

**Extracellular tannase activity on *L. plantarum* strains.** Since the sequences of the four *tanLp2* copies present in the *L. plantarum* strains herein analysed differ, an activity assay was done to determine the functionality of the resulting coded proteins. In this regard, the presence of a putative signal peptide indicated that TanLp2 could be an extracellular protein and in fact, an extracellular tannase produced by a *L. plantarum* strain have been reported (24). *L. plantarum* WCFS1 and the four strains possessing a *tanLp2* copy were grown in a basal medium containing 1 mM tannic acid for 10 days. Tannic acid was chosen as it is a complex gallotannin unable to pass into the cell to be degraded by intracellular TanLp1 tannase. As control, the medium was incubated in the same conditions. From the *L. plantarum* culture, the cells were pelleted, and the tannic acid in the supernatant was extracted and analyzed by HPLC. Figure 2 shows that most of the chromatograms were similar to the control and only small variations were observed among them. However, *L. plantarum* ATCC 14917^T^ showed a chromatogram that clearly indicated hydrolysis of the high molecular weight tannins, suggesting the presence of an active extracellular tannase only in this strain.
Relative expression of *L. plantarum* tannase genes under methyl gallate exposure. As tannase is involved in tannin degradation, the relative expression of both tannase-encoding genes under methyl gallate exposure was studied. Strain WCFS1 was analyzed as a model of strain with only one tannase enzyme, and strain ATCC 14917\(^T\) as model strain having two different active tannase proteins. *L. plantarum* cultures were induced for 10 min by the presence of 15 mM methyl gallate as potential tannase substrate. The gene expression levels obtained were substantially different among both tannase-encoding genes (data not shown), indicating the presence of two different expression patterns for these proteins. The *tanLp2* gene, only present in *L. plantarum* ATCC 14917\(^T\) strain, showed an expression level not affected by the presence of its substrate methyl gallate. However, the *tanLp1* gene expression profiles were affected. In both *L. plantarum* strains, the presence of 15 mM methyl gallate induces about a 3-fold increase in the expression of *tanLp1* gene.

**Biochemical properties of TanLp2 from *L. plantarum* ATCC 14917\(^T\).** From the *L. plantarum* strains possessing two tannase enzymes, only *L. plantarum* ATCC 14917\(^T\) showed extracellular tannase activity, therefore TanLp2 from this strain was biochemically characterized. The *tanLp2* gene lacking the 22-amino acid peptide signal sequence from *L. plantarum* ATCC 14917\(^T\) was expressed in *E. coli* under the control of an inducible promoter. Cell extracts were used to detect the presence of overproduced proteins by SDS-PAGE analysis. Control cells containing the pURI3-TEV vector plasmid did not show protein overexpression; in addition, no tannase activity was observed on this control extract. However, an overproduced protein with an apparent molecular mass around 67 kDa was observed in cells harbouring pURI3-TEV-TanLp2 (Fig. 3). Since the cloning strategy would yield a His-tagged protein variant, *L. plantarum* pURI3-TEV-TanLp2 could be purified on an immobilized metal affinity
chromatography (IMAC) resin. However, unexpectedly, the protein was scarcely purified (2.71 mg/l), with most of the protein being unbound to the resin.

The *L. plantarum* ATCC 14917^T^ TanLp2 enzyme, partially purified by the affinity resin was biochemically characterized. Tannase activity was determined by using methyl gallate as substrate. The specific activity of TanLp2 and TanLp1 (taken as reference) was determined by the rhodanine assay. TanLp2 has a specific activity of 39 U/mg, whereas TanLp1 has 404 U/mg.

Figure 4 shows the optimum temperature, pH and the thermal stability of TanLp2 as determined by the rhodanine assay with methyl gallate as substrate. TanLp2 displays optimal activity within the 20 - 30 ºC temperature range and an optimal pH around 6. Figure 4D shows the effect of various additives (1 mM final concentration) on the enzymatic activity of TanLp2. CaCl₂ greatly increased, and HgCl₂ completely inhibited TanLp2 activity. In relation to TanLp2 substrates, similarly to TanLp1, only the esters derived from gallic and protocatechuic acid were hydrolyzed (Fig. S3). It seems that other cinnamic acids without hydroxyl groups and with substituents other than –H or –OH at position 2 were not metabolized by TanLp2 as well as by TanLp1. Regarding the aliphatic alcohol constituent of the ester bond, lauryl substituent could not be effectively hydrolyzed by TanLp2 (Fig. S3).

**DISCUSSION**

Despite tannase activity has been extensively described in *L. plantarum* strains (12-14), the first bacterial tannase genetically identified was TanA from *S. lugdunensis* (6), and then, by sequence comparison, TanLp1 (Lp_2956) from *L. plantarum* was later identified (7). The first complete genome of a *L. plantarum* strain was from the WCFS1
strain. However, nowadays the sequences of several *L. plantarum* strains are publicly available. In *L. plantarum* ATCC 14917\(^\text{T}\), the locus HMPREF0531_11477 was annotated as “tannase” and designated as TanLp2. TanLp2 from *L. plantarum* ATCC 14917\(^\text{T}\) showed higher sequence identity to TanA from *S. lugdunensis* (50%) than to *L. plantarum* TanLp1 tannase (27%). Despite the low identity shared to TanLp1, TanA and TanLp2 shared TanLp1 motifs involved in tannase activity (10). Therefore, structural data also suggest that TanLp2 from *L. plantarum* ATCC 14917\(^\text{T}\) could be an active tannase.

When the presence of both tannases was studied in 36 *L. plantarum* strains, it was observed that a copy of *tanLp1* gene was present in all the analyzed strains, whereas *tanLp2* was only present in four *L. plantarum* strains (ATCC 14917\(^\text{T}\), NC8, CECT 749, and RM35 strains). The analysis of the TanLp2 protein sequences from these four strains revealed that, as compared to TanLp2 from *L. plantarum* ATCC 14917\(^\text{T}\), TanLp2 from CECT 749 and RM35 possessed amino acid substitutions in a motif conserved in all tannases. Moreover, the sequence of *tanLp2* from *L. plantarum* NC8 posses a frameshift that originates a truncated protein, lacking two of the three residues of the catalytic triad (residues equivalent to His-451 and Asp-419 in TanLp1), and Asp-421, which make contacts with an hydroxyl group of gallic acid.

From the sequence analysis, TanLp2 from *L. plantarum* ATCC 14917\(^\text{T}\) seems to be the only TanLp2 tannase on which the residues important for activity are conserved. In order to verify this hypothesis, and taken into account that TanLp2 seems to be an extracellular tannase, strains possessing a *tanLp2* copy (ATCC 14917\(^\text{T}\), NC8, CECT 749, and RM35) were grown in the presence of tannic acid, a complex gallotannin unable to pass into the cell to be degraded by intracellular TanLp1 tannase. As expected, the presence of an active extracellular tannases was only observed in *L. plantarum*
ATCC 14917\textsuperscript{T}. In this strain, in addition to the functionality of TanLp2, the functionality of TanLp1 was previously demonstrated (8). As this strain possesses two tannase proteins able to hydrolyze gallotannins, the expression of these proteins was studied under methyl gallate exposure. The presence of the substrate methyl gallate did not affect the expression of \textit{tanLp2}, however, it increased the expression of \textit{tanLp1}. This expression behaviour allows assuming that \textit{tanLp1} encodes an inducible tannase in \textit{L. plantarum} ATCC 14917\textsuperscript{T}, as previously observed in the WCFS1 strain under tannic acid challenge (25).

From the results obtained in this study, it seems that both \textit{L. plantarum} tannases exert different physiological roles. This different function could be partially attributed to their different biochemical properties, such as their reported substrate spectrum, which seems to be very similar except that esters having a long aliphatic alcohol were not effectively hydrolyzed by TanLp2. Moreover, TanLp2 from \textit{L. plantarum} ATCC 14917\textsuperscript{T} has a specific activity of 39 U/mg, ten times lower than the specific activity calculated for TanLp1 (404 U/mg) from the same strain. In addition, optimal temperature and pH for TanLp2 (20-30 °C, and pH 6) differed from those described for TanLp1 (40 °C, pH 7-8) (7-8). Interestingly, optimal activity of TanLp2 is similar to optimal tannase activity showed by cell-free extracts from \textit{L. plantarum} ATCC 14917\textsuperscript{T} (26). It is noteworthy to mention that \textit{L. plantarum} ATCC 14917\textsuperscript{T} cell-extracts were obtained from cultures grown in a medium devoid of possible tannase inductors, on which TanLp1 tannase could not be induced. Therefore, it seems that in the absence of a substrate, the biochemical characteristics of tannase activity showed by cell-free extracts of \textit{L. plantarum} ATCC 14917\textsuperscript{T}, containing both tannase genes, are more similar to those exhibited by TanLp2 than those of TanLp1. This could indicate that, in absence of a substrate, TanLp2 activity predominates in \textit{L. plantarum} strains having two tannase
enzymes. The gene expression results indicated that TanLp2 is not inducible by the presence of methyl gallate; however, its basal expression level could be enough to be detected in *L. plantarum* cell extracts. In complex tannins, such as tannic acid, the presence of an extracellular and low active TanLp2 tannase in some *L. plantarum* strains could provide then an enzymatic activity able to partially degrade tannic acid outside the cell. The less complex tannins originated by TanLp2 action, could be able to induce the expression of *tanLp1* and pass into the cell to be degraded by TanLp1. In addition, TanLp2 biochemical properties are more convenient than those from TanLp1 for an extracellular enzyme acting on plant substrates. Temperatures around 20 - 30 °C and acidic pH could be environmental conditions present on these plant substrates. The specific catabolic capacity of *L. plantarum* against gallotannins suggests that they provide a selective advantage to this species for life in environments where compounds of plant origin are abundant. The presence in some *L. plantarum* strains of a second active tannase will provide them an additional advantage. *L. plantarum* should be able to degrade them by TanLp2 and does not depend on other microorganisms for the initial degradation of these compounds. This second tannase TanLp2 is an extracellular enzyme able to hydrolyze complex gallotannins, which are unable to pass into the cell to be degraded by TanLp1. Moreover, the presence of TanLp2 will provide to *L. plantarum* strains an additional response mechanism to overcome the adverse effects of tannins present in their environment.

**ACKNOWLEDGEMENTS**

This work was supported by grants AGL2011-22745, BFU2010-17929/BMC, Consolider INGENIO 2010 CSD2007-00063 FUN-C-FOOD (MINECO), S2009/AGR-
1469 (ALIBIRD) (Comunidad de Madrid), and RM2012-00004 (Instituto Nacional de
Investigación Agraria y Alimentaria). We are grateful to M. V. Santamaría and J. M.
Barcenilla. N. Jiménez is a recipient of a FPI fellowship from the MINECO.

REFERENCES

1. Shahidi F, Naczk M. 2003. Phenolics in food and nutraceuticals. CRC Press,
   London, United Kingdom.


   and ellagitanins. 46:68-84.

5. Chávez-González M, Rodríguez-Durán LV, Balagurusamy N, Pardo-Barragán
   A, Rodríguez R, Contreras JC, Aguilar CN. 2012. Biotechnological advances and

6. Noguchi N, Ohashi T, Shiratori T, Narui K, Hagiwara T, Ko M, Watanabe K,
   Miyahara T, Taira S, Moriyasu F, Sasatsu M. 2007. Association of tannase-
   producing Staphylococcus lugdunensis with colon cancer and characterization of a

   of a gene enconding tannase (tannin acylhydrolase) from Lactobacillus plantarum.


Legend to Figures

FIG 1 Genetic organization of the L. plantarum WCFS1 chromosomal region containing the gallate decarboxylase and tannase encoding genes (accession NC_004567, positions 243093-252815, 1743368-1746325, and 2618290-2635122). The insertion of the tanLp2 gene in L. plantarum ATCC 14917<sup>T</sup> is also represented (accession ACGZ02000013.1, positions c/26013-27893). Arrows indicate genes. Genes coding for putative tannase proteins are represented by black arrows. Genes encoding gallate decarboxylase subunits (Lpd, LpdC, and LpdD) are also represented. The location of a putative tanLp2 promoter (vertical bent arrow) and transcription terminator region (ball and stick) are also indicated.

FIG 2. HPLC analysis of tannic acid degradation by L. plantarum cultures. Modified basal media containing 1 mM tannic acid was inoculated with L. plantarum strains (WCFS1, ATCC 14917<sup>T</sup>, NC8, CECT 749, and RM 35) and incubated for 10 days. A non-inoculated control medium was incubated in the same conditions. Detection was performed at 280 nm.

IPTG-induced *E. coli* BL21(DE3) (pURI3-TEV) (1) or *E. coli* BL21(DE3) (pURI3-TEV-TanLp2) (2), or fractions eluted after His affinity resin (3-7). The arrow indicated the overproduced and purified protein. The 12.5% gel was stained with Coomassie blue. Molecular mass markers are located at the left (SDS-PAGE Standards, Bio-Rad).

**FIG 4.** Some biochemical properties of TanLp2 protein. (A) Relative activity of TanLp2 versus temperature. (B) Relative activity versus pH. (C) Thermal stability of TanLp2 after preincubation at 22 ºC (filled diamond), 30 ºC (filled square), 37 ºC (filled triangle), 45 ºC (cross), 55 ºC (star), and 65 ºC (filled circle) in phosphate buffer (50 mM, pH 6.5); at indicated times, aliquots were withdrawn, and analyzed as described in the Materials and methods section. The experiments were done in triplicate. The mean value and the standard error are showed. The observed maximum activity was defined as 100%. (D) Relative activity of TanLp2 after incubation with 1 mM concentrations of different additives. The activity of the enzyme incubated in the absence of additives was defined as 100%.
Figure 1
Figure 2
Figure 3
Figure 4