**Genetic and biochemical characterization of an oligo- α -1,6-glucosidase from
*Lactobacillus plantarum***

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Key words: *Lactobacillus plantarum*, lactic acid bacteria, α -glucosidase, glycosyl
hydrolase, cloning, heterologous expression

Short title: An oligo- α -1,6-glucosidase from *Lactobacillus plantarum*

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Abstract

Although encoded in the genome of many *Lactobacillus* spp. strains, α -glucosidases have received little attention compared to other glycosyl hydrolases. In this study, a putative oligosaccharide(oligo)- α -1,6-glucosidase-encoding gene (*malL*) was identified in the genome of *Lactobacillus plantarum* LL441. *malL* coded for 572 amino acid residues with a calculated total molecular mass of 66.31 kDa. No predicted signal peptide was observed, suggesting this enzyme to be localized within the cytoplasm of the cell. Homology studies of the deduced amino acid sequence in the area of its active sites classified the enzyme as a member of the α -amylase (AmyAC) superfamily of glycosyl hydrolases (GH), family 13 (GH13), subfamily 31 (GH13_31). *malL* was cloned in *Escherichia coli* and the coded enzyme overexpressed as a histidine-tagged protein (MalL_{His}). It was then purified and characterized. MalL_{His} protein showed strong hydrolytic activity towards 4-nitrophenyl- α -D-glucopyranoside (*p*NP- α -Glu) but not to other *p*NP- α -D- or *p*NP- β -D- derivatives. When using *p*NP- α -Glu as a substrate, MalL_{His} showed similar specific activities between pH 5.0 and 6.0, and between 20 and 42°C (optimum 30°C). With this substrate, the V_{max} and K_m were 24.45 U/mg and 0.37 mM respectively. Among the natural carbohydrates assayed, MalL_{His} showed high specificity towards isomaltose and isomaltulose. However, under the conditions of the assay, the enzyme showed no transglycosylation activity. Characterization of the entire complement of glycosidases in *L. plantarum* might reveal how strains of this species could be used in new biotechnological applications or in the development of functional foods.

1. Introduction

Lactobacillus plantarum is a lactic acid bacterium (LAB) present in a wide range of environments, including plant materials, meat, fish and dairy products, and the gut of humans and animals (Siezen et al., 2010). As a LAB species, *L. plantarum* requires a fermentable carbohydrate as an energy source, from which it produces lactic acid as the major end-product. The remarkable adaptation of *L. plantarum* to different ecological niches reflects its capacity to ferment a wide range of carbohydrates, including monosaccharides, disaccharides and polysaccharides (Bringel et al., 2001; Di Cagno et al., 2010). Its versatility in this respect is confirmed by the presence in its genome of a vast array of genes coding for sugar uptake and utilization (Siezen and van Hylckama-Vlieg, 2011; Siezen et al., 2010). Except for monosaccharides, the post-transport utilization of other carbon sources requires the action of one or more glycosyl hydrolases (glycosidases) (LeBlanc et al., 2004; Silvestroni et al., 2002; Spano et al., 2005).

The glycosidases form a large group of enzymes that hydrolyse the glycosidic bond between two or more sugars, or between a sugar and some other residue (Davies et al., 2005). They are classified into families according to their amino acid sequence similarity, and these families clustered into “clans” based on their folded protein structures. At present, 133 families of glycosyl hydrolases grouped into 14 clans are recognized (http://www.cazypedia.org/index.php/Glycoside_Hydrolase_Families), with many others awaiting classification. A wide range of glycolytic activities permits the occupation of different niches. Some strains can be grown selectively on specific substrates (Garbe and Collin, 2012).

The α -glucosidases of *Lactobacillus* have been little studied (Gänzle and Follador, 2012) compared to other glycosidases. Nonetheless, α -glucosidase activity has been reported for whole cells and cell-free extracts of *Lactobacillus acidophilus* (Li and Chang, 1983), and an intracellular α -glucosidase has been purified and characterized from *Lactobacillus brevis* isolated from lambic beer (de Cort et al., 1994). More recently, α -glucosidases from *Lactobacillus johnsonii* (Kang et al., 2009) and *Lactobacillus acidophilus* (Møller et al., 2012) have been characterized at the biochemical and genetic level. RAST annotation has shown the draft genome sequence of *L. plantarum* LL441 (a strain isolated as a majority organism from the microbiota of a traditional, starter-free cheese made from raw milk; GenBank Accession number LWKN000000000) to contain 29 open reading frames (ORFs) encoding glucosidases belonging to different glycosyl hydrolase families (Flórez et al., unpublished). These ORFs include genes coding for one α -phosphotrehalase, one glucan-1,4- α -maltohydrolase, two α -rhamnosidases, two α -amylases, four α -galactosidases, four α -glucosidases, one phospho- β -galactosidase, four β -galactosidases, three β -glucosidases, six phospho- β -glucosidases, and the oligo- α -1,6-glucosidase examined in the present work.

The characterization of the *L. plantarum* LL441 gene coding for oligo- α -1,6-glucosidase is here reported. This gene was cloned into a polyhistidine-tagged vector in *E. coli*, overexpressed, and the recombinant protein purified by affinity chromatography. This allowed the enzyme to be characterized biochemically and its substrate specificity determined.

2. Material and Methods

2.1. Plasmids, bacterial strains and culture conditions

Table 1 shows the bacterial strains and plasmid vectors used in the present work. *Lactobacillus plantarum* was grown statically in MRS medium (Merck, Darmstadt, Germany) at 32°C. *Escherichia coli* was grown in Luria Bertani (LB) broth (peptone 10 g, yeast extract 5 g, sodium chloride 5 g) at 37°C with shaking. When plates were required, agar was added to the medium at 2% (w/v). For the selection of transformants and plasmid maintenance, kanamycin (40 µg/ml) was added where appropriate to both liquid and solid media. 5-bromo-4-chloro-3-indolyl- α -D-glucopyranoside (X-Glu; a chromogenic substrate) and isopropyl- β -D-thiogalactopyranoside (IPTG; an inducer of the T7 polymerase in *E. coli* BL21(DE3) (Novagen, San Diego, Ca., USA) (both from Sigma-Aldrich, St. Louis, Mo., USA) were used at 0.004% (wt/vol) and 0.5 mM respectively.

2.2. Screening of lactobacilli for oligo- α -1,6-glucosidase genes

Total genomic DNA was extracted and purified from lactobacilli using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich) following the manufacturer's instructions for Gram-positive bacteria. The presence of *malL* in *L. plantarum* and other lactobacilli strains of different origin was tested using primers malL-Plan-F and malL-Plan-R and malL-Lact-F and mal-Lact-R, respectively (Table 1). Southern blot analysis was also performed as described by Sambrook and Russell (2001). Chromosomal DNA was digested with either EcoRI or HindIII (Fermentas; Thermo Fisher Scientific, Waltham, Ma., USA). After transferring to a membrane, DNA was hybridized with a probe (obtained by PCR) embracing the coding part of LL441 *malL*. A digoxigenin

nucleic acid labelling and detection system (The DIG System, Roche, Mannheim, Germany) was used to mark the probe, according to the manufacturer's instructions.

2.3. Protein sequence comparison

Protein sequences of representative α -glucosidase subfamilies were retrieved from the GenBank database (<http://www.ncbi.nlm.nih.gov/protein/>) and aligned with that of *L. plantarum* LL441 using Clustal X 2.0 software (Larkin et al., 2007). A phylogenetic tree was constructed with the DNAMAN program (Lynnon, San Ramon, Ca., USA) using the neighbour-joining algorithm.

2.4. Cloning of *malL* in *E. coli* and purification of the recombinant protein

To construct the plasmid expressing a His-tagged version of MalL (MalL_{His}), chromosomal DNA from *L. plantarum* LL441 was used as a template in PCR amplifications employing the primer pair malL-NheI-F and malL-BamHI-R (Table 1) plus the high-fidelity Platinum Pfx DNA polymerase (Invitrogen, Barcelona, Spain). The PCR product was digested with NheI and BamHI and ligated with T4 DNA ligase (Roche) to pET28a(+) (Novagen) digested with the same enzymes. The ligation mixture was electrotransformed into *E. coli* DH10B using a Gene Pulser apparatus (Bio-Rad, Richmond, CA, USA). The final construct, pET-Glu, was verified via digestion with restriction enzymes and sequencing. Purified DNA of pET-Glu, obtained using the High Pure Plasmid Isolation Kit (Roche), was transferred for overexpression into *E. coli* BL21(DE3).

An overnight culture of *E. coli* BL21(DE3) harbouring pET-Glu was re-inoculated into fresh LB broth supplemented with kanamycin (40 μ g/ml) and grown at 37°C. At

mid-exponential phase ($A_{600\text{ nm}}$, 0.7), the expression of MalL_{His} was induced by the addition of 1 mM IPTG. To avoid the formation of inclusion bodies, cells were then incubated at 18°C for 24 h. They were then harvested by centrifugation, suspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl) and disrupted at 2.3 bars using a FastPrep® FP120 cell disruptor (Constant Systems, Daventry, UK). MalL_{His} was purified at 4°C via Ni-nitrilotriacetic acid affinity chromatography, as recommended by the supplier of the column used (Qiagen, Valencia, CA., USA). For verification purposes, the purified protein was electrophoresed under denaturing conditions in 6% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) in a Mini-Protean apparatus (Bio-Rad). The protein was visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad).

The α -glucosidase activity of constructs was measured in cell-free extracts using *p*-nitrophenyl(*p*NP)- α -D-glucopyranoside (*p*NP- α Glu) (Sigma-Aldrich) as a substrate. To produce these cell-free extracts, cells were harvested by centrifugation, suspended in lysis buffer and disrupted as above. The reaction mixture was composed of 800 μ l of 40 mM sodium acetate buffer (pH 5.5), 100 μ l of 10 mM of *p*NP- α Glu and 100 μ l of the cell-free extract. After 30 min at 37 °C, the reaction was stopped by addition of 1 ml of cold 1 M sodium carbonate, and clarified by centrifugation at 19,000 $\times g$ for 10 min at 4°C. The *p*NP released was determined spectrophotometrically, measuring the increase in absorbance at $A_{410\text{ nm}}$ against a calibration curve. Protein concentration was determined by the Bradford method using the BCA Protein Assay Kit (Pierce, Rockford, IL., USA) using bovine serum albumin as a standard. One unit of activity was defined as the amount that releases 1 μ mol of *p*NP per min.

2.5. Biochemical characterization of the purified enzyme

To determine the pH dependence of the purified enzyme (60 µg/ml of Mall_{His}), its activity was measured against the substrate *p*NP-αGlu between pH 3.5 and 8.0, using sodium acetate (50 mM, pH 3.5 to 5.5) and sodium phosphate (50 mM, pH 6.0 to 8.0) as buffers. The temperature dependency of the oligo-α-1,6-glucosidase activity was measured against *p*NP-αGlu over the range 10-65°C. The kinetic parameters of hydrolysis were determined using *p*NP-αGlu (0.1-8.0 mM), stopping the reactions at 30 min and measuring the *p*NP released as above. Additionally, the substrate specificity of Mall_{His} was evaluated using the following *p*NP-derivatives: *p*NP-β-D-glucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-D-manopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-α-D-fucopyranoside, *p*NP-β-D-arabinopyranoside, *p*NP-α-D-arabinopyranoside and *p*NP-α-D-maltohexaoside (all from Sigma-Aldrich).

2.6. Glycosidation and transglycosylation activity

Glycosidation was performed in 50 mM phosphate buffer (pH 6.5) with a 4 U of the purified Mall_{His} (concentration 0.5 mg/ml) and 20 mM sugars in a final volume of 130 µl. The specificity of the enzyme was assayed using disaccharides, trisaccharides and polysaccharides, including trehalose [α-D-glucosyl-(1-1)-α-D-glucose], maltose [α-D-glucosyl-(1-4)-D-glucose], isomaltose [α-D-glucosyl-(1→6)-α-D-glucose], isomaltulose [α-D-(1→6)-glucopyranosyl-D-fructose], melibiose [α-D-galactosyl-(1-6)-D-glucose] isomaltotriose [α-D-glucosyl-(1→6)-α-D-glucosyl-(1→6)-D-glucose], raffinose [O-α-D-galactosyl-(1-6)-α-D-glucosyl-(1-2)-β-D-fructose], panose [O-α-D-glucosyl-(1→6)-O-α-D-glucosyl-(1→4)-D-glucose] (all from Sigma-Aldrich). After overnight (16 h) incubation at 37°C, the mixture was heated at 95°C for 5 min, and cooled on ice.

Transglycosylation was assayed with 200 mM glucose plus 15 mM sacarose, maltose, gentibiose, panose, and isomaltulose in 50 mM phosphate buffer (pH 6.5) and 5 µg of MalL_{His} in a final reaction volume of 50 µl. Reactions were incubated overnight at 30°C and terminated by boiling the samples for 5 min before cooling on ice. As a control, 10 µl of the reaction mixture were removed immediately after its preparation, boiled, and frozen at -20°C until use.

Thin layer chromatography (TLC), performed using 60 F254 silica gel plates (Merck), was used to reveal glycosylation and transglycosylation reactions. Samples (1 µl aliquots) were loaded onto the TLC plates, allowed to dry, and developed using a solvent system involving ethyl acetate, acetic acid and water (2:1:1) in a vertical development chamber. The plates were sprayed with 5% sulphuric acid in ethanol, allowed to dry, and then heated at 120°C for 10 min to make the carbohydrate spots visible.

Glycosidase activity was further verified by HPLC analysis in a Waters liquid chromatograph controlled by Millenium 32 Software (Waters, Milford, Ma., USA). Reactions were filtered through a 0.45 µm sterile membrane and the separation of glucose and other sugars performed using an ICSep ICE-ION-300 ion-exchange column (mobile phase 0.0085 N H₂SO₄, operating temperature 65°C, flow rate 0.4 ml/min). Sugars were detected using a 410 differential refractometer (Waters) at a detection wavelength of 280 nm. Quantification was performed using calibration curves for the component di- tri- and polysaccharides.

3. Results

3.1. Characterization and genomic context of the oligo- α -1,6-glucosidase gene, and its ubiquity in lactobacilli

A gene coding for a putative oligo- α -1,6-glucosidase (A6B36_09870) was identified in the genome of *L. plantarum* LL441. The nucleotide and deduced protein sequences of this gene showed a complete match with the corresponding sequences from *L. plantarum* ATCC14917 (NZ_ACGZ000000000.2) - annotated as *malL* in the genome of ATCC14917 (EFK27917).

Figure 1 shows *malL* in the *L. plantarum* LL441 genome to be flanked upstream by genes coding for the components (IIB, IIC, IID and IIA) of a carbohydrate phosphotransferase transport system (PTS) belonging to the mannose/fructose/sorbose family, and downstream by a transcription regulator belonging to the ArsR family and an integral membrane protein. Closer inspection of the sequence suggested that the expression of *malL* might be transcriptionally coupled to the last PTS component (IIA), as the start and stop codons of the two genes (respectively) overlapped. Four consecutive start codons (ATG) were observed in the *malL* gene, the first of which overlapped partially with a putative ribosome binding site (RBS) sequence (GGATG). An imperfect inverted repeat was found 12 nucleotides downstream of the stop codon of *malL*, followed by a T-rich DNA stretch (AGGCTAACAGTGCAAC AATCGA GTTGTCCAGTGTTAGCCT TTTATTTT). This sequence resembles that of transcriptional, rho-independent terminators.

malL encoded a protein (MalL) 572 amino acids long with a calculated molecular mass of 66.31 kDa. No predicted signal peptide was observed, suggesting an intracellular localization for the enzyme. Alignment of the protein with representative α -glucosidases from databases identified conserved motifs in MalL at positions 102

(DLVVNH), 170 (QPDLNW), 199 (GFRMDVIS), 240 (MTVGEMPG), 260 (ETPH) and 330 (YWSNHD) (conserved amino acids underlined). These conserved blocks of amino acids are typical of the α -amylase (AmyAC) superfamily, family 13 (glycosyl hydrolases [GH]), subfamily 31 (GH13_31). (Majzlová et al., 2013). A phylogenetic tree constructed with representative bacterial α -glucosidases of family GH13 (Fig. 2) also revealed MalL to be a member of GH13_31.

PCR and hybridization experiments using an internal segment of 700 nt of *malL* as a probe and amplifying with primers malL-Pro-F and malL-Pro-R (Table 1), showed the gene to be present in four out of the nine *L. plantarum* strains studied (CECT748T, CECT749, C3.8, and NCDO1193). Using degenerate primers (Table 1), the gene was also found in strains of lactobacillus species other than *L. plantarum*, such as *L. murinus* G64, *L. paracasei* LP71, *L. reuteri* LR31, and *L. ruminis* B1411. BLAST analysis (January 2016) of *malL* (1704 nt) against genomes in the NCBI Genome database revealed the presence of the gene in most (but not all) of the *L. plantarum* strains sequenced. Homologous genes were also noted in the genomes of eight lactobacillus species (*L. casei*, *L. paracasei*, *L. reuteri*, *L. johnsonii*, *L. gasseri*, *L. hilgardii*, *L. jensenii* and *L. ruminis*).

3.2. Overexpression, purification and enzyme characterization

To overexpress *malL* and purify the GH13_31 enzyme from *L. plantarum* LL441, the gene (1.7 kbp) was amplified with malL-NheI-F and malL-XhoI-R primers and cloned into pET28a(+). MalL was purified as a histidine tagged protein, MalL_{His}, by Ni-nitrilotriacetic acid affinity chromatography to electrophoretic homogeneity (Fig. 3). The purification yield of MalL_{His} was 24.6 mg/l of culture with a specific activity of

478.9 $\mu\text{M}/\text{mg}/\text{min}$. Compared to the mass molecular markers, the purified protein showed an approximate mass of 68 kDa, which agrees well with the calculated molecular mass of the deduced protein (66.31 kDa) plus the histidine tag.

The purified MalL_{His} showed activity against $p\text{NP-}\alpha\text{Glu}$ but not against $p\text{NP-}\beta\text{-D-glucopyranoside}$. The purified enzyme did not act against other $p\text{NP}$ derivatives, such as $p\text{NP-}\alpha\text{-D-galactopyranoside}$, $p\text{NP-}\beta\text{-D-galactopyranoside}$, $p\text{NP-}\alpha\text{-D-manopyranoside}$, $p\text{NP-}\beta\text{-D-fucopyranoside}$, $p\text{NP-}\alpha\text{-D-fucopyranoside}$, $p\text{NP-}\beta\text{-D-arabinopyranoside}$, $p\text{NP-}\alpha\text{-D-arabinopyranoside}$ or $p\text{NP-}\alpha\text{-D-maltohexaoside}$. Using $p\text{NP-}\alpha\text{Glu}$, MalL_{His} showed high specific activity between pH 5.0 and 6.0, with no clear maximum, and at a temperature range between 20 and 40°C (optimum 30°C) (Fig. 4), declining sharply after 40°C. The Michaelis-Menten saturation plot with $p\text{NP-}\alpha\text{Glu}$ showed the V_{max} and K_m to be 24.45 U/mg and 0.37 mM, respectively.

Among the several natural substrates assayed, TLC assays showed the enzyme degraded isomaltose and isomaltulose, but not isomaltotriose, melibiose, panose and raffinose (Fig. 5). No activity was recorded against trehalose, sucrose, turanose or maltose, i.e., sugars with glycosidic bonds other than 1→6. The glycosidase activity of MalL_{His} was also measured via HPLC analysis. Isomaltose and isomaltulose were the preferred substrates; the hydrolysing percentages of these two disaccharides were 54.06 and 37.30% respectively. Weak activity was also seen with this technique against isomaltotriose (1.90% hydrolysis), and marginal activity against melibiose (0.52%), trehalose (0.33%) and maltose (0.14%). No transglycosylation activity of MalL_{His} was seen with respect to the disaccharides sacarose, maltose, gentibiose, panose and isomaltulose.

4. Discussion

Several glycosidases from *L. plantarum* have been chemically and/or genetically characterized. These include: 1) a β -galactosidase, an enzyme involved both in the development of *L. plantarum* in milk and dairy products (Mayo et al., 1994) and in the synthesis of galactooligosaccharides from lactose (Iqbal et al., 2010); 2) two α -galactosidases involved in the catabolism of plant-derived α -galactosides (Acebrón et al., 2009; Silvestroni et al., 2002); 3) two α -rhamnosidases that catalyze the hydrolysis of rhamnosides containing α -glycosidic bonds (Ávila et al., 2009); 4) an amylopullulanase found in starch-hydrolyzing *L. plantarum* strains (Kim et al., 2008; Olympia et al., 1995); and 5) a β -glucosidase, which might in wine contribute to the release of flavour compounds from odourless β -glucoside and di-glucoside conjugates (Spano et al., 2005). Though α -glucosidase activity has been shown in *Lactobacillus* spp., such as *Lactobacillus acidophilus* (Li and Chan, 1983) and *Lactobacillus brevis* (De Cort et al., 1994), the α -glucosidases of lactobacilli have been very little studied.

According to gene ontology annotation, the molecular function of oligo- α -1,6-glucosidases is the hydrolysis of the α -(1 $\rightarrow$ 6)-D-glucosidic bonds of isomaltose and other oligosaccharides produced from starch and glycogen through the action of α -amylases (Majzolvá et al., 2013; van der Maarel et al., 2002). Oligo- α -glucosidases are members of family 13 of the glycosyl hydrolases (GH13) (Henrissat and Bairoch, 1996), thus it was thought that MalL might be an oligo- α -glucosidase belonging to this family. Based on the recognition of homologous amino acid motifs, GH13 α -glucosidases have been subdivided into more than 30 mono- or poly-specific subfamilies (Oslancova and Janecek, 2002; Stam et al., 2006). Homology testing and phylogenetic analysis indicated that MalL from *L. plantarum* belongs to the GH13_31

subfamily. Recently, an α -1,3-glucosidase belonging to GH13_31 from *Lactobacillus johnsonii*, which acts against nigerose and nigeroseoligosaccharides, has been overexpressed and characterized (Kang et al., 2009). Further, a glucan-1,6- α -glucosidase from *Lactobacillus acidophilus* has been studied (Møller et al., 2012) and also found to belong to the GH13_31 subfamily. This latter enzyme confers the ability to utilize isomaltooligosaccharides (IMOs).

The activity of GH13_31 enzymes cannot be deduced directly from their amino acid sequence given the low-level amino acid conservation seen across the sequences in this subfamily (except for those sequences around the active sites) (Majzolvá et al., 2013). It is worth noting that, in addition to oligo-1,6-glucosidase, the activity of GH13_31 glucosidases covers the specificities of α -glucosidase, dextran glucosidase, trehalose-6-phosphate hydrolase, sucrose isomerase, trehalose synthase, and glucan-1,6-glucosidase (Oslancova and Janecek, 2002; Saburi et al., 2006). The MalL enzyme from *L. plantarum* proved to have a rather narrow substrate specificity compared to its closest relatives (Fig. 2). The use of *p*NP derivatives and natural carbohydrates showed the strong activity of MalL to be reduced to *p*NP- α Glu, isomaltose and isomaltulose. This contrasts with the broader activity of related enzymes from *Bacillus* (Yamamoto and Horikoshi, 1990), *Streptococcus* (Hondoh et al., 2008), *Bifidobacterium* (Kelli et al., 2016; Pokusaeva et al., 2009; van den Broek et al., 2003) and *Lactobacillus* (Møller et al., 2012), which act against a larger number of di-, tri- and polysaccharides. Further, the biochemical properties of GH13_31 α -glucosidases from *Streptococcus mutans* (Saburi et al., 2008) and *Lactobacillus acidophilus* (Møller et al., 2012) have been shown specific to glucan-1,6- α -glucosidases - the preferred natural substrates of which are tri- and poly-saccharides rather than di-saccharides. The activity of *L. plantarum*

MalL can therefore be considered exclusive to the oligo-1,6-glucosidase enzyme type (Saburi et al., 2006). Low-level activities that passed undetected by TLC were picked up in HPLC analysis. Judging from the release of glucose, MalL_{His} showed weak activity against isomaltotriose, and even weaker against melibiose, trehalose and maltose. Whether these activities are in fact due to MalL or to a contaminant enzyme deserves closer inspection. Most α -glucosidases show transglycosylation activity, manifested either via the direct coupling of glycosyl moieties causing the reversal of the hydrolysis reaction, or via the transfer of a glycosyl residue from an activated donor to an acceptor (Faijes and Planas, 2007). No MalL_{His} transglycosylation activity was detected under the assay conditions used.

Though the enzyme's activity was notable over a wide pH and temperature range, a slight preference for an acidic pH (maximum pH 5.0) and 30°C was seen; these are the optimum conditions for the growth of *L. plantarum*. In agreement with Watanabe's hypothesis, in which the molecular percentage of proline in α -glucosidases dictates enzyme thermostability (Watanabe et al., 1991), the activity of the oligo- α -1,6-glucosidase, which has 3.9% mol proline, declined sharply from 40°C onwards.

Glycosidic enzymes are thought to have remarkable ecological roles. Thereby, glycosidic activity has been shown critical in the development of *L. plantarum* in cereal-based fermentations (Humblot et al., 2014). The *malL* gene was shown to be absent in some strains isolated from cheese, which suggests that the α -glucosidase activity provided by MalL is not essential for growth and development in the dairy environment. Glucosidases are thought to be paramount for lactobacilli to be competitive in the human gastrointestinal (Saulnier et al., 2007) and genitourinary (Spear et al., 2015) tracts. The provision of such activity is also thought to be the role of

well-characterized glucosidases in bifidobacteria (encoded by *agl* genes) such as *Bifidobacterium breve* UCC2003 (Kelli et al., 2016; Pokusaeva et al., 2009) and *Bifidobacterium adolescentis* DSM20083 (van den Broek et al., 2003). These enzymes hydrolyse α -1,6-glucosidic bonds in IMO, compounds that have been shown to promote the growth of bifidobacteria in the human gut (Goffin et al., 2011). IMOs might therefore be considered promising prebiotics for probiotic strains with oligo- α -1,6-glucosidase activity (Møller et al., 2012). Also of prebiotic interest is the activity of the oligo- α -1,6-glucosidase on isomaltulose, a non-cariogenic sucrose isomer with a low glycaemic index that has been proposed as a potential nutraceutical for the treatment of diabetes (Lina et al., 2002).

5. Conclusions

In summary, this work reports the amplification and cloning of a gene from an *L. plantarum* strain of dairy origin that codes for an oligo- α -1,6-glucosidase in the GH13_31 subfamily. The gene was overexpressed in *E. coli* and the enzyme purified and characterized. As far as we are aware, this is the first report of the purification and characterization of an oligo- α -1,6-glucosidase from lactobacilli with oligo-glycosidase activity. This study contributes to the characterization of the glycoside hydrolase complement of *L. plantarum*, and helps to explain substrate utilization and niche occupation by this bacterium. Characterization of the entire complement of glycosidases in *L. plantarum* might reveal how strains of this species could be used in new biotechnological applications or in the development of functional foods.

Acknowledgments

This study was partially supported by projects from MINECO (Ref. AGL2014-57820-R), INIA (Ref. RM2009-00009) and Asturias Principality (Ref. GRUPIN14-137). A.B. Flórez was supported by a research contract of the JAE-Doc Program from CSIC. L. Guadamuro was supported by a research fellowship of the FPI Program from MINECO (Ref. BES-2012-062502). The skillful technical assistance of Javier Bobadilla is greatly acknowledged.

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Table 1.- Bacterial strains, plasmids and oligonucleotide primers utilized in this work.

Item	Relevant origin, genotype, or sequence	Source or reference
Strains		
<i>Lactobacillus plantarum</i> LL441	Wild type isolate from a traditional, starter-free cheese	Mayo et al. (1989)
<i>L. plantarum</i> CECT748 ^T	Type species, Pickled cabbage	CECT
<i>L. plantarum</i> CECT749	Pickled cabbage	CECT
<i>L. plantarum</i> CECT220	Corn silage	CECT
<i>L. plantarum</i> NCFB1193	Silage of vegetable matter	NCFB
<i>L. plantarum</i> C3.8	Cheese	La Serena cheese
<i>L. plantarum</i> GA10	Cheese	Gamonedo cheese
<i>L. plantarum</i> CTC250	Traditional sausage	CTC
<i>L. plantarum</i> CTC381	Traditional sausage	CTC
<i>L. plantarum</i> E112	Mucosa	Human
<i>Lactobacillus brevis</i> G42	Faeces	Human
<i>Lactobacillus casei</i> BA3	Cheese	Cabrales cheese
<i>Lactobacillus fermentum</i> LF72	Stomach	Human
<i>Lactobacillus gasseri</i> F71	Stomach	Human
<i>L. gasseri</i> LG32	Stomach	Human
<i>L. gasseri</i> LG52	Stomach	Human
<i>Lactobacillus murinus</i> G64	Faeces	Human
<i>Lactobacillus parabuchneri</i> G46	Stomach	Human
<i>Lactobacillus paracasei</i> LP71	Stomach	Human
<i>Lactobacillus reuteri</i> LR31	Stomach	Human
<i>L. reuteri</i> LR32	Stomach	Human
<i>Lactobacillus ruminis</i> B1411	Dairy	Traditional rennet
<i>Lactobacillus vaginalis</i> C32	Faeces	Human
<i>Escherichia coli</i> DH10B	F ⁻ , <i>endA1</i> , <i>deoR</i> ⁺ , <i>recA1</i> , <i>galE15</i> , <i>galK16</i> , <i>nupG</i> , <i>rpsL</i> , $\Delta(lac)X74$, $\phi 80lacZ\Delta M15$, <i>araD139</i> , $\Delta(ara, leu)7697$, <i>mcrA</i> , $\Delta(mrr-hsdRMS-mcrBC)$, Str ^R , λ^{-}	Invitrogen
<i>Escherichia coli</i> BL21(DE3)	F ⁻ , <i>ompT</i> , <i>gal</i> , <i>dcm</i> , <i>lon</i> , <i>hsdSB</i> (<i>rB-mB</i> -), $\lambda(DE3[lacI, lacUV5-T7\ gene\ 1, ind1, sam7, nin5])$	Novagen
Plasmids		
pET-28a(+)	Expression vector under the T7 expression region carrying N- and C-His-Tag sequences, Km ^r , 5,369 bp	Novagen
pET-Glu	pET-28a(+) containing the oligo- α -1,6-galactosidase gene under the T7 promoter region	This work
Oligonucleotides		
MalL-Plan-F	TGTTTGCAAAGCAACAGCCTG	This work
MalL-Plan-R	GATCAAGCAACTCAAAGTCAC	This work
malL-Lact-F	TGGAYTTDGTGTYKTNAAYCAYAC	This work
malL-Lact-R	TCRTGRTTRYTCCARTAVA	This work
MalL-NheI-F	CGAGCTAGCATGATGATGATGAATAAACAGAG	This work
MalL-XhoI-R	CCCTCGAGTTAGCCTCTGTAAATTTTG	This work

Km^r, resistance to kanamycin.

CECT, Colección Española de Cultivos Tipo, Valencia, Spain; NCFB, National Collection of Food Bacteria, Reading, UK; CTC, Irta, Monells, Spain.

Figure 1

Figure 1

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Figure 5

Figure 5

Figure Legends

Figure 1.- Visualization of the genetic context of the *malL* gene (in red) on the *L. plantarum* LL441 genome. Arrows represent open reading frames (ORFs) and their length is proportional to the size of each ORF. The putative function of the ORFs is indicated.

Figure 2.- Phylogenetic tree of representative bacterial α -glucosidases from the GH13 family. The tree is based on the alignment and clustering of protein sequences with Clustal X 2.0 software by using the Neighbor-Joining algorithm. In brackets, the accession number of each of the sequences. The arrow points towards the position of the oligo- α -1,6-glucosidase of *L. plantarum* LL441.

Figure 3.- Coomassie brilliant blue-stained SDS-PAGE of cell-free extracts from *Escherichia coli* BL21(DE3) before and after induction with IPTG and purification and intermediate steps of the histidine-tagged MalL from *L. plantarum* LL441. Order: lane 1, total cell extract of *E. coli* BL21(DE3) containing pET28a(+); lane 2, cell-free extract of *E. coli* BL21(DE3) containing pET-Glu before induction; lane 3, cell-free extract of *E. coli* BL21(DE3) containing pET-Glu after IPTG induction; line 4, protein eluate from the Ni-NTA resin during washing; lanes 5, 6 and 7, purified protein eluted from the Ni-NTA column after one, two or three rounds of purification; M, molecular mass marker (in kDa), as indicated on the left.

Figure 4.- Effect of temperature (A) and pH (B) on the specific activity of MalL as determined with pNP α -Glu as substrate.

Figure 5.- Thin Layer Chromatography (TLC) separation of sugar before and after reacting with the oligo- α -1,6-glucosidase from *L. plantarum* LL441. Key: Lines 1, 3, 5, 7, 9, and 11 control sugars (1 μ l, 10 mM) of panose, isomaltotriose, raffinose, melibiose, isomaltose, and isomaltulose, respectively. Lines 2, 4, 6, 8, 10, and 12, the same sugars (1 μ l, 20 mM) and in the same order after incubation with 4 U of the enzyme for 16 h at 37°C. Arrows pointed towards positive hydrolysis reactions with isomaltose and isomaltulose. Lines G, F, and Ga, molecular markers of glucose, fructose, and galactose, respectively.