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Genetic and biochemical characterization of 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). Among the natural carbohydrates assayed, $MalL_{His}$ showed specificity towards isomaltose (V_{max} and K_m values of 40.64 μmol min^{-1} mg^{-1} and 6.22 mM) and much less to isomaltulose (V_{max} and K_m values of 168.86 μmol min^{-1} mg^{-1} and 244.52 mM). 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 (Henrissat, 1991), 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

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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 no. LWKN00000000.1) 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 and its deduced protein 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

In order to test whether *malL* correlated with dairy ecosystems, PCR and hybridization experiments were performed to detect its presence in *L. plantarum* and other lactobacilli strains of different origin. For this, 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* 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 *Eco*RI or *Hin*dIII (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.

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

Item	Relevant origin, genotype, or sequence	Source or reference
Strains		
Lactobacillus plantarum LL441	Wild type isolate from a traditional, starter-free cheese	Mayo et al. (1994)
L. plantarum CECT748^T	Type species, Pickled cabbage	CECT
L. plantarum CECT749	Pickled cabbage	CECT
L. plantarum CECT220	Corn silage	CECT
L. plantarum NCFB1193	Silage of vegetable matter	NCFB
L. plantarum C3.8	Cheese	La Serena cheese
L. plantarum GA10	Cheese	Gamonedo cheese
L. plantarum CTC250	Traditional sausage	CTC
L. plantarum CTC381	Traditional sausage	CTC
L. plantarum E112	Mucosa	Human
Lactobacillus brevis G42	Faeces	Human
Lactobacillus casei BA3	Cheese	Cabrales cheese
Lactobacillus fermentum LF72	Stomach	Human
Lactobacillus gasseri F71	Stomach	Human
L. gasseri LG32	Stomach	Human
L. gasseri LG52	Stomach	Human
Lactobacillus murinus G64	Faeces	Human
Lactobacillus parabuchneri G46	Stomach	Human
Lactobacillus paracasei LP71	Stomach	Human
Lactobacillus reuteri LR31	Stomach	Human
L. reuteri LR32	Stomach	Human
Lactobacillus ruminis B1411	Dairy	Traditional rennet
Lactobacillus vaginalis C32	Faeces	Human
Escherichia coli DH10B	F$^-$, *endA1, deoR*$^+$, *recA1, galE15, galK16, nupG, rpsL, Δ(lac)X74, φ80lacZΔM15, araD139, Δ(ara,leu)7697, mcrA, Δ(mrr-hsdRMS-mcrBC)*, Str^R, λ$^-$	Invitrogen
Escherichia coli BL21(DE3)	F-, *ompT, gal, dcm, lon, hsdSB*(*rB-mB-*), λ(*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, 5369 bp	Novagen
pET-Glu	pET-28a(+) containing the oligo-α-1,6-galactosidase gene under the T7 promoter region	This work
Oligonucleotides	(5′-3′)	
MalL-Plan-F	TGTTTGCAAAGCAACAGCCTG	This work
MalL-Plan-R	GATCAAGCAACTCAAAGTCAC	This work
malL-Lact-F	TGGAYTTDGTYKTNAAYCAYAC	This work
malL-Lact-R	TCRTGRTTRYTCCARTAVA	This work
MalL-*Nhe*I-F	CGAGCTAGCATGATGATGATGAATAAAACAGAG	This work
MalL-*Xho*I-R	CCCTCGAGTTAGCCTCTGTTAAATTTTG	This work

Km^r, resistance to kanamycin.
CECT, Colección Española de Cultivos Tipo (Spanish Collection of Type Cultures), Valencia, Spain; NCFB, National Collection of Food Bacteria, Reading, UK; CTC, Irta, Monells, Spain.