Title: Cloning and expression of the β-galactosidase genes from *Lactobacillus reuteri* in *Escherichia coli*

Authors: Thu-Ha Nguyen, Barbara Splechtna, Montarop Yamabhai, Dietmar Haltrich, Clemens Peterbauer

PII: S0168-1656(07)00109-5
DOI: doi:10.1016/j.jbiotec.2007.01.034
Reference: BIOTEC 4575

To appear in: *Journal of Biotechnology*

Received date: 21-11-2006
Revised date: 17-1-2007
Accepted date: 29-1-2007

Please cite this article as: Nguyen, T.-H., Splechtna, B., Yamabhai, M., Haltrich, D., Peterbauer, C., Cloning and expression of the β-galactosidase genes from *Lactobacillus reuteri* in *Escherichia coli*, *Journal of Biotechnology* (2007), doi:10.1016/j.jbiotec.2007.01.034

Cloning and expression of the β-galactosidase genes from *Lactobacillus reuteri* in *Escherichia coli*

THU-HA NGUYEN[1, 2], BARBARA SPLECHTNA[1, 2], MONTAROP YAMABHAI[3], DIETMAR HALTRICH[2], AND CLEMENS PETERBAUER[2 *]

[1] *Research Centre Applied Biocatalysis, Petersgasse 14, A-8010 Graz, Austria*,

[2] *Division of Food-Biotechnology, Department of Food Sciences and Technology, University of Natural Resources and Applied Life Sciences Vienna, Muthgasse 18, A-1190 Vienna, Austria,*

[3] *School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Nakhon Ratchasima, 30000, Thailand*

Manuscript number: JBIOTEC-D-06-00953 *Revised version*

* Corresponding author (e-mail: clemens.peterbauer@boku.ac.at). Address: Abteilung Lebensmittelbiotechnologie, Department für Lebensmittelwissenschaften und -technologie, Universität für Bodenkultur Wien, Muthgasse 18, A-1190 Vienna, Austria. Phone: 43-1-36006 6274. Fax: 43-1-36006 6251.

Abstract

Heterodimeric β-galactosidase of *Lactobacillus reuteri* L103 is encoded by two overlapping genes, *lacL* and *lacM*. The *lacL* (1887 bp) and *lacM* (960 bp) genes encode polypeptides with calculated molecular masses of 73,620 Da and 35,682 Da, respectively. The deduced amino acid sequences of *lacL* and *lacM* show significant identity with the sequences of β-galactosidases from other lactobacilli and *Escherichia coli*. The coding regions of the *lacLM* genes were cloned and successfully overexpressed in *E. coli* using an expression system based on the T7 RNA polymerase promoter. Expression of *lacL* alone and coexpression of *lacL* and *lacM* as well as activity staining of both native and recombinant β-galactosidases suggested a translational coupling between *lacL* and *lacM*, indicating that the formation of a functional β-galactosidase requires both genes. Recombinant β-galactosidase was purified to apparent homogeneity, characterized and compared with the native β-galactosidase from *L. reuteri* L103.

1. Introduction

β-Galactosidases (β-gal; EC 3.2.1.23) catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides (such as lactose). β-Galactosidase-catalyzed hydrolysis reactions are widely known for applications in the dairy industries to improve digestibility, solubility and sweetness of lactose, the principle milk carbohydrate (Nakayama and Amachi, 1999). Transgalactosylation reactions catalyzed by β-galactosidases when using lactose or other structurally related galactosides as the substrate yield galacto-oligosaccharides (GOS) (Nakayama and Amachi, 1999; Pivarnik *et al.*, 1995). Galacto-oligosaccharides are classified as prebiotics (Rastall and Maitin, 2002) that beneficially affect host health by stimulating selectively the growth of

specific species of bacteria such as bifidobacteria and lactobacilli in the gut (Gibson and Roberfroid, 1995; Cummings *et al*., 2001).

Lactobacillus reuteri is a dominant strain of the hetero-fermentative lactobacilli in the gastrointestinal tract of human and animals (Benno *et al.*, 1989, Kabuki *et al*., 1997, Sung *et al*., 2003). Apart from our recent studies, no information about β-galactosidases from *L. reuteri*, especially regarding their enzymatic and molecular properties, was available. Our previous studies revealed that the intracellular β-galactosidase enzymes from the potentially probiotic isolates of *L. reuteri*, strains L103 and L461, are heterodimers with a molecular mass of 105 kDa, consisting of a 35 kDa and a 72 kDa subunit (Nguyen *et al*., 2006). Both enzymes were found to be very well suited for the production of galacto-oligosaccharides, components that are of great interest because of their use in functional food (Splechtna *et al*., 2006).

Hence, it was our interest to study the molecular properties of these interesting β-galactosidases from *L. reuteri* in more detail. β-Galactosidase from *L. reuteri* L103 was selected for this study and we identified two partially overlapping genes encoding for this enzyme. In this paper, we describe the cloning of β-gal from *L. reuteri* L103 and its expression in *E. coli*, furthermore, some properties of the recombinant enzyme are also reported.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Lactobacillus reuteri strain L103 was obtained from Lactosan (Starterkulturen GmbH & Co KG, Kapfenberg, Austria). The strain was grown anaerobically overnight at 37°C in MRS broth containing peptone 10 g/l, di-potassium hydrogen phosphate 2 g/l, meat extract 8 g/l, di-ammonium hydrogen citrate 2 g/l, yeast extract 4 g/l, sodium

acetate 5 g/l, magnesium sulfate 0.2 g/l, Tween 80 1 g/l, manganese sulfate 0.04 g/l. Glucose 2% w/v served as the C-source (Lactobacillus broth according to De Man, Rogosa and Sharpe, 1960). *Escherichia coli* TOP10 (Invitrogen Corporation, Carlsbad, CA, USA) was used in the transformation experiments involving the subcloning of the DNA fragments. *Escherichia coli* BL21 Star (DE3) (Invitrogen), which carries the gene for T7 RNA polymerase under control of the *lac*Z-promoter, was used as expression host for the vector carrying the target DNA fragment encoding both large and small subunits (*lacLM*) of β-galactosidase. The *E.coli* strains were grown in Luria broth (LB) containing appropriate antibiotics (100 µg/ml ampicillin or 50 µg/ml kanamycin) required for maintaining the plasmids.

2.2. Chemicals and enzymes

All chemicals were purchased from Sigma (St. Louis, MO., USA) unless otherwise stated and were of the highest quality available. MRS broth powder was obtained from Merck (Darmstadt, Germany). All restriction enzymes, *Pfu* DNA polymerase, T4 DNA ligase and shrimp alkaline phosphatase (SAP) were purchased from Fermentas (Vilnius, Lithuania). GoTaq DNA polymerase was from Promega (WI, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) and agarose were purchased from Roth (Karlsruhe, Germany).

2.3. DNA preparation

Chromosomal DNA was extracted from *L. reuteri* L103 as described by Germond *et al.* (2003) with modifications. The strain was grown anaerobically at 37°C in MRS broth to the mid-log phase. Cells were harvested by centrifugation (4500 rpm, 10 minutes, 4°), washed twice with 0.8% w/v NaCl and once with 60 mM EDTA. The cells

were then subjected to one freeze-and-thaw cycle, resuspended in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) containing lysozyme (8 mg/ml) and mutanolysin (40 U/ml), and incubated at 37°C for 1 hour. One volume of 0.5% w/v sodium dodecyl sulphate (SDS) was added to lyse the cells, and proteinase K was added to a final concentration of 200 µg/ml. After incubating the mixture at 65 °C for 10 minutes, the DNA was extracted with phenol, precipitated with isopropanol and washed with 70% cold ethanol. The DNA was then dissolved in TE buffer. After DNA was dissolved, RNase A was added to a final concentration of 200 µg/ml and the solution was incubated at 35°C for 30 minutes. The final yield of DNA obtained was approximately 0.5 µg/µl.

Plasmid DNA from *E. coli* was purified using the Wizard Plus Miniprep DNA Purification System (Promega) and PureLink™ Quick Plasmid Miniprep Kit (Invitrogen).

2.4. DNA amplification procedure and subcloning of lacL (large subunit)

The degenerated oligonucleotides β-gal F1 and β-gal R1 (table 1) used for PCR amplification of the *L. reuteri lacL* gene were designed by sequence comparison of β-galactosidase large subunit (*lacL*) gene products from *Lactobacillus spp.*, namely *Lactobacillus acidophilus* (GenBank accession number AB004867), *Lactobacillus helveticus* (GenBank accession number AJ512877), and *Lactobacillus sakei* (GenBank accession number X82287). The primers were obtained from VBC-Biotech (Vienna, Austria). The amplifications were performed using a T3 Thermocycler (Biometra; Goettingen, Germany) in a total volume of 50 µl of reaction mixtures containing 0.2 mM of each deoxynucleotide triphosphate, 25 pmol of each primer, 5 µl of 10× *Pfu* buffer with $MgSO_4$ (final concentration of $MgSO_4$ was 2.5 mM), 1.25 U of *Pfu* DNA

polymerase, and 1 µl of diluted genomic DNA (about 50 ng). The initial denaturation step at 95°C for 2 minutes was followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 42°C for 40 s, and extension at 72°C for 4 minutes. The final cycle was followed by additional 10 minutes elongation at 72°C. The amplified products were visualized by gel electrophoresis at 5 $V \cdot cm^{-1}$ in a 0.8% agarose gel (containing 1 µg/ml ethidium bromide) in 1×TBE (Tris-Borate-EDTA) electrophoresis buffer (10.8 g/l Tris base, 5.5 g/l boric acid, 0.9 g/l sodium EDTA; pH 8) and photographed under UV light. The amplified product was purified from the agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega). The vector pCR-Blunt II-TOPO (Invitrogen) was used for subcloning PCR-amplified product and the resulting plasmid pHA1030 was prepared for DNA sequencing.

2.5. Rapid Amplification of Genomic Ends (RAGE) for direct sequencing of lacM (small subunit)

RAGE-PCR for direct sequencing of *lacM* was carried out as described in Mizobuchi and Frohman (1993) with modifications. Five µg each of genomic DNA from L103 and pBluescript II SK (-) plasmid DNA (Stratagene, La Jolla, CA, USA) were digested by single enzyme restriction digests using *Pst*I, *Kpn*I, *Hind*III, *Xho*I, *Xba*I, *EcoR*V at 37°C. After complete digestion, the restriction enzymes were heat inactivated. Digested pBluescript plasmid DNA was then dephosphorylated using shrimp alkaline phosphatase. Digested genomic DNA and pBluescript plasmid DNA were cleaned up using the Wizard® SV Gel and PCR Clean-Up System (Promega) and then ligated with T4 DNA ligase at 16°C for 15 h. These ligation mixtures were stored at -20°C until use.

The primers β-gal F2, β-gal F3, pBS R1 and pBS R2 (table 1) were designed based on the sequence of *lacL* (large subunit) obtained from previous experimental procedures and the sequence of pBluescript II SK (-) vector (GenBank accession number X52330).

The first round of PCR amplification was performed in a total volume of 50 µl of reaction mixtures containing 1 µl of the ligation mixture (as template), 0.2 mM of each deoxynucleotide triphosphate, 25 pmol of each primer (β-gal F2 and pBS R1), 10 µl of 5× colourless GoTaq reaction buffer, and 2.5 U of GoTaq DNA polymerase (Promega). The initial denaturation step at 95°C for 2 min was followed by 35 cycles of amplification (94°C, 40 s; 53-57°C, 40 s; 72°C, 2 min) and a final elongation at 72°C for 10 min. The second round of PCR amplification was carried out using 1 µl of the first PCR reaction mix as template and nested primers β-gal F3 and pBS R2 (table 1) under the same conditions described for the first round of PCR amplification. The positive product of RAGE-PCR was cloned into pCR 2.1-TOPO vector (Invitrogen) and the plasmid was prepared for DNA sequencing.

The product of first RAGE-PCR did not cover the complete *lacM* sequence. Hence, a second RAGE-PCR was performed using different pairs of primers, β-gal F4 and pBS R1, and nested primers β-gal F5 and pBS R2 (table 1) under the same conditions as described above.

2.6. DNA amplification procedure and subcloning of β-galactosidase

The degenerated oligonucleotides β-gal F6 and β-gal R6 (table 1) used for PCR amplification of *L. reuteri* β-galactosidase genes (*lacLM*) were designed based on the sequences of *lacL* and *lacM* obtained from the experimental procedures described above. The amplification procedure was performed as described above for DNA amplification of *lacL* (large subunit) with some modifications (annealing at 57°C for 40 s, and extension at 72°C for 6 min). The vector pCR-Blunt II-TOPO (Invitrogen) was used for subcloning the PCR-amplified product. The resulting plasmid pHA1031 contains the complete genes (*lacL* and *lacM*) of β-galactosidase from *L. reuteri*, which was confirmed by sequencing.

2.7. Nucleotide sequencing and sequence analysis.

The nucleotide sequence was determined by VBC-Biotech (Vienna, Austria). Assembly and analysis of DNA sequences were done by using ChromasPro (version 1.33) (Technelysium, Australia). The basis local alignment tool (BLAST) from the National Center for Biotechnology Information BLAST website was used for database searches. The comparison of β-galactosidases from *Lactobacillus spp*. with homologous proteins was carried out using the programs ClustalX (version 1.81) (Thompson *et al.*, 1997) and GeneDoc (version 2.6.002) (Nicholas *et al.*, 1997).

2.8. Expression of β-galactosidase

Upstream and downstream primers β-gal F7 and β-gal R7 (table 1) were used to amplify the fragment containing β-galactosidase genes from pHA1031. These primers created a restriction site at each end of the gene fragment, *Nco*I and *Xho*I, respectively. The PCR-amplified fragment was digested with *Nco*I and *Xho*I and inserted into the respective sites of the expression vector pET21d (Novagen, Darmstadt, Germany) resulting in the overexpression plasmid pHA1032. The expressed protein carries a C-terminal His-Tag encoded by the vector. *E.coli* BL21 Star (DE3) carrying pHA1032 was grown at 37°C in LB medium containing 100 µg/ml ampicilin until an optical density at 600 nm of 0.6 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM) was then added to the culture medium and the cultures were incubated further at 25°C for 12 hours. The induced cells were then harvested, washed once with sodium phosphate buffer (50 mM, pH 6.5), and disrupted by using a French press (AMINCO, Maryland, USA). Debris was removed by centrifugation (16,000 rpm, 30 minutes, 4°C) to obtain the crude extract.

2.9. Protein purification

The crude extract was loaded on a HisTrap HP column (Ni Sepharose High Performance, 5 ml, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) that was pre-equilibrated with buffer A (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 6.5). The protein was eluted at a rate of 2.5 ml min^{-1} with a 75 ml linear gradient from 0 to 100 % buffer B (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 6.5). Active fractions were pooled, desalted and concentrated for further analysis.

2.10. Protein determination

Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard.

2.11. Gel electrophoresis and active staining

Gel electrophoresis and active staining were carried out using 4-methylumbelliferyl β-D-galactoside as the substrate as previously described (Nguyen *et al.* 2006).

2.12. Enzyme assays

β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) and lactose as the substrates as described previously (Nguyen *et al.* 2006). When chromogenic *o*NPG was used as the substrate, the reaction was initiated by adding 20 µl of enzyme solution to 480 µl of 22 mM *o*NPG in 50 mM sodium phosphate buffer (pH 6.5) and stopped after 10 min of incubation at 30°C by adding 750 µl of 0.4 M Na_2CO_3. The release of *o*-nitrophenol (*o*NP) was measured by determining the absorbance at 420 nm. One unit of *o*NPG activity was defined as the amount of enzyme releasing 1 µmol of *o*NP per minute under the described conditions.

When lactose was used as the substrate, 20 µl of enzyme solution was added to 480 µl of 600 mM lactose solution in 50 mM sodium phosphate buffer, pH 6.5. After 10 minutes of incubation at 30°C, the reaction was stopped by heating the reaction mixture at 99°C for 5 minutes. The reaction mixture was cooled to room temperature, and the release of D-glucose was determined colorimetrically using the GOD/POD assay (Kunst *et al*., 1988). One unit of lactase activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions.

2.13. Characterization of the recombinant β-galactosidase enzyme

Steady-state kinetic measurements, pH and temperature dependency of activity and stability were obtained following the methods described in our previous report (Nguyen *et al*., 2006).

2.14. Nucleotide sequence accession numbers

The GenBank accession numbers are DQ493596 (nucleotide sequence of both genes), ABF72116 (lacL, large subunit) and ABF72117 (lacM, small subunit), respectively.

3. Results

3.1. Cloning of β-galactosidase genes from L. reuteri and nucleotide sequence analysis

Degenerated oligonucleotides were designed for PCR amplification of the gene encoding the large subunit (*lacL*) of *L. reuteri* L103 β-galactosidase. One complete open reading frame (ORF) of 1887 bp encoding 628 amino acid residues with a calculated molecular mass of 73,620 Da (http://au.expasy.org/tools/protparam.html) was obtained. Based on its similarity to the sequences of published *lacL* genes of β-

galactosidases from other *Lactobacillus* spp. it was assumed to be the *lacL* gene of *L. reuteri* L103 β-galactosidase.

To identify the location of the gene encoding the small subunit, RAGE-PCR was carried out for direct sequencing of the flanking region of the 3′ end of *lacL*. A second reading frame which partially overlaps the 3′ end of *lacL* was obtained. The start codon ATG of this open reading frame starts at position 1870 of *lacL* and its 960 bp encode a protein of 319 amino acids with a calculated molecular mass of 35,682 Da. It was designated as *lacM* based on its similarity to the sequences of published *lacM* genes of β-galactosidases from *Lactobacillus* spp. The gene *lacM* was found downstream of *lacL* and the two genes overlap for 17 base pairs (Figure 1). From our previous study, L103 β-galactosidase appeared to be a heterodimer consisting of a 35 kDa and a 72 kDa subunit (Nguyen *et al.*, 2006), hence this was in agreement with the deduced amino acid sequences.

3.2. Comparison of amino acid sequences

The alignments of the deduced amino acid sequences of *lacL* and *lacM* genes of β-galactosidase from *L. reuteri* L103 with other β-galactosidases from *Lactobacillus* spp. and *Leuconostoc lactis* are presented in figure 2 (A,B). The *lacL* and *lacM* genes of β-galactosidase from *L. reuteri* L103 show significant similarity to the reported sequences of β-galactosidases from *L. acidophilus*, 74% and 71%, respectively (accession numbers BAA20536 and BAA20537) (Suzuki *et al.*, 1997); *L. helveticus*, 73% and 70%, respectively (accession numbers CAD55499 and CAD55500) (Fortina *et al.*, 2003); *L. johnsonii*, 69% and 65%, respectively (accession numbers AAS08676 and AAS08675) (Pridmore *et al.*, 2004); *L. plantarum*, 64% and 68%, respectively (accession numbers CAD65569 and CAD65570) (Kleerebezem *et al.*, 2003); *Leuconostoc lactis*, 64% and

67%, respectively (accession numbers Q02603 and Q02604) (David *et al.*, 1992); and *L. sakei*, 58% and 57%, respectively (accession numbers CAA57730 and CAA57731) (Obst *et al*., 1995). The region showing the lowest conservation in the *lacL* genes of these β-galactosidases is from nucleotide positions 240 to 300, while very high conservation was observed in the region of the 3′ end of *lacL* (figure 2A).

The amino acid sequence deduced from *L. reuteri* L103 *lacL* shows 33% identity to the *lacZ* gene encoding β-galactosidase from *E. coli* (accession number V00296) (Kalnins *et al*., 1983). Previous studies suggested that Glu-461 is a general acid-base recognition site (Cupples *et al*., 1990) and Glu-537 is a nucleophile in the active site of β-galactosidase from *E.coli* (Gebler *et al*., 1992). Tyr-503 was also reported as a general acid and base catalyst (Ring and Huber, 1990). Glu-416, His-418, Glu-461 appear to coordinate a bound magnesium ion in *E. coli* β-galactosidase (Jacobson *et al*., 1994). The amino acid sequence alignment of the regions flanking these catalytically important residues in *L. reuteri* L103 *lacL* and *lacZ* from *E.coli* is shown in figure 3. These regions in *lacL* of *L. reuteri* L103 show high similarity to the corresponding regions in *lacZ* from *E. coli*, with the exception of the region flanking a tyrosine residue at position 502 with significant lower similarity. The deduced amino acid sequence of *lacM* showed similarity to 180 residues at the 3′ end of the *lacZ* gene from *E.coli*. As judged from the sequences of the *lacL* and *lacM* genes from *L. reuteri* L103, they possibly belong to the glycosyl hydrolase family 2 according to the structural classification by Henrissat (Henrissat, 1991; Nakayama and Amachi, 1999).

3.3. Overexpression of β-galactosidase from L. reuteri in E. coli

To further study the two gene products of *lacLM*, a T7 RNA polymerase expression system was used for the joint overexpression of the *lacL* and *lacM* genes in *E. coli*. The

coding region for both genes, *lacL* and *lacM*, was cloned into pET21d resulting in the overexpression plasmid pHA1032. Gene expression was induced by 0.1 mM IPTG with 12 h induction at 25°C (figure 4A). β-Galactosidase activity produced by *E. coli* strain BL21 Star (DE3) carrying pHA1032 was determined to be 55 U/mg of protein and approximately 110 kU per liter of fermentation broth. The enzyme was purified with a single-step purification using a HisTrap HP column which gave an overall yield of 75%, and approximately 83 kU of purified recombinant enzyme per liter of fermentation broth with a specific activity of 180 U/mg of protein was obtained.

The recombinant β-galactosidase overexpressed in *E. coli* had a molecular mass of approximately 108 kDa and consisted of a ~35 kDa and a ~73 kDa subunit. This is in agreement with the wild-type β-galactosidase from *L. reuteri* L103 (figure 4B). Active staining of both purified wild-type and recombinant β-galactosidases directly on the SDS-PAGE gel after pre-incubating the enzymes with denaturing SDS buffer at 60°C for 5 minutes and using 4-methylumbelliferyl β-D-galactoside as the substrate showed that one band corresponding to a polypeptide of the size of the larger subunit exhibited activity with this substrate. In contrast, the smaller subunit did not show any activity (Figure 4B). As was reported in our previous study, active staining of wild-type β-galactosidase L103 on native PAGE yielded two bands with β-galactosidase activity, one band of approximately 105 kDa, corresponding to the intact heterodimer, and a second band representing a degradation product containing components of both subunits (Nguyen *et al.*, 2006). This observation was also made with recombinant β-galactosidase overexpressed in *E. coli* (data not shown).

3.4. Expression of lacL gene product alone did not yield functional β-galactosidase activity

Based on these observations with active staining of β-galactosidase L103 using 4-methylumbelliferyl β-D-galactoside as the substrate, it was of interest to determine whether the gene product of *lacL* gene represents an active, monomeric β-galactosidase. To this end, the coding region of only *lacL* was cloned into pET21d resulting in plasmid pHA1033, and this plasmid was introduced into *E. coli*. Gene expression was induced by IPTG, analyzed by SDS-PAGE, and the expressed protein was purified using a HisTrap HP column. A protein with a molecular mass of ~73 kDa, which is in agreement with the molecular mass of the large subunit, was obtained, which confirmed that the gene was expressed. However, no detectable β-galactosidase activity was found. This indicates that both genes, *lacL* and *lacM*, are required for the production of active β-galactosidase.

3.5. Properties of recombinant β-galactosidase EL103

To differentiate from the native β-galactosidase from *L. reuteri* L103, the β-galactosidase overexpressed in *E. coli* was denoted as recombinant β-galactosidase EL103. The steady-state kinetic constants were determined for the hydrolysis of lactose, the natural substrate. Kinetic analysis of recombinant β-galactosidase EL103 with increasing concentrations of lactose as the substrate showed Michaelis-Menten kinetics with the following parameters obtained by nonlinear regression using SigmaPlot (SPSS Inc., Illinois, USA): V_{max} = 38 (µmol D-glucose released/min/mg protein) and K_m = 12 ± 2 (mM). In case of native β-galactosidase L103, the V_{max} and K_m values for lactose were 34 (µmol D-glucose released/min/mg protein), and 13 ± 2 (mM), respectively (Nguyen *et al*., 2006).

The optimum pH of native L103 β-galactosidase is pH 8.0 for both lactose and *o*NPG hydrolysis. The optimum pH of recombinant EL103 β-galactosidase shifts to pH

7.0 for *o*NPG and remains at pH 8.0 for lactose hydrolysis (Figure 5A, B). However, both native L103 β-gal and recombinant EL103 β-gal are most stable at pH 6.0, retaining more than 90% and 80% of it activity, respectively, when incubated at pH 6.0 and 37°C for 3 hours (Figure 6). EL103 β-gal was slightly less stable than L103 β-gal at pH 6.0, although the difference was not significant. Surprisingly, the recombinant EL103 β-gal showed significant activity at pH 7.0 after 3 hours incubation at 37°C, retaining 26% of its activity, whereas the native L103 β-gal was inactive at this pH.

The optimum temperature of both native and recombinant β-galactosidases L103 and EL103 was 50°C when using *o*NPG as the substrate under standard assay conditions (pH 6.5, 10 min). For lactose hydrolysis, the optimum temperatures of L103 and EL103 β-galactosidases were found to be in the range of 45-50°C, and 50-60°C, respectively (Figure 5C, D). Both native and recombinant enzymes, L103 β-gal and EL103 β-gal, are very stable at 4°C in the presence of 1 mM 1,4-dithiothreitol (DTT), retaining their full activity after weeks of storage. L103 and EL103 β-galactosidases retained 30% and 35% of their activities, respectively, when kept at 37°C for 48 hours. The effect of $MgCl_2$ on the thermal stability of native L103 β-gal was reported in our previous study (Nguyen *et al*., 2006) and this observation was also found with recombinant EL103 β-gal. In the presence of 10 mM $MgCl_2$ both enzymes retained 90% of its activity after 6 h incubation at 42°C, and their half-life time ($t_{1/2}$) of activity at this temperature was increased to approximately 24 h.

4. Discussion

Heterodimeric β-galactosidase from *L. reuteri* is encoded by two overlapping genes, *lacL* and *lacM*. As was reported in our previous study (Nguyen *et al*., 2006), the larger subunit of *L. reuteri* β-gal showed activity after subunit dissociation and separation by

SDS-PAGE while the smaller subunit was inactive. This active staining of the purified β-galactosidase L103 was done directly on the SDS-PAGE gel after pre-incubating the enzymes with denaturing SDS buffer at 60°C for 5 min (a 'milder' form of denaturation resulting predominantly in subunit dissociation but not denaturation) and using 4-methylumbelliferyl β-D-galactoside as the substrate. After pre-incubating the enzymes with denaturing SDS buffer at 99°C (presumably resulting in complete denaturation), both subunits exhibited no activity (data not shown).

When both genes *lacLM* were expressed in *E. coli*, functional β-galactosidase was obtained, whereas the expression of *lacL* alone did not yield an active protein. These observations suggest that both *lacL* and *lacM* are required for the synthesis of functional and active β-galactosidase. The precise reason for this is unclear at present, but it can be speculated that the simultaneous presence of polypeptides representing both subunits influences the correct folding of the protein, while a misfolded and therefore inactive protein is formed when only the large subunit is expressed. Translational coupling between *lacL* and *lacM* was also found previously in *Leuconostoc lactis* (David *et al*., 1992).

E. coli BL21 Star (DE3) was used as the expression host for the expression of β-galactosidase genes. Although this strain has background β-galactosidase activity, the expressed recombinant protein is histidine-tagged and can be easily purified with Ni Sepharose High Performance column and separated from the native β-galactosidase. Heterologous expression of the tagged protein resulted in the production of 110 kU of β-galactosidase activity, which could be efficiently purified by metal affinity chromatography in one single step, and approximately 83 kU of purified recombinant enzyme per liter of fermentation broth with a specific activity of 180 U/mg of protein was obtained. When using the natural source *L. reuteri* L103 for the production of β-

galactosidase, 2.5 kU per liter of fermentation broth was formed, of which 175 U (~ 7%) purified enzyme with a specific activity of 158 U/mg was obtained after a three-step-purification procedure (Nguyen *et al*., 2006). The β-galactosidase activities described here are given as activity with the chromogenic model substrate *o*NPG, which is approximately 4.6-fold higher than the activity with the natural substrate lactose.

Unexpectedly, the optimum pH of recombinant EL103 β-galactosidase shifted 1 pH value for *o*NPG lysis. Recombinant EL103 β-gal also exhibited a slightly broader and higher temperature optimum range for lactose hydrolysis compared to native L103 β-gal. The reason for these changes is not clear, but can probably be attributed to slightly aberrant folding of the polypeptide chains by *E. coli* as compared to *L. reuteri*. However, these changes did not affect the spectrum and the yield of galacto-oligosaccharides (GOS) from lactose using recombinant β-gal EL103. Recombinant β-gal EL103 formed a similar GOS spectrum as native β-gal L103 (data not shown) with the main products, identified being β-D-Gal*p*-(1→6)-D-Glc (allolactose), β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-Lac, β-D-Gal*p*-(1→3)-Lac (Splechtna *et al*., 2006).

In conclusion, this work presents the cloning and expression of the genes encoding heterodimeric β-galactosidase from *L. reuteri* in *E. coli*. The cloning of these genes into the expression vector pET21d enables efficient production of the protein of interest in this study in gram scale. The overproduction of this enzyme in yeast or other food-grade expression system, which is ongoing in our project, is also of interest for the applications in food industry.

Finally, it should be mentioned that at the time the experimental work of this research was carried out, no sequence of *L. reuteri* β-galactosidases was yet published.

Genome shotgun sequence of *L. reuteri* JCM 1112 (GenBank accession number NZ_AAOV01000035) was published during the preparation of this manuscript.

Acknowledgements

This research work was supported by the Research Centre Applied Biocatalysis (Graz, Austria). The authors also wish to thank Roman Kittl and Christoph Sygmund (BOKU, Vienna) for the fruitful discussions during the experimental work of this research.

References

Benno, Y., Endo, K., Mizutani, T., Namba, Y., Komori, T., Mituoka, T., 1989. Comparison of fecal microflora of elderly persons in rural and urban areas of Japan. Appl. Environ. Microbiol. 55, 1100-1105.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cummings, J.H., Macfarlane, G.T., Englyst H.N., 2001. Prebiotic digestion and fermentation. Am. J. Clin. Nutr. 73(suppl), 415S-420S.

Cupples, C.G., Miller, J.H., Huber, R.E., 1990. Determination of the roles of Glu-461in β-galactosidase (*Escherichia coli*) using site-specific mutagenesis. J. Biol. Chem. 265, 5512-5518.

David, S., Stevens, H., van Riel, M., Simons, G., de Vos, W. M., 1992. Leuconostoc lactis β-galactosidase is encoded by two overlapping genes. J. Bacteriol. 174, 4475-4481.

De Man, J.D., Rogosa, M., Sharpe, M.E., 1960. A Medium for the Cultivation of Lactobacilli. J. Appl. Bact. 23, 130-135.

Fortina, M.G., Ricci, G., Mora, D., Guglielmetti, S., Manichini, P.L., 2003. Unusual organization for lactose and galactose gene clusters in *Lactobacillus helveticus*. Appl. Environ. Microbiol. 69, 3238-3243.

Gebler, J.C, Aebersold, R., Withers, S.G., 1992. Glu-537, not Glu-461, is the nucleophile in the active site of (lacZ) β-galactosidase from *Escherichia coli*. J. Biol. Chem. 267, 11126-11130.

Germond, J.-E, Lapierre, L., Delley, M., Mollet, B., Felis, G.E., Dellaglio, F., 2003. Evolution of the bacterial species *Lactobacillus delbrueckii*: a partial genomic study with reflections on prokaryotic species concept. Mol.Biol. Evol. 20, 93-104

Gibson, G.R., Roberfroid, M.B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutri. 125,1401-1412.

Henrissat, B., 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 280: 309-316

Jacobson, R.H., Zhang, X-J., DuBose, R.F., Matthews, B.W., 1994. Three-dimensional structure of β-galactosidase from *E. coli*. Nature. 369, 761-766.

Kabuki, T., Saito, T., Kawai, Y., Uemura, J., Itoh, T., 1997. Production, purification and characterization of reutericin 6, a bacteriocin with lytic activity produced by *Lactobacillus reuteri* LA6, Int. J. Food Microbiol. 34, 145-156.

Kalnins, A., Otto, K., Ruther, U., Muller-Hill, B., 1983. Sequence of the lacZ gene of *Escherichia coli*. EMBO J 2, 593-597.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W., Stiekema, W., Lankhorst, R.M., Bron, P.A., Hoffer, S.M., Groot, M.N., Kerkhoven, R., de Vries, M., Ursing, B., de Vos, W.M. and Siezen, R.J., 2003. Complete genome sequence of Lactobacillus plantarum WCFS1. Proc. Natl. Acad. Sci. U.S.A. 100, 1990-1995.

Kunst, A., Draeger, B., Ziegenhorn, J., 1988. Colorimetric methods with glucose oxidase and peroxidase. In: Bergmeyer, H.U., Bergmeyer, J., Graßl, M. (Eds.), Methods of enzymatic analysis. 3rd edition, Weinheim, Germany: VCH Publishers, pp178-185.

Mizobuchi M., Frohman, L.A., 1993. Rapid amplification of genomic DNA ends. Biotechniques 15, 214-216.

Nicholas, K.B., Nicholas H.B.Jr., Deerfield, D.W. II., 1997. GeneDoc: Analysis and Visualization of Genetic Variation. EMBNEW.NEWS 4, 14. http://www.psc.edu/biomed/genedoc.

Nakayama, T., Amachi, T., 1999. β-galactosidase, enzymology. In: Flickinger, M.C., Drew, S.W. (Eds.), Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation. John Wiley and Sons, New York, NY, vol. 3, pp. 1291-1305.

Nguyen, T.-H, Splechtna, B., Steinböck, M., Kneifel, W., Lettner, H.P., Kulbe, K.D. and Haltrich, D., 2006. Purification and characterization of two novel β-galactosidases from *Lactobacillus reuteri*. J. Agric. Food Chem. 54, 4989-4998.

Obst, M., Meding, E.R., Vogel, R.F., Hammes, W.P., 1995. Two genes encoding the β-galactosidase of *Lactobacillus sake*. Microbiology 141, 3059-3066.

Pivarnik, L.F., Senegal, A.G., Rand, A.G., 1995. Hydrolytic and transgalactosylic activities of commercial β-galactosidase (lactates) in food processing. In: Kinsella, J.E., Taylor, S.L. (Eds.), Advances in Food and Nutrition research. Academic Press, vol. 38, pp. 1-102.

Pridmore, R.D., Berger, B., Desiere, F., Vilanova, D., Barretto, C., Pittet, A.-C., Zwahlen, M.-C., Rouvet, M., Barrangou, R., Altermann, E., Mollet, B., Mercenier, A., Klaenhammer, T., Arigoni, F. and

Schell, M.A., 2004. The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. Proc. Natl. Acad. Sci. U.S.A. 101, 2512-2517.

Rastall, R.A., Maitin, V., 2002. Prebiotics and synbiotics: towards the next generation. Curr. Opin. Biotechnol. 13, 490-496.

Ring, M., Huber, R.E., 1990. Multiple replacements establish the importance of Tyrosine-503 in β-galactosidase (*Escherichia coli*). Arch. Biochem. Biophys. 283, 342-350.

Splechtna, B., Nguyen, T.-H., Steinböck, M., Kulbe, K.D., Lorenz, W., Haltrich, D. 2006. Production of prebiotic galacto-oligosaccharides from lactose using β-galactosidases from *Lactobacillus reuteri*. J. Agric. Food Chem. 54, 4999-5006.

Sung, H.W., Chen, C.N., Liang, H.F., Hong, M.H., 2003. A natural compound (reuterin) produced by *Lactobacillus reuteri* for biological-tissue fixation. Biomaterials 24, 1335-1347.

Suzuki, M., Saito, T., Konno, K., Kamio, Y., Itoh, T., 1997. β-Galactosidase from *Lactobacillus acidophilus* JCM1229 is consisted of two nonidentical subunits (unpublished). EMBL/GenBank/DDBJ databases, link: http://us.expasy.org/cgi-bin/niceprot.pl?O07684.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876-4882. (link: http://bips.u-strasbg.fr/en/Documentation/ClustalX/)

FIGURE CAPTIONS

Fig. 1. Overlapping coding region of the *lacL* and *lacM* genes of *Lactobacillus reuteri* L103 β-galactosidase.

Fig. 2. Amino acid sequence alignment of (A) *lacL* and (B) *lacM* genes of the β-galactosidases from *Lactobacillus reuteri* L103 (GenBank accession no. ABF72116 and ABF72117, respectively) and some other *Lactobacillus* spp. and *Leuconostoc lactis*. GenBank accession numbers are indicated.

Fig. 3. Amino acid alignment of the flanking regions of the presumed acid-base catalyst, nucleophilic recognition site and ligands of the magnesium ion of *lacZ* from *E. coli* and *lacL* from *L. reuteri* L103 (GenBank accession no. ABF72116).

Fig. 4. (A) SDS-PAGE of β-galactosidase (*lacLM*) from *Lactobacillus reuteri* L103 overexpressed in *E. coli* BL21 Star (DE3). Lane 1, recombinant molecular weight markers (Biorad); lanes 2 and 3, Coomassie blue staining of whole-cell lysates of *E. coli* containing pHA1032 without induction (lane 2), and with 0.1 mM IPTG induction at 25°C for 12 hours (lane 3); lane 4, Coomassie blue staining of purified recombinant β-galactosidase EL103 (B) SDS-PAGE of purified native and recombinant β-galactosidases. Lane 1, recombinant molecular weight markers (Biorad), lanes 2 and 3, Coomassie blue staining of purified native β-galactosidase from *L. reuteri* L103 (lane 2) and recombinant β-galactosidase EL103 (lane 3); lanes 4 and 5, active staining with 4-methylumbelliferyl β-D-galactoside of native β-galactosidase L103 (lane 4) and recombinant β-galactosidase EL103 (lane 5).

Fig. 5. pH optimum (A, B) and temperature optimum (C, D) of native β-galactosidase from *L. reuteri* L103 and recombinant β-galactosidase EL103 (A, C: *o*NPG as the substrate; B, D: lactose as the substrate). The buffers used as shown in (A,B) were

sodium citrate (50 mM, pH 4.0-5.5) (circles), sodium phosphate (50 mM, pH 6.0-7.5) (triangles) and borate (50 mM, pH 8.0-9.0) (squares).

Fig. 6. pH stability of native L103 and recombinant EL103 β-galactosidases incubated at 37°C in sodium citrate buffer (pH 4-5.5), sodium phosphate buffer (pH 6.0 – 7.5) and borate buffer (pH 8.0 -9.0) with residual activity measured after 3 h.

TABLES

Table 1. Sequences of the primers used in this study

Primer	Sequence (5′→3′)	Location	Reference sequence accession no.
β-gal F1	ATGCAAGCAAAYATMAAWTGG	β-galactosidase (*lacL*)	DQ493596
β-gal F2	GGTGGTGATTTCGATGATCGTCAC	β-galactosidase (*lacL*)	DQ493596
β-gal F3	GGGAGATGGCTTGATGTTTGCTG	β-galactosidase (*lacL*)	DQ493596
β-gal F4	CCTGGTCTACCAGTAGTAGGAATGC	β-galactosidase (*lacM*)	DQ493596
β-gal F5	CCAACAGTTGCTACCGGCTTTG	β-galactosidase (*lacM*)	DQ493596
β-gal F6	ATGCAAGCAAATATAAAATGGCTTGATGAACCG	β-galactosidase (*lacL*)	DQ493596
β-gal F7	TCGCCCCCATGGAAGCAAATATAAAA	[a]	
β-gal R1	TTATTTGTGTAAKCCATARTA	β-galactosidase (*lacL*)	DQ493596
β-gal R6	TTATTTTGCATTCAATACAAACGAAAACTCAAC	β-galactosidase (*lacM*)	DQ493596
β-gal R7	GGAATTCCTCGAGTGATTTTGCATTCAATAC	[b]	
pBS R1	GTTGTGTGGAATTGTGAGCGG	pBluescript II SK (-)	X52330
pBS R2	ACGCCAAGCGCGCAATTAACC	pBluescript II SK (-)	X52330

F: denotes forward primers; R: denotes reverse primers.

[a] Upstream primer to amplify *lacLM* from pHA1031 with *Nco*I site (underlined)

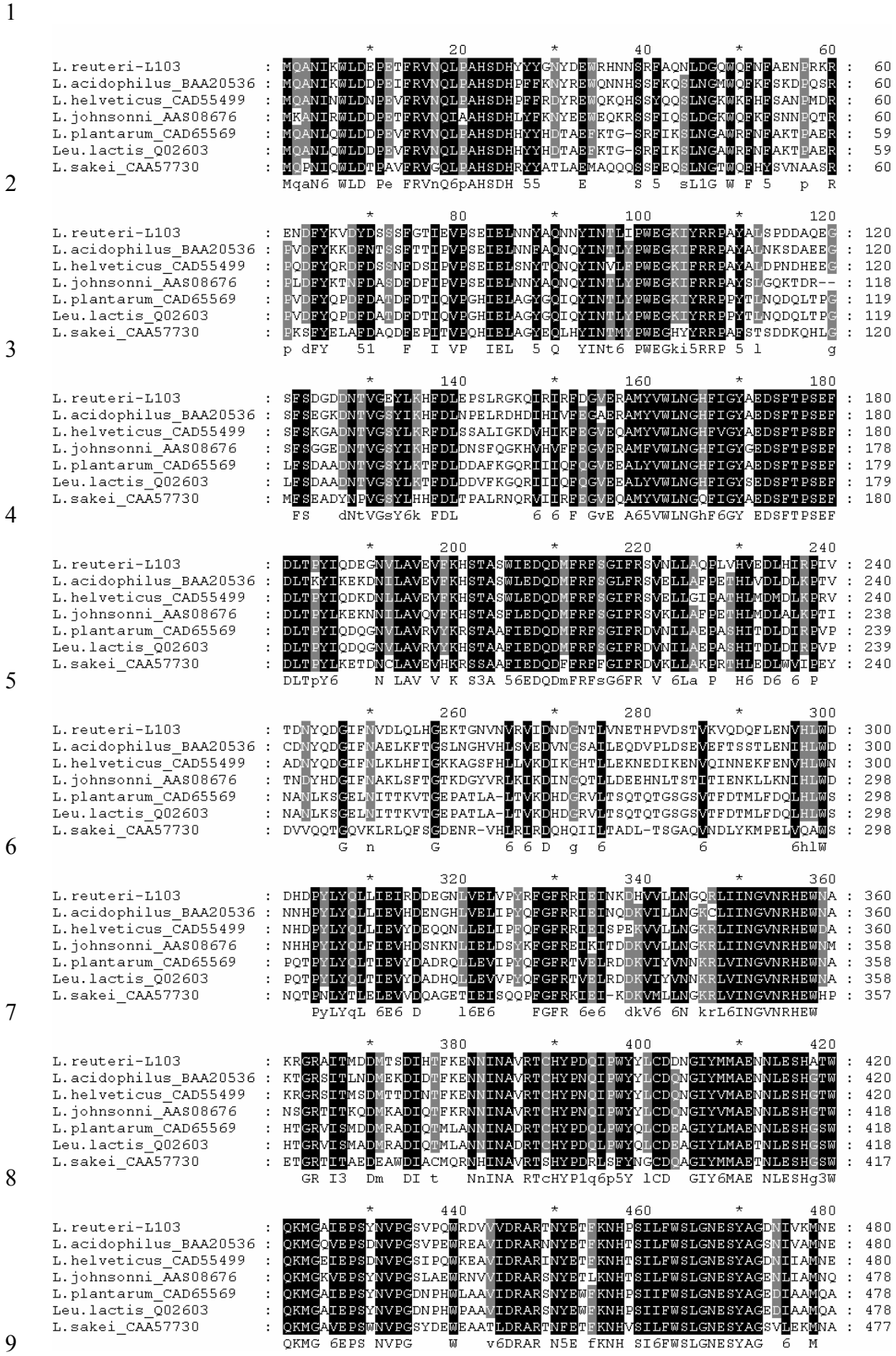
[b] Downstream primer to amplify *lacLM* from pHA1031 with *Xho*I site (underlined)

1 **FIGURES**

2

3

4 **Fig. 1.**



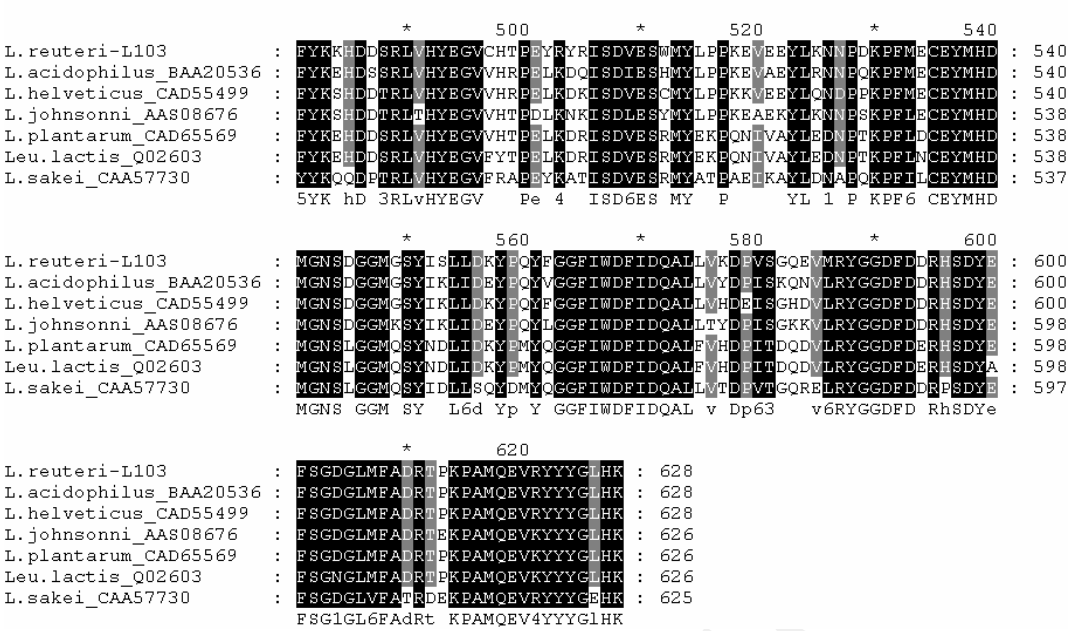


Fig. 2 (A)

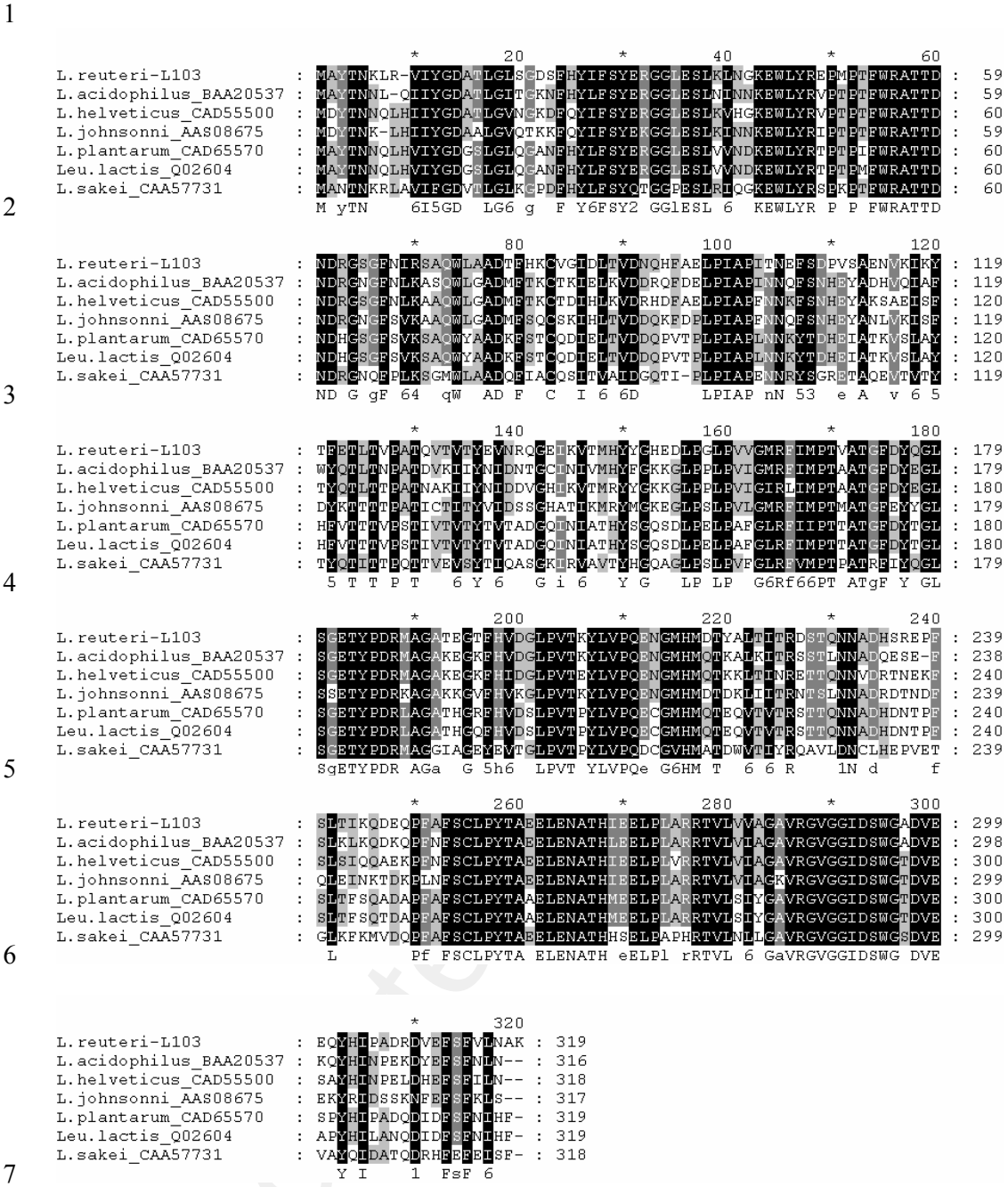


Fig. 2 (B)

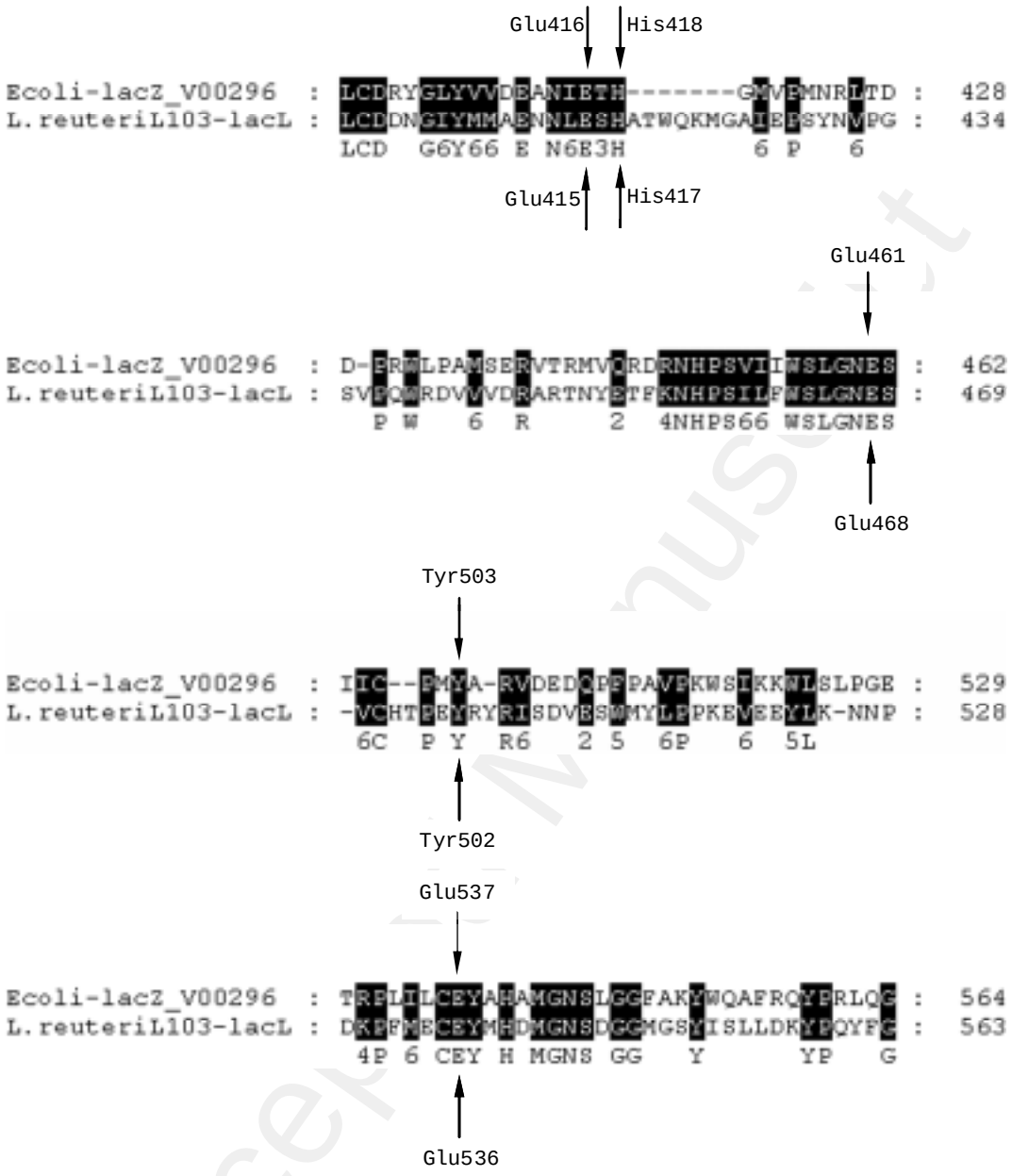
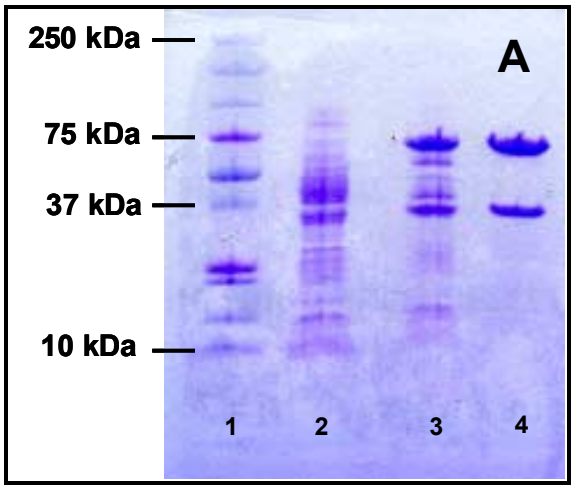


Fig. 3.



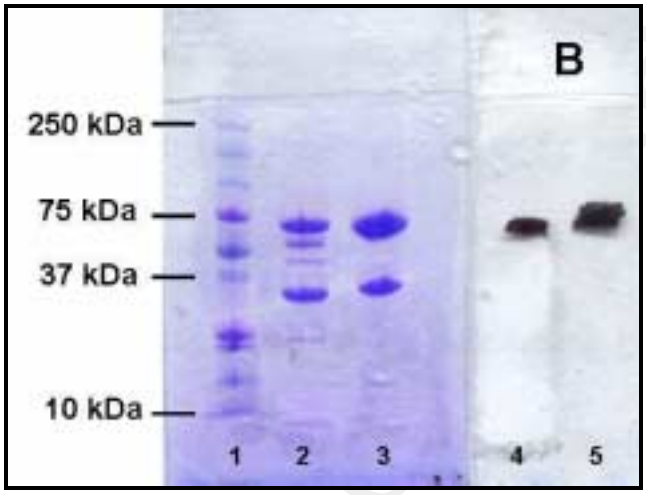
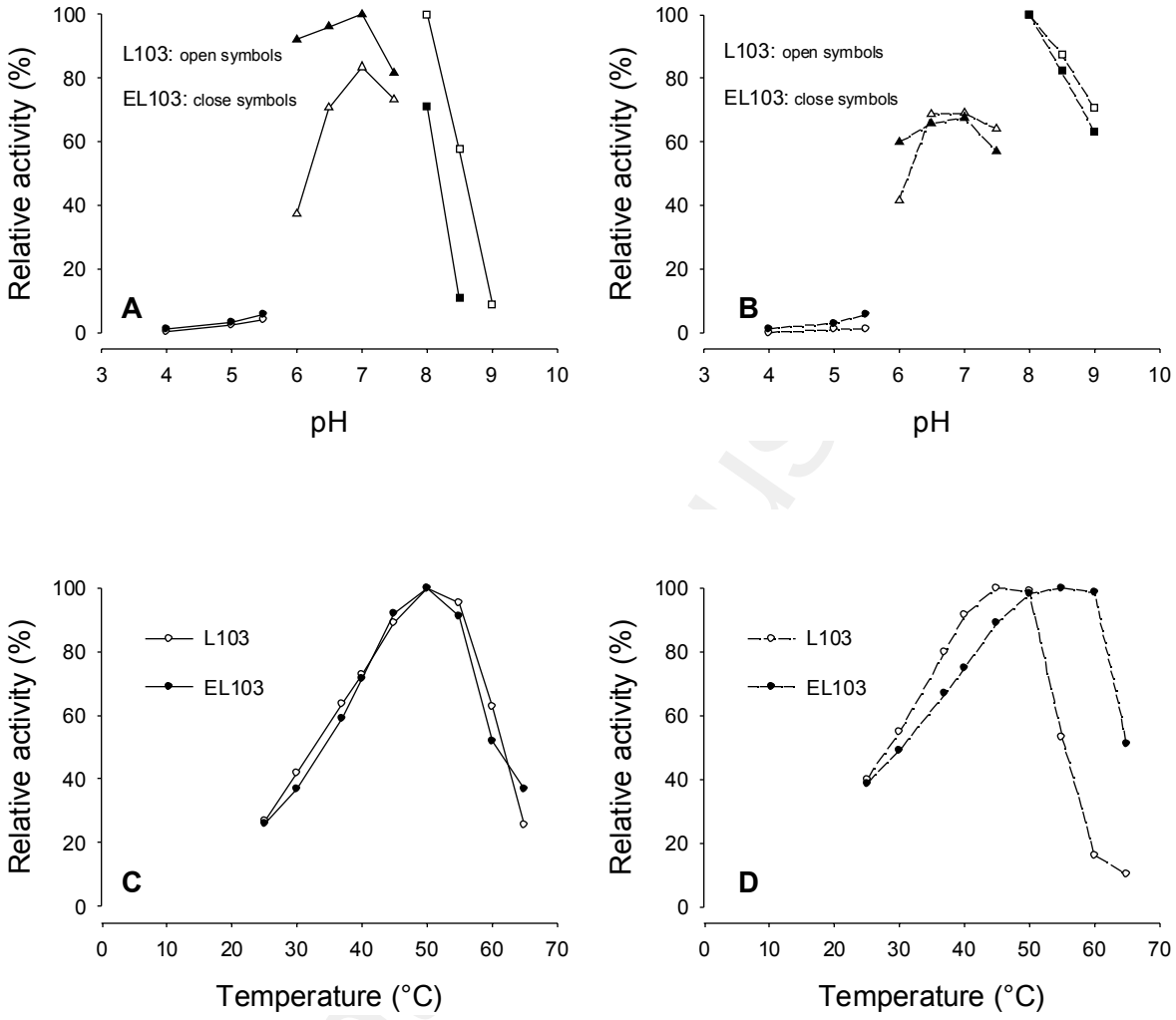


Fig. 4.

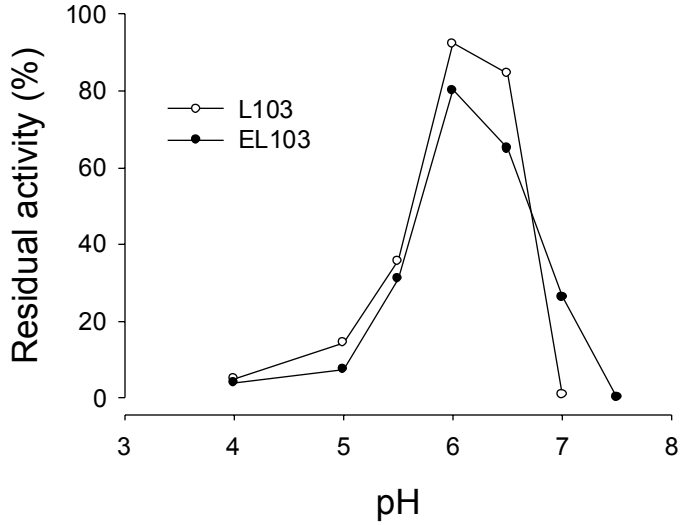
1



2
3
4
5

6 **Fig. 5.**

1



2

3

4 **Fig. 6.**