Physiological and biochemical characterization of the two α -L-rhamnosidases of *Lactobacillus plantarum* NCC245

Marta Ávila,¹ Muriel Jaquet,² Deborah Moine,² Teresa Requena,¹ Carmen Peláez,¹ Fabrizio Arigoni² and Ivana Jankovic²

Correspondence

Ivana Jankovic

ivana.jankovic@rdls.nestle.com

¹Departamento de Ciencia y Tecnología de Productos Lácteos, Instituto del Frío (CSIC), José Antonio Novais 10, Madrid 28040, Spain

²Nestlé Research Center, Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland

This work is believed to be the first report on the physiological and biochemical characterization of α -L-rhamnosidases in lactic acid bacteria. A total of 216 strains representing 37 species and eight genera of food-grade bacteria were screened for α -L-rhamnosidase activity. The majority of positive bacteria (25 out of 35) were *Lactobacillus plantarum* strains, and activity of the *L. plantarum* strain NCC245 was examined in more detail. The analysis of α -L-rhamnosidase activity under different growth conditions revealed dual regulation of the enzyme activity, involving carbon catabolite repression and induction: the enzyme activity was downregulated by glucose and upregulated by L-rhamnose. The expression of the two α -L-rhamnosidase genes *rhaB1* and *rhaB2* and two predicted permease genes *rhaP1* and *rhaP2*, identified in a probable operon *rhaP2B2P1B1*, was repressed by glucose and induced by L-rhamnose, showing regulation at the transcriptional level. The two α -L-rhamnosidase genes were overexpressed and purified from *Escherichia coli*. RhaB1 activity was maximal at 50 °C and at neutral pH and RhaB2 maximal activity was detected at 60 °C and at pH 5, with high residual activity at 70 °C. Both enzymes showed a preference for the α -1,6 linkage of L-rhamnose to β -D-glucose, hesperidin and rutin being their best substrates, but, surprisingly, no activity was detected towards the α -1,2 linkage in naringin under the tested conditions. In conclusion, we identified and characterized the strain *L. plantarum* NCC245 and its two α -L-rhamnosidase enzymes, which might be applied for improvement of bioavailability of health-beneficial polyphenols, such as hesperidin, in humans.

Received 23 January 2009

Revised 22 April 2009

Accepted 30 April 2009

INTRODUCTION

α -L-Rhamnosidase (EC 3.2.1.40) catalyses the hydrolysis of rhamnosides containing an α -glucosidic bond to liberate L-rhamnose. It is produced by a number of animal tissues, plants, fungi, bacteria and bacteriophage (Hashimoto *et al.*, 2003). The physiological role of α -L-rhamnosidase is not well understood but is probably linked to the broad distribution of L-rhamnose as a component in bacterial and plant cell walls, glycosides, biofilms and glycolipids (Bader *et al.*, 1998; Giavasis *et al.*, 2000). α -L-Rhamnosidase has been implicated in invasion processes of plant-pathogenic fungi, infection of bacteria by bacteriophages and meta-

bolism of bacterial biofilms (Hashimoto *et al.*, 1999; Hashimoto & Murata, 1998; Steinbacher *et al.*, 1996).

Bacterial α -L-rhamnosidase activity was first attributed to the gut bacteria, which together with β -glucosidase convert ingested flavonoid glycosides into their aglycone forms (Griffiths & Barrow, 1972; Macdonald *et al.*, 1983). *Bacteroides* strains able to hydrolyse the plant polyphenols rutin and robinin were isolated from human intestinal microflora (Bokkenheuser *et al.*, 1987). The first bacterial α -L-rhamnosidase was purified from *Bacteroides* JY-6 (Jang & Kim, 1996). To date, the genes encoding α -L-rhamnosidases of *Clostridium stercoarium* (*rhaA*) (Zverlov *et al.*, 2000), *Bacillus* sp. GL1 (*rhaA* and *rhaB*) (Hashimoto *et al.*, 1999, 2003), *Sphingomonas paucimobilis* (*rhaM*) (Miake *et al.*, 2000; Miyata *et al.*, 2005) and *Thermomicrobia* sp. (Birgisson *et al.*, 2004b) have been cloned and characterized. Recently, the crystal structure of α -L-rhamnosidase from *Bacillus* sp. GL1, an enzyme involved in the degradation of bacterial biofilm gellan, was resolved (Cui *et al.*, 2007).

Abbreviations: CCR, carbon catabolite repression; *p*-NP, *p*-nitrophenol; *p*-NPR, *p*-nitrophenyl α -L-rhamnopyranoside; qRT-PCR, quantitative reverse transcriptase PCR.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of the *rha* region of *L. plantarum* is FJ943501.

Two supplementary figures are available with the online version of this paper.

α -L-Rhamnosidases are used for different food applications. Naringinase (containing α -L-rhamnosidase and β -glucosidase activities) is used as a debittering agent for citrus juices (Puri *et al.*, 1996). Hesperetin 7-glucoside, a product of hesperidin hydrolysis by α -L-rhamnosidase, is an important precursor for sweetener production (Manzanares *et al.*, 2001). Furthermore, there is an increasing interest in the use α -L-rhamnosidases to enhance grape juice and wine flavour by release of free mono-terpenes from terpenyl glycosides (Williams *et al.*, 1982). Finally, α -L-rhamnosidases can be used to improve the bioavailability of polyphenols, such as hesperidin (Nielsen *et al.*, 2006). Hesperidin is a component extracted from citrus pith that exerts several potential health-beneficial effects, which have been demonstrated in animal and *in vitro* studies. These include the promotion of bone health (Kalu, 1991), lipid-lowering effects (Park *et al.*, 2001), antioxidant properties (Miyake *et al.*, 1998), cardio-protective effects (Ohtsuki *et al.*, 2002), and anti-cancer (Tanaka *et al.*, 2000) and anti-inflammatory properties (Guardia *et al.*, 2001). However, the bioavailability of hesperidin is low and occurs only in the distal part of the gastrointestinal tract in humans due to the lack of the enzyme α -L-rhamnosidase in the small intestine (Nielsen *et al.*, 2006). Colonic commensal bacteria remove L-rhamnose and glucose moieties of hesperidin, leaving the aglycone form (hesperetin) to be absorbed (Griffiths & Barrow, 1972; Macdonald *et al.*, 1983). In contrast to hesperidin, a product of hesperidin hydrolysis by α -L-rhamnosidase, hesperetin 7-glucoside, is deglycosylated in the small intestine by endogenous β -glucosidase (with exohydrolase activity), after which the absorption of the aglycone (hesperetin) is very fast (Nielsen *et al.*, 2006). Due to its better bioavailability, hesperetin 7-glucoside yielded higher total serum concentrations of hesperetin than equivalent amounts of hesperidin (Nielsen *et al.*, 2006). Hesperidin can be converted into hesperetin 7-glucoside using purified enzyme or with the help of α -L-rhamnosidase-producing micro-organisms.

The purpose of this work was to identify bacterial enzymes able to convert hesperidin into hesperetin 7-glucoside in the small intestine. We report the selection of a food-grade lactic acid bacterium with α -L-rhamnosidase activity and characterization of its enzyme production. Detailed analysis of *L. plantarum* NCC245 revealed that the enzyme is induced by L-rhamnose and repressed in the presence of glucose, indicating that α -L-rhamnosidase activity is controlled via carbon catabolite repression. The two genes of NCC245 encoding α -L-rhamnosidase were cloned and the corresponding enzymes characterized.

METHODS

Bacterial source and growth conditions. The bacteria tested in this study originated from the Nestlé Culture Collection (NCC). The strains were grown in the presence of sugars as indicated in the text. Lactobacilli, leuconostocs and pediococci were grown aerobically at

30 °C as static cultures in MRS fermentation broth (De Man *et al.*, 1960), which contains neither glucose nor meat extract. Bifidobacteria were grown anaerobically at 37 °C in the same MRS medium supplemented with 0.5% cysteine (Fluka). Streptococci and lactococci were grown at 37 °C and 30 °C, respectively, in M17 broth (Difco) as static cultures. Staphylococci were grown under agitation at 30 °C in M17.

Analysis of α -L-rhamnosidase activity. α -L-Rhamnosidase activity was measured by employing *p*-nitrophenyl α -L-rhamnopyranoside (*p*-NPR; Sigma) as substrate. Enzyme extracts and conditions of the assay depended on the stage of the study: (i) screening of strains, (ii) quantitative determination in *L. plantarum* NCC245 and (iii) characterization of recombinant enzymes.

(i) The screening method for α -L-rhamnosidase activity was carried out with cells obtained from an overnight culture of each strain grown in its appropriate medium. The cells were harvested, resuspended in citrate/phosphate buffer (CPB), pH 6, and disrupted with 106 μ m glass beads in a Mini-Beadbeater-8 cell disrupter (Biospec Products) for three intervals of 30 s each. Crude extract was then incubated at 37 °C for 3 h in a 5 mM *p*-NPR solution (CPB, pH 6). The pH of the reaction was then adjusted to pH 9 with NaOH and yellow reactions were scored positive. As a positive control we used 10 μ g hesperidinase ml⁻¹ (Amano Enzyme Inc.) in 5 mM *p*-NPR (CPB, pH 6).

(ii) The quantitative determination of α -L-rhamnosidase activity in *L. plantarum* NCC245 was done in crude bacterial extracts obtained as described above. Extracts prepared from 30 ml cells adjusted to OD₆₀₀ 1 were used. The enzyme activities of cell extracts were measured in 5 mM *p*-NPR (CPB, pH 6) at 420 nm and 37 °C every minute for 30 min. The activity of the enzyme was expressed as nmol of released *p*-nitrophenol (*p*-NP) per minute per mg of protein of the crude extract. The absorption coefficient of *p*-NP was calculated from a standard curve made using 0.02–0.06 μ mol *p*-NP ml⁻¹ (Sigma). The concentration of protein was determined spectrophotometrically using the Bradford method (Sigma).

(iii) Standard enzymic reactions (final volume 800 μ l) with the recombinant α -L-rhamnosidases were performed in 50 mM sodium phosphate buffer, pH 7, for RhaB1, and in 50 mM sodium acetate buffer, pH 5, for RhaB2, containing 1 mM *p*-NPR and appropriately diluted enzyme solutions (93 ng RhaB1 and 2.2 μ g RhaB2), unless stated otherwise. Samples were incubated for 10 min at 37 °C (RhaB1) or 55 °C (RhaB2); the reaction was then stopped on ice by adding 200 μ l 0.1 M NaOH and absorbance was measured at 405 nm. The molar absorption coefficient for *p*-NP at 405 nm was taken as ϵ = 18.5 mM⁻¹ cm⁻¹ (Birgisson *et al.*, 2004a). The enzymic activity was expressed as units per mg protein. One unit (U) of enzyme activity was defined as the amount of recombinant enzyme that releases 1 μ mol of product (*p*-NP or other substrate) per minute.

RNA preparation. Isolation of total RNA was performed by the method of Kuipers *et al.* (1993), with the following modifications: macaloid/phenol/sodium dodecyl sulfate/bacteria suspensions were disrupted in a Mini-Beadbeater-8 for three intervals of 1 min each and centrifuged to remove glass beads and cell debris. After phenol extraction, total RNA was precipitated from the aqueous phase with ethanol, suspended in 100 μ l diethylpyrocarbonate-treated water, and stored at -80 °C. A 2 h treatment with 20 U DNase I (Ambion) at 37 °C was performed, followed by RNA purification with the RNeasy kit (Qiagen). RNA quality was controlled using an Agilent 2100 bioanalyser by looking at the integrity of 16S and 23S rRNAs.

Sequencing of the *rha* region in *L. plantarum* NCC245. The *rha* region of *L. plantarum* NCC245 was PCR amplified from genomic DNA of this strain, using the Expand High Fidelity PCR System

(Roche) with the primer pairs pWCFS_F1/pWCFS_R1 and pWCFS_F3/pWCFS_R4 (Table 1). The primers were designed using the genome sequence of *L. plantarum* WCFS1 (Kleerebezem *et al.*, 2003). The amplified fragments were sequenced by Fasteris SA.

Quantitative reverse transcriptase PCR (qRT-PCR). *L. plantarum* NCC245 was grown on 1 % glucose or galactose as carbon source and in the presence of 0.5 % L-rhamnose, 0.05 % rutin, 0.05 % hesperidin or 0.05 % naringenin. The RNA cDNAs were synthesized from RNAs using random hexamers and TaqMan reverse transcription reagents (Applied Biosystems) according to the supplied protocol. Real-time PCR was performed in an ABI PRISM 7000 machine (Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems) following the supplied protocol. PCR cycling conditions were: 50 °C 2 min; 95 °C 10 min; 95 °C 15 s; 60 °C 1 min; 40 cycles. Mean relative gene expression values were calculated from two independent biological replicates using ΔC_t (comparative critical threshold method) after normalization with 16S rRNA signal. The primers were designed with the PRIMEREXPRESS software and are listed in Table 1.

Cloning of *rhaB1* and *rhaB2* into *Escherichia coli*. DNA manipulations, plasmid isolation and *E. coli* TG1 (Stratagene) transformation were performed in accordance with standard procedures (Sambrook & Russell, 2001). The *rhaB* genes were PCR amplified from genomic DNA of *L. plantarum* NCC245 using the Expand High Fidelity PCR System (Roche) with the primer pair prhaB1_F1/prhaB1_R1 for *rhaB1* and prhaB2_F1/prhaB2_R1 for *rhaB2* (Table 1). The primers were designed to provide a coding region of the genes in-frame with the start codon and with C-terminal histidine tag of pET24d (Novagen). The two PCR fragments of 1955 bp for *rhaB1* and 1578 bp for *rhaB2* were cloned into the *NcoI* and *XhoI* sites of pET24d to produce the plasmids pDM19 and pDM20, respectively. The plasmids were transformed into *E. coli* BL21 S1 (Life Technologies), in which the expression of T7 polymerase is under the control of a salt-inducible promoter.

Overexpression and purification of RhaB1 and RhaB2 recombinant proteins. Expression of the α -L-rhamnosidase genes was induced by salt present in LB growth medium and by adding IPTG (final concentration 1 mM) to *E. coli* BL21 S1(pDM19) and *E. coli* BL21 S1(pDM20) fresh cultures grown to an OD₆₀₀ of 0.5. To optimize enzyme expression, 0.8 mM glucose was also added to LB medium and cultures were incubated at 30 °C. After 3 h of further incubation, the cells were harvested by centrifugation. The production of the target proteins was verified by the analysis of the soluble cytoplasmic fractions. For enzyme purification, harvested cells from expression cultures were frozen at –80 °C, resuspended in 1 ml binding buffer (0.02 M sodium phosphate, pH 7.4, 0.5 M NaCl, 0.01 M imidazole), and then mixed (1:1, w/v) with glass beads to obtain supernatant fractions containing soluble cytoplasmic proteins. Recombinant enzymes were purified from the supernatant fractions by immobilized metal-affinity chromatography, employing 1 ml HiTrap Chelating HP columns (Amersham Biosciences) according to the manufacturer's instructions. The enzymes were eluted with 0.5 M imidazole and the solution was desalted (PD-10 column) and stored in 50 mM sodium phosphate, pH 7, at –80 °C. IPTG-induced culture of *E. coli* BL21 S1(pET24d), with only the expression vector, was used as a negative control.

The apparent molecular mass of the purified enzymes was estimated by native PAGE on 4–12 % pre-cast Tris/glycine gels using a non-denatured protein high molecular mass marker (Amersham Biosciences) as standard. The result was confirmed by gel filtration chromatography with a Superdex 200 HR 10/30 column (Amersham Biosciences), previously calibrated with thyroglobulin, urease, bovine serum albumin, albumin chicken egg and carbonic anhydrase (Sigma).

Biochemical characterization of RhaB1 and RhaB2 recombinant enzymes. The temperature optimum of α -L-rhamnosidase activity was assayed from 5 °C to 70 °C in standard conditions. The pH optimum for the two recombinant enzymes' activity was investigated in sodium acetate buffer (pH 3–5.5), sodium phosphate

Table 1. Real-time PCR and cloning primers

Primer name	Sequence 5'–3'
prhaP2_F1_realtime	TCAAGCACATGCGAGTCAAAG
prhaP2_R1_realtime	GCCAACAACGAGATAAAGTTTAGGA
prhaB2_F1_realtime	TGGCGAGCTGTTTTAGCAA
prhaB2_R1_realtime	GTTGATGAACTGTCGCAGTGTCTA
prhaP1_F1_realtime	GGTTATTTTCGGAACGGATGTCT
prhaP1_R1_realtime	CAAAATGCCCATCGTCCAA
prhaB1_F1_realtime	CGCGCTGTGAATGGAACGTG
prhaB1_R1_realtime	CGGACTCCCCGTAACATAGTG
p16S_F1_realtime	TGATCCTGGCTCAGGACGAA
p16S_R1_realtime	TGCAAGCACCAATCAATACCA
prhaB1_F1 (<i>NcoI</i> site underlined)	AAAGGGCCATGGTGTGCGAAAGAGGCTGTTTGGT
prhaB1_R1 (<i>XhoI</i> site underlined)	AAAGGGCTCGAGCACTGGGACCACCGCAGTTCT
prhaB2_F1 (<i>NcoI</i> site underlined)	AAAGGGCCATGGCGTTTACTTTTCAAATCAATA
prhaB2_R1 (<i>SalI</i> site underlined)	AAAGGGCTCGACAACGAGGTACTTATTAATCAA
pWCFS_F1	GCTAATGATCCGATGGCA
pWCFS_R1	TTCAAAATCACCAGGATACCA
pWCFS_F3	TGGTTGGATGTACGGTGC
pWCFS_R4	ACCGTAACGTTCCGCCAACT

buffer (pH 6–7.5) and Tris/HCl buffer (pH 8–9), each at 50 mM, in standard conditions. Temperature and pH stabilities were evaluated after incubating the enzymes at different temperatures (RhaB1 at 37 °C; RhaB2 at 45 °C) and pH values (RhaB1 at pH 7; RhaB2 at pH 5) for different times up to 5 h. Purified enzymes were preincubated with 1 or 10 mM concentrations of several metal ions and chemical agents at 37 °C (RhaB1) or 45 °C (RhaB2) for 20 min. The residual α -L-rhamnosidase activities were measured under standard conditions for each α -L-rhamnosidase.

Substrate specificity. Hydrolysis of naringin, hesperidin, rutin, quercitrin, gellan gum, proscillaridin A, vitexin 2-O-rhamnoside and α -chaconine were measured using the 3,5-dinitrosalicylic acid method for detection of reducing ends of released L-rhamnose (Wood, 1988). Enzymic reactions were performed in 50 mM sodium phosphate buffer, pH 7, for RhaB1 and in 50 mM sodium acetate buffer, pH 5, for RhaB2, containing 2.0 mM of each substrate except gellan gum, which was at 0.15% (w/v), in a final volume of 0.3 ml. All the reaction mixtures contained 2% (v/v) DMSO (Probus). Samples were incubated for 10 min at 37 °C (RhaB1) or 55 °C (RhaB2). When examining substrate specificity, 17.5 μ g RhaB1 was added for all substrates except for rutin (1.75 μ g) and 16.4 μ g RhaB2 was added for all substrates except for rutin (1.64 μ g) and hesperidin (0.16 μ g).

Kinetics. Kinetic parameters of the α -L-rhamnosidases were calculated using *p*-NPR, hesperidin and rutin at concentrations ranging from 0.1 to 2 mM for *p*-NPR and 0.5 to 2.5 mM for hesperidin and rutin. K_m and V_{max} values were calculated by fitting data to the Lineweaver–Burk linear transformation of the Michaelis–Menten equation. Enzyme activity inhibition was studied using L-rhamnose at concentrations ranging from 0 to 15 mM and at fixed concentration of the substrate *p*-NPR (0.5 mM, 1 mM and 2 mM) and results analysed by fitting data to linear regression in the Dixon plot.

RESULTS

Selection of bacteria with α -L-rhamnosidase activity

We screened 216 strains representing 37 species and eight genera of food-grade bacteria from the Nestlé Culture Collection (NCC) for α -L-rhamnosidase activity (Table 2). Preliminary analysis revealed that none of the tested strains was able to grow on hesperidin and most of them were not able to grow on L-rhamnose as a carbon source. Thus, the bacteria were grown overnight in medium containing 0.3% glucose (lactobacilli, leuconostocs, pediococci, bifidobacteria and staphylococci) or 0.2% lactose (streptococci and lactococci) and 0.5% L-rhamnose, and α -L-rhamnosidase was tested as described in Methods. Under these conditions glucose (or lactose) was completely depleted after overnight growth, and the only residual sugar present in the medium was L-rhamnose (data not shown). The absence of glucose/lactose was chosen to avoid glucose repression (carbon catabolite repression, CCR), which often regulates carbohydrate utilization genes (Deutscher, 2008). L-Rhamnose, which has been shown to induce expression of α -L-rhamnosidase genes in some bacteria (Hashimoto *et al.*, 1999; Miyata *et al.*, 2005), was added as a potential inducer.

Thirty-five strains, representing seven of the 37 species tested, exhibited α -L-rhamnosidase activity (Table 2), with

Table 2. Species screened for α -L-rhamnosidase activity

Species	Tested*	Positive†
<i>Bacillus subtilis</i>	1	0
<i>Bifidobacterium breve</i>	2	0
<i>Bifidobacterium bifidum</i>	2	0
<i>Bifidobacterium adolescentis</i>	2	0
<i>Bifidobacterium infantis</i>	1	0
<i>Bifidobacterium longum</i>	5	0
<i>Bifidobacterium catenulatum</i>	2	0
<i>Bifidobacterium pseudocatenulatum</i>	2	0
<i>Enterococcus durans</i>	3	1
<i>Enterococcus faecium</i>	10	1
<i>Lactobacillus acidophilus</i>	12	3
<i>Lactobacillus brevis</i>	4	0
<i>Lactobacillus buchneri</i>	3	0
<i>Lactobacillus crispatus</i>	7	2
<i>Lactobacillus curvatus</i>	4	0
<i>Lactobacillus delbrueckii</i>	7	0
<i>Lactobacillus farciminis</i>	3	0
<i>Lactobacillus fermentum</i>	4	0
<i>Lactobacillus gasseri</i>	7	2
<i>Lactobacillus helveticus</i>	4	0
<i>Lactobacillus johnsonii</i>	9	0
<i>Lactobacillus paracasei</i>	10	0
<i>Lactobacillus plantarum</i>	33	25
<i>Lactobacillus reuteri</i>	8	0
<i>Lactobacillus rhamnosus</i>	10	0
<i>Lactobacillus sakei</i>	4	0
<i>Lactobacillus salivarius</i>	5	0
<i>Lactococcus lactis</i>	10	0
<i>Leuconostoc mesenteroides</i>	12	1
<i>Pediococcus acidilactici</i>	2	0
<i>Pediococcus cerevisiae</i>	2	0
<i>Pediococcus pentosaceus</i>	2	0
<i>Streptococcus carnosus</i>	3	0
<i>Streptococcus macedonicus</i>	5	0
<i>Streptococcus salivarius</i>	5	0
<i>Streptococcus thermophilus</i>	8	0
<i>Streptococcus xylophilus</i>	3	0
Total	216	35

*Number of tested strains.

†Number of α -L-rhamnosidase-positive strains.

the majority being strains of *L. plantarum* (25 out of 35). In the remaining six species, α -L-rhamnosidase activity was detected only in a few strains. Among the *L. plantarum* strains, NCC245 showed the highest activity in the preliminary screening and was selected for further characterization of α -L-rhamnosidase activity.

Influence of sugars on α -L-rhamnosidase activity

Aiming to achieve the maximal α -L-rhamnosidase activity, we studied the influence of different L-rhamnose-containing glycosides and sugars on the enzyme activity. *L. plantarum* NCC245 was not able to grow on hesperidin,

rutin or naringenin as carbon source. When the strain was grown on different mono- and disaccharides, the enzyme activity varied considerably, suggesting that the production or activity of the enzymes is regulated at the molecular level.

Starting with the hypothesis that one of the mechanisms involved in the regulation of α -L-rhamnosidase expression is CCR, we first looked for a carbon source that did not repress the enzyme activity. α -L-Rhamnosidase activity of the cells grown in the presence of 1 % of the carbon source (glucose, sucrose, galactose or maltose) and 0.5 % of L-rhamnose (as a potential inducer) to the mid-exponential, late exponential, early stationary and late stationary growth phases was measured (Fig. 1). The highest α -L-rhamnosi-

Fig. 1. Influence of carbon source on α -L-rhamnosidase activity of *L. plantarum* NCC245. (a) Non-repressing carbon source. The strain was grown in Sixfors fermenters on 1 % glucose (Glc), 1 % sucrose (Suc), 1 % galactose (Gal) or 1 % maltose (Mal) as a carbon source and in the presence of 0.5 % L-rhamnose (Rha) as an inducer. The cells were harvested at four stages of growth as indicated with filled symbols. α -L-Rhamnosidase activity of cell crude extracts was determined as described in Methods. (b) Inducer compounds. The strains were grown in Sixfors fermenters on 1 % galactose (Gal) as a carbon source and in the presence of 0.5 % L-rhamnose (Rha), 0.05 % rutin (Rut), 0.05 % hesperidin (Hes) and 0.05 % naringin (Nar) as inducers. Glucose (Glc)-grown cells were used as control. The cells were harvested in the late stationary phase, after 16 h growth, and α -L-rhamnosidase activity of cell crude extracts was determined as described in Methods. The data are the means of at least three biological replicates.

dase activity was obtained when the strain was grown in the presence of galactose as carbon source (Fig. 1a). The activity reached up to 45-fold higher levels compared to the activity of cells grown on glucose. The activities of the cells grown on maltose and sucrose were 10- and 25-fold higher, respectively, than the activity of the cells grown on glucose. Unusually for carbohydrate catabolic enzymes, the activity of α -L-rhamnosidase was the highest in the late stationary phase. Thus, all further analyses were done with cells from late stationary phase cultures and the fermentation time was reduced from 24 h to 16 h after confirming that the enzyme activity was the same at these two time points.

In previous experiments, we used L-rhamnose as an inducer for α -L-rhamnosidase. However, other sugars or glycosides containing L-rhamnosyl moieties, such as hesperidin, rutin, naringin or quercitrin, could also be potential inducers of α -L-rhamnosidase. To compare the α -L-rhamnosidase induction by L-rhamnose with other potential inducers, we grew bacteria in the presence of 1 % galactose as carbon source and 0.5 % L-rhamnose, 0.05 % hesperidin, 0.05 % naringin and 0.05 % rutin as inducers and measured α -L-rhamnosidase activity (Fig. 1b). Due to the low solubility of polyphenols, it was not possible to use the concentration of 0.5 % as used in previous experiments with L-rhamnose. After confirming that 0.05 % L-rhamnose induces the α -L-rhamnosidase to the same level, we continued using 0.5 % L-rhamnose in further experiments. The only sugar able to induce α -L-rhamnosidase of *L. plantarum* NCC245 was L-rhamnose, with maximal specific activity of 22 nmol *p*-NP min⁻¹ (mg protein)⁻¹, which is three times higher than the activity of the cells grown on galactose without inducer. Compared to cells grown on glucose or glucose plus L-rhamnose, this activity was increased 100- and 45-fold, respectively (Fig. 1b).

Nucleotide sequence of the *L. plantarum* NCC245 *rha* region

Two genes encoding α -L-rhamnosidase were identified in the publicly available genome of *L. plantarum* WCFS1 (Kleerebezem *et al.*, 2003) based on the sequence similarity with *rhaB* from *Bacillus* sp. GL1 (Hashimoto *et al.*, 2003). Using the primers developed on the *L. plantarum* WCFS1 genome sequence, we amplified the *rha* region and determined the nucleotide sequence in *L. plantarum* NCC245 (Fig. 2; accession number FJ943501). The gene organization in *L. plantarum* NCC245 was identical to that of *L. plantarum* WCFS1. The two α -L-rhamnosidase genes, *rhaB1* and *rhaB2*, are preceded by two genes encoding putative permeases of the major facilitator superfamily, *rhaP1* and *rhaP2*. The *rha* genes are flanked by two potential transcriptional regulators: a gene similar to *araC*, located upstream, and a gene similar to *lacI* downstream of the *rha* genes. LacR is probably the regulator of the lactose operon located upstream of *lacR*, and AraC is a transcriptional regulator of the AraC/XylS family

Fig. 2. Genomic organization of *rhaB1/rhaB2* region in *L. plantarum* NCC245 (accession no. FJ943501). The sizes and orientations of the genes were deduced from the nucleotide sequences. The predicted transcriptional terminators are indicated. A promoter region is presented above the genetic organization, showing a putative ribosome-binding site (RBS), a putative -10 promoter region and a putative catabolite-responsive element (*cre*).

(Gallegos *et al.*, 1997). Some members of this family are known to be L-rhamnose-responsive transcription activators, regulating L-rhamnose catabolic genes (Wickstrum *et al.*, 2005). Thus, *araC* is a potential transcriptional regulator of the *rha* operon in *L. plantarum*. However, its precise role remains to be clarified.

The deduced amino acid sequences of the two α -L-rhamnosidases *rhaB1* and *rhaB2* share 26 % identity with each other. Among experimentally characterized α -L-rhamnosidases, RhaB1 shares 23 % identity with RhaB of *Bacillus* sp. GL1 (accession no. BAB62315) and 22 % with RhaB of *Thermomicrobia* PRI-1686 (accession no. AAR96047). RhaB2 shares 23 % identity with RhaB of *Bacillus* sp. GL1 (accession no. BAB62315) and 23 % with RhaB of *Thermomicrobia* PRI-1686 (accession no. AAR96047). No similarity of the two proteins was found with RhaA of *Bacillus* sp. GL1 (BAB62314) or RhaA of *Thermomicrobia* PRI-1686 (accession no. AAR96046). The highest BLASTP scores of the RhaB1 sequence were 99 % identity with the putative α -L-rhamnosidase Ram1 of *L. plantarum* WCFS1 (accession no. NP_786680) and 58 % with the putative α -L-rhamnosidase of *Enterococcus faecium* (accession no. ZP_00602679). RhaB2 shares 98 % identity with Ram2 *L. plantarum* WCFS1 (accession no. NP_786682) and 45 % with the putative protein of *Paenibacillus* sp. JDR-2 (accession no. ZP_02845099).

The putative transporters RhaP1 and RhaP2 share 32 % identity with each other and belong to the major facilitator superfamily. Although the BLASTP analysis did not give a clear indication of the potential substrate for these transporters, their genetic location suggests that they might be transporters of L-rhamnose-containing molecules. The *araC-rhaP2* intergenic region harbours a potential *cre* (catabolite responsive element) 30 bp upstream of the predicted *rhaP2* coding region (Fig. 2). This *cis*-acting element (Miwa *et al.*, 2000) is a binding site of the major CCR regulator CcpA (Warner & Lolkema, 2003).

Analysis of the available lactic acid bacteria and bifido-bacteria genomes revealed only five strains with α -L-rhamnosidase-like genes: *L. plantarum* WCFS1, *Ent. faecium* DO, *Lactobacillus rhamnosus* HN001, *Lactobacillus acidophilus* NCFM and *Bifidobacterium dentium* ATCC 27678.

Expression of the *rha* locus genes in *L. plantarum* NCC245

Expression of the *rha* locus genes *rhaP2*, *rhaP1*, *rhaB2* and *rhaB1* was examined by qRT-PCR as described in Methods. The RNA samples were obtained from cells grown on glucose or galactose as carbon source and in the presence of L-rhamnose, rutin, hesperidin or naringenin as inducers (Fig. 3). Relative gene expression of all four genes using cells grown in the presence of glucose as a reference depicted the same profile in all tested conditions, suggesting probable co-expression of the *rha* genes. The expression of *rha* genes was fourfold higher in glucose-plus-L-rhamnose-grown cells, 22-fold higher in galactose- and 42-fold higher in galactose-plus-L-rhamnose-grown cells compared to glucose-grown cells. Addition of rutin or hesperidin to galactose did not influence expression of the *rha* genes, while naringenin caused a weak induction.

Fig. 3. qRT-PCR analysis of the *rha* locus genes. The strains were grown in Sixfors fermenters on 1 % glucose (Glc) or 1 % galactose (Gal) as carbon source, with 0.5 % L-rhamnose (Rha), 0.05 % rutin (Rut), 0.05 % hesperidin (Hes) and 0.05 % naringenin (Nar) as inducers. The cells were harvested in the late stationary phase, after 16 h growth, and gene expression was determined by quantitative PCR as described in Methods. The data are presented as fold induction using glucose grown cells as a reference and represent the mean value of at least three biological replicates.

This expression pattern correlates to a large extent with the pattern of enzyme activities of the cells grown on different sugars (Fig. 1), indicating that α -L-rhamnosidase gene expression is regulated at the transcriptional level.

Molecular mass of recombinant RhaB1 and RhaB2 α -L-rhamnosidases

The two α -L-rhamnosidase genes were cloned and expressed in *E. coli* cells as His-tagged proteins that were purified as described in Methods. The proteins were purified from the soluble, cytoplasmic fractions of induced *E. coli*. The apparent molecular mass of the recombinant α -L-rhamnosidases, calculated from SDS-PAGE, were 73 kDa for RhaB1 and 57 kDa for RhaB2, which is in agreement with their theoretical molecular mass (74.558 kDa and 59.540 kDa, respectively, without the His-tag), deduced from the predicted amino acid sequences. On native PAGE, RhaB1 and RhaB2 migrated as single bands, with a molecular mass of 155 kDa and 100 kDa, respectively (Fig. 4). These results, comparable with those obtained by gel-filtration chromatography (data not shown), suggest that both α -L-rhamnosidases probably associate as dimers with two identical subunits.

Fig. 4. Native PAGE of purified protein fraction of IPTG-induced *E. coli* BL21 S1(pDM19) and *E. coli* BL21 S1(pDM20) harbouring the recombinant α -L-rhamnosidases RhaB1 and RhaB2, respectively. Lanes: 1, high molecular mass protein standard; 2, recombinant RhaB1 purified from soluble cytoplasmic fraction from *E. coli* BL21 S1(pDM19); 3, recombinant RhaB2 purified from soluble cytoplasmic fraction from *E. coli* BL21 S1(pDM20). Numbers on the left indicate the molecular mass (kDa) of the standard proteins.

Biochemical characterization of recombinant RhaB1 and RhaB2

Maximal activity (determined at pH 7) for RhaB1 was detected at 50 °C and it showed relative activities over 64 % from 30 to 60 °C. RhaB2 was more thermophilic than RhaB1, with optimal temperature at 60 °C (determined at pH 5), and was most active at temperatures between 50 and 70 °C, still showing 70 % relative activity at 70 °C. The optimal pH was found to be 7 for RhaB1 and 5 for RhaB2. The α -L-rhamnosidases maintained over 60 % of their activity in the pH range from 5 to 7.5 (RhaB1), and in the pH range from 4.4 to 5.5 (RhaB2). RhaB2 was rather thermostable, retaining 43 % and 25 % activity after 5 h incubation at 55 °C and 65 °C, respectively (Fig. 5). Regarding pH stability, RhaB1 remained active over a broad range of pH values, showing 90–100 % remaining relative activities from pH 4 to 7.5, and over 40 % at pH 3 and 9, after 5 h at 37 °C. Stability of RhaB2 was lower than that of RhaB1, with 80–85 % enzymic activity from pH 5 to 7, and only 17 % and 12 % at pH 3 and 9, respectively.

The effects of metal ions and potential inhibitory agents on the recombinant enzymes were studied. The most effective inhibitor of both α -L-rhamnosidases was pHMB, with an oxidizing effect on thiol groups, which reduced the activity of RhaB1 to 10.9 % and RhaB2 to 36.0 % residual activity, suggesting the presence of thiol groups at the active site or involved in maintaining the tertiary structure of the enzymes. RhaB1 and RhaB2 activity was also reduced by phosphoramidon (23.8 % and 42.5 % residual activity, respectively), an agent known to completely inhibit some metalloendopeptidases (Turner *et al.*, 2001). On the other hand, phosphoramidon contains an α -rhamnosyl residue which might take part in the inhibition of the α -L-rhamnosidases activity. The specific metalloenzyme inhibitors thiorphan, 1,10-phenanthroline and EDTA were less effective in the inhibition of the *L. plantarum* NCC245 α -L-rhamnosidases at 1 mM. When the 1,10-phenanthroline concentration was raised to 10 mM, the activity of both α -L-rhamnosidases decreased by 50 % and a slight activating influence on RhaB1 was observed for EDTA at 10 mM. An ionic stimulation of the α -L-rhamnosidases was observed with Ca^{2+} and Co^{2+} , which could act as potential cofactors of the enzymes, while Mn^{2+} , Fe^{2+} and Cu^{2+} diminished the activity of both enzymes, to different extents. DTT and β -mercaptoethanol had no or little effect on any of the enzymes. In general, RhaB2 was more susceptible than RhaB1 to a higher number of chemicals and cations (results not shown). SDS produced partial inactivation of RhaB1 and RhaB2, most probably due to dissociation of the dimeric structure of the enzymes.

Substrate specificity and kinetics of recombinant RhaB1 and RhaB2

For further characterization, the α -L-rhamnosidases were incubated with L-rhamnose-containing substrates, mainly flavonoids of plant origin. In rutin and hesperidin the L-

Fig. 5. Stability of recombinant RhaB1 and RhaB2 at different temperatures. Residual activity was monitored under standard conditions after different times of incubation at 37 °C (▲), 40 °C (△), 45 °C (■), 50 °C (□), 55 °C (●) and 65 °C (○). The initial activity was set as 100 %.

rhamnose residue is α -1,6 linked; in naringin and vitexin 2-O-rhamnoside it is α -1,2 linked; and in quercitrin and procillaridin A it is linked to the aglycone directly at the C-1 position of the L-rhamnose moiety. Gellan gum is a linear tetrasaccharide with the L-rhamnose residue α -1,3 linked. The glycoalkaloid α -chaconine contains a trisaccharide attached to the 3-OH position of solanidine, with two L-rhamnose residues α -1,2 and α -1,4 linked to the same glucosyl residue. The structures of naringin, hesperidin and rutin are illustrated in Supplementary Fig. S1 (available with the online version of this paper). In 10 min reaction intervals, RhaB1 was able to release L-rhamnose from rutin (maximum activity, taken as 100 %), hesperidin (11.2 %) and procillaridin A (4.5 %). RhaB2 released L-rhamnose from hesperidin (maximum activity, taken as 100 %), rutin (10.1 %) and procillaridin A (0.4 %). Activity of RhaB1 and RhaB2 towards the other substrates was below the lowest quantifiable level ($0.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$). The lowest activity for RhaB1 and RhaB2 was detected on α -1 linkage of L-rhamnose to the aglycone of procillaridin A, and no activity was detected on α -1 linkage of quercitrin, probably because α -L-rhamnosidase action appeared to be hindered by the steric structure of both compounds. Overall, these results suggest that RhaB1 and RhaB2 activities are influenced by both the type of L-rhamnose linkage in the substrate and its structure.

The $V_{\max}$, K_m and $V_{\max}/K_m$ values were determined for hesperidin and rutin, the best of the tested natural substrates for *L. plantarum* NCC245 α -L-rhamnosidases and for *p*-NPR, and are presented in Table 3. The K_m values of RhaB1 and RhaB2 on *p*-NPR were within the range previously described for fungal and bacterial α -L-rhamnosidases (Manzanares *et al.*, 2007). RhaB1 hydrolysed *p*-NPR most efficiently ($V_{\max}/K_m$ 133.3), while RhaB2 exhibited high activity against hesperidin ($V_{\max}/K_m$ 214.1). End-product inhibition by L-rhamnose was also studied. RhaB1 was slightly inhibited by L-rhamnose, showing non-competitive inhibition, with a K_i value of 19.88 mM. Inhibition of RhaB2 could not be clearly established. Enzymes were tested adding different concentrations of inhibitor, L-rhamnose, to fixed concentrations of substrate, *p*-NPR; when evaluation was made at 0.5 mM and 1 mM *p*-NPR, very low competitive inhibition was observed, with a K_i value of 92.18 mM. But when the *p*-NPR concentration was raised to 2 mM, the inhibition of RhaB2 seemed to be uncompetitive.

DISCUSSION

In this study we identified a food-grade lactic acid bacterium with α -L-rhamnosidase activity that could be

Table 3. $V_{\max}$, K_m and $V_{\max}/K_m$ values of recombinant RhaB1 and RhaB2 α -L-rhamnosidases from *L. plantarum* NCC245

Substrate	$V_{\max}$ ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)		K_m (mM)		$V_{\max}/K_m$	
	RhaB1	RhaB2	RhaB1	RhaB2	RhaB1	RhaB2
<i>p</i> -NPR	76	1	0.57	0.51	133.3	1.96
Hesperidin	5	167	3.39	0.78	1.47	214.1
Rutin	22	24	1.77	1.06	12.4	22.6

used for the bioconversion of polyphenols. Production of α -L-rhamnosidase was rather rare among the lactic acid bacteria tested, being present in only a minority of strains of seven species out of 37 examined (Table 2). The exception was *L. plantarum*, where the majority of strains produced the enzyme. Among the *L. plantarum* strains, NCC245 showed the highest activity in the preliminary screening and was selected for further characterization of α -L-rhamnosidase activity.

Our results strongly suggest that the α -L-rhamnosidases of *L. plantarum* NCC245 are subjected to the control by the mechanisms of CCR and induction. We have shown that enzyme activity is strongly repressed in the presence of glucose as a carbon source whereas the repression was relieved in the cells grown on galactose (Fig. 1). The regulation of α -L-rhamnosidase by CCR has not to our knowledge been reported in the literature so far. Of the potential inducers tested (rutin, hesperidin, naringenin and L-rhamnose), only L-rhamnose was able to induce α -L-rhamnosidase activity. Thus, maximal α -L-rhamnosidase activity, obtained with cells grown on galactose and L-rhamnose, was 100-fold higher than with cells grown on glucose. qRT-PCR experiments indicate that both regulation mechanisms occur at the level of transcription. Similarly, the α -L-rhamnosidase expression of *S. paucimobilis* FP2001 was induced by the addition of L-rhamnose (Miyata *et al.*, 2005) and the α -L-rhamnosidase activity of *Bacillus* sp. GL1 was strongly induced by its substrate gellan (Hashimoto *et al.*, 1999). Conversely, *Bacteroides* strains isolated from human faeces seem to produce flavonoid-glucoside-hydrolysing enzymes constitutively (Bokkenheuser *et al.*, 1987).

The CCR in *L. plantarum* NCC245 probably acts through the principal transcriptional regulator CcpA (Warner & Lolkema, 2003), which binds to its operator site, *cre*. A typical *cre* element (Miwa *et al.*, 2000) was identified in the *rha* promoter region (Fig. 2). The function of CcpA as a carbon catabolite regulator and *cre* as its operator site has been demonstrated in *L. plantarum* (Marasco *et al.*, 2002; Muscariello *et al.*, 2001).

The two recombinant α -L-rhamnosidases from *L. plantarum* NCC245, RhaB1 and RhaB2 were characterized and shown to be homodimers in their native form, exhibiting different biochemical characteristics. α -L-Rhamnosidase RhaB2 stands out for its thermophilic character and thermostability, and registered an acidic pH optimum, unusual in bacteria, which has only been described for α -L-rhamnosidase RhaB from *Thermomicrobia* sp. (Birgisson *et al.*, 2004b) and α -L-rhamnosidase from *Fusobacterium* K-60 (Park *et al.*, 2005). In addition, the $V_{\max}$ value of RhaB2 towards hesperidin, 167 U mg⁻¹, is the highest found to date for an α -L-rhamnosidase, followed by 109 U mg⁻¹ for *Bacteroides* JY-6 α -L-rhamnosidase (Jang & Kim, 1996), and this enzyme shows a quite high $V_{\max}/K_m$ value of 214.1 for hesperidin. Noteworthy is the preference of *L. plantarum* NCC245 RhaB1 and RhaB2 for the α -1,6 linkage of L-

rhamnose to β -D-glucose and their lack of activity towards the α -1,2 linkage in naringin under the conditions used. To our knowledge, this has not been reported to date for bacterial α -L-rhamnosidases, but reactions with natural substrates have been performed, when indicated, for longer times ranging from 2 h to 36 h (Birgisson *et al.*, 2004b; Manzanares *et al.*, 2001; Miake *et al.*, 2000). In bacteria, only α -L-rhamnosidase from *S. paucimobilis* FP2001 hydrolysed hesperidin more efficiently than other flavonoids (Miake *et al.*, 2000). This was also the case for *L. plantarum* NCC245 RhaB2, whereas *L. plantarum* NCC245 RhaB1 is the first α -L-rhamnosidase shown to hydrolyse rutin better than other flavonoids. Amino acid sequence comparison of RhaB1 and RhaB2 with bacterial glycoside hydrolase family 78 α -L-rhamnosidases indicates that Asp567, Trp576, Asp579 and Glu841 are conserved but not Glu572, although both RhaB1 and RhaB2 contain an Asp residue at this position (see Supplementary Fig. S2, available with the online version of this paper). This amino acid substitution could be related to the observed RhaB1 and RhaB2 substrate specificities, since these negatively charged residues have been demonstrated to be crucial for the α -L-rhamnosidase activity of RhaB from *Bacillus* sp. GL1 (Cui *et al.*, 2007).

L. plantarum is a versatile and widespread micro-organism found in environments ranging from vegetable, dairy and meat fermentations to the human gastrointestinal tract (Kleerebezem *et al.*, 2003). Thus, natural substrates for bacterial α -L-rhamnosidase could be flavonoids of plant origin. Our results indicate that one of the two purified α -L-rhamnosidases from *L. plantarum* NCC245 efficiently converts hesperidin to hesperetin 7-glucoside. However, the strain is not able to grow on hesperidin as carbon source, nor is the enzyme induced in the presence of hesperidin. Together with the fact that the maximal enzyme activity was observed in the late stationary phase, these findings suggest that the primary role of α -L-rhamnosidase in *L. plantarum* NCC245 is not to provide the cells with energy. In contrast to *L. plantarum*, other bacteria with α -L-rhamnosidase activity, such as *S. paucimobilis* FP2001 or *Bacillus* GL1, can use their substrates L-rhamnose and gellan, respectively, as carbon sources (Hashimoto *et al.*, 1999; Miake *et al.*, 2000).

Most lactic acid bacteria use L-rhamnose as a major component of cell-wall polysaccharides or extracellular polysaccharides (EPS) (Boels *et al.*, 2004) and are equipped with the biosynthetic enzymes for precursor molecule dTDP-rhamnose (as analysed using KEGG biosynthetic pathway tools, <http://www.genome.ad.jp/kegg/pathway.html>). It is not clear whether bacteria can use exogenous sources of L-rhamnose as building blocks for its polysaccharide structures, but it could be a possibility to avoid endogenous biosynthesis. For example, *S. paucimobilis* FP2001 forms flagella only when it is grown in the presence of L-rhamnose (Miake *et al.*, 1995). It is also possible that *L. plantarum* NCC245 recycles L-rhamnose molecules from its own surface polysaccharide structures. It is known that

some lactobacilli degrade their own EPS structures after prolonged fermentation (Pham *et al.*, 2000), in stationary phase, when α -L-rhamnosidase of *L. plantarum* NCC245 is the most active. Perpetual modification of the surface polysaccharide structures might also be a strategy of intestinal bacteria to survive the selective pressure of the adaptive immune system of the host and remain an entrenched resident of the community (Peterson *et al.*, 2007). Finally, it is possible that the natural substrate for α -L-rhamnosidases of *L. plantarum* has not yet been identified.

This is the first extensive characterization of α -L-rhamnosidases from lactic acid bacteria. The different but complementary features of the two enzymes make them of interest for developing industrial applications with *L. plantarum* NCC245, where temperature and pH may vary through the process. This bacterium might also be applied for improvement of bioavailability of health-beneficial polyphenols, such as hesperidin in humans.

ACKNOWLEDGEMENTS

The authors thank M. P. Fernández de Palencia for their valuable assistance with FPLC, B. Berger for the analysis of real-time PCR results and R. D. Pridmore and R. Brückner for critical reading of the manuscript. This work was partly supported by the Spanish Ministry of Science and Innovation (grants: AGL2004-07285-C02-01, AGL2006-12100 and Consolider FUN-C-FOD CSD2007-063) and Comunidad Autónoma de Madrid (ALIBIRD: S-0505/AGR-0153). M.A. was funded by a *Juan de la Cierva* postdoctoral contract from the Spanish Ministry of Science and Innovation.

REFERENCES

- Bader, G., Wray, V., Just, U. & Hiller, K. (1998). Enzymatic hydrolysis of the cytotoxic triterpenoid glycoside virgaureasaponin 1. *Phytochemistry* **49**, 153–156.
- Birgisson, H., Fridjonsson, O., Bahrani-Mougeot, F. K., Hreggvidsson, G. O., Kristjansson, J. K. & Mattiasson, B. (2004a). A new thermostable α -L-arabinofuranosidase from a novel thermophilic bacterium. *Biotechnol Lett* **26**, 1347–1351.
- Birgisson, H., Hreggvidsson, G. O., Fridjonsson, O. H., Mort, A., Kristjansson, J. K. & Mattiasson, B. (2004b). Two new thermostable α -L-rhamnosidases from a novel thermophilic bacterium. *Enzyme Microb Technol* **34**, 561–571.
- Boels, I. C., Beerthuyzen, M. M., Kusters, M. H., Van Kaauwen, M. P., Kleerebezem, M. & de Vos, W. M. (2004). Identification and functional characterization of the *Lactococcus lactis* *rfb* operon, required for dTDP-rhamnose biosynthesis. *J Bacteriol* **186**, 1239–1248.
- Bokkenheuser, V. D., Shackleton, C. H. & Winter, J. (1987). Hydrolysis of dietary flavonoid glycosides by strains of intestinal *Bacteroides* from humans. *Biochem J* **248**, 953–956.
- Cui, Z., Maruyama, Y., Mikami, B., Hashimoto, W. & Murata, K. (2007). Crystal structure of glycoside hydrolase family 78 α -L-rhamnosidase from *Bacillus* sp. GL1. *J Mol Biol* **374**, 384–398.
- De Man, J. C., Rogosa, M. & Sharpe, E. (1960). A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130–135.
- Deutscher, J. (2008). The mechanisms of carbon catabolite repression in bacteria. *Curr Opin Microbiol* **11**, 87–93.
- Gallegos, M. T., Schleif, R., Bairoch, A., Hofmann, K. & Ramos, J. L. (1997). Arac/XylS family of transcriptional regulators. *Microbiol Mol Biol Rev* **61**, 393–410.
- Giavasis, I., Harvey, L. M. & McNeil, B. (2000). Gellan gum. *Crit Rev Biotechnol* **20**, 177–211.
- Griffiths, L. A. & Barrow, A. (1972). Metabolism of flavonoid compounds in germ-free rats. *Biochem J* **130**, 1161–1162.
- Guardia, T., Rotelli, A. E., Juarez, A. O. & Pelzer, L. E. (2001). Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Farmacol* **56**, 683–687.
- Hashimoto, W. & Murata, K. (1998). α -L-rhamnosidase of *Sphingomonas* sp. R1 producing an unusual exopolysaccharide of sphingane. *Biosci Biotechnol Biochem* **62**, 1068–1074.
- Hashimoto, W., Nankai, H., Sato, N., Kawai, S. & Murata, K. (1999). Characterization of α -L-rhamnosidase of *Bacillus* sp. GL1 responsible for the complete depolymerization of gellan. *Arch Biochem Biophys* **368**, 56–60.
- Hashimoto, W., Miyake, O., Nankai, H. & Murata, K. (2003). Molecular identification of an α -L-rhamnosidase from *Bacillus* sp strain GL1 as an enzyme involved in complete metabolism of gellan. *Arch Biochem Biophys* **415**, 235–244.
- Jang, I. S. & Kim, D. H. (1996). Purification and characterization of α -L-rhamnosidase from *Bacteroides* JY-6, a human intestinal bacterium. *Biol Pharm Bull* **19**, 1546–1549.
- Kalu, D. N. (1991). The ovariectomized rat model of postmenopausal bone loss. *Bone Miner* **15**, 175–191.
- Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O. P., Leer, R., Turchini, R., Peters, S. A., Sandbrink, H. M. & other authors (2003). Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc Natl Acad Sci U S A* **100**, 1990–1995.
- Kuipers, O. P., Beerthuyzen, M. M., Siezen, R. J. & de Vos, W. M. (1993). Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*. Requirement of expression of the *nisA* and *nisI* genes for development of immunity. *Eur J Biochem* **216**, 281–291.
- Macdonald, I. A., Mader, J. A. & Bussard, R. G. (1983). The role of rutin and quercitrin in stimulating flavonol glycosidase activity by cultured cell-free microbial preparations of human feces and saliva. *Mutat Res* **122**, 95–102.
- Manzanares, P., van den Broeck, H. C., De Graaff, L. H. & Visser, J. (2001). Purification and characterization of two different α -L-rhamnosidases, RhaA and RhaB, from *Aspergillus aculeatus*. *Appl Environ Microbiol* **67**, 2230–2234.
- Manzanares, P., Valles, S., Ramon, D. & Orejas, M. (2007). α -L-Rhamnosidases: old and new insights. In *Industrial Enzymes: Structure, Function, and Applications*, pp. 117–140. Edited by J. Polaina & A. P. MacCabe. Dordrecht: Springer.
- Marasco, R., Muscariello, L., Rigano, M. & Sacco, M. (2002). Mutational analysis of the *bglH* catabolite-responsive element (*cre*) in *Lactobacillus plantarum*. *FEMS Microbiol Lett* **208**, 143–146.
- Miake, F., Murata, K., Kuroiwa, A., Kumamoto, T., Kuroda, S., Terasawa, T., Tone, H. & Watanabe, K. (1995). Characterization of *Pseudomonas paucimobilis* FP2001 which forms flagella depending upon the presence of rhamnose in liquid medium. *Microbiol Immunol* **39**, 437–442.
- Miake, F., Satho, T., Takesue, H., Yanagida, F., Kashige, N. & Watanabe, K. (2000). Purification and characterization of intracellular α -L-rhamnosidase from *Pseudomonas paucimobilis* FP2001. *Arch Microbiol* **173**, 65–70.

- Miwa, Y., Nakata, A., Ogiwara, A., Yamamoto, M. & Fujita, Y. (2000). Evaluation and characterization of catabolite-responsive elements (*cre*) of *Bacillus subtilis*. *Nucleic Acids Res* **28**, 1206–1210.
- Miyake, Y., Yamamoto, K., Tsujihara, N. & Osawa, T. (1998). Protective effects of lemon flavonoids on oxidative stress in diabetic rats. *Lipids* **33**, 689–695.
- Miyata, T., Kashige, N., Satho, T., Yamaguchi, T., Aso, Y. & Miake, F. (2005). Cloning, sequence analysis, and expression of the gene encoding *Sphingomonas paucimobilis* FP2001 α -L-rhamnosidase. *Curr Microbiol* **51**, 105–109.
- Muscariello, L., Marasco, R., De Felice, M. & Sacco, M. (2001). The functional *ccpA* gene is required for carbon catabolite repression in *Lactobacillus plantarum*. *Appl Environ Microbiol* **67**, 2903–2907.
- Nielsen, I. L., Chee, W. S., Poulsen, L., Offord-Cavin, E., Rasmussen, S. E., Frederiksen, H., Enslen, M., Barron, D., Horcajada, M. N. & Williamson, G. (2006). Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. *J Nutr* **136**, 404–408.
- Ohtsuki, K., Abe, A., Mitsuzumi, H., Kondo, M., Uemura, K., Iwasaki, Y. & Kondo, Y. (2002). Effects of long-term administration of hesperidin and glucosyl hesperidin to spontaneously hypertensive rats. *J Nutr Sci Vitaminol (Tokyo)* **48**, 420–422.
- Park, Y. B., Do, K. M., Bok, S. H., Lee, M. K., Jeong, T. S. & Choi, M. S. (2001). Interactive effect of hesperidin and vitamin E supplements on cholesterol metabolism in high cholesterol-fed rats. *Int J Vitam Nutr Res* **71**, 36–44.
- Park, S., Kim, J. & Kim, D. (2005). Purification and characterization of quercitrin-hydrolyzing α -L-rhamnosidase from *Fusobacterium* K-60, a human intestinal bacterium. *J Microbiol Biotechnol* **15**, 519–524.
- Peterson, D. A., McNulty, N. P., Guruge, J. L. & Gordon, J. I. (2007). IgA response to symbiotic bacteria as a mediator of gut homeostasis. *Cell Host Microbe* **2**, 328–339.
- Pham, P. L., Dupont, I., Roy, D., Lapointe, G. & Cerning, J. (2000). Production of exopolysaccharide by *Lactobacillus rhamnosus* R and analysis of its enzymatic degradation during prolonged fermentation. *Appl Environ Microbiol* **66**, 2302–2310.
- Puri, M., Marwaha, S., Kothari, R. M. & Kennedy, J. F. (1996). Biochemical basis of bitterness in citrus fruit juices and biotech approaches for debittering. *Crit Rev Biotechnol* **16**, 145–155.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Steinbacher, S., Baxa, U., Miller, S., Weintraub, A., Seckler, R. & Huber, R. (1996). Crystal structure of phage P22 tailspike protein complexed with *Salmonella* sp. O-antigen receptors. *Proc Natl Acad Sci U S A* **93**, 10584–10588.
- Tanaka, T., Kohno, H., Murakami, M., Shimada, R., Kagami, S., Sumida, T., Azuma, Y. & Ogawa, H. (2000). Suppression of azoxymethane-induced colon carcinogenesis in male F344 rats by mandarin juices rich in beta-cryptoxanthin and hesperidin. *Int J Cancer* **88**, 146–150.
- Turner, A. J., Isaac, R. E. & Coates, D. (2001). The neprilysin (NEP) family of zinc metalloendopeptidases: genomics and function. *Bioessays* **23**, 261–269.
- Warner, J. B. & Lolkema, J. S. (2003). CcpA-dependent carbon catabolite repression in bacteria. *Microbiol Mol Biol Rev* **67**, 475–490.
- Wickstrum, J. R., Santangelo, T. J. & Egan, S. M. (2005). Cyclic AMP receptor protein and RhaR synergistically activate transcription from the L-rhamnose-responsive *rhaSR* promoter in *Escherichia coli*. *J Bacteriol* **187**, 6708–6718.
- Williams, P. J., Strauss, C. R., Wilson, B. & Massy-Westropp, R. A. (1982). Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes and wines. *Phytochemistry* **21**, 2013–2020.
- Wood, T. M. B. K. M. (1988). Methods for measuring cellulase activity. In *Methods in Enzymology*, pp. 91–92. Edited by A. K. S. T. Wood. San Diego: Academic Press.
- Zverlov, V. V., Hertel, C., Bronnenmeier, K., Hroch, A., Kellermann, J. & Schwarz, W. H. (2000). The thermostable α -L-rhamnosidase RamA of *Clostridium stercorarium*: biochemical characterization and primary structure of a bacterial α -L-rhamnoside hydrolase, a new type of inverting glycoside hydrolase. *Mol Microbiol* **35**, 173–179.

Edited by: D. A. Mills