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Characterization of Rhamnosidases from *Lactobacillus plantarum* and *Lactobacillus acidophilus*▽

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Lactobacilli are known to use plant materials as a food source. Many such materials are rich in rhamnose-containing polyphenols, and thus it can be anticipated that lactobacilli will contain rhamnosidases. Therefore, genome sequences of food-grade lactobacilli were screened for putative rhamnosidases. In the genome of *Lactobacillus plantarum*, two putative rhamnosidase genes (*ram1*$_{Lp}$ and *ram2*$_{Lp}$) were identified, while in *Lactobacillus acidophilus*, one rhamnosidase gene was found (*ramA*$_{La}$). Gene products from all three genes were produced after introduction into *Escherichia coli* and were then tested for their enzymatic properties. Ram1$_{Lp}$, Ram2$_{Lp}$, and RamA$_{La}$ were able to efficiently hydrolyze rutin and other rutinosides, while RamA$_{La}$ was, in addition, able to cleave naringin, a neohesperidoside. Subsequently, the potential application of *Lactobacillus* rhamnosidases in food processing was investigated using a single matrix, tomato pulp. Recombinant Ram1$_{Lp}$ and RamA$_{La}$ enzymes were shown to remove the rhamnose from rutinosides in this material, but efficient conversion required adjustment of the tomato pulp to pH 6. The potential of Ram1$_{Lp}$ for fermentation of plant flavonoids was further investigated by expression in the food-grade bacterium *Lactococcus lactis*. This system was used for fermentation of tomato pulp, with the aim of improving the bioavailability of flavonoids in processed tomato products. While import of flavonoids into *L. lactis* appeared to be a limiting factor, rhamnose removal was confirmed, indicating that rhamnosidase-producing bacteria may find commercial application, depending on the technological properties of the strains and enzymes.

Lactobacilli such as *Lactobacillus plantarum* have been used for centuries to ferment vegetables such as cabbage, cucumber, and soybean (34). Fruit pulps, for instance, those from tomato, have also been used as a substrate for lactobacilli for the production of probiotic juices (38). Recently, the full genomic sequences of several lactobacilli have become available (1, 22). A number of the plant-based substrates for lactobacilli are rich in rhamnose sugars, which are often conjugated to polyphenols, as in the case of cell wall components and certain flavonoid antioxidants. Utilization of these compounds by lactobacilli would involve α-L-rhamnosidases, which catalyze the hydrolytic release of rhamnose. Plant-pathogenic fungi such as *Aspergillus* species produce the rhamnosidases when cultured in the presence of naringin, a rhamnosilated flavonoid (24, 26). Bacteria such as *Bacillus* species have also been shown to use similar enzyme activities for metabolizing bacterial biofilms which contain rhamnose (17, 40).

In food processing, rhamnosidases have been applied primarily for debittering of citrus juices. Part of the bitter taste of citrus is caused by naringin (Fig. 1), which loses its bitter taste upon removal of the rhamnose (32). More recently, application of rhamnosidases for improving the bioavailability of flavonoids has been described. Human intake of flavonoids has been associated with a reduced risk of coronary heart disease in epidemiological studies (19). Food flavonoids need to be absorbed efficiently from what we eat in order to execute any beneficial function. Absorption occurs primarily in the small intestine (12, 37). Unabsorbed flavonoids will arrive in the colon, where they will be catabolized by the microflora, which is then present in huge quantities. Therefore, it would be desirable for flavonoids to be consumed in a form that is already optimal for absorption in the small intestine prior to their potential degradation. For the flavonoid quercetin, it has been demonstrated that the presence of rhamnoside groups inhibits its absorption about fivefold (20). A number of flavonoids which are present in frequently consumed food commodities, such as tomato and citrus products, often carry rutinoside (6-β-L-rhamnosyl-D-glucose) or neohesperidoside (2-β-L-rhamnosyl-D-glucose) residues (Fig. 1). Therefore, removal of the rhamnose groups from such flavonoid rutinosides and neohesperidosides prior to consumption could enhance their intestinal absorption. With this aim, studies were recently carried out toward the application of fungal enzyme preparations as a potential means to selectively remove rhamnoside moieties (16, 30).

In view of the frequent occurrence of lactobacilli on decaying plant material and fermented vegetable substrates, one could anticipate that their genomes carry one or more genes encoding enzymes capable of utilizing rhamnosilated com-

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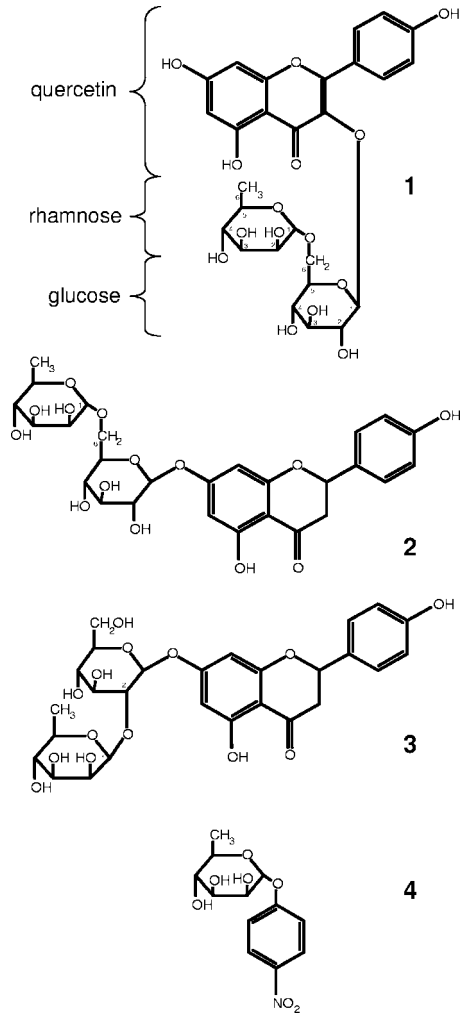

FIG. 1. Chemical structures of rhamnose-containing flavonoids from plants. Relevant carbon atoms in glycoside moieties are numbered. (1) Rutin (quercetin-3-glucoside-1→6-rhamnoside); (2) narirutin (naringenin-7-glucoside-1→6-rhamnoside); (3) naringin (naringenin-7-glucoside-1→2-rhamnoside); (4) *p*-nitrophenol-rhamnose.

pounds. In the work reported here, we describe the identification of three putative rhamnosidase genes in lactobacillus genomes. We expressed these genes in *Escherichia coli* and characterized their gene products. The activities of all three lactobacillus rhamnosidases on flavonoids naturally present in tomato pulp were then assessed. One of the *L. plantarum* genes, which encoded the enzyme with the highest activity and stability in *E. coli*, was then also expressed in *Lactococcus lactis*, with the aim of investigating the potential use of such a recombinant organism to improve the bioavailability of fruit flavonoids and thus their efficacy in common foodstuffs.

MATERIALS AND METHODS

Strains and chemicals. *L. plantarum* DSM20205 and *Lactobacillus acidophilus* DSM9126 were obtained from DSMZ. Strain *L. lactis* NZ9000 came from the NIZO food research collection. All chemicals were obtained from Sigma (St. Louis, MO), except for rutin, quercetin-3-glucoside, kaempferol-3-rutinoside, kaempferol-3-glucoside, naringenin-7-glucoside, narirutin, and naringin, which were obtained from Apin (Oxon).

Sequence analyses. Protein sequences of experimentally verified α-L-rhamnosidases (EC 3.2.1.40) were obtained from the Braunschweig enzyme database (BRENDA) (5; http://www.brenda-enzymes.org) and the carbohydrate-active enzymes (CAZY) database (9; http://www.cazy.org/). Homologous sequences were searched using BLAST (E values of $<e-2$) (2), and the search was continued (E values of $<e-10$) with the hits until no new sequences were retrieved. ClustalX (35) was used to generate multiple sequence alignments and, based on those, bootstrapped (n = 1,000) neighbor-joining trees. The trees were visualized with LOFT (36). All sequence information was extracted from the ERGO bioinformatic suite (31). This resource contained sequence information on the following lactobacilli: *L. acidophilus* (accession no. NC006814), *Lactobacillus brevis* (NC008497), *Lactobacillus casei* (NC008526), *Lactobacillus delbrueckii* (NC008054), *Lactobacillus fermentum* (NC010610), *Lactobacillus gasseri* (NC008530), *Lactobacillus helveticus* (NC010080), *Lactobacillus johnsonii* (NC005362), *L. plantarum* (NC004567), *Lactobacillus reuteri* (NC009513), *Lactobacillus sakei* (NC007576), and *Lactobacillus salivarius* (NC007929). The initial analysis was performed in February 2007, and the final analysis was performed in October 2008.

Gene cloning. For amplification of gene sequences, bacteria were boiled in water for 5 min. After centrifugation, the DNA-containing supernatant was diluted and used as a template for amplification. Amplification was carried out using Phusion polymerase (Finnzyme) according to the manufacturer's instructions. *L. plantarum* Ram1 ($Ram1_{Lp}$) was amplified from *L. plantarum* DSM20205, using the oligonucleotides 5′TATATAGATCTCATGTCGAAAG AGGCTGTTTGG and 5′TATATCTCGAGTCACACTGGGACCACCGCAG TTG. $Ram2_{Lp}$ was amplified from the same strain, using the oligonucleotides 5′ATATGAATTCGGAAGAGATGGCGTTTACTTTTC and 5′TATATGCG GCCGCTCCTCCTAAACGAGGTACT. *L. acidophilus* RamA ($RamA_{La}$) was amplified from *L. acidophilus* DSM9126, using the oligonucleotides 5′TTATG GATCCTATGAAAATTACAAATATTTTAGTTAACCAAATGG and 5′AAT AACTGCAGCTGCTGCACATAGATTGCTAG. Amplified fragments were digested with restriction enzymes (for $Ram1_{Lp}$, BglII and XhoI; for $Ram2_{Lp}$, EcoRI and NotI; and for $RamA_{La}$, BamHI and PstI) and cloned into digested pACYC-DUET1 (Novagen) (for $Ram1_{Lp}$, BamHI and SalI; for $Ram2_{Lp}$, EcoRI and NotI; and for $RamA_{La}$, BamHI and PstI). This generated plasmids pDM2 ($Ram1_{Lp}$), pDM6 ($Ram2_{Lp}$), and pDM9 ($RamA_{La}$). Sequences were confirmed by analyses in both orientations.

To introduce $Ram1_{Lp}$ into lactococci, pDM2 was digested with HindIII and NcoI and ligated into vector pNZ8148. The ligation mixture was directly introduced into *L. lactis* NZ9000 by electroporation (27).

Protein production and extraction. Plasmids pACYCDUET-1, pDM2 ($Ram1_{Lp}$), pDM6 ($Ram2_{Lp}$), and pDM9 ($RamA_{La}$) were introduced into *E. coli* BL21(DE3). For protein expression, fresh overnight cultures were diluted 1:100 in 2× YT medium (16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter) supplemented with 10 mg/liter chloramphenicol and grown at 37°C and 250 rpm to an optical density at 600 nm (OD_{600}) of 0.5. At that point, 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added, and the culture was incubated overnight at 30°C and 250 rpm.

Individual transformants of *E. coli* were inoculated into LB medium with 1% glucose and 30 mg/liter chloramphenicol and grown overnight at 37°C. These overnight cultures were diluted 1:100 in 50 ml 2× YT medium with 15 mg/liter chloramphenicol and further incubated at 37°C. At an OD_{600} of 0.4, IPTG was added to give a final concentration of 1 mM, and cultures were grown overnight at 28°C and 250 rpm. The next day, the cultures were centrifuged and cell pellets were resuspended in 2 ml buffer (50 mM Tris, pH 7, 300 mM NaCl). After sonication and centrifugation, the supernatant was either used as the intracellular fraction or used for metal affinity purification with Ni-nitrilotriacetic acid spin columns (Qiagen) according to the manufacturer's instructions. Purified $Ram1_{Lp}$ was dialyzed and stored at −20°C in 100 mM Tris, pH 7, 15% glycerol, and 5 mM β-mercaptoethanol.

Fresh overnight cultures of *L. lactis* strains harboring the plasmids pNZ8148 and pNZ-DM2 were diluted 1 to 20 in M17 medium with 0.5% glucose and chloramphenicol (10 mg/liter) to a final volume of 25 ml and incubated at 30°C without agitation. Expression was induced at an OD_{600} of 0.3 by the addition of 5 ng/ml nisin (27), and incubation was continued overnight.

L. lactis cultures were centrifuged, and cell pellets were resuspended in 2 ml buffer (50 mM Tris, pH 7, 300 mM NaCl). To 1 ml cell suspension, 0.3 g zirconium sand (Biospec) was added, and the suspension was subjected to FastPrep treatment (speed 4; twice for 20 s each time) (Bio101 Savant) followed by centrifugation (5 min, 13,000 × g, 4°C).

Enzyme assays. Rhamnosidase activity was measured using the substrate *p*-nitrophenyl rhamnose, in which the *p*-nitrophenyl group is attached to the 1 position of rhamnose, as described by Romero et al. (33). Briefly, protein was mixed with 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 6.5, and

1.7 mM substrate (final concentration), unless otherwise indicated in the text. After 1 h of incubation at 30°C, the reaction was stopped and color was developed following the addition of an equal volume of a 0.2 M sodium carbonate solution. Product formation was measured at 405 nm. One unit (U) was defined as the amount of enzyme that liberates 1 μmol nitrophenol/min.

Flavonoids were dissolved in dimethyl sulfoxide and digested at 30°C in 50 mM MOPS, pH 6.5, and 5% dimethyl sulfoxide at 30°C at 0.05 mg/ml in the presence of 30 μU enzyme. After 1 h, the reaction was stopped by the addition of 3 volumes of methanol with 0.13% formic acid. From each extract, 10 μl was injected into a high-performance liquid chromatography (HPLC) system with a Luna 3u $C_{18(2)}$ 150- by 4.6-mm column (Phenomenex). The HPLC setup was composed of a Waters 600 controller and a Waters 996 photo diode array detector. The column was maintained at 40°C with a flow rate of 1 ml/min. The products were eluted using a linear gradient going from 95% buffer A (0.1% formic acid in water) and 5% buffer B (100% acetonitrile) to a 50-50 mixture in 37 min. Product formation was calculated by comparing the peak surface area to those for dilutions of standard compounds of known concentration. Product identification was performed using a QTOF Ultima mass spectrometer, as described before (7).

Kinetic constants were determined by supplying 1 mg/liter enzyme with various concentrations of rhamnose-pNP (from 0 to 0.14 M) and rutin (from 0 to 1 mM) and measuring product formation after 1 h.

Tomato digestion. Ripe tomatoes were purchased at the local market and finely pulped using a blender. The pulp was separated into two portions, one of which was left untreated (pH 3.9), while the other was adjusted to pH 6.0 by the addition of a 1 M disodium hydrogen phosphate solution. For digestion of 700 μl tomato pulp, 20 μl of a 3-mU/ml enzyme solution was added, and the mixture was incubated overnight at 37°C. The reaction was stopped by the addition of 1.5 ml methanol with 0.13% formic acid to 500 μl reaction mixture, which was followed by 10 min of sonication and filtration through a 0.2-μm Anotop filter. Product identification was performed on an HPLC system coupled to a QTOF Ultima mass spectrometer and by comparison to authentic standards, as described before (7). Rutin apioside was identified using the MOTO database (28).

Tomato fermentation. Fresh overnight cultures of *L. lactis* strains harboring plasmids pNZ8148 and pNZ-DM2 were diluted 1:20 in M17 medium containing 2% glucose and 10 μg/ml chloramphenicol to a final volume of 50 ml and incubated at 30°C without agitation. Expression was induced at an OD_{600} of 0.3 by the addition of 5 ng/ml nisin and was continued until an OD_{600} of 2.0 was achieved. Subsequently, 20 ml of the culture was centrifuged, and the pellet was washed with phosphate-buffered saline and finally dissolved in 20 ml phosphate-buffered saline with 5 ng/ml nisin. This was transferred to 200 ml of commercial, shop-bought tomato paste (Royalty gezeefde tomaten), mixed at a 1:1 ratio with a phosphate buffer to reach a pH of 4.6, and incubated for 48 h at 30°C. The number of CFU per ml increased from 10^8 to 10^{12}, and the substrate acidified to pH 4.0. Subsequently, the material was deep-frozen, and analysis was performed as described above.

RESULTS

Comparative sequence analyses of lactoballus α-rhamnosidases. A comprehensive search for homologs of experimentally characterized α-L-rhamnosidases yielded the following four lactic acid bacterial proteins: Lba1473 of *Lactobacillus acidophilus* NCFM (1), Lp_3471 and Lp_3473 of *Lactobacillus plantarum* WCFS1 (22), and sequence 5806 of *Enterococcus faecium* ATCC 35667 (13) (see Materials and Methods). The sequences were aligned, aberrant sequences were removed, and a bootstrapped neighbor-joining tree was generated. The tree, which is depicted in Fig. 2A, indicates that orthologs of the *L. acidophilus* protein exist in some bacilli (e.g., RhaA of *Bacillus* sp. strain GL1 [17]), in *Clostridium stercorarium*, in *Rhizobium etli*, and in *Opitutus terrae.* Considering the sequence conservation, Lba1473 probably displays a substrate preference that is highly similar to that of RamA of *Clostridium stercorarium* (40). The protein is therefore referred to as $RamA_{La}$. Orthologs of the *L. plantarum* and *E. faecium* putative rhamnosidases were found only in phylogenetically distant species, such as *Victivaldis vadensis*, *Rhuminococcus obeum*, *Flavobacterium johnsonii*, several *Bacteroides* species, and *Salmonella bongori*. These sequences appeared most similar to those of some of the experimentally characterized α-rhamnosidases of *Aspergillus* spp. (e.g., *Aspergillus nidulans* [6] and *Aspergillus kawachii* [24]), though the distinct differences in the alignment imply that their substrate specificities could well differ. The proteins are therefore referred to as $Ram1_{Lp}$ (Lp_3471), $Ram2_{Lp}$ (Lp_3473), and Ram_{Ef} (sequence 5806).

$Ram1_{Lp}$, $Ram2_{Lp}$, and Ram_{Ef}, together with the orthologous putative rhamnosidase in *S. bongori*, share two striking genomic associations, namely, with a gene encoding a transport protein and a gene encoding a transcription factor of the AraC family (Fig. 2B). The transport proteins could be involved in (di)saccharide transport, whereas the transcription factor could be rhamnose responsive for *S. bongori* and *N*-acetylglucosamine responsive for *L. plantarum* and *E. faecium* (see the legend to Fig. 2).

The genomic arrangement of the two genes in *L. plantarum* is suggestive of a specific association with a transporter-encoding gene for each of the two, as follows: lp_3471 with lp_3472 and lp_3473 with lp_3474 (Fig. 2B). Furthermore, the orthologous relationships imply more substrate similarity between $Ram1_{Lp}$ and Lp_3472 and the respective proteins in *E. faecium*, on the one hand, and between $Ram2_{Lp}$ and Lp_3474 and the respective proteins in *S. bongori* on the other. Clearly, the associations with the transporter and transcription factor genes suggest that the putative rhamnosidases from lactobacilli are involved in (di)glycoside utilization. However, detailed characteristics of the enzymes cannot be revealed by in silico analyses, and their potential involvement in the utilization of (di) saccharides coupled to phenolic compounds would remain to be proven. Therefore, we set out to characterize these enzymes.

Production and activity of *Lactobacillus* rhamnosidase enzymes. The $ram1_{Lp}$ and $ram2_{Lp}$ genes (*L. plantarum*) and the $ramA_{La}$ gene (*L. acidophilus*) were amplified from chromosomal DNA and expressed as fusions to an N-terminal His_6 tag in *E. coli* BL21 to test their proposed rhamnosidase activity. After protein expression, the cells and culture supernatant were separated by centrifugation. The cell pellet was subsequently lysed and clarified by centrifugation. Rhamnosidase activity was assayed both in the culture supernatant and in the intracellular soluble protein fraction from the lysed cells. The results given in Table 1 indicate that the activities of the soluble intracellular protein fractions from *E. coli* clones expressing $Ram1_{Lp}$, $Ram2_{Lp}$, and $RamA_{La}$ proteins were very similar on the substrate rhamnose-*p*-nitrophenyl. The activities of $Ram1_{Lp}$ and $RamA_{La}$ preparations did not change much after 1 day of incubation at 4°C, whereas the activity of $Ram2_{Lp}$ was strongly reduced after overnight incubation under these conditions (not shown). This shows that $ram1_{Lp}$, $ram2_{Lp}$, and $ramA_{La}$ indeed encode enzymes with rhamnosidase activity, in agreement with the expectation based on sequence analysis. Notably, about 30% of the total activity of $Ram1_{Lp}$ was found in the culture supernatant. The other enzyme activities were restricted to the intracellular fractions.

Specificity of rhamnosidases. To assess the specificities of the $Ram1_{Lp}$, $Ram2_{Lp}$, and $RamA_{La}$ enzymes, four substrates (*p*-nitrophenyl α-L-rhamnopyranoside [rhamnose-pNP], *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl α-D-galactopyranoside, and *p*-nitrophenyl β-D-glucoronide) were tested. For

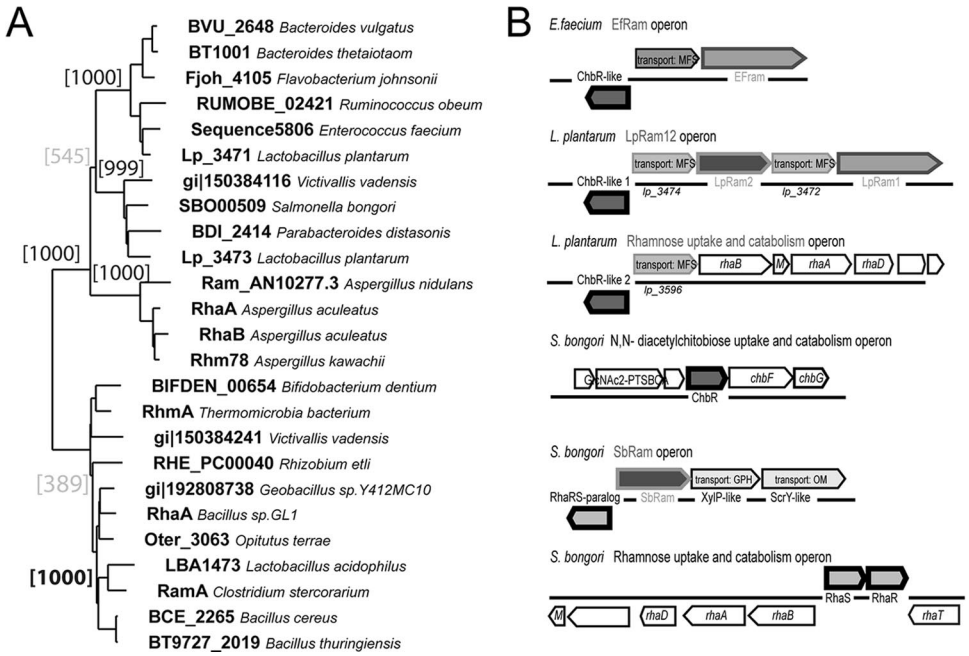

FIG. 2. (A) Neighbor-joining tree of experimentally characterized α-L-rhamnosidases and some of their homologs. Though enzyme activity has been reported for enzymes of three different glycoside hydrolase families (i.e., families 28, 78, and 106 in the CAZY reference database [18]), clear homologs (as judged from sequence similarity) were retrieved only for glycoside hydrolase family 78. The sequences for RhaB of *Bacillus* sp. strain GL1 and RhmB of *Thermomicrobia bacterium* PRI-1686 were left out of the comparison (due to clear sequence differences with the others). The comprehensive search for homologs yielded various additional homologs with respect to the CAZY reference database entries, including lp_3473 of *L. plantarum* and SBO00509 of *S. bongori*. (B) Gene context information related to the putative α-L-rhamnosidase-encoding genes in *E. faecium* ATCC 35667, *L. plantarum* WCFS1, and *S. bongori* 12419. The genes showed conserved associations with an AraC family transcription factor and a disaccharide transporter. Orthologous genes are coded by identical shading. In *E. faecium*, the transcription factor is orthologous to ChbR, the regulator of the *N*,*N*′-diacetylchitobiose [$(GlcNAc)_2$] utilization operon in *E. coli*, which is induced by $(GlcNAc)_2$ (21) or the monosaccharide. The transporter is a protein of the MFS superfamily and can be related to carbohydrate transport. In *L. plantarum*, the putative rhamnosidase-encoding genes are located in one operon, together with two paralogous MFS family transporter-encoding genes, whereas a third transporter paralog, lp_3596, is found in association with the rhamnose utilization operon that is equivalent to the one found in *E. coli* (29). The transporters are orthologous to IolF of *Bacillus subtilis*, which should transport carbohydrates that are somehow similar but not identical to *myo*-inositol (39). Finally, in *S. bongori*, the transcription factor is orthologous to RhaS and RhaR (see reference 23 and references cited therein) and thus probably induced by rhamnose. The two transporter-encoding genes are related to a GPH family periplasmic membrane protein that is similar to XylP, the isoprimeverose transporter of *Lactobacillus pentosus* (10), and to an outer membrane channel protein that is similar to ScrY (sucrose transport) (3).

each of the three enzymes, only rhamnose-pNP gave any detectable activity, indicating that the enzymes act as rhamnosidases. In a second experiment, the ability to degrade rhamnosylated flavonoids was tested, using the potential substrates rutin (quercetin-3-glucoside-6-β-1-rhamnoside), nicotiflorin (kaemferol-3-glucoside-6-β-1-rhamnoside), narirutin (naringenin-7-glucoside-6-β-1-rhamnoside) and naringin (naringenin-7-glucoside-2-β-1-rhamnoside) (Fig. 1). As shown in Table 2, $Ram1_{Lp}$ and $Ram2_{Lp}$ efficiently converted the rutinosides rutin, nicotiflorin, and narirutin into quercetin glucoside, kaempferol glucoside, and naringenin glucoside, respectively. Naringin, a neohesperidoside isomer of narirutin, was hardly deglycosylated by the $Ram1_{Lp}$ and $Ram2_{Lp}$ enzymes. $RamA_{La}$, on the other hand, readily converted all rutin, nicotiflorin, and

TABLE 1. Rhamnosidase activities of intracellular fluid (intracellular) and culture medium (extracellular) collected after expression in *E. coli*, as measured by release of *p*-nitrophenol from rhamnose-pNP in a 50 mM MOPS, pH 6.0, buffer

Enzyme	Fraction	Activity (mU per μg protein)
$Ram1_{Lp}$	Intracellular	7.1 ± 2.1
	Extracellular	0.020 ± 0.005
$Ram2_{Lp}$	Intracellular	3.1 ± 0.5
$RamA_{La}$	Intracellular	8.5 ± 0.9

TABLE 2. Rhamnosidase activities of $Ram1_{Lp}$, $Ram2_{Lp}$, and $RamA_{La}$ on flavonoid substrates[a]

Substrate ([M − H])	Product ([M − H])	Rhamnosidase activity (% conversion of substrate)		
		LpRam1	LpRam2	LaRamA
Rutin (609 *m/z*)	Quercetin-3-glucoside (463 *m/z*)	100	100	100
Nicotiflorin (593 *m/z*)	Kaempferol-3-glucoside (447 *m/z*)	100	100	100
Narirutin (579 *m/z*)	Naringenin-7-glucoside (433 *m/z*)	100	100	80
Naringin (579 *m/z*)	Naringenin-7-glucoside (433 *m/z*)	2	0	100

[a] Compounds (50 μg/ml) were incubated overnight at 37°C in 50 mM morpholineethanesulfonic acid, pH 6.1, with 5 mM β-mercaptoethanol and in the presence or absence of 10 μg protein of crude *E. coli* cell extract. Substrate formation was monitored using HPLC QTOF mass spectrometry. The masses of the compounds, as measured in negative mode ([M − H]), are indicated behind the substrate and product names.

TABLE 3. Activities of $Ram1_{Lp}$ and $RamA_{La}$ in the presence of different salts, as measured by release of *p*-nitrophenol from rhamnose-pNP in a 50 mM MOPS, pH 6.0, buffer[a]

Salt at 250 mM	Activity (%)	
	$Ram1_{Lp}$	$RamA_{La}$
NaCl	100 ± 15	65 ± 14
KCl	115 ± 18	50 ± 15
$CaCl_2$	60 ± 10	35 ± 6
$MgSO_4$	50 ± 8	20 ± 7
$(NH_4)_2SO_4$	80 ± 30	25 ± 12

[a] Activity in the absence of additional salts was set at 100%.

naringin and the majority of narirutin. Thus, $Ram1_{Lp}$ and $Ram2_{Lp}$ liberated 1→6-conjugated rhamnose residues, like those present in rutinosides, but not 1→2-conjugated rhamnose residues, like those in neohesperidosides. $RamA_{La}$ acted on both 1→6- and 1→2-conjugated rhamnose residues.

Properties of *Lactobacillus* rhamnosidases. For further investigations, $Ram1_{Lp}$ and $RamA_{La}$ were produced in 100-ml recombinant *E. coli* cultures. $Ram2_{Lp}$ was not used for further studies because it was instable during storage. $Ram1_{Lp}$ was then purified from the culture medium to apparent homogeneity by Ni-nitrilotriacetic acid chromatography, using the N-terminal His_6 tag. For as yet unknown reasons, a similar purification strategy did not work for $RamA_{La}$ in the soluble intracellular protein fraction of *E. coli* cells. The purified $Ram1_{Lp}$ protein (6.7 U/ml) and a cell lysate of $RamA_{La}$ (1.6 U/ml) were filtered through a 0.2-μm filter, and after the addition of 5 mM β-mercaptoethanol and 15% glycerol, they were stored at −20°C. Monthly activity assays showed that the enzymes could be stored in this way for at least 6 months without a significant loss of activity.

The tolerance of the $Ram1_{Lp}$ and $RamA_{La}$ enzymes to a number of conditions relevant to their application in foods was tested. Both enzymes showed the most activity at temperatures from 37 to 45°C. Higher temperatures irreversibly inactivated the enzymes. The presence of all five tested salts had a deleterious effect on the performance of $RamA_{La}$. For $Ram1_{Lp}$, the effects were salt dependent: while $CaCl_2$ and $MgSO_4$ significantly reduced activity, the other three salts had essentially no effect (Table 3).

The effect of pH on rhamnosidase activity was measured and is displayed in Fig. 3. The specific pH dependence of enzyme activity has a major impact on potential future applications in view of the generally low pH of fruit material. Since the detection of the synthetic substrate is sensitive to pH, rutin was used as a substrate. For both enzymes, the optimal pH was around 6. $Ram1_{Lp}$ was active from pH 4.5 to pH 8.0, while $RamA_{La}$ was active from pH 4.5 to pH 6.5. At pHs of <4.0, the activities of both enzymes dropped to background levels.

Kinetic constants for both enzymes were determined (Table 4). The synthetic substrate rhamnose-pNP and the flavonoid rutin were tested as substrates. For $Ram1_{Lp}$, the K_m value for rhamnose-pNP (6 mM) was much higher than that for rutin (50 μM). For $RamA_{La}$, K_m values were much closer for both substrates (0.7 mM and 0.3 mM for rhamnose-pNP and rutin, respectively). For the $Ram1_{Lp}$ enzyme, the k_{cat} value was much higher for rhamnose-pNP (64 s^{-1}) than for rutin (1 s^{-1}). The

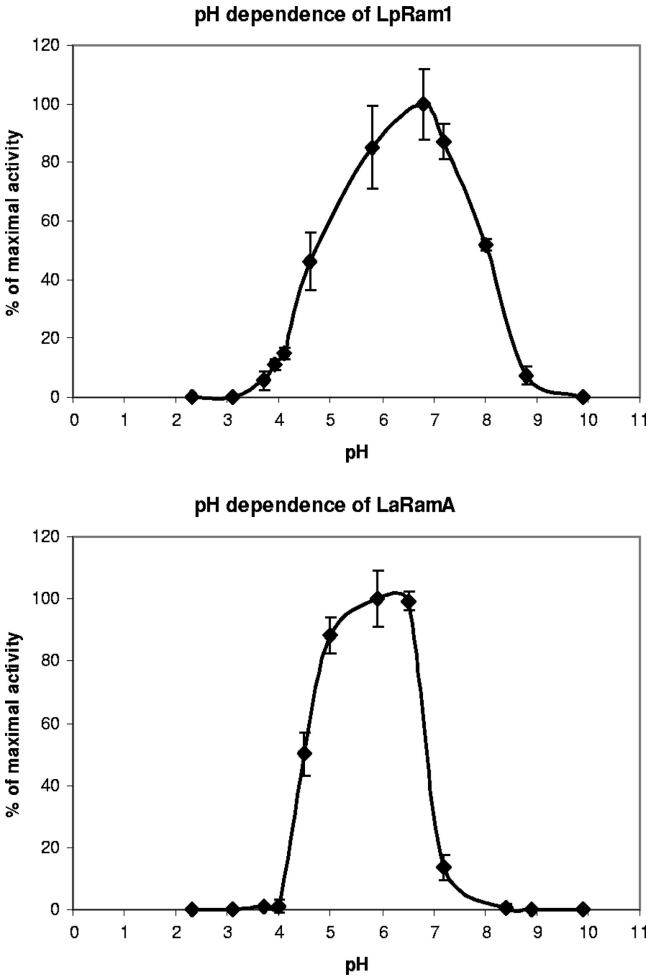

FIG. 3. Activities of $Ram1_{Lp}$ and $RamA_{La}$ at various pHs. The activities of rhamnosidases were measured using rutin as a substrate. Buffers were 100 mM sodium citrate (pH 2.3 to 3.7), 100 mM sodium acetate (pH 3.9 to 5.8), 100 mM MOPS (pH 6.5 to 7.3), and 100 mM Tris-HCl (pH 7.6 to 9.9). The reported values are the averages for three technical replications.

k_{cat} values could not be calculated for $RamA_{La}$ because the enzyme could not be purified from the total *E. coli* soluble protein extract. V_{max} values indicated that the activity of $RamA_{La}$ with rhamnose-pNP was also much higher than that with rutin. Due to the higher affinity of $Ram1_{Lp}$ for rutin, its catalytic efficiency (k_{cat}/K_m) with this natural rhamnoside substrate is on the same order of magnitude as that with the synthetic substrate.

Activities of rhamnosidases in tomato pulp. The activities of $Ram1_{Lp}$ and $RamA_{La}$ (at 85 μU/ml) on naturally occurring

TABLE 4. Kinetic constants for $Ram1_{Lp}$ and $RamA_{La}$

Enzyme	Substrate	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
$Ram1_{Lp}$	Rhamnose-pNP	$6.3 \times 10^{-3} \pm 3.1 \times 10^{-3}$	64 ± 12	1.0 ± 10^5
	Rutin	$5.0 \times 10^{-5} \pm 2.2 \times 10^{-5}$	1.0 ± 0.3	2.0 ± 10^5
$RamA_{La}$	Rhamnose-pNP	$7.0 \times 10^{-4} \pm 4.2 \times 10^{-4}$		
	Rutin	$3.1 \times 10^{-4} \pm 1.3 \times 10^{-4}$		

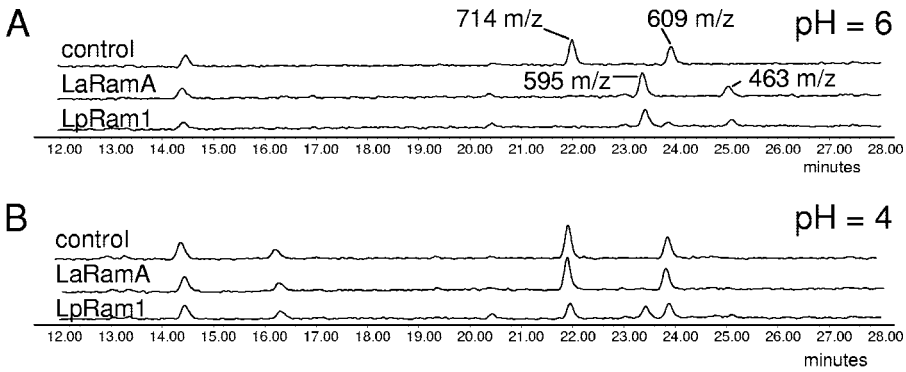

FIG. 4. Flavonoid content of tomato pulp upon rhamnosidase treatment. The data shown are HPLC chromatograms recorded at 360 nm following enzyme treatment of tomato pulp which had first been adjusted to pH 6.0 (A) or had not been adjusted (B). The masses of the compounds at the absorption peaks, as determined by mass spectrometry in negative mode, are indicated.

flavonoids in tomato pulp were tested. In buffered pulp (at pH 6), both enzymes were highly active and showed a complete turnover of rutinosides into glucosides. In Fig. 4, the HPLC chromatograms show that two peaks with elution times similar to those for rutin occurred in the control samples. By mass spectrometry, it was determined that the peak at 22.0 min represents rutin apioside ([M − H] = 741 *m/z*), while the peak at 24.0 min corresponds to rutin ([M − H] = 609 *m/z*). After complete conversion, two new peaks with the same absorption intensities appeared, at 23.6 and 25.2 min. Mass spectrometry analysis revealed that these peaks corresponded to quercetin-glucoside-pentoside ([M − H] = 595 *m/z*) and quercetin-glucoside ([M − H] = 463 *m/z*), respectively. These compounds represent the expected products of rhamnosidase activity on rutin apioside and rutin, respectively. At pH 6, incubation with $RamA_{La}$ as well as $Ram1_{Lp}$ resulted in (almost) complete conversion of rutin and rutin-apioside (Fig. 4A). In contrast, at pH 4.0, only 60% of the rutin and rutin-apioside was converted by $Ram1_{Lp}$, and only 10% was converted in the case of $RamA_{La}$ (Fig. 4B).

Expression of rhamnosidase in *L. lactis*. For application in food production strategies, rhamnosidases need to be produced in food-grade systems. As a first experiment, *L. plantarum* WCFS1 was used to ferment tomato pulp and then tested for rhamnosidase production. However, no conversion of tomato flavonoids was observed, nor could any rhamnosidase activity be detected in bacterial lysates using the synthetic substrate (data not shown). This indicated that the rhamnosidase operon was not expressed in *L. plantarum* under the circumstances tested. Therefore, we switched to a different food-grade expression system. A good example of such a system is the nisin-controlled expression (NICE) system based on *Lactococcus lactis* (27). To express $Ram1_{Lp}$ in the NICE system, the gene was cloned into pNZ8148 and subsequently transferred to *L. lactis* NZ9000. Control *L. lactis* did not show a background rhamnosidase activity (Table 5). Subsequently, we tested whether $Ram1_{Lp}$ was produced and if this activity was secreted from the cells. As detailed above, the intracellular fluid of *L. lactis* expressing $Ram1_{Lp}$ (at 5 ng/ml nisin inducer) showed a rhamnosidase activity (using rhamnose-pNP) of 0.67 μU per μg protein. No activity was detected in the culture medium or in the intracellular fraction of nonexpressing strains (uninduced or with empty plasmid). The *L. lactis* strain expressing $Ram1_{Lp}$ was used for the fermentation of tomato paste. After 48 h of fermentation, the changes in the concentration of rutin or rutin apioside were analyzed by HPLC. Unexpectedly, the levels of these compounds were unaltered relative to those in the original paste and after control fermentations using *L. lactis* harboring a control plasmid, uninduced *L. lactis* harboring $Ram1_{Lp}$, and induced, $Ram1_{Lp}$-expressing *L. lactis*. In addition, no formation of the reaction products quercetin-3-glucoside and quercetin-3-glucoside-apioside could be detected.

Potential reasons for the lack of activity of rhamnosidase-expressing *L. lactis* cells on tomato rutinosides were investigated. The *L. lactis* culture was calculated to produce 324 μU rhamnosidase per ml, which is ±4 times the enzyme load used when the activity of purified enzyme on tomato pulp was positively tested (see above) (85 μU rhamnosidase per ml). Therefore, it was concluded that sufficient enzyme should be available. Subsequently, it was tested if the flavonoid substrate rutin is accessible to the intracellular compartment of *L. lactis* in comparison with the artificial substrate rhamnose-pNP. To this end, cultures of *L. lactis* expressing $Ram1_{Lp}$ and control cultures of *L. lactis* (with an empty plasmid, pNZ8148) were generated. Equal volumes of these cultured cells were transferred to a small volume of phosphate buffer (pH 7) and were left as intact cells, or the intracellular fluid was released by

TABLE 5. Rhamnosidase activity of *L. lactis* expressing $Ram1_{Lp}$ compared to a control *L. lactis* strain (pNZ) lacking the $Ram1_{Lp}$ gene[a]

Enzyme prepn	Activity (% of substrate converted)	
	Rutin	Rhamnose-pNP
L. lactis expressing $Ram1_{Lp}$, intracellular	13.2	9.8
L. lactis expressing pNZ, intracellular	0.0	0.0
L. lactis expressing $Ram1_{Lp}$, whole cells	0.6	22.8
L. lactis expressing pNZ, whole cells	0.0	0.0

[a] Activity measurements were made using both a clarified cell lysate (intracellular) and unlysed cells (whole cells) on the substrates rutin (measured after 24 h by HPLC at 360 nm) and rhamnose-pNP (measured after 2 h as the release of pNP at 405 nm).

FastPrep treatment and then cleared by centrifugation. Subsequently, the rhamnosidase activities of both whole cells and the intracellular fluid were tested on rutin and pNP-rhamnose. The results are summarized in Table 5. Whole cells containing $Ram1_{Lp}$ converted rhamnose-pNP at a similar rate to that for intracellular fluid. In contrast, the conversion of rutin by whole cells was much lower (>20-fold) than that for the intracellular fluid. Thus, in comparison to rhamnose-pNP, rutin seems to have very poor access to intracellular rhamnosidases as a substrate in whole *L. lactis* cells.

DISCUSSION

In this paper, we describe the identification, isolation, and characterization of three rhamnosidases from *L. plantarum* and *L. acidophilus*. First, the evolutionary relationships between these sequences and those of known rhamnosidases were established. Unexpectedly, the enzyme of *L. acidophilus* mapped to a distinctly different branch of the rhamnosidase glycoside hydrolase family from that for the two *L. plantarum* enzymes. Furthermore, the analysis implied that $RamA_{La}$ is the *L. acidophilus* functional equivalent of RamA (40) of *Clostridium stercorarium.* Indeed, both enzymes are active on the 1→2-linked rhamnose in naringin.

In contrast, the enzymes of *L. plantarum* seem to be related more closely to rhamnosidase A of *Aspergillus*, although the sequences are distinctly different. The genomic association of the *L. plantarum* genes with a putative (di)saccharide transporter and a transcription factor that is induced by an *N*-acetylglucosamine-like molecule could indicate a possible role in assimilation of gellan-like substrates, much like the role of the rhamnosidases in the soil bacterium *Bacillus* sp. strain GL1. Gellan is a rhamnose-containing heteropolysaccharide produced by soil bacteria (15). The *Bacillus* rhamnosidases are proposed to act intracellularly on disaccharide breakdown products of gellan. Though the lactobacilli are not known to live in association with roots and thus with gellan, they likely encounter disaccharide breakdown products of plant cell wall constituents, produced, for instance, in the colon part of the digestive system. A role in this gut compartment is further suggested by the homology of $Ram1_{Lp}$ and $Ram2_{Lp}$ to rhamnosidases from bacteria occurring mainly in the colon (*Bacteroides*, *Ruminococcus*, and *Salmonella* species) (Fig. 2A). Moreover, previous data (8) indicate that expression of $Ram2_{Lp}$ is induced in the colon. Colonization of the colon by lactobacilli needs to be established among massive numbers of enteric bacteria in an environment that has been deprived of readily available monosaccharides. Under such conditions, means for utilization of rhamnose-containing oligosaccharides may be useful. Especially in woody plant material, polysaccharides are often coupled to phenolic compounds, e.g., flavonoids (Fig. 1). In fermentation of cabbage and tomato by *Lactobacillus*, breakdown of rhamnose-containing oligosaccharides is probably less important, since these are quite rich in fermentable monosaccharides (1.8% and 5% of the fresh weight, respectively) (11, 14).

Application of rhamnosidases. The rhamnosidases described in this paper have been shown to convert flavonoid rutinosides (such as rutin from tomato) into well-absorbed glucosides, and one of the enzymes is also active on naringin, a neohesperoside from citrus. Consequently, the activities of the *Lactobacillus* rhamnosidases are potentially useful for the enhancement of the health benefits of naturally occurring plant flavonoid rhamnosides. This could imply that probiotic lactobacilli such as *L. plantarum*, when present in the gut microflora, may enhance flavonoid bioavailability. However, while flavonoid absorption occurs mainly in the small intestine (12, 37), *L. plantarum* will predominantly colonize the colon. This implies that rhamnosidase activity expressed by probiotic lactic acid bacteria such as *L. plantarum* will occur too late in the digestive system to be of direct benefit. Therefore, an efficient application of rhamnosidases will most likely rely on the treatment of foodstuffs prior to ingestion.

In this paper, we show that *Lactobacillus* rhamnosidases are able to degrade compounds such as rutin and naringin present in a food matrix (Fig. 4). However, a strong limitation to the application of $Ram1_{Lp}$ and $RamA_{La}$ in the processing of fruit juices is that these enzymes act efficiently only at pHs of >4.5 (Fig. 3 and 4), while many fruit juices have a pH of 4.0 (tomato) or even lower (citrus). Adjustment of the fruit pulp matrix to pH 6 resulted in full conversion, which indicates that apart from its pH, this matrix enabled the enzyme to act on the flavonoids. However, pH adjustment is often not feasible during fruit juice production, and therefore an application of $Ram1_{Lp}$ in industrial fruit juice processing is probably not realistic. Alternative applications may be found in fermentation of less acidic flavonoid sources, such as cabbages and beans, and during extraction of flavonoids from plant waste material, such as olive mill residue (4). Application in soy processing, a material which is typically rich in polyphenols, could also be considered.

A novel way to deploy the rhamnosidase activity of *Lactobacillus* enzymes could be to ferment fruit juices with whole food-grade bacteria expressing rhamnosidases. No expression of rhamnosidase activity in *L. plantarum* could be observed. Therefore, $Ram1_{Lp}$ was expressed in *L. lactis*, using the NICE system. Food fermentations using recombinant microbes will not easily find commercial application. It has been shown that *Saccharomyces cerevisiae* can express and secrete a rhamnosidase from *Aspergillus* during vinification (25), with the aim to increase the content of volatile terpenoid compounds in wine, but this system has not been deployed commercially. However, recombinant systems do provide a platform to identify the required components for such a system. From this perspective, it is interesting that *L. lactis*, while fermenting tomato pulp, can functionally express sufficient amounts of $Ram1_{Lp}$ to convert tomato rutin into the more bioavailable quercetin-3-glucoside. However, this conversion does not occur during fermentation due to the fact that the substrate fails to be internalized by *L. lactis*. From Table 5, it is evident that although a synthetic substrate like rhamnose-pNP is readily taken up and degraded by intact *L. lactis* cells, a natural substrate (the rhamnosilated flavonoid rutin) is not available to the intracellular enzyme. This focuses our interest on the role of the transporter genes flanking the *L. plantarum* $Ram1_{Lp}$ and $Ram2_{Lp}$ genes. Bioinformatic analysis clearly implies that they are involved in (oligo)carbohydrate import. Possibly they mediate absorption of rhamnose-containing molecules such as flavonoids. If this is the case, they may be deployed to facilitate internalization of rhamnosilated flavonoids into lactobacilli, which may, by the

action of one of the rhamnosidases described in this paper, remove the rhamnose and produce flavonoids with improved bioavailability to the human digestive system.

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