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High-Level Expression of Recombinant β-Galactosidases in *Lactobacillus plantarum* and *Lactobacillus sakei* Using a Sakacin P-Based Expression System

ELISABETH HALBMAYR,[†] GEIR MATHIESEN,[‡] THU-HA NGUYEN,[†,§] THOMAS MAISCHBERGER,[†,§] CLEMENS K. PETERBAUER,*[,†] VINCENT G. H. EIJSINK,[‡] AND DIETMAR HALTRICH[†]

Division of Food Biotechnology, Department of Food Sciences and Technology, BOKU University of Natural Resources and Applied Life Sciences Vienna, Muthgasse 18, A-1190 Vienna, Austria, Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway, and Research Centre Applied Biocatalysis, Petersgasse 14, A-8010 Graz, Austria

This work presents the cloning and expression of the genes encoding heterodimeric β-galactosidases from *Lactobacillus reuteri* L103, *Lactobacillus acidophilus* R22, *Lactobacillus plantarum* WCFS1, and *Lactobacillus sakei* Lb790. These enzymes consist of two subunits of approximately 73 and 35 kDa, which are encoded by two overlapping genes, *lacL* and *lacM*, respectively. We have cloned these genes into the lactobacillal expression vectors pSIP403 and pSIP409, which are based on the sakacin P operon of *L. sakei* (Sørvig et al. *Microbiology* 2005, *151*, 2439−2449), and expressed them in the host strains *L. plantarum* WCFS1 and *L. sakei* Lb790. Results varied considerably, ranging from 2.23 to 61.1 U/mg of β-galactosidase activity, depending on the origin of the *lacLM* genes, the host strain, and the expression vector used. Highest expression levels were obtained in a laboratory cultivation of *L. plantarum* WCFS1 harboring the plasmid pEH3R containing the *lacLM* gene from *L. reuteri* L103. These cultivations yielded approximately 23 000 U of β-galactosidase activity per liter, corresponding to the formation of roughly 100 mg of recombinant protein per liter of fermentation medium, and β-galactosidase levels amounted to 55% of the total intracellular protein of the host organism. To further verify the suitability of this expression system, recombinant β-galactosidase from *L. reuteri* was purified to apparent homogeneity. The properties of the purified enzyme were essentially identical with the properties of purified native β-galactosidase from *L. reuteri* L103. The presented results lead the way to efficient overproduction of β-galactosidase in a food-grade expression system, which is of high interest for applications in food industry.

KEYWORDS: Inducible expression of proteins; β-galactosidase; sakacin; *Lactobacillus*; lactic acid bacteria

INTRODUCTION

At present, most host strains for the production of recombinant enzymes for food applications comprise a relatively small number of bacterial and fungal species, primarily *Bacillus subtilis*, *Bacillus licheniformis*, *Aspergillus niger*, or *Aspergillus oryzae*. These microorganisms have a long history of use as safe sources of native and recombinant enzymes in food biotechnology, and there is ample documentation of efficient growth under industrial production conditions. Even though these strains are used traditionally as a source of food-relevant enzymes and are considered nonpathogenic and nontoxigenic, some of these, e.g., *A. oryzae* or *A. niger*, are known to produce certain secondary metabolites under specific growth conditions. This can be circumvented by selecting strains where these pathways are inactivated, or by using appropriate cultivation conditions (*1*). Thus, it is desirable to develop alternative, safe, "food-grade" production hosts in certain food applications (*2*). In this respect, *Lactobacillus* spp. or other lactic acid bacteria (LAB) are of major interest as potential food-grade cell factories (*3*–*8*).

Many *Lactobacillus* spp. are traditionally used in food and feed industries in a wide variety of processes and products (*9*),

* Corresponding author: Department für Lebensmittelwissenschaften and -technologie, Universität für Bodenkultur Wien. Phone: +43-1-36006 6274. Fax: +43-1-36006 6251. E-mail: clemens.peterbauer@boku.ac.at.

[†] BOKU University of Natural Resources and Applied Life Sciences Vienna.

[‡] Norwegian University of Life Sciences.

[§] Research Centre Applied Biocatalysis.

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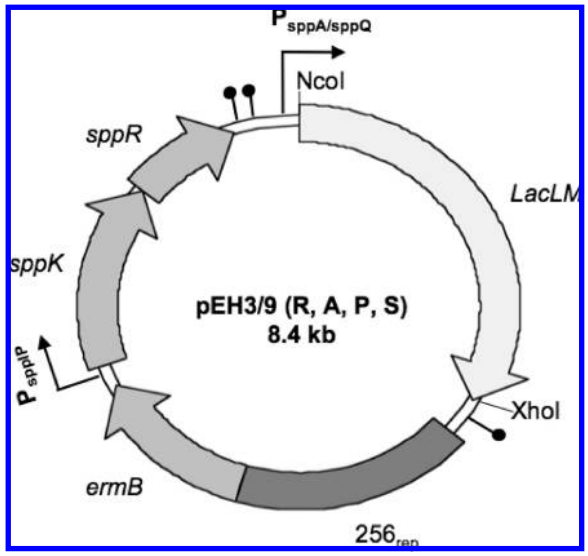

Figure 1. Schematic overview of the pEH plasmids used in this study. Dark-gray region, replication determinants (256_{rep}); light-gray region, structural gene (*lacLM*); gray regions, erythromycin resistance marker (ermB), histidine kinase (*sppK*), and response regulator (*sppR*) genes; black arrows, inducible bacteriocin promoters, inducible P_{sppA} promoter for pEH3, and P_{sppQ} (formerly P_{orfX}) promoter for pEH9; lollypop structures, transcriptional terminator.

and they have been generally recognized as safe (GRAS) status. A number of lactobacilli have furthermore been proposed and are applied as probiotic strains, i.e., live microorganisms that are used as a food supplement to benefit health (*10–12*). Probiotics show several diverse beneficial effects, among others a normalizing effect on the intestinal microbiota by inhibiting growth or adhesion of pathogenic microorganisms (*13*). This is achieved by production of lactic acid lowering the pH, by displacement of competing bacteria on binding surfaces such as human mucosa (*12*), and by secretion of antimicrobial peptides called bacteriocins (*14*).

It has been shown that production of bacteriocins may be regulated via quorum-sensing mechanisms based on secreted peptide pheromones with no or little bacteriocin activity (*15, 16*). The pheromone activates a two-component regulatory system consisting of a membrane-bound histidine kinase that senses the pheromone, and an intracellular response regulator that, when activated by the histidine kinase, induces the promoters of the operons involved in bacteriocin production. Using these regulatory mechanisms, several inducible gene expression systems have been developed for efficient and regulated overproduction of heterologous proteins in LAB, using, for example, *Lactococcus lactis* (*4–17*), *Lactobacillus brevis* (*8*), *Streptococcus thermophilus* (*18*), or *Lactobacillus sakei* and *L. plantarum* (*3, 19–21*) as expression hosts. One of the systems for *L. sakei* and *L. plantarum* employs the so-called pSIP expression vectors that are based on promoters and regulatory genes involved in the production of the class II bacteriocins sakacin A [*sap* gene cluster (*22*)] or sakacin P [*spp* gene cluster (*23, 24*)] (**Figure 1**). In these vectors expression of the gene of interest is placed under control of a strong inducible bacteriocin promoter and gene expression is induced by an externally added peptide pheromone (*21*). The applicability and usefulness of this expression system has been shown in previous studies using β-glucuronidase from *Escherichia coli* (*gusA*) and aminopeptidase N from *Lactococcus lactis* (*pepN*) as reporter genes (*20, 25*). Furthermore, Straume et al. (*26*) found that production of a membrane-associate lactobacillal protein in its soluble form was more efficient when using the pSIP system than when using standard *E. coli*-based methods.

The goal of the present study was to exploit and compare the pSIP expression vectors, using two different promoters and two different host strains, for overproduction of β-galactosidases from *Lactobacillus spp.* β-Galactosidases (β-gal, EC 3.2.1.23) catalyze both the hydrolysis and transgalactosylation of β-D-galactopyranosides. β-Galactosidase-catalyzed hydrolysis reactions are widely known for applications in the dairy industries where they are used to improve digestibility, solubility, and sweetness of lactose, the principal milk carbohydrate (*27*). Transgalactosylation reactions involving lactose or structurally related galactosides yield galacto-oligosaccharides (*27, 28*). These compounds attract an increasing amount of attention because of their prebiotic effects (*29–31*). Lactobacillal β-galactosidases are heterodimeric proteins consisting of two subunits of approximately 35 and 73 kDa, which are encoded by two overlapping genes, *lacL* and *lacM*, respectively (*32–36*). We describe for the first time the homologous and heterologous expression of the genes (*lacLM*) encoding β-galactosidases from four different *Lactobacillus* species, *L. acidophilus* R22, *L. reuteri* L103, *L. plantarum* WCFS1, and *L. sakei* Lb790, in *Lactobacillus* expression systems. These four source strains are among the best known probiotic lactobacilli (*12, 13 , 37*), and β-galactosidases from these strains are attractive for various applications in food technology. Our goal was to obtain high and inducible production of these important enzymes using expression systems that can be easily adapted to the need of the food industry for the production of food-grade enzymes.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated, and were of the highest quality available. MRS broth (*Lactobacillus* broth according to De Man, Rogosa, and Sharpe (*38*)) was obtained from Oxoid (Hampshire, UK) and Merck (Darmstadt, Germany). BHI (Brain Heart Infusion) broth was purchased from Oxoid, 1,4-dithiothreitol (DTT) was from Roth (Karlsruhe, Germany), and glucose oxidase (GOD) from *A. niger* (lyophilized, 205 U/mg enzyme preparation) was from Fluka (Buchs, Switzerland). Horseradish peroxidase (POD) (lyophilized, 210 U/mg) and the test kit for the determination of D-glucose were from Boehringer (Mannheim, Germany). All restriction enzymes and Quick T4 DNA ligase were purchased from New England Biolabs (Beverly, MA), while Phusion DNA Polymerase was from Finnzymes OY (Espoo, Finland).

Bacterial Strains and Culture Conditions. *L. reuteri* strain L103, isolated from calf, was obtained from Lactosan (Starterkulturen GmbH & Co. KG, Kapfenberg, Austria). *L. acidophilus* R22, originating from probiotic yogurt, was obtained from the culture collection of the Division of Food Microbiology, BOKU University of Natural Resources and Applied Life Sciences, Vienna. *L. plantarum* WCFS1, isolated from human saliva as described in Kleerebezem et al. (*35*) and *L. sakei* Lb790, isolated from meat as described in Schillinger and Lücke (*39*), were from the culture collection of the Norwegian University of Life Sciences, Ås, Norway. *E. coli* One Shot TOP10 chemical competent cells used for chemical transformation and electroporation were purchased from Invitrogen (Carlsbad, CA, USA). *E. coli* was grown in BHI medium at 37 °C with shaking. *Lactobacillus* strains were grown in MRS medium at 30 °C in tightly capped flasks and without shaking. Agar plates were solidified by adding 1.5% (w/v) agar. When appropriate, antibiotics were added as follows: 100 μg/mL kanamycin or 200 μg/mL erythromycin for *E. coli,* and 5 μg/mL erythromycin for lactobacilli.

Plasmids. The construction of the plasmids pHA1031 and pHAR22 containing the complete genes (*lacL* and *lacM*) of β-galactosidases from *L. reuteri* L103 and *L. acidophilus* R22, respectively, has been described previously by Nguyen et al. (*32, 34*). These plasmids were used as

Table 1. Sequences of the Primers Used in This Study[a]

primer	sequence (5′ → 3′)	ref sequence accession no.
LacReuF	**GGTCTCAC** ATGCAAGCAAATATAAAAT	DQ493596
LacReuR	CAAACATAACTTACGTTTTATT **GAGCTC**	DQ493596
LacAciF	**GGTCTCTC** ATGCAAGCAAACATAAAAT	EF053367
LacAciR	TGCTTAAAAGAAAGTTAGAATTAATT **CTTAAG** GG	EF053367
LacLMPlaF	**GGTCTCTC** ATGCAAGCTAATCTTCAAT	AL935262
LacLMPlaR	TCGAAATTATAAGTAAAGATT **GAGCTC**	AL935262
Lb790LacF	**GGTCTCTC** ATGCAACCTAATATTCAAT	X82287
Lb790LacR	TAAAACTTAAACTTTAAAGCAAATT **GAGCTC**	X82287

[a] Restriction sites are underlined and in boldface. F denotes forward primers, R denotes reverse primers; LacReu stands for β-galactosidase (LacLM) from *L. reuteri* L103, LacAci for β-galactosidase from *L. acidophilus* R22, LacPla for β-galactosidase from *L. plantarum* WCFS1, and Lb790Lac for β-galactosidase from *L. sakei* Lb790.

templates for amplifying the *lacLM* operon from these strains. Chromosomal DNA from *L. plantarum* WCFS1 and *L. sakei* Lb790 was extracted using the E.ZNA Bacterial DNA kit (Omega Bio-Tek, Doraville, GA) and used as a template for amplifying *lacLM* from these strains. Expression vectors pSIP403, pSIP409, and pSIP405 were prepared as described previously in Sørvig et al. (*3, 21*) and Mathiesen et al. (*40*). Plasmid DNA was purified using the E.ZNA Plasmid Miniprep Kit I (Omega Bio-Tek).

Cloning of *lacLM* Genes. The oligonucleotides LacReuF and LacReuR, LacAciF and LacAciR, LacLMPlaF and LacLMPlaR, and Lb790LacF and Lb790LacR (**Table 1**) used for PCR amplification of the *L. reuteri lacLM*, *L. acidophilus lacLM*, *L. plantarum lacLM*, and *L. sakei lacLM* genes, respectively, were designed based on the sequences of β-galactosidases from *L. reuteri* L103 (GenBank accession number DQ493596), *L. acidophilus* R22 (GenBank accession number EF053367), *L. plantarum* WCFS1 (GenBank accession number AL935262), and *L. sakei* subsp. *sakei* (GenBank accession no. X82287). *Bsa*I restriction sites were added to the 5′-ends of the forward primers, and *Xho*I restriction sites were added at the 5′-end of reverse primers, except for LacAciR, which contains an *Eco*RI site instead. All primers were purchased from Operon Biotechnologies GmbH (Cologne, Germany). DNA amplification was performed with Phusion High-Fidelity DNA Polymerase using an Eppendorf Mastercycler (Eppendorf; Hamburg, Germany) and standard procedures. Standard PCR reactions were conducted in a total volume of 50 μL containing 0.2 mM of each deoxynucleotide triphosphate, 0.5 μM of each primer, 10 μL of 5 × Phusion HF buffer (final concentration of $MgCl_2$ was 1.5 mM), 1 U of Phusion DNA Polymerase, and approximately 50 ng of genomic DNA. The initial denaturation step at 98 °C for 30 s was followed by 35 cycles of denaturation at 98 °C for 5 s, annealing at 49/53 °C for 10 s, and extension at 72 °C for 1 min. The final cycle was followed by an additional 10-min elongation step at 72 °C. The amplified products were visualized by gel electrophoresis at 7 V/cm in a 1.2% agarose gel (containing ethidium bromide at 0.2 μg/mL) in 1 × TAE (Tris-Acetate) electrophoresis buffer (Tris base 4.8 g/L, acetic acid 1.2 g/L, 1 mM EDTA pH 8.0). All PCR products were purified from the agarose gel using the NucleoSpin Extract II (Macherey-Nagel; Bethlehem, PA), and directly subcloned into the pCRII-Blunt-TOPO vector (Invitrogen; Carlsbad, CA) according to the manufacturers instructions, resulting in the plasmids pTReu, pTAci, pTLacLMPlant, and pTLacLMSak containing the complete genes (*lacL* and *lacM*) of β-galactosidases from *L. reuteri*, *L. acidophilus*, *L. plantarum*, and *L. sakei*, respectively. The sequence of the inserts was verified by DNA sequencing, using a Big Dye sequencing kit obtained from Applied Biosystem and an ABI Prism 377 DNA sequencer (PE Applied Biosystems, Foster City, CA).

Construction of *lacLM* Expression Vectors. The operons encoding for the complete heterodimeric β-galactosidase (*lacLM*) were translationally fused to the P_{sspA} promoter in pSIP403, or the P_{sspQ} (formerly called P_{orfX}) promoter in pSIP409 (*3, 41*) using the *Nco*I site (**Figure 1**). To achieve this, the *lacLM* gene-fragments were excised from the TOPO plasmids pTReu, pTAci, pTLacLMPlant, and pTLacLMSak using the restriction enzymes *Bsa*I (*Nco*I compatible) and *Xho*I (*Eco*RI instead of *Xho*I for pTAci). The resulting fragments were ligated into the 5.6 kb *Nco*I-*Xho*I fragment (or the 5.6 kb *Nco*I-*Eco*RI fragment for *lacLM* from *L. acidophilus*) of pSIP403 and pSIP409 (*2, 16*), yielding pEH3R, pEH9R, pEH3A, pEH9A, pEH3P, pEH9P, pEH3S, and pEH9S. All plasmids were transformed into *E. coli* One Shot TOP10 chemical competent cells (Invitrogen) following the manufacturers protocol.

Expression of β-Galactosidases. To generate 16 expression strains carrying the newly constructed plasmids pSIP403LacLM (pEH3R, pEH3A, pEH3P, pEH3S) and pSIP409LacLM (pEH9R, pEH9A, pEH9P, pEH9S) were transformed into electro-competent *L. plantarum* WCFS1 and *L. sakei* Lb790 cells according to the protocol by Aukrust and Blom (*42*), and transformants were selected on MRS agar containing 5 μg/mL erythromycin.

Overnight cultures of *Lactobacillus* harboring plasmids with *lacLM* genes were diluted in 50 mL of fresh MRS medium containing 5 μg/mL erythromycin to an OD_{600} of ∼0.1 and incubated at 30 °C to an OD_{600} of 0.3. If induction was desirable the cultures were then induced with 25 ng/mL peptide pheromone IP-673 (supplied by the Molecular Biology Unit, University of Newcastle-upon-Tyne, UK). Cells were harvested at an OD_{600} of approximately 1.8 by centrifugation at 3300 × g for 5 min at 4 °C and washed once with a 0.9% sodium chloride solution. The cell pellet was resuspended in 1 mL of sonication buffer (50 mM sodium phosphate buffer, pH 6.5, 20% glycerol, 1 mM DTT) and disrupted using a sonicator (Sonics vibra-cell; Sonics and Materials, Newtown, CT) set at an amplitude of 25%, pulse 5 s on, 5 s off, and 3 min sonication time. Cell debris was removed by centrifugation (16 000 × g, 5 min, 4 °C) to obtain the crude enzyme extract. Background expression of native β-galactosidases was examined with *L. plantarum* WCFS1/pSIP405 and *L. sakei* Lb790/pSIP405, which carry a variant of pSIP403 harboring a *cat* (chloramphenicol acetyltransferase) gene instead of the *lacLM* genes (*40*).

Fermentation of the Selected *Lactobacillus* Strain. *L. plantarum* WCFS1 carrying pSIP403LacLM-Reu, the plasmid containing the genes (*lacL* and *lacM*) for the β-galactosidase from *L. reuteri* L103, was selected for further studies. The strain was grown in MRS broth containing 5 μg/mL erythromycin in a 1-L glass reactor at 30 °C. Precultures were grown overnight in 5 mL of MRS broth medium containing 5 μg/mL erythromycin and diluted in 1000 mL of fresh medium to an optical density at 600 nm of 0.1 and incubated at 30 °C until OD_{600} reached 0.3. At this point, pheromone (25 ng/mL) was added to the culture medium and the cultures were incubated further for 20 h to an OD_{600} of approximately 6.0. The induced cells were harvested, washed once with 0.9% sodium chloride, and resuspended in 50 mM sodium phosphate buffer (pH 6.0). The cells were disrupted using a French press (Aminco; Jessup, MD), and the cell debris was removed by centrifugation (16 000 × g, 45 min, 4 °C) to obtain the crude protein extract.

Protein Purification. The crude protein extract was loaded onto an affinity column (10 mL of *p*-aminobenzyl 1-thio-β-D-galactopyranoside immobilized on cross-linked 4% beaded agarose; Sigma) that was pre-equilibrated with 50 mM sodium phosphate buffer pH 6.0 (buffer A). The enzyme was eluted at a rate of 0.6 mL/min by using a linear gradient of 0 to 2 M sodium chloride in buffer A (15 column volumes). The active fractions were pooled, desalted, and concentrated (Amicon Ultra Centrifugal filter tubes; 10 kDa cutoff; Millipore, Billeria, MA). The purified enzyme was stored in 50 mM sodium phosphate buffer (pH 6.0) containing 1 mM DTT at 4 °C. Protein concentrations were determined by the method of Bradford (*43*) using bovine serum albumin as standard.

Gel Electrophoresis and Activity Staining. To compare the expression of the different β-galactosidases in *L. plantarum* WCFS1 and *L. sakei* Lb790 the crude protein extracts were analyzed by running 10% NuPAGE Novex Bis-Tris gels (Invitrogen). The sample preparation, running buffer, and running conditions were as described in the NuPAGE manual from Invitrogen with slight modifications (samples were heated at 99 °C for 5 min). Coomassie blue staining was performed for visualization of the protein bands. Activity staining was carried out using 4-methylumbelliferyl β-D-galactoside as the substrate, as previously described (*33*).

Enzyme Assays. β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) and lactose as the sub-

Table 2. Expression Plasmids Used in This Study

name	alternate name	derived from	promoter	source of β-galactosidase gene
pEH3R	pSIP403LacLM-Reu	pSIP403	P_{sppA}	*L. reuteri*
pEH9R	pSIP409LacLM-Reu	pSIP409	P_{sppQ}	*L. reuteri*
pEH3A	pSIP403LacLM-Aci	pSIP403	P_{sppA}	*L. acidophilus*
pEH9A	pSIP409LacLM-Aci	pSIP409	P_{sppQ}	*L. acidophilus*
pEH3P	pSIP403LacLM-Plan	pSIP403	P_{sppA}	*L. plantarum*
pEH9P	pSIP409LacLM-Plan	pSIP409	P_{sppQ}	*L. plantarum*
pEH3S	pSIP403LacLM-Sak	pSIP403	P_{sppA}	*L. sakei*
pEH9S	pSIP409LacLM-Sak	pSIP409	P_{sppQ}	*L. sakei*

strates, as described previously (*33*). When chromogenic *o*NPG was used as the substrate, the reaction was initiated by adding 20 μL of enzyme solution to 480 μL of 22 mM *o*NPG in 50 mM sodium phosphate buffer (pH 6.5) and stopped after 10 min of incubation at 30 °C and shaking at 600 rpm by adding 750 μL of 0.4 M Na_2CO_3. The release of *o*-nitrophenol (*o*NP) was measured by determining the absorbance at 420 nm. One unit of *o*NPG activity was defined as the amount of enzyme releasing 1 μmol of *o*NP per minute under the described conditions.

When lactose was used as the substrate, 20 μL of enzyme solution was added to 480 μL of 600 mM lactose solution in 50 mM sodium phosphate buffer, pH 6.5. After 10 min of incubation at 30 °C, the reaction was stopped by heating the reaction mixture at 99 °C for 5 min. The reaction mixture was cooled to room temperature, and the release of D-glucose was determined colorimetrically using the GOD/POD assay (*44*). One unit of lactase activity was defined as the amount of enzyme releasing 1 μmol of D-glucose per minute under the given conditions.

Characterization of Recombinant β-Galactosidase. Steady-state kinetic data for the substrate lactose were obtained following the methods described in a previous report (*33*). Lactose concentrations of 9.6−576 mM were employed, and data were analyzed with SigmaPlot (SPSS Inc.; Chicago, IL). The pH dependence of the enzymatic release of *o*-nitrophenol (*o*NP) from *o*NPG and D-glucose from lactose was measured between pH 4 and 9 using Britton-Robinson buffer (containing 20 mM each of phosphoric, acetic, and boric acid adjusted to the required pH with NaOH). The temperature dependence of enzyme activity (both *o*NPG and lactase activity) was determined by measuring activity over the range of 25−70 °C for 10 min.

RESULTS

Cloning and Expression of β-Galactosidase Genes from *L. reuteri*, *L. acidophilus*, *L. plantarum*, and *L. sakei* in *Lactobacillus* Expression Systems. In the present study the entire *lacLM* operons encoding both the large and the small subunits of β-galactosidase from *L. reuteri* L103, *L. acidophilus* R22, *L. plantarum* WCFS1, and *L. sakei* Lb790 were cloned into the pSIP expression vectors pSIP403 and pSIP409. This implies that the operons were cloned such as to translationally fuse the *lacL* gene to either the P_{sppA} or P_{sppQ} promoter, resulting in eight expression plasmids: pSIP403LacLM-Reu, pSIP409LacLM-Reu, pSIP403LacLM-Aci, pSIP409LacLM-Aci, pSIP403LacLM-Plan, pSIP409LacLM-Plan, pSIP403LacLM-Sak, and pSIP409LacLM-Sak (pEH3R, pEH9R, pEH3A, pEH9A, pEH3P, pEH9P, pEH3S and pEH9S; "3" denotes that the expression plasmid is derived from pSIP403, "9" denotes that pSIP409 was used; "R" indicates the *lacLM* genes are from *L. reuteri*, "A" those from *L. acidophilus*, "P" those from *L. plantarum*, and "S" those from *L. sakei*, see **Table 2** for a compilation). **Table 3** shows the size of the respective genes as well as the calculated molecular masses for the large and the small subunits of the β-galactosidases. The plasmids were transformed into *L. plantarum* and *L. sakei*, and expression of the introduced β-galactosidase genes was studied. Gene expression was induced by adding peptide pheromone at a concentration (25 ng/mL) that is known to give maximum expression in these systems (*20*). Induced cells were harvested at $OD_{600} \approx 1.8$ (late stationary phase), which is known from previous studies with similar expression systems to give the maximum level of recombinant protein expression under these growth conditions (*21, 45*), and cell-free protein extracts were prepared and analyzed by SDS-PAGE (**Figure 2**) and determination of specific enzyme activity in the extracts (**Table 4**; **Figure 3**).

Table 3. Number of Base Pairs and Calculated Molecular Masses for the Large (*lacL*) and the Small (*lacM*) Subunits of β-Galactosidases from Different Lactobacilli

	lacL		*lacM*		
source of β-galactosidase	base pairs	molecular mass [Da]	base pairs	molecular mass [Da]	accession no.
L. reuteri L103	1887	73 492	960	35 683	DQ493596
L. acidophilus R22	1887	73 254	951	35 590	EF053367
L. plantarum WCFS1	1881	72 180	960	35 223	AL935262
*L. sakei*Lb790	1878	72 457	993	36 742	X82287

Two of the β-galactosidases (from *L. reuteri* and *L. plantarum*) were produced in high amounts, as illustrated by the presence of additional bands at approximately 75 kDa in the SDS-PAGE gel corresponding to the expected size of LacL (**Figure 2**). Activity data (**Table 4**, **Figure 3**) confirmed the high production levels. The β-galactosidases from *L. sakei* and *L. acidophilus* were produced in 5-fold to 10-fold lower amounts (**Figure 3**, **Table 4**) and are not as clearly identifiable on SDS-PAGE. With the exception of the β-galactosidase from *L. acidophilus*, all enzymes were produced more efficiently in *L. plantarum* than in *L. sakei.* Generally, exchange of the P_{sspA} promoter (pSIP403 derivatives) with the P_{sppQ} promoter (pSIP409 derivatives) did not significantly affect the levels of β-galactosidase activity formed in both host strains.

Noninduced cells of *L. plantarum* WCFS1 and *L. sakei* Lb790 harboring the expression plasmids were also grown and harvested as described above to check for basal expression ("leakage") from the promoters and to calculate the degree of induction obtained with the pheromone. In addition, the specific β-galactosidase activities in the wild-type host strains were determined and found to be 0.07 U/mg in both organisms. The results (**Table 4**) show that all expression strains show basal activities that are clearly above the wild-type background levels, but that are still low compared to the levels obtained upon induction. Generally, the *L. plantarum* strains showed higher basal activities than the *L. sakei* strains. Furthermore, cells harboring pSIP409-derived plasmids containing the P_{sppQ} promoter showed lower basal activities than cells harboring pSIP403-derived plasmids (P_{sppA} promoter). Consequently, the highest induction factors were obtained with *L. sakei* as expression host and constructs based on pSIP409.

For comparison, we determined the levels of β-galactosidase activity produced by wild-type strains *L. reuteri*, *L. acidophilus*, and *L. plantarum* grown on MRS containing lactose instead of glucose. Cells were harvested at an OD_{600} of ∼2.0, and the intracellular β-galactosidase activity was determined in cell-free extracts. The activities were 7.90 ± 0.78, 4.00 ± 0.56, and 2.22 ± 0.14 U/mg for *L. reuteri*, *L. acidophilus*, and *L. plantarum*, respectively. For the β-galactosidases from *L. reuteri* and *L. plantarum* these levels are much lower than the levels obtained with the expression vectors (**Table 4**).

The highest specific activity was obtained with *L. plantarum* WCFS1/pSIP403LacLM-Reu (carrying the *lacLM* gene from *L. reuteri*). Thus, this strain and the recombinant *L. reuteri* β-galactosidase (*Lp*L103) it produces were selected for further studies.

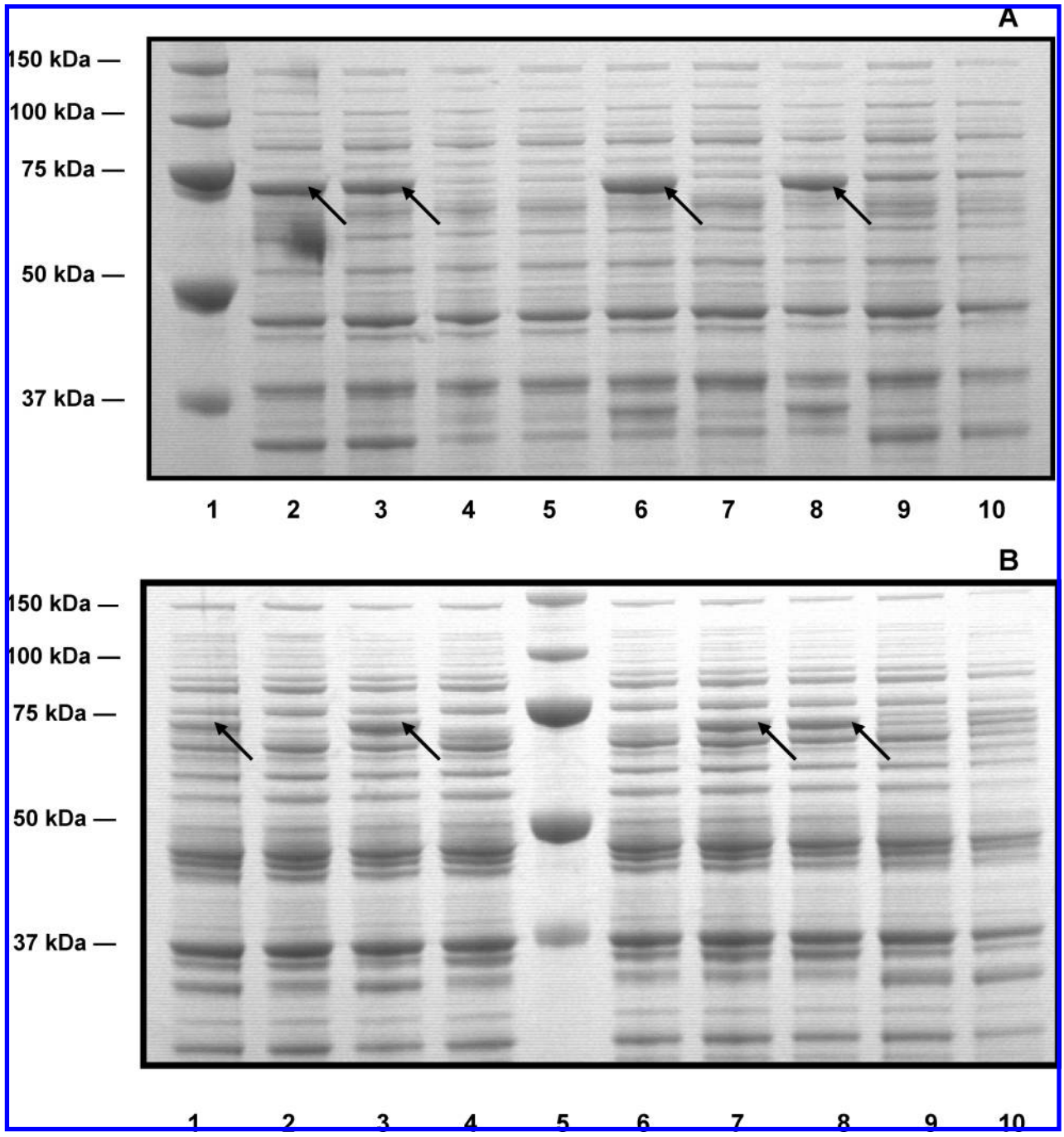

Figure 2. SDS-PAGE of whole-cell lysates of *L. plantarum* WCFS1 (panel **A**) and *L. sakei* Lb790 (panel **B**) carrying plasmids containing the complete β-galactosidase operon (*lacL* and *lacM*) from *L. reuteri* L103 (R), *L. acidophilus* R22 (A), *L. plantarum* WCFS1 (P), and *L. sakei* Lb790 (S). Gels were stained with Coomassie blue. Panel A: lane 1, benchmark protein ladder (Invitrogen); lane 7, *L. plantarum* carrying pEH9A without induction; other lanes, induced *L. plantarum* carrying pEH3R (lane 2), pEH9R (lane 3), pEH3A (lane 4), pEH9A (lane 5), pEH3P (lane 6), pEH9P (lane 8), pEH3S (lane 9), and pEH9S (lane 10). Panel B: lane 2, *L. sakei* carrying pEH9A, without induction; lane 5, Precision Plus Protein standard (BioRad); other lanes, induced *L. sakei* carrying pEH3R (lane 1), pEH9R (lane 3), pEH3A (lane 4), pEH9A (lane 6), pEH3P (lane 7), pEH9P (lane 8), pEH3S (lane 9), and pEH9S (lane 10). Identifyable additional bands in induced extracts representing LacL (pEH3R and pEH9R, pEH3P and pEH9P) are marked with arrows.

Overexpression of β-Galactosidase Genes from *L. reuteri* in *L. plantarum*. The *L. reuteri* β-galactosidase was overproduced using a 1-L bioreactor cultivation of *L. plantarum* WCFS1 carrying pEH3R, which yielded a specific activity 136 U/mg of protein (23 kU per liter of fermentation broth). This indicated that the β-galactosidase constituted approximately 55% of the total amount of intracellular soluble proteins as calculated from the specific activities. Furthermore, this high level of expression was also obvious by the two strong bands of LacM and LacL visible in SDS-PAGE for the crude cell extract (**Figure 4**). The enzyme was purified in one single step to apparent homogeneity (**Figure 4**) using an affinity column (*p*-aminobenzyl 1-thio-β-D-galactopyranoside immobilized in cross-linked 4% beaded agarose), which gave an overall yield of 12% and a specific activity of 245 U/mg of protein. The recombinant β-galactosidase overexpressed in *L. plantarum* consisted of subunits with apparent molecular masses of 73 and 35 kDa (**Figure 4**). This compares well to the calculated molecular masses of the two subunits (73 492 and 35 683 Da, respectively). Activity staining on the SDS-PAGE gel (sample treatment was done at 60 °C)

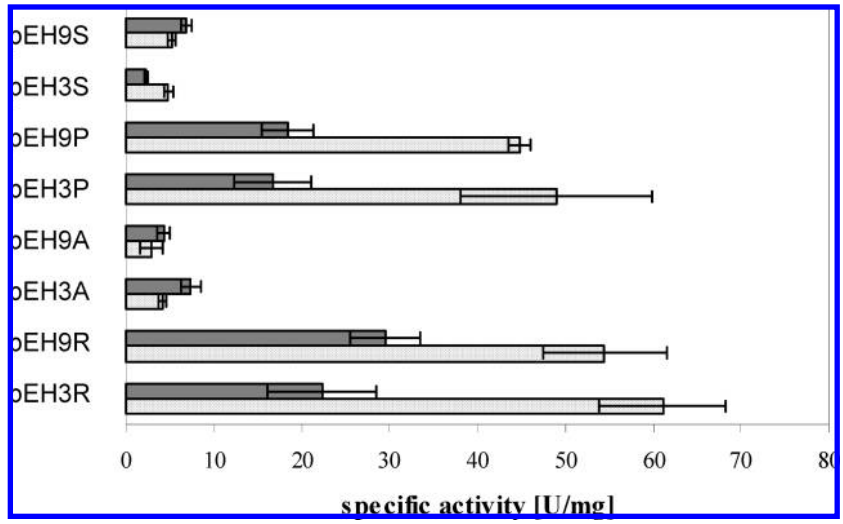

Figure 3. Specific β-galactosidase activities in cell-free extracts of *L. sakei* Lb790 (dark bars) and *L. plantarum* WCFS1 (light bars) harboring plasmids for the expression of β-galactosidase from *L. reuteri* L103 (R), *L. acidophilus* R22 (A), *L. plantarum* WCFS1 (P), and *L. sakei* Lb790 (S). All data are the mean value of three independent experiments; the error bars indicate the standard deviations.

Table 4. Specific β-Galactosidase Activity in Cell-Free Extracts of Noninduced and Induced Cultures of *L. plantarum* WCFS1 and *L. sakei* Lb790 Containing Various Expression Plasmids[a]

	specific activity (U/mg)		
strain (plasmid)	without induction	with induction	induction factor
WCFS1 (pEH3R)	1.610 ± 0.195	61.1 ± 7.2	38.0
Lb790 (pEH3R)	0.213 ± 0.061	22.3 ± 6.2	104
WCFS1 (pEH9R)	0.290 ± 0.031	54.5 ± 7.0	183
Lb790 (pEH9R)	0.123 ± 0.006	29.5 ± 4.0	240
WCFS1 (pEH3A)	0.273 ± 0.015	4.21 ± 0.47	15.4
Lb790 (pEH3A)	0.130 ± 0.000	7.42 ± 1.06	57.1
WCFS1 (pEH9A)	0.057 ± 0.006	2.96 ± 1.22	51.9
Lb790 (pEH9A)	0.043 ± 0.006	4.32 ± 0.81	100
WCFS1 (pEH3P)	1.203 ± 0.049	49.1 ± 10.9	40.8
Lb790 (pEH3P)	0.267 ± 0.021	10.7 ± 4.4	40.1
WCFS1 (pEH9P)	0.360 ± 0.026	44.8 ± 1.3	124
Lb790 (pEH9P)	0.230 ± 0.017	18.5 ± 2.9	80.4
WCFS1 (pEH3S)	0.376 ± 0.065	4.87 ± 0.48	13.0
Lb790 (pEH3S)	0.060 ± 0.010	2.23 ± 0.22	37.2
WCFS1 (pEH9S)	0.320 ± 0.010	5.23 ± 0.39	16.3
Lb790 (pEH9S)	0.106 ± 0.006	6.91 ± 0.63	65.2

[a] Data given are the average and standard deviation of three experiments. The induction factor was calculated from the β-galactosidase activity obtained under induced conditions divided by the activity under noninduced conditions in cells harvested at $OD_{600} \approx 1.8$. The specific β-galactosidase activity in cell-free extracts of the nontransformed host strains was 0.07 U/mg.

using 4-methylumbelliferyl β-D-galactoside as the substrate showed activity for the band corresponding to a polypeptide the size of the larger subunit, while the smaller subunit did not show any activity (**Figure 5A**). This is in agreement with previous reports (*33, 34*), in which we also showed that activity staining of native β-galactosidase L103 and recombinant β-galactosidase *Ec*L103 expressed in *E. coli* after native PAGE yields two bands with β-galactosidase activity, one band of approximately 105 kDa, corresponding to the intact heterodimer, and a second band representing a degradation product containing components of both subunits as shown by using mass spectrometry (*33*). This observation was also made with recombinant β-galactosidase overexpressed in *L. plantarum* (**Figure 5B**).

Properties of Recombinant β-Galactosidase *Lp*L103. Enzymatic properties of purified *L. reuteri* β-galactosidase overexpressed in *L. plantarum* (*Lp*L103) were compared with those of β-galactosidase purified from *L. reuteri* (L103) (*33*) and purified recombinant β-galactosidase overexpressed in *E. coli*

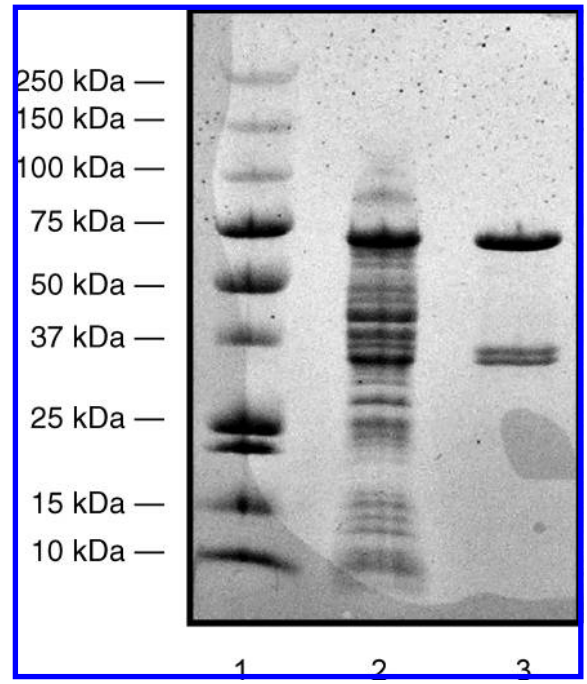

Figure 4. SDS-PAGE of β-galactosidase (*lacLM*) from *L. reuteri* L103 overexpressed in *L. plantarum* WCFS1. Lane 1, molecular weight markers (dual color, Biorad); lane 2, crude extract of *L. plantarum* cells after induction and cultivation in a bioreactor to an OD_{600} of approximately 6; lane 3, purified *Lp*L103 β-galactosidase. The double band for the small subunit apparently results from sample treatment.

(*Ec*L103) (*34*). Steady-state kinetic constants were determined for the hydrolysis of lactose, the natural substrate of this enzyme. The kinetic parameters for *Lp*L103 were the following: v_{max} = 43 (μmol D-glucose released per min and per mg protein) and K_m = 12 ± 2 mM. For native L103 and the recombinant *Ec*L103, the v_{max} values for lactose were 34 and 38 (μmol D-glucose released per min and per mg protein), and the K_m values were 13 ± 2 and 12 ± 2 mM, respectively (*33, 34*). Thus, the three enzyme preparations showed highly similar kinetic properties.

The optimum pH of *Lp*L103 was found to be pH 7.0 for *o*NPG hydrolysis and pH 6.5−7.5 for lactose hydrolysis. Again, this compares very well to the data obtained for both L103 and

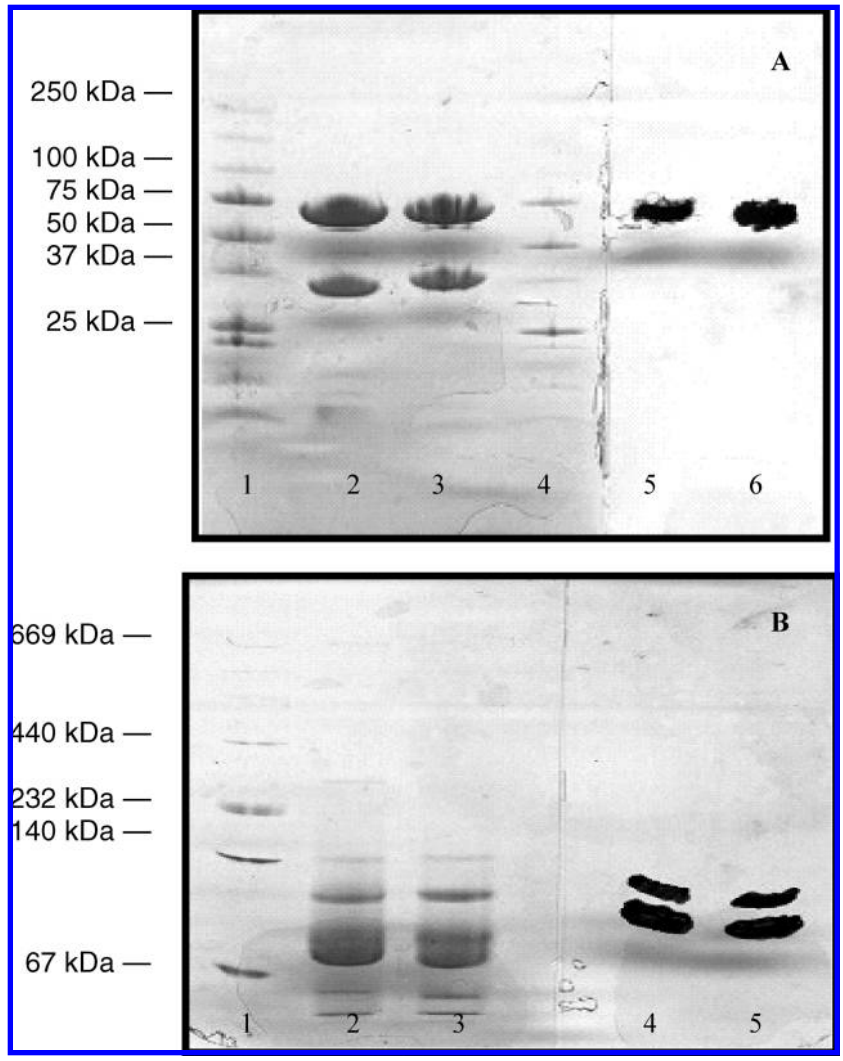

Figure 5. (**A**) SDS-PAGE of β-galactosidase (*lacLM*) from *L. reuteri* L103 overexpressed in *L. plantarum* WCFS1 and *E. coli* BL21 Star (DE3). Lane 1, molecular weight markers (dual color, Biorad); lane 2, Coomassie blue staining of purified *Lp*L103 β-galactosidase overexpressed in *L. plantarum*; lane 3, Coomassie blue staining of purified *Ec*L103 β-galactosidase overexpressed in *E. coli*; lane 4, molecular weight markers (unstained, Biorad). Lane 5 (*Ec*L103) and lane 6 (*Lp*L103) show a gel stained by activity with 4-methylumbelliferyl β-D-galactoside. (**B**) Native PAGE of purified β-galactosidase (*lacLM*) from *L. reuteri* L103 overexpressed in *L. plantarum* WCFS1 and *E. coli* BL21 Star (DE3). Lane 1, high molecular weight marker (Amersham); lanes 2 and 3, Coomassie blue staining of purified recombinant *Lp*L103 (lane 2) and *Ec*L103 β-galactosidases (lane 3); lanes 4 and 5, activity staining with 4-methylumbelliferyl β-D-galactoside of recombinant *Lp*L103 (lane 4) and *Ec*L103 (lane 5) β-galactosidases.

*Ec*L103 (*33, 34*). The optimum temperature for activity of *Lp*L103 was 50 °C when using *o*NPG as the substrate under standard assay conditions (pH 6.5 and 10 min), which is the same as for L103 and *Ec*L103 (*33, 34*). For lactose hydrolysis, the optimum temperature was found to be 55 °C. On the basis of these characterization studies, *L. reuteri* β-galactosidase overexpressed in *L. plantarum* has essentially identical properties as the wild-type enzyme.

DISCUSSION

Escherichia coli is, besides being the preferred host for gene cloning, widely used for recombinant protein production, as a large number of tools such as promoters, tags, fusion partners, and mutant expression strains are available. It is, however, not the best choice for every application (*2*). Gram-positive bacteria such as *Bacillus* spp. are preferred hosts for extracellular proteins, as they have natural high secretion capacity and export proteins directly into the growth medium rather than the periplasmic space (*46*). For food-related applications and the production of food-relevant enzymes expression systems based on food-grade microorganisms, which have the "generally recognized as safe" status, are of great interest (*47*). Lactic acid bacteria (LAB) are widely used in industrial fermentations, and therefore a wealth of information is available about their growth conditions and nutrient requirements. Furthermore, significant progress has been made with respect to the availability of genetic engineering tools and molecular characterization (*48, 49*) of these organisms. Therefore, LAB are highly attractive cell-factories for the production of recombinant enzymes but also as delivery vehicles for proteins such as antibodies or antigens (*50*). Several inducible and controlled expressions systems have been developed for LAB, of which the nisin-controlled gene expression system (NICE) derived from *Lactococcus lactis* is probably the best known (*17, 51*). In this paper we studied a related bacteriocin-based expression system for lactobacilli (*3, 20, 21*) using *L. plantarum* WCFS1 and *L. sakei* Lb790 as expression hosts. The background β-galactosidase activities of the chromosomal β-galactosidases of these two strains when grown on

glucose were found to be negligible, hence the activities reported in this paper can be considered as originating exclusively from homologous or heterologous expression of the introduced β-galactosidase genes. The β-galactosidase genes, originating from four *Lactobacillus* species, were inserted into two different expression vectors, pSIP403 and pSIP409, differing in the promoters P_{sppA} and P_{sppQ} (formerly P_{orfX}). No significant differences were found when comparing the levels of β-galactosidase activity obtained with these two promoters (**Figure 3**). This is in contrast to recent results, where the P_{sppQ} (P_{orfX}) promoter led to considerably higher levels of β-glucuronidase (GusA) from *E. coli* and aminopeptidase (PepN) from *L. lactis*. It has been noted previously that the performance of this expression system strongly depends on the origin of the gene that is being expressed and also on the host strain used (*3*).

In all cases, addition of the IP-673 peptide pheromone induced expression of the recombinant β-galactosidases (**Table 4**). Without induction, all constructs showed some basal formation of β-galactosidase, at levels that were always somewhat higher in *L. plantarum* than in *L. sakei*. This is in accordance with previous studies, which showed that the expression system is in general more tightly regulated in *L. sakei* than in *L. plantarum* (*3*). As a consequence, the *L. sakei* strains showed higher induction factors, ranging from 37 to 240, compared to values of 13 to 183 obtained for the *L. plantarum* strains (**Table 4**). The P_{sppQ} promoter seemed to be more tightly regulated than the P_{sppA} promoter as strains containing pSIP409-derived constructs consistently gave higher induction factors (**Table 4**). These high induction factors indicate that β-galactosidase expression is tightly controlled by the two promoters used.

Interestingly, the apparent production levels of the four β-galactosidase genes differed considerably, despite the fact that the genes are almost identical in size (**Table 1**), code for proteins with high sequence identities (58−74%),and have similar GC contents (36.8−47.2%; we observed no correlation between GC content and expression levels). The four heterodimeric β-galactosidases are very similar with respect to their activity (*32, 33*). Hence the observed differences in β-galactosidase activities are most probably the result of varying levels of protein. This is confirmed by the SDS-PAGE gels of **Figure 2**: although these are difficult to interpret in quantitative terms, they do show that intensity of the LacL bands correlates reasonably well with the activity levels reported in **Table 4**. This result adds to the results of other studies that show that effectiveness of expression systems for one particular gene of interest is difficult to predict. Apparently, effectiveness depends on subtle properties that are defined by the particular combination of the promoter, the gene of interest, nd the expression host. In the present case, differences in mRNA stability may be one factor determining effectiveness; protein folding efficiency and stability may be another factor. Remarkably, overexpression of the (homologous) *L. sakei lacLM* operon in *L. sakei* was among the least successful in this study.

The highest expression levels were found with *L. plantarum* WCFS1 harboring pSIP403 containing the *lacLM* gene from *L. reuteri*, and therefore this system was studied in more detail. A laboratory cultivation in a 1-L bioreactor yielded approximately 23 kU of β-galactosidase activity per liter. The β-galactosidase level amounted to approximately 55% of the total intracellular protein of *L. plantarum*, which is one of the highest expression levels ever obtained with gene expression systems in lactic acid bacteria (*3, 17, 20*). In terms of activity, the yield of 23 kU per liter is considerably (9-fold) higher than the maximal yield of 2.5 kU per liter of fermentation broth reported for the wild-type *L. reuteri* L103 strain (*33*). These results further illustrate the usefulness of the pSIP system for efficient protein production in lactic acid bacteria. By further optimizing the growth and induction conditions one can expect that these yields in recombinant protein production can still be increased.

Purification of the recombinant β-galactosidase was straightforward. Because of the high specific activity of the crude recombinant enzyme of 136 U/mg, one single step of purification using affinity chromatography on *p*-aminobenzyl 1-thio-β-D-galactopyranoside agarose was sufficient for the purification of the protein to apparent homogeneity. This single purification step of recombinant β-galactosidase *Lp*L103 is clearly more efficient than the three-step-purification protocol based on ammonium sulfate precipitation, hydrophobic interaction chromatography, and affinity chromatography that needed to be used for the wild-type β-galactosidase from *L. reuteri* L103. In the latter case, the specific activity in the starting material was only 9.6 U/mg (*33*).

Properties of the recombinant β-galactosidase produced in *L. plantarum* were found to be essentially the same as those of the enzyme purified from the wild-type strain or the recombinant enzyme produced in *E. coli*. Recently, we reported the application of β-galactosidase from *L. reuteri* for the production of galacto-oligosaccharides (GOS; *30, 52*). The spectrum and yield of GOS produced from lactose using the recombinant *Lp*L103 β-galactosidase were also similar to GOS formed by native β-galactosidase L103 (data not shown).

In conclusion, we showed the efficient and inducible expression of recombinant β-galactosidases in *L. plantarum* and *L. sakei* using expression vectors based on bacteriocin operons found in *L. sakei* strains. These vectors are simple to use and can easily be adapted for a particular purpose, such as production of food-grade enzymes, by exchanging one or more of the "cassettes" in the vector such as the promoter, the gene of interest, the replicon, or the selection marker. As lactobacilli are widely used in the food industry, these efficient and tightly regulated expression systems for the overproduction of enzymes and proteins in food-grade host strains can be of great interest.

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