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High-Level Expression of *Lactobacillus* β-Galactosidases in *Lactococcus lactis* Using the Food-Grade, Nisin-Controlled Expression System NICE

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In this work the overlapping genes (*lacL* and *lacM*) encoding heterodimeric β-galactosidases from *Lactobacillus reuteri*, *Lb. acidophilus*, *Lb. sakei*, and *Lb. plantarum* were cloned into two different nisin-controlled expression (NICE) vectors and expressed using *Lactococcus lactis* NZ9000 and NZ3900 as hosts. The *lacL* gene, encoding the large subunit of the β-galactosidases, was fused translationally downstream of the nisin-inducible promoter *nisA*. Chloramphenicol was employed as selection marker for the standard system using *L. lactis* NZ9000, whereas lactose utilization based on the complementation of the *lacF* gene was used as a dominant selection marker for the food-grade system employing *L. lactis* NZ3900. Comparison of the standard and the food-grade expression system, differing only in their selection markers, gave considerable differences in volumetric β-galactosidase activity, ranging from 1.17 to 14 kU/L of fermentation broth, depending on both the origin of the *lacLM* genes and the selection marker used. The occurrence of codons less frequently used by *L. lactis* especially at the beginning of the *lacL* gene could be an explanation for the significant differences between the expression levels of *lacLM* from different origins, while plasmid stability might cause the difference obtained when employing the different selection markers.

KEYWORDS: β-Galactosidase; inducible gene expression; nisin-controlled expression system; NICE; *Lactococcus lactis*; *Lactobacillus*; lactic acid bacteria; food grade

INTRODUCTION

The enzyme β-galactosidase occurs in a wide variety of organisms ranging from animals and plants to microorganisms, including fungi, yeast, and bacteria. The latter have been the best studied and most important enzymes for industrial applications. β-Galactosidases (EC 3.2.1.23) catalyze both the hydrolysis and transgalactosylation reactions of β-D-galactopyranosides: e.g., the milk sugar lactose. Enzymatic hydrolysis of the disaccharide lactose by microbial β-galactosidases has been described and characterized extensively in the literature (*1*). Hydrolysis of lactose to the monosaccharides glucose and galactose is of high interest with respect to nutrition and food technology, and some enzymes are commercially available and currently used in large-scale bioconversions (*2*). The formation of oligosaccharides through the transglycosylation reaction catalyzed by certain glycosidases has been known for several decades (*3*). While these reactions were first considered unwanted side reactions (*4*), they have now been exploited as interesting biocatalytic reactions of commercial importance, leading to the formation of oligosaccharides with prebiotic properties. Galacto-oligosaccharides as an economically important example of prebiotics are produced in Europe by Friesland Campina (Vivinal GOS), while the market is better established in Japan, with products such as galacto-, xylo-, and isomalto-oligosaccharides available (*5*, *6*).

At present, a relatively small number of fungal and bacterial hosts are used for recombinant enzyme production in food applications. *Aspergillus niger*, *A. oryzae*, *Bacillus subtilis*, and *B. licheniformis*, as well as various lactic acid bacteria (LAB), including lactobacilli or lactococci, are used for overproduction of food-relevant products (*7*). *Lactococcus lactis* is a homofermentative Gram-positive bacterium that has been used for thousands of years in the production of fermented milk products. Because of this long history of safe use, *L. lactis* carries the "generally recognized as safe" (GRAS) status. During the past two decades LAB, and in particular *L. lactis*, were of major interest as potential food-grade cell factories (*8*−*11*). Recent advances in the development of *L. lactis* compatible genetic engineering tools and expression plasmids have simplified the use of this alternative, safe, and "food-grade" host organism for recombinant protein production (*12*, *13*). In 1995 Kuipers et al. (*14*) published a study on the autoinduction of the expression of the bacteriocin nisin in lactococci. This was the starting point for one of the most widely used gene expression systems in

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Table 1. Strains and Plasmids

strain or plasmid	description	reference or source
Lactococcus lactis NZ9000	MG1363 *pepN*::*nisRK*; host strain for plasmids pTM50R/A/S/P; selection based on *Cm*^{r}	*44*
L. lactis NZ3900	NZ3000 derivative; Δ*lacF*, *pepN*::*nisRK*; host strain for food-grade use with plasmids pTM51R/A/S/P; selection based on the ability to grow on lactose (*lacF*)	*45*
E. coli TOP10		Invitrogen
Lactobacillus plantarum WCFS1		*46*
pNZ8150	*Cm*^{r}, P_{nisA}	*19*
pTM50R	*Cm*^{r}, pNZ8150 derivative containing *Lb. reuteri lacLM* genes downstream of P_{nisA}	this work
pTM50A	*Cm*^{r}, pNZ8150 derivative containing *Lb. acidophilus lacLM* genes downstream of P_{nisA}	this work
pTM50S	*Cm*^{r}, pNZ8150 derivative containing *Lb. sakei lacLM* genes downstream of P_{nisA}	this work
pTM50P	*Cm*^{r}, pNZ8150 derivative containing *Lb. plantarum lacLM* genes downstream of P_{nisA}	this work
pTM51R	l*acF*, pNZ8151 derivative containing *Lb. reuteri lacLM* genes downstream of P_{nisA}	this work
pTM51A	l*acF*, pNZ8151 derivative containing *Lb. acidophilus lacLM* genes downstream of P_{nisA}	this work
pTM51S	l*acF*, pNZ8151 derivative containing *Lb. sakei lacLM* genes downstream of P_{nisA}	this work
pTM51P	*lacF*, pNZ8151 derivative containing *Lb. plantarum lacLM* genes downstream of P_{nisA}	this work
pCR-Blunt	Kan^r, Zeo^r	Invitrogen
pTMlacF	Kan^r, Zeo^r, pCR-Blunt derivative containing *lacF* PCR product	this work
pNZ8149	*lacF*, P_{nisA}, pNZ8148 derivative for food grade expression	*19*
pNZ7565	*Cm*^{r}, *alr* gene downstream of P_{pepV}	Mierau (unpublished data)
pEH3R	Ery^r, pSIP403 derivative containing *Lb. reuteri lacLM* genes downstream of P_{sppA}	*24*
pEH3A	Ery^r, pSIP403 derivative containing *Lb. acidophilus lacLM* genes downstream of P_{sppA}	*24*
pEH3S	Ery^r, pSIP403 derivative containing *Lb. sakei lacLM* genes downstream of P_{sppA}	*24*
pEH3P	Ery^r, pSIP403 derivative containing *Lb. plantarum lacLM* genes downstream of P_{sppA}	*24*

Gram-positive bacteria, termed NICE (*ni*sin-*c*ontrolled gene *e*xpression). Nisin, an antimicrobial peptide with 34 amino acids, is used for the induction of a regulatory cascade starting with its binding to the membrane-bound histidine protein kinase (*NisK*), which upon binding autophosphorylates a conserved His residue in the cyctoplasmic domain of the *NisK* for its activation. In the next step the phosphate group from activated *NisK* is transferred to the intracellular response regulator (*NisR*), thereby activating this regulator. Activated *NisR* then induces the nisin operon at the nisin A promoter (P_{nisA}) controlling the expression of the genes involved in nisin biosynthesis or a gene of interest when using the NICE expression system. In this system, the food-grade compound nisin is an efficient inducer, which fully activates the tightly regulated promotor P_{nisA} already at nanogram per milliliter levels. This expression system has been used to produce homologous as well as heterologous proteins, among them intracellular, extracellular, and cell wall bound enzymes, to express toxic proteins, or bacterial and viral antigens (see ref *15* and references therein). Genes of closely related bacteria, including the genes encoding lipase of *Staphylococcus hycius* (*16*), NADH oxidase of *Streptococcus mutans* (*17*), or lysostaphin of *Staphylococcus simulans* biovar. Staphylolyticus (*18*), are typically expressed very efficiently when using this nisin-inducible system. However, the product and protein yields can vary considerably and hence are strongly case-dependent (*19*). Several studies reported the application of the NICE system: e.g., for the production of food-related peptidases and esterases used to affect flavor formation in dairy fermentations (*20*−*22*). This NICE system can be easily adopted to "food-grade" production of recombinant proteins by, for example, exchanging the cassettes for antibiotic resistance routinely used as selection markers by other selectable markers, such as the complementation of genes that have been deleted from the host strain and that are essential for growth under certain conditions. This approach ensures that neither an antibiotic resistance gene nor an antibiotic is present in the enzyme preparations obtained from these recombinant systems (*7*).

In the present study we describe the high-level production of heterodimeric β-galactosidases, derived from four different *Lactobacillus* species (*Lb. reuteri*, *Lb. acidophilus*, *Lb. sakei*, and *Lb. plantarum*) and encoded by the partially overlapping genes *lacL* and *lacM*, in *Lactococcus lactis* NZ9000 and NZ3900 using the NICE system. These heterodimeric β-galactosidases of the LacLM type are frequently found in various LAB. Typically, the large subunit LacL consists of ∼625−630 amino acid residues (molecular mass of roughly 73 kDa), while LacM is composed of ∼325 amino acid residues (molecular mass of approximately 35 kDa). In general, these sequences show high sequence similarity when compared to other lactobacillal β-galactosidases of the LacLM type (*40*). LacL carries the amino acids necessary for catalysis and forms the active site; very high conservation is found in the region of the 3′ end of the *lacL* genes of these β-galactosidases, while the region showing the lowest conservation in *lacL* is from nucleotide positions 240−300. Furthermore, the amino acid sequences deduced from *lacL* genes of various lactobacillal origins show significant similarity to the *lacZ* gene encoding β-galactosidase from *E. coli* (GenBank accession number V00296). Our main objective in this study was the expression of four different LacLM-type β-galactosidases in *L. lactis* and the comparison of the gene expression efficiencies using two different NICE expression plasmids and selection markers for a group of very similar and closely related proteins.

MATERIALS AND METHODS

Bacterial Strains, Media, and Plasmids. The bacterial strains and plasmids used in this study are given in **Table 1**. *E. coli* TOP10 (Invitrogen; Carlsbad, CA) was grown in Luria broth (*23*) at 37 °C with shaking, and *Lb. plantarum* WCFS1 harboring the plasmids pEH3R, pEH3A, pEH3S, and pEH3P was grown at 30 °C under anaerobic conditions in MRS medium containing 2% glucose and 5 μg/mL of erythromycin (*24*). *L. lactis* was grown statically in M17 medium (*25*) at 30 °C in tightly capped flasks supplemented with 0.5% glucose unless otherwise stated. The ability to ferment lactose was tested on indicator agar plates based on Elliker broth (*26*) supplemented with 0.5% lactose and 0.004% bromocresol purple. Histochemical screening for *lacLM*-positive clones was performed with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The following antibiotics were added when appropriate for the selection of plasmid-containing bacteria: 50 μg/mL of kanamycin and 5 μg/mL of chloramphenicol.

The construction of the plasmids pEH3R, pEH3A, pEH3S, and pEH3P containing the complete genes *lacLM* encoding heterodimeric

Table 2. Sequences of the Primers Used in This Study[a]

primer	sequence (5′ → 3′)	reference sequence GenBank accession no.
SIPReuF	ATGCAAGCAAATATAAAATGGCT	DQ493596
SIPReuR	CGATTCACTAGTTTATTTTGCATTCA	DQ493596
SIPAciF	ATGCAAGCAAACATAAAATGGCT	EF053367
SIPAciR	CGGGTAACTAGTTTAATTAAGATTG-AAAG	EF053367
SIPSakF	ATGCAACCTAATATTCAATGGTTAG	AL935262
SIPSakR	CGATTCACTAGTTTAAAACGAAATT	AL935262
SIPPlanF	ATGCAAGCTAATCTTCAATGG	X82287
SIPPlanR	CGATTCACTAGTTTAGAAATGAATA-TTAAAGCT	X82287
8149lacFF	GGTTCTTGATGCTGAAACGG	
8149lacFR	GTTATAAGACTAGAGATCTATCCGTCGT	

[a] Legend: F denotes forward primers; R denotes reverse primers; SIPReu stands for the *lacLM* genes (encoding heterodimeric β-galactosidase) from *Lactobacillus reuteri*, SIPAci for *lacLM* from *Lb. acidophilus*, SIPSak for *lacLM* from *Lb. sakei*, and SIPPlan for *lacLM* from *Lb. plantarum*. Restriction sites are underlined.

β-galactosidase from *Lb. reuteri*, *Lb. acidophilus*, *Lb. sakei*, and *Lb. plantarum*, respectively, was described previously (*24*). The expression vector pNZ8150 was prepared as described in Mierau et al. (*19*).

Molecular Cloning. *Construction of the Four "Standard Expression Systems".* The plasmids pEH3R, pEH3A, pEH3S, and pEH3P were used as templates for amplifying the *lacLM* genes from *Lb. reuteri*, *Lb. acidophilus*, *Lb. sakei*, and *Lb. plantarum*, respectively. The oligonucleotides SIPReuF, SIPReuR, SIPAciF, SIPAciR, SIPSakF, SIPSakR and SIPPlanF, SIPPlanR (**Table 2**) used for PCR amplification of the *Lb. reuteri lacLM*, *Lb. acidophilus lacLM*, *Lb. sakei lacLM*, and *Lb. plantarum lacLM* genes, respectively, were designed on the basis of the sequence published in GenBank (accession numbers DQ493596, EF053367, X82287, and AL935262, respectively). *Spe*I restriction sites were added at the 5′-ends of the reverse primers. All primers were purchased from Sigma (St. Louis, MO). DNA amplification was performed with KOD Hot Start DNA Polymerase (Novagen; Darmstadt, Germany) using a Biometra Mastercycler (Göttingen, Germany) and standard procedures. After the initial denaturation step (120 s at 95 °C) the genes of interest were amplified using 30 cycles of denaturation (30 s at 95 °C), annealing at 60 °C (*Lb. reuteri lacLM*), 56.4 °C (*Lb. acidophilus lacLM*), 55 °C (*Lb. sakei lacLM*), and 62 °C (*Lb. plantarum lacLM*) for 10 s, and extension at 70 °C for 70 s. The PCR products were digested with *Spe*I, and the resulting fragments encoding the β-galactosidases were translationally fused to the P_{nisA} promoter in pNZ8150 using the *Sca*I and *Spe*I site. This yielded pTM50R, pTM50A, pTM50S, and pTM50P. All plasmids were transformed into *Lactococcus lactis* NZ9000 following the protocol of Holo and Nes (*27*) and selected on M17 agar containing 0.5% glucose and 5 μg/mL of chloramphenicol.

Construction of the Four Food-Grade Expression Systems. For the construction of food-grade vectors—i.e., systems that do not rely on antibiotic resistance markers for selection—based on pTM50R, pTM50A, pTM50S, and pTM50P, the gene coding for chloramphenicol acetyltransferase (*cat*) was deleted by restriction digestion with *Sal*I and *Bgl*II and replaced with the *lacF* gene encoding the soluble carrier enzyme IIA^{Lac} (*13*). pNZ8149 (*19*) was used as a template to amplify this gene using KOD Hot Start DNA Polymerase (initial denaturation step of 120 s at 95 °C, 30 cycles of 20 s at 95 °C, 10 s at 59 °C, and 7 s at 70 °C) using the oligonucleotides 8149lacFF and 8149lacFR (**Table 2**). Each reaction contained 1 x buffer (Novagen), 1 U of KOD Hot Start DNA polymerase, 10 ng of plasmid DNA, 0.2 mM of each dNTP, 1.5 mM of $MgSO_4$, and 0.4 μM of each primer. The *Bgl*II restriction site was added at the 5′-end of the reverse primer 8149lacFR. The PCR product was subcloned into *E. coli* TOP10 cells using the pCR-Blunt vector (Invitrogen) according to the manufacturers instructions, resulting in the plasmid pTMlacF. The *cat* gene from the pTM50 series and the *lacF* gene were both excised using *Sal*I and *Bgl*II, and the resulting fragments (pTM50 with excised *cat* gene and the *lac F* gene) were ligated, yielding pTM51R, pTM51A, pTM51S, and pTM51P. These plasmids were transformed into *L. lactis* NZ3900, carrying the *lacF* deletion (*27*). Positive clones were selected on the basis of their ability to grow on Elliker agar supplemented with lactose.

The new plasmids were verified by restriction enzyme digestion, and the correct sequence of the insert was confirmed by DNA sequencing (BaseClear; Leiden, The Netherlands).

Expression of β-Galactosidase. For the heterologous expression of the four β-galactosidases, overnight cultures of *L. lactis* NZ9000 harboring the plasmids containing the *lacLM* genes were inoculated into 40 mL of fresh M17 medium containing 1% glucose and 5 μg/mL of chloramphenicol. The plasmid-carrying *L. lactis* NZ3900 strains were transferred into 40 mL of fresh M17 medium containing 1% lactose. The lactococcal strains were incubated at 30 °C without shaking until the optical density at 600 nm reached values between 0.8 and 0.9 (the starting OD_{600} value at the time point of inoculation was ∼0.14). At this point the culture was divided into two equal subsamples, and nisin was added to a final concentration of 10 ng/mL to one of these subsamples. The other sample was used as a noninduced control. Cells were harvested at an OD_{600} value of approximately 2.4 by centrifugation at 6200*g* for 10 min. The pellets were resuspended in 2 mL of sodium phosphate buffer (50 mM, pH 6.5), and crude extracts were prepared by disrupting 1 mL of the cell suspension by bead beating (FastprepTM FP120 beater; 4 times for 20 s each) using 1 g of silica sand. Cell debris was removed by centrifugation (16 200*g*, 10 min, 4 °C), and the cell-free crude enzyme extracts were used immediately for activity measurement and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme Assay. β-Galactosidase activity was determined using the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) as described previously (*28*). The reaction was started by adding 20 μL of crude enzyme extract to 480 μL of 22 μM *o*NPG in 50 mM sodium phosphate buffer (pH 6.5). The reaction was stopped by adding 750 μL of 0.4 M Na_2CO_3 after 10 min of incubation at 30 °C and shaking at 600 rpm, and the release of *o*-nitrophenol (*o*NP) was measured by determining the absorbance at 420 nm. One unit of *o*NPG activity is defined as the amount of enzyme releasing 1 μmol of *o*NP per minute under the assay conditions described above.

Protein Analysis and Gel Electrophoreses. Protein concentrations were determined by the method of Bradford (*29*) using bovine serum albumin as the standard. The level of heterologous expression of the different β-galactosidases was compared by SDS-PAGE using 12% polyacrylamide gels. Samples were treated as described by Laemmli (*30*), diluted to 1−2 mg of protein per mL, and aliquots of 15 μL were loaded per lane. Molecular masses were estimated using the Precision Plus Protein Unstained standard (BioRad). Protein bands were stained using Coomassie blue.

Codon Usage Analysis. For the calculation of codon usage preference of *L. lactis cremoris* MG1363 all proteinogenic genes from this strain were analyzed using data from the NCBI database (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Lactococcus_lactis_cremoris_MG1363/NC_009004.ffn). The usage frequency of the 61 codons was calculated on the basis of the entire *L. lactis* genome and was expressed as the relative frequency (in percent) one codon is used for the same amino acid. A codon usage between 10−20% for a particular codon is considered as "rather low" and below 10% as a "very low" preference. These data were then used to calculate the codon usage frequency of the four heterologously expressed β-galactosidases.

RESULTS

Construction of Standard and Food-Grade Expression Vectors. In this study the standard plasmid pNZ8150, which carries the gene *cat* for chloramphenicol acetyltransferase conferring resistance to this antibiotic (**Table 1**), was used for the expression of the *lacLM* genes encoding both the large and the small subunits of β-galactosidase from *Lb. reuteri*, *Lb. acidophilus*, *Lb. sakei*, and *Lb. plantarum*. The *lacL* gene was fused translationally to the nisin-inducible promotor P_{nisA}, resulting in the four expression plasmids pTM50R, pTM50A, pTM50S, and pTM50P (**Figure 1**; "R" indicates the *lacLM* genes from *Lb. reuteri*, "A" from *Lb. acidophilus*, "S" from *Lb. sakei*, and "P" from *Lb. plantarum*). The construction of these vectors was performed in *L. lactis* NZ9000 by selection for chloramphenicol-resistant clones. Subsequently, a replacement of the chloramphenicol acetyltransferase gene in these

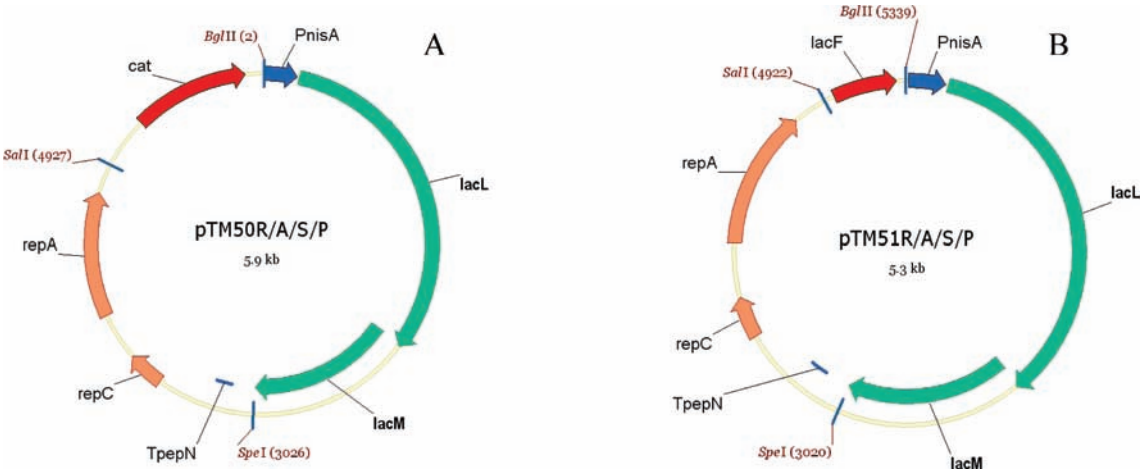

Figure 1. Vector map of pTM plasmids used in this study: *lacLM*, structural gene; *cat*, chloramphenicol resistance marker; *lacF*, *lacF* gene for selection on lactose; *repA* and *repC*, replication determinants; P_{nisA}, inducible promoter; T_{pepN}, transcriptional terminator.

Table 3. Volumetric and Specific β-Galactosidase Activities in Cell Free Extracts of Induced and Noninduced Cultures of *Lactococcus lactis* Containing Different Expression Plasmids[a]

	volumetric activity (kU/L of fermentation broth)		specific activity (U/mg)		
L. lactis (plasmid)	induced	uninduced	induced	uninduced	induction factor
NZ9000 (pTM50R)	5.94 ± 0.01	0.026 ± 0.002	77.8 ± 5.3	0.261 ± 0.021	298
NZ9000 (pTM50A)	9.64 ± 0.24	0.042 ± 0.007	103.7 ± 2.6	0.460 ± 0.082	225
NZ9000 (pTM50S)	1.39 ± 0.001	0.014 ± 0.001	17.87 ± 0.007	0.148 ± 0.006	121
NZ9000 (pTM50P)	5.12 ± 0.058	0.042 ± 0.008	58.0 ± 0.66	0.497 ± 0.093	117
NZ3900 (pTM51R)	8.42 ± 0.016	0.852 ± 0.003	67.5 ± 0.13	6.81 ± 0.025	9.9
NZ3900 (pTM51A)	13.96 ± 0.59	0.849 ± 0.006	118.0 ± 5.0	6.72 ± 0.05	18
NZ3900 (pTM51S)	1.17 ± 0.062	0.039 ± 0.002	10.33 ± 0.55	0.303 ± 0.012	34
NZ3900 (pTM51P)	7.49 ± 0.074	0.365 ± 0.014	81.1 ± 0.80	3.38 ± 0.13	24

[a] Data given are the mean together with the standard deviation calculated from two independent experiments. The induction factor is the quotient of specific β-galactosidase activity obtained under induced conditions (10 ng/mL of nisin) and the activity obtained under noninduced conditions. Cells were harvested 18 h after induction.

four plasmids with the promotorless lactococcal *lacF* gene, encoding the soluble carrier enzyme IIA^{Lac}, was carried out so that *lacF* was then under control of the *repC* promotor. This resulted in the expression vectors pTM51R, pTM51A, pTM51S, and pTM51P (**Figure 1**), which again harbor the four different *lacLM* genes. *L. lactis* NZ3900, carrying an in-frame deletion of the chromosomal *lacF* gene, was the host for these newly constructed vectors, which could easily be selected by complementation of this strain for growth on lactose. Chloramphenicol-resistant transformants (NZ9000(pTM50R/A/S/P)) as well as lactose-utilizing clones (NZ3900(pTM51R/A/S/P)) gave rise to blue colonies on plates containing X-Gal.

Expression of β-Galactosidase Genes. To study the expression of the different yet closely related lactobacillal β-galactosidase genes, gene expression was induced by the addition of nisin to a final concentration of 10 ng/mL when an optical density at 600 nm of the culture of 0.3−0.4 was reached (midlog growth phase). The induced cells were harvested after approximately 5 h (early stationary phase), which is known to give maximum expression levels in these systems (*15*, *31*, *32*) and additionally after 18 h (late stationary phase), Recombinant β-galactosidase formation was evaluated by both enzyme activity measurements and SDS-PAGE analysis of the crude cell extracts (**Table 3** and **Figures 2** and **3**). Five hours after induction the highest volumetric activities were obtained for NZ9000(pTM50R) and NZ3900-(pTM51R), ranging from 6.5 to 7.1 kU β-galactosidase activity per L fermentation broth (corresponding to 36−38 mg recombinant protein per L). At this time point the expression levels in NZ9000 and NZ3900 were more or less the same, and the three β-galactosidases from *Lb. reuteri*, *Lb. acidophilus*, and *Lb. plantarum* were formed at similar levels of approximately 2.7−7.1 kU/L. Interestingly, β-galactosidase from *Lb. sakei* was produced in roughly 6−9-fold lower amounts in both the NZ9000 and the NZ3900 expression system.

After 18 h of induction the *lacLM* genes from *Lb. acidophilus* were overexpressed to maximal values of 9.6 and 14 kU/L of fermentation broth using the NZ9000 and the NZ3900 expression hosts, respectively. From the first (5 h after induction) to the second (18 h after induction) measurement the *Lb. acidophilus* and *Lb. plantarum* β-galactosidase activities increased by factors of 1.9 and 2.2 for the expression system NZ9000 and NZ3900, respectively, whereas no further increase in β-galactosidase activity of *Lb. reuteri* was measured in both systems. In general, higher β-galactosidase levels were found when using the NZ3900-(pTM51R/A/S/P) system obtaining up to 70 mg recombinant protein per L fermentation broth. Again, three out of four β-galactosidases (i.e., those from *Lb. reuteri*, *Lb. acidophilus*, and *Lb. plantarum*) were overexpressed in high amounts, regardless of the expression system used, as is evident from additional protein bands in the SDS-PAGE gel at a molecular mass of 73 kDa, corresponding to the expected size of the large β-galactosidase subunit LacL (**Figure 2**), as well as from enzyme activity data (**Table 3**).

Noninduced cells of *L. lactis* NZ9000 and NZ3900 harboring the expression plasmids were also grown and harvested as above to check for basal expression ("leakage") from the promoter in the absence of the inducer nisin and to calculate the degree of induction: i.e., the induction factor (**Table 3**). Noninduced

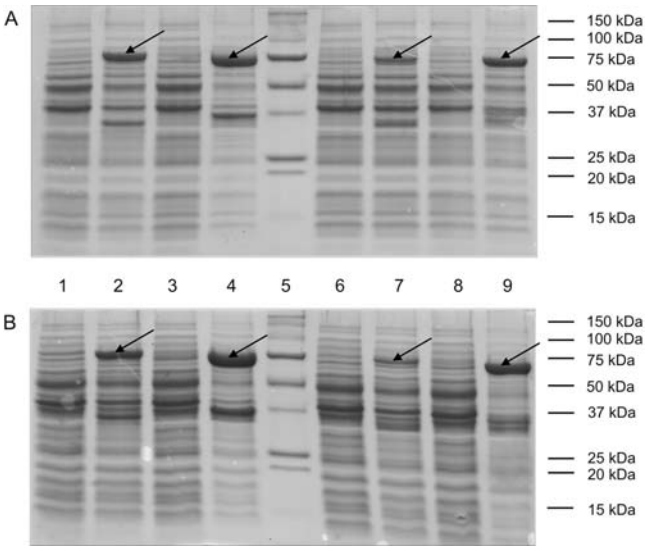

Figure 2. SDS-PAGE of whole-cell lysates of *L. lactis* NZ9000 (**A**) and NZ3900 (**B**) carrying plasmids containing the *lacLM* genes from *Lb. reuteri*, *Lb. acidophilus*, *Lb. sakei*, and *Lb. plantarum*. Panel **A**: Precision Plus Protein Unstained standard (BioRad) (lane 5); NZ9000 carrying pTM50R (lane 1), pTM50A (lane 3), pTM50S (lane 6), and pTM50P (lane 8) without induction; induced NZ9000 carrying pTM50R (lane 2), pTM50A (lane 4), pTM50S (lane 7) and pTM50P (lane 9). Panel **B**: Precision Plus Protein Unstained standard (lane 5); NZ3900 carrying pTM50R (lane 1), pTM50A (lane 3), pTM50S (lane 6) and pTM50P (lane 8) without induction; induced NZ3900 carrying pTM50R (lane 2), pTM50A (lane 4), pTM50S (lane 7) and pTM50P (lane 9). Arrows indicate additional bands in induced cell extracts representing the large β-galactosidase subunit LacL.

NZ9000 transformants showed very low basal activities with specific activities of less than 0.49 U of β-galactosidase activity per milligram of protein, which is negligible compared to the specific activities produced upon nisin induction (10.3−118.0 U/mg). As a result, induction factors of up to 298 were obtained. When using the NZ3900 host for expression of the *lacLM* genes, the basal activities were considerably higher, with values of up to 6.8 U/mg, resulting in significantly lower induction factors of up to 34 (**Table 3**).

Codon Usage Analysis. It is well-documented that low efficiencies of translation are often caused by an accumulation of rare codons in heterologous gene expression (*33*, *34*). Adapting the codons of the gene of interest to the most frequently used codons occurring in the genes of the host strain can improve expression yields significantly (*35*). In accordance, unfavorable codon usage similarity could be an explanation for the low expression of the *lacLM* genes of *Lb. sakei* in *L. lactis*. An analysis of the codon usage showed that 14 out of the first 50 codons of the *Lb. sakei lacLM* gene are in fact of "low" and "very low" usage frequency as defined in Materials and Methods, while these corresponding figures range from 5 to 8 out of the first 50 codons for the other three *lacLM* genes (*Lb. reuteri*, *Lb. acidophilus*, and *Lb. plantarum*) that expressed well in *L. lactis*. **Figure 4** shows a more detailed analysis of the codon usage, giving the number of codons that fall into the "low" and "very low" usage frequency category per 50 codons over the entire lactobacillal *lacLM* genes. It is obvious that a high frequency of unfavorable codons occurs between positions 650 and 700, which corresponds to the start of the small subunit, as well as for most parts of the small subunit (700−950). When the percentage of "low" and "very low" codon usage was calculated over the entire *lacLM* genes, the lowest frequencies of these unpreferred codons were calculated for *Lb. acidophilus* (18.9%), followed by *Lb. reuteri* (25.0%). *Lb. sakei*

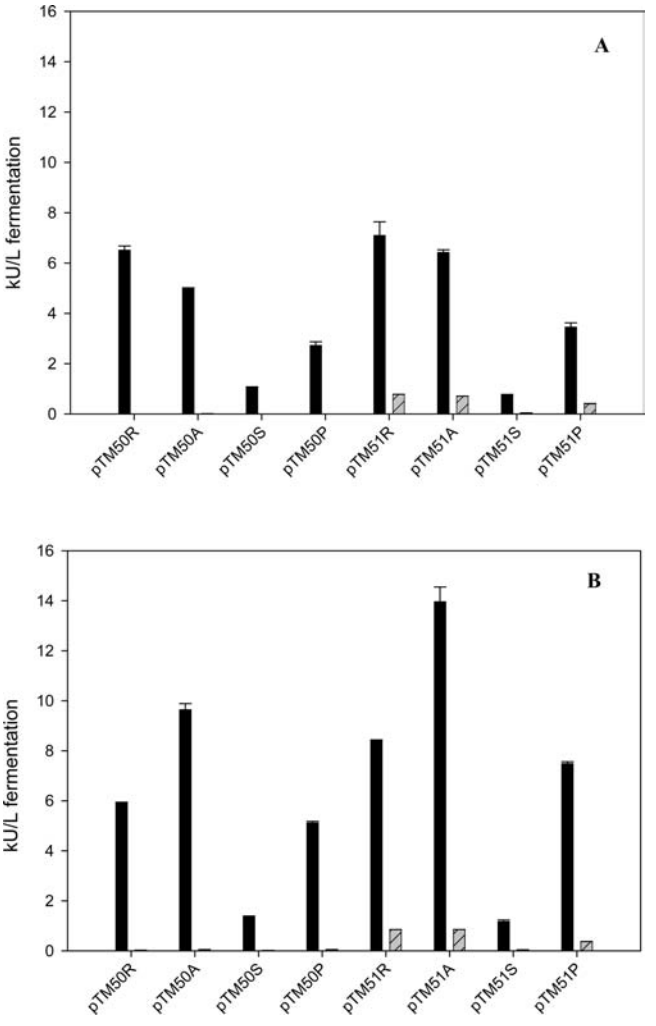

Figure 3. Volumetric β-galactosidase activity (kU/L of fermentation broth) in cell-free extracts of induced (dark bars) and noninduced (light bars) NZ9000 harboring plasmids pTM50R/A/S/P and NZ3900 harboring pTM51R/A/S/P for the expression of β-galactosidase from *Lactobacillus reuteri* (R), *Lb. acidophilus* (A), *Lb. sakei* (S), and *Lb. plantarum* (P). Cells were harvested 5 (**A**) and 18 h (**B**) after induction. All data are mean values of two independent experiments; error bars indicate standard deviation.

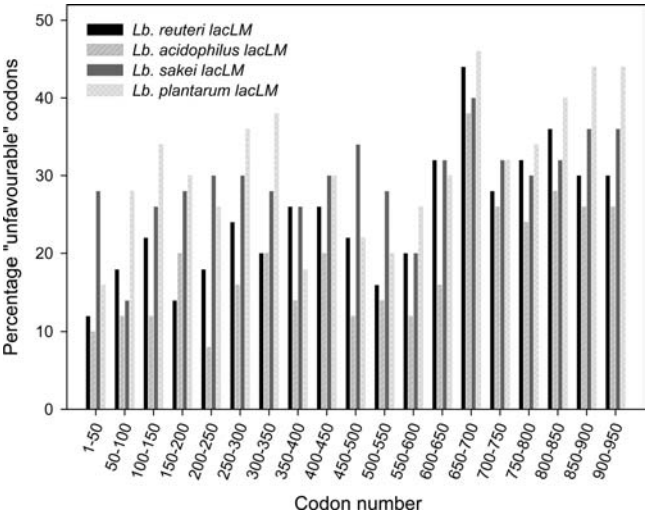

Figure 4. Percentage of "unfavorable" codons per 50 base triplets calculated over the entire *lacLM* genes from *Lactobacillus reuteri*, *Lb. acidophilus*, *Lb. sakei*, and *Lb. plantarum*.

Table 4. Homologous and Heterologous Overexpression of Various Microbial β-Galactosidases

enzyme source	expression host	reference
Bacillus megaterium ATCC 14581	*E. coli*	*47*
Bifidobacterium infantis	*E. coli*	*48*, *49*
Kluyveromyces lactis	*E. coli*	*50*
Lactobacillus bulgaricus	*E. coli*	*51*
Lactobacillus casei	*L. lactis* CNRZ 1123	*52*
Lactobacillus plantarum	*L. lactis*, *E. coli*	*53*
Lactobacillus reuteri	*E. coli*	*40*
Lactobacillus sakei	*E. coli*	*54*
Lb. reuteri, *Lb. acidophilus*, *Lb. sakei*, *Lb. plantarum*	*Lb. plantarum*, *Lb. sakei*	*24*
Leuconostoc lactis	*E. coli*	*55*
Pseudoalteromonas sp. 22b	*E. coli*	*56*
Rhizobium meliloti	*E. coli*	*57*
Streptococcus thermophilus	*E. coli*	*58*
Thermus sp.	*E. coli*	*59*

and *Lb. plantarum* gave nearly the same values of 29.5% and 30.8%, respectively.

DISCUSSION

Gram-positive bacteria have been receiving increased attention for recombinant protein production as an alternative to the dominant organism in this field, *Escherichia coli*, since this organism is not the best choice for every application. Various *Bacillus* spp. are preferred hosts for extracellular proteins, on the basis of their natural high secretion capacity, and they can be used for food-related applications or for the production of food-relevant enzymes. For these latter applications, expression systems based on food-grade microorganisms having the "generally recognized as safe" status are of considerable interest. Lactic acid bacteria (LAB) are widely used in industrial fermentations, and hence abundant information is available about nutrient requirements, cultivation conditions, etc. In addition, significant progress has been made pertaining to the development of novel genetic engineering tools and the characterization of molecular pathways in LAB (*12*, *19*, *36*). Because of this, LAB are becoming very attractive microbial cell factories for engineering of novel pathways or the production of recombinant proteins such as enzymes (*37*). Several inducible and controlled expression systems for LAB have been developed, of which the nisin-controlled gene expression system NICE in *L. lactis* is probably the best known and studied (*20*−*22*) and for which a wide variety of tools, including expression hosts and vectors, are already available. As some of these tools, food-grade expression approaches were developed with molecular working techniques, modification tools, and genetic elements derived from self-cloning. Unwanted selection markers, such as antibiotic resistance genes, which might be transferred to other microorganisms when the producing organism remains in the final product, the use of antibiotics in fermentations, and the production of harmful compounds should also be avoided in these food-grade approaches. Finally, food-grade expression systems require stable processes in large industrial-scale applications (*38*).

In this paper we describe the expression of four closely related β-galactosidase genes derived from different *Lactobacillus* species in *L. lactis,* using two different NICE systems and comparing their respective expression characteristics. To this end, the overlapping *lacLM* genes encoding the large and small β-galactosidase subunits were cloned downstream of the strong inducible promoter *nisA,* and subinhibitory amounts of nisin (10 ng/mL) were used for the induction of recombinant enzyme production. A number of previous studies described the overproduction of β-galactosidases using different expression systems and host organisms (summarized in **Table 4**). In most of these studies, however, *E. coli* was used as expression host, and they focused on cloning and characterization of the recombinant β-galactosidases, while the establishment of a food-grade expression system for the efficient overproduction of β-galactosidase was not the main aim. To the best of our knowledge, only one publication can be seen as a direct step toward food-grade overproduction of this enzyme, describing the expression of different β-galactosidases in *Lactobacillus* species (*24*). Halbmayr and co-workers could inducibly overexpress β-galactosidases in *Lb. plantarum* and *Lb. sakei,* using a bacteriocin-based system that is related to NICE. However, selection of positive constructs was still based on antibiotic (erythromycin) resistance in that study (*24*).

The present *L. lactis* based overexpression of β-galactosidases relates to a certain extent to the study carried out by Halbmayr et al. mentioned above, as we were using the same four lactobacillal genes for heterologous expression in *L. lactis*. Interestingly, considerably varying production levels of the respective β-galactosidases were obtained when comparing both expression systems studied here—NZ9000(pTM50R/A/S/P) and NZ3900(pTM51R/A/S/P)—even though they only differed in the selection marker. With the exception of the *lacLM* gene from *Lb. sakei,* the expression host NZ3900 harboring the expression plasmids pTM51R/A/P was able to produce higher β-galactosidase activities compared to NZ9000 after 5 h (**Figure 3A**) and 18 h of induction (**Figure 3B**, **Table 3**). The expression system NZ9000 together with the pTM50 series of plasmids derived from pNZ8150 is based on antibiotic resistance as a selection marker. Here, the *cat* gene encoding chloramphenicol acetyltransferase is part of the expression plasmid. This enzyme inactivates the antibiotic through covalent modification; hence, the effective concentration of chloramphenicol is decreasing during the cultivation and especially after a prolonged incubation this could result in exhaustion of the antibiotic in its active form. However, the presence of chloramphenicol is important to maintain plasmid stability in the cells. When working with closely related LAB expression systems, we observed that in the absence of a suitable selection pressure, these expression plasmids are rapidly lost (unpublished results). In contrast, such a loss in selection pressure is not expected to occur with the second system that is based on complementation of the carrier protein IIA^{Lac}. Hence, a possible explanation for the difference between the two expression systems differing only with respect to the selection marker could be plasmid stability. This effect of the selection marker used was more pronounced when comparing the activity data obtained 21 h after induction with those after 5 h, which is in agreement with this explanation.

In all cases studied, addition of nisin induced the expression of the recombinant β-galactosidases, i.e., expression levels were significantly higher than under noninduced conditions. The basal

Table 5. Amino Acid Comparison of the Large and Small Subunits of β-Galactosidase from *Lactobacillus reuteri* (β-galReu), *Lb. acidophilus* (β-galAci), *Lb. sakei* (β-galSak), and *Lb. plantarum* (β-galPlan)

	β-galReu	β-galAci	β-galSak	β-galPlan	GenBank accession no.
β-galReu	100[a]/100[b]	75[a]/72[b]	58[a]/56[b]	65[a]/68[b]	DQ493596
β-galAci		100[a]/100[b]	59[a]/57[b]	64[a]/64[b]	EF053367
β-galSak			100[a]/100[b]	59[a]/58[b]	AL935262
β-galPlan				100[a]/100[b]	X82287

[a] Percentage of amino acid identity for the large subunit. [b] Percentage of amino acid identity for the small subunit.

formation of β-galactosidase by noninduced transformants was considerably higher in the NZ3900 constructs. Thus, these show consistently lower induction factors ranging from only 9.9 to 34 as compared to values of 116 to 298 calculated for the NZ9000 strains. The low induction factors and higher basal formation of recombinant proteins for NZ3900 might be explained by nonspecific induction of the *nisA* promoter that was used in both expression systems. Chandrapaty and Sullivan (*39*) reported on the nisin-independent induction of the *nisA* promoter using galactose and lactose. To test this hypothesis, a simple experiment was carried out by adding either lactose or galactose at two different concentrations (10 and 60 g/L) to the NZ9000 clones (grown on media containing glucose) in the absence of nisin. In contrast to the aforementioned study, however, no increased β-galactosidase activity than the very low basal expression levels was measured (data not shown). Hence, the reason for these different basal expression levels is yet unknown.

The four heterodimeric β-galactosidases studied here are encoded by two partially overlapping genes, *lacL* and *lacM*. As we also found for other bacterial hosts (*24*, *40*), recombinant expression of these overlapping genes to functional, active β-galactosidases was not a problem in *L. lactis,* while various *Pichia pastoris* constructs did not result in the expression of active β-galactosidase (unpublished results). Since the four *lacLM* genes are almost identical in size (nucleotide lengths for the *lacL* and *lacM* genes are 1887 + 951 for *Lb. acidophilus*, 1881 + 960 for *Lb. plantarum*, 1887 + 960 for *Lb. reuteri,* and 1878 + 993 for *Lb. sakei*), code for proteins with high sequence similarities (**Table 5**), and were isolated from closely related Gram-positive organisms we also expected comparable expression levels for these different systems. Yet, the apparent production levels of the four β-galactosidase genes differed considerably with lowest values obtained for LacLM from *Lb. sakei*, regardless of the expression system used. The four heterodimeric β-galactosidases are very similar with respect to their activity (*28*, *41*); hence, the differences in levels of β-galactosidase activity obtained (**Table 3**) most probably result from different levels of recombinant protein. This is also corroborated by SDS-PAGE (**Figure 2**), with the intensity of the band of the larger subunit corresponding reasonably well with the activity levels shown in **Table 3**. Interestingly, expression of the *Lb. sakei lacLM* gene was also poor in *Lb. plantarum* and even in *Lb. sakei* (*24*).

A possible explanation for the low expression of the *lacLM* genes from *Lb. sakei* could be the high frequency of "unfavorable" codons occurring especially within the first 50 base triplets of this gene. The frequency of codons designated as "low" and "very low" preference is approximately 2−3 times higher within the first 50 codons for *Lb. sakei lacLM* as compared to the other *lacLM* genes (14 versus 5−8). Interestingly, the overall frequencies of "low" and "very low" preference codons in the complete *lacLM* genes of *Lb. sakei* and *Lb. plantarum* are almost identical (29.5 and 30.8% of all codons belonging to "low" and "very low" preference, respectively). Despite this similarity, expression of the latter enzyme is approximately 10-fold higher, as judged from the activity levels obtained. Thus it seems that especially the first codons are important for efficient translation of a recombinant protein. Codon optimization (*42*, *43*) or supplementation of rare tRNAs (*31*) might therefore help to improve expression yields significantly. Furthermore, it is evident from **Figure 4** that less frequently used codons are accumulated around the start of the *lacM* gene for all four β-galactosidases studied here (codons 650−700). Apparently, this does not hamper the efficient expression of these heterodimeric enzymes.

The highest levels of enzyme activity and recombinant protein obtained in these *L. lactis* expression systems are approximately 9.6−14 kU of *Lb. acidophilus* β-galactosidase activity with a specific activity of 103−118 U/mg depending on the system used. This corresponds to 50−70 mg of recombinant protein produced per liter of fermentation medium for the highest yields, with roughly 50−60% of the total soluble intracellular protein being active recombinant β-galactosidase, which is one of the highest expression levels obtained with gene expression systems in lactic acid bacteria (*19*).

The heterologous overexpression of the *Lb. reuteri* β-galactosidase genes using the expression host *L. lactis* NZ3900 resulted in a volumetric activity of 8.4 kU/L of fermentation medium with a specific activity of 67.5 U/mg of protein. This is significantly higher than the maximum yield of 2.5 kU/L obtained with the wild-type strain of *Lb. reuteri* (*28*). When expressing the identical *lacLM* gene of *Lb. reuteri* in *E. coli* BL21 Star (DE3), using a pET21d-based expression plasmid and 0.1 mM IPTG as inducer, we obtained approximately 110 kU/L β-galactosidase activity with a specific activity of ∼55 U/mg (*24*, *40*). However, 100 μg/mL of ampicillin had to be used in these cultivations as selection marker. Expression of the *Lb. reuteri lacLM* gene in *Lb. plantarum* WCFS1 under comparable cultivation conditions (50 mL flask cultivations) using a pSIP403-based expression plasmid resulted in 3.7 kU/L β-galactosidase activity (specific activity of 61 U/mg), yet again an antibiotic (5 μg/mL erythromycin) had to be added to maintain selection pressure (*24*). Hence, the productivities obtained in the food-grade system that does not rely on an antibiotic for a selection pressure compare well to other microbial expression systems. It should be pointed out that these results on the expression in *L. lactis* were carried out under nonoptimized conditions using simple flasks for the cultivation. Fermentation of this strain under controlled conditions (e.g., pH control) as in a bioreactor might increase the productivities that can be attained by these different systems further.

To the best of our knowledge, we presented the first "true" heterologous food-grade overexpression of the enzyme β-galactosidase in prokaryotic microbial cell factories. This was also the first study comparing two different NICE systems using four highly related enzymes as target genes with respect to expression levels of recombinant enzymes. We could show that not only the origin of the gene of interest but also the selection marker had considerable effects on the expression characteristics. Due to the easy exchange of the "cassettes" (target gene, promoter, selection marker) these vectors are simple to use, but it is still very hard to predict the expression properties, when parts of the vector are exchanged. Finally, after a carefully designed optimization procedure is performed concerning the induction and fermentation conditions, the food-grade overproduction of β-galactosidases in *L. lactis* may find application in industrial fermentation processes, enabling the cost-efficient production of β-galactosidases that show some favorable properties compared to the microbial enzymes currently available on the market: e.g., significantly lower K_m values and increased thermostability as compared to

the *Kluyveromyces* or *Aspergillus* enzymes dominating in commercial applications (*28*, *41*). The use of a GRAS expression host together with a food-grade expression system will make possible the use of these enzymes that are derived from various probiotic and therefore safe strains in various applications: e.g., the production of lactose-free products or novel prebiotic galactooligosaccharides (*60*), to name a few.

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