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Identification and Cloning of *gusA*, Encoding a New β-Glucuronidase from *Lactobacillus gasseri* ADH†

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The *gusA* gene, encoding a new β-glucuronidase enzyme, has been cloned from *Lactobacillus gasseri* ADH. This is the first report of a β-glucuronidase gene cloned from a bacterial source other than *Escherichia coli*. A plasmid library of *L. gasseri* chromosomal DNA was screened for complementation of an *E. coli gus* mutant. Two overlapping clones that restored β-glucuronidase activity in the mutant strain were sequenced and revealed three complete and two partial open reading frames. The largest open reading frame, spanning 1,797 bp, encodes a 597-amino-acid protein that shows 39% identity to β-glucuronidase (GusA) of *E. coli* K-12 (EC 3.2.1.31). The other two complete open reading frames, which are arranged to be separately transcribed, encode a putative bile salt hydrolase and a putative protein of unknown function with similarities to MerR-type regulatory proteins. Overexpression of GusA was achieved in a β-glucuronidase-negative *L. gasseri* strain by expressing the *gusA* gene, subcloned onto a low-copy-number shuttle vector, from the strong *Lactobacillus* P6 promoter. GusA was also expressed in *E. coli* from a pET expression system. Preliminary characterization of the GusA protein from crude cell extracts revealed that the enzyme was active across an acidic pH range and a broad temperature range. An analysis of other lactobacilli identified β-glucuronidase activity and *gusA* homologs in other *L. gasseri* isolates but not in other *Lactobacillus* species tested.

The action of bacterial β-glucuronidase in the gastrointestinal (GI) tract is an important component in the enterohepatic circulation of many hydrophobic xenobiotics and endogenous waste compounds. The human body detoxifies a variety of these compounds via conjugation to D-glucuronic acid, rendering them more water soluble and subject to excretion in the bile or urine (18, 22). As long as these compounds remain conjugated, they are poorly reabsorbed into the bloodstream and are efficiently eliminated from the body. However, reduction of these β-D-glucuronides in the GI tract by bacterial β-glucuronidase activity frees the aglycone components, allowing them to reenter the bloodstream. This cycling of compounds means that rather than being eliminated from the body all at once, many physiologically important compounds, including endogenous steroid hormones and exogenously acquired xenobiotics, are maintained in the body for longer periods of time. Additionally, bacterial β-glucuronidase activity has been implicated in the generation of toxic and carcinogenic metabolites which may be important precursors to tumor initiation and large bowel cancer in the gut (6, 36).

Bacterial β-glucuronidase activity has been considered for many years to be almost unique to *Escherichia coli* and closely related *Enterobacteriaceae* (53). However, evidence has slowly been accumulating to indicate that β-glucuronidase activity can also be found in a limited number of other bacteria, particularly gram-positive inhabitants of the GI tract (3, 4, 23, 36). Despite reports of enzyme activity associated with other bacteria and the physiological importance of β-glucuronidase to human health, only the genetic elements for the *E. coli* enzyme have been identified and studied (26, 53).

Lactobacillus gasseri ADH is a human intestinal isolate that was identified by its ability to adhere to intestinal epithelial cells (30). *L. gasseri* is one of a number of indigenous lactobacilli that are commonly associated with the microflora of a healthy human GI tract (38, 45). A number of these lactobacilli are currently under investigation to determine the mechanistic basis of a variety of proposed probiotic activities (29). It remains an important objective to characterize the physiological and enzymatic activities of this group of organisms and ultimately to identify the genetic factors responsible for those activities. Studies with various *Lactobacillus* species, including *L. gasseri*, have consistently shown their ability to reduce the amount of fecal β-glucuronidase activity and lower the occurrence of cancer indicators present in the GI tract (15, 27, 33, 37, 41). The mechanisms by which lactobacilli lower the amount of β-glucuronidase activity in the gut remain unknown but may be the reflection of a variety of activities including, but not limited to, the exclusion or antagonism of typically β-glucuronidase-positive enterobacteria. Because lactobacilli colonize the proximal region of the small intestine, it is reasonable to expect them to be frequently exposed to β-D-glucuronides excreted via bile into the GI tract. Indeed, their frequent exposure to bile is reflected in the common occurrence of conjugated bile acid hydrolysis among different species (12, 19). Lactobacilli themselves have not traditionally been associated with β-glucuronidase activity, however, and there have been, to date, only two reports of β-glucuronidase-like activity in lactobacilli (37, 42). It has been unclear, however, whether this β-glucuronidase activity was the result of a true β-glucuronidase enzyme or reflected the activity of some other enzyme. It

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid[a]	Origin or relevant characteristics	Source and/or reference
Bacterial strains		
L. acidophilus ATCC 4356	Type strain; human pharynx	ATCC[b]
L. acidophilus NCFM	Human intestinal isolate	7
L. casei ATCC 393	Type strain; cheese isolate	ATCC
L. delbrueckii subsp. *bulgaricus* 1489	Cheese isolate	55
L. gasseri ADH	Human intestinal isolate	30 (called strain MS02)
L. gasseri ATCC 33323	Type strain; human isolate	ATCC
L. gasseri NCK 1338	Human endoscopy isolate	32
L. gasseri NCK 1340	Human endoscopy isolate	32
L. gasseri NCK 1341	Human fecal isolate	32
L. gasseri NCK 1342	Human endoscopy isolate	32
L. gasseri NCK 1343	Human fecal isolate	32
L. gasseri NCK 1344	Human endoscopy isolate	32
L. gasseri NCK 1345	Human endoscopy isolate	32
L. gasseri NCK 1346	Human endoscopy isolate	32
L. gasseri NCK 1347	Human endoscopy isolate	32
L. gasseri NCK 1348	Human fecal isolate	32
L. gasseri NCK 1349	Human fecal isolate	32
L. helveticus CNRZ 32	Cheese isolate	Steele[c] (8)
L. johnsonii VPI 11088	ATCC 11506; origin unknown	VPI[d]
L. plantarum 299v	Human intestinal isolate	1
L. reuteri DSM 20016	ATCC 53609, type strain; human fecal isolate	ATCC
L. rhamnosus GG	ATCC 53103; human fecal isolate	ATCC
E. coli DH10B	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*Δ*M15* Δ*lacX74 endA1 recA1 deoR*	Gibco-BRL[e]
E. coli DH5α	F^- ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*) *U169 endA1 recA1 hsdR17* ($r_K^- m_K^+$) *deoR*	Gibco-BRL
E. coli KW1	Δ(*add-gus-man*) *hsdR hsdM*$^+$ *metB strA purB*	54
E. coli Tuner(DE3)	F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm lacY1* (DE3)	Novagen[f]
Plasmids		
pET28a(+)	Kan^r; *E. coli* expression vector	Novagen
pTRK665	pET28a(+) with cloned *gusA* gene	This study
pUC18	Ap^r; cloning vector	Amersham[g]
pTRK666	pUC18 with 4.5-kb *L. gasseri* ADH chromosomal insert; GusA$^+$	This study
pTRK667	pUC18 with 4.9-kb *L. gasseri* ADH chromosomal insert; GusA$^+$	This study
pGK12	ori (pWV01), Em^r, Cm^r; gram-positive shuttle vector	31
pTRK563	Em^r; Δ*cat*-derivative of pGK12 with *lacZ* from pBluescript II KS(+)	This study
pTRK668	pTRK563 with 4.5-kb *Eco*RI-*Pst*I insert from pTRK666; GusA$^+$	This study
pTRK664	pTRK563 with T7 terminator-P6 promoter-*gusA* gene; GusA$^+$	This study

[a] Intermediate plasmids not shown.
[b] American Type Culture Collection.
[c] James Steele, Dept. of Food Science, University of Wisconsin—Madison.
[d] Virginia Polytechnic Institute.
[e] Gibco-BRL, Grand Island, N.Y.
[f] Novagen, Inc., Madison, Wis.
[g] Amersham Pharmacia Biotech, Piscataway, N.J.

was the objective of this study to identify genetic determinants for β-glucuronidase-like activity in lactobacilli.

This study describes the identification and cloning of a β-glucuronidase gene, *gusA*, from *L. gasseri* ADH. The nucleotide and deduced amino acid sequences of the *gusA* gene and surrounding DNA are described. Additionally, GusA was expressed from the cloned *gusA* gene by a constitutive promoter in *L. gasseri* ATCC 33323 and by a regulated promoter in *E. coli*. Initial characterization of GusA enzyme activity is also reported.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Lactobacilli were propagated statically at 37°C in MRS (Difco, Detroit, Mich.) or on MRS supplemented with 1.5% agar. When appropriate, erythromycin (ERY) was added at a concentration of 5 μg/ml, and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-glu) (Gold Biotechnologies, St. Louis, Mo.) was added at a concentration of 100 or 200 μg/ml. For induction studies with X-glu and methyl-β-D-glucuronide, *L. gasseri* ADH cells were also grown in modified MRS (per liter: 10.0 g of BactoPeptone, 5.0 g of yeast extract, 2.0 g of K_2HPO_4, 5.0 g of sodium acetate, 2.0 g of sodium citrate, 0.2 g of $MgSO_4$, 0.05 g of $MnSO_4$, 1.0 g of Tween 80 [pH 6.5]) supplemented with 2.0% of the appropriate sugar.

E. coli cells were propagated at 37°C in Luria-Bertani (LB) broth with shaking or on LB broth supplemented with 1.5% agar. When appropriate, ampicillin was added at a concentration of 200 μg/ml, carbenicillin was added at 200 μg/ml, kanamycin was added at 40 μg/ml, and X-glu was added at 50 μg/ml. Carbenicillin was used during the initial genomic library construction and screening to reduce the growth of satellite colonies; thereafter, ampicillin was used to maintain selection for clones. ERY-resistant *E. coli* clones were selected on brain heart infusion agar (Difco) supplemented with 150 μg of ERY/ml and were maintained afterwards in LB broth supplemented with 200 μg of ERY/ml.

DNA isolation, manipulations, and transformations. *Lactobacillus* genomic DNA was isolated according to the method of Walker and Klaenhammer (50). Small-scale *E. coli* plasmid preparations for screening transformants were performed by alkaline sodium dodecyl sulfate lysis (43). Larger preparations of plasmid DNA for sequencing and for transformation of lactobacilli were performed using the QIAprep Spin Miniprep kit (Qiagen, Valencia, Calif.). All of the DNA manipulations in this study were performed according to standard procedures (43). Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals (Indianapolis, Ind.). Calf intestinal phosphatase and T4 DNA polymerase were purchased from New England Biolabs, Inc. (Beverly, Mass.). All enzymes were used according to the manufacturers' specifications. DNA fragments were purified from agarose gels using the QIAEX II

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gel extraction kit (QIAGEN). All PCRs were performed according to standard procedures (52) using either *Taq* DNA polymerase or the Expand High Fidelity PCR system (Roche Molecular Biochemicals). PCR primers were synthesized by Integrated DNA Technologies (Coralville, Iowa). When appropriate, restriction sites were designed in the 5′ end of the primers to facilitate future cloning steps. PCR products were purified using the QIAquick PCR purification kit (Qiagen).

Electrocompetent *E. coli* ElectroMAX DH10B cells were purchased from Gibco-BRL and transformed with a gene pulser (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's specifications. Electrocompetent *E. coli* DH5α and KW1 cells were prepared and transformed as described by Dower et al. (17). Electrocompetent *Lactobacillus gasseri* ATCC 33323 cells were prepared using 3.5× SMEB as described by Luchansky et al. (35).

Construction and screening of *Lactobacillus gasseri* ADH genomic library. *L. gasseri* ADH chromosomal DNA was randomly sheared by nebulization and separated on a 1% agarose gel. Four- to six-kilobase bands were gel purified and treated with T4 DNA polymerase to create blunt ends. These fragments were then ligated to *Sma*I-bacterial alkaline phosphatase-treated pUC18. This ligation reaction was first transformed into *E. coli* DH10B to create an initial plasmid library. Transformants were selected on LB plates plus 200 μg of carbenicillin/ml. PCR and restriction analysis of 50 random transformants revealed that >90% of the transformants contained a plasmid with an average insert size of 4.5 kb. In order to avoid a competitive enrichment step, plasmid DNA was isolated from approximately 9,000 DH10B transformants that were pooled directly from outgrowth plates. This plasmid library was then screened for the presence of genes involved in β-glucuronidase activity by transformation of electrocompetent *E. coli* KW1 cells. KW1 transformants were plated on LB plates supplemented with 200 μg of carbenicillin/ml and 50 μg of X-glu/ml. Positive clones were identified by the formation of blue colonies.

DNA sequencing and analysis. DNA sequencing was performed at the University of California—Davis Automated DNA Sequencing Facility on an ABI Prism 377 DNA sequencer with a 96-lane upgrade (Applied Biosystems, Foster City, Calif.). Assembly and analysis of DNA sequences were performed with DNASIS for Windows (Hitachi Software) and Clone Manager 5 (Scientific & Educational Software). Protein homology searches were performed with the Basic Local Alignment Search Tool (BLAST), version 2.1, at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), protein motifs were identified by comparison to the PROSITE database (http://www.expasy.ch/prosite/), and amino acid alignments were performed with ClustalW (http://www.ebi.ac.uk/clustalw/).

Construction of the expression vector pTRK664. Plasmid pTRK563 was created by the ligation of a *Bgl*II-*Nhe*I PCR product amplified from pGK12 with primers 5′-AGTCAGATCTACAGCTCCAGATCGATTCAC-3′ and 5′-AGTCGCTAGCTTACGAACTGGCACAGATGG-3′ to a *Bgl*II-*Nhe*I PCR product amplified from pBluescript II KS(+) with primers 5′-AGTCAGATCTTTAATGCGCCGCTACAGG-3′ and 5′-AGTCGCTAGCAATGCAGCAGCTGGCACGACAGG-3′ (restriction sites are underlined). For the creation of plasmid pTRK664, the T7 terminator, *Lactobacillus* P6 promoter, and *gusA* gene were cloned sequentially into plasmid pTRK563. The T7 terminator was amplified from pET28a(+) as an *Xho*I-*Sal*I fragment as described previously (51) and cloned into the *Sal*I site. The *Lactobacillus* P6 promoter was amplified from pLA6 (16) using the primers 5′-AGAGTCGACTAATGAAGCTTGTTTTGTTTCAG-3′ and 5′-ACTGAATTCTTCTTTAGTTAATGGCTCAG-3′ and cloned as a *Sal*I-*Eco*RI fragment. The *gusA* gene including the putative RBS was amplified using the primers 5′-GTCGAATTCTACTAGAAAGGAAAATCATC-3′ and 5′-TGCTCTAGATAATTGAGCACGATTATTTG-3′ and cloned as an *Eco*RI-*Xba*I fragment.

Enzyme assays. For lactobacilli, β-glucuronidase activity in cell extracts (CFEs) was measured by the hydrolysis of *para*-nitrophenyl-β-D-glucuronide (PNPG) (Sigma, St. Louis, Mo.) Cultures (10.0 ml each) were washed twice in 10.0 ml of GUS buffer (100 mM sodium phosphate–2.5 mM EDTA [pH 6.0]) and resuspended in 1.0 ml of the same. Cell suspensions were then added to chilled tubes with silica beads and subjected to three 1-min cycles at the highest setting in a Mini Bead Beater (Biospec Products, Bartlesville, Okla.) with 1 min on ice in between cycles. Following centrifugation to pellet beads and cell debris, the CFE was collected and kept temporarily on ice until the start of the assays. Protein concentrations were determined by the method of Bradford (10) using the Sigma protein determination kit. For each assay, the CFEs were warmed to the assay temperature and 200 μl of sample was added to 800 μl of GUS buffer containing 12.5 mM PNPG and incubated at 37°C (except during temperature experiments). The pH of the GUS buffer was 6.0 except during pH experiments, when sodium phosphate buffer at different pHs was used to prepare the GUS buffer. The final concentration of PNPG in the assay buffer was 10.0 mM. At appropriate time intervals, usually 5, 10, and 15 min, 100 μl of the reaction

TABLE 2. Screening of various lactobacilli for β-glucuronidase activity

Strain	β-Glucuronidase activity[a]
L. acidophilus ATCC 4356	−
L. acidophilus NCFM	−
L. casei ATCC 393	−
L. delbrueckii subsp. *bulgaricus* 1489	−
L. gasseri ADH	+
L. gasseri ATCC 33323	−
L. gasseri NCK 1338	−
L. gasseri NCK 1340	−
L. gasseri NCK 1341	−
L. gasseri NCK 1342	−
L. gasseri NCK 1343	+
L. gasseri NCK 1344	+
L. gasseri NCK 1345	+
L. gasseri NCK 1346	−
L. gasseri NCK 1347	+
L. gasseri NCK 1348	+
L. gasseri NCK 1349	+
L. helveticus CNRZ 32	−
L. johnsonii VPI 11088	−
L. plantarum 299v	−
L. reuteri DSM 20016	−
L. rhamnosus GG	−

[a] As determined by the ability (+) or inability (−) of cells to hydrolyze X-glu in MRS media.

mixture was added to 800 μl of 1.0 M Na_2CO_3, and the optical density was measured at 405 nm (OD_{405}). One unit of activity is defined as 1 nmol of *p*-nitrophenol liberated per min per milligram of protein. For the measurement of activity in *E. coli* cells, assays were performed nearly identically, except that whole cells disrupted with chloroform were used instead of cell extracts and assays were done at a pH of 4.0 to reduce any potential interference by the native *E. coli* β-glucuronidase. Enzyme activity for *E. coli* experiments is represented per OD_{600}. Each value presented is the average of results from at least three independent experiments.

Expression of *gusA* in *E. coli*. In order to create the plasmid pTRK665, the *gusA* gene was amplified using the primers GUS7F 5′-AGTCCATGGAATCTGCACTATATCCAATTC-3′ and GUS6R 5′-ACTGGAATTCTAATTGAGCACGATTATTTG-3′. An *Nco*I site (underlined) was designed in primer GUS7F to include the start codon sequence. Cloning into the *Nco*I-*Eco*RI sites of pET28a(+) resulted in the translational fusion of the *gusA* gene to the T7 promoter and *E. coli* ribosome binding site of the plasmid. Plasmid pTRK665 was created in *E. coli* DH5α and transformed into *E. coli* Tuner(DE3) to perform the induction experiments. For induction experiments, cells at an OD_{600} of 0.6 were induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. Samples were removed at appropriate time points to measure growth and β-glucuronidase activity.

Southern hybridization. Southern hybridization of genomic DNA was performed using the Roche Molecular Biochemicals DIG nonradioactive nucleic acid labeling and detection system. A 776-bp internal *gusA* PCR product was amplified in the presence of digoxigenin-11-dUTP with the primers 5′-ACAGTTGACGAATACACAGAT-3′ and 5′-AGGCGATGAGAAGAAGATAATG-3′. Hybridization was performed according to the manufacturer's specifications at 42°C in standard hybridization buffer plus 50% formamide, and detection was performed with a CSPD chemiluminescent substrate.

Nucleotide sequence accession numbers. The nucleotide sequences presented in this paper have been submitted to GenBank and assigned the accession number AF305888.

RESULTS

Screening and identification of β-glucuronidase-positive lactobacilli. The *Lactobacillus* spp. listed in Table 2 were screened for β-glucuronidase activity by measuring their ability to hydrolyze the chromogenic substrate X-glu on MRS plates (100 μg/ml) or in MRS broth (200 μg/ml). Initially, only *L. gasseri*

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strains ADH and ATCC 33323 were included in the screening; however, upon observation of β-glucuronidase activity in strain ADH but not in ATCC 33323, 11 additional *L. gasseri* strains of human origin were screened. These 11 strains had been previously identified as unique strains of *L. gasseri* by 16S rRNA sequencing and pulsed-field gel electrophoresis (32). In addition to strain ADH, six other *L. gasseri* strains exhibited activity towards X-glu as evidenced by the formation of blue colonies or a blue color in broth media. None of the other *Lactobacillus* species tested showed activity (Table 2). *L. gasseri* ADH was chosen to be the subject of further experiments because it was the best characterized of all the β-glucuronidase-positive strains.

The amount of enzyme activity of *L. gasseri* ADH cells was determined by measuring the ability of CFEs to hydrolyze PNPG. The activity of log-phase and stationary-phase cells grown in MRS broth was found to be 5.3 ± 0.3 and 97.7 ± 10.7 U, respectively. It is possible that this level of expression in MRS reflects some form of negative regulation by the cell due to the abundance of glucose and the absence of glucuronide substrates. Therefore, attempts were made to increase β-glucuronidase activity by induction with 10.0 mM X-glu or 10.0 mM methyl-β-D-glucuronide, both of which are known inducers of the *E. coli* β-glucuronidase enzyme (53). In addition, to address the possibility that catabolite repression by glucose is involved in regulating β-glucuronidase expression, these experiments were performed on cultures grown in MRS or in modified MRS with the alternative carbon source galactose, lactose, or sucrose. Each inducer was added at different times during growth and allowed to incubate for time periods ranging from 15 min to overnight. However, even in the absence of glucose, no induction of β-glucuronidase activity was observed for any treatment with X-glu or methyl-β-D-glucuronide (data not shown).

Cloning of the *gusA* gene. In order to identify the gene(s) responsible for β-glucuronidase activity in *L. gasseri* ADH, a random genomic library was created in pUC18 and screened in *E. coli* KW1 (Δ*gus*) cells. Eight positive clones were identified by the formation of dark blue colonies on LB plates supplemented with 200 μg carbenicillin/ml and 50 μg of X-glu/ml. Each clone contained a single plasmid which complemented the Δ*gus* mutation upon retransformation into strain KW1. Preliminary restriction analysis revealed that each plasmid contained one of four different overlapping inserts (data not shown). Further confirmation of one clone, designated pTRK666, was obtained by subcloning its 4.5-kb insert as an *Eco*RI-*Pst*I fragment into the gram-positive shuttle vector, pTRK563, and introducting the resulting plasmid (pTRK668) into *L. gasseri* ATCC 33323 by electroporation. ERY-resistant transformants, plated on MRS plus X-glu (100 μg/ml), were converted to a β-glucuronidase-positive phenotype as evidenced by the formation of blue colonies. The 4.5-kb insert of pTRK666 was then completely sequenced on both strands. Another plasmid, pTRK667, containing an overlapping fragment of 4.9 kb, was later used to determine the nucleotide sequence of an additional 1,013 bp of flanking DNA (Fig. 1).

Analysis of nucleotide and amino acid sequences. In all, a total of 5,438 bp was sequenced from plasmids pTRK666 and pTRK667 to reveal the presence of three complete and two partial open reading frames (ORFs) (Fig. 1). The predominant ORF of 1,797 bp begins with an ATG initiation codon at nucleotide 1663 and ends with a TAA codon at nucleotide 3457. The ATG codon is preceded at a distance of 9 bp by the putative ribosome binding site AGAAAGGA. The sequence is capable of coding for a polypeptide of 597 amino acids with a predicted molecular mass of 69.8 kDa. Comparison of the predicted amino acid sequence to previously known sequences by BLAST analysis revealed the highest similarities to β-glucuronidase (GusA) of *E. coli* K-12 (39% identity) (26) and to several synthetic GUS constructs. High similarities (30 to 35% identity) were also found to many mammalian β-glucuronidase enzymes. An alignment of the predicted GusA amino acid sequence with the *E. coli* K-12 GusA sequence revealed the largest areas of homology in the carboxy-terminal end of the enzymes (Fig. 2). Recently Islam et al. (25), studying active site residues of human β-glucuronidase, presented evidence for Glu^{540} as the nucleophile residue, for Glu^{451} as the acid-base residue, and for the involvement of Tyr^{504} in the active site. Analysis of the predicted amino acid sequence encoded by *gusA* reveals that the residues corresponding to the human β-glucuronidase Glu^{540}, Glu^{451}, and Tyr^{504} residues are conserved in the *L. gasseri* ADH enzyme (Fig. 2). All of the currently known β-glucuronidase enzymes from *E. coli*, plant, and animal sources belong to the glycosyl hydrolase family 2. Comparison of the predicted amino acid sequence with the PROSITE database revealed the presence of two motifs that could be identified as the two signature sequences proposed for this family (Fig. 2). The first motif showed 100% identity to signature 1 (PS00719), and the second motif, 49 residues downstream, showed identity at 12 out of 15 positions to signature 2 (PS00608). More information about these signatures can be found at the ExPASy Molecular Biology Server (http://expasy.cbr.nrc.ca/cgi-bin/nicedoc.pl?PDOC00531). Immediately downstream of the *gusA* gene is a large, imperfect inverted repeat capable of forming a hairpin structure with a predicted Δ*G* of −37.6 kcal/mol. The presence of this potential terminator structure and the arrangement of genes around *gusA* indicate that it is most likely transcribed as a monocistronic unit. Lack of a sequence resembling a signal sequence in the amino-terminal region of the enzyme implies that it has an intracellular location.

A second ORF, designated ORF-R, begins on the opposite

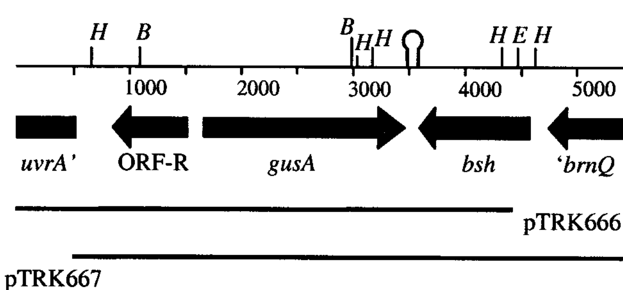

FIG. 1. Organization of genetic elements on the cloned region of *L. gasseri* ADH genomic DNA. The positions and orientations of genes and ORFs are indicated by arrows and boxes. The large inverted repeat downstream of *gusA*, capable of forming a stem-loop structure, is indicated by a hairpin. The positions of *Bam*HI (*B*), *EcoRI* (*E*), and *Hin*dIII (*H*) sites are indicated. The solid lines below the arrows indicate the positions of the inserts of plasmids pTRK666 and pTRK667.

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---MLRPVETPTREIKKLDGLWAFSLDRENCGIDQRWWESALQESRAIAVPGSFNDQFAD 57
MESALYPIQNKYRFNTLMNGTWQFETDPNSVGLDEGWNK-ELPDPEEMPVPGTFAELTTK 59
    * *::.  *   . ::* * *. * :. *:*: * :   * :.. :.***:* :  :.

ADIRNYAGNVWYQREVFIPKGWAGQRIVLRFDAVTHYGKVWVNNQEVMEHQGGYTPFEAD 117
RDRKYYTGDFWYQKDFFIPSFLKKKELYIRFGSVTHRAKVFINGHEVGQHEGGFLPFQVK 119
 * : *:*:.***::.***.     :.: :**.:*** .**::*.:** :*:**: **:..

VTPYVIAGKSVRITVCVNNELNWQTIPPGMVITDENGKKKQSYFHDFFNYAGIHRSVMLY 177
ISNYINYDQTNRVTVLVNNELSEKAIPCGTEEILDNGQKLAQPYFDFFNYSGIMRNVWLL 179
:: *:  .:: *:** *****. ::** *      :**:*  . :.*****:** *.* *

TTPNTWVDDITVVTHVAQDCNHASVDWQVVANGDVSVELRDADQQVVATGQGTSGTLQVV 237
ALPQSQITNFKLNYQLANNKATITYNIEANNNAEFKVTLFDNQKEVACATSKNTSSLTIK 239
: *:: : ::.:  ::*::    : : :.  *.:..* * * :::*..: . .:.:* :

NPHLWQPGEGYLYELCVTAKS-QTECDIYPLRVGIRSVAVKGEQFLINHKPFYFTGFGRH 296
NPHLWSPNDPYSYKIKIEMLEDGKTVDEYTDKIGIRTVKIVNDKILLNNHPIYLKGFGKH 299
*****.*.: * *:: :    .    .  * *. ::***:* : .:::*:*::*:*:.***:*

EDADLRGKGFDNVLMVHDHALMDWIGANSYRTSHYPYAEEMLDWADEHGIVVIDETAAVG 356
EDFNVLGKAVNESIIKRDYECMKWIGA[NCFRSSHYPYAEEWYQYADKYGFLII]DEVPAVG 359
** :: **..:: :: :*:   *.*****.:*:********  ::**::*:::***..***

                                                       451
FNLSLGIGFEAGNKPKELYSEEAVNGETQQAHLQAIKELIARDKNHPSVVMWSIANEPDT 416
LNRSITNFLNVTNSNQSHFFASKTVPELKKVHEQEIKEMIDR[DQRHPSVIAWSLFNE]PES 419
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                                           ††       †
                                                    504
RPQGAREYFAPLAEATRKLDP-TRPITCVNVMFCDAHTDTISDLFDVLCLNRYYGWYVQS 475
TTQESYDYFKDIFAFARKLDPQNRPYTGTLVMGSGPKVDKLHPLCDFVCLNRYYGWYVAG 479
 .* : :**  :   :***** .** *  . ** ...:.*.:   * *.:********** .

                              540
G-DLETAEKVLEKELLAWQ-EKLHQPIIITEYGVDTLAGLHSMYTDMWSEEYQCAWLDMY 533
GPEIVNAKKMLEDELDGWQNLKLNKPFVFTEFGADTLSSSHRLPDEMWSQEYQNEYYQMY 539
* :: .*:*:**.** .**  **::*:::**:*.***:. * :  :***:***  : :**

HRVFDRVSAVVGEQVWNFADFATSQGILRVGGNKKGIFTRDRKPKSAAFLLQKRWTGMNF 593
FDIFKKYPFICGELVWNFADFKTSEGIMRVGGNDKGIFTRDREPKDIAFTLKKRWQQLN- 598
. :*.: . : ** ******* **:**:*****.********:**. ** *:***  :*

GEKPQQGGKQ 603
----------
```

FIG. 2. Comparison of the amino acid sequence of *E. coli* K-12 GusA (top) with the deduced amino acid sequence of *L. gasseri* ADH GusA (bottom). Identical residues are indicated by an asterisk, conserved residues by a colon, and semiconserved residues by a dot. The three residues corresponding to human β-glucuronidase, Glu^{540}, Glu^{451}, and Tyr^{504}, are indicated by bold type (see the text). The amino acid residues corresponding to the PROSITE glycosyl hydrolase family 2 signature sequences are indicated by boxes. † indicates a residue that differs from the currently proposed signature sequence.

strand, 152 nucleotides upstream of *gusA*, with an ATG start codon at nucleotide 1510, and ends with a TAG codon at nucleotide 886. BLAST analysis of the 208 amino acids encoded by ORF-R revealed similarities in the amino-terminal end to a number of transcriptional regulators of the MerR family. The strongest of these similarities were to the transcriptional activator encoded by the *bltR* gene of *Bacillus subtilis* (27% identity over 155 residues) (2) and the *merR* gene of *Synechococcus* sp. strain PCC7942 (38% identity over 68 residues) (GenPept no. 1841790). The nature of this gene and its arrangement with respect to *gusA* may indicate a role in the regulation of *gusA*.

The third complete ORF encoding a protein of 325 amino acids, is located downstream of *gusA* on the opposite strand and is designated *bsh*. It begins with an ATG codon at nucleotide 4573 and ends 138 bp downstream of *gusA* with a TAA codon at nucleotide 3598. The predicted amino acid sequence coded by *bsh* shows strong similarities to the bile salt hydrolase genes of *Lactobacillus plantarum* (46% identity) (12), *Clostridium perfringens* (35% identity) (13), *Bifidobacterium longum* (35% identity) (48), and *Lactobacillus johnsonii* (34% identity) (19).

Two incomplete ORFs were also identified on the fragment, both in the opposite orientation from that of *gusA* (Fig. 1). The first, *'uvrA*, located upstream of *gusA* beyond ORF-R, begins with an ATG start codon at nucleotide 515 and contains 171 codons. The deduced amino acid sequence from *uvrA'* shows similarities to several *uvrA* gene products (ABC excision nuclease subunit A) from organisms including *Haemophilus influenzae* (31% identity) (14) and *Neisseria gonorrhoeae* (37% identity) (9). The second, *'brnQ*, contains 226 codons for the carboxy-terminal end of a protein whose amino acid sequence shows 40 and 31% identity to branched-chain amino acid transport system carrier proteins from *Lactobacillus delbrueckii* (46) and *Pseudomonas aeruginosa* (24), respectively.

Overexpression of GusA in *L. gasseri* ATCC 33323. A significant barrier to the preliminary characterization of the *gusA* gene product was its weak expression in wild-type ADH cells. Since no conditions were initially found to induce *gusA* expression in *L. gasseri* ADH, GusA was overproduced in *L. gasseri* ATCC 33323 by using the strong *Lactobacillus* P6 promoter (16). In order to facilitate cloning and expression in *L. gasseri*, plasmid pTRK563 was first created by modification of the low-copy-number, broad-host-range plasmid pGK12. The removal of the *cat* gene and addition of the *lacZ* multiple cloning site from pBluescript II KS(+) resulted in a smaller plasmid that had more cloning sites than the original plasmid pGK12. Using pTRK563 as a base vector, plasmid pTRK664 was then constructed for the heterologous, unregulated expression of GusA in lactobacilli.

Plasmid pTRK664 was introduced by electroporation into *L. gasseri* ATCC 33323, which was identified in this study as a β-glucuronidase-negative strain. GusA expression was observed in Ery-resistant transformants by the formation of blue colonies on MRS plates supplemented with 100 μg of X-glu/ml. The amount of β-glucuronidase activity was determined for cells grown in MRS broth. The activity of log-phase and stationary-phase cells was 1468 ± 97 and 2532 ± 220 U, representing a 277-fold and 26-fold increase in activity, respectively, over that of *L. gasseri* ADH. No hydrolysis of PNPG was observed in ATCC 33323 cells harboring the plasmid pTRK563.

Characterization of GusA. CFEs of *L. gasseri* ATCC 33323 cells harboring plasmid pTRK664 were used to measure the effects of pH, temperature, and saccharic acid 1,4-lactone on β-glucuronidase activity. Figure 3 shows the results of pH and temperature optimization experiments. The maximum activity was found at approximately pH 6.0 and at 65°C. While the activity dropped off quickly at pH values above 6.0, the enzyme retained more than 50% activity at a pH of 4.0 and approximately 33% activity at pH 3.0. An approximately twofold increase in activity was observed as the temperature was raised from 37 to 65°C.

Saccharic acid 1,4-lactone (SAL) is a specific inhibitor of all β-glucuronidases examined to date from *E. coli*, plants, and mammals (21). To determine the sensitivity of *L. gasseri* GusA to SAL, β-glucuronidase assays were performed on CFEs in the presence of 0.5 or 1.0 mM SAL at 37°C and pH 6.0. The addition of 0.5 or 1.0 mM SAL resulted in the reduction of β-glucuronidase activity of the cell extracts by 80 and 88%, respectively.

Controlled expression of *gusA* in *E. coli*. In order to further correlate β-glucuronidase activity with *gusA* expression, plasmid pTRK665 was constructed to contain a translational fusion between the *gusA* gene and the T7 promoter and ribosome binding site of pET28a(+). Plasmid pTRK665 was

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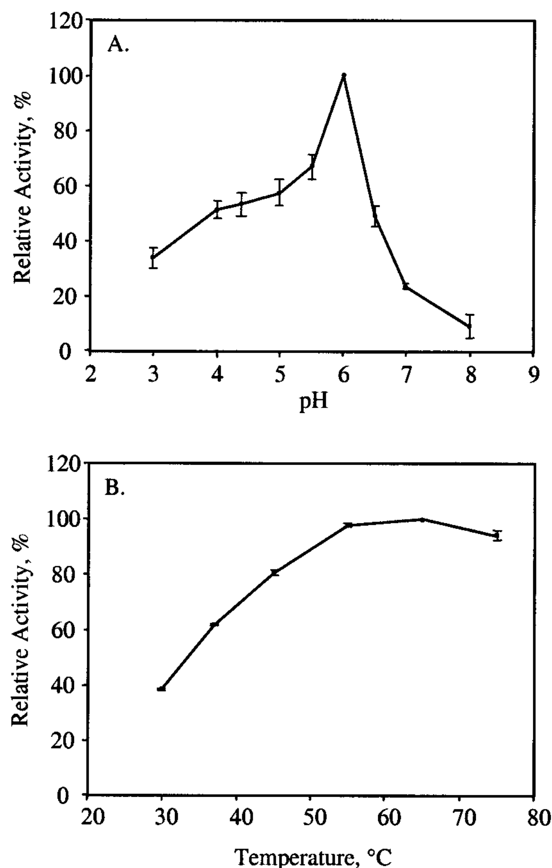

FIG. 3. Effect of pH (A) and temperature (B) on β-glucuronidase activity. CFEs of late-log-phase *L. gasseri* ATCC 33323 cells harboring plasmid pTRK664 were assayed under the various conditions for the hydrolysis of PNPG. pH experiments were performed at 37°C, and temperature experiments were performed at pH 6.0.

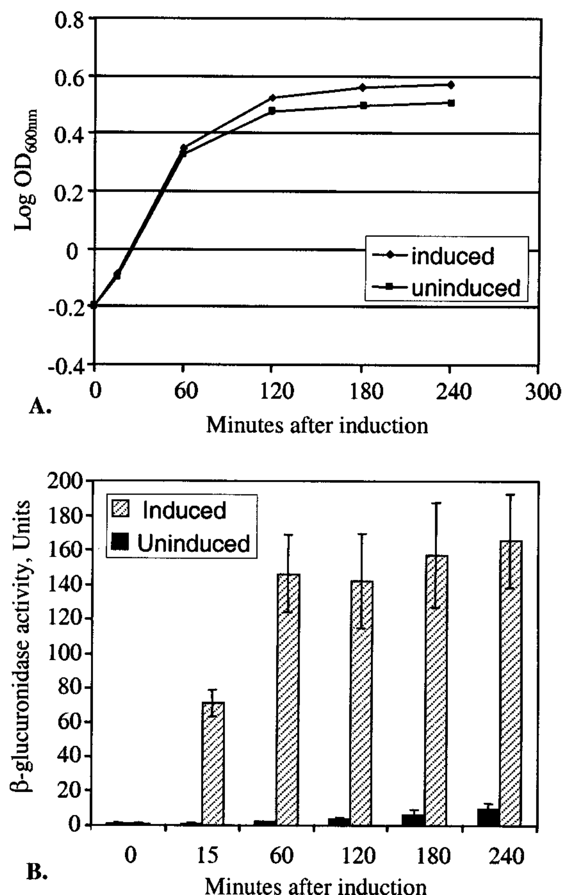

FIG. 4. Growth (A) and expression (B) of β-glucuronidase for *E. coli* Tuner(DE3)::pTRK665 cells following induction with 1.0 mM IPTG.

transformed into *E. coli* Tuner(DE3), which carries a chromosomal copy of the T7 polymerase gene under the control of the inducible *lac* promoter. GusA expression was induced in *E. coli* Tuner(DE3)::pTRK665 over 4 h by the addition of 1.0 mM IPTG (Fig. 4). β-Glucuronidase activity peaked in induced cells between 15 and 60 min and stayed relatively constant over the time course of 4 h. The growth of induced cells was not significantly different from that of uninduced cells.

Screening other *L. gasseri* strains for *gusA* homologues. Out of 13 strains of *L. gasseri* screened in this study for β-glucuronidase activity, only 7 showed activity towards the chromogenic substrate X-glu. In order to examine the distribution of *gusA* genes among these 13 strains, a 776-bp internal region of the *gusA* gene was labeled with digoxigenin and used as a probe in a Southern blot analysis. Genomic digests from each of the strains were separated by electrophoresis and transferred to a nylon membrane. The membrane was then hybridized at mild stringency with the labeled *gusA* probe. With the exception of ATCC 33323, all of the strains tested showed a positive hybridization to the *gusA* probe (Fig. 5).

DISCUSSION

In this work we report the identification and cloning of the *gusA* gene of *L. gasseri* ADH by its ability to complement an *E. coli* Δ*gus* mutant. The view that β-glucuronidase activity is a trait unique to *E. coli* has been supported in large part by the lack of solid evidence proving the existence of other β-glucuronidase enzymes. Previous studies have observed β-glucuronidase activity in some gram-positive inhabitants of the GI tract, including three species of lactobacilli, but in no case have the

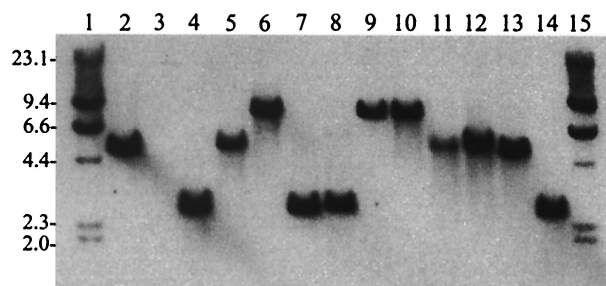

FIG. 5. Southern hybridization of genomic DNA from *L. gasseri* strains. Genomic DNA from each strain was digested with *Eco*RI, separated on a 1.0% agarose gel, and transferred to a nylon membrane prior to hybridization with the *gusA* probe. Lanes: 1 and 15, DIG-labeled molecular weight marker; 2, strain ADH; 3, ATCC 33323; 4, NCK 1340; 5, NCK 1344; 6, NCK 1345; 7, NCK 1342; 8, NCK 1341; 9, NCK 1346; 10, NCK 1347; 11, NCK 1348; 12, NCK 1349; 13, NCK 1343; 14, NCK 1338. Sizes of the molecular weight marker bands are indicated in kilobases.

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genes responsible for these activities been identified. Of the three *Lactobacillus* species previously studied, *L. delbrueckii*, L. fermentum, and *L. rhamnosus*, only the enzyme activity from *L. rhamnosus* has been partially characterized (37, 42). Thus, this report describes the first *gusA* gene to be cloned from a bacterial source other than *E. coli*. The discovery of this new bacterial *gusA* gene, some 20 years after the identification of the *E. coli* gene, will provide a new context in which to study the effects of bacterial β-glucuronidase on gastrointestinal health and disease.

The genetic organization of the *L. gasseri gusA* gene and the surrounding region is not conserved with respect to the *E. coli gusA* gene (53). The G+C composition (35%) of the *gusA* gene reflects the overall G+C composition of the *L. gasseri* chromosome (33 to 35%), indicating that it was not acquired recently by lateral transfer from enterobacteria. In *E. coli*, the *gusA* gene is transcribed as part of the *gusRABC* operon together with the genes coding for the repressor (*gusR*), the transporter (*gusB*), and a membrane-associated protein (*gusC*) of unknown function. However, in *L. gasseri*, the arrangement of the *gusA* gene with respect to flanking ORFs indicates that unlike the *E. coli* gene, it is transcribed as a monocistronic unit. Due to its position and nature, ORF-R is a good candidate for a regulatory protein with a role in the regulation of *gusA*; however, it is separately transcribed and cannot be directly linked to *gusA* without further study. The DNA surrounding the *gusA* gene did not reveal a gene encoding an obvious transport protein, indicating that *L. gasseri* utilizes either a specific transporter located elsewhere on the chromosome or an alternative transporter for glucuronide uptake. The close proximity of the *bsh* gene to the *gusA* gene is also very interesting and suggests that this region of the chromosome may be involved in the cellular response to bile secretion in the GI tract.

The reasons for a low level of expression of β-glucuronidase in ADH cells grown in MRS are still unknown, but this may result from transcriptional regulation of the *gusA* gene. The regulation of *gusA* has been well studied with *E. coli*, in which the predominant mechanisms are similar to those of the well-known *lac* operon. Expression is induced by a variety of glucuronides and is repressed in the presence of alternative carbon sources. Regulation is controlled at the level of transcription by the specific repressor GusR (40) and by catabolite-responsive elements (26). Similar regulatory mechanisms have been identified for a variety of metabolic enzymes in lactobacilli. In *L. pentosus*, α-xylosidase expression is induced by growth on xylose and is negatively regulated by the repressor protein XyIR and the catabolite control protein, CcpA (34). CcpA is a global regulator of low-G+C gram-positive organisms that binds to catabolite-responsive elements (*cre*) in promoter regions to control carbon catabolite repression (47). A *ccpA* gene has been identified for a number of lactobacilli, including *L. pentosus*, *L. casei*, and *L. delbrueckii* (34, 39, 44), but not for *L. gasseri*. Additionally, analysis of the region preceding the *L. gasseri gusA* gene did not reveal sequences with high levels of similarity to the currently proposed *cre* consensus, but regions of dyad symmetry were observed in the promoter region.

Because of the paucity of versatile cloning vectors for organisms in the *Lactobacillus acidophilus* complex, a new plasmid, pTRK563, was constructed for cloning and expressing the *gusA* gene in *L. gasseri* ATCC 33323. This plasmid, based on the well-characterized pWV01 replicon (49), maintains the disadvantages of being low copy number and utilizing a rolling circle mechanism of replication. However, it offers the benefits of a broad host range, small size, multiple cloning site, and a *lacZ* gene for screening transformants in *E. coli*. The creation of this new plasmid offered more flexibility in creating the expression vector pTRK664. In addition to *L. gasseri* ATCC 33323, expression of GusA from plasmid pTRK664 was observed in *L. acidophilus*, *L. johnsonii*, and *L. lactis*, all of which were shown previously to be β-glucuronidase negative.

The pH and temperature profiles for *L. gasseri* GusA are similar to those obtained by Pham et al. (42) with partially purified enzyme fractions from *L. rhamnosus*. The results indicate that GusA is a very stable enzyme that is more active at acidic pHs and higher temperatures. The *Lactobacillus* enzyme is considerably more active at acidic pHs than what has been reported for the *E. coli* enzyme (5, 28). This difference is not entirely surprising considering that the lactic acid bacteria generally inhabit a more acidic environment and maintain a lower intracellular pH than the enterobacteria. It does, however, imply that the two β-glucuronidases are active in different microenvironments in the GI tract. This new information suggests that for detecting β-glucuronidase activity, the use of fecal contents may not adequately reflect the contributions of all organisms in vivo, especially when assays are performed at higher pH values.

The discovery of GusA in *L. gasseri* is expected to stimulate further research on the distribution of β-glucuronidase enzymes among other organisms. The unexpected discovery that a number of β-glucuronidase-negative *L. gasseri* strains possess a *gusA* homologue raises further questions. In fact, the only strain to lack both β-glucuronidase activity and a *gusA* homologue was the type strain, ATCC 33323. These results can be explained by a number of hypotheses. Feng and Lampel (20) showed that isolates of *E. coli* O157:H7 that did not exhibit β-glucuronidase activity still carried nucleotide sequences for the *uidA* (*gusA*) gene. These genes carried a small number of mutations that resulted in the production of an inactive β-glucuronidase enzyme which was still reactive with the anti-β-glucuronidase antibody. In a study of β-glucuronidase activity among human fecal *E. coli* isolates, Chang et al. (11) found three isolates in which activity was minimal at 37°C but significant at 44.5°C. In the case of *E. coli* K-12, β-glucuronidase activity is poorly induced by X-glu because of the failure of the glucuronide-specific permease to concentrate it from the media (53). These and other issues will have to be studied in order to elucidate the true distribution and functionality of *gusA* homologs.

Finally, it has yet to be determined what advantage GusA expression provides *L. gasseri*. There is currently no evidence to support the ability of lactobacilli to utilize glucuronides for energy production or to support the existence of a D-glucuronate catabolic pathway in lactobacilli. It also remains to be determined what the natural substrates of *L. gasseri* GusA are. A number of important natural compounds, including bilirubin and endogenous hormones, are routinely excreted as glucuronide conjugates via bile into the GI tract. Perhaps knowing this information will provide clues to the significance of β-glucuronidase activity, in vivo, to *L. gasseri* and to the host.

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