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Distinct Roles of β-Galactosidase Paralogues of the Rumen Bacterium *Mannheimia succiniciproducens*

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***Mannheimia succiniciproducens*, a rumen bacterium belonging to the family *Pasteurellaceae*, has two putative β-galactosidase genes, *bgaA* and *bgaB*, encoding polypeptides whose deduced amino acid sequences share 56% identity with each other and show approximately 30% identity to the *Escherichia coli* gene for LacZ. The *M. succiniciproducens bgaA* (*MsbgaA*) gene-deletion mutant was not able to grow on lactose as the sole carbon source, suggesting its essential role in lactose metabolism, whereas the *MsbgaB* gene-deletion mutant did not show any growth defect on a lactose medium. Furthermore, the expression of the *MsbgaA* gene was induced by the addition of lactose in the growth medium, whereas the *MsbgaB* gene was constitutively expressed independently of a carbon source. Biochemical characterization of the recombinant proteins revealed that MsBgaA is more efficient than MsBgaB in hydrolyzing *o*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-galactopyranoside. MsBgaA was highly specific for the hydrolysis of lactose, with a catalytic efficiency of 46.9 s^{-1} mM^{-1}. However, MsBgaB was more efficient for the hydrolysis of lactulose than lactose, and the catalytic efficiency was 10.0 s^{-1} mM^{-1}. Taken together, our results suggest that the β-galactosidase paralogues of *M. succiniciproducens* BgaA and BgaB play a critical role in lactose metabolism and in an unknown but likely specific function for rumen bacteria, respectively.**

Ruminant animals digest cellulosic biomass through their digestive tract and metabolize this material to produce milk, meat, wool, and hides. The rumen is the first of four stomachs in ruminant animals (5). Plant biomass is fermented by ruminal bacteria in the rumen under a relatively stable environment, characterized by a stable temperature of 39°C, a near neutral pH, and a constant oxidation-reduction potential (35, 37). More than 200 species of microorganisms in the rumen, through symbiosis or commensalism, convert plant polysaccharides to monosaccharides or disaccharides (5, 16). The resulting carbohydrates are further metabolized during fermentation by rumen bacteria into organic acids, including acetic, lactic, propionic, butyric, and succinic acids, and into methane and CO_2 (37). Among these organic acids, succinic acid is an attractive chemical building block for the manufacture of various substances, such as biodegradable polymers, surfactants, synthetic resins, and additives in food and pharmaceuticals (36).

Several microorganisms, including *Actinobacillus succinogenes*, which enable the biotechnological production of succinic acid have been isolated from the rumen and in other habitats (8). Recently, *Mannheimia succiniciproducens*, which produces a large amount of succinic acid with a 67.5% yield from glucose under an anaerobic condition, was isolated from a bovine rumen (23). The Gram-negative rod, nonmotile, mesophilic, and capnophilic bacterium *M. succiniciproducens* belongs to the family *Pasteurellaceae* (23). *M. succiniciproducens* is unable to degrade the complex polysaccharides associated with dietary plant cell wall components. However, the genome sequence of *M. succiniciproducens* contains several genes that are involved in the transport of external carbon sources, such as *mtlA*, *fruA*, *xylE*, and *malX*, suggesting that *M. succiniciproducens* utilizes soluble carbohydrates released from initial hydrolysis of these polymers by other ruminal bacteria (14). In fact, growth test experiments have indicated that *M. succiniciproducens* can use glucose, fructose, xylose, sucrose, maltose, lactose, or mannitol as the sole carbon and energy source (14).

Interestingly, two genes encoding β-galactosidase homologues (MS0806, *M. succiniciproducens bgaA* [*MsbgaA*], encoding the protein with GenBank accession no. AAU37413, and MS0749, *MsbgaB*, encoding the protein with GenBank accession no. AAU37356) were found in the genome sequence of *M. succiniciproducens* MBEL55E (14). β-Galactosidase (EC 3.2.1.23) catalyzes the hydrolysis of lactose as well as the transgalactosylation of galacto-oligosaccharides. The biochemical properties or physiological roles of β-galactosidases in a number of microorganisms, plants, and animal tissues have been characterized, and some of their crystal structures have been solved (17, 27, 32, 34). In bacteria, β-galactosidases play a role in the initiation of the metabolism of lactose. The bacteria usually uptake lactose via a permease and then hydrolyze it to galactose and glucose with an inducible β-galactosidase (17, 27). It has been well characterized in the model organism *Escherichia coli* that the machinery for lactose metabolism, including the repressor (LacI), permease (LacY), and β-galactosidase (LacZ), is catabolically repressed by the cyclic AMP receptor protein (CRP) complex and strongly induced in the

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presence of lactose (38). However, the initial lactose metabolism mediated by β-galactosidase has not yet been characterized in any ruminal bacteria. Thus, the functional characterization of two β-galactosidase paralogues of *M. succiniciproducens* will provide information both on the general features of β-galactosidase in rumen bacteria and on the physiological roles of each β-galactosidase paralogue.

In this paper, we characterized the two β-galactosidase genes, *bgaA* and *bgaB*, in *M. succiniciproducens* by analyzing the growth phenotypes of mutant strains, the expression patterns of each gene, and the biochemical properties of the corresponding recombinant proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth condition. *M. succiniciproducens* MBEL55E (KCTC0769BP) was obtained from the South Korean Collection for Type Cultures (KCTC). *M. succiniciproducens* was cultivated in Trypticase soy (TS) broth (Difco Laboratories, Detroit, MI) or in MH3 medium (23), which contained (per liter) 5.0 g D-glucose, 5.0 g yeast extract, 3.0 g K_2HPO_4, 2.0 g NaCl, 2.0 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgCl_2 \cdot 6H_2O$, and 1 g $Na_2S \cdot 9H_2O$. The cultures were grown in a sidearm flask containing 50 ml of MH3 medium at 39°C without shaking in a 10% CO_2 atmosphere. Growth was monitored spectrophotometrically at 600 nm. β-Galactosidase induction experiments were carried out in a modified MH3 medium by omitting D-glucose and supplementing with each carbon source (5 g/liter), specifically, L-arabinose, D-fructose, D-glucose, D-galactose, D-mannose, lactose, sucrose, D-maltose, or D-raffinose. *E. coli* strains DH5α and BL21(DE3) were used as hosts for the construction of the recombinant plasmids and for the overexpression of the recombinant β-galactosidases, respectively. *E. coli* cells were grown in an LB medium supplemented with an appropriate antibiotic at 37°C. The bacterial strains used in this study are summarized in Table S1 in the supplemental material.

Construction of *M. succiniciproducens bgaA* and *bgaB* mutants and their complemented strains. For the construction of the gene-deletion mutant strain, the markerless gene knockout system for *M. succiniciproducens* based on the temperature-sensitive plasmid (21) was employed, as described below. First, a target deletion vector was constructed using the pSacHR06 vector (33) containing the *sacB* gene by cloning the PCR-amplified up- and downstream flanking fragments of the target gene and a *lox66*-Cm^r-*lox71* cassette between them. The resulting knockout plasmid was used to construct a *lox66*-Cm^r-*lox71* cassette insertion mutant by homologous recombination. The *lox66*-Cm^r-*lox71* cassette was then removed using the Cre-*lox* system, which employs the temperature-sensitive plasmid pCRX5 (21). The single-gene-deletion mutant strains for *bgaA* and for *bgaB* were termed EL101 and EL102, respectively. The *bgaA* and *bgaB* double-gene-deletion mutant, EL103, was constructed by introducing the *bgaB* mutation into the EL101 strain by using the method described above.

Complemented strains were prepared by the transformation of an expression vector for each gene into the EL103 strain. To construct the expression vectors for *MsbgaA* and *MsbgaB*, each gene was amplified and cloned into the pMS3 vector containing the fumarate reductase operon promoter (18). The pMS3-bgaA and pMS3-bgaB vectors were transformed into the EL103 strain, resulting in the EL105 and EL106 strains, respectively. The empty vector pMS3 was also transformed into EL103 as a negative control, resulting in the EL104 strain.

RNA isolation and semiquantitative reverse transcriptase PCR (RT-PCR). The total RNAs were extracted from the *M. succiniciproducens* cells using the phenol-chloroform extraction method. The RNA samples were treated with RNase-free DNase I to remove any contaminating DNA and were cleaned with an RNeasy MinElute cleanup kit (Qiagen, Hilden, Germany). cDNA synthesis was performed as follows: a reverse transcription reaction was carried out in a total volume of 10 μl with 10 μg RNA, 50 pmol random hexamer primer, and 20 U Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A second PCR was carried out in a total volume of 20 μl with 10 ng cDNA and 10 pmol of appropriate primer pairs (see Table S2 in the supplemental material) using a Maxime PCR PreMix kit (i-Taq; Intron, Seongnam, Republic of Korea). The *polA* gene, a housekeeping gene, was used as a positive control. The amplified DNAs were analyzed by 0.8% agarose gel electrophoresis.

Cloning, overexpression, and purification of recombinant MsBgaA and MsBgaB. The *MsbgaA* and *MsbgaB* genes were PCR amplified and cloned into the pET28a vector (Novagen, Madison, WI), generating pET28-bgaA and pET28-bgaB, respectively. These vectors were transformed into *E. coli* BL21(DE3) to overexpress 6× His-tagged recombinant MsBgaA and MsBgaB proteins. The *E. coli* strains harboring pET28-bgaA or pET28-bgaB were cultivated in the LB medium supplemented with 50 μg ml^{-1} of kanamycin at 37°C until the optical density at 600 nm (OD_{600}) reached 0.6 to 0.8, at which point 1.0 mM isopropyl-β-D-thiogalactopyranoside was added to the culture broth to induce gene expression. After induction at 18°C for 28 h with shaking, the cells were harvested and washed with 10 mM Tris-HCl (pH 7.6) containing 10 mM EDTA, 10 mM β-mercaptoethanol (BME), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation, the washed cells were suspended in 50 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl and were then disrupted by sonication. The crude extracts were clarified by centrifugation (12,000 × *g*, 30 min, 4°C). The clarified homogenates were loaded onto a 1-ml HiTrap FF column (GE Healthcare) and washed with 20 bed volumes of 50 mM Tris-HCl (pH 8.0) containing 250 mM NaCl and 10 mM imidazole, followed by a linear gradient of 10 to 500 mM imidazole. Fractions of 0.5 ml were collected at a flow rate of 1.0 ml min^{-1}. The fractions containing the recombinant protein were pooled and loaded onto a Sephacryl S-300 column (1.0 by 35 cm) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl. The enzyme was eluted using the equilibrium buffer. Fractions of 1 ml were collected at a flow rate of 0.5 ml min^{-1}. The fractions containing the recombinant protein were pooled, concentrated by ultrafiltration, and then desalted by dialysis.

Protein molecular weight determination. The purified proteins were chromatographed at a flow rate of 0.5 ml min^{-1} on a Sephacryl S-300 column (1.0 by 35 cm) using an HMW gel filtration calibration kit (GE Healthcare). The buffer used for column equilibration and elution was 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl. The molecular mass standards used were aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa), and blue dextran (2,000 kDa). The absorbance of the eluted proteins was monitored, and the β-galactosidase activities were measured using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate, as described below. The molecular weights of the two MsBgaA and MsBgaB β-galactosidases were calculated by interpolation on a plot of the log molecular weight against the protein elution volume.

β-Galactosidase activity staining. The purified proteins (15 μg), mixed with a sample loading buffer which did not contain SDS or any reducing agents, were loaded on 6% native polyacrylamide gel without a boiling step for protein denaturation. After gel running, the gel was soaked three times for 5 min in distilled water and then incubated in 100 mM potassium phosphate buffer (pH 7.6) containing 0.04% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The gel was incubated for 2 h at 37°C. The in-gel β-galactosidase activity staining was stopped by soaking the gel in a destaining solution containing 40% methanol and 10% acetic acid.

Enzyme assays and determination of kinetic parameters. The β-galactosidase activity with ONPG as a substrate was determined by measuring the amount of *o*-nitrophenol released by the galactosidase-catalyzed hydrolysis process. The hydrolysis of the substrate was carried out at 37°C for 10 min in 100 mM sodium phosphate buffer (pH 8.0). The addition of 1 M Na_2CO_3 to a final concentration of 0.4 M terminated the enzyme reaction. The amount of liberated *o*-nitrophenol was determined

at 420 nm (molar extinction coefficient [ε] = 4.5 mM^{-1} cm^{-1}) using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). One unit of galactosidase activity was defined as the amount of enzyme that produced 1 μmol of *o*-nitrophenol per minute at 37°C. The background hydrolysis of the substrate was deduced using a reference sample with a composition identical to that of the reaction mixture but without the enzyme. For β-galactosidase activity toward lactose, lactulose, and arabinogalactan, the amount of galactose released after the enzyme reaction was measured by using a galactose dehydrogenase assay (Sigma-Aldrich, St. Louis, MO), as described below. First, the reaction was carried out in a total volume of 900 μl containing an appropriate concentration of lactose, lactulose, or arabinogalactan and 100 mM Tris-HCl buffer (pH 8.0). To initiate the enzyme reaction, 72 mM β-NAD and 0.3 U galactose dehydrogenase were added to the reaction mixture, which was then incubated at 37°C for 20 min. After the enzyme reaction, the amount of NADH produced from NAD by galactose dehydrogenase was measured at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) using a spectrophotometer (Shimazu, Kyoto, Japan). All enzyme activities were determined in triplicate. The kinetic parameters of β-galactosidase were determined using ONPG and *p*-nitrophenyl-β-D-galactopyranoside (PNPG; 0.1 mM to 10 mM) as well as lactose and lactulose (0.5 mM to 500 mM) as substrates. The apparent V_{max} and K_m values (means $\pm$ standard deviations) were calculated by fitting the initial rate data to the Michaelis-Menten equation with a nonlinear regression analysis program (Sigma Plot, version 10.0). The amount of protein was determined by the Bradford method with bovine serum albumin as the standard.

Biochemical characterizations of recombinant MsBgaA and MsBgaB. The substrate specificities of recombinant MsBgaA and MsBgaB were determined at 37°C for 10 min in a final volume of 200 μl of 100 mM sodium phosphate buffer (pH 8.0) containing 2 mM *o*-nitrophenyl–glycoside (*o*NP-glycoside) or *p*-nitrophenyl–glycoside (*p*NP-glycoside). The *o*NP- and *p*NP-glycoside substrates used in this study were as follows: *p*NP-α-L-arabinopyranoside, *p*NP-β-L-arabinopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-L-fucopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-α-D-mannopyranoside, *p*NP-*N*-acetyl-β-D-glucosaminide, *p*NP-α-D-*N*-acetylneuraminic acid, *p*NP-2-acetamido-2-deoxy-3-*o*-β-D-galactopyranosyl-β-D-glucopyranoside (*p*NP-β-*N*-acetyllactosaminide), *o*NP-β-D-cellobioside, and *p*NP-β-D-lactopyranoside. The effects of the pH on the activities of MsBgaA and MsBgaB were determined at 37°C in 100 mM sodium acetate buffer (pH 4.0 to 6.0), 100 mM potassium phosphate buffer (pH 6.4 to 8.8), and 100 mM sodium carbonate-borate buffer (pH 8.0 to 10.0) using 10 mM ONPG as a substrate by the standard method described above. The temperature profiles for the β-galactosidase activity (10 μg ml^{-1} of BgaA and 10 μg ml^{-1} of BgaB) in 100 mM sodium phosphate buffer (pH 8.0) were determined at reaction temperatures of between 10°C and 70°C. At each temperature, the enzyme activities were determined using 10 mM ONPG as a substrate under the standard method. The effects of divalent metal ions (Mn^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, and Hg^{2+}) and EDTA on the β-galactosidase activities were determined by measuring the enzyme activity after preincubation in 100 mM sodium phosphate buffer (pH 8.0) at 37°C for 1 h in the presence of 1 mM each of these divalent metal ions or EDTA. The effects of thiol reagents (β-mercaptoethanol, dithiothreitol, and glutathione) and thiol modification compounds (iodoacetamide and *p*-hydroxymercuribenzoic acid) on the recombinant β-galactosidases were assayed using the same procedure described above with thiol reagent instead of divalent metal ions.

RESULTS

***M. succiniciproducens* β-galactosidase paralogues as members of the novel β-galactosidase family in rumen bacteria.** BLAST searches using the *M. succiniciproducens* β-galactosidase paralogues BgaA and BgaB were performed against protein databases. The amino acid sequence of MsBgaA shares 56% identity with that of MsBgaB. A database search with these two proteins revealed a large number of sequences with apparent similarity to bacterial β-galactosidases in two phylogenetic domains of *Proteobacteria* and *Firmicutes*. The phylogenetic tree shows that the sequences of MsBgaA and MsBgaB are grouped only with the β-galactosidase homologues of capnophilic rumen bacteria, specifically, three *Actinobacillus* species (Fig. 1): *A. succinogenes*, *Actinobacillus minor*, and *Actinobacillus pleuropneumoniae*. *A. succinogenes* Bgal (AsBgal), *A. minor* Bgal (AmBgal), and *A. pleuropneumoniae* Bgal (ApBgal) have 61%, 62%, and 63% amino acid sequences identities with the amino acid sequence of MsBgaA, respectively. Interestingly, MsBgaA and MsBgaB paralogues are not separated on the phylogenetic tree; instead, they form one group with the β-galactosidases of capnophilic rumen bacteria, classified as members of *Actinobacillus-Haemophilus-Pasteurella* (28). Moreover, as shown in Table S3 in the supplemental material, these two proteins are not close to any of the well-known β-galactosidases from the proteobacteria family, including *E. coli*, *Salmonella enterica*, or *Vibrio vulnificus*, but they are clustered with the β-galactosidase homologues of the facultatively commensal bacteria *Neisseria lactamica* and *Aggregatibacter aphrophilus*, which are capable of fermenting lactose (15, 29). These data would imply that the origin of the β-galactosidases of capnophilic bacteria is separate from the origins of other proteobacterial β-galactosidases.

The multiple-amino-acid-sequence alignments of the *M. succiniciproducens* β-galactosidase paralogues with other bacterial homologues revealed several conserved residues in the active site of β-galactosidase (see Fig. S1 in the supplemental material). In a comparison with the well-characterized *E. coli* β-galactosidase LacZ protein, *M. succiniciproducens* BgaA shows a well-conserved Glu528 residue, which is a catalytic nucleophile that forms a covalent bond with the galactosyl moiety in lactose (4, 6, 25). Other amino acid residues known to be involved in the formation of hydrogen bonds to lactose, Asp201, Glu456, Tyr505, His531, and Phe590, are also conserved in MsBgaA, with the exception of Glu593, which corresponds to Asn605 of *E. coli* LacZ. In addition, the Glu381 and His383 residues for the binding of a divalent metal ion are well conserved in MsBgaA (see Fig. S1 in the supplemental material) (11, 19, 26). Furthermore, the variations found in *M. succiniciproducens* β-galactosidases (BgaA and BgaB) compared to *E. coli* LacZ were also detected in the β-galactosidases of other capnophilic rumen bacteria, *A. succinogenes*, *A. minor*, and *A. pleuropneumoniae*, which were grouped together in the phylogenetic tree. This may reflect an evolutionary relationship between these two groups of β-galactosidases.

Role of MsBgaA and MsBgaB in growth on lactose. To determine the function of BgaA and BgaB for lactose metabolism in *M. succiniciproducens*, each gene in the chromosome was disrupted using the Cre-*lox* system as described in Materials and Methods, resulting in two single-gene-deletion mutants, EL101 for Δ*bgaA* and EL102 for Δ*bgaB*, and a double-gene-deletion mutant, EL103 for Δ*bgaA* Δ*bgaB*. Each mutant was cultivated in MH3 medium supplemented with 0.5% lactose as the sole carbon source (Fig. 2). The growth of the EL101 and the EL103 strains was reduced considerably compared to that of the wild-type strain (Fig. 2A). On the other hand, the growth of the EL102 strain was not very different from that of the wild-type strain.

As expected, in the EL105 strain, the *M. succiniciproducens* Δ*bgaA* Δ*bgaB* double-gene-deletion mutant strain, complementation with pMS3-bgaA restored the normal cell growth pheno-

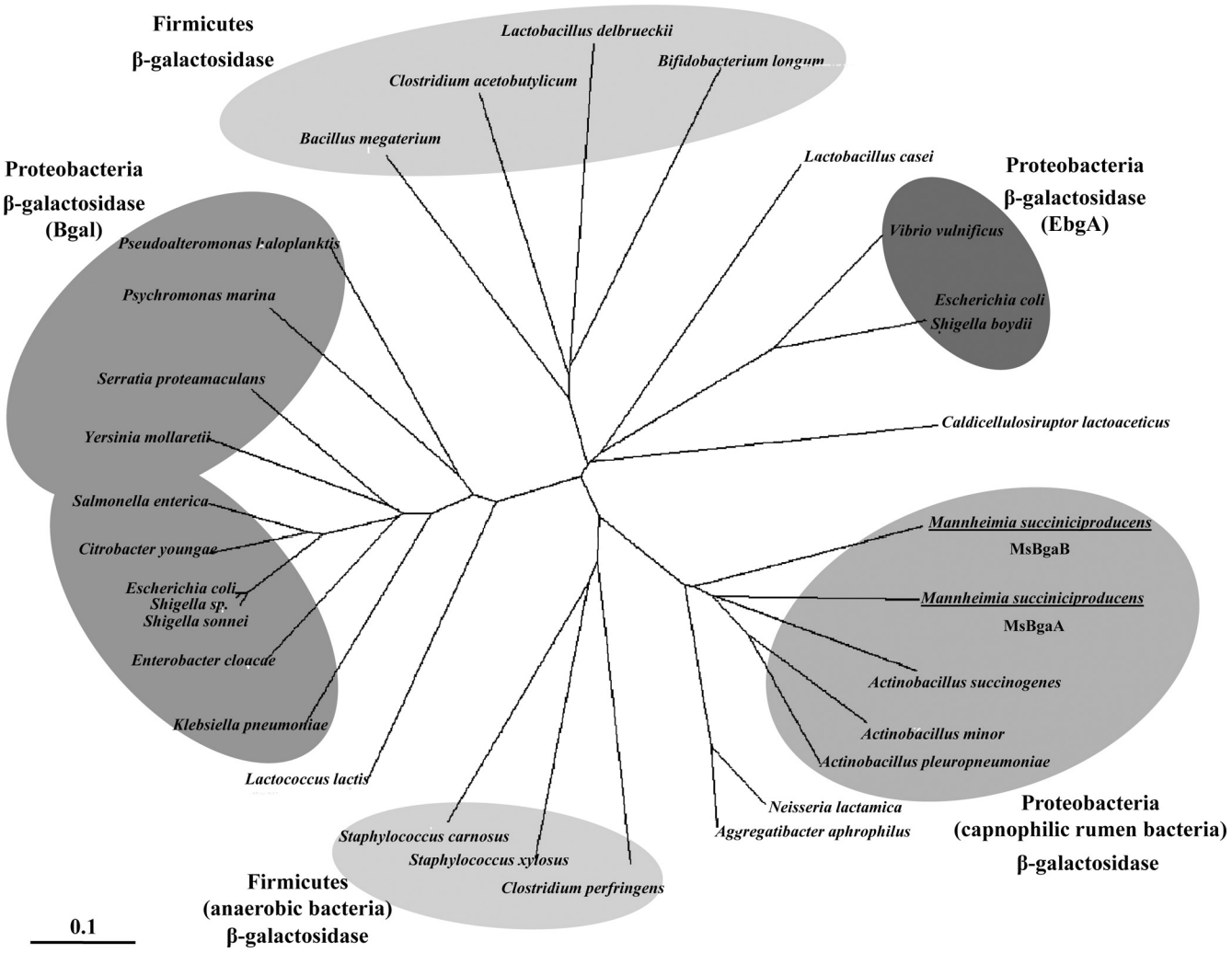

FIG 1 Phylogenetic analysis and amino acid sequence alignment of *M. succiniciproducens* β-galactosidases and other related proteins. Unrooted neighbor-joining bootstrap tree constructed from the alignments of MsBgaA and MsBgaB with other bacterial β-galactosidase homologues: *Mannheimia succiniciproducens* BgaA (GenBank accession no. AAU37413), *M. succiniciproducens* BgaB (AAU37356), *Actinobacillus succinogenes* (YP_001344692), *Actinobacillus minor* (ZP_04753333.1), and *Actinobacillus pleuropneumoniae* (P70753); β-galactosidases of the *Firmicutes* (anaerobic bacteria) *Staphylococcus carnosus* (AAM26297), *Staphylococcus xylosus* (O33815), and *Clostridium perfringens* (NP_561687); β-galactosidases (Bgal) of the *Proteobacteria Pseudoalteromonas haloplanktis* (P81650), *Psychromonas marina* (BAA76741), *Serratia proteamaculans* (YP_001479401), *Yersinia mollaretii* (ZP_04639172), *Salmonella enterica* (YP_001571822), *Citrobacter youngae* (ZP_06354081), *Escherichia coli* (P00722; Ecol_BGAL), a *Shigella* sp. (ZP_05434185), *Shigella sonnei* (YP_309314), *Enterobacter cloacae* (Q47077), and *Klebsiella pneumoniae* (P06219; Kpne_BGAL); β-galactosidases of the *Firmicutes Bacillus megaterium* (O52847), *Clostridium acetobutylicum* (P24131; Cace_BAL), *Lactobacillus delbrueckii* (P20043), and *Bifidobacterium longum* (AAL02052); EbgA of the *Proteobacteria Vibrio vulnificus* (NP_935435), *Escherichia coli* (AP_003625: Ecol_EbgA), and *Shigella boydii* (YP_409278); and other proteins from *Neisseria lactamica* (ZP_05987025: Nlac_GAL), *Aggregatibacter aphrophilus* (YP_003007428), *Lactococcus lactis* (AAC63020), *Lactobacillus casei* (ABJ69242), and *Caldicellulosiruptor lactoaceticus* (CAC50563).

type (Fig. 2B). Interestingly, in the EL106 strain, the *M. succiniciproducens* Δ*bgaA* Δ*bgaB* double-gene-deletion mutant strain, complementation with pMS3-bgaB also restored the normal growth pattern. This suggests that the expression of the single copy of the *bgaB* gene on the chromosome in the EL101 Δ*bgaA* mutant strain was not enough to support growth on lactose medium, whereas the expression of the same gene cloned in the pMS3 vector under the strong *frdA* gene promoter of the fumarate reductase operon (18) was high enough to support the growth of the EL106 double-gene-deletion strain on lactose medium.

Lactose-dependent inducibility of *MsbgaA* and *MsbgaB* genes. To evaluate the effects of different carbon sources on the expression of *MsbgaA* and *MsbgaB* genes, RT-PCR was performed from the cells grown on a variety of sole carbon sources. The wild-type strain was cultivated in MH3 medium supplemented with arabinose, fructose, glucose, galactose, mannose, lactose, sucrose, maltose, or raffinose, and the cells were then harvested at an early exponential phase to isolate the total RNA for use as a template for the RT-PCR analysis. As a control, the expression of the *polA* gene in each total RNA sample was analyzed by RT-PCR. The RT-PCR results showed different expression profiles between the *MsbgaA* and the *MsbgaB* genes (Fig. 3A). The expression levels of the *MsbgaA* gene varied depending on the type of carbon source added to the medium. The *MsbgaA* gene was highly expressed in

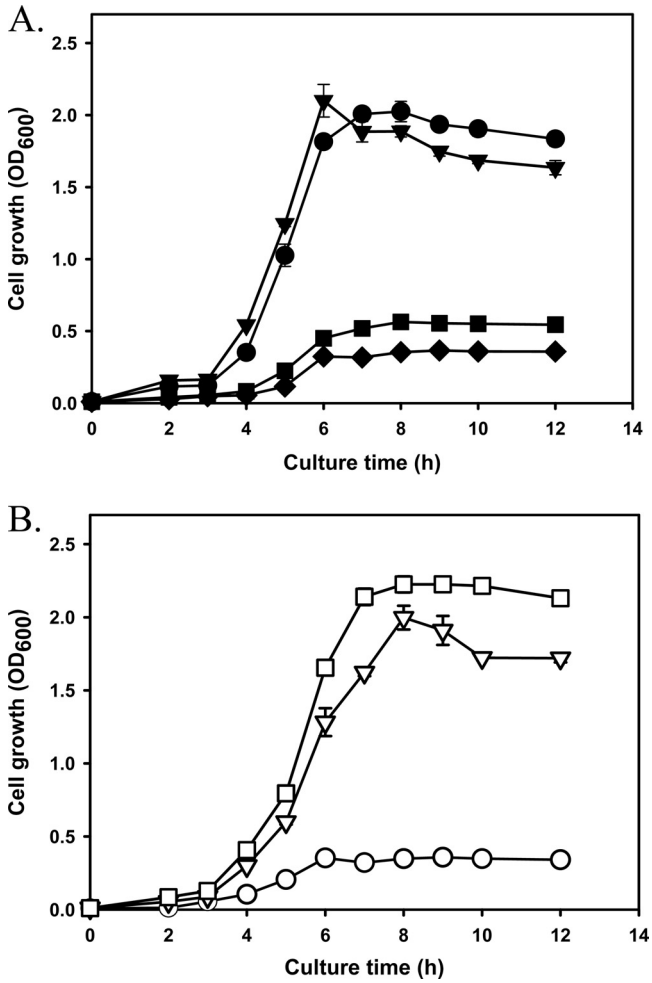

FIG 2 The *bgaA* and *bgaB* genes influence *M. succiniciproducens* growth. (A) Cell growth of the wild-type strain MBEL55E (filled circles), the *bgaA*-deletion strain EL101 (filled squares), the *bgaB*-deletion strain EL102 (filled inverted triangles), and the *bgaA bgaB* double-gene-deletion strain EL103 (filled diamonds). (B) Complementation of growth in the *bgaA bgaB* double-gene-deletion strains EL104 harboring an empty vector (open circles), EL105 harboring pMS3-bgaA (open squares), and EL106 harboring pMS3-bgaB (open inverted triangles). All strains were cultivated in a modified MH3 medium supplemented with 0.5% lactose as the sole carbon source.

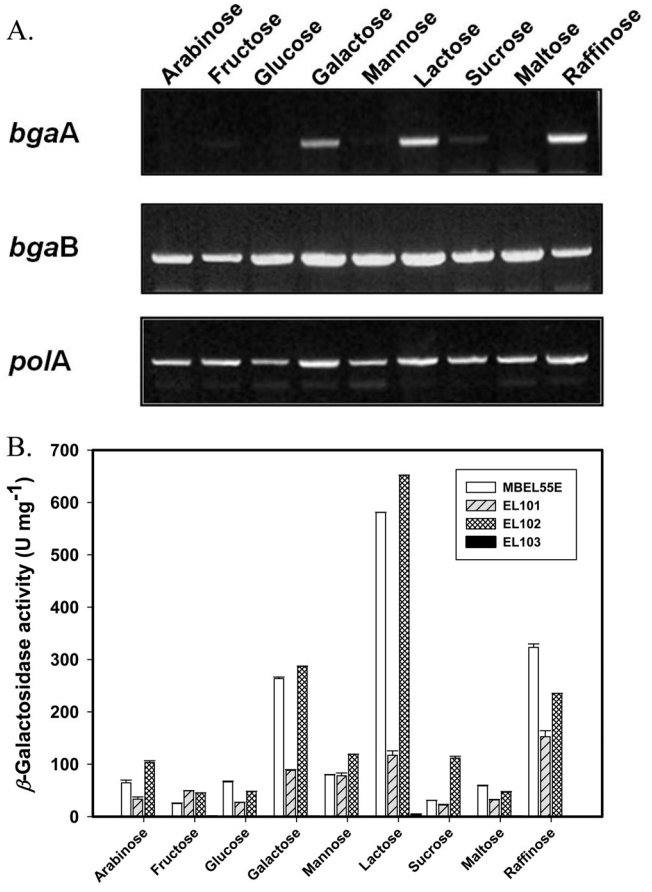

FIG 3 Semiquantitative RT-PCR analysis of the regulation of *bgaA* and *bgaB* gene transcriptions during growth on various carbon sources of *M. succiniciproducens* (A) and β-galactosidase activities of the corresponding cell lysates (B). (A) Ten micrograms of total RNA was used as the template in each experiment. Cells were harvested after 20 h of growth, after being transferred to media containing 5% (wt/vol) of the indicated carbon sources. The RT-PCR product of *polA* served as a control. (B) The specific enzyme activities were measured from cells that were grown to the mid-exponential phase in a modified MH3 medium containing each carbon source. The enzyme assay was carried out in triplicate (± standard error), using ONPG as a substrate, under the standard method described in Materials and Methods.

cells cultured on medium supplemented with galactose, lactose, or raffinose, while its expression levels with other carbon sources were very low or undetectable under the conditions used in this study. In contrast, the expression of the *MsbgaB* gene was detected at a similar level in all cells cultured on medium supplemented with each sole carbon source. The expression of the *MsbgaA* gene was therefore strongly induced by galactose, lactose, and raffinose, suggesting a carbon-source-dependent manner of regulation, while the *MsbgaB* gene was expressed constitutively independently of the type of carbon source.

For a further confirmation of the carbon-source-dependent expressions of the *MsbgaA* and *MsbgaB* genes, the β-galactosidase activities were measured in the single-gene-deletion or the double-gene-deletion mutant strains (Fig. 3B). As expected, no β-galactosidase activity was observed in the double-gene-deletion strain, but other tested strains exhibited variable ranges of β-galactosidase activity depending on the carbon source. In the wild-type cells, relatively higher activities were observed when cultivated in the presence of galactose, lactose, or raffinose than in the presence of the other carbon sources. In the lactose-supplemented medium, the enzyme activity was highest, while in the galactose medium, the enzyme activity decreased by nearly 50%. In the Δ*bgaB* mutant strain, the activity levels and expression patterns of β-galactosidase were quite similar to those of the wild-type strain. However, in the Δ*bgaA* mutant strain, the β-galactosidase activities decreased dramatically compared to those in the wild-type strain and the carbon-source-dependent expression patterns were also changed. These observations, based on both RT-PCR analysis and β-galactosidase activity assay of wild-type and mutant strains, indicated that BgaA was mainly responsible for the β-galactosidase activity in the wild-type *M. succiniciproducens* strain under most of our experimental conditions, whereas BgaB contributed to a lesser extent. Furthermore, it suggests that MsBgaA is involved in lactose-related metabolism and that MsBgaB is involved in some lactose-independent function in the cell.

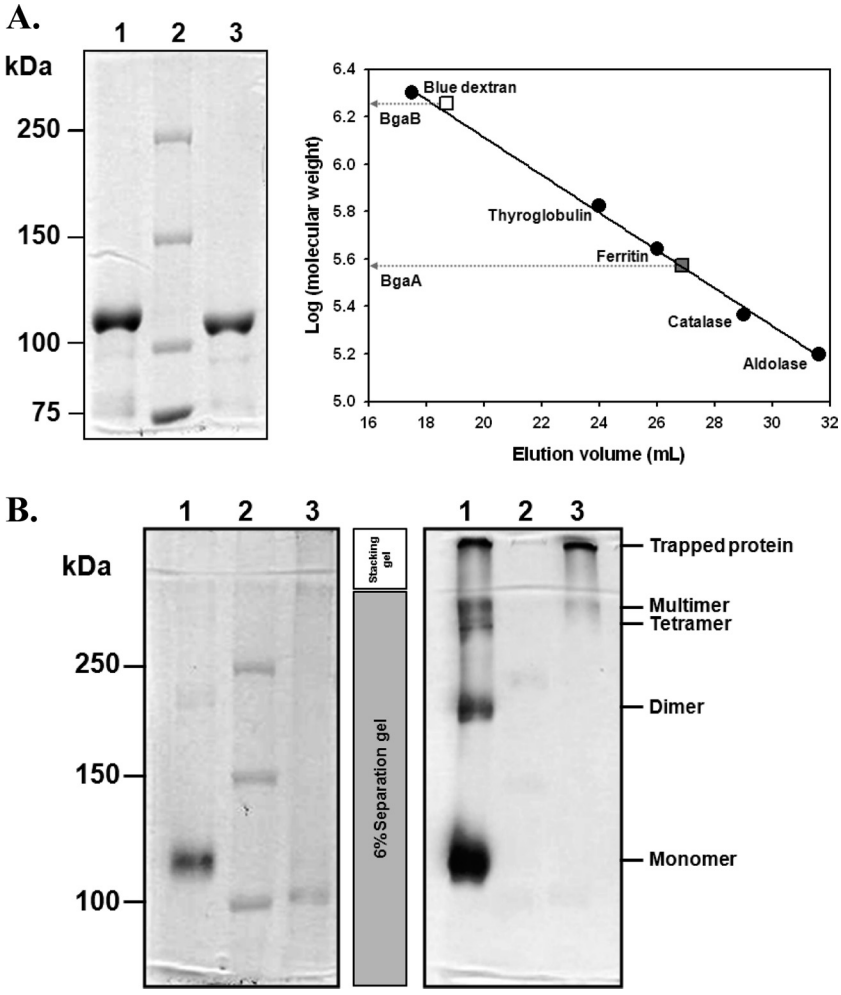

FIG 4 Purified recombinant MsBgaA and MsBgaB proteins (A) and their β-galactosidase activities (B). (A) Purified recombinant MsBgaA and MsBgaB proteins (left) and their molecular masses estimated by size-exclusion chromatography (right). The eluates from Sephacryl S-300 chromatography were analyzed by 6% SDS-PAGE, which was followed by Coomassie brilliant blue G-250 staining. Lane 1, MsBgaA; lane 2, molecular mass marker; lane 3, MsBgaB. The molecular masses of BgaA and BgaB were calculated from the retention times of the peak absorbance by calibration standards (aldolase, 158 kDa; catalase, 232 kDa; ferritin, 440 kDa; thyroglobulin, 669 kDa; blue dextran, 2,000 kDa) using an HMW gel filtration calibration kit (GE Healthcare). (B) The purified proteins were analyzed by 6% native PAGE, followed by the staining mentioned above (left). In-gel β-galactosidase activity staining was performed by the native gel incubated with 0.04% X-gal for 2 h at 37°C (right). Lane 1, MsBgaA; lane 2, molecular mass marker; lane 3, MsBgaB.

Cloning, expression, and purification of recombinant MsBgaA and MsBgaB. To obtain recombinant BgaA and BgaB proteins, *bgaA* and *bgaB* genes amplified from *M. succiniciproducens* genomic DNA by PCR were cloned into the pET28a vector under the T7 promoter and expressed in *E. coli* BL21(DE3) at 37°C. Unfortunately, the recombinant proteins were produced in insoluble forms at this temperature. To produce soluble proteins, the induction temperature was decreased to 18°C. After 28 h of induction, both MsBgaA and MsBgaB proteins were successfully produced in soluble forms. We employed this condition to produce the recombinant proteins to characterize their biochemical properties. The N-terminal hexahistidine-fused recombinant MsBgaA and MsBgaB proteins were purified from the cell lysates of recombinant *E. coli* by two-step column chromatography using a nickel-nitrilotriacetic acid affinity column and a Sephacryl S-300 column.

The molecular masses of the denatured recombinant MsBgaA and MsBgaB proteins, separated by SDS-PAGE, appeared to be approximately the same size, at approximately 110 kDa. In contrast, the molecular masses of the native MsBgaA and MsBgaB were estimated to be 450 ± 75 kDa and 1,807 ± 120 kDa, respectively, according to gel permeation chromatography (Fig. 4A). These results suggest that MsBgaA and MsBgaB in their native conformations are a homotetramer and a homohexadecamer, respectively. However, in staining for hydrolysis activity of a β-galactosidase substrate, X-gal, using a native gel, MsBgaA and MsBgaB displayed different patterns depending on their molecular weight (Fig. 4B): MsBgaA activity was detected at heterogeneous oligomeric forms as a monomer, a dimer, or a tetramer in a native gel. However, MsBgaB did not have any enzyme activity as a monomer form. The multimer forms of MsBgaB in the gel harbored less activity than those of MsBgaA. The MsBgaB trapped in the 4% stacking gel showed strong activity. The molecular weight of MsBgaB would be too high to migrate into a 6% acrylamide separation gel.

Substrate specificities of *M. succiniciproducens* BgaA and BgaB. The relative hydrolysis activities of recombinant MsBgaA

TABLE 1 Relative hydrolysis activity of MsBgaA and MsBgaB toward *o*NP- and *p*NP-glycosides

Substrate	Relative activity[a] (%)	
	MsBgaA	MsBgaB
*o*NP-β-D-galactopyranoside	100.0 ± 0.1	100.0 ± 0.1
*p*NP-β-D-galactopyranoside	242.0 ± 7.1	139.7 ± 4.5
*p*NP-α-L-arabinopyranoside	27.6 ± 0.3	8.4 ± 0.7
*p*NP-β-L-arabinopyranoside	ND	ND
*p*NP-α-L-arabinofuranoside	ND	ND
*p*NP-α-L-fucopyranoside	ND	ND
*p*NP-β-D-fucopyranoside	1.7 ± 0.3	0.4 ± 0.1
*p*NP-α-D-galactopyranoside	ND	ND
*p*NP-α-D-mannopyranoside	ND	ND
*p*NP-β-*N*-acetylglucosaminide	ND	ND
*p*NP-α-*N*-acetylneuraminic acid	ND	ND
*p*NP-β-D-lactopyranoside	ND	ND
*p*NP-β-D-acetyllactosaminide	ND	ND
*p*NP-β-D-cellobioside	ND	ND

[a] The relative hydrolysis activities were determined for purified MsBgaA and MsBgaB after 10 min of incubation with 2 mM (each) *o*NP- or *p*NP-glycoside at 37°C. The ONPG hydrolysis activities of MsBgaA and MsBgaB were regarded as 100%. ND, not detectable.

TABLE 3 Effect of divalent cations and EDTA on the activity of β-galactosidase[a]

Metal ion	Relative enzyme activity[b] (%)	
	MsBgaA	MsBgaB
None[c]	100.0 ± 0.1	100.1 ± 0.1
Mn^{2+}	312.6 ± 3.0	44.3 ± 3.6
Mg^{2+}	170.4 ± 0.6	454.9 ± 35.2
Ca^{2+}	35.5 ± 1.5	79.1 ± 6.3
Fe^{2+}	16.6 ± 1.7	5.6 ± 0.6
Co^{2+}	24.9 ± 1.5	10.8 ± 0.6
Ni^{2+}	38.9 ± 1.4	36.5 ± 3.3
Cu^{2+}	2.9 ± 0.7	9.4 ± 1.0
Zn^{2+}	34.9 ± 0.6	16.1 ± 0.2
Cd^{2+}	15.6 ± 0.4	9.2 ± 1.0
Hg^{2+}	ND	0.4 ± 0.1
EDTA	1.6 ± 0.7	4.8 ± 0.8

[a] The purified protein (10 μg ml^{-1}) was preincubated with 1 mM each metal solution for 0.5 h at 37°C. Enzyme activity was assayed as described in Materials and Methods.
[b] The ONPG hydrolysis activities of MsBgaA and MsBgaB preincubated in a buffer without the addition of any metal ions or EDTA were regarded as 100%. ND, not detectable.
[c] The purified protein was preincubated with 10 mM EDTA for 12 h and then dialyzed in high-pressure liquid chromatography-grade water overnight.

and MsBgaB proteins toward various *o*NP- or *p*NP-conjugated glycosides were analyzed and compared to the hydrolysis activity toward ONPG. Both enzymes showed hydrolysis activities toward ONPG, PNPG, *p*NP-α-L-arabinopyranoside, and *p*NP-β-D-fucopyranoside among the tested *o*NP- and *p*NP-conjugated substrates (Table 1). Interestingly, the hydrolysis activities of the two enzymes were much higher on the *p*NP substrate than on the *o*NP substrate: the activities of MsBgaA and MsBgaB on PNPG were approximately 2.4 and 1.4 times higher, respectively, than those on ONPG. In addition, a comparative analysis of the hydrolysis activities on the substrates showed that MsBgaA was more active than MsBgaB (Table 1).

To compare the hydrolysis activity between MsBgaA and MsBgaB, the apparent kinetic parameters were measured using ONPG, PNPG, lactose, lactulose, or arabinogalactan as a β-galactosidase substrate. After an enzyme reaction, the amounts of *o*-nitrophenol or *p*-nitrophenol released were measured by a spectrophotometer at 420 nm. The amounts of galactose hydrolyzed from lactose or lactulose by the β-galactosidases were determined by a galactose dehydrogenase enzyme assay. The kinetic parameters of MsBgaA and MsBgaB are summarized in Table 2. *M. succiniciproducens* BgaA and BgaB can hydrolyze lactose and lactulose as the substrate. The substrate preference of these β-galactosidases is PNPG ≫ ONPG > lactose. Interestingly, these two β-galactosidases showed different substrate specificities: the MsBgaA protein displays 20.9 times higher hydrolysis activity toward lactose than the MsBgaB protein, whereas the MsBgaB protein shows 2.7 times higher enzyme activity toward lactulose than the MsBgaA protein. Although both enzymes harbor β-galactosidase activities, their catalytic efficiency (k_{cat}/K_m) values imply that MsBgaA and MsBgaB have different roles as regards the hydrolysis of their preferable substrates in the cells.

Physicochemical properties of *M. succiniciproducens* BgaA and BgaB. It was reported that monovalent or divalent cations influence the hydrolysis activity of β-galactosidase (20). The effects of divalent cations on the MsBgaA and MsBgaB activity levels were analyzed by measuring the ONPG hydrolysis activities of each enzyme after preincubation in 100 mM sodium phosphate buffer (pH 8.0) at 37°C for 1 h in the absence or presence of 1 mM each divalent ion (Table 3). Individual metal ions influenced the substrate hydrolysis activities of MsBgaA and MsBgaB differently: the MsBgaA activity was enhanced by Mn^{2+} and Mg^{2+}, and the MsBgaB activity was enhanced by Mg^{2+} but decreased by Mn^{2+}. Interestingly, the catalytic activity of MsBgaA was increased by threefold in the presence of Mn^{2+}, while Mg^{2+} increased the ac-

TABLE 2 Apparent kinetic parameters of MsBgaA and MsBgaB

Substrate	MsBgaA			MsBgaB		
	K_m (mM)	V_{max} (μmol min^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	V_{max} (μmol min^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
ONPG[a]	1.3 ± 0.2	8.8 ± 0.5[a]	2,741.3 ± 161.3	1.1 ± 0.2	6.2 ± 0.4[a]	535.8 ± 34.6
PNPG[a]	0.7 ± 0.1	12.5 ± 0.5[a]	7,387.2 ± 326.7	2.4 ± 0.7	11.0 ± 1.5[a]	732.2 ± 100.9
Lactose[b]	25.5 ± 2.0	15.8 ± 0.4[b]	46.9 ± 1.2	170.3 ± 32.0	5.5 ± 0.6[b]	2.3 ± 0.3
Lactulose[b]	181.6 ± 27.8	9.0 ± 0.6[b]	3.8 ± 0.3	112.4 ± 16.2	17.5 ± 0.8[b]	10.0 ± 0.5
Arabinogalactan[b]	ND[c]	ND	ND	ND	ND	ND

[a] The amount of nitrophenol liberated from *o*NP- or -*p*NP-β-galactopyranoside was measured at 420 nm by a spectrophotometer (ε = 4.5 mM^{-1} cm^{-1}).
[b] The amount of galactose hydrolyzed from lactose and lactulose was determined by a galactose dehydrogenase enzyme assay (ε = 6.22 mM^{-1} cm^{-1}).
[c] ND, not detectable.

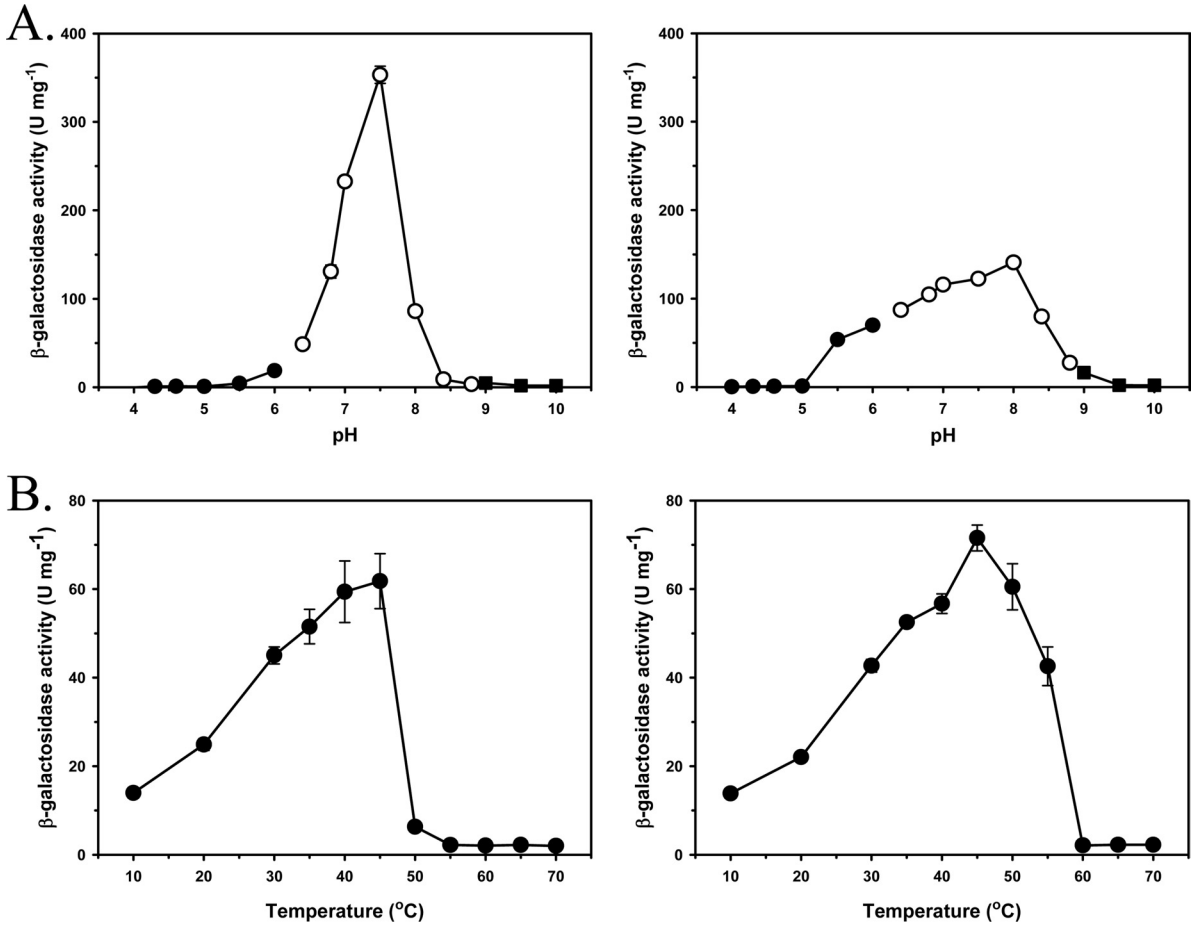

FIG 5 Effect of pH (A) and temperature (B) on activities of MsBgaA and MsBgaB. (A) Effect of the pH on the galactosidase activities of MsBgaA (left) and MsBgaB (right). The enzyme assay was carried out in 100 mM sodium acetate buffer at pH 4.0 to 6.0 (filled circles), 100 mM potassium phosphate buffer at pH 6.4 to 8.8 (open circles), and 100 mM sodium carbonate-borate buffer at pH 9.0 to 10.0 (filled squares). The hydrolysis activities were determined using ONPG as a substrate. (B) Optimum temperature of the hydrolysis activities of MsBgaA (left) and MsBgaB (right). The temperature profiles for activities of the β-galactosidases (10 μg ml^{-1} MsBgaA or 10 μg ml^{-1} MsBgaB in 100 mM sodium phosphate buffer, pH 8.0) were determined at between 10°C and 70°C. At each temperature, the enzyme activities were determined using ONPG as the substrate under the standard method described in Materials and Methods.

tivity of MsBgaB by 4.5 times the original level. On the other hand, most metal ions, except Mn^{2+} and Mg^{2+}, had a negative effect on the activities of both enzymes. Moreover, of the metal ions tested, the Cu^{2+} and Hg^{2+} ions resulted in a significant loss of enzyme activity. In addition, the monovalent metal ions Na^{+} and K^{+} did not have a positive effect on the catalytic activities of MsBgaA and MsBgaB (data not shown). The activities of the enzymes treated with EDTA were less than 5% of the control levels.

Thiol compounds or other reagents for specific modification of amino acid residues have effects on the β-galactosidase activity (7). The residual activities on ONPG were measured after treatment with 1 mM β-mercaptoethanol, dithiothreitol (DTT), glutathione, iodoacetamide, diethylenepryocarbonate, PMSF, or *p*-hydroxymercuribenzoic acid in an enzyme solution. β-Mercaptoethanol and DTT had a slightly negative effect on BgaA and BgaB and resulted in 10% and 5% reductions of their residual activities, respectively. Glutathione and iodoacetamide did not affect the catalytic activity of either enzyme. On the other hand, *p*-hydroxymercuribenzoic acid, a reagent for irreversible thiol-group modification in an amino acid, strongly inhibited the hydrolysis activities of BgaA and BgaB, decreasing their activities by more than 90% (data not shown). It seems that both BgaA and BgaB have some amino acid residues with the SH group essential for catalytic activity.

The effect of the pH on the MsBgaA and MsBgaB activities was examined at 37°C in 100 mM sodium acetate buffer (pH 4.0 to 6.0), 100 mM potassium phosphate buffer (pH 6.4 to 8.8), and 100 mM sodium carbonate-borate buffer (pH 9.0 to 10.0) using ONPG as a substrate. Two β-galactosidases showed optimal hydrolysis activities in the neutral or slightly alkaline pH ranges between 7.0 and 8.0 (Fig. 5A). MsBgaA displayed enzyme activity around the neutral pH range of 6.4 to 8.0, whereas MsBgaB maintained high activity over a broad pH range of 5.5 to 9.0.

The effect of the temperature on the MsBgaA and MsBgaB activity was tested with 10 μg ml^{-1} of each enzyme in 100 mM sodium phosphate buffer (pH 8.0) using 10 mM ONPG as a substrate. The activities were measured in the range of 10°C to 70°C. The enzymes showed the highest activities at 45°C (Fig. 5B). The ONPG hydrolysis activities of both enzymes showed a gradual increase when the reaction temperature was increased to 45°C. Both the MsBgaA and MsBgaB β-galactosidases, however, were deactivated at a temperature higher than 45°C. Moreover, the

MsBgaA activity decreased dramatically in the range of 45°C and 50°C, whereas the MsBgaB activity decreased gradually in the range of 45°C and 60°C.

DISCUSSION

β-Galactosidases belong to glycoside hydrolase (GH) families 1, 2, 35, and 42 in the CAZy database (3). According to the amino acid sequences and the protein structure prediction, BgaA and BgaB from *M. succiniciproducens* share the highest homologies with the β-galactosidases (EC 3.2.1.23) in the GH-2 family, which also contains β-mannosidases (EC 3.2.1.25), β-glucuronidases (EC 3.2.1.31), mannosylglycoprotein endo-β-mannosidases (EC 3.2.1.152), and exo-β-glucosaminidases (EC 3.2.1.165). The hydrolysis activities of *M. succiniciproducens* BgaA and BgaB are comparable to those of other microbial β-galactosidases (32). Although many β-galactosidases harbor broad substrate specificities toward *p*NP- or *o*NP-conjugated glycoside substrates, the *M. succiniciproducens* BgaA and BgaB show rather specific hydrolysis activities toward ONPG and PNPG (Table 1). Many β-galactosidases have higher specific hydrolysis activity toward ONPG than PNPG (22). However, MsBgaA and MsBgaB show higher activity toward PNPG than toward ONPG. In addition, both enzymes show low hydrolysis activities toward *p*NP-α-L-arabinopyranoside as well as *p*NP-β-D-fucopyranoside (Table 1). These substrate promiscuities of the β-galactosidases have been observed with corresponding enzymes of several bacteria and plants: *Clostridium cellulovorans* BgaA, *Sterigmatomyces elviae* β-galactosidase, bell pepper (*Capsicum annuum*) PBG1, and mung bean (*Vigna radiate*) β-galactosidases are able to hydrolyze *p*NP-α-L-arabinopyranoside and *p*NP-β-D-fucopyranoside (22, 24, 30, 31).

The well-known organism *E. coli* also harbors two β-galactosidase paralogues: one is the lactose-inducible β-galactosidase (LacZ), and the other is the evolved β-galactosidase (Ebg) (9, 10). The *E. coli* Ebg protein shows characteristics different from those of the classic β-galactosidase, LacZ (2, 11, 12, 13, 27): (i) Ebg β-galactosidase has insufficient catalytic activity toward lactose compared to the LacZ enzyme. In addition, the K_m value of Ebg toward the synthetic substrate ONPG is four times higher than that of LacZ. (ii) The enzyme activity of Ebg requires potassium but is inactivated by sodium. (iii) The heteromultimeric Ebg enzyme has a higher molecular weight than the homotetrameric LacZ enzyme. (iv) The Ebg enzyme is unable to convert lactose to allolactose, which is the natural inducer for the *lac* operon.

Like *E. coli* LacZ and Ebg β-galactosidases, the apparent kinetic parameters of both MsBgaA and MsBgaB for the hydrolysis of lactose, a natural substrate for β-galactosidase, are similar to each other. MsBgaA shows high hydrolysis activity toward lactose, whereas MsBgaB shows very poor activity toward the same substrate. The k_{cat}/K_m value of MsBgaA toward lactose was 20.9 times higher than that of MsBgaB. However, MsBgaA showed relatively low hydrolysis activity toward lactulose, whereas MsBgaB showed very high activity toward the same substrate. The k_{cat}/K_m value of MsBgaB toward lactulose was 2.7 times higher than that of MsBgaA. In terms of the substrate specificities in both enzymes, these data would imply that BgaA is an enzyme harboring the characteristics of *E. coli* LacZ for lactose hydrolysis and BgaB is an Ebg β-galactosidase-like enzyme in *M. succiniciproducens*.

According to a test of the metal ion effect on the β-galactosidase activity level, each enzyme exhibited a clear preference for metal ions. The ONPG hydrolysis activity of MsBgaA was dramatically enhanced by the Mn^{2+} ion, whereas the activity of MsBgaB was significantly enhanced by addition of the Mg^{2+} ion (Table 3). In contrast to the metal ion effects on the enzyme activities observed in *E. coli* LacZ and EbgA, the potassium ion required for the *E. coli* LacZ and Ebg activities (13, 27) negatively affected the MsBgaA and MsBgaB activities. Sodium ions also decreased the activities of *M. succiniciproducens* enzymes.

Activity straining, SDS-PAGE, and a size-exclusion chromatography analysis of purified recombinant β-galactosidases revealed that MsBgaA and MsBgaB are tetrameric and hexadecameric proteins, respectively (Fig. 4). This feature appears to be common for bacterial cytosolic β-galactosidases with predominantly oligomeric structures (13, 27). It has been reported that secretory bacterial β-galactosidases largely have a monomeric form (22, 32). Interestingly, MsBgaA had a tetrameric form, like *E. coli* LacZ, whereas MsBgaB showed a hexadecameric structure. This suggests that MsBgaA harbors biochemical properties corresponding to those of *E. coli* LacZ. However, unlike *E. coli* EbgA, the molecular weight of MsBgaB is very high and it has yet to be reported in any bacterial β-galactosidases. It therefore still needs to be investigated whether MsBgaB interacts with another protein to form a heteromultimer.

Both β-galactosidases showed the highest level of activity at 45°C (Fig. 5B). Although the MsBgaA activity decreased dramatically when the temperature exceeded 50°C, MsBgaB maintained 83% and 55% activity levels at 50°C and at 55°C, respectively. The optimum temperature of these enzymes, ranging from 40°C to 45°C, is slightly higher than the growth temperature of *M. succiniciproducens* MBEL55E isolated from a bovine rumen maintaining a relatively constant temperature near 39°C (23, 37). Despite the weakly acidic condition (pH 5.3 to pH 6.7) in a bovine rumen, unlike other rumen bacterial enzymes, these enzymes showed the highest hydrolysis activity levels at approximately neutral pH ranges (pH 7.5 to pH 8.0) (1).

The phylogenetic analysis of bacterial β-galactosidases revealed that those enzymes of capnophilic rumen bacteria could be grouped onto a branch (Fig. 1). Furthermore, a comparison of the operons comprising the genes involved in the initial lactose metabolism in rumen bacteria indicated that these bacteria, except for *A. pleuropneumoniae* harboring a gene cluster of the Leloir pathway for the metabolism of galactose (1), could share a similar operon structure which is clearly distinguished from the *lac* operon of *E. coli* (Fig. 6). In the *M. succiniciproducens* genome, the *bgaA* gene (MS0806), encoding a LacZ homologue, is located downstream of MS0807, which encodes a putative lactose transporter. A putative repressor gene (MS0808) encoding the LacI homologue, oriented in the opposite direction, is located upstream of the operon of the MS0807-*bgaA* genes. The intergenic region between MS0808 and MS0807-*bgaA* contains two putative promoters (in Fig. 6, the black box for the forward direction of the MS0807-*bgaA* operon and the gray box for the reverse direction of the MS0808 gene) containing −35 and −10 elements and two ribosome binding sites, AGGA. These putative promoter regions share a homology with the LacI transcript regulator binding site of the *A. succinogenes* 130ZT genome (NCBI accession number CP000746). A dyad symmetry composed of nucleotide sequences between −90 and −109 (TTAAATTTTACAATTGCAAT) and −165 to −183 (ATTGCAATTGTAAAATTTA), which may function as a *cis* regulatory element, was also detected in this intergenic

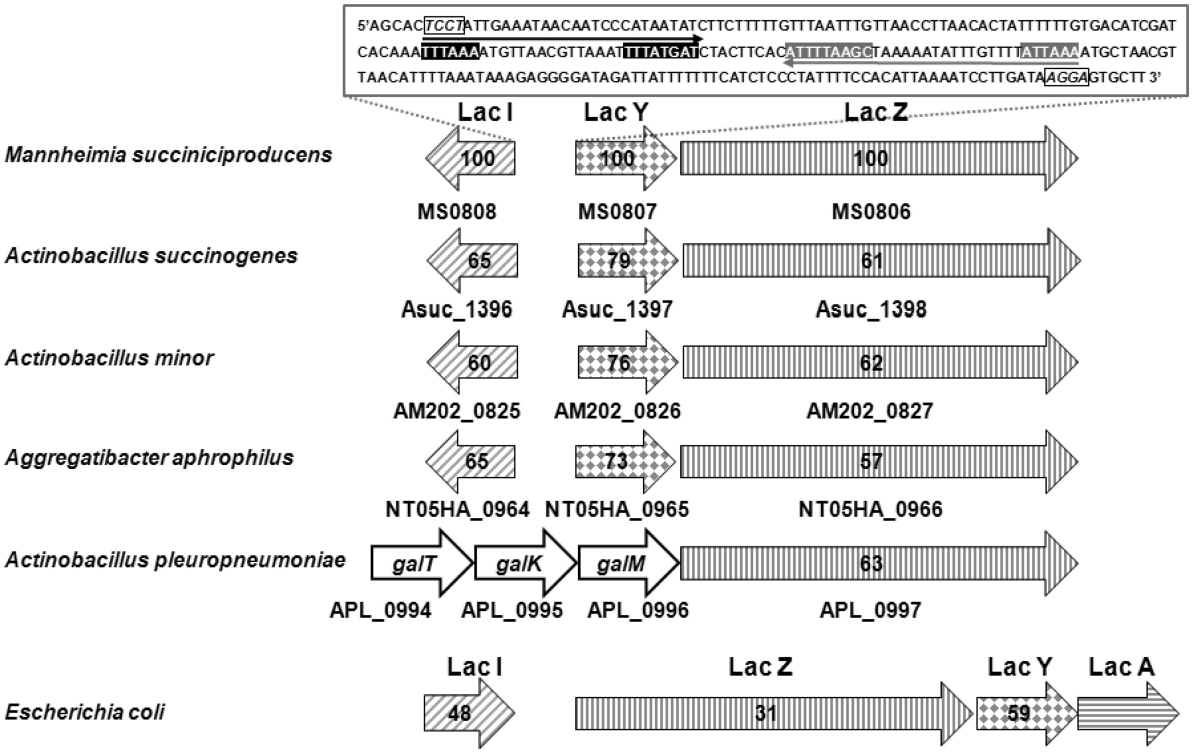

FIG 6 Genomic organization and flanking regions of the putative lactose metabolism gene operon in *M. succiniciproducens* and other lumen bacteria. Arrows represent identified or putative genes and their directions. The numbers in the arrows indicate the amino acid sequence homologies. The enlargement shows the promoter region and the upstream region of the putative genes encoding a lactose transporter protein (LacY) and a lactose repressor (LacI) with promoter (−10 and −35 elements; black and gray boxes) and ribosome binding sites (open boxes). The black and gray arrows indicate the forward and reverse transcription directions, respectively.

region. In addition, the short palindromic repeated sequence CCTCTTTACTCCCGGTAAAGAGG, a putative transcription termination signal, was detected downstream of the termination codon of the *MsbgaA* gene. In *M. succiniciproducens*, these putative genetic elements may indicate that the *bgaA* gene is involved in the initial stage of lactose catabolism. Moreover, it can be expected that the expression of the *bgaA* gene is regulated in a lactose-dependent manner as a part of the MS0807-*bgaA* operon by the action of the putative LacI repressor encoded by MS0808. In fact, it was shown in a reporter gene fusion expression analysis that the expression from the promoter of the MS0807-*bgaA* operon was tightly repressed in the presence of MS0808 and strongly induced by addition of lactose in a culture medium (data not shown). These results indicate that the gene context and regulation of the MS0807-*bgaA* operon in *M. succiniciproducens* may represent a novel regulatory feature for lactose metabolic genes in capnophilic rumen bacteria. Surprisingly, the *MsbgaB* gene was detected as a monocistron with a single promoter and a terminator. The flanking region upstream of *MsbgaB* did not share a homology with binding sequences of any known repressors.

In the present study, we were able to characterize some of the molecular and the biochemical properties of two β-galactosidase paralogues in the capnophilic rumen bacterium *M. succiniciproducens* in comparison with those of *E. coli* enzymes. The results obtained here clearly indicate that the *MsbgaA*-encoded enzyme is involved in lactose metabolism. Nonetheless, the function of the *MsbgaB*-encoded enzyme remains unknown. Further physicochemical and kinetic studies of both wild-type and mutant enzymes will be required for a better understanding of the substrate specificities, tertiary structure formation, and distinct physiological roles of MsBgaA and MsBgaB enzymes.

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