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Glycosulfatase-Encoding Gene Cluster in *Bifidobacterium breve* UCC2003

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ABSTRACT

Bifidobacteria constitute a specific group of commensal bacteria typically found in the gastrointestinal tract (GIT) of humans and other mammals. *Bifidobacterium breve* strains are numerically prevalent among the gut microbiota of many healthy breast-fed infants. In the present study, we investigated glycosulfatase activity in a bacterial isolate from a nursling stool sample, *B. breve* UCC2003. Two putative sulfatases were identified on the genome of *B. breve* UCC2003. The sulfated monosaccharide *N*-acetylglucosamine-6-sulfate (GlcNAc-6-S) was shown to support the growth of *B. breve* UCC2003, while *N*-acetylglucosamine-3-sulfate, *N*-acetylgalactosamine-3-sulfate, and *N*-acetylgalactosamine-6-sulfate did not support appreciable growth. By using a combination of transcriptomic and functional genomic approaches, a gene cluster designated *ats2* was shown to be specifically required for GlcNAc-6-S metabolism. Transcription of the *ats2* cluster is regulated by a repressor open reading frame kinase (ROK) family transcriptional repressor. This study represents the first description of glycosulfatase activity within the *Bifidobacterium* genus.

IMPORTANCE

Bifidobacteria are saccharolytic organisms naturally found in the digestive tract of mammals and insects. *Bifidobacterium breve* strains utilize a variety of plant- and host-derived carbohydrates that allow them to be present as prominent members of the infant gut microbiota as well as being present in the gastrointestinal tract of adults. In this study, we introduce a previously unexplored area of carbohydrate metabolism in bifidobacteria, namely, the metabolism of sulfated carbohydrates. *B. breve* UCC2003 was shown to metabolize *N*-acetylglucosamine-6-sulfate (GlcNAc-6-S) through one of two sulfatase-encoding gene clusters identified on its genome. GlcNAc-6-S can be found in terminal or branched positions of mucin oligosaccharides, the glycoprotein component of the mucous layer that covers the digestive tract. The results of this study provide further evidence of the ability of this species to utilize mucin-derived sugars, a trait which may provide a competitive advantage in both the infant gut and adult gut.

The genus *Bifidobacterium* represents one of the major components of the intestinal microbiota of breastfed infants (1–5) while also typically constituting between 2% and 10% of the adult intestinal microbiota (6–11). Bifidobacteria are saccharolytic microorganisms whose ability to colonize and survive in the large intestine is presumed to depend on the ability to metabolize complex carbohydrates present in this environment (12, 13). Certain bifidobacterial species, including *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium adolescentis*, and *Bifidobacterium breve*, utilize a range of plant/diet-derived oligosaccharides such as raffinose, arabinoxylan, galactan, and cellodextrins (14–20). Bifidobacterial metabolism of human milk oligosaccharides (HMOs) is also well described, with the typically infant-derived species *B. longum* subsp. *infantis* and *Bifidobacterium bifidum* being particularly well adapted to utilizing these carbon sources in the infant gut (21–23). However, the ability to utilize mucin, the glycoprotein component of the mucous layer that covers the epithelial cells of the gastrointestinal tract, is limited to members of the *B. bifidum* species (21, 24). Approximately 60% of the predicted glycosyl hydrolases encoded by *B. bifidum* PRL2010 are predicted to be involved in mucin degradation, most of which are conserved exclusively within the *B. bifidum* species (21).

Host-derived glycoproteins such as mucin and proteoglycans (e.g., chondroitin sulfate and heparan sulfate), which are found in the colonic mucosa and/or human milk, are often highly sulfated (25–29). Human colonic mucin is heavily sulfated, which is in contrast to mucin from the stomach or small intestine, the presumed purpose of which is to protect mucin against degradation by bacterial glycosidases (30–32). Despite this apparent protective measure, glycosulfatase activity has been identified in various members of the gut microbiota, e.g., *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, and *Prevotella* sp. strain RS2 (33–38).

Prokaryotic and eukaryotic sulfatases uniquely require a 3-oxoalanine (typically called C_α-formylglycine or FGly) residue at their active site (39–41). Prokaryotic sulfatases carry either a conserved cysteine (Cys) or a serine (Ser) residue, which requires posttranslational conversion to FGly in the cytosol in order to convert the enzyme to an active state (42–44). In bacteria, two

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant feature(s)	Reference or source
Strains		
Escherichia coli		
EC101	Cloning host; *repA*$^+$ *kmr*	92
EC101-pNZ-M.Bbrll+Bbr1	EC101 harboring a pNZ8048 derivative containing the methyltransferase-encoding genes *bbrIIM* and *bbrIIIM* from *B. breve* UCC2003	57
XL1-Blue	*supE44 hsdR17 recA1 gyrA96 thi relA1 lac* F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(Tet^r)]	Stratagene
XL1-Blue-pBC1.2-atsProm	XL1-Blue harboring pBC1.2-atsProm	This study
L. lactis		
NZ9000	MG1363 *pepN*::*nisRK*; nisin-inducible overexpression host	65
NZ9000-pNZ8048	NZ9000 containing pNZ8048	This study
NZ9000-pNZ-atsR2	NZ9000 containing pNZ-atsR2	This study
NZ97000	Nisin A-producing strain	65
B. breve		
UCC2003	Isolate from a nursling stool sample	58
UCC2003-atsR2	pORI19-tetW-atsR2 insertion mutant of *B. breve* UCC2003	This study
UCC2003-atsT	pORI19-tetW-atsT insertion mutant of *B. breve* UCC2003	This study
UCC2003-atsA2	pORI19-tetW-atsA2 insertion mutant of *B. breve* UCC2003	This study
UCC2003-atsR2-pBC1.2-atsProm	pORI19-tetW-atsR2 insertion mutant of UCC2003 containing pBC1.2-atsProm	This study
Plasmids		
pAM5	pBC1-pUC19-Tc^r	64
pNZ8048	Cm^r; nisin-inducible translational fusion vector	65
pNZ-atsR2	Cm^r; pNZ8048 derivative containing a translational fusion of an *atsR2*-containing DNA fragment to a nisin-inducible promoter	This study
pORI19	Em^r *repA*-negative *ori*$^+$; cloning vector	92
pORI19-tetW-atsR2	Internal 408-bp fragment of *atsR2* and *tetW* cloned into pORI19	This study
pORI19-tetW-atsT	Internal 416-bp fragment of *atsT* and *tetW* cloned into pORI19	This study
pORI19-tetW-atsA2	Internal 402-bp fragment of *atsA2* and *tetW* cloned into pORI19	This study
pBC1.2	pBC1-pSC101-Cm^r	64
pBC1.2-atsProm	AtsR2 promoter region cloned into pBC1.2	This study

distinct systems have been described for the posttranslational modification of sulfatase enzymes. In *Mycobacterium tuberculosis*, the conversion of the Cys_{58} residue to FGly is catalyzed by an FGly-generating enzyme (FGE), which requires oxygen as a cofactor (45). In *Klebsiella pneumoniae*, the conversion of the Ser_{72} residue of the *atsA*-encoded sulfatase is catalyzed by the AtsB enzyme, which is a member of the *S*-adenosyl-L-methionine (AdoMet)-dependent family of radical enzymes (43, 46). Similar enzymes have also been characterized from *Clostridium perfringens* and *Ba. thetaiotaomicron*, which are active on both Cys- and Ser-type sulfatases (37, 38, 47). Crucially, these enzymes are active under anaerobic conditions and were thus designated anaerobic sulfatase-maturing enzymes (anSMEs) (38). Sulfatase activity has yet to be described for bifidobacteria. In the present study, we identify two predicted sulfatase- and anSME-encoding gene clusters in *B. breve* UCC2003 (and other *B. breve* strains) and demonstrate that one such cluster is required for the metabolism of the sulfated monosaccharide *N*-acetylglucosamine-6-sulfate (GlcNAc-6-S).

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *B. breve* UCC2003 was routinely cultured in reinforced clostridial medium (RCM; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). Carbohydrate utilization by bifidobacteria was examined by using modified De Man-Rogosa-Sharpe (mMRS) medium made from first principles (48), excluding a carbohydrate source, supplemented with 0.05% (wt/vol) L-cysteine HCl (Sigma-Aldrich, Steinheim, Germany) and a particular carbohydrate source (0.5%, wt/vol). The carbohydrates used were lactose (Sigma-Aldrich), GlcNAc-6-S (Dextra Laboratories, Reading, United Kingdom) (see below), *N*-acetylglucosamine-3-sulfate (GlcNAc-3-S), *N*-acetylgalactosamine-3-sulfate (GalNAc-3-S), and *N*-acetylgalactosamine-6-sulfate (GalNAc-6-S) (see below). In order to determine bacterial growth profiles and final optical densities, 10 ml of freshly prepared mMRS medium, supplemented with a particular carbohydrate, was inoculated with 100 μl (1%) of a stationary-phase culture of a particular strain. Uninoculated mMRS medium was used as a negative control. Cultures were incubated anaerobically for 24 h, and the optical density at 600 nm (OD_{600}) was recorded. Bifidobacterial cultures were incubated under anaerobic conditions in a modular atmosphere-controlled system (Davidson and Hardy, Belfast, Ireland) at 37°C. *Escherichia coli* was cultured in Luria-Bertani broth (LB) at 37°C with agitation (49). *Lactococcus lactis* strains were grown in M17 medium supplemented with 0.5% (wt/vol) glucose at 30°C (50). Where appropriate, growth media contained tetracycline (Tet) (10 μg ml^{-1}), chloramphenicol (Cm) (5 μg ml^{-1} for *E. coli* and *L. lactis* and 2.5 μg ml^{-1} for *B. breve*), erythromycin (Em) (100 μg ml^{-1}), or kanamycin (Kan) (50 μg ml^{-1}). Recombinant *E. coli* cells containing pORI19 were selected on LB agar containing Em and Kan and supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg ml^{-1}) and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

Chemical synthesis of sulfated monosaccharides. In brief, the 6-*O*-sulfated GlcNAc structure (structure 1) (Fig. 1A) was synthesized in four steps from GlcNAc in an overall 40% yield, while the other three target structures, 3-*O*-sulfated GlcNAc structure 2 (Fig. 1A), 3-*O*-sulfated GalNAc, and 6-*O*-sulfated GalNAc (structures 3 and 4, respectively) (Fig. 1B), were synthesized from their corresponding benzyl β-glycoside

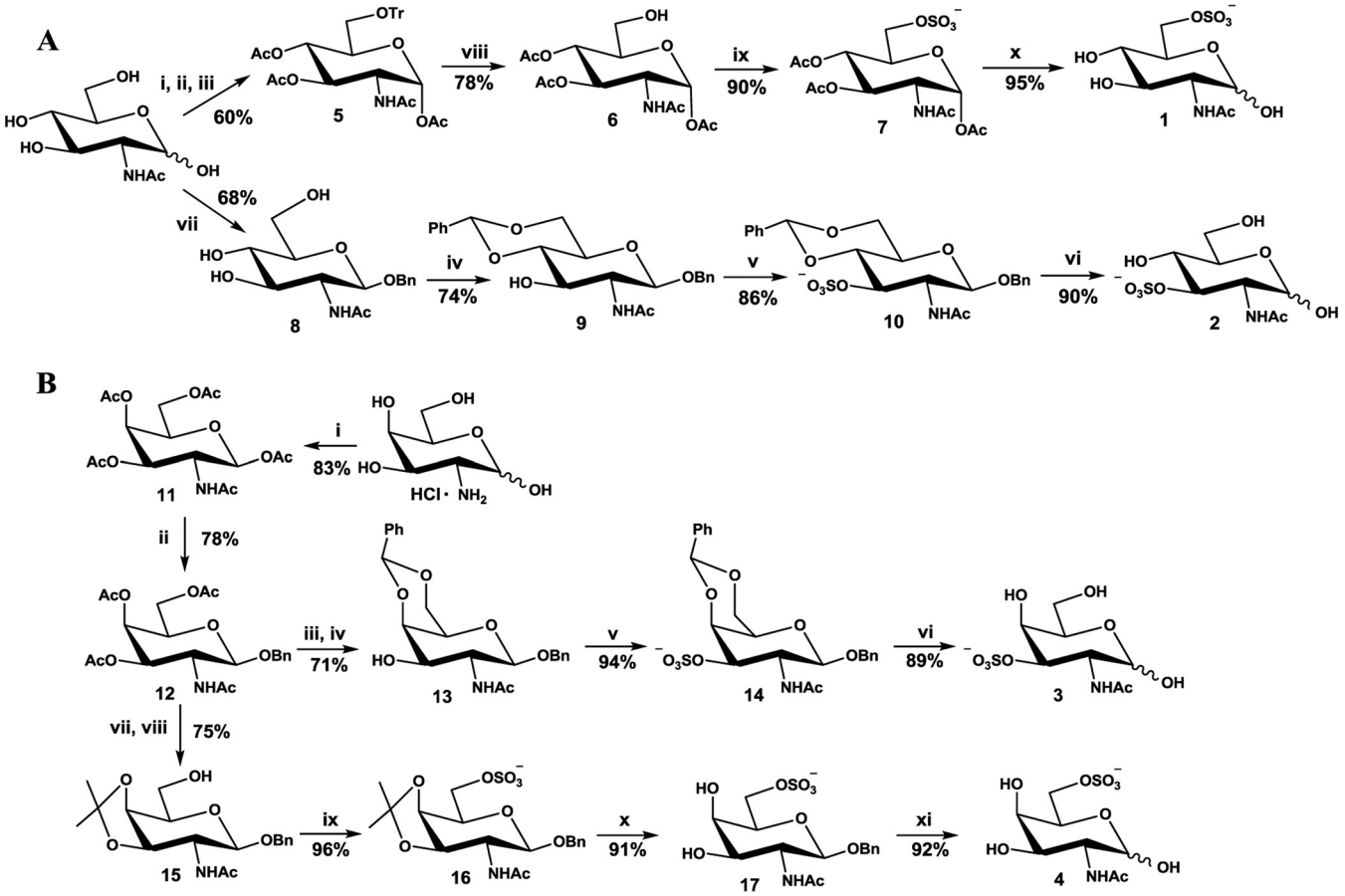

FIG 1 (A) Synthesis of 6-*O*- and 3-*O*-sulfate-2-acetamido-2-deoxy-D-glucose (structures 1 and 2). (i) BnBr, NaH, LiBr, and DMF; (ii) Ac_2O and Py; (iii) NaOMe and methanol (MeOH); (iv) $PhCH(OMe)_2$ and HCOOH; (v) $SO_3{\cdot}NEt_3$ and Py at 85°C; (vi) 10% palladium on carbon (Pd/C), ethanol (EtOH), and 1,500 kPa H_2; (vii) TrCl, $CaSO_4$, and Py at 100°C (structure 1) and Ac_2O (structure 2); (viii) AcOH and HBr; (ix) $SO_3{\cdot}NEt_3$ and DMF at 55°C; (x) NaOMe and MeOH. (B) Synthesis of 3-*O*- and 6-*O*-sulfate-2-acetamido-2-deoxy-D-galactose (structures 3 and 4). (i) Ac_2O and Py; (ii) BnOH, $BF_3{\cdot}OEt_2$, CH_2Cl_2, 3 Å molecular sieve; (iii) NaOMe and MeOH; (iv) $PhCH(OMe)_2$ and HCOOH; (v) $SO_3{\cdot}NEt_3$ and DMF at 55°C; (vi) 10% Pd/C, EtOH, and 1,500 kPa H_2; (vii) NaOMe and MeOH; (viii) $Me_2C(OMe)_2$, p-toluenesulfonic acid (p-TSA), and DMF at 65°C; (xi) $SO_3{\cdot}NEt_3$ and DMF at 55°C; (x) CF_3COOH and H_2O; (xi) 10% Pd/C, EtOH, and 1,000 kPa H_2.

(structures 8 and 12) (Fig. 1A and B) in three or four steps, with an overall yield of about 60%. The benzyl glycoside was obtained either by direct alkylation of a hemiacetal (structure 8, GlcNAc) (Fig. 1A) or by glycosylation of a peracetylated precursor (structure 12, GalNAc) (Fig. 1B). Sulfations were performed by using a $SO_3{\cdot}NEt_3$ complex in pyridine or dimethylformamide (DMF) (yields, 86 to 96%). Direct regioselective 6-*O*-tritylation of GlcNAc followed by *in situ* acetylation afforded compound 5, from which the trityl group was removed by using aqueous acetic acid, without any acetyl migration being detected, to yield the 6-OH derivative compound 6, the sulfation of which gave compound 7, which was subsequently deacetylated under Zemplen conditions to afford target structure 1 (Fig. 1A). Benzylidenation of compounds 8 and 12 gave 3-OH compounds 9 and 13, respectively. Sulfation (resulting in structures 10 and 14) followed by deprotection through catalytic hydrogenolysis yielded target structures 2 and 3. Isopropylidenation of compound 12 gave 6-OH compound 15, which was sulfated (resulting in structure 16) and then deprotected through acetal hydrolysis (resulting in structure 17) followed by catalytic hydrogenolysis to afford target structure 4 (Fig. 1). The experimental methods are described in further detail in the supplemental material.

Nucleotide sequence analysis. Sequence data were obtained from the Artemis-mediated genome annotations of *B. breve* UCC2003 (51, 52). Database searches were performed by using the nonredundant sequence database accessible at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/), using BLAST (53). Sequence analysis was performed by using the SeqBuilder and SeqMan programs of the DNASTAR software package (DNASTAR, Madison, WI, USA). Inverted repeats were identified by using the PrimerSelect program of the DNASTAR software package, and a graphical representation of the identified motifs was obtained by using WebLogo software (54).

DNA manipulations. Chromosomal DNA was isolated from *B. breve* UCC2003 as previously described (55). Plasmid DNA was isolated from *E. coli*, *L. lactis*, and *B. breve* by using the Roche High Pure plasmid isolation kit (Roche Diagnostics, Basel, Switzerland). An initial lysis step was performed by using 30 mg ml^{-1} of lysozyme for 30 min at 37°C prior to plasmid isolation from *L. lactis* or *B. breve* (56). Single-stranded oligonucleotide primers used in this study were synthesized by Eurofins (Ebersberg, Germany) (Table 2). Standard PCRs were performed by using *Taq* PCR master mix (Qiagen GmbH, Hilden, Germany). *B. breve* colony PCRs were carried out as described previously (57). PCR fragments were purified by using the Roche High Pure PCR purification kit (Roche Diagnostics). Electroporation of plasmid DNA into *E. coli*, *L. lactis*, or *B. breve* was performed as previously described (49, 58, 59).

Construction of *B. breve* UCC2003 insertion mutants. Internal fragments of Bbr_0849, designated here *atsR2* (fragment encompassing 408 bp, representing codons 134 through 271 of the 395 codons of this gene);

TABLE 2 Oligonucleotide primers used in this study

Purpose	Primer	Sequence[a]
Cloning of a 408-bp fragment of *atsR2* into pORI19	AtsR2F	GACTAG*AAGCTT*GCCATCACGATCGACGACG
	AtsR2R	TAGCAT*TCTAGA*GCATCCCGGACGTCCACAG
Cloning of a 416-bp fragment of *atsT* into pORI19	AtsTF	GACTAG*AAGCTT*GATCTCCTTCCGCCAGCTC
	AtsTR	TAGCAT*TCTAGA*CGTTGGTGCCGGTCAGCTG
Cloning of a 402-bp fragment of *atsA2* into pORI19	AtsA2F	GACTAG*AAGCTT*GAATACGTCGCCTGGCTCAAG
	AtsA2R	TAGCAT*TCTAGA*CCTCCACTGGTCGTTGTCG
Amplification of *tetW*	TetWF	TCAGCT*GTCGAC*ATGCTCATGTACGGTAAG
	TetWR	GCGACG*GTCGAC*CATTACCTTCTGAAACATA
Confirmation of site-specific homologous recombination	AtsR2confirm	CATCGACACGGCATACTGG
	AtsTconfirm	CATCTTCGGCGCGTTATG
	AtsA2confirm	GGAAACCGACTGGACCTACAC
Cloning of *atsR2* into pNZ8048	AtsR2FOR	TACGTA*CCATGG*TGCATTTCGCATCGG
	AtsR2REV	GCTAGC*TCTAGA*GTGGAATATGCGGTGCGTG
Cloning of the *atsR2* promoter into pBC1.2	AtsRPromF	GTACTA*AAGCTT*CCAGTATGCCGTGTCGATG
	AtsRPromR	TAGCTA*TCTAGA*CGCAATGCCAGAAACTCAGC
IRD-labeled primers	AtsR2R1F	CATCGTGTTATTGGCGCGG
	AtsR2R1R	GACGCCATATCACAGAGGGTTG
	AtsR2R2F	GCATGCGGCGTGAACTCC
	AtsR2R2R	CGCAATGCCAGAAACTCAGC
	AtsR2R3F	GATGTTGCCTTGCGGTATG
	AtsR2R3R	CAACGGCTGCCCACTGG
	AtsR2T1F	GGTCCTCCTTCGTCTGTGTGG
	AtsR2T1R	GTCGTGGCATATCGTTCGG
	AtsR2T2F	GGGCCGACGAAGTTGTTG
	AtsR2T2R	CGATGAGACCGCCGATG
	AtsR2T3F	CTAGCGGCATTCAGTATCGAG
	AtsR2T3R	GCGGCAGAACAGCAGGAAC

[a] Restriction sites incorporated into oligonucleotide primer sequences are indicated in italics.

Bbr_0851, designated *atsT* (fragment encompassing 416 bp, representing codons 149 through 288 of the 476 codons of this gene); and Bbr_0852, designated *atsA2* (fragment encompassing 402 bp, representing codons 148 through 281 of the 509 codons of this gene) were amplified by PCR using *B. breve* UCC2003 chromosomal DNA as a template and primer pairs atsR2F and atsR2R, atsTF and atsTR, and atsA2F and atsA2R, respectively (Table 2). The insertion mutants were constructed as described previously (57). Site-specific recombination of potential Tet-resistant mutants was confirmed by colony PCR using primer combination TetWF and TetWR to verify *tetW* gene integration and primers atsR2confirm, atsTconfirm, and atsA2confirm (positioned upstream of the selected internal fragments of *atsR2*, *atsT*, and *atsA2*, respectively) in combination with primer TetWF to confirm integration at the correct chromosomal location.

Analysis of global gene expression using *B. breve* DNA microarrays. Global gene expression was determined during log-phase growth (OD_{600} of ~0.5) of *B. breve* UCC2003 in mMRS medium supplemented with 0.5% GlcNAc-6-S, and the obtained transcriptome was compared to that obtained from *B. breve* UCC2003 grown in mMRS medium supplemented with 0.5% ribose. Similarly, global gene expression of the insertion mutant strain *B. breve* UCC2003-atsR2 was determined during log-phase growth (OD_{600} of ~0.5) of the mutant in mMRS medium supplemented with 0.5% ribose, and the transcriptome was also compared to that obtained from *B. breve* UCC2003 grown in 0.5% ribose. DNA microarrays containing oligonucleotide primers representing each of the 1,864 identified open reading frames of the genome of *B. breve* UCC2003 were designed and obtained from Agilent Technologies (Palo Alto, CA, USA). RNA was isolated and purified from bifidobacterial cells by using a combination of the "Macaloid" method and the Roche High Pure RNA isolation kit, as previously described (60). The RNA level was quantified spectrophotometrically as described previously by Sambrook et al. (49). Methods for cDNA synthesis and labeling were performed as described previously (61). Hybridization, washing of the slides, and processing of the DNA microarray data were also performed as previously described (62).

Plasmid constructions. For the construction of plasmid pNZ-atsR2, a DNA fragment encompassing the complete coding region of the predicted transcriptional regulator *atsR2* (Bbr_0849) was generated by PCR amplification from chromosomal DNA of *B. breve* UCC2003 using *Pfu*Ultra II DNA polymerase (Agilent Technologies) and primer combination atsR2FOR and atsR2REV (Table 2). The generated amplicon was digested with NcoI and XbaI and ligated into the similarly digested nisin-inducible translational fusion plasmid pNZ8048 (63). The ligation mixture was introduced into *L. lactis* NZ9000 by electrotransformation, and transformants were selected based on Cm resistance. The plasmid content of a number of Cm^r transformants was screened by restriction analysis, and the integrity of positively identified clones was verified by sequencing.

To clone the Bbr_0849 promoter region, a DNA fragment encompassing the intergenic region between the Bbr_0849 and Bbr_0850 genes was generated by PCR amplification employing *B. breve* UCC2003 chromosomal DNA as a template and using PfuUltra II DNA polymerase in combination with primer pair atsRPromF and atsRPromR (Table 2). The PCR

product was digested with HindIII and XbaI and ligated into similarly digested pBC1.2 (64). The ligation mixture was introduced into *E. coli* XL1-Blue by electrotransformation, and transformants were selected based on Tet and Cm resistance. Transformants were checked for plasmid content by restriction analysis, and the integrity of several positively identified recombinant plasmids was verified by sequencing. One of these verified recombinant plasmids, designated pBC1.2-atsProm, was introduced into *B. breve* UCC2003-atsR2 by electrotransformation, and transformants were selected based on Tet and Cm resistance.

Heterologous protein production. For the heterologous expression of AtsR2, 25 ml of M17 broth supplemented with 0.5% (wt/vol) glucose was inoculated with a 2% inoculum of a culture grown overnight for 16 h of *L. lactis* NZ9000 harboring either pNZ-atsR2 or the empty vector pNZ8048 (used as a negative control), followed by incubation at 30°C until an OD_{600} of ~0.5 was reached, at which point protein expression was induced by the addition of the cell-free supernatant of a nisin-producing strain (65), followed by continued incubation for a further 2 h. Cells were harvested by centrifugation, resuspended in 10 mM Tris-HCl (pH 8.0), and disrupted with glass beads in a mini-bead beater (BioSpec Products, Bartlesville, OK). Cellular debris was removed by centrifugation to produce an AtsR2-containing crude cell extract.

Electrophoretic mobility shift assays. DNA fragments representing different portions of each of the promoter regions upstream of the *atsR2* and *atsT* genes were prepared by PCR using 5′ IRDye 700-labeled primer pairs synthesized by Integrated DNA Technologies (Coralville, IA) (Table 2). Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously (66). In all cases, the binding reactions were performed in a final reaction mixture volume of 20 μl in the presence of poly(dI-dC) in binding buffer (20 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], 1 mM EDTA, 50 mM KCl, and 10% glycerol at pH 7.0). Various amounts of the crude cell extract of *L. lactis* NZ9000 containing pNZ-atsR2 or pNZ8048 were mixed on ice with a fixed amount of a DNA probe (0.1 pmol) and subsequently incubated for 30 min at 37°C. Samples were loaded onto a 6% nondenaturing polyacrylamide (PAA) gel prepared in TAE buffer (40 mM Tris-acetate [pH 8.0], 2 mM EDTA) and run in a 0.5× to 2.0× gradient of TAE at 100 V for 120 min in an Atto Mini PAGE system (Atto Bioscience and Biotechnology, Tokyo, Japan). Signals were detected by using an Odyssey infrared imaging system (Li-Cor Biosciences, United Kingdom, Ltd., Cambridge, United Kingdom), and images were captured by using the supplied Odyssey software v3.0. To identify the effector molecule of AtsR2, either GlcNAc or GlcNAc-6-S was added to the binding reaction mixture in concentrations ranging from 2.5 mM to 20 mM.

Primer extension analysis. Total RNA was isolated from exponentially growing cells of *B. breve* UCC2003-atsR2 or *B. breve* UCC2003-atsR2-pBC1.2-atsRProm in mMRS medium supplemented with 0.5% ribose, as previously described (61). Primer extension was performed by annealing 1 pmol of an 5′ IRDye 700-labeled synthetic oligonucleotide to 20 μg of RNA, as previously described (67), using primer AtsR2R1F or AtsR2T1R (Table 2). Sequence ladders of the presumed *atsR2* and *atsT* promoter regions were produced by using the same primer as the one used for the primer extension reaction and a DNA cycle sequencing kit (Jena Bioscience, Germany) and were run alongside the primer extension products to allow precise alignment of the transcriptional start site with the corresponding DNA sequence. Separation was achieved on a 6.5% Li-Cor Matrix KB Plus acrylamide gel. Signal detection and image capture were performed with a Li-Cor sequencing instrument (Li-Cor Biosciences).

Accession number(s). The microarray data obtained in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) database and are accessible through GEO series accession number GSE81240.

RESULTS

Genetic organization of the sulfatase gene clusters in *B. breve* UCC2003. Based on the presence of a sulfatase-associated PFAM domain, PF00884, and the previously described N-terminally located sulfatase signature (CxPxR, where x represents a variable amino acid) (68, 69), two putative Cys-type sulfatase-encoding genes were identified on the genome of *B. breve* UCC2003. The first, represented by the gene with the associated locus tag Bbr_0352 (and designated here *atsA1*), is located in a cluster of four genes, designated the *ats1* cluster, which also includes a gene encoding a predicted hypothetical membrane-spanning protein (Bbr_0349); a gene specifying a putative anSME, which contains the signature motif CxxxCxxC characteristic of the radical AdoMet-dependent superfamily (Bbr_0350, designated here *atsB1*) (70); and a gene specifying a predicted LacI-type transcriptional regulator (Bbr_0351, designated *atsR1*). Adjacent to these four genes, but oppositely oriented, three genes that encode a predicted ABC-type transport system (corresponding to locus tags Bbr_0353 through Bbr_0355) are present (Fig. 2).

The second predicted sulfatase-encoding gene, Bbr_0852 (designated here *atsA2*), is located in a cluster of four genes (Bbr_0851 through to Bbr_0854, designated here *ats2*). Bbr_0851, designated *atsT*, encodes a predicted transporter from the major facilitator superfamily. Bbr_0853 (designated *atsB2*) encodes a putative anSME, which contains the signature CxxxCxxC motif. Bbr_0854 encodes a predicted membrane-spanning protein, which shares 75% amino acid identity with the deduced protein encoded by Bbr_0349 of the *ats1* gene cluster (Fig. 2). The AtsA1 and AtsA2 proteins share 28% amino acid identity, while the AtsB1 and AtsB2 proteins exhibit 74% identity between each other. Interestingly, the *ats2* gene cluster has a notably different GC content (63.96%) compared to the *B. breve* UCC2003 genome average (58.73%), whereas the GC content of the *ats1* cluster (57.6%) is comparable to that of the genome.

Based on the comparative genome analysis presented in Fig. 2, we found that the putative sulfatase clusters are well conserved among the *B. breve* strains whose genomes were recently reported (71). Of currently available complete *B. breve* genomes, *B. breve* NCFB2258, *B. breve* 689B, *B. breve* 12L, and *B. breve* S27 encode clear homologues of both identified putative sulfatase gene clusters described above. In contrast, the genomes of *B. breve* JCM7017, *B. breve* JCM7019, and *B. breve* ACS-071-V-Sch8b contain just a single but variable putative sulfatase cluster (Fig. 2). A clear homologue of the *ats1* gene cluster was also identified in the recently reported genome of *B. longum* subsp. *infantis* BT1 (GenBank accession number CP010411). No other homologues of either sulfatase-encoding gene cluster were identified within the available bifidobacterial genome sequences by BLASTP analysis.

Growth of *B. breve* UCC2003 on sulfated monosaccharides. The presence of two putative sulfatase-encoding clusters in the genome of *B. breve* UCC2003 suggests that this gut commensal is capable of removing a sulfate ester from a sulfated compound, possibly a sulfated carbohydrate. In mMRS medium supplemented with 0.5% GlcNAc-6-S as the sole carbon source, the strain was capable of substantial growth (final OD_{600} values following growth overnight varied between 0.6 and 0.8). However, no appreciable growth was observed on GlcNAc-3-S, GalNAc-3-S, or GalNAc-6-S. For growth on the positive control, 0.5% lactose, the strain reached an OD_{600} of almost 2, which is comparable to data from previous studies with this strain (17, 72, 73) (Fig. 3A).

Genome response of *B. breve* UCC2003 to growth on GlcNAc-6-S. In order to investigate which genes are responsible for GlcNAc-6-S metabolism in *B. breve* UCC2003, global gene expression was determined by microarray analysis during growth of the

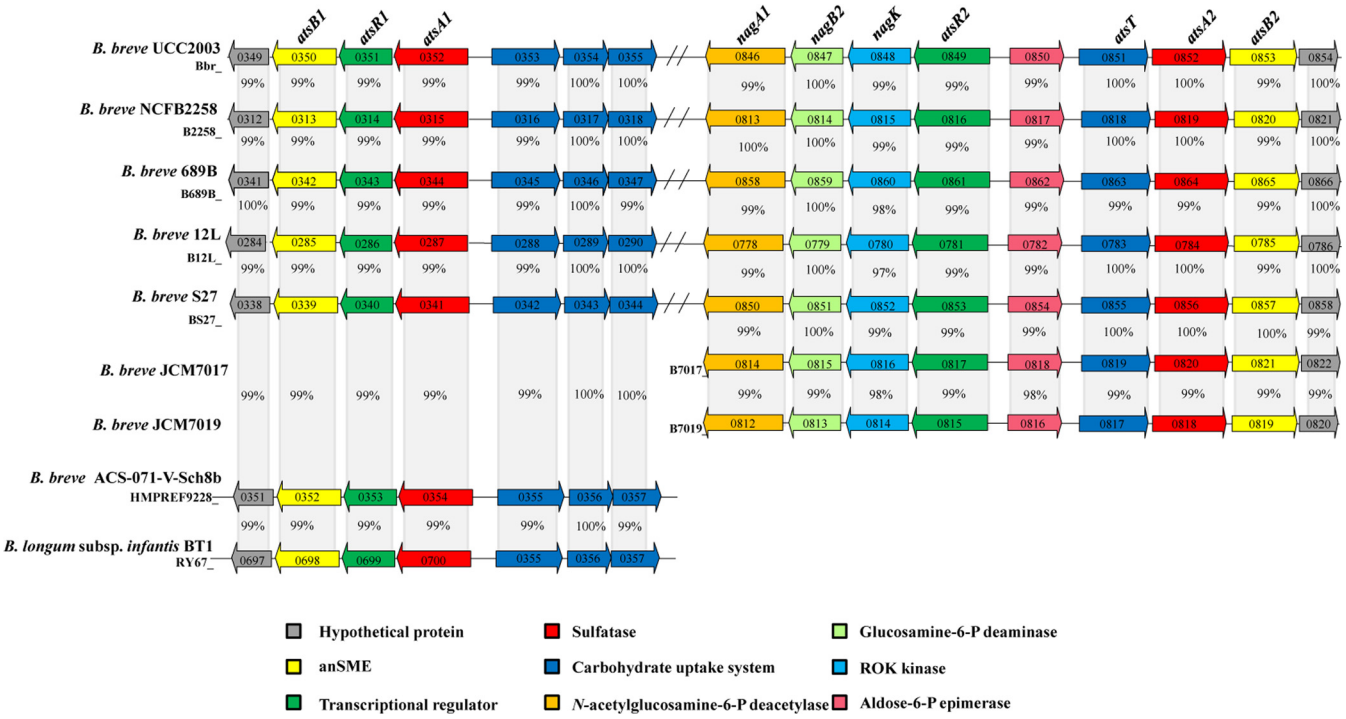

FIG 2 Comparison of the sulfatase- and anSME-encoding gene clusters of *B. breve* UCC2003 with corresponding loci in the currently available complete *B. breve* genome sequences and *B. longum* subsp. *infantis* BT1. Each solid arrow represents an open reading frame. The length of the arrows (which show the locus tag) is proportional to the size of the open reading frame. The corresponding gene name, which is indicative of putative function, is given above relevant arrows at the top. Orthologues are marked with the same color. The amino acid identity of each predicted protein to its equivalent protein encoded by *B. breve* UCC2003, expressed as a percentage, is given above each arrow.

strain in mMRS medium supplemented with GlcNAc-6-S and compared with the gene expression of the strain grown in mMRS medium supplemented with ribose. Ribose was considered an appropriate carbohydrate for comparative transcriptome analysis because the genes involved in ribose metabolism are known, and furthermore, it has successfully been used in a number of transcriptome studies of this strain (17, 18, 72–74). Of the two predicted sulfatase- and anSME-encoding gene clusters of *B. breve* UCC2003 (see above), transcription of the *ats2* gene cluster was significantly upregulated (fold change of >3.0; *P* value of <0.001) during growth on GlcNAc-6-S, while no (significant) difference in the level of transcription was observed for the *ats1* gene cluster (Table 3). Interestingly, three other gene clusters were also significantly upregulated (corresponding to locus tags Bbr_0846 through Bbr_0849 [Bbr_0846–0849 gene cluster], Bbr_1585 through Bbr_1590, and Bbr_1247 through Bbr_1249) (Fig. 4 and Table 3).

Within the Bbr_0846–0849 gene cluster, which is separated from the *ats2* cluster by a single gene (Fig. 3), Bbr_0846 (*nagA1*) and Bbr_0847 (*nagB2*) are predicted to encode an *N*-acetylglucosamine-6-phosphate (GlcNAc-6-P) deacetylase and a glucosamine-6-phosphate deaminase, respectively. Bbr_0848 (designated here *nagK*) encodes a predicted repressor open reading frame kinase (ROK) family kinase, which contains the characteristic DxGxT motif at its N-terminal end (75). The *B. breve* UCC2003-encoded NagK protein exhibits 42% similarity at the protein level to the previously characterized *E. coli* K-12-encoded, ROK family NagK protein, which phosphorylates GlcNAc to produce GlcNAc-6-P (76). Therefore, this cluster is predicted to encode enzymes for the complete GlcNAc catabolic pathway, as previously described for *E. coli*, whereby GlcNAc is first phosphorylated by NagK, producing GlcNAc-6-P, followed by NagA-mediated deacetylation to produce glucosamine-6-phosphate and NagB-mediated deamination and isomerization to produce fructose-6-phosphate (76, 77). Bbr_0849 encodes a predicted transcriptional regulator from the ROK family (designated here *atsR2*).

The Bbr_1585–1590 cluster includes a predicted UDP-glucose-4-epimerase (Bbr_1585 [*galE*]), a predicted *N*-acetylhexosamine-1-kinase (Bbr_1586 [*nahK*]), and a predicted lacto-*N*-biose (LNB) (Galβ1-3GlcNAc) phosphorylase (Bbr_1586 [*lnbP*]), representing three of the four enzymes required for the degradation of galacto-*N*-biose (GNB) (Galβ1-3GalNAc), which is found in mucin, or LNB, a known HMO (78, 79). The other three genes of this cluster, Bbr_1588–1590, encode a predicted ABC transport system, including two predicted permease proteins and a solute-binding protein, respectively (Fig. 4). This gene cluster was previously shown to be transcriptionally upregulated when *B. breve* UCC2003 was grown in coculture with *B. bifidum* PRL2010 in mucin (80).

Finally, the Bbr_1247–1249 cluster contains an *N*-acetylglucosamine-6-phosphate deacetylase (Bbr_1247)-encoding gene and a glucosamine-6-phosphate deaminase (Bbr_1248)-encoding gene, designated *nagA2* and *nagB3*, respectively. These genes were previously shown to be upregulated during *B. breve* UCC2003 growth on sialic acid (72). The NagA1 protein shares 74% identity with NagA2, while the NagB2 protein shares 84% identity with NagB1 of the *nan-nag* cluster for sialic acid metabolism (72) and 84%

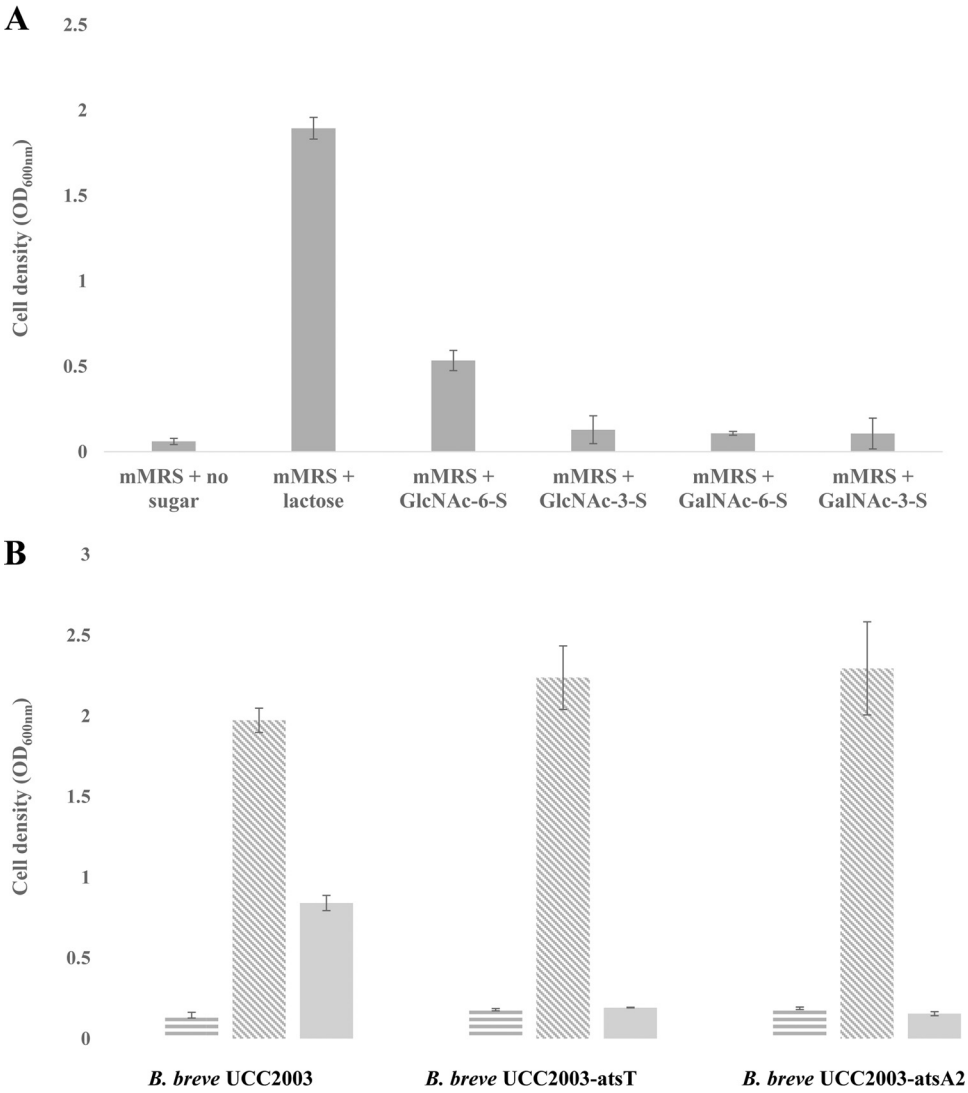

FIG 3 (A) Final OD_{600} values obtained following 24 h of growth of *B. breve* UCC2003 on mMRS medium without supplementation with a carbon source (negative control) or containing 0.5% (wt/vol) lactose, GlcNAc-6-S, GlcNAc-3-S, GalNAc-6-S, or GalNAc-3-S as the sole carbon source. (B) Final OD_{600} values obtained following 24 h of growth of *B. breve* UCC2003, *B. breve* UCC2003-atsT, and *B. breve* UCC2003-atsA2 in mMRS medium without supplementation with a carbon source (negative control) (horizontally striped bars) or containing 0.5% (wt/vol) lactose (diagonally striped bars) or GlcNAc-6-S (solid gray-filled bars) as the sole carbon source. The results are the mean values obtained from two separate experiments. Error bars represent the standard deviations.

identity with NagB3. Bbr_1249 encodes a predicted transcriptional ROK family regulator (Fig. 4).

Disruption of the *atsT* and *atsA2* genes. In order to investigate if the disruption of individual genes from the *ats2* gene cluster would affect the ability of *B. breve* UCC2003 to utilize GlcNAc-6-S, insertion mutations were constructed in the *atsT* and *atsA2* genes, resulting in *B. breve* strain UCC2003-atsT and *B. breve* strain UCC2003-atsA2, respectively (see Materials and Methods). The insertion mutants were analyzed for their ability to grow in mMRS medium supplemented with GlcNAc-6-S compared to *B. breve* UCC2003. As expected, and in contrast to the wild type, there was a complete lack of growth of *B. breve* UCC2003-atsT and *B. breve* UCC2003-atsA2 in medium containing GlcNAc-6-S as the sole carbon source (Fig. 3B), thus demonstrating the involvement of the disrupted genes in GlcNAc-6-S metabolism. Growth of the insertion mutants was not impaired on lactose, where all strains reached final OD_{600} values comparable to that reached by the wild-type strain (Fig. 3B).

Transcriptome of *B. breve* UCC2003-atsR2. The Bbr_0846–0849 gene cluster, which is upregulated when *B. breve* UCC2003 is grown on GlcNAc-6-S, and the *ats2* gene cluster are separated by just a single gene (Fig. 2). An insertion mutant was constructed in the predicted ROK-type transcriptional regulator-encoding gene Bbr_0849 (*atsR2*). It was hypothesized that if this gene encoded a repressor, mutation of the gene would lead to increased transcription levels of the genes that it controls, even in the absence of the inducing carbohydrate. Microarray data revealed that in comparison to *B. breve* UCC2003, the genes of the *ats2* cluster were indeed significantly upregulated (>3.0-fold change; $P < 0.001$) in the mutant strain, thus identifying *atsR2* as a transcriptional repressor (Table 4). Transcription of the Bbr_0846–0849 gene cluster was downregulated in the mutant strain compared to the wild type

TABLE 3 Effect of GlcNAc-6-S on the transcriptome of *B. breve* UCC2003[a]

Locus tag (gene)	Predicted function	Level of upregulation (fold)
Bbr_0846 (*nagA1*)	*N*-Acetylglucosamine-6-phosphate deacetylase	12.63
Bbr_0847 (*nagB2*)	Glucosamine-6-phosphate isomerase	6.17
Bbr_0848 (*nagK*)	Sugar kinase, ROK family	9.85
Bbr_0849 (*atsR2*)	Transcriptional regulator, ROK family	8.58
Bbr_0851 (*atsT*)	Carbohydrate transport protein	96.75
Bbr_0852 (*atsA2*)	Sulfatase	35.36
Bbr_0853 (*atsB2*)	anSME	31.25
Bbr_0854	Hypothetical membrane-spanning protein	4.175
Bbr_1247 (*nagA2*)	*N*-Acetylglucosamine-6-phosphate deacetylase	10.84
Bbr_1248 (*nagB3*)	Glucosamine-6-phosphate isomerase	11.88
Bbr_1249	Transcriptional regulator, ROK family	3.07
Bbr_1585 (*galE*)	UDP-glucose 4-epimerase	3.09
Bbr_1586 (*nahK*)	*N*-Acetylhexosamine kinase	4.96
Bbr_1587 (*lnbP*)	Lacto-*N*-biose phosphorylase	6.58
Bbr_1588	Permease protein of the ABC transporter system	6.24
Bbr_1589	Permease protein of the ABC transporter system	8.27
Bbr_1590	Solute-binding protein of the ABC transporter system	23.97

[a] The cutoff point is 3-fold, with a *P* value of <0.001.

when both strains were grown on ribose. It is speculated that since *atsR2* represents the first gene of this presumed operon (Fig. 2), the insertion mutation caused a (negative) polar effect on the transcription of the genes located downstream.

Electrophoretic mobility shift assays. In order to determine if the AtsR2 protein directly interacts with promoter regions of the *ats2* gene cluster, crude cell extracts of *L. lactis* NZ9000-pNZ-atsR2 were used to perform EMSAs, with crude cell extracts of *L. lactis* NZ9000-pNZ8048 (empty vector) being used as a negative control. As expected, the negative control did not alter the electrophoretic behavior of any of the tested DNA fragments (Fig. 5B). The results obtained with crude cell extracts expressing AtsR2 demonstrate that this presumed regulator specifically binds to DNA fragments encompassing the upstream regions of *atsR2* and *atsT* (Fig. 5A and B). Dissection of the promoter region of *atsR2* showed that AtsR2 binding required a 184-bp region, within which a 21-bp imperfect inverted repeat was identified. Similarly, dissection of the *atsT* promoter region revealed that AtsR2 binding required a 192-bp region, which also includes a 21-bp imperfect repeat, similar to that identified upstream of *atsR2*. When either of the inverted repeats was excluded, binding of AtsR2 to such DNA fragments was abolished, suggesting that these inverted repeats contained the operator sequence of AtsR2 (Fig. 5A and B).

To demonstrate if the binding of AtsR2 to its DNA target is affected by the presence of a carbohydrate effector molecule, GlcNAc and GlcNAc-6-S were tested for their effects on the formation of the AtsR2-DNA complex. The ability of AtsR2 to bind to the promoter region of *atsR2* or *atsT* was eliminated in the presence of 2.5 mM GlcNAc-6-S, the lowest concentration used in this assay. The presence of GlcNAc was shown to inhibit the binding of AtsR2 to the *atsR2* and *atsT* promoter regions but only at GlcNAc concentrations above 5 mM (Fig. 5C). This finding suggests that while GlcNAc-6-S has the highest affinity for the regulator and is therefore the most likely effector of this repressor protein, the structurally similar GlcNAc is also able to bind this regulator but at concentrations that are probably not physiologically relevant.

Identification of the transcription start sites of *atsR2* and *atsT*. Based on the EMSA results and the transcriptome of *B. breve* UCC2003-atsR2, it was deduced that an AtsR2-dependent promoter is located upstream of both *atsR2* and *atsT* (Fig. 2). In order to determine the transcriptional start site of these presumed promoters, primer extension analysis was performed by using RNA

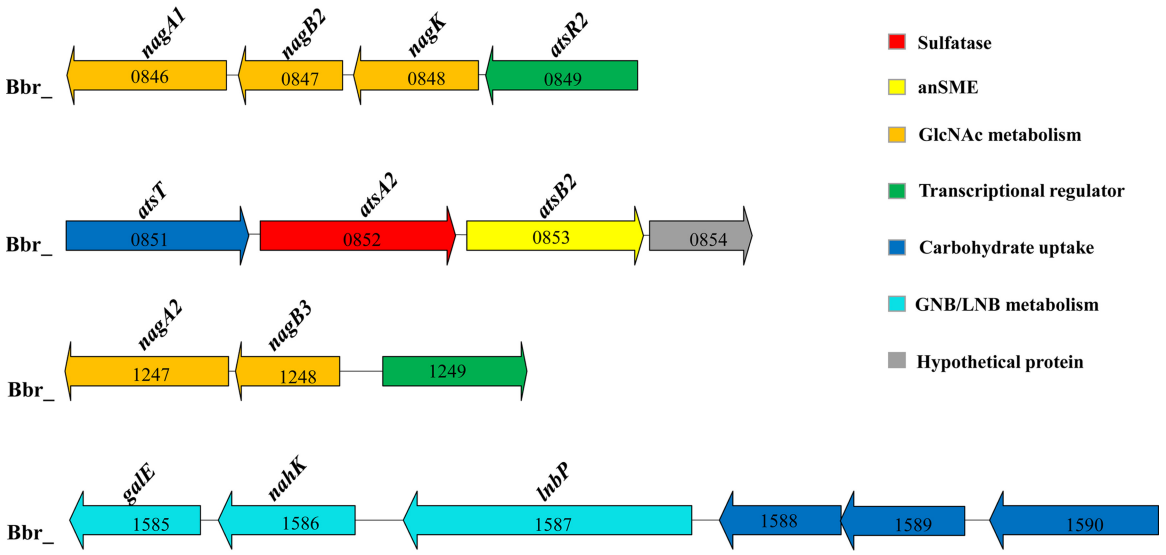

FIG 4 Schematic representation of the four *B. breve* UCC2003 gene clusters upregulated during growth on GlcNAc-6-S as the sole carbon source. The length of the arrows (which show the locus tag) is proportional to the size of the open reading frame, and the gene locus name, which is indicative of its putative function, is given at the top. Genes are grouped by color based on their predicted function in carbohydrate metabolism.

TABLE 4 Transcriptome analysis of *B. breve* UCC2003-atsR2 compared to *B. breve* UCC2003 grown on 0.5% (wt/vol) ribose[a]

Locus tag (gene)	Predicted function	Fold upregulation	Fold downregulation
Bbr_0846 (*nagA1*)	*N*-Acetylglucosamine-6-phosphate deacetylase	–	3.77
Bbr_0847 (*nagB2*)	Glucosamine-6-phosphate isomerase	–	3.35
Bbr_0848 (*nagK*)	Sugar kinase, ROK family	–	4.45
Bbr_0850	Aldose-1-epimerase	4.58	–
Bbr_0851 (*atsT*)	Carbohydrate transport protein	106.28	–
Bbr_0852 (*atsA2*)	Sulfatase	59.58	–
Bbr_0853 (*atsB2*)	anSME	15.57	–
Bbr_0854	Hypothetical membrane-spanning protein	9.09	–

[a] The cutoff point is 3-fold, with a *P* value of <0.001; values below the cutoff are indicated by a minus.

extracted from *B. breve* UCC2003-atsR2 grown in mMRS medium supplemented with 0.5% ribose. Microarray analysis had shown that the expression levels of *atsT* were high when the *B. breve* UCC2003-atsR2 strain was grown on ribose (Table 4). For this reason, the mutant strain was considered the most suitable for primer extension analysis. For the *atsR2* promoter region, initial attempts to attain a primer extension product from mRNA isolated from *B. breve* UCC2003-atsR2 cells were unsuccessful. In an attempt to increase the amount of mRNA transcripts of this promoter region, a DNA fragment encompassing the deduced promoter region was cloned into pBC1.2 and introduced into *B. breve* UCC2003-atsR2, generating *B. breve* strain UCC2003-atsR2-pBC1.2-atsRProm. A primer extension product was obtained for the *atsT* promoter region using mRNA isolated from *B. breve* UCC2003-atsR2; therefore, it was not necessary to clone this promoter. Single extension products were identified upstream of *atsR2* and *atsT* (Fig. 6). Potential promoter recognition sequences resembling consensus −10 and −35 hexamers were identified upstream of each of the transcription start sites (Fig. 6). The deduced operator sequences of AtsR2 overlap the respective −35 or −10 sequences, consistent with our findings that AtsR2 acts as a transcriptional repressor.

DISCUSSION

A large-scale metagenomic analysis of fecal samples from 13 individuals of various ages revealed that genes predicted to encode anSMEs are enriched in the gut microbiomes of humans compared to microbial communities not in the gut (81). Interestingly, in that same study, it was found that such genes are found more commonly in members of the gut microbiota of adults and weaned children than in unweaned infants. The present study describes two gene clusters in a bacterium isolated from an infant, namely, *B. breve* UCC2003, each encoding a (predicted) sulfatase and the accompanying anSME as well as an associated transport system and transcriptional regulator. The *ats2* gene cluster was shown to be required for the metabolism of GlcNAc-6-S, while GlcNAc-3-S, GalNAc-3-S, and GalNAc-6-S did not support the growth of *B. breve* UCC2003. The substrate(s) for the sulfatase encoded by the *ats1* gene cluster is as yet unknown. However, as recently shown in a study of sulfatases from *Ba. thetaiotaomicron*, these enzymes can vary quite significantly in their substrate specificities. It is therefore possible that, similar to the recently characterized BT_3349 and BT_1596 enzymes from *Ba. thetaiotaomicron*, the AtsA1 sulfatase might be active on sulfated di- or oligosaccharides rather than monosaccharides (35) or that the transport system encoded by the *ats1* cluster is specific for an as-yet-unknown sulfated substrate. However, at present, this is mere speculation, and further study is required to expand this premise.

Interestingly, the two gene clusters *ats1* and *ats2* are quite dissimilar in terms of their genetic organizations. The gene order and composition of the *ats1* cluster resemble those of a typical bifidobacterial carbohydrate utilization cluster, as it includes genes encoding a predicted ABC-type transport system, a LacI-type repressor (*atsR1*), and the carbohydrate-active *atsA1*-encoded sulfatase and *atsB*-encoded anSME, which in this case replace the typical glycosyl hydrolase-encoding gene(s) (16, 82). In the *ats2* cluster, the *atsT* gene encodes a predicted transporter of the major facilitator superfamily, while the *atsA2* and *atsB2* genes are adjacent, as is also the case for their homologous genes in *K. pneumoniae* and *Prevotella* strain RS2 (83, 84). We obtained compelling evidence that the *ats2* cluster is coregulated with the Bbr_0846–0849 cluster by the ROK family transcriptional repressor AtsR2. The only previously characterized bifidobacterial ROK family transcriptional regulator is RafA, the transcriptional activator of the raffinose utilization cluster in *B. breve* UCC2003 (73). The Bbr_0846–0848 genes are presumed to be involved in the metabolism of GlcNAc following the removal of the sulfate residue from GlcNAc-6-S. The fructose-6-phosphate produced from GlcNAc by the combined activities of NagK, NagA, and NagB is expected to enter the fructose-6-phosphate phosphoketolase pathway or bifid shunt, the central metabolic pathway of bifidobacteria (85). It is interesting that *B. breve* UCC2003 is capable of growth on GlcNAc-6-S, but apparently not on GlcNAc, as a sole carbon source (16). Since the *B. breve* UCC2003 genome seems to encode the enzymes required to metabolize GlcNAc, this suggests that the *atsT* transporter has (high) affinity for only the sulfated form of this N-acetylated carbohydrate.

A novel method of desulfating mucin that does not require a sulfatase enzyme has been characterized for *Prevotella* strain RS2, whereby a sulfoglycosidase removes GlcNAc-6-S from purified porcine gastric mucin (86). The presence of a signal sequence on this glycosulfatase (86), thus indicating extracellular activity, is interesting in relation to the present study, as it presents a source of GlcNAc-6-S to *B. breve* strains, which is suggestive of a cross-feeding opportunity for members of this species. This is particularly noteworthy considering that the sulfatase enzymes produced by *B. breve* UCC2003 are intracellular, implying that *B. breve* UCC2003 is reliant on the extracellular glycosyl hydrolase activity of other members of the gut microbiota in order to gain access to mucin-derived sulfated monosaccharides. Recent studies have shown that *B. breve* UCC2003 employs a cross-feeding strategy to great effect, as it can utilize components of 3′ sialyllactose (a HMO) and mucin following the degradation of these sugars by *B.*

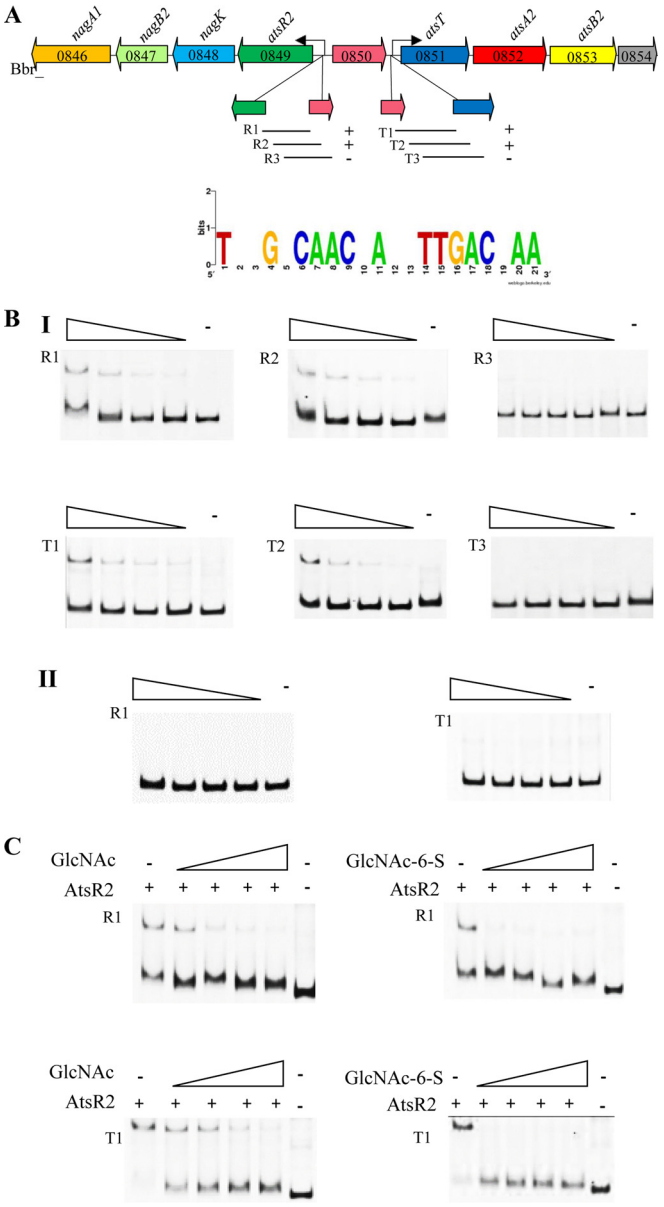

FIG 5 (A) Schematic representation of the *ats2* gene cluster of *B. breve* UCC2003 and DNA fragments used in EMSAs for the *atsR2* and *atsT* promoter regions, together with a WebLogo representation of the predicted operator of AtsR2. Plus or minus signs indicate the ability or inability of AtsR2 to bind to the DNA fragment. The bent arrows represent the position and direction of the proven promoter sequences (Fig. 6). (B) EMSAs showing the interactions of a crude cell extract containing pNZ-AtsR2 with DNA fragments R1, R2, R3, T1, T2, and T3 (I) and of a crude cell extract containing pNZ8048 (empty vector) with DNA fragments R1 and T1 (II). The minus symbol indicates reaction mixtures to which no crude cell extract was added, while the remaining lanes represent binding reactions with the respective DNA probes incubated with increasing amounts of the crude cell extract. Each successive lane from right to left represents a doubling of the amount of the crude cell extract. (C) EMSAs showing AtsR2 interactions with DNA fragments R1 and T1 with the addition of GlcNAc or GlcNAc-6-S at concentrations ranging from 2.5 mM to 20 mM.

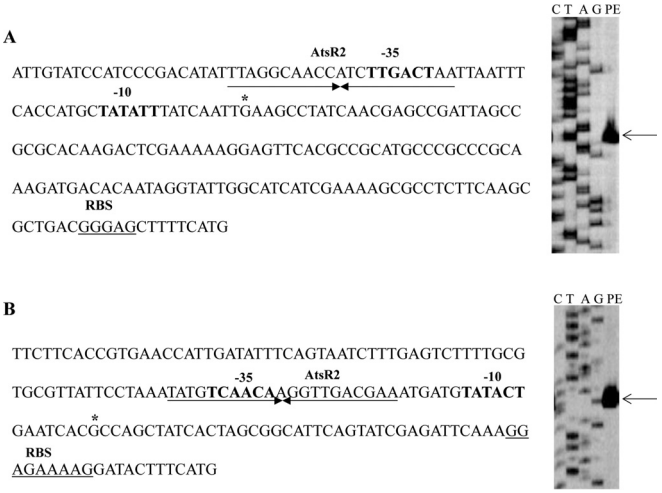

FIG 6 Schematic representation of the *atsR2* (A) and *atsT* (B) promoter regions. Boldface type and underlining indicate −10 and −35 hexamers (as deduced from the primer extension results) and the ribosomal binding site (RBS); the transcriptional start site is indicated by an asterisk. The arrows below the indicated DNA sequences indicate the inverted repeats that represent the presumed AtsR2-binding site. The arrows in the right panels indicate the primer extension products. C, cytosine; T, thymine; A, adenine; G, guanine; PE, primer extension product.

bifidum PRL2010, whereas in the absence of *B. bifidum* PRL2010, it is not capable of utilizing either of these sugars as a sole carbon source (72, 80). A recent study provided further transcriptomic evidence for carbohydrate cross-feeding between bifidobacterial species. Four bifidobacterial strains, namely, *B. bifidum* PRL2010, *B. breve* 12L, *B. adolescentis* 22L, and *B. longum* subsp. *infantis* ATCC 25697, were cultivated either in pairs (biassociation) or as a combination of all four strains (multiassociation) under *in vivo* conditions in a murine model. In all strains, transcription of predicted glycosyl hydrolase-encoding genes, particularly those involved in xylose or starch utilization, was affected by co- or multiassociation. In relation to xylose metabolism, the authors of that study speculated that in co- or multiassociation, the combined glycosyl hydrolase activities of the strains may allow them to degrade xylose-containing polysaccharides that would otherwise be inaccessible (87).

For *Ba. thetaiotaomicron*, the *in vivo* contribution of sulfatase activity to bacterial fitness has been well established. In previous studies of chondroitin sulfate and heparan sulfate metabolism by this species, mutagenesis of a gene designated *chuR*, which was first predicted to encode a regulatory protein but was later found to encode an anSME, resulted in an inability to compete with wild-type *Ba. thetaiotaomicron* in germfree mice (37, 88). In a recent study, 28 predicted sulfatase-encoding genes were identified in the genome of *Ba. thetaiotaomicron*, 20 of which are predicted extracellular enzymes, yet the previously described *chuR* gene is the sole anSME-encoding gene (36, 89, 90). Recently, this anSME was shown to be of significant importance in this strain's ability to colonize the gut, as an isogenic derivative of this strain (designated ΔanSME) carrying a deletion in the anSME-encoding gene displayed reduced fitness *in vivo* (36). The authors of that study speculated that anSME activity and associated sulfatase activities are important as the bacterium adapts to the gut environment (36). Given that sulfatase activity within the *Bifidobacterium* genus is limited to the *B. breve* species and a single strain of *B. longum* subsp. *infantis* (at least based on currently available genome sequences), it is interesting to speculate on the effect that this activity may have on bacterial fitness in the large intestine. It is

intriguing that human intestinal mucins increase in acidity along the intestinal tract, with more than half of mucin oligosaccharide structures in the distal colon containing either sialic and/or sulfate residues (91). We recently showed that 11 of 14 strains of *B. breve* tested were capable of growth on sialic acid, while sialic acid utilization genes can also be found in the genomes of *B. longum* subsp. *infantis* strains (20, 22, 72). The ability of *B. breve* strains and possibly certain *B. longum* subsp. *infantis* strains to utilize both sialic acid and sulfated GlcNAc-6-S may provide them with a competitive advantage over other members of the *Bifidobacterium* genus and other members of the gut microbiota, thus contributing to their successful colonization ability in this highly competitive environment.

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