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PHYSIOLOGY

Bifidobacterium breve UCC2003 Employs Multiple Transcriptional Regulators To Control Metabolism of Particular Human Milk Oligosaccharides

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ABSTRACT Bifidobacterial carbohydrate metabolism has been studied in considerable detail for a variety of both plant- and human-derived glycans, particularly involving the bifidobacterial prototype strain *Bifidobacterium breve* UCC2003. We recently elucidated the metabolic pathways by which the human milk oligosaccharide (HMO) constituents lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT) and lacto-*N*-biose (LNB) are utilized by *B. breve* UCC2003. However, to date, no work has been carried out on the regulatory mechanisms that control the expression of the genetic loci involved in these HMO metabolic pathways. In this study, we describe the characterization of three transcriptional regulators and the corresponding operator and associated (inducible) promoter sequences, with the latter governing the transcription of the genetic elements involved in LN(n)T/LNB metabolism. The activity of these regulators is dependent on the release of specific monosaccharides, which are believed to act as allosteric effectors and which are derived from the corresponding HMOs targeted by the particular locus.

IMPORTANCE Human milk oligosaccharides (HMOs) are a key factor in the development of the breastfed-infant microbiota. They function as prebiotics, selecting for a specific range of microbes, including a number of infant-associated species of bifidobacteria, which are thought to provide a range of health benefits to the infant host. While much research has been carried out on elucidating the mechanisms of HMO metabolism in infant-associated bifidobacteria, to date there is very little understanding of the transcriptional regulation of these pathways. This study reveals a multicomponent transcriptional regulation system that controls the recently identified pathways of HMO metabolism in the infant-associated *Bifidobacterium breve* prototype strain UCC2003. This not only provides insight into the regulatory mechanisms present in other infant-associated bifidobacteria but also provides an example of a network of sequential steps regulating microbial carbohydrate metabolism.

KEYWORDS bifidobacteria, probiotic, prebiotic, transcriptional regulation, HMO, carbohydrate metabolism

Bifidobacteria represent high-G+C, Gram-positive, anaerobic members of the phylum *Actinobacteria* and are common commensals of the mammalian, avian, and insect guts. In humans, they are particularly abundant and prevalent among the gut microbiota of healthy, vaginally delivered, breastfed infants (1) and are thought to confer a multitude of benefits to the neonatal host (2–4). For this reason, as well as because of their purported health-promoting activities in adults, bifidobacteria are used as functional ingredients in a variety of foods and therapeutic products. The use of prebiotics is also becoming commonplace for the improvement of both adult and infant (gut) health. A prebiotic has been defined as "a nondigestible food ingredient

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that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (5).

Fascinatingly, the archetypal prebiotic would appear to be human breast milk, in particular its bifidogenic constituents known as human milk oligosaccharides (HMOs). HMOs represent specific glycans present in human breast milk that are thought to shape, at least partly, the compositional structure of the neonatal gut microbiota (6, 7). HMOs represent, after lactose, the second-largest carbohydrate component of breast milk (6, 8) and constitute a heterogeneous mix of at least 200 distinct glycan structures (9). The majority of complex HMO structures can be classified into one of two types, depending on their backbone composition. The more abundant type I HMOs contain the core tetrasaccharide lacto-*N*-tetraose (LNT) within their structure (Galβ1-3GlcNAcβ1-3Galβ1-4Glc). Type II HMOs contain lacto-*N*-neotetraose (LNnT), a stereoisomer of LNT, within their backbone (Galβ1-4GlcNAcβ1-3Galβ1-4Glc). Lacto-*N*-biose (LNB) (Galβ1-3GlcNAc) is a subunit of LNT and other type I HMO structures and can be released by the degradation of these sugars (10).

The effects of specific human breast milk components on the prevalence, abundance, and activity of members of the infant gut microbiota are currently enjoying a great deal of scientific and commercial attention due to the beneficial roles that they are believed to play in infant health and development (11, 12). Understanding the pathways by which specific HMOs are metabolized by particular microbial species that inhabit the infant gut is important, although our knowledge regarding these processes is still in its infancy, particularly with regard to the manner in which they affect microbiota development.

It is hardly surprising that the dominant *Bifidobacterium* species found among the neonatal gut microbiota can utilize various HMO components as their sole carbohydrate source (9). These species chiefly include strains of *Bifidobacterium bifidum*, *Bifidobacterium longum* subsp. *infantis*, and *Bifidobacterium breve*. HMO utilization by *B. bifidum* and *B. longum* subsp. *infantis* is relatively well characterized. *B. bifidum* extracellularly hydrolyzes complex HMO structures, including LNT and LNnT, by employing secreted glycosyl hydrolases, followed by the internalization and intracellular degradation/metabolism of (most of) the resulting mono- and disaccharides, such as LNB (10, 13–19). *B. longum* subsp. *infantis* internalizes intact LNT, LNnT, and LNB and uses a series of sequential hydrolytic/phosphorolytic reactions acting from the nonreducing end of the carbohydrate structures to degrade them into their monosaccharide components for further metabolic processing (9, 10, 20–23). However, *B. infantis* has also been demonstrated to take up and utilize fucosyl- and sialyl-lactose (24–26).

The metabolic pathways of LNT, LNnT, and LNB have recently been elucidated for the prototype strain *B. breve* UCC2003 (27). In that study, converging pathways of LNT and LNnT catabolism were identified, where monosaccharide moieties are sequentially released from the nonreducing end of either sugar by hydrolytic reactions. The genetic units responsible for the uptake and breakdown of these structures are the *lnt* locus (corresponding to locus tags Bbr_0526 to Bbr_0530) and the *nah* locus (locus tags Bbr_1554 to Bbr_1560) (Table 1 and Fig. 1). The *lnt* locus encodes proteins that are responsible for the internalization of LNT and the intracellular hydrolysis of both LNT and LNnT, releasing a galactose (Gal) moiety from their nonreducing end and at the same time liberating the trisaccharide lacto-*N*-triose (GlcNAcβ1-3Galβ1-4Glc). The *nah* locus specifies an LNT/LNnT uptake system, while it furthermore encodes a glycosyl hydrolase that liberates *N*-acetylglucosamine (GlcNAc) from the nonreducing end of lacto-*N*-triose, leaving lactose, which itself is further degraded by lactose-specific glycosyl hydrolases. Additionally, the gene products of the *lnp-glt* locus (corresponding to locus tags Bbr_1585 to Bbr_1590) (Table 1 and Fig. 1) are responsible for the internalization and subsequent phosphorolysis of free LNB, releasing its constituent monosaccharides Gal-1-phosphate and GlcNAc (27, 28). We also identified the transcriptional upregulation of genes in the *nag* locus (locus tags Bbr_1247 to Bbr_1252) (Table 1 and Fig. 1) during growth on LNT, LNnT, and LNB, indicating their role in the utilization of these sugars, specifically in the multistep metabolism of GlcNAc. The *nag*

TABLE 1 *B. breve* UCC2003 regulator mutant genes with upregulated transcription during growth in mMRS medium supplemented with 1% ribose as the sole carbohydrate, compared to the wild type (control)

Locus tag	Gene	Function	Fold upregulation[a] during growth of:		
			UCC2003-lntR	UCC2003-nahR	UCC2003-nagR1
Bbr_0526	*lntR*	Transcriptional regulator, LacI family	NA	—	—
Bbr_0527	*lntP1*	Permease protein of the ABC transporter system for sugars	3.84	—	—
Bbr_0528	*lntP2*	Permease protein of the ABC transporter system for sugars	3.77	—	—
Bbr_0529	*lntA*	GH42 beta-galactosidase	2.78	—	—
Bbr_0530	*lntS*	Solute-binding protein of the ABC transporter system for sugars	5.66	—	—
Bbr_1247	*nagA2*	CE9 *nagA2 N*-acetylglucosamine-6-phosphate deacetylase	—	—	6.70
Bbr_1248	*nagB3*	*nagB3* glucosamine-6-phosphate isomerase	—	—	9.11
Bbr_1249	*nagR1*	Transcriptional regulator, ROK family	—	—	NA
Bbr_1250	*nagK*	Sugar kinase, ROK family	—	—	2.29
Bbr_1251	*nagR2*	Transcriptional regulator, ROK family	—	—	—
Bbr_1252	*nagK2*	Sugar kinase, *pfkB* family	—	—	—
Bbr_1554	*nahS*	Solute-binding protein of the ABC transporter system (lactose)	—	17.44	—
Bbr_1555	*nahR*	NagC/XylR-type transcriptional regulator	—	NA	—
Bbr_1556	*nahA*	GH20 *nagZ* beta-*N*-acetylhexosaminidase	—	—	—
Bbr_1558	*nahP*	Permease protein of the ABC transporter system	—	—	—
Bbr_1559	*nahT1*	ATP-binding protein of the ABC transporter system	—	—	—
Bbr_1560	*nahT2*	ATP-binding protein of the ABC transporter system	—	—	—
Bbr_1585	*lnpD*	UDP-glucose 4-epimerase	—	—	3.06
Bbr_1586	*lnpB*	Phosphotransferase family protein	—	—	3.36
Bbr_1587	*lnpA*	GH112 lacto-*N*-biose phorylase	—	—	2.90
Bbr_1588	*gltC*	Permease protein of the ABC transporter system for sugars	—	—	2.91
Bbr_1589	*gltB*	Permease protein of the ABC transporter system for sugars	—	—	3.02
Bbr_1590	*gltA*	Solute-binding protein of the ABC transporter system for sugars	—	—	5.07

[a]The levels of transcription are shown as fold increases in transcription levels on each carbohydrate, compared to a ribose control. Data are based on comparative transcriptome analysis using *B. breve* UCC2003-lntR, *B. breve* UCC2003-nahR, and *B. breve* UCC2003-nagR1 grown on 1% ribose, compared to wild-type *B. breve* UCC2003 grown under the same conditions as a control. Two independent biological replicates were used for each array, using a Cy3/Cy5 dye swap. The cutoff point is 2.0-fold, with a *P* value of 0.001. —, value below the cutoff. NA indicates that the fold increase in the transcription level of this gene is not included, as this is the gene in which the mutation was made, and thus, it does not accurately represent its natural transcription under these conditions. The level of transcription is not given for the regulator-encoding genes containing the mutations in their respective arrays, as their transcription has been interrupted and thus cannot be considered reliable.

locus was previously implicated in the metabolism of sialic acid and mucin-derived N-glycans, both of which contain GlcNAc as well (29, 30). While the degradation routes of these key HMO structures have thus been identified, the regulatory mechanisms that control the expression of these pathways have remained unexplored, for both *B. breve* and HMO-utilizing *Bifidobacterium* species as a whole.

In this study, we identified and characterized the genes encoding transcriptional

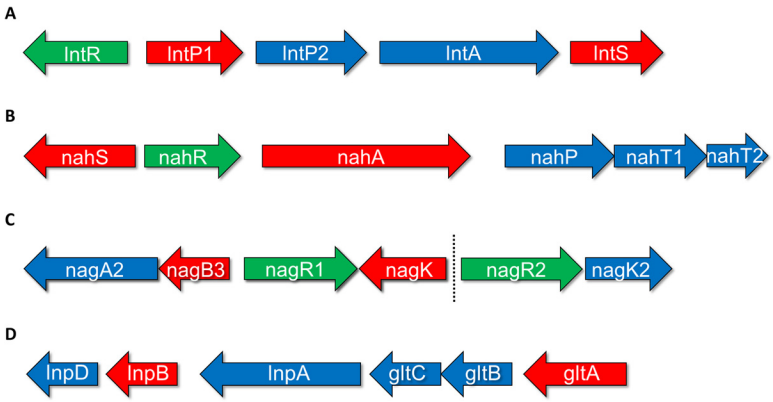

FIG 1 Schematic representation of HMO metabolism-associated loci in *B. breve* UCC2003, as identified previously (21). (A) The genes of the *lnt* locus; (B) the genes of the *nah* locus; (C) the genes of the *nag* locus and the adjacent genes *nagR2* and *nagK2*; (D) the genes of the *lnp-glt* locus. The length of the arrows is proportional to the size of the open reading frame. Genes shown in red possess a predicted promoter in their upstream intergenic region. Genes shown in green are predicted to encode a regulator protein. Genes shown in blue were identified as not possessing a predicted promoter in their upstream intergenic region.

regulators responsible for the control of gene expression in four key HMO-associated loci in *B. breve* UCC2003 during growth on LNT, LNnT, or LNB.

RESULTS

Identification of putative transcriptional regulator-encoding genes in the vicinity of HMO utilization loci. In a previous study, we observed that genes within four chromosomal loci exhibit transcriptional induction during the growth of *B. breve* UCC2003 on LNT, LNnT, or LNB as the sole carbohydrate source (27). This indicates that these genes are subject to transcriptional regulation, which was presumed to be either directly or indirectly controlled by the presence of these HMO substrates. The four loci concerned are the *lnt* locus (Bbr_0526 to Bbr_530), the *nah* locus (Bbr_1554 to Bbr_1560), the *nag* locus (Bbr_1247 to Bbr_1250), and the *lnp-glt* locus (Bbr_1585 to Bbr_1590) (Fig. 1; see Table 1 for a description of [predicted] functions). Detailed scrutiny of these four loci and neighboring regions showed that the *lnt* and *nah* loci are flanked by or contain a predicted regulator-encoding gene, respectively: *lntR* (Bbr_0526), encoding a LacI-type repressor, and *nahR* (Bbr_1555), encoding a NagC/XylR-type repressor (Fig. 1A and B). The *nag* locus is associated with two genes, *nagR1* (Bbr_1249) and *nagR2* (Bbr_1251), both of which are predicted to encode repressor open reading frame kinase (ROK)/NagC family-type repressors, while no regulator-encoding gene was observed in the close vicinity of the *lnp-glt* locus (Fig. 1C and D). NagC/XylR-type and ROK/NagC-type repressors are both members of the large family of ROK-type transcriptional regulators (31). The four identified putative regulator-encoding genes were thus selected as candidates for mutagenesis in order to ascertain their role, if any, in the transcriptional regulation of the *lnt*, *nah*, *nag*, and *lnp-glt* loci.

Generation and transcriptomic analysis of insertional mutants in putative HMO-associated regulator-encoding genes. Individual insertional mutants were constructed in *lntR*, *nahR*, *nagR1*, and *nagR2*, resulting in *B. breve* strains UCC2003-lntR, UCC2003-nahR, UCC2003-nagR1, and UCC2003-nagR2, respectively (see Materials and Methods). In order to identify promoters/genes that are subject to the transcriptional control of these predicted regulators, global gene transcription data were obtained from microarray-based analyses performed on the *B. breve* UCC2003-lntR, UCC2003-nahR, UCC2003-nagR1, and UCC2003-nagR2 insertion mutants grown in modified de Man-Rogosa-Sharpe (mMRS) medium supplemented with ribose and compared to the transcriptome of the UCC2003 wild-type strain grown under the same conditions.

Transcriptome analysis of the *lntR* mutant revealed the upregulation of the adjacent *lntP1*, *lntP2*, *lntA*, and *lntS* genes of the *lnt* locus (Table 1 and Fig. 1), when this mutant was grown on ribose (compared to wild-type UCC2003), all of which were also previously found to be upregulated during the growth of wild-type UCC2003 on LNT or LNnT (27). This corroborates the notion that LntR is a LacI-type repressor and that this protein negatively regulates the LNT/LNnT-dependent transcription of genes within the *lnt* cluster. Conversely, the array data obtained for the *nahR* mutant only revealed the transcriptional upregulation (compared to the UCC2003 control) of the *nahS* gene (Table 1), when grown on ribose. This is consistent with previously observed expression patterns for UCC2003, with the exception of *nahA*, which may have been expected to exhibit transcriptional upregulation in the *nahR* mutant, as its expression was increased during growth on LNT and LNnT in *B. breve* UCC2003 (27). These results suggest that NahR, a NagC/XylR-type repressor, is responsible for the transcriptional regulation of at least one gene of the *nah* cluster. For the *nagR1* mutant, upregulation of *nagA2*, *nagB3*, and *nagK* (but not *nagR2* or *nagK2*), as well as all of the genes of the *lnp-glt* locus (Table 1), was observed when cells were grown on ribose (compared to the UCC2003 control). These results suggest that NagR1, a ROK/NagC family-type repressor, is responsible for the transcriptional regulation of (part of) the *nag* and *lnp-glt* clusters. This is consistent with transcriptomic data previously obtained for wild-type UCC2003 during growth on LN(n)T and LNB, which demonstrated the transcriptional upregulation of genes in both of these loci (27). When the transcriptome of UCC2003-nagR2 was compared to that of UCC2003 grown on ribose, the *nagR2* mutant exhibited increased transcription levels of

genes in the *mal* locus (locus tags Bbr_0118 to Bbr_0123), which is known to be involved in maltooligosaccharide metabolism (32, 33), and Bbr_1719 to Bbr_1721 (predicted to function in fatty acid metabolism) (34) (data not shown), none of which are predicted to function in HMO metabolism, nor were they shown to be upregulated in our previous wild-type arrays for growth on LNT, LNnT, or LNB (27). These results thus show that NagR2 is not involved in the transcriptional control of the loci responsible for LNT, LNnT, or LNB metabolism, and no further investigation of this regulator was carried out. The *lntR*, *nahR*, and *nagR1* genes, however, were selected for further study, as described below, in order to further elucidate their regulatory activities and specificities.

Promoter mapping through identification of transcription start sites. Based on the transcriptome findings, we presumed that LntR, NahR, and NagR1 act as transcriptional regulators of (certain genes of) the *lnt*, *nah*, and *nag-lnp-glt* loci, respectively. Gene expression patterns observed for the regulator gene mutants and examination of the genetic layout and transcriptome profiles of these loci allowed us to assign putative promoter-containing regions within each locus. In order to verify these predicted promoter regions, the associated transcription start sites (TSSs) were experimentally determined by primer extension analyses.

The *lnt* locus was deduced to contain at least two promoters: one just upstream of *lntP1* (Fig. 2a) and one in front of *lntS* (Fig. 2b). The *lntP1* and *lntS* genes on the *B. breve* UCC2003 genome encode a permease and a solute-binding protein of an ABC transporter system, respectively, and exhibit increased transcription upon growth on LNT, LNnT, LNB, lactosamine, or lactose (27). The TSSs of the presumed *lntP1* and *lntS* promoters were determined by primer extension analysis using RNA extracted from *B. breve* UCC2003 grown in mMRS medium supplemented with 1% LNnT. An extension product was identified 41 nucleotides 5′ of the predicted translational start site of the *lntP1* gene (see Fig. S1A in the supplemental material), while the TSS for the *lntS* gene was identified 154 nucleotides 5′ of the predicted translational start site (Fig. S1B). In both cases, the TSS was preceded by −10 and −35 hexamers that resemble (bifidobacterial) consensus vegetative promoter recognition sequences (35, 36).

The *nah* locus was deduced to contain at least two promoters: one just upstream of *nahS* (Fig. 2c) and one in front of *nahA* (Fig. 2d). The *nahS* and *nahA* genes on the *B. breve* UCC2003 genome encode a solute-binding protein of an ABC transporter system and a GH20 *N*-acetylhexosaminidase, respectively. While an increase in transcription was observed only for *nahS* in the *nahR* mutant-based array, both this gene and *nahA* were found to be subject to transcriptional induction when wild-type UCC2003 was grown on LNT, LNnT, or lactosamine (27). The TSSs of the presumed *nahS* and *nahA* promoters were determined by primer extension analysis using RNA extracted from *B. breve* UCC2003 grown in mMRS medium supplemented with 1% LNnT. An extension product was identified 59 nucleotides 5′ of the predicted translational start site for the *nahS* gene (Fig. S1C), while the TSS upstream of *nahA* was identified 74 nucleotides 5′ of the predicted *nahA* translational start site (Fig. S1D). The *nahS* upstream region contained −10 and −35 hexamers just upstream of the TSS, resembling bifidobacterial promoter sequences (35, 36), while in the case of the *nahA* promoter region, the TSS is preceded by a sequence that resembles a canonical −10 promoter sequence, although no associated −35 hexamer could be identified.

The *nag* and *lnp-glt* loci were each deduced to contain at least two promoters, just upstream of the *nagB3* (Fig. 2e) and *nagK* (Fig. 2f) genes and the *lnpB* (Fig. 2g) and *gltA* (Fig. 2h) genes, respectively, based on the associated genetic layout coupled to transcription patterns of the *nagR1* mutant or when UCC2003 was grown on LNB (27). The TSSs of the presumed *nagB3*, *nagK*, *lnpB*, and *gltA* promoters were determined by primer extension analysis using RNA extracted from *B. breve* UCC2003 grown in mMRS medium supplemented with 1% LNB. An extension product was identified 155 nucleotides 5′ of the predicted translational start site for the *nagB3* gene (Fig. S1E), while the transcriptional start site of *nagK* was identified 35 nucleotides 5′ of the predicted translational start site (Fig. S1F). An extension product was identified 43 nucleotides 5′

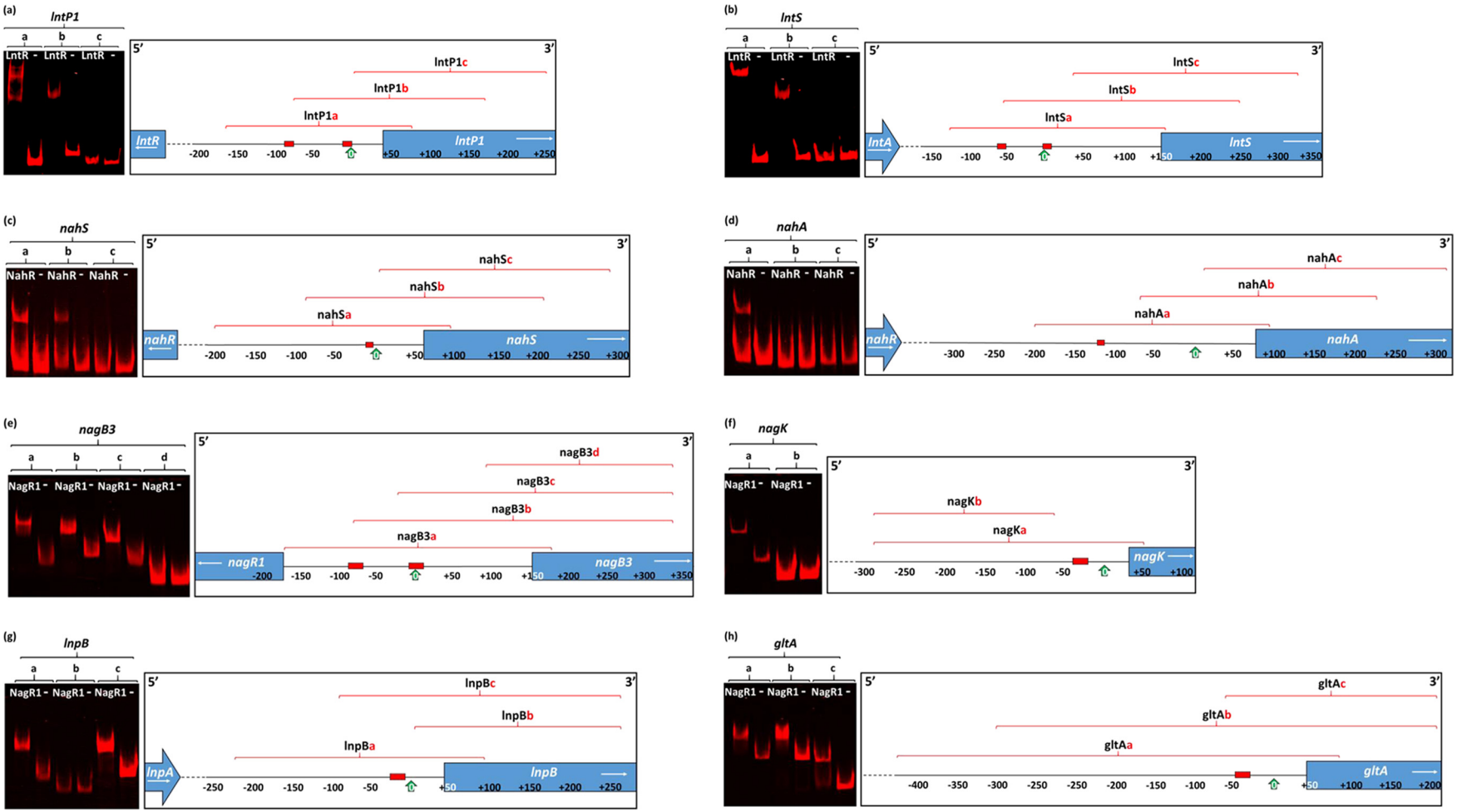

FIG 2 EMSA images showing LntR (a and b), NahR (c and d), and NagR1 (e to h) interactions with a range of DNA fragments from the regions in the proximity of their predicted target promoters, in order to identify their approximate locations. The locations and sizes of fragments used, in relation to the promoter regions' respective transcription start sites, are given in Table S1 in the supplemental material. The panels at the right schematically represent the locations of the DNA fragments used in relation to the locations of the putative operator sequences (red boxes), transcription start sites (green arrows), and genes (arrows in blue boxes). In each panel, "−" indicates a negative control, where an equivalent amount of the crude cell extract from NZ9000 harboring empty plasmid pNZ8150 was added instead of the crude extract from the regulator-expressing NZ9000 strain.

of the predicted translational start site for the *lnpB* gene (Fig. S1G), while the transcription start site for the *gltA* gene was identified 44 nucleotides 5′ of the predicted translational start site (Fig. S1H). All four regions contained −10 and −35 hexamers just upstream of the TSS that resembled bifidobacterial vegetative promoter recognition sequences.

Identification of regulator-operator interactions by using electromobility shift assays and *in silico* analyses. In order to establish if the LntR, NahR, and NagR1 proteins directly and specifically interact with operator sequences within the identified promoter regions of the *lnt*, *nah*, and *nag-lnp-glt* gene clusters, respectively, electrophoretic mobility shift assays (EMSAs) were performed. For the purpose of performing EMSAs, the *lntR*, *nahR*, and *nagR1* genes were first individually cloned into the nisin-inducible vector pNZ8150 with an N-terminal His tag-encoding sequence to facilitate protein expression and purification in *Lactococcus lactis* NZ9000 (see Materials and Methods). As was noted previously for other regulators from bifidobacteria (37–39), LntR, NahR, and NagR1 could be obtained as purified proteins but had lost their DNA-binding activity during some stage of the purification process. Thus, instead of purified protein, crude cell extracts of (nisin-induced) *L. lactis* NZ9000/pNZ-lntR$_{His}$, *L. lactis* NZ9000/pNZ-nahR$_{His}$, and *L. lactis* NZ9000/pNZ-nagR1$_{His}$ were used to carry out the EMSAs. A crude cell extract obtained from nisin-induced *L. lactis* NZ9000/pNZ8150 (empty vector) incubated with the respective DNA fragments was used as a negative control. The DNA fragments used were various short amplicons representing different segments of the putative promoter regions (Fig. 2; see also Table S1 in the supplemental material).

The LntR-containing crude extract was shown to specifically bind to the IRD700 (infrared dye)-labeled DNA fragments lntP1a and lntP1b but not to lntP1c (Fig. 2a and Table S1). A double mobility shift was observed for fragment lntP1a, which is indicative of two distinct LntR-binding sites being present on this fragment, while a single mobility shift was visible for fragment lntP1b. Similarly, LntR was able to bind to IRD700-labeled DNA fragments lntSb and lntSa, in the latter case being visible as a double mobility shift (suggesting the presence of two distinct LntR-binding sites), while no binding was observed with lntSc (Fig. 2b and Table S1). Inspection and comparison of the four fragments in which binding was observed revealed the presence of at least one complete conserved sequence, representing an inverted repeat, in all four fragments, while two such conserved sequences were observed in fragments lntP1a and lntSa (consistent with the observed double mobility shift). Comparative analysis of these inverted repeats identified a 14-nucleotide consensus sequence (Fig. 3a) containing conserved "CG" nucleotides at its center, which is a well-documented conserved feature of operator sequences bound by LacI-type regulators (40, 41). This consensus sequence furthermore contains conserved 5′ "TG" and 3′ "CA" nucleotides at its flanking ends, a feature previously documented for operator sequences identified for other LacI-type regulators encoded by *B. breve* UCC2003 (38, 42, 43). In both promoter regions, one such presumed operator sequence was found closely downstream of or partially overlapping the predicted −10 element of the promoter region, while the second was found closely upstream of the predicted −35 element of the promoter region (Fig. S1A and S1B). The positions of these identified operators are consistent with LntR acting as a repressor for the identified *lnt* promoters (44, 45).

The results obtained with the *L. lactis* NZ9000/pNZ-nahR$_{His}$ crude extract demonstrated specific binding to the IRD700-labeled DNA fragments nahSa and nahSb but not to nahSc (Fig. 2c and Table S1). Furthermore, binding was observed for IRD700-labeled DNA fragment nahAa but not for fragment nahAb or nahAc (Fig. 2d and Table S1). Sequence inspection and comparison of the NahR-bound DNA fragments revealed the presence of an inverted-repeat sequence, which was common to these fragments yet not present in fragments to which NahR did not bind. These inverted-repeat elements therefore represent putative operator sequences required for the NahR protein. Further analysis of these inverted repeats identified a 10-nucleotide consensus sequence (Fig. 3b). Conserved 5′ C and 3′ G nucleotides at the extreme flanks of this consensus

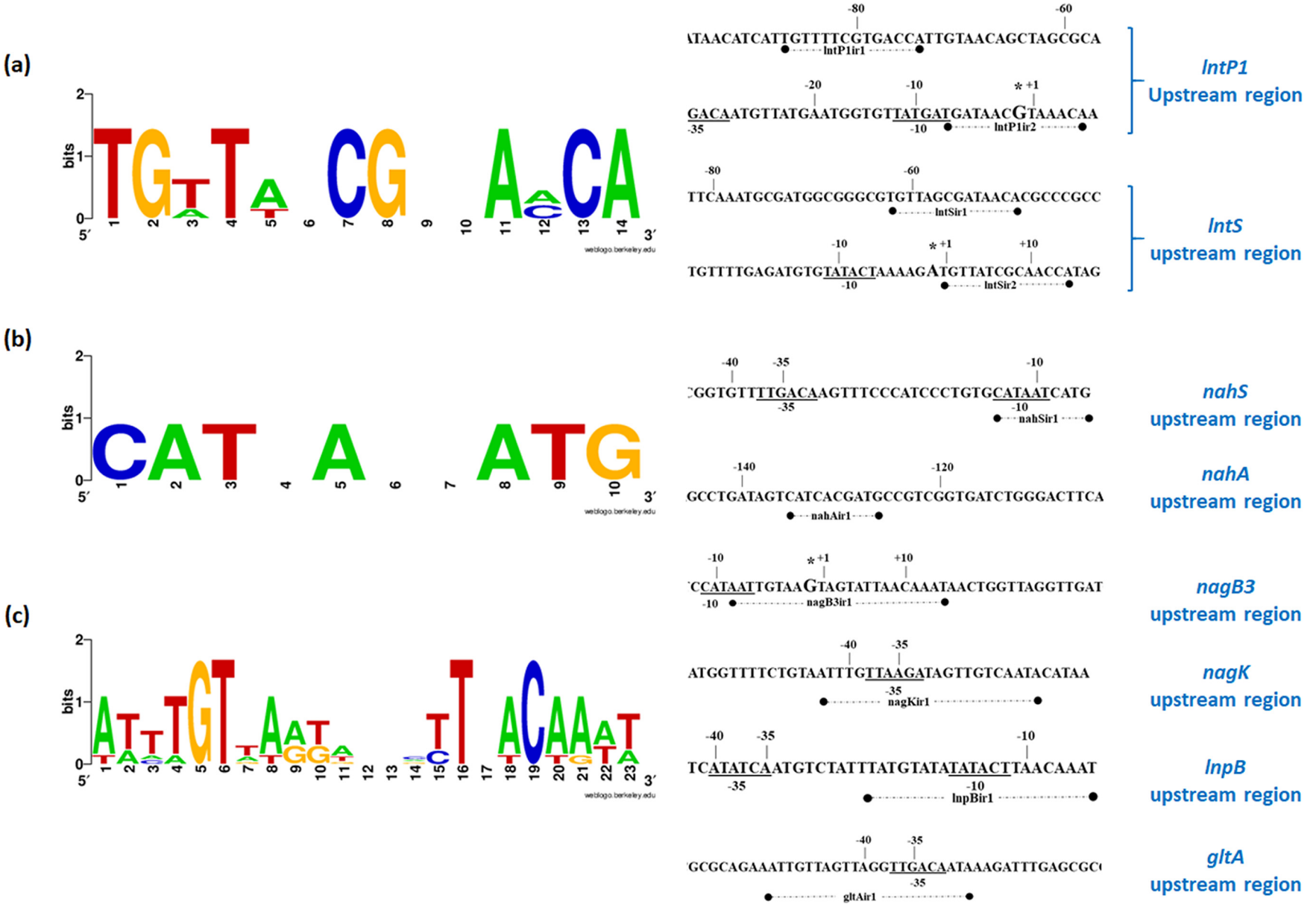

FIG 3 WebLogo representation of the operator motif consensus sequences of the LacI-type regulator LntR (a), the NahC/XylR-type regulator NahR (b), and the ROK/NagC-type regulator NagR1 (c), predicted by using *in silico* analysis. Predicted operator sequences were identified in the intergenic regions containing the coregulated promoters for each regulator by using the MEME online tool. Motif consensuses were generated by inputting these predicted operator sequences to the WebLogo online tool. The locations and sequences of each operator are shown alongside their respective consensus sequences.

sequence were previously observed for operator sequences of certain NagC/XylR-type regulators (46). The presumed operator upstream of *nahS* overlaps the downstream end of the predicted −10 promoter element (Fig. S1C), while the *nahA*-associated operator was found to be roughly 110 bp upstream of the predicted −10 element (Fig. S1D). The position of the identified *nahS* operator and the consensus obtained between this and the putative operator identified for *nahA* confirm the function of NahR as a repressor of *nahS*. While binding of NahR may occur at the *nahA* operator, this binding does not appear to directly interfere with the *nahA* promoter. This agrees with the lack of upregulation of *nahA* expression observed for the *nahR* mutant, although the transcriptional role, if any, of NahR in this case is not clear. The mapped locations of the operator and −10 and −35 sequences are shown in Fig. S1 in the supplemental material.

The results obtained with the crude extract obtained from nisin-induced *L. lactis* NZ900/pNZ-nagR1$_{His}$ revealed specific binding to the IRD700-labeled DNA fragments nagB3a, nagB3b, and nagB3c (with a weak apparent double shift observed for nagB3a) but not to fragment nagB3d (Fig. 2e and Table S1). Specific binding was identified for IRD700-labeled DNA fragment nagKa, while no binding was detected when fragment nagKb was used (Fig. 2f and Table S1). Binding of the NagR protein was also demonstrated for the IRD700-labeled DNA fragments lnpBa and lnpBc but not for lnpBb (Fig. 2g and Table S1). Finally, NagR1 was shown to bind IRD700-labeled DNA fragments gltAa, gltAb, and gltAc (Fig. 2h and Table S1). Inspection and comparison of the *nagB3*-, *nagK*-, *lnpB*-, and *gltA*-associated fragments in which binding was observed revealed the presence of a common sequence, representing an inverted repeat (with two repeats present in fragment nagB3a, consistent with the observed double shift), which was absent within fragments for which no binding was observed. These sequence motifs are presumed to act as operator sequences for the NagR1 protein. *In silico* analysis of these inverted-repeat sequences revealed a 23-nucleotide consensus motif (Fig. 3c). Interestingly, while this obtained consensus motif bears little resemblance to many previously proposed binding motifs for ROK/NagC family-type repressors from other bacteria (47), a substantial degree of similarity to motifs identified previously for other ROK/NagC-type regulators encoded by *B. breve* UCC2003 can be observed (30, 38). The putative *nagB3*, *nagK*, *lnpB*, and *gltA* operators were all found to overlap or encompass the predicted −10 or −35 elements (Fig. S1E to S1H). The positions of these identified operators corroborate the notion that NagR1 acts as a transcriptional repressor of its target genes (i.e., *nagB3*, *nagK*, *lnpB*, and *gltA*).

Identification of transcriptional effectors. In order to identify effectors that control the binding activity of LntR, NahR, and NagR1, we performed EMSAs with fragments containing the binding motifs for each regulator, in the presence of a range of carbohydrates, including lactose, LNB, LNT, LNnT, galactose, galactose-6-phosphate (Gal-6-P), galactose-1-phosphate (Gal-1-P), GlcNAc, *N*-acetylglucosamine-6-phosphate (GlcNAc-6P), GalNAc, or glucose (at a standard concentration of 20 mM) (see Fig. S2 in the supplemental material). These carbohydrates were chosen as they include both the complete structures and various components (or breakdown products) of LNT, LNnT, or LNB. Carbohydrates that did not elicit any effect on fragment binding by the regulator (at a concentration of 20 mM) were assumed not to represent transcriptional effectors for that particular regulator. If inhibition of binding was observed at 20 mM, the EMSA was repeated with a range of descending concentrations (or in some cases, higher concentrations were used for a related molecule [e.g., Gal, Gal-1-P, and Gal-6-P]). For LntR, galactose was found to reduce the binding of this regulator to its DNA targets at a concentration of 10 mM or lower (Fig. 4a). Gal-6-P and Gal-1-P were also found to reduce target DNA binding of LntR but at considerably higher, and perhaps biologically irrelevant, concentrations of ≥20 mM (Fig. S3). For NahR, only GlcNAc was found to reduce the interaction between NahR and its DNA target at a minimum concentration of 0.0625 mM (Fig. 4b), while in the case of NagR1, GlcNAc-6-P was found to prevent NagR1-binding activity at a minimum concentration of 1 mM (Fig. 4c and d).

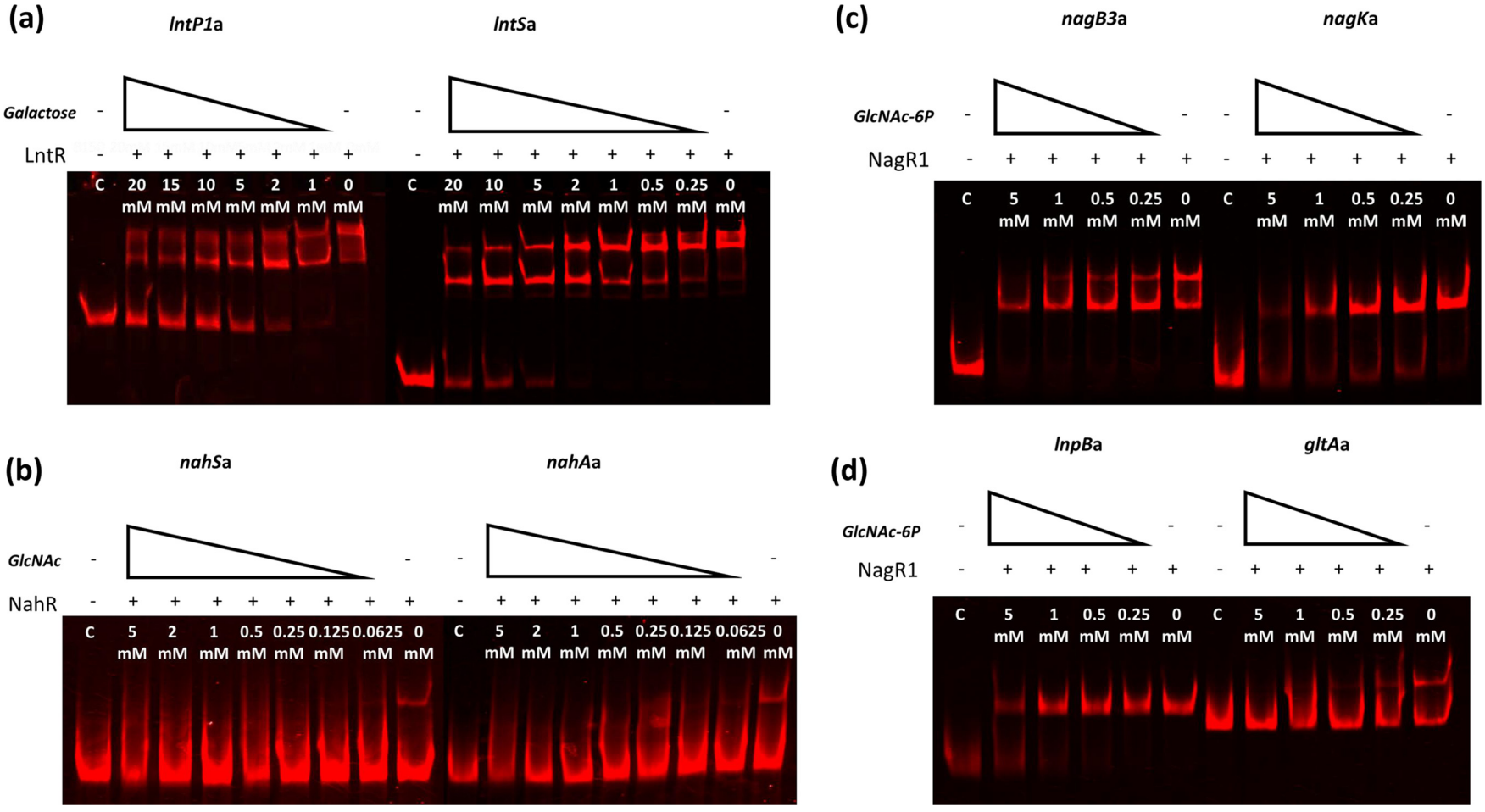

FIG 4 EMSA images showing LntR (a), NahR (b), and NagR1 (c and d) interactions with promoter-containing DNA fragments, with the addition of a gradient of their respective inducers ranging from 0 mM to 20 mM. In each panel, "C" indicates a negative control, where an equivalent amount of the crude cell extract from NZ9000 harboring empty plasmid pNZ8150 was added instead of the crude extract from the regulator-expressing NZ9000 strain.

DISCUSSION

The dominance of (certain) bifidobacteria within the breastfed neonatal gut microbiota (1) is substantially aided by the ability of these infant-associated species to utilize indigestible HMO residues as a carbon source (9). Our previous work demonstrated that the consumption and utilization of LNT, LNnT, or LNB by *B. breve* UCC2003 are facilitated by interrelated catabolic pathways (27). While pathways for HMO utilization in other *Bifidobacterium* species have been identified and elucidated (13, 21, 22), very little work has been carried out with regard to their regulation. Our results reveal molecular details of the transcriptional regulation of *B. breve* UCC2003 loci responsible for LN(n)T/LNB metabolism and provide insights into how the metabolism of these HMOs is controlled in *B. breve* UCC2003.

In this study, we identified four transcriptional regulators, three of which were shown to be involved in regulating LN(n)T/LNB metabolism in UCC2003 (Fig. 5). Microarray analysis of insertional mutants of *lntR*, *nahR*, *nagR1*, and *nagR2* identified genes under the regulation of each encoded regulator. LntR and NahR were shown to represent "local" regulators, i.e., controlling the transcription of genes adjacent to *lntR* and *nahR*, respectively. In contrast, NagR1 regulates the transcription of not only the local *nag* locus but also the genetically unlinked *lnp-glt* locus. We also investigated the transcriptome effect of a mutation in Bbr_1251 (*nagR2*); however, the affected genes are not believed to be involved in HMO metabolism but apparently are involved in maltooligosaccharide and fatty acid metabolism. While LacI-type, NagC/XylR-type, and ROK/NagC-type regulators have all previously been identified and characterized in *B. breve* UCC2003 (30, 37, 38, 42), functional analysis of regulators in other bifidobacteria is comparatively undocumented. However, a recent study identified transcription factors homologous to those of LntR, NagR1, and NagR2 in a range of different *Bifidobacterium* species (48).

Details of promoter and operator sequences specific to the LntR, NahR, and NagR1 regulators were elucidated by using a combination of electromobility shift and primer extension analyses. These operator results, for the most part, agree with those predicted previously by Khoroshkin et al. (48). The operator sequences predicted in their study concur with our experimentally determined data, in both approximate location and number, for both LntR and NagR1, with the exception of one additional predicted operator for LntR and two for NagR1. An additional NagR1 operator sequence was predicted to be upstream of *gltA*; however, this did not appear to be functional, based on the lack of a double mobility shift in the EMSAs of this region. This operator may indeed be a nonfunctional relic resulting from a duplication event. Khoroshkin et al. (48) also predicted an operator sequence upstream of the Bbr_1884 gene for NagR1 binding, although we did not examine this. However, based on the predicted functions of this gene in the bifid shunt, it may also be tied into the overall regulation of LNB and LacNAc metabolism carried out by NagR1. An additional LntR operator was predicted to be upstream of *lntR* itself, which may function in *lntR* transcriptional autoregulation. The observed lack of upregulation of *nahA* transcription for the *nahR* mutant appears discordant with the increase in the transcriptional level of this gene that was previously observed for wild-type UCC2003 during growth on LN(n)T (27) as well as the presence of the functional *nahA* operator sequence for NahR binding identified in this study. However, this may be explained if the transcriptional induction of *nahA* is mediated by both LntR and NahR. This possibility is corroborated by the presence of an inverted-repeat sequence resembling an LntR operator, and this intriguing possibility merits further experimental investigation.

Perhaps most interesting of all was the identification of the effectors for each transcriptional regulator. The binding of LntR to its targets is impeded by Gal, and NahR-mediated operator binding is prevented by the presence of GlcNAc, while the NagR1-operator interaction is prevented by the presence of GlcNAc-6-P. In each case, the genes under the transcriptional control of their respective regulators encode the metabolic machinery responsible for the release (and/or generation) of the effector

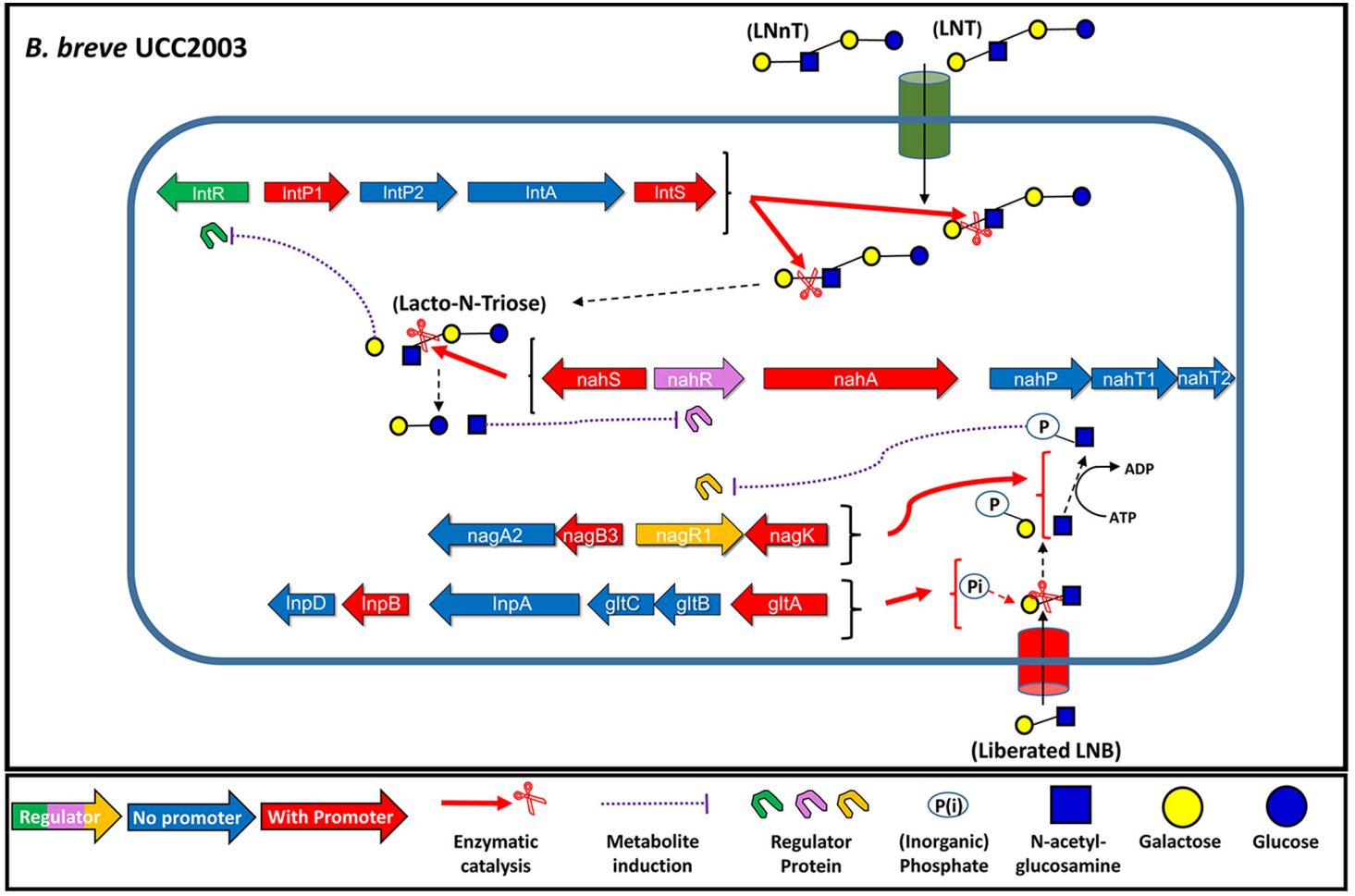

FIG 5 Schematic representation of the proposed model for the transcriptomic regulation of LNT, LNnT, and LNB metabolism by *B. breve* UCC2003. LNT and LNnT are internalized and intracellularly degraded through the sequential hydrolysis and release of monosaccharides from their nonreducing ends. These released monosaccharides (or their metabolites) act as effectors to relieve the transcriptional repression of the loci encoding the cellular components responsible for the liberation of these glycans. As such, liberated galactose relieves the transcriptional repression of the *Int* locus, and *N*-acetylglucosamine relieves the transcriptional repression of the *nah* locus. Similarly, the intracellular degradation of LNB (derived from the extracellular hydrolysis of complex HMO structures by other infant gastrointestinal tract microbes) releases *N*-acetylglucosamine and galactose-1-phosphate. GlcNAc is further converted into GlcNAc-6-P, which relieves the transcriptional repression of the *lnp-glt* and *nag* loci.

monosaccharide from the substrate at that metabolic step. For example, Gal is released from the nonreducing end of LN(n)T through the hydrolytic activity of LntA, which is encoded by the *lnt* locus (27). The transcriptional repression of this locus is thus believed to be relieved by the presence of the released monosaccharide, which is presumed to interact with the allosteric effector site typical of LacI-type repressors (38, 44, 49, 50). A similar scenario applies to GlcNAc release, which acts as the effector for the NahR regulator that controls the transcription of *nahS*, and to GlcNAc-6-P, which governs the activity of NagR1, the presumed transcriptional regulator of the *lnp-glt* and *nag* loci. The possible dual regulation of *nahA* transcription, as mentioned above, would mean that the presence of both the *lnt* locus activity product (and LntR effector) galactose and the *nah* locus activity product (and NahR effector) GlcNAc is required for the induction of *nahA* expression. This provides an extra level of transcriptional and, thus, metabolic control, ensuring the expression of *nahA* strictly during LN(n)T metabolism despite GlcNAc release during the metabolism of other sugars, such as LNB, sialic acid, and sulfated GlcNAc (27, 29, 30). Interestingly, in the case of GlcNAc-6-P and NagR1, the *lnp-glt* locus is required for the degradation of LNB, while the activity of the *nag* locus results in the generation of GlcNAc-6-P from liberated GlcNAc, during metabolism of both HMO and sialic acid (29). This may not be surprising, as sialic acid residues are commonly found in HMOs (6), and more importantly, GlcNAc is a breakdown product of LNB (as well as LNT and LNnT).

Interestingly, previous work showed that transcriptional induction takes place at the *lnt* locus during the growth of UCC2003 on galacto-oligosaccharides (GOSs) (51). This would appear to disagree with the high degree of specificity of transcriptional induction by effectors of these HMO-associated loci. However, it is worth noting that GOSs consist mainly of galactose (52, 53) and that the intracellular release of galactose during GOS metabolism by UCC2003 would be sufficient to cause the transcriptional induction of the *lnt* locus.

Thus, the presence and initial degradation of such a structure (i.e., LNT, LNnT, or LNB) indirectly induce the further expression of the locus required for its degradation, until the sugar is no longer available, at which point the absence of inducers will cause a return to transcriptional repression. Initial internalization and degradation are likely facilitated by a low level of "leaky" gene expression of the locus. In the case of LNT and LNnT degradation, this regulation is a two-step process, first at the level of LN(n)T degradation (by the *lnt* locus) and then at the level of [LN(n)T breakdown product] lacto-*N*-triose degradation (by the *nah* locus). The regulation of LNB metabolism is managed in a single step, at the level of LNB phosphorolysis and GlcNAc phosphorylation (by the *lnp-glt* locus and the *nag* locus, respectively). We see that all three regulators in this transcriptional control network belong to distinct families of regulator proteins despite functioning in similar roles as saccharide-controlled repressors. In conclusion, our results reveal a tightly controlled system for the transcriptional regulation of genes encoding the metabolic machinery required for (certain) HMO metabolism in *B. breve* UCC2003. Such tight regulation is necessary for infant-associated bifidobacteria such as *B. breve*, where switching metabolic processing to and from milk-derived sugars, such as HMO and lactose, and plant-derived carbohydrate sources (54) is a regular occurrence during the weaning period. Moreover, this suggests the evolution of specific catabolic responses to the presence of and for the utilization of specific HMO moieties by *B. breve* and poses the question of whether such regulatory systems have similarly evolved in other infant-associated *Bifidobacterium* species.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 2. *B. breve* UCC2003 was routinely cultured in either de Man-Rogosa-Sharpe (MRS) medium (Difco, BD, Le Pont de Claix, France) supplemented with 0.05% cysteine-HCl or reinforced clostridial medium (RCM; Oxoid Ltd., Basingstoke, England). Growth of bifidobacterial strains for transcriptional and primer extension analyses was carried out with mMRS medium, which was prepared from

TABLE 2 Bacterial plasmids and strains used in this work[a]

Strain or plasmid	Relevant feature(s)	Reference
Strains		
Escherichia coli		
EC101	Cloning host; *repA*$^+$ Km$^+$	42
EC101/pNZ-M.BbrII+M.BbrIII	EC101 harboring pNZ8048 derivative containing *bbrIIM* and *bbrIIIM*	40
Lactococcus lactis		
NZ9000	MG1363 *pepN*::*nisRK*; nisin-inducible overexpression host	54
NZ9700	Nisin-producing strain	54
NZ9000/pNZ-lntR	NZ9000 containing pNZ-lntR	This study
NZ9000/pNZ-nahR	NZ9000 containing pNZ-nahR	This study
NZ9000/pNZ-nagR1	NZ9000 containing pNZ-nagR1	This study
Bifidobacterium breve		
UCC2003	Isolate from nursling stool	41
UCC2003-lntR	pORI19-tet-bbr_0526 insertion mutant of UCC2003	This study
UCC2003-nahR	pORI19-tet-bbr_1555 insertion mutant of UCC2003	This study
UCC2003-nagR1	pORI19-tet-bbr_1249 insertion mutant of UCC2003	This study
UCC2003-nagR2	pORI19-tet-bbr_1251 insertion mutant of UCC2003	This study
Plasmids		
pAM5	pBC1-puC19-Tet^r	82
pORI19	Em^r Δ*repA ori*$^+$; cloning vector	42
pORI19-tet-lntR	Internal 367-bp fragment of Bbr_0526 and *tetW* cloned into pORI19	This study
pORI19-tet-nahR	Internal 448-bp fragment of Bbr_1554 and *tetW* cloned into pORI19	This study
pORI19-tet-nagR1	Internal 502-bp fragment of Bbr_1249 and *tetW* cloned into pORI19	This study
pORI19-tet-nagR2	Internal 507-bp fragment of Bbr_1251 and *tetW* cloned in pORI19	This study
pNZ8150	Cm^r; nisin-inducible translational fusion vector	50
pNZ-lntR	Cm^r; pNZ8150 derivative containing a translational fusion of the Bbr_0526 DNA fragment to the nisin-inducible promoter	This study
pNZ-nahR	Cm^r; pNZ8150 derivative containing a translational fusion of the Bbr_1555 DNA fragment to the nisin-inducible promoter	This study
pNZ-nagR1	Cm^r; pNZ8150 derivative containing a translational fusion of the Bbr_1249 DNA fragment to the nisin-inducible promoter	This study

[a]Cm^r, Em^r, Km^r, and Tet^r indicate resistance to chloramphenicol, erythromycin, kanamycin, and tetracycline, respectively. UCC, University College Cork Culture Collection.

first principles (using individual components) (55) and which does not contain a fixed carbohydrate source. Prior to inoculation, mMRS medium was supplemented with cysteine-HCl (0.05%, wt/vol) and a particular carbohydrate source (1%, wt/vol). It was previously shown that mMRS medium does not support the growth of *B. breve* UCC2003 in the absence of an added carbohydrate (56). Carbohydrates used were ribose (Sigma-Aldrich, Steinheim, Germany), LNB (Elicityl Oligotech, Crolles, France), and LNnT (Glycom, Lyngby, Denmark). A 1% (wt/vol) concentration of the carbohydrate was considered sufficient to encourage adequate growth for RNA harvesting. The addition of these carbohydrates did not significantly alter the pH of the medium, and therefore, subsequent pH adjustment was not required.

B. breve cultures were incubated under anaerobic conditions in a modular atmosphere-controlled system (Davidson and Hardy, Belfast, Ireland) at 37°C. *Lactococcus lactis* strains were cultivated in M17 broth (Oxoid Ltd., Basingstoke, England) containing 0.5% glucose (57) at 30°C. *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth (58) at 37°C with agitation. Where appropriate, growth media contained tetracycline (Tet) (10 μg ml^{-1}), chloramphenicol (Cm) (5 μg ml^{-1} for *L. lactis* and *E. coli* 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* EC101 cells containing (derivatives of) 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).

Nucleotide sequence analysis. Sequence information was obtained from the Artemis-mediated (59) genome annotations of *B. breve* UCC2003 (60). Database searches were performed by using nonredundant sequences accessible at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), using the basic local alignment search tool (BLAST) (61, 62). Sequences were verified and analyzed by using the SeqMan and SeqBuilder programs of the DNAStar software package (version 10.1.2; DNAStar, Madison, WI, USA).

DNA manipulations. Chromosomal DNA was isolated from *B. breve* UCC2003 as previously described (63). Plasmid DNA was isolated from *Escherichia coli*, *Lactococcus 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*. Procedures for DNA manipulations were performed essentially as described previously (58). All restriction enzymes and T4 DNA ligase were used according to the supplier's instructions (Roche Diagnostics, Basel, Switzerland). Synthetic single-stranded oligonucleotide primers used in this study (Table 3) were synthesized by Eurofins (Ebersberg, Germany). Standard PCRs were performed by using *Taq* PCR master mix (Qiagen) or Extensor Hi-Fidelity PCR master mix (Thermo Scientific, Waltham, MA, USA) in a Biometra

TABLE 3 Oligonucleotide primers used in this work

Purpose	Primer	Sequence (5′–3′)[a]
Cloning of Bbr_0526 in pNZ8150	526F	TGCATC**CCCGGG**ATG*CATCACCATCACCATCACCATCACCATCAC*GCGAGACCAACACAGGTTTCC
	526R	TGCGCA**TCTAGA**CGTTTCCCGTATACCATTAATCAG
Cloning of Bbr_1555 in pNZ8150	1555F	TGCATC**GATATC**ATG*CATCACCATCACCATCACCATCACCATCAC*TACGCTAAATCCAATCCC
	1555R	TGCGCA**TCTAGA**CGGCGGCACGGTGATCTG
Cloning of Bbr_1249 in pNZ8150	1249F	TGCATC**CAGCTG**ATG*CATCACCATCACCATCACCATCACCATCAC*TCGTATCCCGGTCTTGCC
	1249R	TGCATC**CAGCTG**ATGTCGTATCCCGGTCTTGCC
Cloning of an internal 465-bp fragment of Bbr_0526 in pORI19	IM526F	CTGGTC**AAGCTT**CGTTGAAGCCGCGATGGA
	IM526R	CTGGTC**TCTAGA**GTCAACGGTGGGGCAGTG
Cloning of an internal 488-bp fragment of Bbr_1555 in pORI19	IM1555F	CTGGTC**AAGCTT**GCTGGCCATCGATACGGAC
	IM1555R	CTGGTC**TCTAGA**CTCGTCGTTCAGCAGCAC
Cloning of an internal 443-bp fragment of Bbr_1249 in pORI19	IM1249F	CTGGTC**AAGCTT**CGAAGAAGGCCTATTGCG
	IM1249R	CTGGTC**TCTAGA**CAGCAGAATCGCCGAACC
Cloning of an internal 488-bp fragment of Bbr_1251 in pORI19	IM1251F	CTGGTC**AAGCTT**GAAGAGACCGGCGACCTGG
	IM1251R	CTGGTC**TCTAGA**GCCATTGTCGATGACGCC
Amplification of *tetW*	tetWFw	TCAGCTGTCGACATGCTCATGTACGGTAAGGAAGCA
	tetWRv	GCGACGGTCGACCATAACTTCTGATTGTTGCCG
Confirmation of site-specific homologous recombination	526confirm1	GCGCTAGCTGTTACAATGGTC
	526confirm2	GCCATTTCCAACCCCTCTC
	1555confirm1	TACGCTAAATCCAATCCC
	1555confirm2	GACGCAAGGGCCAACAACCGC
	1249confrim1	CATACAGCCGCCACGGCAC
	1249confrim2	TCGTATCCCGGTCTTGCC
	1251confrim1	GCAGACGATACTGCACGCG
	1251confrim2	GTCAAGCATCTCTACCAC
Amplification of Bbr_0527 promoter fragments with IRD700-labeled oligonucleotides	527IRDfa	CTCGCCCCTCGCTTGTCTCTC
	527IRDra	GCATAGGCACGGCAGCGAC
	527IRDfb	ATTGTTTTCGTGACCATTG
	527IRDrb	GAATAATGAACACGAACACG
	527IRDfc	CAATTTTGGTCAACCTTCG
	527IRDrc	CGCGCGTAGTTCTCGAC
Amplification of Bbr_0530 promoter fragments with IRD700-labeled oligonucleotides	530IRDfa	GCCGAACGGTGTGCTGGTGG
	530IRDra	CTTCATCGTTCTGTTCTCCTTC
	530IRDfb	CGATAACACGCCCGCCATC
	530IRDrb	GCTGGACTTGCCGCTATC
	530IRDfc	CTTCATAGAGCCACTTC
	530IRDrc	CTCGAAGTCCTTGGCAAC
Amplification of Bbr_1554 promoter fragments with IRD700-labeled oligonucleotides	1554IRDfa	GTCGCTGGGATTGGATTTAGCG
	1554IRDra	GTGGCTATGACTGCGCGC
	1554IRDfb	CGGCTTTCAGGATAACACCCA
	1554IRDrb	GGATTTGGCGGCGCGATC
	1554IRDfc	CCAAACAAAATAGTTGCTACGGC
	1554IRDrc	GTTGAGTGCGGTGTAGGTCTCC
Amplification of Bbr_1556 promoter fragments with IRD700-labeled oligonucleotides	1556IRDfa	CTGGACGGCTGCTCAAAGC
	1556IRDra	GCAGAGATGTTTGACCGTTCAT
	1556IRDfb	GGTGACGACGCCACTCTGC
	1556IRDrb	GTGGTTTGCGGTTGCCCT
	1556IRDfc	GCCATCTCAGGACCGAACG
	1556IRDrc	GGTCGTCAAGGTGATGAATCC
Amplification of Bbr_1248 promoter fragments with IRD700-labeled oligonucleotides	1248IRDfa	CCTCCTGCCTGAACGATG
	1248IRDra	GACAATGATGATTTCCGGC
	1248IRDfb	GTTAGGGAACTTCACTAATACATTCC
	1248IRDrb	CCTGGGAGATGTCGATCGACTC

(Continued on next page)

TABLE 3 (Continued)

Purpose	Primer	Sequence (5′–3′)[a]
	1248IRDfc	GTCCGTACGTCCATAATTGTAAGTAG
	1248IRDrc	CCTGGGAGATGTCGATCGACTC
	1248IRDfd	GATGGGCGGCTTTGGGCAG
	1248IRDrd	CCTGGGAGATGTCGATCGACTC
Amplification of Bbr_1250 promoter fragments with IRD700-labeled oligonucleotides	1250IRDfa	GATGCCGTTGTGGTAGAGATG
	1250IRDra	GGTGTTATCAGTCATTGCCTATCC
	1250IRDfb	GCGTGTCGCGTATGAGGC
	1250IRDrb	GGTGTTATCAGTCATTGCCTATCC
Amplification of Bbr_1586 promoter fragments with IRD700-labeled oligonucleotides	1586IRDfa	CGGTTCGTCGAAAATCCAAG
	1586IRDra	CAGTGCGAAGTGTGAGGCG
	1586IRDfb	GCCGCTTATTGCGGCTTTATAG
	1586IRDrb	CTTTGAGGGCAGAAGTAACTAGTTC
	1586IRDfc	GTATGCGCGTTCGTCCAC
	1586IRDrc	CTTTGAGGGCAGAAGTAACTAGTTC
Amplification of Bbr_1590 promoter fragments with IRD700-labeled oligonucleotides	1590IRDfa	GGCCCGCTGGCAGATTAG
	1590IRDra	GGCAAGAGCAGCCACGATG
	1590IRDfb	GACAGATGTCTGAGCGGTC
	1590IRDrb	GAATCGGGCAGACGGTGC
	1590IRDfc	CGCGCAGAAATTGTTAGTTAGG
	1590IRDrc	GAATCGGGCAGACGGTGC
Amplification of a region containing the Bbr_0527 promoter region for sequencing ladders	527promF	GCATTGCTGTCATTCGCCACAC
	527promR	GAATAATGAACACGAACACG
Amplification of a region containing the Bbr_0530 promoter region for sequencing ladders	530promF	GCGTGCGGATGAAACTGG
	530promR	GTCTGGAACGGCTTGGCGC
Amplification of a region containing the Bbr_1554 promoter region for sequencing ladders	1554promF	CGTTTCCTCGACCCCAGTTC
	1554promR	GAATGTGTCCTTGAGCTTGGC
Amplification of a region containing the Bbr_1556 promoter region for sequencing ladders	1556promF	CTGGACGGCTGCTCAAAGC
	1556promR	GGTCGTCAAGGTGATGAATCC
Amplification of a region containing the Bbr_1248 promoter region for sequencing ladders	1248promF	GGAGGCTTTGGCGGTACGG
	1248promR	CCTGGGAGATGTCGATCGACTC
Amplification of a region containing the Bbr_1250 promoter region for sequencing ladders	1250promF	GATGCCGTTGTGGTAGAGATG
	1250promR	GGTGCCACCCACATCAACAC
Amplification of a region containing the Bbr_1586 promoter region for sequencing ladders	1586promF	GCGAGACCTTCGACCTTCAGCC
	1586promR	CGGCACGAGATTGTAAGACAC
Amplification of a region containing the Bbr_1590 promoter region for sequencing ladders	1590promF	GGCCCGCTGGCAGATTAG
	1590promR	GAATCGGGCAGACGGTGC
527 promoter for primer extension analysis	527PE	GCATAGGCACGGCAGCGAC
530 promoter for primer extension analysis	530PE	CTTCATCGTTCTGTTCTCCTTC
1554 promoter for primer extension analysis	1554PE	GTTCATGTTGGTCTTCTTTCC

(Continued on next page)

TABLE 3 (Continued)

Purpose	Primer	Sequence (5′–3′)[a]
1556 promoter for primer extension analysis	1556PE	GCAGAGATGTTTGACCGTTCAT
1248 promoter for primer extension analysis	1248PE	CTGCCCAAAGCCGCCCATC
1250 promoter for primer extension analysis	1250PE	GGTGTTATCAGTCATTGCCTATCC
1586 promoter for primer extension analysis	1586PE	GCGATGTCAAATAGTGTTTCC
1590 promoter for primer extension analysis	1590PE	GGCAAGAGCAGCCACGATG

[a]Restriction sites incorporated into oligonucleotide primer sequences are indicated in boldface type, and His-tagged sequences incorporated into nucleotide primer sequences are indicated in italic type.

T3000 thermocycler (Biometra, Göttingen, Germany) or a Life Technologies Proflex PCR system (Thermo Scientific, Waltham, MA, USA). PCR products were visualized by ethidium bromide (EtBr) staining following agarose gel electrophoresis (1% agarose). *B. breve* colony PCRs were performed as described previously (64). PCR fragments were purified by using the Roche High Pure PCR purification kit (Roche Diagnostics, Basel, Switzerland). Plasmid DNA was isolated by using the Roche High Pure plasmid isolation kit (Roche Diagnostics, Basel, Switzerland). Plasmid DNA was introduced into *E. coli* by electroporation, as described previously (58). *B. breve* UCC2003 (65) and *L. lactis* (66) were transformed by electroporation according to previously reported protocols. The correct orientation of DNA inserts and the integrity of all plasmid constructs (see also below) were verified by DNA sequencing performed at Eurofins (Ebersberg, Germany).

Construction of *B. breve* UCC2003 insertion mutants. Internal fragments of Bbr_0526 (designated here *lntR*) (367 bp representing codons 40 through 162 of the 320 codons of this gene), Bbr_1249 (designated here *nagR1*) (502 bp representing codons 64 through 231 of the 375 codons of this gene), Bbr_1251 (designated here *nagR2*) (507 bp representing codons 62 through 230 of the 405 codons of this gene), and Bbr_1555 (designated here *nahR*) (448 bp representing codons 74 through 223 of the 380 codons of this gene) were amplified by PCR using *B. breve* UCC2003 chromosomal DNA as a template and primer pairs 526LacIInsFHindIII and 526LacIInsRXbaI, 1249LacIInsFHindIII and 1249LacIInsRXbaI, 1251LacIInsFHindIII and 1251LacIInsRXbaI, and 1555LacIInsFHindIII and 1555LacIInsRXbaI (Table 3), respectively. The insertion mutants were constructed by using a previously described approach (64), generating mutant strains *B. breve* UCC2003-lntR, *B. breve* UCC2003-nagR1, *B. breve* UCC2003-nagR2, and *B. breve* UCC2003-nahR, which carried disrupted *lntR*, *nagR1*, *nagR2*, and *nahR* genes, respectively (Table 2). The site-specific recombination of potential Tet-resistant mutant isolates was confirmed by colony PCR using primer pair TetWF and TetWR to verify *tetW* gene integration and primers Bbr_526ConfirmP1 or Bbr_526ConfirmP2, Bbr_1249ConfirmP1 or Bbr_1249ConfirmP2, Bbr_1251ConfirmP1 or Bbr_1251ConfirmP2, and Bbr_1555ConfirmP1 or Bbr_1555ConfirmP2 (positioned upstream of the selected internal fragments of Bbr_0526, Bbr_1249, Bbr_1251, and Bbr_1555, respectively) in combination with primer TetWF to confirm integration at the correct chromosomal location (Table 3).

Analysis of global gene expression using *B. breve* DNA microarrays. Global gene expression levels were determined during log-phase growth of the insertional mutant strains *B. breve* UCC2003-lntR, *B. breve* UCC2003-nagR1, *B. breve* UCC2003-nagR2, and *B. breve* UCC2003-nahR in mMRS medium supplemented with ribose. The generated transcriptome data sets were compared to the transcriptome information obtained for log-phase wild-type *B. breve* UCC2003 cells grown in mMRS medium supplemented with ribose. Ribose was selected as a suitable transcriptomic reference as the metabolic pathway and gene expression profile for the growth of UCC2003 on ribose are known and were employed previously (43, 67). DNA microarrays containing oligonucleotide primers representing each of the 1,864 identified open reading frames on the genome of *B. breve* UCC2003 were designed by and obtained from Agilent Technologies (Palo Alto, CA, USA). Methods for cell disruption, RNA isolation, RNA quality control, and cDNA synthesis and labeling were performed as described previously (68). Two independent biological replicates were used for each array, using a Cy3/Cy5 dye swap, as described previously (68). Labeled cDNA was hybridized by using the Agilent gene expression hybridization kit (part no. 5188-5242), as described in the Agilent Two-Color Microarray-Based Gene Expression Analysis v4.0 manual (publication number G4140-90050). Following hybridization, microarrays were washed in accordance with Agilent's standard procedures and scanned by using an Agilent DNA microarray scanner (model G2565A). The generated scans were converted to data files with Agilent Feature Extraction software (version 9.5). DNA microarray data were processed as previously described (69–71). Differential expression tests were performed with the Cyber-T implementation of a variant of the *t* test (72).

Construction of overexpression vectors and protein overproduction and purification. For the construction of plasmids pNZ-lntR, pNZ-nagR1, and pNZ-nahR, DNA fragments encompassing *lntR*,

nagR1, and *nahR* were generated by PCR amplification from chromosomal DNA of *B. breve* UCC2003 using Q5 High-Fidelity DNA polymerase and primer pairs 526PurFSmaI and 526PurRXbaI, 1249PurFPvuII and 1249PurRXbaI, and 1555PurFEcoRV and 1555PurXbaI, respectively (Table 3). An in-frame N-terminal His_{10}-encoding sequence was incorporated into forward primers 526PurFSmaI, 1249PurFPvuII, and 1555PurFEcoRV to facilitate downstream protein purification. The generated amplicons were digested with SmaI and XbaI, PvuII and XbaI, and EcoRV and XbaI, respectively, and ligated into the ScaI- and XbaI-digested, nisin-inducible translational fusion plasmid pNZ8150 (73). The ligation mixtures were introduced into *L. lactis* NZ9000 by electrotransformation, and transformants were then selected based on Cm resistance. The plasmid content of a number of Cm-resistant transformants was screened by restriction analysis, and the integrity of positively identified clones was verified by sequencing.

Nisin-inducible gene expression and protein overproduction were performed as described previously (37, 42, 74). In brief, 50 ml of M17 broth supplemented with 0.5% (wt/vol) glucose was inoculated with a 2% inoculum of a particular *L. lactis* strain, followed by incubation at 30°C until an optical density at 600 nm (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 (75), followed by continued incubation for a further 2 h. Cells were harvested by centrifugation, and the crude cell extract was obtained as described previously (38). Although protein purification of LntR-His, NahR-His, and NagR1-His was achieved by using His tag affinity chromatography, the purification procedure appeared to render the proteins inactive in subsequent EMSAs. For this reason, crude cell extracts, prepared in 10 mM Tris-HCl lysis buffer (pH 7.0), were adopted for the EMSAs (see below).

Electrophoretic mobility shift assays. DNA fragments representing different portions of the promoter regions upstream of *lntP1* (locus tag Bbr_0527) and *lntS* (locus tag Bbr_0530), *nagB3* (locus tag Bbr_1248) and *nagK* (locus tag Bbr_1250), *lnpB* (locus tag Bbr_1586) and *gltA* (locus tag Bbr_1590), and *nahS* (Bbr_1554) and *nahA* (Bbr_1556) were prepared by PCR using IRD700-labeled primer pairs (Integrated DNA Technologies, Coralville, IN, USA) (Table 3). EMSAs were performed essentially as described previously (42, 76). In all cases, binding reactions were carried out with a final 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, 100 mM KCl, 10% glycerol). Various amounts of the crude protein extract, ranging from 140 ng to 180 ng, of the constructed LntR-, NahR-, or NagR1-(over)producing *L. lactis* NZ9000 strain and a fixed amount of a DNA probe (0.1 pmol) were mixed on ice and subsequently incubated for 15 min at 37°C. In order to assess if the binding activity of LntR, NahR, or NagR1 is modulated by a carbohydrate ligand, various carbohydrates, including galactose, galactose-1-phosphate, galactose-6-phosphate (all from Sigma-Aldrich, Steinheim, Germany), LNT (Glycom, Lyngby, Denmark), LNnT (Glycom, Lyngby, Denmark), LNB (Elicityl Oligotech, Crolles, France), glucose, *N*-acetylglucosamine, *N*-acetylglucosamine-6-phosphate, or lactose (all from Sigma-Aldrich, Steinheim, Germany), with concentrations ranging from 50 to 0.0625 mM, were included in the binding reaction buffer. Samples were loaded onto a 6% nondenaturing phosphonoacetic acid (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 90 min in an Atto Mini PAGE system (Atto Bioscience and Biotechnology, Tokyo, Japan). Signals were detected by using the Odyssey infrared imaging system (Li-Cor Biosciences UK Ltd., Cambridge, UK) and captured by using the supplied Odyssey V3.0 software.

Primer extension analysis. Total RNA was isolated from *B. breve* UCC2003 cells grown in mMRS medium supplemented with 1% LNnT or 1% LNB to early exponential phase, using a previously described Macaloid method (77). RNA samples were treated with RNase-free DNase (Ambion). Primer extension was performed by annealing 1 pmol of IRD700 synthetic 18-mer oligonucleotides to 15 μg of RNA, as described previously (78). Sequence ladders of the presumed promoter regions immediately upstream of *lntP1*, *lntS*, *nagB3*, *nagK*, *lnpB*, *gltA*, *nahS*, or *nahA*, amplified from UCC2003 genomic DNA, which were run alongside the primer extension products, were produced by using the same primer as the one used for the primer extension reaction and by employing the Thermo Sequenase primer cycle sequencing kit (Amersham). Separation was achieved on a 6.5% Li-Cor Matrix KB Plus acrylamide gel. Signal detection and image capture were performed by means of a Li-Cor sequencing instrument (Li-Cor Biosciences).

Operator consensus sequence prediction using MEME and WebLogo online software tools. Coregulated promoter regions were assessed for the presence of operator sequences by the use of the MEME (Multiple Em for Motif Elicitation) online tool (http://meme-suite.org/tools/meme) (79) and then visualized by using the WebLogo online tool (http://weblogo.berkeley.edu/logo.cgi) (80, 81). Sequences used for consensus sequence prediction are given in Table S2 in the supplemental material.

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 no. GSE105108.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02774-17.

SUPPLEMENTAL FILE 1, PDF file, 1.6 MB.

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D.V.S., K.J., and M.O.M. conceived the experiments. K.J., with the assistance of C.P. and R.L.O., conducted the experiments. All authors analyzed the results and contributed to writing the manuscript.

We declare that, to the best of our knowledge, there are no competing financial interests.

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