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Bifidobacterium breve UCC2003 employs multiple transcriptional regulators to control metabolism of particular human milk oligosaccharides.

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Running Title: Control of bifidobacterial HMO metabolism

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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 *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 expression of the genetic loci involved in these HMO metabolic pathways. In the current study, we describe the characterization of three transcriptional regulators and corresponding operator and associated (inducible) promoter sequences, the latter governing 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, there is to date very little understanding of the transcriptional regulation of these pathways. The current study reveals a multi-component 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.

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Introduction

Bifidobacteria represent high-G+C, Gram-positive, anaerobic members of the phylum Actinobacteria, and are common commensals of the mammalian, avian and insect gut. In humans, they are particularly abundant and prevalent among the gut microbiota of healthy, vaginally-delivered, breastfed infants (2), and are thought to confer a multitude of benefits to the neonatal host (3-5). 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 non-digestible food ingredient 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' (6).

Fascinatingly, the archetypal prebiotic would appear to be human breastmilk, and in particular its bifidogenic constituents known as Human Milk Oligosaccharides (HMOs). HMOs represent specific glycans present in human breastmilk that are thought to shape, at least partly, the compositional structure of the neonatal gut microbiota (7, 8). HMOs represent, after lactose, the second-largest carbohydrate component of breastmilk (7, 9), and constitute a heterogeneous mix of at least 200 distinct glycan structures (10). 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 (11).

The effects of the specific human breastmilk 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 they are believed to play in infant health and development (12, 13). 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 regards to the manner in which they affect microbiota development.

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It is hardly surprising that the dominant *Bifidobacterium* species found among the neonatal gut microbiota can utilise various HMO components as their sole carbohydrate source (10). 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 characterised. *B. bifidum* extracellularly hydrolyses complex HMO structures, including LNT and LNnT, employing secreted glycosyl hydrolases, followed by the internalisation and intracellular degradation/metabolism of (most of) the resulting mono- and di-saccharides, such as LNB (11, 14-20). *B. longum* subsp. *infantis* internalises intact LNT, LNnT and LNB, and uses a series of sequential hydrolytic/phosphorolytic reactions acting from the non-reducing end of the carbohydrate structures to degrade them into their monosaccharide components for further metabolic processing (10, 11, 21-24). However, *B. infantis* has also been demonstrated to take up and utilise fucosyl- and sialyl-lactose (25-27).

The metabolic pathways of LNT, LNnT and LNB have recently been elucidated in the prototype strain *B. breve* UCC2003 (28). In the latter study, converging pathways of LNT and LNnT catabolism were identified, where monosaccharide moieties are sequentially released from the non-reducing 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_0526-0530) and the *nah* locus (locus tags Bbr_1554-1560) (Table 3 and Fig. 1). The *lnt* locus encodes proteins that are responsible for the internalisation of LNT and intracellular hydrolysis of both LNT and LNnT, releasing a galactose (Gal) moiety from their non-reducing 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 which liberates GlcNAc from the non-reducing 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_1585-1590; Table 3 and Fig. 1) are responsible for the internalisation and subsequent phosphorolysis of free LNB, releasing its constituent monosaccharides Gal-6-phosphate and GlcNAc (28, 29). We also identified the transcriptional upregulation of genes in the *nag* locus (locus tags Bbr_1247-1252; Table 3 and Fig. 1) during growth on LNT, LNnT and LNB, indicating their role in the utilisation of these sugars, specifically in the multi-step metabolism of GlcNAc. The *nag* locus has previously been implicated in the metabolism of sialic acid and mucin-derived N-glycans, both of which contain GlcNAc as well (30, 31).

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While the degradation routes of these key HMO structures have thus been identified, the regulatory mechanisms that control expression of these pathways have remained unexplored, both for *B. breve* and HMO-utilising *Bifidobacterium* species as a whole.

In the current study, we identified and characterized the genes encoding transcriptional regulators responsible for 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-utilisation loci. In a previous study, we had observed that genes within four chromosomal loci exhibit transcriptional induction during growth of *B. breve* UCC2003 on LNT, LNnT or LNB as the sole carbohydrate source (28). 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_0526-530), the *nah* locus (Bbr_1554-1560), the *nag* locus (Bbr_1247-1250) and the *lnp/glt* locus (Bbr_1585-1590) (Fig. 1; see Table 3 for a description of [predicted] functions). Detailed scrutiny of these four loci and neighbouring 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 1B). The *nag* locus is associated with two genes, *nagR1* (Bbr_1249) and *nagR2* (Bbr_1251), both predicted to encode ROK/NagC family-type repressors, while no regulator-encoding gene was observed in close vicinity of the *lnp/glt locus* (Fig. 1C and 1D). NagC/XylR-type and ROK/NagC-type repressors are both members of the large family of ROK-type transcriptional regulators (32). 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

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141 to identify promoters/genes that are subject to transcriptional control of these predicted
142 regulators, global gene transcription data was obtained from microarray-based analyses
143 performed on the *B. breve* UCC2003-lntR, UCC2003-nahR, UCC2003-nagR1 and
144 UCC2003-nagR2 insertion mutants, grown in mMRS supplemented with ribose, as compared
145 to the transcriptome of the UCC2003 wild type strain grown under the same conditions.

146 Transcriptome analysis of the *lntR* mutant revealed the upregulation of the adjacent *lntP1*,
147 *lntP2*, *lntA* and *lntS* genes of the *lnt* locus (Table 3 and Fig. 1), when this mutant was grown
148 on ribose (as compared to wild type UCC2003), all of which were also previously found to be
149 upregulated in expression during growth of wild type UCC2003 on LNT or LNnT (28). This
150 corroborates the notion that LntR is a LacI-type repressor and that this protein negatively
151 regulates the LNT/LNnT-dependent transcription of genes within the *lnt* cluster. Conversely,
152 the array data obtained for the *nahR* mutant only revealed transcriptional upregulation
153 (compared to the UCC2003 control) of the *nahS* gene (Table 3), when grown on ribose. This
154 is consistent with previously observed expression patterns in UCC2003, with the exception of
155 *nahA*, which may have been expected to exhibit transcriptional upregulation in the *nahR*
156 mutant, as its expression was increased during growth on LNT and LNnT in *B. breve*
157 UCC2003 (28). These results suggest that NahR, a NagC/XylR-type repressor, is responsible
158 for the transcriptional regulation of at least one gene of the *nah* cluster. For the *nagR1*
159 mutant, upregulation of *nagA2*, *nagB3* and *nagK* (but not *nagR2* or *nagK2*), as well as all of
160 the genes of the *lnp/glt* locus (Table 3) was observed when grown on ribose (as compared to
161 the UCC2003 control). These results suggest that NagR1, a ROK/NagC family-type
162 repressor, is responsible for the transcriptional regulation of (part of) the *nag* and *lnb* clusters.
163 This is consistent with transcriptomic data previously obtained for wild type UCC2003
164 during growth on LN(n)T and LNB, which demonstrated the transcriptional upregulation of
165 genes in both of these loci (28). When the transcriptome of UCC2003-nagR2 was compared
166 to that of UCC2003 when grown on ribose, the *nagR2* mutant exhibited increased
167 transcription of genes in the *mal* locus (locus tags Bbr_0118-0123), which is known to be
168 involved in maltooligosaccharide metabolism (33, 34), and Bbr_1719-1721 (predicted to
169 function in fatty acid metabolism) (35) (data not shown), none of which are predicted to
170 function in HMO metabolism, nor were shown to be upregulated in our previous wild type
171 arrays on LNT, LNnT or LNB (28). These results thus show that NagR2 is not involved in
172 the transcriptional control of the loci responsible for LNT, LNnT or LNB metabolism, and no
173 further investigation of this regulator was carried out. The *lntR*, *nahR* and *nagR1* genes,

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however, were selected for further study, as described below, in order to further elucidate their regulatory activity and specificity.

Promoter mapping through identification of the 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 (TSS) 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 an increase in transcription upon growth on LNT, LNnT, LNB, lactosamine or lactose (28). The transcription start sites (TSS) of the presumed *lntP1* and *lntS* promoters were determined by primer extension analysis using RNA extracted from *B. breve* UCC2003 grown in mMRS supplemented with 1 % LNnT. An extension product was identified 41 nucleotides 5' of the predicted translational start site of the *lntP1* gene (Supplemental Fig. S1A), while the TSS for the *lntS* gene was identified 154 nucleotides 5' of the predicted translational start site (Supplemental Fig. S1B). In both cases, the TSS was preceded by -10 and -35 hexamers that resemble (bifidobacterial) consensus vegetative promoter recognition sequences (36, 37).

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 only observed 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 is grown on LNT, LNnT or lactosamine (28). 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 supplemented with 1 % LNnT. An extension product was identified 59 nucleotides 5' of the

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predicted translational start site for the *nahS* gene (Supplemental Fig. S1C), while the TSS upstream of *nahA* was identified 74 nucleotides 5' of the predicted *nahA* translational start site (Supplemental Fig. S1D). The *nahS* upstream region contained a -10 and a -35 hexamer just upstream of the TSS resembling bifidobacterial promoter sequences (36, 37), 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 genes: *nagB3* (Fig. 2E) and *nagK* (Fig. 2F), and *lnpB* (Fig. 2G) and *gltA* (Fig. 2H), respectively, based on the associated genetic lay-out coupled to transcription patters of the *nagR1* mutant or when UCC2003 was grown on LNB (28). The transcription start sites (TSS) 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 supplemented with 1 % LNB. An extension product was identified 155 nucleotides 5' of the predicted translational start site for the *nagB3* gene (Supplemental Fig. S1E), while the transcriptional start site of *nagK* was identified 35 nucleotides 5' of the predicted translational start site (Supplemental Fig. S1F). An extension product was identified 43 nucleotides 5' of the predicted translational start site for the *lnpB* gene (Supplemental Fig. S1G), while the transcription start site for the *gltA* gene was identified 44 nucleotides 5' of the predicted translational start site (Supplemental 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 using electromobility shift assays and *in silico* analysis. 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 *L. lactis* NZ9000 (see Materials and Methods). As had been noted previously for other regulators from bifidobacteria (38-40), 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*$_{His}$

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were used to carry out the EMSAs. 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, Supplemental Table S1).

LntR-containing crude extract was shown to specifically bind to the IRD700-labelled DNA fragments lntP1a and lntP1b, but not with lntP1c (Fig. 2A, Supplemental Table S1). A double mobility-shift was observed for fragment lntP1a, 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-labelled 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, Supplemental 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 (being consistent with the observed double mobility-shift). Comparative analysis of these inverted repeats identified a 14-nucleotide consensus sequence (Fig. 3A), containing a conserved 'CG' at its centre, which is a well-documented conserved feature of operator sequences bound by LacI-type regulators (41, 42). This consensus sequence furthermore contains a conserved 5' 'TG' and 3' 'CA' at its flanking ends, a feature previously documented in operator sequences identified for other LacI-type regulators encoded by *B. breve* UCC2003 (39, 43, 44). 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 (Supplemental Fig. S1A, S1B). The positions of these identified operators are consistent with LntR acting as a repressor for the identified *lnt* promoters (45, 46).

The results obtained with the *L. lactis* NZ9000 pNZ-*nahR*$_{His}$ crude extract demonstrated specific binding to the IRD700-labelled DNA fragments nahSa and nahSb, but not with nahSc (Fig. 2C, Supplemental Table S1). Furthermore, binding was observed for IRD700-labelled DNA fragment nahAa, but not to fragment nahAb or nahAc (Fig. 2D, Supplemental 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

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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 sequence have previously been observed for operator sequences of certain NagC/XylR-type regulators (47). The presumed operator upstream of *nahS* overlaps with the downstream end of the predicted -10 promoter element (Supplemental Fig. S1C), while the *nahA*-associated operator was found to be roughly 110 bp upstream of the predicted -10 element (Supplemental 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 for *nahS*. While binding of NahR may occur at the *nahA* operator, its binding does not appear to directly interfere with the *nahA* promoter. This agrees with the lack of upregulation in *nahA* expression observed for the *nahR* mutant, though the transcriptional role of NahR in this case, if any, is not clear. The mapped locations of the operator, -10 and -35 sequences are shown in Supplemental Figure S1.

The results obtained with the crude extract obtained from nisin-induced *L. lactis* NZ900 pNZ-*nagR1*$_{His}$ revealed specific binding to the IRD700-labelled DNA fragments nagB3a, nagB3b and nagB3c (with a weak apparent double-shift observed for nagB3a), but not to fragment nagB3d (Fig. 2E, Supplemental Table S1). Specific binding was identified for IRD700-labelled DNA fragment nagKa, while no binding was detected when fragment nagKb was used (Fig. 2F, Supplemental Table S1). Binding of the NagR protein was also demonstrated for the IRD700-labelled DNA fragments lnpBa and lnpBc, but not with lnpBb (Fig. 2G, Supplemental Table S1). Finally, NagR1 was shown to bind IRD700-labelled DNA fragments gltAa, gltAb and gltAc (Fig. 2H, Supplemental 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 the fragment nagB3a, consistent with the observed double-shift), while being 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 (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 (48), a substantial degree of similarity can be observed to motifs identified previously for other ROK/NagC-type regulators encoded by *B. breve* UCC2003 (31, 39). The putative *nagB3*, *nagK*, *lnpB* and *gltA*

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operators were all found to be overlapping with or encompassing the predicted -10 or -35 elements (Supplemental Fig. S1E, S1F, S1G, 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, GalNAc or glucose (at a standard concentration of 20 mM) (Supplemental Fig. S2). These carbohydrates were chosen as they include both the complete structures and various components of LNT, LNnT or LNB. Carbohydrates which 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 an inhibition in binding was observed at 20 mM, the EMSA was repeated at 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 binding of this regulator to its DNA targets at a concentration of 10 mM or less (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 (Supplemental Figure S3). For NahR, only GlcNAc was found to reduce interaction between NahR and its DNA target at a minimum concentration of 0.0625 mM (Fig. 4B), while in the case of NagR1, Gal-6-P was found to prevent NagR1-binding activity at a minimum concentration of 1 mM (Fig. 4C, 4D).

Discussion

The dominance of (certain) bifidobacteria within the breastfed neonatal gut microbiota (2) is substantially aided by the ability of these infant-associated species to utilise indigestible HMO residues as a carbon source (10). Our previous work demonstrated that consumption and utilisation of LNT, LNnT or LNB by *B. breve* UCC2003 is facilitated by interrelated catabolic pathways (28). While pathways for HMO utilisation in other *Bifidobacterium* species have been identified and elucidated (14, 22, 23), very little work has been carried out with regard to their regulation. Our results reveal molecular details of the transcriptional

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regulation of *B. breve* UCC2003 loci responsible for LN(n)T/LNB metabolism, and provide insights into how metabolism of these HMOs is controlled in *B. breve* UCC2003.

In the current study, we identified four transcriptional regulators, three of which were shown to be involved in regulating LN(n)T/LNB metabolism in UCC2003. Microarray analysis of insertional mutants in *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 transcription of genes adjacent to *lntR* and *nahR*, respectively. In contrast, NagR1 regulates transcription of not only the 'local' *nag* locus, but also of 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 in malto-oligosaccharide and fatty acid metabolism. While LacI-type, NagC/XylR-type and ROK/NagC-type regulators have all previously been identified and characterised in *B. breve* UCC2003 (31, 38, 39, 43), functional analysis of regulators in other bifidobacteria is comparatively undocumented. However, a recent study identified homologous transcription factors for those of LntR, NagR1and NagR2 in a range of different *Bifidobacterium* species (49).

Details of promoter and operator sequences specific to the LntR, NahR and NagR1 regulators were elucidated using a combination of electromobility shift and primer extension analyses. These operator results, for the most part, agree with those predicted by Khoroshkin et al. (49). The operator sequences predicted in their study concur with our experimentally determined data, both in 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 upstream of *gltA*, however, this did not appear to be functional, based on the lack of a double mobility-shift in the EMSA's for that region. This operator may indeed be a non-functional relic resulting from a duplication event. Khoroshkin et al. (49) also predicted an operator sequence upstream of the gene Bbr_1884 for NagR1 binding, though 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 upstream of *lntR* itself, which may function in *lntR* transcriptional auto-regulation. The observed lack of upregulation in *nahA* transcription for the *nahR* mutant appears discordant with the transcriptional increase of this gene that was previously observed in wild type UCC2003 during growth on LN(n)T (28), as well as the presence of the functional *nahA* operator

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sequence for NahR binding identified in this study. However, this may be explained if 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 interestingly of all was the identification of the effectors for each transcriptional regulator. Binding of LntR to its targets is impeded by Gal, NahR-mediated operator binding is prevented by the presence of GlcNAc, while NagR1-operator interaction is prevented by the presence of Gal-6-P. In each case, the genes under transcriptional control by their respective regulator encode the metabolic machinery responsible for the release of the effector monosaccharide from the substrate at that metabolic step. For example, Gal is released from the non-reducing end of LN(n)T through the hydrolytic activity of LntA, which is encoded by the *lnt* locus (28). 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 (39, 45, 50, 51). A similar scenario applies to GlcNAc release which acts as the effector for the NahR regulator that controls transcription of *nahS*, and to Gal-6-P which governs the activity of the 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 both the presence of the *lnt* locus activity product (and LntR effector) galactose, and the *nah* locus activity product (and NahR effector) GlcNAc, are 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 metabolism of other sugars, such as LNB, sialic acid and sulphated GlcNAc (28, 30, 31). Interestingly, in the case of Gal-6-P and NagR1, the *lnp/glt* locus is required for the degradation of LNB, though the *nag* locus is not directly involved in this, yet plays a role in N-acetylglucosamine (GlcNAc) and sialic acid metabolism (30). This may not be surprising, as sialic acid residues are commonly found in HMO (7), and more importantly, GlcNAc is a breakdown product of LNB (as well as LNT and LNnT).

Interestingly, previous work has shown that transcriptional induction takes place of the *lnt* locus during growth of UCC2003 on galacto-oligosaccharides (GOS) (52). 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 GOS consist mainly of galactose

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(53, 54), and that the intracellular release of galactose during GOS metabolism by UCC2003 would be sufficient to cause transcriptional induction of the *lnt* locus.

Thus, the presence and initial degradation of such a structure (i.e. LNT, LNnT or LNB) indirectly induces 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 internalisation and degradation is 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, at the level of LN(n)T degradation first (by the *lnt* locus), and then at the level of (LN(n)T breakdown product) lacto-N-triose degradation (by the *nah* locus). Regulation of LNB metabolism is managed in a single step, at the level of LNB phosphorolysis (by the *lnp/glt* locus). 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 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 (55) is a regular occurrence during the weaning period. Moreover, this suggests the evolution of specific catabolic responses to the presence of and for the utilisation of specific HMO moieties by *B. breve*, and poses the question as to 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 1. *B. breve* UCC2003 was routinely cultured in either de Man Rogosa and Sharpe medium (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 in modified de Man Rogosa and Sharpe (mMRS) medium, which was prepared from first principles (56), and which does not contain a fixed carbohydrate source. Prior to inoculation, the mMRS medium was supplemented with cysteine-HCl (0.05 %, wt/vol) and a particular carbohydrate source (1 %, wt/vol). It has

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previously been shown that mMRS does not support growth of *B. breve* UCC2003 in the absence of an added carbohydrate (57). Carbohydrates used were ribose (Sigma Aldrich, Steinheim, Germany), LNB (Elicityl Oligotech, Crolles, France) and LNnT (Glycom, Lyngby, Denmark). A 1 % wt/vol concentration of 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 (58) at 30°C. *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth (59) 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*, 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-galactopyranoside).

Nucleotide sequence analysis. Sequence information was obtained from the Artemis-mediated (60) genome annotations of *B. breve* UCC2003 (61). Database searches were performed using non-redundant sequences accessible at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the basic local alignment search tool (BLAST) (62, 63). Sequences were verified and analysed 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 (64). Plasmid DNA was isolated from *Escherichia coli*, *Lactococcus lactis* and *B. breve* using the Roche High Pure Plasmid Isolation kit (Roche Diagnostics, Basel, Switzerland). An initial lysis step was performed using 30 mg ml^{-1} of lysozyme for 30 minutes at 37°C prior to plasmid isolation from *L. lactis* or *B. breve*. Procedures for DNA manipulations were essentially performed as described previously (59). All restriction

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466 enzymes and T4 DNA ligase were used according to the supplier's instructions (Roche
467 Diagnostics, Basel, Switzerland). Synthetic single stranded oligonucleotide primers used in
468 this study (Table 2) were synthesized by Eurofins (Ebersberg, Germany). Standard PCRs
469 were performed using Taq PCRmaster mix (Qiagen) or Extensor Hi-Fidelity PCR Master
470 Mix (Thermo Scientific, Waltham, United States) in a Biometra T3000 thermocycler
471 Biometra, Göttingen, Germany) or a Life Technologies Proflex PCR System (Thermo
472 Scientific, Waltham, United States). PCR products were visualized by ethidium bromide
473 (EtBr) staining following agarose gel electrophoresis (1 % agarose). *B. breve* colony PCR
474 reactions were performed as described previously (65). PCR fragments were purified using
475 the Roche high Pure PCR purification kit (Roche Diagnostics, Basel, Switzerland). Plasmid
476 DNA was isolated using the Roche High Pure Plasmid Isolation kit (Roche Diagnostics,
477 Basel, Switzerland). Plasmid DNA was introduced into *E. coli* by electroporation as
478 described previously (59). *B. breve* UCC2003 (66) and *L. lactis* (67) were transformed by
479 electroporation according to published protocols. Correct orientation of DNA inserts and
480 integrity of all plasmid constructs (see also below) were verified by DNA sequencing,
481 performed at Eurofins (Ebersberg, Germany).

482

483 **Construction of *B. breve* UCC2003 insertion mutants.** Internal fragments of Bbr_0526
484 (designated here as *lntR*) (367 base pairs [bp] representing codon numbers 40 through to 162
485 of the 320 codons of this gene), Bbr_1249 (designated here as *nagR1*) (502 bp representing
486 codon numbers 64 through to 231 of the 375 codons of this gene), Bbr_1251 (designated here
487 as *nagR2*) (507 bp representing codon numbers 62 through to 230 of the 405 codons of this
488 gene), and Bbr_1555 (designated here as *nahR*) (448 bp representing codon numbers 74
489 through to 223 of the 380 codons of this gene) were amplified by PCR using *B. breve*
490 UCC2003 chromosomal DNA as a template and primer pairs 526LacIInsFHindIII and
491 526LacIInsRXbaI, 1249LacIInsFHindIII and 1249LacIInsRXbaI, 1251LacIInsFHindIII and
492 1251LacIInsRXbaI, or 1555LacIInsFHindIII and 1555LacIInsRXbaI (Table 2), respectively.
493 The insertion mutants were constructed using a previously described approach (65),
494 generating mutant strains *B. breve* UCC2003-lntR, *B. breve* UCC2003-nagR1, *B. breve*
495 UCC2003-nagR2 and *B. breve* UCC2003-nahR, which carried disrupted *lntR*, *nagR1*, *nagR2*
496 and *nahR* genes, respectively (Table 1). Site-specific recombination of potential tet-resistant
497 mutant isolates was confirmed by colony PCR using primer combinations TetWF and TetWR
498 to verify *tetW* gene integration, and primers Bbr_526ConfirmP1 or Bbr_526ConfirmP2,

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499 Bbr_1249ConfirmP1 or Bbr_1249ConfirmP2, Bbr_1251ConfirmP1 or Bbr_1251ConfirmP2,
500 and Bbr_1555ConfirmP1 or Bbr_1555ConfirmP2 (positioned upstream of the selected
501 internal fragments of Bbr_0526, Bbr_1249, Bbr_1251 and Bbr_1555, respectively) in
502 combination with primer TetWF to confirm integration at the correct chromosomal location
503 (Table 2).

504

505 **Analysis of global gene expression using *B. breve* DNA microarrays.** Global gene
506 expression was determined during log-phase growth of the insertional mutant strains *B. breve*
507 UCC2003-*lntR*, *B. breve* UCC2003-*nagR1*, *B. breve* UCC2003-*nagR2* and *B. breve*
508 UCC2003-*nahR* in mMRS supplemented with ribose. The generated transcriptome data sets
509 were compared to the transcriptome information obtained for log-phase wild-type *B. breve*
510 UCC2003 cells when grown in mMRS supplemented with ribose. Ribose was selected as a
511 suitable transcriptomic reference, as the metabolic pathway and gene expression profile for
512 growth of UCC2003 on ribose is known and has been employed previously as a reference
513 (44, 68). DNA microarrays containing oligonucleotide primers representing each of the 1864
514 identified open reading frames on the genome of *B. breve* UCC2003 were designed and
515 obtained from Agilent Technologies (Palo Alto, Ca., USA). Methods for cell disruption,
516 RNA isolation, RNA quality control, complementary DNA synthesis and labelling were
517 performed as, described previously (69). Two independent biological replicates were used for
518 each array using a Cy3/Cy5 dye-swap, as described previously (69). Labelled cDNA was
519 hybridized using the Agilent Gene Expression hybridization kit (part number 5188-5242) as
520 described in the Agilent Two-Colour Microarray-Based Gene Expression Analysis v4.0
521 manual (publication number G4140-90050). Following hybridization, microarrays were
522 washed in accordance with Agilent's standard procedures and scanned using an Agilent DNA
523 microarray scanner (model G2565A). Generated scans were converted to data files with
524 Agilent's Feature Extraction software (Version 9.5). DNA-microarray data were processed as
525 previously described (70-72). Differential expression tests were performed with the Cyber-T
526 implementation of a variant of the t-test (73).

527

528 **Construction of overexpression vectors, protein overproduction and purification.** For
529 the construction of plasmids pNZ-lntR, pNZ-nagR1 and pNZ-nahR, DNA fragments
530 encompassing *lntR*, *nagR1* and *nahR* were generated by PCR amplification from

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chromosomal DNA of *B. breve* UCC2003 using Q5 High-Fidelity DNA polymerase and primer combinations 526PurFSmaI and 526PurRXbaI, 1249PurFPvuII and 1249PurRXbaI, or 1555PurFEcoRV and 1555PurXbaI, respectively (Table 2). An in-frame N-terminal His10-encoding sequence was incorporated into the forward primers 526PurFSmaI, 1249PurFPvuII and 1555PurFEcoRV to facilitate downstream protein purification. The generated amplicons were digested with SmaI and XbaI, PvuII and XbaI, or EcoRV and XbaI, respectively, and ligated into the ScaI and XbaI digested, nisin-inducible translational fusion plasmid pNZ8150 (74). The ligation mixtures were introduced into *L. lactis* NZ9000 by electrotransformation and transformants were then selected based on chloramphenicol (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 was performed as described previously (38, 43, 75). 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 OD600 of 0.5 was reached, at which point protein expression was induced by addition of cell-free supernatant of a nisin-producing strain (76), followed by continued incubation for a further 2 hours. Cells were harvested by centrifugation, and crude cell extract was obtained as described previously (39). Although protein purification of LntR-His, NahR-His and NagR1-His was achieved using His tag affinity chromatography, the purification procedure appeared to render the proteins inactive in subsequent electrophoretic mobility shift assays (EMSAs). For this reason, crude cell extracts, prepared in a 10 mM Tris-HCl lysis buffer (pH 7.0), were adopted for the EMSAs (see below).

Electrophoretic mobility shift assay (EMSA). 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-labelled primers pairs (Integrated DNA Technologies, Coralville, Indiana, United States) (Table 2). EMSAs were performed essentially as described previously (43, 77). In all cases, binding reactions were carried out in a final volume of 20 µl in the presence of poly[d(I-C)] in binding buffer (20 mM Tris-HCl, 5 mM MgCl2, 0.5 mM DTT, 1 mM EDTA, 100 mM KCl, 10 % glycerol). Varying amounts of crude protein extract, ranging from 140 ng to 180 ng, of the LntR-, NahR-, or NagR1-(over)producing *L. lactis*

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NZ9000 strain constructed, and a fixed amount of DNA probe (0.1 pmol) was 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 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 Sigma Aldrich, Steinheim, Germany) ranging in concentration from 50 to 0.0625 mM, were included to the binding reaction buffer. Samples were loaded onto a 6 % non-denaturing 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 x gradient of TAE at 100 V for 90 min in an Atto Mini PAGE system (Atto Bioscience and Biotechnology, Tokyo, Japan). Signals were detected using Odyssey Infrared Imaging System (Li-Cor Biosciences UK Ltd, Cambridge, UK) and captured using the supplied software Odyssey V3.0.

Primer extension analysis. Total RNA was isolated from *B. breve* UCC2003, grown in mMRS supplemented with 1 % LNnT or 1 % LNB, to early exponential phase, using a previously described Macaloid method (78). 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 (79). Sequence ladders of the presumed promoter regions immediately upstream of *lntP1*, *lntS*, *nagB3*, *nagK*, *lnpB*, *gltA*, *nahS* or *nahA*, amplified from both UCC2003 genomic DNA which were run alongside the primer extension products, were produced using the same primer as the primer extension reaction and 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 was performed by means of a Li-Cor sequencing instrument (Li-Cor Biosciences).

Operator Consensus Sequence Prediction, using MEME and WEBLOGO online software tools. Co-regulated 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) (80), which were the visualized by the WebLogo online tool (http://weblogo.berkeley.edu/logo.cgi) (81, 82). Sequences used for consensus sequence prediction are given in Supplemental Table S2.

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Microarray data accession number. The microarray data obtained in this study have been deposited in NCBI's Gene Expression Omnibus database and are accessible through GEO Series accession number GSE105108.

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Figure 1. Schematic representation of HMO metabolism-associated loci in *B. breve* UCC2003, as identified preciously [22]. (a) The genes of the *lnt* locus. (b) The genes of the *nah* locus. (c) The genes of the *nag* locus, and 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. 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.

Figure 2. EMSA images showing (a) LntR, (b) NahR, and (c) and (d) NagR1 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 Supplemental Table S1. The panels below 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 arrow) and genes (blue box arrows). In each panel, '-' indicates a negative control, where an equivalent amount crude cell extract from NZ9000 harbouring empty pNZ8150 was added instead of crude extract from the regulator-expressing NZ9000 strain.

Figure 3. WebLogo representation of the operator motif consensus sequences for (a) the LacI-type regulator LntR, (b) the NahC/XylR-typr regulator NahR and (c) the ROK/NagC-type regulator NagR1, predicted using *in silico* analysis. Predicted operator sequences identified in the intergenic regions containing the co-regulated promoters for each regulator 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 sequence.

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

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Figure 5. Schematic representation of the proposed model for transcriptomic regulation of LNT, LNnT and LNB metabolism by *B. breve* UCC2003. LNT and LNnT are internalised and intracellularly degraded through sequential hydrolysis and release of monosaccharides from their non-reducing ends. These released monosaccharides act as effectors relieving transcriptional repression of the loci encoding the cellular components responsible for liberation of these glycans. As such, liberated galactose relieves transcriptional repression of the *lnt* locus, and N-acetylglucosamine relieves transcriptional repression of the *nah* locus. Similarly, intracellular degradation of LNB (derived from the extracellular hydrolysis of complex HMO structures by other infant GIT microbes) releases N-acetylglucosamine and galactose-6-phosphate, the latter of which relieves transcriptional repression of the *lnp*/*glt* and *nag* loci.

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Author Contributions Statement: D.v.S., K.J. and M.O.C.M. conceived the experiments. K.J., with the assistance of C.P. and R.O'B., conducted the experiments. All authors analysed the results and contributed to writing the manuscript.

Additional Information: The microarray data obtained in this study have been deposited in NCBI's Gene Expression Omnibus database and are accessible through GEO Series accession number GSE105108.

The authors declare that, to the best of their knowledge, there are no competing financial interests.

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Strain or plasmid	Relevant Features	Reference or Source
Strains		
Escherichia coli strains		
E. coli EC101	Cloning host, repA$^+$ km$^+$	(43)
E. coli EC101-pNZ-M.BbrII + M.BbrIII	EC101 harbouring pNZ8048 derivative containing *bbrIIM* and *bbrIIIM*	(41)
Lactococcus lactis strains		
L. lactis NZ9000	MG1363, pepN::nisRK, nisin-inducible overexpression host	(55)
L. lactis NZ9700	Nisin-producing strain	(55)
L. lactis NZ9000-pNZ-lntR	NZ9000 conatining pNZ-lntR	This study
L. lactis NZ9000-pNZ-nahR	NZ9000 conatining pNZ-nahR	This study
L. lactis NZ9000-pNZ-nagR1	NZ9000 conatining pNZ-nagR1	This study
Bifidobacterium sp. Strains		
B. breve UCC2003	Isolate from nursling stool	(42)
B. breve UCC2003-lntR	pORI19-tet-bbr_0526 insertion mutant of UCC2003	This study
B. breve UCC2003-nahR	pORI19-tet-bbr_1555 insertion mutant of UCC2003	This study
B. breve UCC2003-nagR1	pORI19-tet-bbr_1249 insertion mutant of UCC2003	This study
B. breve UCC2003-nagR2	pORI19-tet-bbr_1251 insertion mutant of UCC2003	This study
Plasmids		
pAM5	pBC1-puC19-Tet^r	(1)
pORI19	Em^r, repA$^-$, ori$^+$, cloning vector	(43)
pORI19-tet-lntR	Internal 367 bp fragment of *bbr_0526* and tetW cloned in pORI19	This study
pORI19-tet-nahR	Internal 448 bp fragment of *bbr_1554* and tetW cloned in pORI19	This study
pORI19-tet-nagR1	Internal 502 bp fragment of *bbr_1249* and tetW cloned in 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	(51)
pNZ-lntR	Cm^r, pNZ8150 derivative containing translational fusion of Bbr_0526 encoding DNA fragment to nisin inducible promoter	This study
pNZ-nahR	Cm^r, pNZ8150 derivative containing translational fusion of Bbr_1555 encoding DNA fragment to nisin inducible promoter	This study
pNZ-nagR1	Cm^r, pNZ8150 derivative containing translational fusion of Bbr_1249 encoding DNA fragment to nisin inducible promoter	This study

Table 1: Bacterial plasmids and strains used in this work.

Cm^r, Em^r,Km^r and Tet^r, resistance to chloramphenicol, erythromycin, kanamycin and tetracycline, respectively.

UCC, University College Cork Culture Collection.

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Purpose	Primer	Sequence (5'-3')
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 internal 465bp fragment of Bbr_0526 in pORI19	IM526F	ctggtc**aagctt**cgttgaagccgcgatgga
	IM526R	ctggtc**tctaga**gtcaacggtggggcagtg
Cloning of internal 488bp fragment of Bbr_1555 in pORI19	IM1555F	ctggtc**aagctt**gctggccatcgatacggac
	IM1555R	ctggtc**tctaga**ctcgtcgttcagcagcac
Cloning of internal 443bp fragment of Bbr_1249 in pORI19	IM1249F	ctggtc**aagctt**cgaagaaggcctattgcg
	IM1249R	ctggtc**tctaga**cagcagaatcgccgaacc
Cloning of internal 488bp fragment of Bbr_1251 in pORI19	IM1251F	ctggtc**aagctt**gaagagaccggcgacctgg
	IM1251R	ctggtc**tctaga**gccattgtcgatgacgcc
Amplification of *tet*W	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-labelled oligonucleotides	527IRDfa	ctcgcccctcgcttgtctctc
	527IRDra	gcataggcacggcagcgac
	527IRDfb	attgttttcgtgaccattg
	527IRDrb	gaataatgaacacgaacacg
	527IRDfc	caattttggtcaaccttcg
	527IRDrc	cgcgcgtagttctcgac
Amplification of Bbr_0530 promoter fragments with IRD700-labelled oligonucleotides	530IRDfa	gccgaacggtgtgctggtgg
	530IRDra	cttcatcgttctgttctccttc
	530IRDfb	cgataacacgcccgccatc
	530IRDrb	gctggacttgccgctatc
	530IRDfc	cttcatagagccacttc
	530IRDrc	ctcgaagtccttggcaac
Amplification of Bbr_1554 promoter fragments with IRD700-labelled oligonucleotides	1554IRDfa	gtcgctgggattggatttagcg
	1554IRDra	gtggctatgactgcgcgc
	1554IRDfb	cggctttcaggataacaccca
	1554IRDrb	ggatttggcggcgcgatc
	1554IRDfc	ccaaacaaaatagttgctacggc
	1554IRDrc	gttgagtgcggtgtaggtctcc
Amplification of Bbr_1556 promoter fragments with IRD700-labelled oligonucleotides	1556IRDfa	ctggacggctgctcaaagc
	1556IRDra	gcagagatgtttgaccgttcat
	1556IRDfb	ggtgacgacgccactctgc
	1556IRDrb	gtggtttgcggttgccct
	1556IRDfc	gccatctcaggaccgaacg
	1556IRDrc	ggtcgtcaaggtgatgaatcc
Amplification of Bbr_1248 promoter fragments with IRD700-labelled oligonucleotides	1248IRDfa	cctcctgcctgaacgatg
	1248IRDra	gacaatgatgatttccggc
	1248IRDfb	gttagggaacttcactaatacattcc
	1248IRDrb	cctgggagatgtcgatcgactc
	1248IRDfc	gtccgtacgtccataattgtaagtag
	1248IRDrc	cctgggagatgtcgatcgactc
	1248IRDfd	gatgggcggctttgggcag
	1248IRDrd	cctgggagatgtcgatcgactc

Table 2: Oligonucleotide primers used in this work.

Restriction sites incorporated into oligonucleotide primer sequences are indicated in bold, and His-tag sequences incorporated into nucleotide primer sequences are indicated in italics.

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Table 2: Oligonucleotide primers used in this work.

Purpose	Primer	Sequence (5'-3')
Amplification of Bbr_1250 promoter fragments with IRD700-labelled oligonucleotides	1250IRDfa	gatgccgttgtggtagagatg
	1250IRDra	ggtgttatcagtcattgcctatcc
	1250IRDfb	gcgtgtcgcgtatgaggc
	1250IRDrb	ggtgttatcagtcattgcctatcc
Amplification of Bbr_1586 promoter fragments with IRD700-labelled oligonucleotides	1586IRDfa	cggttcgtcgaaaatccaag
	1586IRDra	cagtgcgaagtgtgaggcg
	1586IRDfb	gccgcttattgcggctttatag
	1586IRDrb	ctttgagggcagaagtaactagttc
	1586IRDfc	gtatgcgcgttcgtccac
	1586IRDrc	ctttgagggcagaagtaactagttc
Amplification of Bbr_1590 promoter fragments with IRD700-labelled oligonucleotides	1590IRDfa	ggcccgctggcagattag
	1590IRDra	ggcaagagcagccacgatg
	1590IRDfb	gacagatgtctgagcggtc
	1590IRDrb	gaatcgggcagacggtgc
	1590IRDfc	cgcgcagaaattgttagttagg
	1590IRDrc	gaatcgggcagacggtgc
Amplification of region containing Bbr_0527 promoter region for sequencing ladders	527promF	gcattgctgtcattcgccacac
	527promR	gaataatgaacacgaacacg
Amplification of region containing Bbr_0530 promoter region for sequencing ladders	530promF	gcgtgcggatgaaactgg
	530promR	gtctggaacggcttggcgc
Amplification of region containing Bbr_1554 promoter region for sequencing ladders	1554promF	cgtttcctcgaccccagttc
	1554promR	gaatgtgtccttgagcttggc
Amplification of region containing Bbr_1556 promoter region for sequencing ladders	1556promF	ctggacggctgctcaaagc
	1556promR	ggtcgtcaaggtgatgaatcc
Amplification of region containing Bbr_1248 promoter region for sequencing ladders	1248promF	ggaggctttggcggtacgg
	1248promR	cctgggagatgtcgatcgactc
Amplification of region containing Bbr_1250 promoter region for sequencing ladders	1250promF	gatgccgttgtggtagagatg
	1250promR	ggtgccacccacatcaacac
Amplification of region containing Bbr_1586 promoter region for sequencing ladders	1586promF	gcgagaccttcgaccttcagcc
	1586promR	cggcacgagattgtaagacac
Amplification of region containing 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
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

Restriction sites incorporated into oligonucleotide primer sequences are indicated in bold, and His-tag sequences incorporated into nucleotide primer sequences are indicated in italics.

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Table 3. *B. breve* UCC2003 regulator mutant genes upregulated in transcription during growth in mMRS medium supplemented with 1 % ribose as the sole carbohydrate, as compared to the wild type (control).

Gene ID	Gene name	Function	Fold upregulation[a,b] during growth on:		
			UCC2003-lntR	UCC2003-nahR	UCC2003-nagR1
Bbr_0526	lntR	Transcriptional regulator, LacI family	N/A	-	-
Bbr_0527	lntP1	Permease protein of ABC transporter system for sugars	3.84	-	-
Bbr_0528	lntP2	Permease protein of ABC transporter system for sugars	3.77	-	-
Bbr_0529	lntA	GH42 Beta-galactosidase	2.78	-	-
Bbr_0530	lntS	Solute-binding protein of 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	-	-	N/A
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 ABC transporter system (lactose)	-	17.44	-
Bbr_1555	nahR	NagC/XylR-type transciptional regulator	-	N/A	-
Bbr_1556	nahA	GH20 nagZ Beta-N-acetylhexosaminidase	-	-	-
Bbr_1558	nahP	Permease protein of ABC transporter system	-	-	-
Bbr_1559	nahT1	ATP-binding protein of ABC transporter system	-	-	-
Bbr_1560	nahT2	ATP-binding protein of 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 ABC transporter system for sugars	-	-	2.91
Bbr_1589	gltB	Permease protein of ABC transporter system for sugars	-	-	3.02
Bbr_1590	gltA	Solute-binding protein of ABC transporter system for sugars	-	-	5.07

The level of transcription is shown as a fold-value of increase in transcription on each carbohydrate, as compared to a ribose control, with a cut-off of a minimum 2.0-fold increase in transcription.

a Based on comparative transcriptome analysis using B. breve UCC2003-lntR, B. breve UCC2003-nahR and B. breve UCC2003-nagR1 grown on 1% ribose, as 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.

b The cutoff point is 2.0-fold, with a P value of _0.001. —, value below the cutoff.

N/A indicates that the fold-value for increase in transcription for this gene is not included, as this is the gene in which the mutation was made, and thus 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 as reliable.

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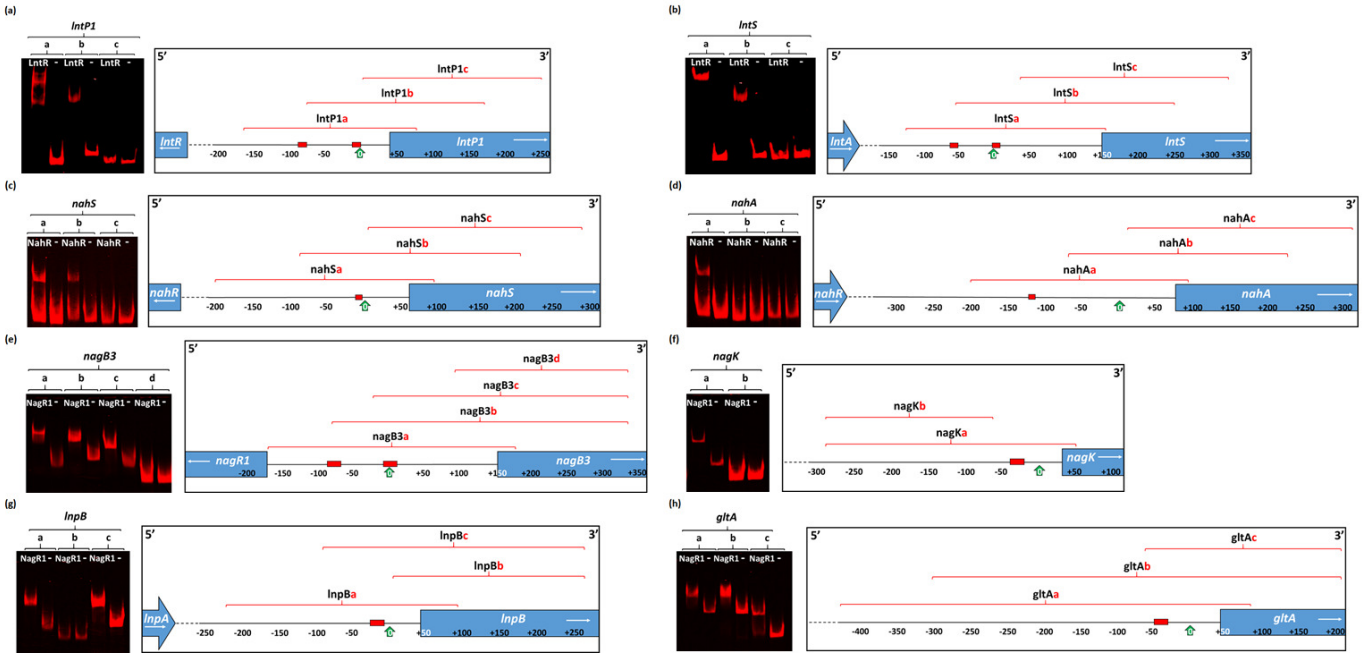
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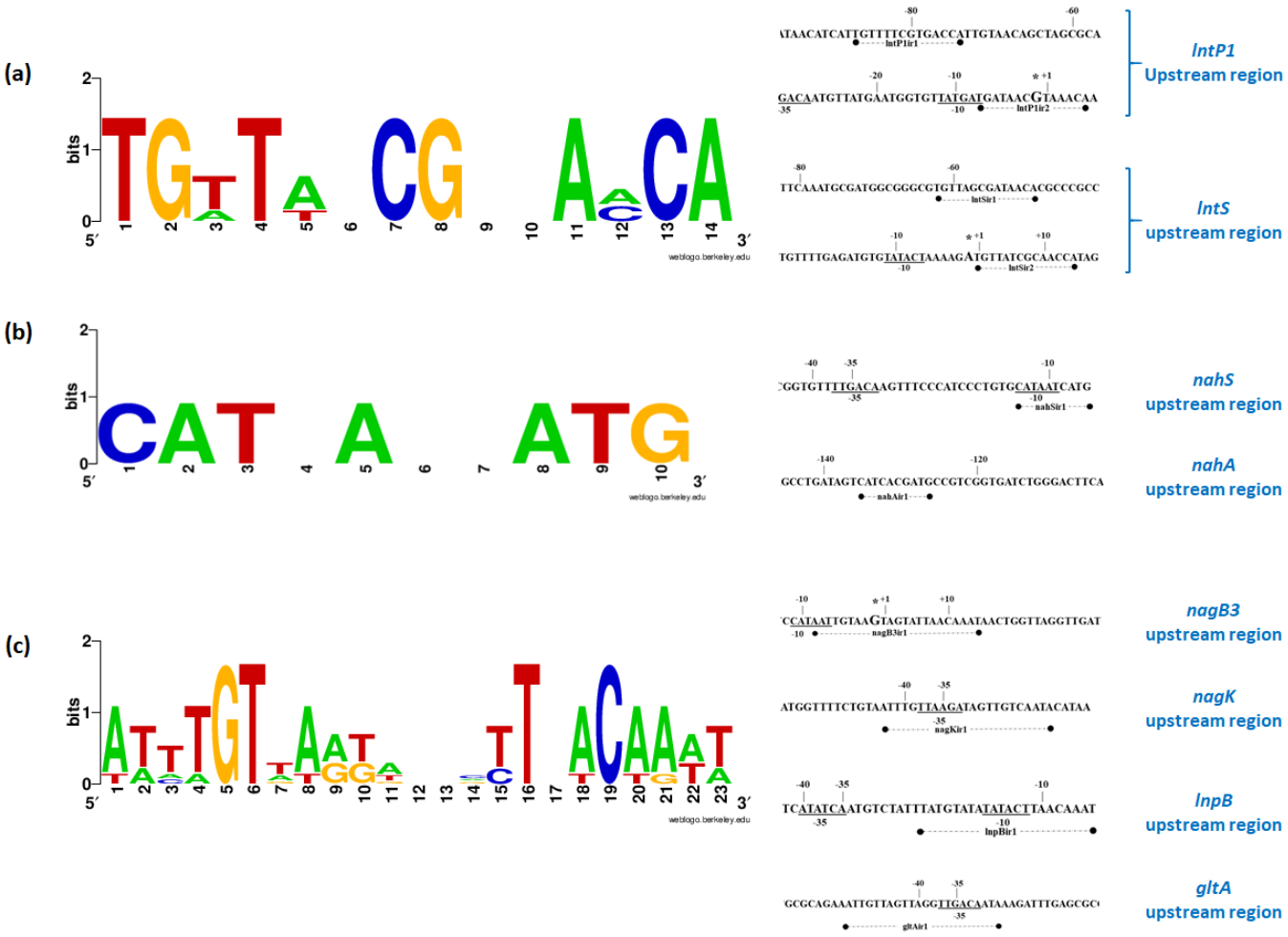
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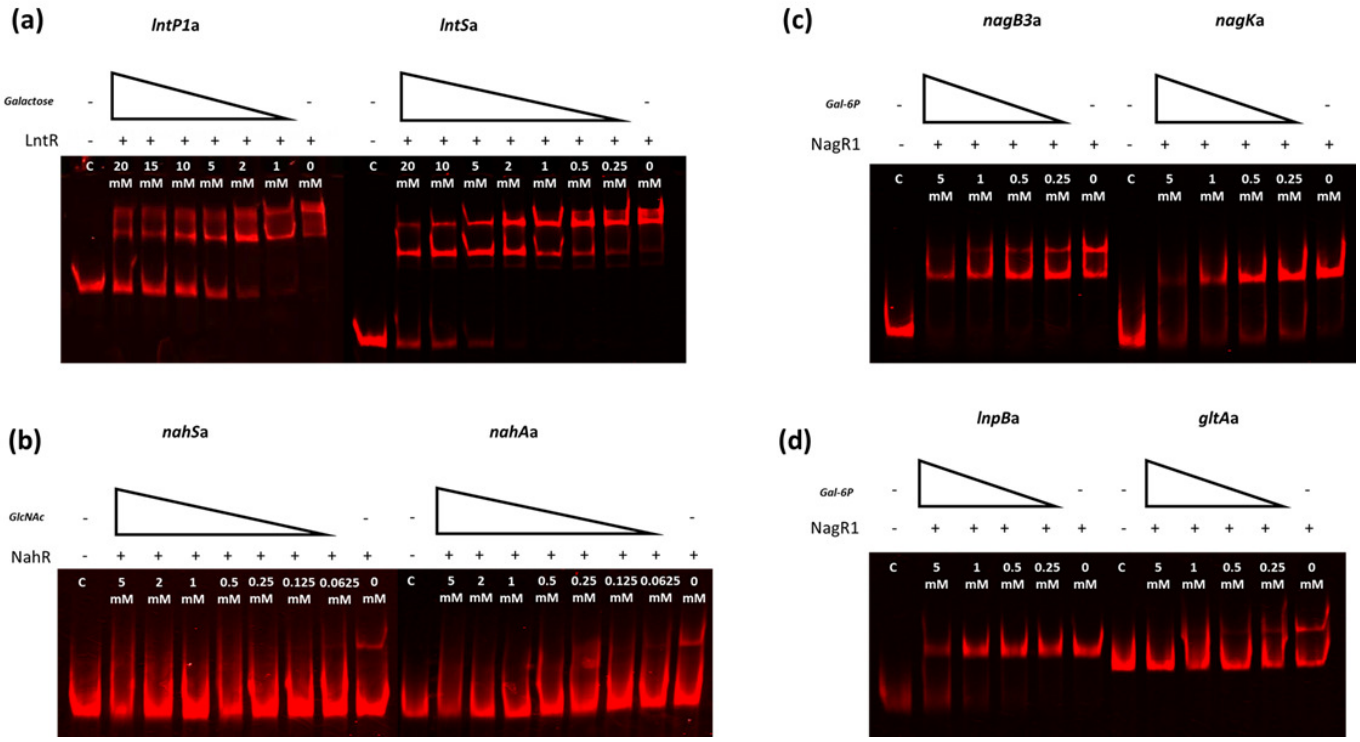
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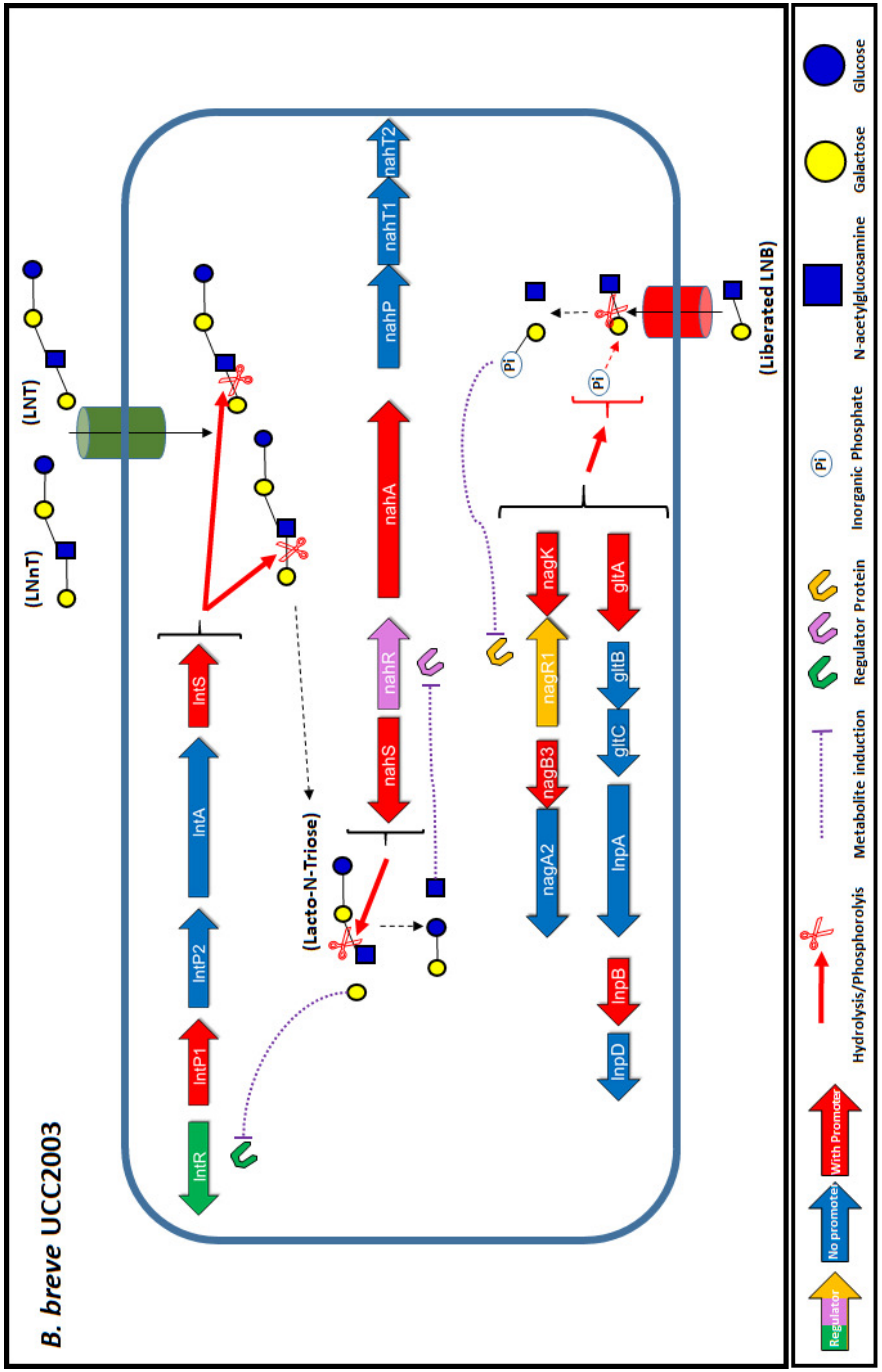
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