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Substrate preference of an ABC importer corresponds to selective growth on β-(1,6)-galactosides in
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(SPR).

Abstract
Bifidobacteria are exposed to substantial amounts of dietary β-galactosides. Distinctive preferences for
growth on different β-galactosides are observed within Bifidobacterium members, but the basis of
these preferences remains unclear. We previously described the first β-(1,6)/(1,3)-galactosidase from
Bifidobacterium animalis subsp. lactis Bl-04. This enzyme is relatively promiscuous, exhibiting only 5-
fold higher efficiency on the preferred β-(1,6)-galactobiose than the β-(1,4) isomer. Here, we
characterize the solute-binding protein (Bal6GBP) that governs the specificity of the ABC transporter
encoded by the same β-galactoside–utilization locus. We observed that although Bal6GBP recognizes both
β-(1,6)- and β-(1,4)-galactobiose, Bal6GBP has a 1630-fold higher selectivity for the former, reflected
in dramatic differences in growth, with several hours lag on less preferred β-(1,4)- and β-(1,3)-
galactobiose. Experiments performed in the presence of varying proportions of β-(1,4)/ β-(1,6)-
galactobioses indicated that the preferred substrate was preferentially depleted from the culture supernatant. This established that the poor growth on
the non-preferred β-(1,4) was due to inefficient uptake. We solved the structure of Bal6GBP in complex with β-(1,6)-galactobiose at 1.39 Å
resolution, revealing the structural basis of this strict selectivity. Moreover, we observed a close
evolutionary relationship with the human milk disaccharide lacto-N-biose–binding protein from
Bifidobacterium longum, indicating that the recognition of the non-reducing galactosyl is
essentially conserved, whereas the adjacent position is diversified to fit different glycosidic linkages and
monosaccharide residues. These findings indicate that oligosaccharide uptake has a pivotal role in
governing selectivity for distinct growth substrates and have uncovered evolutionary trajectories that
shape the diversification of sugar-uptake proteins within Bifidobacterium.

Introduction
The human gut microbiota (HGM) is increasingly acknowledged as a major contributor to human health as well as a modulator of host metabolism (1) and immune-homeostasis (2). Specific microbiota signatures are associated with serious disease states including inflammatory disorders (3), obesity, type 2 diabetes (4) and colorectal cancer (5), underscoring the crucial impact of a balanced HGM composition. Diet is a major external factor affecting the composition of the microbiota (6), and oligo- as well as polysaccharides that are non-digestible to the human host are instrumental in shaping this community (7). The abundance and the diversity of dietary glycans have driven the evolution of different microbial strategies to harvest energy from these metabolic resources. Dominant commensals from the Bacteroides genus are considered generalists that target a broad range of glycans via outer membrane bound extracellular enzymes (7,8). Other taxonomic groups, e.g. bifidobacteria, typically possess fewer extracellular enzymes compared to Bacteroides. Instead, bifidobacteria rely on effective transport systems, mainly ATP-binding cassette (ABC) transporters, to access oligosaccharides that are either naturally present in diet (9) or produced by hydrolytic enzymes from other HGM members, thereby facilitating cross-feeding (10,11). Genomes of bifidobacteria typically encode a multitude of oligosaccharide specific ABC transporters (12), which mediate high affinity capture of oligosaccharides via their extracellular lipid anchored solute-binding proteins (SBPs). Uptake of oligosaccharides by these transporters is likely to confer an important advantage in the competitive human gut niche, particularly in the colon that harbors the highest bacterial density. Despite this key role, our insight into oligosaccharide uptake in the human gut niche remains limited.

β-Galactosides are ubiquitous in human diet, e.g. in both human and animal milk (13) and in fruits and vegetables where they are omnipresent in primary cell-wall pectic polysaccharides (14). Oligomeric β-galactosides of different origin have been shown to selectively promote members of the Bifidobacterium genus that harbor many probiotic health-promoting strains (15). Dietary β-galactosides display source-dependent diversity with respect to degree of polymerization (DP), glycosidic linkages and presence of other monosaccharides. β-Galactosides are also considered prime prebiotic dietary supplements (galactooligosaccharides, GOS) produced by transglycosylation of lactose, partially due to their bifidogenic effect. Notably, commercial GOS preparations typically comprise tens of compounds of DP 2–6 and contain β-(1,3)-, β-(1,4)-, and β-(1,6)-galactosidic linkages (16). Only some of these oligosaccharides are metabolized by bifidobacteria in a species- and strain-specific manner (17-19). Knowledge on β-galactoside preferences of bifidobacteria largely stems from characterization of β-galactosidases (20) or from growth and oligosaccharide consumption profiles (19). The role of oligosaccharide transporters in β-galacto-oligosaccharide utilization selectivity has received less attention, with two recent studies having addressed the identification and binding properties of β-galacto-oligosaccharide transport proteins in B. breve (21,22).

We have previously identified a transcriptionally induced operon during growth of the probiotic strain B. animalis subsp. lactis BI-04 on GOS (a commercial β-galactoside mixture). This operon encodes a transcriptional regulator, a β-galactosidase of glycoside hydrolase family 42 (BlGH42A) and an ABC transporter (23). The β-galactosidase revealed a broad specificity hydrolysing β-(1,6)-, β-(1,3)- and β-(1,4)-galactosidic bonds with a modest 5-fold preference for β-(1,6)-galactobiose (β6Gal2) as compared to the β-1,4 isomer (24). Here, we report the molecular characterization of the β-galactoside specific SBP (Bal6GBP) associated with the ABC transporter. By contrast to the relatively promiscuous β-galactosidase, Bal6GBP displayed selectivity exceeding three orders of magnitude for β6Gal2 as compared to the β-(1,4)-isomers, which was explained by the structure of Bal6GBP in complex with β6Gal2. The high selectivity of Bal6GBP is reflected in a dramatic difference in growth profiles of B. animalis subsp. lactis BI-04 showing several hours of lag phase on less preferred substrates. This was shown to be attributed to the inefficient uptake of ligands that are less preferred by the binding protein.

Structural analyses allowed tracking the evolutionary relationship with the lacto-N-biose
(human milk derived) binding protein from *Bifidobacterium longum* subsp. *longum* (25) that shares only 26% sequence identity with Bal6GBP. Despite the low primary structure identities, these two binding proteins share a similar architecture of the side chains interacting with the non-reducing galactosyl moiety, but diverge in the recognition of the penultimate carbohydrate ring from the non-reducing end.

Altogether, the present study constitutes a unique case enabling the comparison of ligand selectivity of an ABC-associated transport protein and the intracellular glycoside hydrolase mediating uptake and breakdown, respectively, of the same substrates. These findings underscore the leading role of ABC-mediated glycan transport in governing the growth preferences of core gut microbiota members on β-galactosides. This insight paves the way to tailored editing of specific members of the microbiota based on uptake profiles in future interventions to promote health and to alleviate disease.

**Results**

**The ABC transporter associated binding protein from* B. animalis* subsp. *lactis* Bl-04 is highly specific towards β-(1,6)-galactosides**

The β-galactoside SBP from *B. animalis* subsp. *lactis* BI-04 (Bal6GBP) was recombinantly produced and purified (yield = 85 mg L\(^{-1}\) medium). The binding of a panel of di- and oligosaccharides to Bal6GBP was screened using surface plasmon resonance (SPR), which established the specificity towards β-galactopyranosyl linked ligands (Supporting information Table S1). The binding affinities and kinetic parameters of the β-galactoside ligands (Fig. 1) were discerned by surface plasmon analysis (SPR) (Fig. 2A, Table 1). The highest affinity of Bal6GBP was for β6Gal2 (\(K_D \approx 100\) nM) and the selectivity towards this galactosidic linkage was striking as the affinity dropped by 304- and 1630-fold for β-(1,3)-galactobiose (β3Gal2) and β-(1,6)-galactobiose (β4Gal2), respectively. The selectivity was governed by staggering (up to 3×10\(^4\) fold) changes in the association rate constant (\(k_{on}\)), whereas the dissociation rate constants (\(k_{off}\)) varied modestly with glycosidic linkage. The affinity appeared to decrease with size for β-(1,6)-galactosides with a 26-fold drop for β-(1,6)-galactotetraose (β6Gal4) as compared to β6Gal2. This trend was not valid for the less preferred β-(1,4)-galactosides. The affinity of Bal6GBP was highest at pH 6.5, but varied only modestly in the pH range 5.4–8.2 consistent with the slightly acidic pH prevalent in the colon.

Isothermal titration calorimetry (ITC) analyses confirmed the binding magnitude and preference of Bal6GBP for β-(1,6)-galactosides (Fig. 2B, Table 2).

**Overall three-dimensional structure of Bal6GBP in complex with the preferred ligand**

The crystal structure of Bal6GBP in the free open conformation was solved to a resolution of 1.94 Å by SeMet single anomalous dispersion (SAD) phasing. Coordinates of the separate domains from the open conformation were used as molecular replacement (MR) models to solve the structure of Bal6GBP in complex with the preferred ligand β6Gal2 to 1.39 Å (Table 3). Bal6GBP adopts a classical SBP-fold (cluster B type SBP according to structural classification (26)), which comprises two α/β domains of different sizes interconnected by three hinge regions with the ligand binding site located at the domain interface (Fig. 3A). The smaller domain 1 (1–144; 311–362) is formed by six α-helices and five centrally positioned β-strands. Domain 2 (149–306; 374–447) consists of ten α-helices and six β-strands. The architecture of these two domains is similar to other SBP structures (27). The hinge region that connects the two domains and potentiates domain movement between the open and close conformation (Fig. S1), consists of two loop regions (145–148; 311–320) and an anti-parallel β-sheet formed by two short β-strands 363–373 (Fig. 3A). A DALI structure comparison search (28) against the Protein Data Bank (PDB) identified the (galacto/lacto)-N-biose (GNB/LNB)-binding protein (GL-BP) from *B. longum* subsp. *longum* ATCC 15697 (PDB ID: 2Z8D-F; Z-score = 42.0; RMSD = 2.8 Å; 26% sequence identity) as the structurally closest related homologue to Bal6GBP (25). The structure of GL-BP was determined in complex with galacto-N-biose (PDB ID: 2Z8E), lacto-N-biose (PDB ID: 2Z8D) and lacto-N-tetraose (PDB ID: 2Z8F).

The crystal obtained for phasing from the SeMet-labelled protein belongs to the same space
group as the ligand-bound form, but with a different \( \beta \) angle and slightly different cell lengths (Table 3). The structure of the open form of the protein was determined from the Se-Me protein crystals, which revealed high flexibility between domain 1 and domain 2, resulting in an open ligand-free form and a closed ligand-bound form (29). The transition between these two states involves a rotation angle of \( 32.3^\circ \) between the domains of \( \text{Bal6GBP} \) as estimated with the DynDom server (30) (Fig. S1). Thus, closure of the protein involves a rigid body movement of domains with changes mainly occurring in the hinge region.

\( \beta-(1,6) \)-Galactobiose binding site in \( \text{Bal6GBP} \)

\( \text{Bal6GBP} \) in complex with \( \beta6\text{Gal2} \) showed a well-defined density of the ligand that primarily assumes the \( \beta \)-configuration. The ligand is almost completely engulfed in the largely solvent inaccessible binding site (Fig. S2). The non-reducing galactosyl stacks onto Trp275 from domain 2, which defines position 1. A total of 12 potential hydrogen bonds recognise \( \beta6\text{Gal2} \): three from domain 1, six from domain 2, and three from the hinge region (Table S2, Fig. 3B). Five out of the six water molecules, which coordinate the ligand, also mediate hydrogen-bonding networks to residues in the binding pocket. All hydroxy groups of the galactosyl at position 1 are recognised by polar interactions to Glu96, Glu98, Asp145, Asn279, Gly310 and Ser311. This correlates well with the strict requirement for \( \beta \)-galactosyl at position 1 for recognition by \( \text{Bal6GBP} \). By contrast, only the C2-OH and C4-OH of the galactosyl at position 2 are recognized by direct polar contacts to Asn74, Glu98, Asp196 and Asn308. Glucosyl can be accommodated at position 2, albeit with 140-fold reduced affinity for the disaccharide allolactose as compared to \( \beta6\text{Gal2} \) (Table 2), probably due to the recognition of the axial C4-OH in \( \beta6\text{Gal2} \) by Asn74 and Glu98 that also interacts with the C2-OH from the reducing end galactosyl unit. Both hemiacetal ring oxygens in \( \beta6\text{Gal2} \) form hydrogen bonds with indole nitrogens of tryptophans (Trp47 and Trp254; Fig. 3B).

Transporter preference is reflected in the \( \beta \)-galactobiose isomer growth profiles of \( B. \text{animalis} \) \textit{sp. lactis} Bl-04

Only two loci were previously observed to be highly upregulated during growth on galactooligosaccharides: the canonical lactose metabolism locus encoding a LacS transport system and the ABC system characterised in the present study (23), suggesting that this ABC system is the only \( \beta \)-galactoside specific locus. To evaluate the impact of the preference of \( \text{Bal6GBP} \) on growth of \( B. \text{animalis} \) sp. lactis Bl-04, the bacterium was cultivated for 48 hours on the \( \beta-(1,3), \beta-(1,4) \) or \( \beta-(1,6) \) structural isomers of \( \beta \)-galactobiose as carbon source (Fig. 4A). Starting from the same inoculum, the most rapid growth was on \( \beta6\text{Gal2} \) with no observable lag phase. Conversely, growth on \( \beta3\text{Gal2} \) and \( \beta4\text{Gal2} \) commenced after lag phases of 9 and 15 h, respectively. We carried out another growth experiment to verify if the growth delay on less preferred ligands was related to inefficient transcriptional response or due to poor uptake. The growth in this case was performed on 0.5% \( \beta6\text{Gal2} \), 0.2% of each \( \beta4\text{Gal2} \) and \( \beta6\text{Gal2} \) or 0.45% and 0.05% of \( \beta4\text{Gal2} \) and \( \beta6\text{Gal2} \), respectively (all w/v) (Fig. 4B). The growth level was proportional to the amount of the preferred \( \beta6\text{Gal2} \) after 40 h, although the final one-point OD measurement before the culture at about 48 h was higher (data not shown) compared to the 40 h sample, consistent with the delayed growth pattern observed on \( \beta4\text{Gal2} \) (Fig. 4A). The reason for using the lower concentration in the 1:1 galactobiose mixture was to have higher sensitivity in the uptake assay for monitoring uptake during the exponential phase. The uptake preference was evaluated by analysis of supernatants from a culture growing on same equimolar mixture of \( \beta4\text{Gal2} \) and \( \beta6\text{Gal2} \) as in Fig. 4B. The preferred \( \beta6\text{Gal2} \) was depleted after 12 h, while consumption of \( \beta4\text{Gal2} \) was only observed after 24 h and was not completed after 72 h of growth (Fig. 4C). These experiments suggest that the uptake preference is a key factor that governs the onset and the extent of growth on both pure substrates and on mixtures of those.

Discussion

Transporter proteins as sensitive probes of the evolution of microbial glycan preferences in the gut niche

The evolution of bifidobacteria has been shaped by the acquisition of genes that support the
adaptation to the digestive tracts of animals that provide parental care, especially mammals (31,32). The evolution of unique glycan utilization capabilities by *Bifidobacterium* members underscores the impact of metabolic specialization as a driving force for species differentiation and niche adaptation (12). Considerable acquisition of carbohydrate transporters, especially of the ABC-type, has been proposed in *Bifidobacterium* (12). The evolution of oligosaccharide transporter specificities to support the adaptation of bifidobacteria to distinct niches in host guts has not received sufficient attention. Transport proteins are suitable probes of the evolution of glycan metabolic preferences due to their superior selectivities as demonstrated in the present study. Biochemical and structural characterization of carbohydrate transporters is key to enable the assignment of metabolic preferences based on genome sequence data. To illustrate this, we performed structural and multiple sequence alignments of *Bal*6GBP and GL-BP, the homologue conferring uptake of the mucin derived galacto-*N*-biose (GNB) and the human milk derived lacto-*N*-biose (LNB) from *B. longum* sp. *longum* (25), together with closely related sequences that cluster with these two characterized proteins in the phylogenetic analysis (Fig. S3). The aromatic platform, which is the hallmark of carbohydrate recognition by SBPs at position 1, is strictly conserved in both the primary and tertiary structural alignment together with the four residues recognising the non-reducing β-galactosyl (Fig. 5C and 5D). This striking conservation unveils the evolutionary pressure to retain the functionality of binding subsite 1 of these two proteins despite the divergence of primary structure (26% sequence identity). Another tryptophan (*Bal*6GBP Trp254) is also fully conserved in the sequence alignment (Fig. 5A), but this residue serves two different roles at position 2: aromatic stacking onto the *N*-acetyl hexosamine ring in GL-BP, while in *Bal*6GBP the indole ring is orthogonal to the galactosyl plane and forms a polar interaction to the hemiacetal oxygen.

A key difference is the substitution of Trp47 in *Bal*6GBP with Arg49 in GL-BP. Importantly, Arg49 recognises the *N*-acetyl group unique to GL-BP substrates via direct and water mediated hydrogen bonds (Fig. 5B). This residue together with Glu110 also engage the galactosyl at position 1 (ring oxygen and C2-OH) and the C4-OH of the *N*-acetyl hexosamine at position 2. This arginine is strictly conserved in close homologs of GL-BP, thereby constituting a specificity signature. Another facet underpinning the divergence of GL-BP towards LNB/GNB recognition entails amino acid changes that reduce the volume of side chains flanking the bulky *N*-acetyl group to enable its accommodation.

The structure of *Bal*6GBP also gives an explanation for the extreme preference for the longer β-(1,6)-galactosidic bond that allows higher rotational freedom between the two monosaccharide rings as compared to β4Gal2, with β3Gal2 having intermediate rotational freedom. The rotational flexibility of β6Gal2 allows the positioning the sugar planes at an angle of about 140°, a conformation that is enforced by Glu98. This residue locks the orientation of the galactosyl at position 2 through a bidentate polar interaction that connects the galactosyl (C4-OH) at position 2 to the counterpart (C2-OH) at position 1 (Fig. 3B). Notably, the position of Glu98 is fixed by two highly conserved tyrosines (Tyr78 and Tyr100) that from rigid α-helices, which is likely to be important for the high selectivity for β6Gal2. The preference for galactosyl at position 2 is mediated by a polar interaction between the axial C4-OH and Asn74. A 140-fold weaker affinity is observed when the axial C4-OH is replaced by an equatorial one in allolactose, possibly due to the loss of the hydrogen bond to Asn74. The glycosidic bond to an axial OH group in β4Gal2 restricts the rotation rendering this ligand more rigid. Therefore, binding of β4Gal2 is associated with a large penalty due to conformational re-arrangements of the protein and a strained conformation of the ligand. Comparing *Bal*6GBP to the β-galactosyl-(1,4)-lactose specific homolog from *B. breve* (21), reveals that the hydrophobic platform and six out of seven residues that bind the galactosyl at position 1 are also conserved in the primary structure (cluster 5 in Fig. S3). Strikingly, however, the double tyrosine motif and Asn74 discussed above are variable, highlighting the sequence divergence underpinning the evolution of transporters for recognition of β-different galactosides.

*The selectivity of transport binding proteins correlates to rapid growth and competitiveness on preferred substrates*
Dietary glycans are instrumental in shaping the composition of the HGM (7,8). For bacteria that lack extracellular polysaccharide degrading enzymes, high affinity capture and transport of oligosaccharides is crucial to cross-feed on glycan oligomers produced by enzymes of primary degraders. The early dominance of bifidobacteria in the human gut relies partially on their arsenal of ABC transporters that confer the uptake of abundant human milk galactosides (33,34). The competitiveness of bifidobacteria on preferred substrates for their ABC transporters was recently demonstrated in B. animalis subsp. lactis in coculture with the human gut symbiont Bacteroides ovatus during growth on raffinose (9). This transporter is highly conserved in adult human gut bifidobacterial species, consistent with the stimulation of bifidobacteria on α-raffinose family α-galactosides from legumes. Similar observations were recently reported in other taxa, e.g. competitive growth of the xylan degrading symbiont Roseburia intestinalis grown on a preferred ligand for its xylooligosaccharide ABC transporter in a mixed culture with Ba. ovatus (35). Most bifidobacteria lack extracellular xylanases, but are able to cross-feed on shorter arabinoxylooligosaccharides produced by other primary degraders using an efficient ABC system (10,11) further supporting the pivotal role of ABC transporters in bifidobacterial adaptation as secondary degraders that efficiently target oligosaccharides.

Bifidobacteria selectively ferment different dietary β-galactosides, e.g. B. animalis subsp. lactis grows well on β-(1,6)-galactosyl lactose, but not on the β-(1,4) version of this trisaccharide (both from bovine milk) (36). Recent studies have identified an ABC transporter that mediates the uptake of β-(1,6)-galactosyl-lactose from B. breve and characterised the preference of the associated SBP (22,37). Notably, B. breve was able to grow comparably well on the β-(1,3)-, β-(1,4)- and β-(1,6)-galactosyl-lactose (GL) isomers. Different ABC transporters appear to mediate this utilization, and the closest homolog of Bal6GBP (WP_003828494.1; amino acid sequence identity 76%) showed the highest affinity for β-(1,6)-GL (K_D = 0.357 μM). This protein has a markedly different selectivity as compared to Bal6GBP, based on a decrease in affinity of only 16-fold for β-(1,4)-GL and 460-fold lower affinity for β-(1,3)-GL (22). The shared preference for β-(1,6)-galactosides, but the large selectivity differences are consistent with these SBP populating different branches in the same clade in the phylogenetic tree (clade 1, Fig. S3). The notable difference in selectivity despite the relatedness of these homologues highlight the need for more structural and biochemical data for reliable assignments of transport proteins from this niche. The second closest homolog (WP_003828303.1; sequence identity 45%) is specific for only β-(1,4)-galactosides with highest affinity for β4Gal2 (K_D = 23.7 nM) followed by β-(1,4)-GL (K_D = 10.4 μM) (21). β-(1,6)-Galactoside motifs are present in plant cell wall pectic arabinogalactans (types I and II) and arabinogalactan proteins (type II) (AGP) (38,39). We have identified homologs (~80% identity) of galactan β-(1,3)-galactosidase (40) and an endo-β-(1,6)-galactanase (41) in the Human Microbiome Project database (42), suggesting that β-(1,6)-galactosides are likely to be accessible degradation products from especially type II arabinogalactan in the diet. The about 100-fold lower selectivity of the B. breve closest homolog for the β-(1,6)-galactosides, may suggest an adaptation to bovine milk, whereas the strict selectivity of Bal6GBP may suggest an adaptation to different ligands, e.g. from pectin. The selectivity of Bal6GBP for β6Gal2 (1630-fold preference over β4Gal2) demonstrates the role of oligosaccharide transporters in defining metabolic preferences as compared to intracellular enzymes, which may display more promiscuity (24). The preference of Bal6GBP correlates to rapid growth on the preferred ligand, whereas relative delay of several hours was observed on less-preferred ligands (Fig. 4A). The preferential depletion of β6Gal2 before β4Gal2 (Fig. 4C) provides compelling evidence that the large delay in the onset of growth on β4Gal2 is attributed to the inefficient uptake. The impact of the poor uptake is amplified by the lower affinity of the intracellular β-galactosidase. The observed correlation between the selectivity of transport systems and early growth efficiency is likely to be crucial for resource allocation in the competitive human gut niche.

In conclusion, the study gives an unprecedented insight into the selectivity of transport proteins as compared to intracellular enzymes targeting the same substrates, and the large impact of
this selectivity on bacterial growth patterns. Access to the first structure of a β6Gal2 binding protein uniquely allowed analyses of the structural elements that support the evolutionary divergence of bifidobacterial β-galactoside transport proteins, which is otherwise challenging due to low primary structure conservation. Altogether, these findings promote our understanding of the structural and functional aspect of oligosaccharide transport in prevalent core microbiota.

**Experimental procedures**

**Carbohydrate ligands and chemicals**

The carbohydrates tested for binding interactions with Bal6GBP are listed in Table S1 and were 94–99% pure. All other chemicals were of analytical grade.

**Cloning, expression and purification of the galactooligosaccharide-binding protein from B. animalis subsp. lactis Bl-04 (Bal6GBP)**

The gene fragment encoding Bal6GBP (locus tag: Balac_0483; GenBank: NC_012814.1) without the signal peptide (residues 1-21 as predicted by SignalP 4.0 (43)) was amplified from genomic Bifidobacterium animalis subsp. lactis Bl-04 DNA (Kindly provided by DuPont Nutrition & Health, Kantvik Finland) using the sense primer 5´-ATACATATGGCAGCCTGTGGGGGTGGC-3' and the antisense primer 5´-ACTGGATCCCTACTCTTCATCTGAAGCTTG-3' with NdeI and BamHI restriction sites highlighted in bold and the amplicon was cloned into the same sites of pET28a(+) (Novagen). The resulting recombinant plasmid pET28 Balac_0483 was verified by full sequencing and transformed into the strains Escherichia coli Rosetta (DE3) and B834 (DE3) (Novagen) to allow production of the unlabelled and seleno-L-methionine labelled recombinant proteins, respectively. Transformants were grown in LB medium with kanamycin (50 μg mL⁻¹) and chloramphenicol (34 μg mL⁻¹) at 30 °C to OD₆₀₀ = 0.5. Expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG, 250 μM) and cells were harvested after 5 h by centrifugation (10,000 g, 30 min), lysed with Bugbuster® (Millipore) and debris removed by centrifugation (20,000g, 2x30 min). Clarified lysates were sterile filtered (0.45 μM) and purified on a HisTrap HP column (GE Healthcare) as previously described (10). Pure fractions were applied onto a HiLoad 16/600 Superdex 75 prep grade column and the proteins eluted during 1.2 column volumes in 20 mM phosphate, 150 mM NaCl, 0.5 mM dithiothreitol (DTT), pH 6.5, and concentrated by 10 kDa Amicon Ultrafiltration filters (Millipore). The fractions containing pure Bal6GBP were pooled and dialysed against 20 mM Tris-HCl, 150 mM NaCl, pH 7.5. The N-terminal hexa-histidine tag of Bal6GBP was cleaved using human thrombin (Calbiochem), recovered after passing through a 1 mL HisTrap HP column, and buffer exchanged into 10 mM MES buffer, pH 6.5. Protein purity was verified by SDS-PAGE and protein concentration was determined by measuring A₂₈₀ using the theoretical molar extinction coefficient ε₂₈₀nm=93280 M⁻¹cm⁻¹ calculated by the ProtParam on the EXPASY server (http://web.expasy.org/protparam/).

**Surface plasmon resonance (SPR)**

SPR binding analysis was performed using a Biacore T100 (GE Healthcare). Bal6GBP (0.1 mg mL⁻¹) in 10 mM sodium acetate, pH 4.0 was immobilised on a CM5 chip by random amine coupling (GE Healthcare) to 2385–3448 response units (RU). Initial binding screening was conducted at a flowrate of 30 μL min⁻¹ and 10 mM carbohydrate ligand at 25 °C with association and dissociations steps of 60 s and 150 s, respectively. Subsequently, saccharides that displayed binding were analysed using 10 concentrations in the range of 0.05–10 K_D in at least duplicates. The pH and temperature dependency of β-(1,4)-galactobiose binding to Bal6GBP was analysed at eight temperatures (15–40 °C) and at nine pH values (3.6–8.2) in 20 mM phosphate citrate buffer. The Bia-evaluation software (GE Healthcare) was used to analyse the data as previously described (10) to determine dissociation constants (K_D) as well as association (k_on) and dissociation rate (k_off) constants where possible.

**Isothermal titration calorimetry (ITC)**

Binding thermodynamics of Bal6GBP (8–600 μM) and preferred galactosides were measured at 25 °C in 20 mM phosphate citrate, pH 6.5 using an iTC200 microcalorimeter (MicroCal). The titration was initiated by a 0.3 μL injection followed by 19 x
2 μL injections of ligand. The concentrations used were 80 μM β6Gal2 injected into 8 μM Bal6GBP, and 2.6 mM β6Gal4 injected into 222 μM Bal6GBP. A one binding site model was fitted to the ITC data after baseline adjustment and correction for the dilution enthalpy using Origin software 7.0552 supplied with the instrument to determine the equilibrium association constant (K_A), the stoichiometry of binding (N) and the molar binding enthalpy (ΔH).

**Crystallization, data processing and protein structure determination**

Crystallization of Bal6GBP was initially tested with the purified protein in 10 mM MES buffer pH 6.5 at a concentration of 10 mg mL⁻¹ and 2.5 mM β6Gal2 with reservoir from the JCSG+ screen (Molecular Dimensions) set up using a Mosquito® liquid handling robot (TTP Labtech) with 200 nL protein mixed with 100 nL reservoir in MRC 3-well plates and after several months at room temperature (297K), a crystal was obtained in C12 (reservoir consisting of 10 % PEG 1000 and 10 % PEG 8000) that diffracted beyond 1.4 Å resolution. Data was collected at the I911-3 beamline at MAX-II (44), and a total of 390 images (195° of data) were collected to a maximum resolution of 1.5 Å on the edge of the square detector, which impacted the completeness of the data in the outer shell (Table 3). The SeMet-labelled protein was crystallized in MRC 2-well plates using an Oryx 8 liquid-handling robot (Douglas Instruments) at a concentration of 20 mg mL⁻¹. Standard screens JCSG+, PACT and Morpheus (Molecular Dimensions) were initially carried out. Drops consisting of 200 nL protein and 100 nL reservoir or 150 nL of each were setup and kept at room temperature (around 297K). Crystals appeared under several conditions. Data were collected on a crystal obtained with the Morpheus screen, condition E4 (12.5% PEG 1000, 12.5% PEG 3350, 12.5% MDP, 30 mM each of di-,tri-,tetra- and pentaethylene glycol with 0.1M MES/imidazole at pH6.5) at the ESRF on beamline ID29 (45) at a wavelength of 0.979 Å and a temperature of 100K. A total of 360° degrees were collected over 2400 images to a resolution of 2 Å. For both datasets, the diffraction data was processed and scaled with XDS (46) using XDSAPP (47). Both native and SeMet Bal6GBP crystals belong to space group P2₁, but are not isomorphous. Phenix.AutoSol (48) found a total of 19 Se sites with a figure of merit of 0.382, and the density modified map was used in phenix.autobuild (49) to produce a preliminary model for the SeMet-labelled protein. This model was split into domain 1 and domain 2 and used in molecular replacement with phaser. After automated model building with phenix.autobuild, the model was completed using Coot (50) and phenix.refine (51) and analysed with Molprobity (52) (see Table 3). The Bal6GBP structures with β6Gal2 (PDB ID: 6H0H) and in the open SeMet form (PDB ID: 6Q5G) were submitted to the Protein Data Bank.

**Galactobiose uptake preference of B. animalis sp. lactis BI-04**

Growth of BI-04 was performed as previously described (9). Freeze-dried B. animalis subsp. lactis BI-04 was grown anaerobically o/n at 37 °C in Bifidus Selective Medium (BSM) and a 2 % inoculum of the o/n culture was similarly propagated twice in de Man Rogosa Sharpe (MRS) broth supplemented with 0.5 g L⁻¹ L-cysteine, before 2 % (v/v) of this culture was used to inoculate a fresh culture in the modified MRS medium supplemented with either 0.5 % (w/v) β3Gal2, β4Gal2, or β6Gal2 in total volumes of 200 μL in a 96 well microtiter plates. In another experiment, the same procedure was used to inoculate 0.5 % (w/v) β6Gal2 or mixtures of β6Gal2 and β4Gal2 (0.20% + 0.20% or 0.05% + 0.45%, respectively). Growth was continued for 48 h at 37 °C and monitored at A_600 by the 2030 Multilabel Reader Victor™ X4 (PerkinElmer). The growth was performed in five replicates in both cases.

Preferential culture supernatant utilization of the β6Gal2 and β4Gal2 isomers by BI-04 was analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) ( Dionex, California, USA) in an time-dependent manner of a growth experiment, performed in Eppendorf tubes, otherwise as described above with 0.5 % (w/v) equimolar mixture of β4Gal2 and β6Gal2 and aliquots were collected for 72 hours. An injection volume of 10 μL onto a Carbopac PA200 column and a mobile phase (0.35 ml min⁻¹) of constant 100 mM NaOH and a gradient of sodium acetate 0–30 min of 0–100 mM and 30–35 min of 100–400 mM was used. Standards and
samples were diluted 10X in 100 mM NaOH before analysis, and standards of the two isomers (0.58 mM) were used to identify corresponding peaks in the chromatograms.

**Bioinformatic analyses**

A BLAST was performed with the native Bal6GBP sequence (GenBank: ACS45862.1) as the query against all proteins of the *Bifidobacterium* genus. The 795 resulting proteins sequences (length 350-550 aa) with Max Scores ≥100 were aligned by MAFFT with default settings (53). From these sequences, 40 and 123 protein sequences clustered together with Bal6GP and GL-BP (the GNB/LNB binder from *B. longum* sp. *longum*), respectively in a neighbour-joining consensus phylogenetic analysis (S3) performed in the Geneious version 11.1.5 (54) including bootstrap resampling with 1,000 replicates. Amino acid sequence logos of the residues involved in ligand binding were generated by TEXshade (55) for the orthologue groups.
Acknowledgements: This study was funded by The Independent Research Fund Denmark | Natural Sciences (FNU) (Research Project 2, grant 4002-00297) to MAH and the Biacore T100 instrument (Large instrument grant 272-06-0050) to BS; the Carlsberg Foundation is acknowledged for an instrument grant (2011-01-0598) to fund the isothermal titration calorimeter to MAH. The authors would like to thank: Professor Mads Hartvig Clausen (Technical University of Denmark, Department of Chemistry), Dr. Motomitsu Kitaoka (National Food Research Institute, Tsukuba, Japan), Asst. Professor Wataru Saburi (Faculty of Agriculture, Hokkaido University, Sapporo, Japan) for their generous oligosaccharide gifts; Professor Tine Rask Licht and technician Bodil Madsen (National Food Institute, Technical University of Denmark) for help regarding bifidobacterial growth. The MAX IV Laboratory and Maria Håkonsson (MAX IV Laboratory, Lund University) are thanked for use of the Mosquito robot and help during crystallization and Tobias Tandrup (Department of Chemistry, University of Copenhagen) for doing the Oryx8 crystallization setup. Mickael Blaise (currently at Institut de Recherche en Infectiologie de Montpellier, CNRS) and Tobias Tandrup and Jens-Christian Navarro Poulsen (both from Department of Chemistry, University of Copenhagen) are thanked for their help during data collection. The ESRF and the MAX IV Laboratory are thanked for beamtime. LLL acknowledges funding for synchrotron travel and sample transport from the Danish Ministry of Higher Education and Science through the Instrument Center DANSATT.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: MCT, BS and MAH have conceived the idea of the study. MCT performed all experiments except for the crystallography. FF and LLL obtained X-ray crystallography protein structure data and solved the structure. MCT, FF and MAH drafted the first version of the manuscript, and MCT, FF, BS, LLL and MAH finalized the manuscript.
References


Table 1. Surface plasmon resonance (SPR) binding analysis of Bal6GBP to β-galactosides of different glycosidic linkages, degrees of polymerization (DP) and composition. Experiments were performed in at least duplicates.

<table>
<thead>
<tr>
<th>Linkage</th>
<th>Ligand</th>
<th>$K_D$ (M)</th>
<th>$k_{on}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (10$^{-2}$ s$^{-1}$)</th>
<th>$R_{max}$ (RU)$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-(1,6)</td>
<td>β6Gal2</td>
<td>(9.2±0.5)×10$^{-8}$</td>
<td>(6.91 ± 0.02) ×10$^6$</td>
<td>7.39 ± 0.02</td>
<td>11.7 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>β6Gal4</td>
<td>(2.4±0.1)×10$^{-6}$</td>
<td>(2.12±0.04) ×10$^4$</td>
<td>1.28±0.02</td>
<td>5.6 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Allolactose</td>
<td>(1.3±0.3)×10$^{-5}$</td>
<td>ND$^b$</td>
<td>ND$^b$</td>
<td>12.1 ± 0.59</td>
</tr>
<tr>
<td>β-(1,3)</td>
<td>β3Gal2</td>
<td>(2.8±0.2)×10$^{-5}$</td>
<td>(2.48±0.01) ×10$^3$</td>
<td>4.66±0.01</td>
<td>16.2 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Galβ13Glc</td>
<td>(5.7±0.7) ×10$^{-4}$</td>
<td>ND$^b$</td>
<td>ND$^b$</td>
<td>12.1 ± 0.33</td>
</tr>
<tr>
<td>β-(1,4)</td>
<td>β4Gal2</td>
<td>(1.5±0.3)×10$^{-4}$</td>
<td>(3.22±0.1)×10$^2$</td>
<td>5.97±0.08</td>
<td>14.1 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>β4Gal3</td>
<td>(3.4±0.2)×10$^{-5}$</td>
<td>ND$^i$</td>
<td>ND$^i$</td>
<td>22.7 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>β4Gal4</td>
<td>(1.2±0.03)×10$^{-4}$</td>
<td>ND$^i$</td>
<td>ND$^i$</td>
<td>25.6 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>β4Gal5</td>
<td>(1.0±0.1)×10$^{-4}$</td>
<td>(3.20±0.06)×10$^2$</td>
<td>1.55±0.02</td>
<td>8.5 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Lactulose</td>
<td>(4.9±0.3)×10$^{-4}$</td>
<td>(2.71±0.06)×10$^2$</td>
<td>6.61±0.08</td>
<td>8.4 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Lactose</td>
<td>(7.2±0.4)×10$^{-4}$</td>
<td>(2.00±0.02)×10$^2$</td>
<td>6.71±0.03</td>
<td>7.5 ± 0.04</td>
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<tr>
<td></td>
<td>Epilactose</td>
<td>(2.1±0.2)×10$^{-3}$</td>
<td>(6.35±0.06)×10$^1$</td>
<td>8.87 ± 0.06</td>
<td>7.7 ± 0.03</td>
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</tbody>
</table>

$^\dagger$Maximum binding signal level determined from the fits to the data.

$^b$Binding kinetics were not within range for reliable determination.
Table 2. Binding energetics of β6GBP to β-(1,6)-galactobiose (β6Gal2) and β-(1,6)-galactotetraose (β6Gal4) at 25 °C and pH 6.5 using isothermal titration calorimetry (ITC).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>N (Sites)</th>
<th>$K_D$ (M)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
<th>$\Delta H$ (kJ mol$^{-1}$)</th>
<th>$-T\Delta S$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β6Gal2†</td>
<td>0.61±0.0</td>
<td>$(1.4±0.02) \times 10^{-7}$</td>
<td>-39.7</td>
<td>-59.4±0.7</td>
<td>19.8</td>
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<tr>
<td>β6Gal4‡</td>
<td>1.1±0.0</td>
<td>$(4.1±0.2) \times 10^{-6}$</td>
<td>-30.7</td>
<td>-13.2±0.1</td>
<td>-17.5</td>
</tr>
</tbody>
</table>

†Average of three experiments. The deviation of the stoichiometry from 1:1 is likely due to inaccuracy in the ligand concentration, which makes the thermodynamic parameters from this measurement of this ligand less reliable. ‡Average of two experiments.
Table 3. Data collection and refinement statistics of the X-ray crystal structure of native \textit{Bal}6GBP in complex with $\beta$-(1,6)-galactobiose and the selenomethionine labelled protein in open form.

<table>
<thead>
<tr>
<th></th>
<th>6H0H (complex with $\beta$6Gal2)</th>
<th>6Q5G (SeMeth open form)</th>
</tr>
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<tbody>
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<td><strong>Wavelength (Å)</strong></td>
<td>1.000</td>
<td>0.979</td>
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<td><strong>Resolution range (Å)</strong></td>
<td>30.21 - 1.39 (1.44 - 1.39)</td>
<td>46.19 - 1.94 (2.01 - 1.94)</td>
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<tr>
<td><strong>Space group</strong></td>
<td>$P 1 2_1 1$</td>
<td>$P 1 2_1 1$</td>
</tr>
<tr>
<td><strong>$a, b, c$ (Å)</strong></td>
<td>56.35, 71.69, 88.71</td>
<td>62.94, 69.36, 97.30</td>
</tr>
<tr>
<td><strong>$\beta$ (°)</strong></td>
<td>95.04</td>
<td>104.43</td>
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<tr>
<td><strong>Total reflections</strong></td>
<td>489576 (19734)</td>
<td>378068 (21326)</td>
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<tr>
<td><strong>Unique reflections</strong></td>
<td>130307 (8129)</td>
<td>57952 (4289)</td>
</tr>
<tr>
<td><strong>Multiplicity</strong></td>
<td>3.8 (2.4)</td>
<td>6.5 (5.0)</td>
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<tr>
<td><strong>Completeness (%)</strong></td>
<td>91.81 (57.69)</td>
<td>96.38 (71.74)</td>
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<tr>
<td><strong>Mean I/σ(I)</strong></td>
<td>18.59 (2.39)</td>
<td>12.69 (2.64)</td>
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<tr>
<td><strong>Wilson B-factor (Å$^2$)</strong></td>
<td>10.84</td>
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<tr>
<td><strong>$R$-merge</strong></td>
<td>0.04525 (0.3927)</td>
<td>0.09661 (0.3816)</td>
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<td><strong>$R$-meas</strong></td>
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<td><strong>$R$-pim</strong></td>
<td>0.02629 (0.3028)</td>
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<td><strong>CC$_{1/2}$</strong></td>
<td>0.999 (0.799)</td>
<td>0.996 (0.931)</td>
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<tr>
<td><strong>CC</strong></td>
<td>1.000 (0.943)</td>
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<td><strong>Reflections used in refinement</strong></td>
<td>129672 (8129)</td>
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<tr>
<td><strong>Reflections used for $R$-free</strong></td>
<td>6526 (427)</td>
<td>1078 (80)</td>
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<td><strong>$R$-work</strong></td>
<td>0.1161 (0.2066)</td>
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<tr>
<td><strong>$R$-free</strong></td>
<td>0.1464 (0.2344)</td>
<td>0.2035 (0.2848)</td>
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<tr>
<td><strong>CC(work)</strong></td>
<td>0.982 (0.902)</td>
<td>0.971 (0.935)</td>
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<tr>
<td><strong>CC(free)</strong></td>
<td>0.973 (0.866)</td>
<td>0.965 (0.809)</td>
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<td><strong>Macromolecules</strong></td>
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<td>6444</td>
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<tr>
<td><strong>Ligands</strong></td>
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<td>None</td>
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<tr>
<td><strong>Solvent</strong></td>
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<tr>
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<td><strong>RMSD(angles)</strong></td>
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<td>0.96</td>
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<td><strong>Ramachandran outliers (%)</strong></td>
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<td>0.12</td>
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<td><strong>Rotamer outliers (%)</strong></td>
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<td><strong>Clashscore</strong></td>
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<td><strong>Average B-factor</strong></td>
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<tr>
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<td><strong>Number of TLS groups</strong></td>
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</table>
**Figure legends**

**Figure 1.** Schematic representation of β-galactosides that display binding to the transport protein *Bal6GBP* from *B. animalis* subsp. *lactis*. The β-galactosides are ordered according to preferred glycosidic bond by *Bal6GBP*.

**Figure 2.** Binding analyses of *Bal6GBP*. A, Binding of β-(1,6)-galactotetraose to *Bal6GBP* as analysed by surface plasmon resonance at 25°C and pH 6.5. Blank and reference cell corrected sensograms (top) and one binding site steady state analysis (bottom) to seven ligand concentrations (0.2–25.6 μM). B, Isothermal titration calorimetry binding analysis to the same ligand as above to *Bal6GBP* (246 μM) to β-(1,6)-galactobiose (2.4 mM) at 25°C and pH 6.5. The top panel shows the baseline adjusted thermogram and the bottom panel depicts the binding isotherm and a one site binding model fit to the data (solid line).

**Figure 3: *Bal6GBP* protein structure in complex with β-(1,6)-galactobiose (β6Gal2). A, Overall structure of *Bal6GBP* with a semitransparent white surface that visualizes protein surface. Domain 1 (residues 38–144 and 311–362) in brown and domain 2 (residues 149–306 and 374–447) in green, held together by linker regions (residues 145–148, 307–310 and 363–373) in light blue. The non-reducing galactosyl unit of β6Gal2 occupies position 1, which is defined by the aromatic platform provided Trp275. B, The residues and waters (red spheres) that directly interact with the bound β6Gal2 are shown and the dashed orange lines indicate polar interactions. The mFo–DFcalcdifference electron map for β6Gal2 is shown prior to inclusion of the ligand in the model at a contour level of 3σ (blue mesh). The main chain atoms are omitted for clarity unless they participate in polar interactions. The bottom panel is a view rotated 60° along the X-axis.

**Figure 4.** Growth curves and uptake profiles of *Bifidobacterium animalis* subsp. *lactis* BI-04 growing in modified MRS medium supplemented with β-galactobiose. A, Growth on 0.5% w/v of β-(1,6)-galactobiose (β6Gal2, blue), β-(1,3)-galactobiose (β3Gal2, green) or β-(1,4)-galactobiose (β4Gal2, red). B, A different growth experiment on 0.5% w/v β6Gal2 (blue), on 0.2% β6Gal2 + 0.2% β4Gal2 (purple) or 0.05% β6Gal2 + 0.45% β4Gal2 (pink). C, Uptake analysis of culture supernatants of a static growth experiment in Eppendorf tubes, but otherwise similar to panel B with the equimolar mixture of β4Gal2 and β6Gal2 (purple) using high performance anion exchange with peramperometric detection. The experiments in panels A, B and C are from different experiments. Experiments in A and B were performed in five replicates and the data are shown as means±standard deviations, whereas the data in panel C is from a single experiment.

**Figure 5.** Comparison of the β-(1,6)-galactooligosaccharide (β6Gal) and GNB/LNB binding (GL-BP) proteins. A, Multiple sequence alignment logos of the binding site residues of the β6Gal and GL-BP clusters of bifidobacteria accessed by phylogenetic analysis. Amino acid residues of the logos are colored by amino acid category. The sequences of GL-BP and *Bal6GBP* are included, and are colored according to the conservation as compared to their respective clusters (dark blue: all match; pink: similar; blue: ≥50%). B, Structural alignment of *Bal6GBP*:β6Gal2 (domain 1: sand, domain 2: green; linker: light blue) and GL-BP:GNB (grey). The structural alignment is based on the identical β-galactopyranosyl in position 1 of the binding pockets. (C, D) Binding sites of *Bal6GBP*:β6Gal2 and GL-BP:GNB, respectively. The top panels depict the conserved residues, and the bottom panel depicts variant residues (domain 1 residues: sand; domain 2 residues: green; linker residues: light blue).
Figure 1
Figure 2
Figure 4
Figure 5
Substrate preference of an ABC importer corresponds to selective growth on β-(1,6)-galactosides in Bifidobacterium animalis subsp. lactis
Mia Christine Theilmann, Folmer Fredslund, Birte Svensson, Leila Lo Leggio and Maher Abou Hachem

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