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Proteomic Analysis of *Bifidobacterium longum* subsp. *infantis* Reveals the Metabolic Insight on Consumption of Prebiotics and Host Glycans

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Abstract

Bifidobacterium longum subsp. *infantis* is a common member of the intestinal microbiota in breast-fed infants and capable of metabolizing human milk oligosaccharides (HMO). To investigate the bacterial response to different prebiotics, we analyzed both cell wall associated and whole cell proteins in *B. infantis*. Proteins were identified by LC-MS/MS followed by comparative proteomics to deduce the protein localization within the cell. Enzymes involved in the metabolism of lactose, glucose, galactooligosaccharides, fructooligosaccharides and HMO were constitutively expressed exhibiting less than two-fold change regardless of the sugar used. In contrast, enzymes in N-Acetylglucosamine and sucrose catabolism were induced by HMO and fructans, respectively. Galactose-metabolizing enzymes phosphoglucomutase, UDP-glucose 4-epimerase and UTP glucose-1-P uridylytransferase were expressed constitutively, while galactokinase and galactose-1-phosphate uridylyltransferase, increased their expression three fold when HMO and lactose were used as substrates for cell growth. Cell wall-associated proteomics also revealed ATP-dependent sugar transport systems associated with consumption of different prebiotics. In addition, the expression of 16 glycosyl hydrolases revealed the complete metabolic route for each substrate. Mucin, which possesses O-glycans that are structurally similar to HMO did not induced the expression of transport proteins, hydrolysis or sugar metabolic pathway indicating *B. infantis* do not utilize these glycoconjugates.

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Introduction

Bifidobacterium longum subsp. *infantis* (*B. infantis*) is a common member of the gastrointestinal tract (GIT) of breast-fed infants [1]. The establishment of a bifidobacterial-dominant microbiota has received considerable attention regarding the development of the infant [2]. One potential means by which specific bifidobacteria succeed in colonizing the infant GIT is through prebiotic enrichment by human milk oligosaccharides (HMO). Human milk contains a significant amount of oligosaccharides ($\sim$15 g l^{-1}), in contrast to bovine or formula milk [3,4]. HMO is a term that collectively refers to approximately 200 different types of glycans with diverse structures with a length smaller than 20 units of carbohydrates [5,6]. These HMO are composed of hexoses (Hex) and N-Acetylhexosamines (HexNAc) connected through β1-3 or β1-4-glycosidic linkage with additional decoration of fucose (Fuc) and N-Acetylneuraminic acid (NeuAc). Since the human host does not have the enzymatic capacity to degrade these polymers, HMO are not considered to be metabolized by infant, and have been shown to arrive to the lower GIT [7]. HMO are considered part of the innate immune system being used as decoy binding sites for intestinal pathogens in the developing newborn [8]. Another role is the enrichment of bifidobacteria in the GIT [9]. While *B. infantis* and other bifidobacteria can actively utilize HMO as growth substrate, other intestinal bacteria such as streptococci, enterococci, *E. coli* and *Clostridium* sp. cannot use HMO as a carbon source, emphasizing the bifidogenic potential of HMO [10]. Genome sequencing showed that *B. infantis* has a 40 kb gene cluster, termed HMO cluster I, that encodes several enzymes and transport systems required for HMO catabolism including α-fucosidase, α-sialidase, β-hexosaminidase, β-galactosidase and the ABC transport systems with six family 1, extracellular solute binding proteins (SBPs) predicted to bind oligosaccharides [11]. These clusters of HMO-linked genes suggested a broader evolutionary partnership among *B. infantis*, the developing infant and human milk [12].

While current commercial prebiotics used in infant formulas, such as fructooligosaccharides (FOS) and galactooligosaccharides [13], have been shown to be bifidogenic, these commercial prebiotics do not replicate the many different roles that HMO

carry out in the developing infant. Since *B. infantis* displays several mechanistic preferences for HMO consumption, this subspecies represents a useful reference with which to assess the similarity of commercial prebiotics to actual HMO. This in turn will aid in the design of more effective prebiotics for use in infant formulas as well as other conditions where very selective symbiotic applications may be useful.

Global expression analysis of whole cell proteome by LC-MS/MS can provide direct information relative to biological systems such as metabolic pathways and regulatory networks. Despite the advantages of whole cell proteomics, it is still challenging to reliably identify cell wall associated (CWA) proteins due to the fact that these proteins are often sequestered into the insoluble material upon cell lysis. The lack of CWA proteins in proteomic analyses results in significant loss of biological information since membrane proteins or cell wall-anchored proteins also play important roles in transport, exopolysaccharide production, hydrolysis of macromolecules, and sensing of extracellular signals. Several proteomic methods have been developed to specifically identify the CWA proteins, including a 2-D gel based proteomic analysis followed by fractionation [14,15,16], or labeling the cell surface proteins via biotinylation followed by affinity capture [17,18]. An alternative approach is the tryptic digestion of exposed epitopes on cell surface proteins from intact cells in isotonic solution followed by the MS analysis, which often called "shaving and shedding" [19,20].

In this study, we have investigated the impact of various prebiotics on the proteomic expression profiles of *B. infantis* ATCC 15697. The microorganism was cultured with seven different sugars and prebiotics including HMO and mucin, and more than 500 proteins were quantitatively identified enabling us to explore the entire activity cell in a given substrate. Importantly, we developed a proteomic analysis method that allowed reliable determination of cell wall associated proteins. Through this method, the expression of cell surface/membrane associated proteins can be determined, maximizing the total protein identification per cell. This has allowed a more comprehensive comparison of the *B. infantis* physiology on these prebiotics.

Materials and Methods

Cell Culture

Bifidobacterium longum subsp. *infantis* ATCC 15697 was obtained from the American Type Culture Collection (Manassas, VA). Cultures were routinely maintained in de Mann-Rogosa-Sharp (MRS) medium with no carbon source, and supplemented with 0.05% w/v L-cysteine (Sigma-Aldrich, St. Louis, MO), and 2% w/v of either lactose (Sigma Aldrich, MO), purified HMO [21], mucin from porcine stomach type III (Sigma Aldrich, MO), FOS (raftilose Synergy 1, Orafti, Malvern, PA), inulin (raftiline HP, Orafti, Malvern, PA) or GOS (Purimune, GTC Nutrition, Golden, CO). These experiments were performed in duplicates. Cells were anaerobically grown in a vinyl chamber (Coy Laboratory Products, Grass Lake, MI) at 37°C for 24 h, in an atmosphere consisting of 5% carbon dioxide, 5% hydrogen, and 90% nitrogen. Optical density was assayed using a PowerWave microplate spectrophotometer (BioTek Instruments, Inc., Winoosky, VT). Due to the significant high turbidity of medium, cell growth on mucin was evaluated separately. Mucin was autoclaved for 10 minutes and added on the media (50 ml) at a final concentration of 20 g l^{-1}. Cell growth was monitored by measuring optical density of cell at 600 nm in a Shimadzu UV1601 spectrophotometer (Shimadzu Scientific Instruments, Columbia MD). Cell cultures supplemented with 2% (w/v) of glucose and 2% (w/v) of HMO were obtained in similar conditions as control experiments.

Proteomic Sample Preparation

B. infantis cells were taken at the exponential phase of growth and normalized at an OD of 1.0 by dilution or concentration. After centrifugation to remove the spent media, 15 mL of cells were washed three times with phosphate buffer saline (PBS) followed by three more washes with urea buffer (8 M Urea/0.1 M Tris, pH 8.5). The cell pellet was resuspended in 600 μL of urea buffer, then mechanically disrupted by silica beads in a bead-beater (FastPrep; QBiogene, Carlsbad, CA, USA) for eight cycles of 30 s pulses and 30 s on ice. Upon centrifugation, the soluble and the insoluble fractions were stored separately at −80°C until further analysis.

For proteome analysis of the soluble fraction, a sample volume containing 200 mg of protein was transferred to a new micro-centrifuge tube and precipitated by the addition of ethanol (75% (v/v) final) at −20°C. After centrifugation, the protein pellet was resuspended in 100 μL of 0.1 M Tris/1 M urea buffer (pH 8.5). Proteins were then digested using 5 μg of mass spectrometry grade trypsin (Promega, Madison, WI, USA) overnight at 37°C. In order to obtain the proteome of insoluble fraction, the insoluble cell debris pellet was resuspended in 1 mL of PBS and washed three times with urea buffer (8 M Urea/0.1 M Tris). The resulting pellet was resuspended again in 100 μL of 0.1 M Tris/1M urea buffer (pH 8.5) and digested with trypsin overnight. These two fractions, soluble and CWA, were analyzed independently. The tryptic peptides from two fractions were cleaned independently using a Macro Trap with Peptide concentration and Desalting cartridge (Michrom, Aurburn, CA, USA) according to the manufacturer's instructions. The resultant peptides were eluted in 98% acetonitrile in water and then dried prior to mass spectrometry analysis.

Mass Spectrometry Analysis

Peptides were reconstituted in water at concentrations corresponding to between 40 and 200 ng of the original protein per 2 μL injection. Nano LC/MS and nano LC/MS/MS analyses were performed on an Agilent HPLC-Chip Quadrupole Time-of-Flight (Q-TOF) MS system equipped with a microwell plate autosampler (maintained at 6°C), capillary sample loading pump, nano pump, HPLC Chip/MS interface, and Agilent 6520 Q-TOF MS detector. The chip used consisted of a 9×0.075-mm i.d. enrichment column and a 150×0.075-mm i.d. analytical column. For sample loading, the capillary pump delivered 0.1% formic acid in 3.0% acetonitrile/water (v/v) isocratically at 4.0 μL/min. Following sample injection, a nano pump gradient was delivered at 0.4 μL/min using (A) 0.1% formic acid in 3.0% acetonitrile/water (v/v) and (B) 0.1% formic acid in 90.0% acetonitrile/water (v/v). Samples were eluted with 0% B, 0.00–2.50 min; 0 to 16% B, 2.50–10.00 min; 16 to 44% B, 10.00–30.00 min; 44 to 100% B, 30.00–35.00 min; and 100% B, 35.00–45.00 min. This was followed by equilibration at 0% B, 45.01–65.00 min. The drying gas temperature was set at 325°C with a flow rate of 5.0 L/min (2.5 L of filtered nitrogen gas and 2.5 L of filtered dry compressed air).

Single-stage MS spectra were acquired in the positive ionization mode over a mass range of m/z 400–3,000 with an acquisition time of 1 s per spectrum. Mass correction was enabled using reference masses of m/z 322.048, 622.029, 922.010, 1,221.991, and 1,521.971 (ESI-TOF Calibrant Mix G1969-85000, Agilent Technologies, Santa Clara, CA, USA).

Protein Identification

All MS/MS samples were analyzed by X! Tandem (GPM-XE manager, ver. 2.2.1). X! Tandem was set up to search against the proteome of *B. infantis* ATCC 15697 and common contaminant

proteins and searched with a fragment ion mass tolerance of 100 ppm. Oxidation of methionine was specified as a variable modification in X! Tandem. The probability (log(e)) cutoff for peptide assignment was set at −2. For the identification, two conditions were required: (a) more than two unique peptides per protein (P_{uniq}≥2) and (b) the probability cutoff for protein less than −6 (log(e)≤−6). Additional algorithms for protein identification validated the protein identification results by X! Tandem. All proteins identified by the X! Tandem were also assigned by the peptide prophet [22] and protein prophet algorithm [23] with the probability greater than 95% and 99% respectively. Since the experiments were performed on biological duplicates an additional rule was applied for the decision of protein expression. If a protein was identified in one of the duplicates with high probability (P_{uniq}≥2 and log(e)≤−6) then it was considered expressed. If a protein had a log(e) value between −6 and−4 and a P_{uniq}≥2 but was found in both duplicates, it was also considered as expressed.

Protein Quantification

Relative amounts of protein in a sample were calculated by the spectral counting method [24] using the proteome on lactose grown cells as control. Normalized spectral abundance factor (NSAF), which represented the relative abundance of protein in a sample, was estimated from the total number of spectra used for the identification of protein (*SpC*) and the number of aminoacids of the protein (*L*). For accurate estimations, the relative amount of protein was calculated only if the protein NSAF value was higher than 1.0 or *SpC* of both proteins were ≥6.

Determination of Protein Localization

For this purpose, a scoring system was introduced that employed the NSAF values of each protein in the soluble and insoluble fraction (Table S1). If the protein was observed only at the insoluble fraction, or the NSAF value of that protein in the insoluble fraction was more than 3-fold higher than the NSAF value of the same protein in the soluble fraction, the protein had score +1. If the NSAF ratio was between 2- and 3-fold, a protein was given a score of +0.5. When the NSAFs values of the same protein in the soluble and insoluble fractions were within 2-fold difference, the protein was given a score of zero. If the NSAF of a protein at the soluble fraction was larger than that of at insoluble fraction, the scores were attributed using the same criteria above, however the values were negative. Once scores were determined, values from the experiments with six different carbon sources were averaged. If a protein had an average score between +0.5 to +1.0 it was classified as a CWA protein. If the average score of a protein was between −0.5 to −1.0, it was considered as cytosolic (CYT) protein (Table S2).

Results

Cell Growth and Overall Protein Expression Profiles

B. infantis was able to utilize various mono and oligosaccharides as growth substrates. The specific cell growth rates on GOS, FOS, inulin, and HMO were in a range of 0.14∼0.19 (hr^{-1}) which are similar to that of glucose and lactose (Figure S1A). Mucin is a large extracellular protein that is heavily glycosylated. The O-glycan attached on the serine residue of mucin protein is composed of N-Acetylgalactosamine, N-Acetylglucosamine, fucose, galactose and sialic acid and has similar structures to HMO. It has been reported that several intestinal bacteria are able to interact with the mucin glycans [25,26]. Using mucin as the sole carbon source (Figure S1B), an increase in OD_{600} was appreciated for *B. infantis* albeit with the high initial cell density values.

The number of proteins identified from the soluble and insoluble fractions is summarized in Table S3. Between 350 and 400 proteins were found in any individual sample grown on each carbon source, and 100∼170 proteins were commonly observed in both the soluble and insoluble fractions in each sample. A sum total of 540 proteins were identified during growth on the seven different carbon sources among 2416 ORFs encoded by *B. infantis*.

Using the normalized spectrum abundance factor (NSAF) for each protein (representing the normalized protein quantity in a whole proteome of a single sample), quantitative expression profiles of whole proteomes were compared between cells grown on different carbon sources. While growth on most substrates exhibited similar proteome expression patterns, growth on galactooligosaccharides resulted in a separate cluster as witnessed by hierarchical cluster analysis (Figure S2). In addition, the correlation coefficients (Pearson's product) between the GOS and the other proteomes were low (0.6∼0.8), indicating that the protein expression observed during growth on GOS is significantly different from growth on other tested substrates (Table S4).

Validation of Cell Wall Associated (CWA) Proteomics

Most proteins observed in current shotgun whole cell proteomics experiments are cytosolic, while cell wall associated (CWA) proteins (both cell wall attached and membrane proteins) remain in the insoluble fraction with cell debris. Even if released from the soluble fraction, they are often missed due to the low ionization efficiency during MS analysis. Another constraint affecting their detection is their low (and/or variable) abundance by comparison to more readily obtained cytosolic proteins. To better understand the expression of CWA proteins, the insoluble fraction obtained after cell disruption was analyzed separately followed by the extensive washing with a strong denaturing agent (8 M urea/0.1M Tris). After removal of cytosolic proteins by washing, peptides from the CWA proteins were released by tryptic digestion. Protein localization was estimated by the quantitative ratio of their amounts in the soluble fraction and insoluble fraction.

To validate this approach, the expression of representative CWA proteins such as extracellular solute binding proteins (SBPs) from *B. infantis* ATCC 15697 was examined [27]. These are common lipoproteins often associated with the transport of various ligands. Overall, 71 incidences of SBP expression were observed from the proteome of *B. infantis* grown on the seven different carbon sources. Among these, 23 cases of SBP expression were only found in the insoluble fraction (Figure 1, black bar). Moreover, 93% of the observed SBPs exhibited in average 2.9-fold higher NSAF value in the insoluble fraction by comparison to the soluble fraction (Figure 1). In addition to the SBPs, expression of other known cell wall proteins was more clearly, or solely, witnessed in the insoluble fraction. As shown in Table S5, Blon_1259, a V5/Tpx-1 family protein was not identified on the soluble fraction during growth on any carbon source, but it was one of the most abundant proteins observed in insoluble fraction. Also a cell surface protein involved in capsular exopolysaccharide synthesis (Blon_2082) and the NlpA lipoprotein (Blon_1721) exhibited 2 and 14 fold higher NSAF values in the insoluble fractions than that observed in the soluble fractions respectively.

Therefore, by comparing the NSAF values of the soluble and insoluble protein fractions this approach provided insight into the cellular localization of each protein identified (Table 1 and 2). As shown on Table 1, 196 proteins were classified as CWA proteins and 93 were cytosolic (CYT) protein. However, the remaining 281 proteins could not be conclusively classified in either cellular location (UI: unidentified). To further evaluate our method, the number of a theoretical CWA protein was predicted bioinforma-

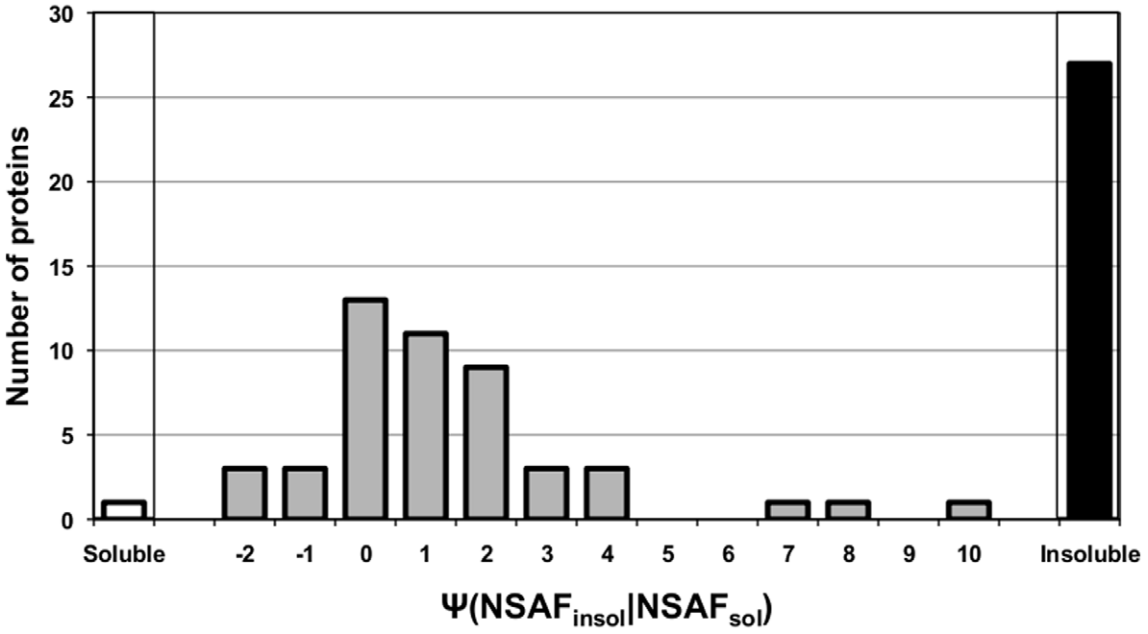

Figure 1. Distribution of the NSAF ratio of each SBP between soluble and insoluble fraction (grey bars; $\psi NSAF_{insol}/NSAF_{sol}$). White bars (soluble: left corner) and black bars (insoluble: right corner) indicate the number of SBP observed solely in soluble and insoluble fraction, respectively.
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tically [28]. Determination of transmembrane domains and signal peptides by TMHMM 2.0 [29] and the SignalP 2.0 HMM [30] indicated that 127 proteins are putative cell surface associated proteins. Among this pool of proteins, 108 were validated as cell wall associated by this proteomic approach. Only two of the cell surface associated proteins identified bioinformatically were categorized as cytosolic. In addition, 17 out of 127 theoretical surface proteins were found in the UI group. Thus while 281 proteins were classified into this group by the above scoring criteria, the latter results on putative cell surface proteins suggests that the UI group is more likely to contain cytosolic proteins.

Carbohydrate Metabolism

Proteins involved in carbohydrate metabolism in *B. infantis* are summarized in Figure 2. Enzymes in the bifid shunt and glycolytic pathway, which are common to the utilization of various carbon sources, exhibited constitutive expression on the substrates used in this study (Figure S3). When compared to lactose-grown cells, the amount of each protein in these pathways varied less than two-fold among the different substrates. Interestingly, the genome of *B. infantis* ATCC 15697 indicates that several enzymes have isozymes. For example it contains seven genes encoding putative phosphoglycerate mutases, but only the expression of a single gene was appreciated, Blon_2152 (Figure S3).

B. infantis metabolizes galactose through the Leloir pathway. Proteins of this pathway were observed in the proteomes of cells grown on all seven carbon sources, albeit their amounts varied. As shown in Figure 2A, galactokinase (*galK*: Blon_2062), Gal-1-P uridylyltransferase (*galT*: Blon_2063) and UDP-glc epimerase (*galE*: Blon_0538) were highly expressed on lactose, HMO and FOS. However, the alternative *galE* (Blon_2171) and phosphoglucomutase (*pgm*: Blon_2184) were constitutively expressed across all substrates.

N-Acetylglucosamine (GlcNAc) is one of the major constituents of HMO. To metabolize this monosaccharide, *B. infantis* possibly converts GlcNAc to fructose-6-P (Fru-6-P) by deacetylation followed by deamidation. GalNAc is another hexosamine found in O-linked glycans, and it could be first isomerized to GlcNAc by an N-Acetylglucosamine 6-phosphate 2-epimerase (*nanE*: Blon_0645). The acetyl and amine groups of GlcNAc-6-P could then be removed by GlcNAc-6-P deacetylase (*nagA*: Blon_0882) and glucosamine-6-P isomerase (*nagB*: Blon_0881), respectively.

Table 1. Distribution of *B. infantis* proteins in cell wall or cytosolic fractions.

Location	Proteins identified[a]	Theoretical cell wall/membrane associated proteins[b]
Cell wall association (CWA)	196	108
Cytosol (CYT)	93	2
Unidentified (UI)	281	17
Total	540	127

[a]Number of the protein for which the location was determined by the score system (See Materials and Methods).
[b]Theoretical cell wall/membrane associated proteins were determined by the Signal prediction by SignalP 2.0 HMM and the transmembrane helices prediction by TMHMM2.0. Bioinformatic information including pfam classification was obtained from the Integrated Microbial Genomes database.
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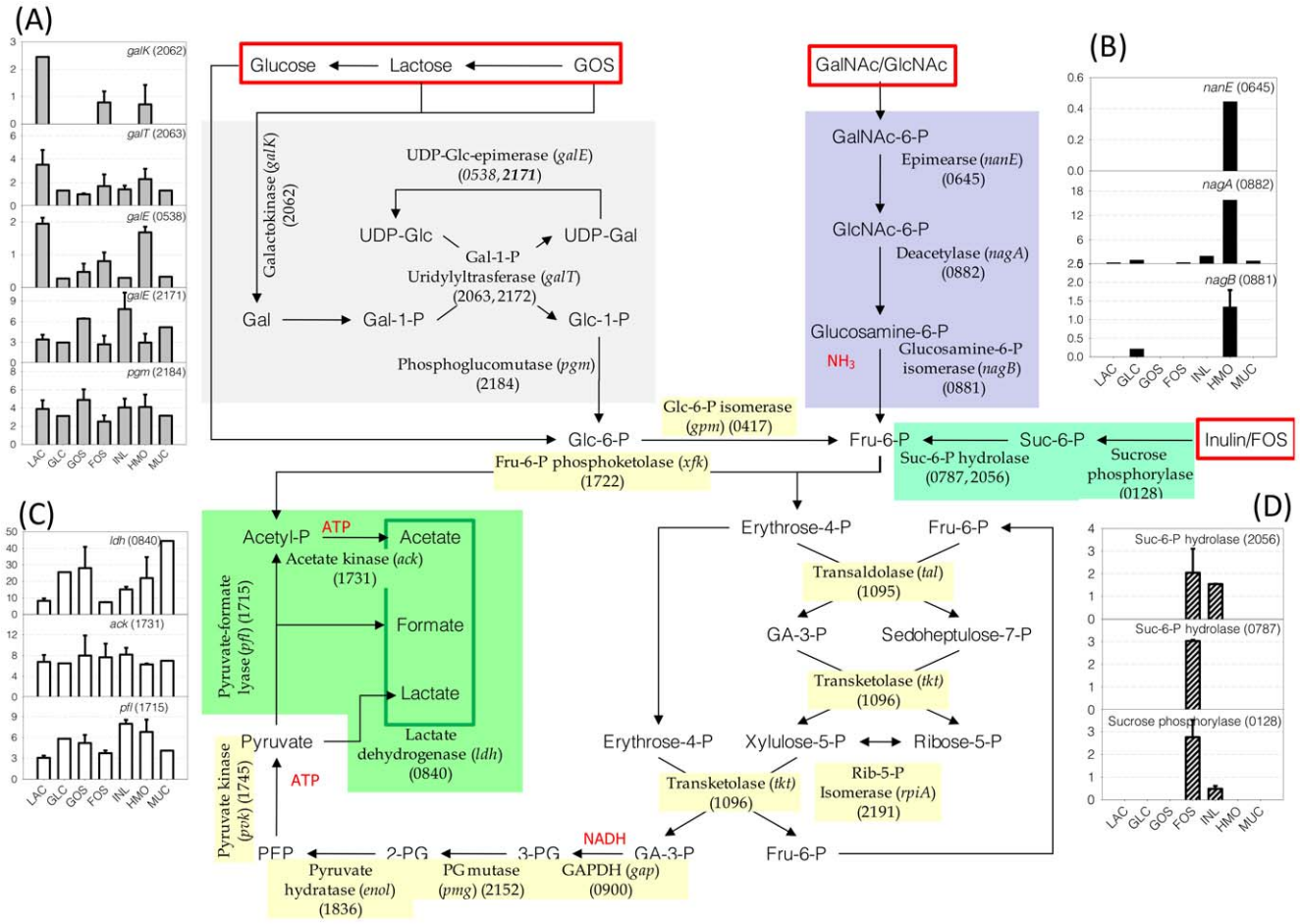

Figure 2. Central metabolic pathways reconstructed from proteomic datasets. Sugar substrates are presented inside the red boxes. Pathways regulated or induced by the presence of a specific sugar are indicated by colored background and their expressions on different sugars are presented next to the pathway as bar graphs: (a) Leloir pathway, (b) HexNAc catabolic pathway, (c) pyruvate fermentation pathway, (d) FOS/Inulin glycosyl hydrolase. The relative amounts of enzymes involved in the bifid shunt are presented in Figure S3. Numbers in the parenthesis next to the enzyme are the locus tag of each protein expressed.
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These three enzymes are expressed significantly only when *B. infantis* was cultivated on HMO, suggesting the specific induction of catabolic pathway for HexNAc (Figure 2B).

While the proteins in the main glycolytic pathway were constitutively expressed, enzymes involved in pyruvate fermentation changed their expression level depending on the carbon source. As shown in Figure 2C, pyruvate is reduced to lactate by lactate dehydrogenase (*ldh*: Blon_0840) with the regeneration of NAD^+. Alternatively, pyruvate is converted to formate and acetate by the combination of pyruvate-formate lyase (*pfl*: Blon_1715) cleaving pyruvate to formate and acetyl CoA, and acetate kinase (*ack*: Blon_1731) converting acetyl CoA to acetate with the production of ATP. Expression of acetate kinase and formate C-acetyltransferse was consistent across all growth substrates. However, lactate dehydrogenase exhibited a different expression pattern depending on the carbon sources used. During growth on lactose or FOS, the amounts of LDH were 2 to 3.5-fold smaller than those when *B. infantis* grew on glucose, GOS, inulin and HMO. Interestingly, LDH was expressed 5.5-fold more on mucin compared to lactose. Alternative route enzymes that can produce acetyl CoA from pyruvate, i.e. pyruvate dehydrogenase or pyruvate oxidase, were not expressed in any of the proteomes (data not shown).

Inulin and FOS are fructose polymers with different degrees of polymerization (DP). When FOS and inulin were used as carbon sources, a specific glycosyl hydrolase (GH) was induced (Figure 2D). Blon_0128 is a family 13 GH annotated as a sucrose phosphorylase. These enzymes cleave sucrose to glucose and fructose-6-phosphate. The NSAF of Blon_0128 in the soluble fraction is higher than insoluble fraction suggesting a cytosolic localization. Two family 32 GH proteins (Blon_2056 and Blon_0787) were also expressed exclusively on FOS and/or inulin. Known activities of the family 32 GH includes inulinase (EC:3.2.1.7), levanase (EC:3.2.1.65), and exo-inulinase (EC:3.2.1.80). While Blon_0128 and Blon_2056 were expressed on both inulin and FOS, the expression of Blon_0787 was observed only in the proteome of FOS grown cells. The localization of Blon_0787 was determined in the UI group but is most likely a cytosolic protein.

Solute Binding Proteins (SBP)

Extracellular SBPs are typically linked to specific ABC transport systems consisting also in two inner membrane transporter components and an ATPase. The *B. infantis* genome contains 39 SBPs of three different classes: Family 1, 3, and 5 [31]. In particular, Family 1 extracellular SBPs are thought to be

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