Sugar source modulates exopolysaccharide biosynthesis in *Bifidobacterium longum* subsp. *longum* CRC 002

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The effect of four sugars (glucose, galactose, lactose and fructose) on exopolysaccharide (EPS) production by *Bifidobacterium longum* subsp. *longum* CRC 002 was evaluated. More EPS was produced when CRC 002 was grown on lactose in the absence of pH control, with a production of 1080 ± 120 mg EPS l⁻¹ ($P < 0.01$) after 24 h of incubation. For fructose, galactose and glucose, EPS production was similar, at 512 ± 63 , 564 ± 165 and 616 ± 93 mg EPS l⁻¹, respectively. The proposed repeating unit composition of the EPS is 2 galactose to 3 glucose. The effect of sugar and fermentation time on expression of genes involved in sugar nucleotide production (*galK*, *galE1*, *galE2*, *galT1*, *galT2*, *galU*, *rmlA*, *rmlB1* and *rmlCD*) and the priming glycosyltransferase (*wblE*) was quantified using real-time reverse transcription PCR. A significantly higher transcription level of *wblE* (9.29-fold) and the genes involved in the Leloir pathway (*galK*, 4.10-fold; *galT1*, 2.78-fold; and *galE2*, 4.95-fold) during exponential growth was associated with enhanced EPS production on lactose compared to glucose. However, *galU* expression, linking glucose metabolism with the Leloir pathway, was not correlated with EPS production on different sugars. Genes coding for dTDP-rhamnose biosynthesis were also differentially expressed depending on sugar source and growth phase, although rhamnose was not present in the composition of the EPS. This precursor may be used in cell wall polysaccharide biosynthesis. These results contribute to understanding the changes in gene expression when different sugar substrates are catabolized by *B. longum* subsp. *longum* CRC 002.

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INTRODUCTION

Bifidobacteria are indigenous members of the anaerobic microbiota of the human colon, and can positively influence gastrointestinal health (Leahy *et al.*, 2005; Picard *et al.*, 2005). Functional foods containing specific strains of bifidobacteria are thus becoming more popular (Roy, 2005). The capacities of bifidobacterial strains to survive food-processing technologies and remain viable during product storage are important selection criteria (Grand *et al.*, 2003; Masco *et al.*, 2007; Roy, 2005; Stanton *et al.*, 1998). Exopolysaccharide (EPS) production could contribute to cell protection and survival as well as having

beneficial effects on human health, although these ecological and health-related roles are not fully understood (Ruas-Madiedo & de los Reyes-Gavilán, 2005; Ruas-Madiedo *et al.*, 2006). As fermentable substrates, bifidobacterial EPSs could contribute to modulating gut microbial populations (Salazar *et al.*, 2008). In *Bifidobacterium animalis* subsp. *lactis*, EPS production seems to be enhanced in the presence of bile (Ruas-Madiedo *et al.*, 2009), as has been found for two pathogenic *Vibrio* species (Hsieh *et al.*, 2003; Hung *et al.*, 2006). Surface polysaccharides are well known as virulence and colonization factors of pathogenic bacteria, but molecules produced by commensal gut micro-organisms are gaining attention for their role in developing a normal immune system through communication with the mammalian host (reviewed by Mazmanian & Kasper, 2006).

EPSs can form a capsule when associated with the cell surface or can be released as slime into the medium (De Vuyst & Degeest, 1999). The production of molecules such as EPS by some bifidobacteria may also enhance the viscosity of fermented food products, contributing to their rheological properties. EPSs produced by bifidobacteria can thus be considered as natural additives, which are

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Abbreviations: EPS, exopolysaccharide; pGTF, priming glycosyltransferase; Q-PCR, quantitative real-time PCR; Q-RT-PCR, quantitative real-time reverse transcription PCR.

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Three supplementary tables are available with the online version of this paper.

preferred by some consumers over stabilizers of plant or animal origin (Boels *et al.*, 2001b).

Although our understanding of EPS production, structure, biosynthesis and gene molecular organization in Gram-positive bacteria has advanced (De Vuyst & Degeest, 1999; Degeest & De Vuyst, 1999, 2000; Degeest *et al.*, 2001; Laws & Marshall, 2001; Looijesteijn *et al.*, 1999), relatively little is known about EPS produced by bifidobacteria. Only 17 % of lactobacilli and bifidobacterial strains isolated from human gastrointestinal microbiota showed EPS production by phenotypic methods (Ruas-Madiedo *et al.*, 2007). *Bifidobacterium longum* strain YIT4028 has been reported to have cell wall polysaccharides consisting of rhamnogalactan (Nagaoka *et al.*, 1995), while three out of four EPSs characterized from *B. longum* also contained rhamnose (Salazar *et al.*, 2009). More recently, the EPS repeating unit from *B. longum* strain JBL05 was shown to be a heptasaccharide containing glucose, galactose and rhamnose (Kohno *et al.*, 2009). However, no rhamnose was reported in the EPS produced by *B. longum* strains ATCC 15707 and BB-79 (Abbad Andaloussi *et al.*, 1995; Roberts *et al.*, 1995). Polymorphism in polymer composition among bifidobacterial EPSs indicates that most can be classified as heteropolysaccharides and that the EPS-specific gene organization differs between *B. longum* strains (Ruas-Madiedo *et al.*, 2007). However, no EPS gene locus from bifidobacteria has yet been functionally demonstrated.

Sugar nucleotides, along with glycosyltransferases, are involved in heteropolysaccharide repeat unit formation (Boels *et al.*, 2001b; De Vuyst *et al.*, 2001; Jolly & Stinglee, 2001). Hence, precursor sugar availability or transcriptional regulation in the biosynthesis and conversion pathways may modulate EPS production by cells grown on diverse sugar sources (Boels *et al.*, 2001a, b, 2003a; Welman *et al.*, 2006). To date, the influence of the sugar source on sugar nucleotide biosynthesis and its effect on EPS production are still unknown in *B. longum*. In *B. longum* subsp. *longum* strain NCC2705, lactose was found to be consumed before glucose (Parche *et al.*, 2006) and a higher biomass productivity on lactose compared to glucose has been noted for *B. longum* subsp. *infantis* ATCC 27920 (Roy *et al.*, 1991). The hypothesis of the current study was that the rate of transcription in cells growing on lactose should be higher than the rate of transcription in cells growing on glucose, resulting in higher expression of genes involved in sugar nucleotide and EPS biosynthesis. Quantitative real-time reverse transcription PCR was used to track specific gene expression at different phases of growth of *B. longum* subsp. *longum* CRC 002 on four sugars.

METHODS

Micro-organisms and storage conditions. *B. longum* subsp. *longum* CRC 002 (our collection) was stored at -80°C in de Man–Rogosa–Sharpe (MRS) medium (EMD Biosciences) supplemented

with 15 % (v/v) glycerol and 0.05 % (w/v) L-cysteine.HCl (Calbiochem). *Escherichia coli* strain TOP10 transformants were grown in Luria Broth supplemented with $100\text{ }\mu\text{g}$ ampicillin ml^{-1} , which was solidified when necessary with 1.5 % Bacto agar (Difco).

EPS production and isolation. For monomer determination by gas–liquid chromatography (GLC), *B. longum* subsp. *longum* CRC 002 was grown at 37°C in modified Norris broth without human milk. This semi-defined medium (Norris *et al.*, 1950; Poupard *et al.*, 1973) minimizes possible polysaccharide contamination from culture broth components. Initially, bacteria were transferred from storage at -80°C to 10 ml modified Norris broth and were subcultured twice for 16 h at 37°C in an AnaeroJar device (Oxoid); anaerobiosis was generated with an Anaerogen sachet (Oxoid). A 1.6 l stirred-tank bioreactor (New Brunswick Scientific) containing 1.25 l modified Norris broth was inoculated at 2 % (v/v) and was used to produce EPS in a 16 h fermentation at 37°C , 125 r.p.m. agitation, at a controlled pH of 6.0 by automatic addition of 4 M NH_4OH and under anaerobic conditions by continuously bubbling CO_2 gas. The EPS was extracted following the method of Cerning *et al.* (1994) and further purified as previously described (Dabour & LaPointe, 2005). Monosaccharide analysis was performed by GLC as described previously (Van Calsteren *et al.*, 2002).

Growth kinetics in small-scale fermentations. *B. longum* subsp. *longum* CRC 002 was grown in no-sugar MRS-based broth (Institut Rosell-Lallemand, Montreal, QC, Canada), which is an MRS broth devoid of glucose. The medium was supplemented with 0.05 % (w/v) L-cysteine.HCl and 0.1 % (v/v) Tween 80 (BDH), and one of four sugars was added at 2 % (w/v): galactose (MRSCG2), glucose (MRSCG2), lactose (MRSL2) or fructose (MRSCF2). Initially, bacteria were transferred from storage at -80°C to each of the four variants of MRS medium containing one of the four sugars and were incubated under anaerobic conditions (10.2 % CO_2 , 10.8 % H_2 and 79 % N_2) in a Forma 1025 anaerobic chamber (Forma Scientific) for 16 h at 37°C . The strain was propagated twice for 8 h in the same conditions and finally added (1 % v/v) to the same four media. Fermentations were monitored for 24 h and 50 ml samples were removed at regular intervals for pH, titratable acidity, viable counts and RNA extraction. The pH measurement was carried out with a pH meter (pHM84; Radiometer) and titratable acidity was determined from 5 ml samples titrated against 0.1 M NaOH to the phenolphthalein end point (pH 8.3) and expressed in % of equivalent lactic acid. Viable counts (c.f.u. ml^{-1}) were determined by plating 10-fold serial dilutions. Four independent fermentations were carried out. ANOVA followed by Tukey–Kramer multiple comparison tests were performed using JMP software (SAS). EPSs were extracted as described above from 10 ml culture samples. The same extraction parameters were applied to the four uninoculated broths, each containing one of the four sugars as controls to correct for contaminating medium components. Total reducing sugar concentration was determined by the phenol/sulfuric method, using glucose as a standard (Dubois *et al.*, 1956).

Detection, cloning and sequencing of genes located in the EPS biosynthesis locus. *B. longum* subsp. *longum* CRC 002 genomic DNA was isolated from 1 ml overnight cultures in MRSCG2 medium as described previously (Vincent *et al.*, 1998), with the following modification. The phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) extraction was performed by adding the mixture to a PhaseLock Gel-Heavy tube (Eppendorf). The priming glycosyltransferase (pGTF) was targeted with previously developed hybrid primers G-Bact-a-F-36 and G-Bact-a-R-27 as previously described (Provencher *et al.*, 2003). Genomic DNA from the EPS-producing strain *B. longum* subsp. *longum* ATCC 15707 was used as a positive control (Abbad Andaloussi *et al.*, 1995). To isolate genes flanking the pGTF gene from strain CRC 002, probe 1 consisted of the 0.2 kb DIG-labelled

PCR fragment (Roche Diagnostics). Hybridizing fragments of the *B. longum* subsp. *longum* CRC 002 digested genomic DNA (Fig. 1c) were cloned and then sequenced using the genome-walking approach. Primers SLAB38 (5'-CTCGAGACGGAATGCGTTTGTG-3') and SLAB40 (5'-GCGTAAATCGTGGAAGTAA-3') were used to create probe 2 in order to detect clones containing the 4.8 kb *KpnI/EcoRI* fragment, which was then sequenced using genome walking (Fig. 1c). All restriction enzymes were purchased from New England Biolabs. Genes *wzb* and *wzx* were amplified by PCR using primers listed in Supplementary Table S1 (available with the online version of this paper).

PCR amplification of sugar nucleotide precursor genes. Specific primers (Supplementary Table S1) were designed from two *B. longum* subsp. *longum* genomes available from the National Center for Biotechnology Information (NCBI) (strain NCC2705 GenBank accession no. AE014295 and strain DJO10A genome GenBank

accession no. CP000605) in order to amplify the *galK*, *galE1*, *galE2*, *galT1*, *galT2*, *galU*, *rmlA*, *rmlB1* and *rmlCD* genes. PCR was performed with a GeneAmp PCR System 2400 (Perkin-Elmer) according to the following protocol: initial hold for 30 s at 94 °C, followed by 30 three-step cycles at 94 °C for 5 s, 58 °C for 30 s and 72 °C for 1 min and a final DNA extension of 5 min at 72 °C. The PCR products were purified using the QIAquick gel extraction kit (Qiagen), cloned into pCR4-TOPO (Invitrogen) and sequenced as described below.

DNA sequencing and bioinformatics analysis. Recombinant plasmid DNA from selected *E. coli* TOP10 transformants was purified using the QIAprep Spin Miniprep kit (Qiagen), and inserts were sequenced on both strands using M13 universal primers and custom primers. Sequencing reactions were carried out using the BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems) as recommended by the manufacturer and were analysed on an

Fig. 1. (a) Detection of the potential pGTF gene by PCR using hybrid primers: lane 1, *B. longum* ATCC 15707^T (*B. longum* subsp. *longum* type strain producing EPS); lane 2, *B. longum* subsp. *longum* CRC 002; M, 100 bp DNA ladder. (b) Hydrophobicity plot of the WblA putative protein product using the Tmpred program. The preferred model predicts that the N-terminus is inside, followed by six strong transmembrane helices. (c) CRC 002 chromosomal DNA restriction map of the putative EPS biosynthesis region. Restriction fragments are designated by numbers indicating the sizes in kb and letters indicating the restriction sites (E, *EcoRI*; H, *HindIII*; K, *KpnI*; X, *XhoI*). (d) Predicted ORFs (arrows) from the sequenced region; gene designations are indicated below the arrows. Potential functions are based on homology searches as described in Methods.

Applied Biosystems 3100 automated DNA sequencer. Nucleotide sequences were assembled and edited with the GCG Wisconsin Package Version 10.3-UNIX (Accelrys). The BLASTX algorithm (<http://www.ncbi.nlm.nih.gov/blast/>) was used to perform similarity searches of the nucleotide and protein databases (Altschul *et al.*, 1997). Protein motif similarity searches were carried out with the Pfam database (<http://pfam.sanger.ac.uk/>) (Finn *et al.*, 2006) and domain architecture similarity searches with CDART (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi/>). Sequence alignments were conducted with the CLUSTAL W server at the European Bioinformatics Institute (<http://www.ebi.ac.uk/clustalw/index.html>) (Thompson *et al.*, 1994). Sequences were deposited in GenBank under accession numbers EU372989 to EU372994.

RNA extraction. Total RNA from *B. longum* subsp. *longum* CRC 002 was extracted at incubation time points 6 h, 10 h and 24 h, which represent the exponential, late-exponential and stationary phases, respectively, in small-scale fermentations. Cell suspensions (0.5 ml) were mixed with RNaprotect Bacteria Reagent (1 ml) (Qiagen). Sample handling and total RNA extraction were performed according to the RNaprotect Bacteria Reagent Handbook with some modifications. The cell pellet was suspended in T buffer (10 mM Tris/HCl pH 8.0) (700 µl), to which lysozyme (0.6 mg) (Sigma), proteinase K (0.4 mg) (Sigma) and mutanolysin (200 U) (Sigma) were added, followed by incubation for 10 min at 37 °C. An RNase-free DNase treatment was carried out directly on the RNeasy column (Qiagen) and a second DNase I (5 µl; Roche Diagnostics) treatment was carried out in the 50 µl eluate. DNase was removed with the RNeasy Mini kit clean-up protocol according to the manufacturer's instructions and Protector RNase Inhibitor (160 U) (Roche Diagnostics) was added. The absence of DNA contamination in RNA samples was confirmed by quantitative real-time PCR (Q-PCR) amplification using the *tufA* primers prior to cDNA synthesis. All RNA samples were visualized on gel and verified for quality by measurement of absorbance at 260 nm and 280 nm, then quantified with RiboGreen (Invitrogen). RNA (1 µg) was reverse transcribed into cDNA in a final volume of 50 µl with the High Capacity cDNA Archive kit (Applied Biosystems) as recommended by the manufacturer.

Quantitative real-time reverse transcription PCR (Q-RT-PCR). The primers for Q-RT-PCR were all designed with Primer Express 2.0 (Applied Biosystems) using strain CRC 002-specific sequences for target genes *galK*, *galE1*, *galE2*, *galT1*, *galT2*, *galU*, *wblE*, *rmlA*, *rmlB1* and *rmlCD* and reference genes 16S rRNA, *atpD*, *ldh*, *rpoB*, *tufA* and *recA* (Supplementary Table S1). Melting temperature (T_m) values were maintained between 58 and 60 °C, while total amplicon size was limited to between 54 and 61 bp with optimized conditions for SYBR Green detection. Primer secondary structures and dimer formation were controlled with Oligo Analyser V3.0 software (<http://scitools.idtdna.com>). Primers were synthesized by Invitrogen Canada. Q-RT-PCR was performed using the ABI PRISM 7500 system (Applied Biosystems). PCR products were detected with SYBR Green fluorescent dye and amplified according to the following protocol: the initial hold for 10 min at 95 °C was followed by 40 two-step cycles at 95 °C for 15 s and 60 °C for 60 s. Each PCR contained the following: 12.5 µl 2 × SYBR Green master mix with ROX passive reference dye (Applied Biosystems), 1 µl of cDNA dilutions, each of the forward and reverse primers at optimized concentrations (Supplementary Table S2), and nuclease-free water to obtain a final volume of 25 µl. In each run, negative controls without cDNA for each primer set were included. After each amplification, a melting curve was performed by increasing the temperature by 1 °C every 20 s from 65 to 94 °C, to confirm primer specificity. Melting curve analysis showed clear melting peaks without non-specific products or artefacts for all genes.

Relative quantification of gene expression and statistical analysis. Gene transcription was evaluated during the consumption

of fructose, galactose or lactose versus the control condition (glucose) using the Relative Expression Software Tool software (REST2005) (Pfaffl *et al.*, 2002). REST2005 considers efficiency (E) for each gene primer set (Peirson *et al.*, 2003) and multiple reference genes to calculate transcription levels (Pfaffl, 2001). Relative expression ratios were established by comparing the concentration for each target gene transcript from treated samples versus control samples, which were then divided by the geometric mean of the most stable reference gene concentration values (Supplementary Table S3) to obtain normalized relative expression levels. The transcription ratio of the 16 genes of interest at 6 h, 10 h and 24 h in fructose, galactose or lactose versus the control condition (glucose) was tested for significance by randomization and bootstrapping techniques carried out as described by Pfaffl *et al.* (2002), using 50 000 random reallocations.

RESULTS

Identification and sequence analysis of genes involved in EPS biosynthesis

The C-terminal domain of the pGTF (priming glycosyltransferase), which catalyses the addition of the first sugar 1-phosphate to a lipophilic carrier molecule for initiation of EPS repeating unit assembly, was amplified from the genomic DNA of *B. longum* subsp. *longum* CRC 002 with previously developed hybrid primers (Fig. 1a). The predicted amino acid sequence of the 0.2 kb PCR product exhibits similarity with the C-terminal region of the *cpsD* gene from NCC2705, a putative pGTF. By successive hybridizations (Fig. 1c), a 15.9 kb contiguous sequence was assembled from overlapping fragments (Fig. 1d), giving a G + C content of 53.1 mol% for the targeted region.

Sequence analysis revealed 11 ORFs (Table 1, Fig. 1d), which are all highly homologous to ORFs found in the genome of *B. longum* subsp. *infantis* CCUG 52486 (GenBank accession no. DS990238; Table 1, Fig. 2). Four ORFs (*orf8*, *mntH*, *orf10* and *orf11*) showed 99% amino acid identity with sequences from *B. longum* subsp. *longum* strains NCC2705 (GenBank accession no. AE014295) and DJO10A (CP000605). Genes *wblE* and *wblD* share only moderate similarity with sequences from strain NCC2705 while *orf6* shares moderate similarity with both NCC2705 and DJO10A (Fig. 2). The *wblA* gene is predicted to be involved in EPS repeat unit polymerization based on sequence similarity. Analysis of hydrophobicity by TMpred indicates that WblA has six highly probable transmembrane helices (Fig. 1b).

The gene products of *wblB*, *wblC* and *wblE* are predicted to be glycosyltransferases that could be involved in biosynthesis of the repeating unit. No equivalent to the pGTF gene *wblE* was found in the DJO10A genome (Fig. 2). The predicted WblE protein is classified in the conserved domain database (CDD) within the multi-domain model of EPS biosynthesis polyprenyl glycosylphosphotransferases (TIGR03025). pGTFs from *Bifidobacterium* spp., *Streptococcus thermophilus* and pathogenic streptococci as well as enterobacteria include a poorly conserved N-terminus that is not found in equivalent proteins from the

Table 1. Analysis of ORFs flanking the pGTF gene in *B. longum* subsp. *longum* CRC 002

ORF	Mol% G+C	Amino acids	Similar protein	Protein identity (%)/ Similarity (%)	Bacterium with the most relevant identity	Motif	Potential function	GenBank/ UniProtKB/ TrEMBL
<i>orf1</i>	45	307	BLIG_00241	98/99	<i>B. longum</i> subsp. <i>infantis</i> CCUG 52486		Uncharacterized	ZP_03647580
<i>wblA</i>	36	240	BLIG_00242	95/97	<i>B. longum</i> subsp. <i>infantis</i> CCUG 52486	Polymerase	Hypothetical protein	ZP_03647581
<i>wblB</i>	40	315	BLIG_00243	98/98	<i>B. longum</i> subsp. <i>infantis</i> CCUG 52486	pf00535: Glycosyltransferase family 2; COG0463: Glycosyltransferases involved in cell wall biogenesis	β -1,4-Galactosyltransferase	ZP_03647582
<i>wblC</i>	51	378	BLIG_00244	97/99	<i>B. longum</i> subsp. <i>infantis</i> CCUG 52486	pf00534: Glycosyltransferases group 1; COG0438: Glycosyltransferase	Putative glycosyltransferase	ZP_03647583
<i>wblD</i>	56	492	BLIG_00245	98/98	<i>B. longum</i> subsp. <i>infantis</i> CCUG 52486	pf02706: Chain length determinant protein; COG3944: Capsular polysaccharide biosynthesis protein; COG2035: Predicted membrane protein	Etk-like tyrosine kinase	ZP_03647584
<i>orf6</i>	57	642	BLIG_00246	95/97	<i>B. longum</i> subsp. <i>infantis</i> CCUG 52486		Hypothetical protein	ZP_03647585
<i>wblE</i>	56	525	BLIG_00247	97/98	<i>B. longum</i> subsp. <i>infantis</i> CCUG 52486	pf02397: Bacterial sugar transferase; COG2148: Sugar transferases involved in lipopolysaccharide synthesis	pGTF CpsD	ZP_03647586
<i>orf8</i>	61	545	BLIG_00248	99/100	<i>B. longum</i> subsp. <i>infantis</i> CCUG 52486	COG0477: Permeases of the major facilitator superfamily; pf07690: Major Facilitator Superfamily; COG2814: Arabinose efflux permease	Putative permease	ZP_03647587
<i>mntH</i>	61	455	MntH	99/100	<i>B. longum</i> NCC2705	pf01566: Natural resistance-associated macrophage protein; COG1914: Mn^{2+} and Fe^{2+} transporters of the NRAMP family	NRAMP family Mn^{2+} Fe^{2+} transporter	Q8G7L2
<i>orf10</i>	50	456	BL0253	99/100	<i>B. longum</i> NCC2705	COG1373: Predicted ATPase (AAA + superfamily)	Predicted ATPase (AAA + superfamily)	Q8G7L1
<i>orf11</i>	63	247	BL0254	99/100	<i>B. longum</i> NCC2705	pf09362: Domain of unknown function (DUF1996)	Uncharacterized protein	Q8G7L0

Fig. 2. Comparison of the sequenced region from *B. longum* subsp. *longum* CRC 002 with *B. longum* subsp. *longum* NCC 2705 (GenBank accession no. AE014295), *B. longum* subsp. *longum* DJO10A (CP000605) and *B. longum* subsp. *infantis* CCUG 52486 (DS990238). Arrows with the same colour share the same putative function.

lactobacilli and lactococci. The sequence of WblD has a domain architecture resembling that of the Gram-negative tyrosine kinase involved in the production of group 1 and 4 capsule types (Whitfield, 2006). This protein is expected to potentially be involved in chain length determination. Two further genes were amplified by PCR using primers designed from the highly similar *B. longum* subsp. *infantis* CCUG 52486 sequences (GenBank accession no. DS990238). Gene *wzx*, located 3 kb from *wblA*, encodes a product with the topology of a 'flippase'-type transporter, having 14 transmembrane sequences. Gene *wzb* is predicted to encode the protein tyrosine phosphatase, completing the functions necessary for chain length determination.

Identification and sequence analysis of genes involved in the biosynthesis of sugar nucleotides as EPS precursors

The genes and their predicted protein products involved in the biosynthesis of galactose 1-phosphate and glucose 1-phosphate and their conversion to EPS precursors (UDP-glucose, UDP-galactose and dTDP-rhamnose) were identified from the sequenced *B. longum* subsp. *longum* NCC2705 and DJO10A genomes (Table 2). Gene organization in *B. longum* subsp. *longum* CRC 002 was verified by PCR. The *galK* gene (galactokinase) is located immediately downstream of *galT1* (galactose-1-phosphate uridylyltransferase). The *galT2* and *galE1* (UDP-glucose 4-epimerase) genes are also located together in that order, while *galE2* and *galU* (UTP-glucose-1-phosphate uridylyltransferase) are located separately. The *galE2* gene is present in the NCC2705 genome, but no homologous gene was found in the DJO10A genome (Table 2). The *rml*

genes required for dTDP-rhamnose biosynthesis are organized in a cluster in the order *rmlB1-rmlCD-rmlA*.

Effect of sugar source on bacterial growth and EPS production

Galactose, fructose, lactose and glucose were fermented by *B. longum* subsp. *longum* CRC 002 with comparable growth profiles (Fig. 3). However, viable plate counts at 24 h were significantly lower ($P < 0.05$) on fructose versus galactose (Fig. 3). *B. longum* subsp. *longum* CRC 002 produced less acid when metabolizing galactose than the other sugars; the final values of pH (4.55 ± 0.02) and acidity expressed in % of equivalent lactic acid (1.34 ± 0.08) were significantly different ($P < 0.05$). CRC 002 produced significantly more EPS when metabolizing lactose, yielding a total of 1080 ± 120 mg EPS l^{-1} ($P < 0.01$) after 24 h of incubation. For fructose, galactose and glucose, EPS production was similar, at 512 ± 63 , 564 ± 165 and 616 ± 93 mg EPS l^{-1} , respectively. The monosaccharide composition of EPS from *B. longum* subsp. *longum* CRC 002 was 40.0 ± 1.3 % galactose and 58.5 ± 2.1 % glucose, giving a proposed repeating unit with a molar ratio of 2 galactose to 3 glucose.

Effect of sugar source on differential gene expression

Glucose was selected as the control sugar to evaluate differential gene transcription when fructose, lactose and galactose substrates were fermented. The 10 target genes involved in sugar nucleotide biosynthesis and conversion can roughly be divided into three groups: the *galK*, *galT*

Table 2. Genes proposed to be involved in the formation of sugar nucleotides (EPS precursors) by *B. longum* subsp. *longum* strain CRC 002 and comparison with homologous genes from *B. longum* subsp. *longum* strains NCC2705 and DJO10A

A, Gene absent.

Gene	Length (bp)	Proposed gene product	Motif	DNA identity (%)	
				NCC2705*	DJO10A†
<i>galE1</i>	1022	UDP-glucose 4-epimerase	pf01370: NAD dependent epimerase/dehydratase family; COG1087: UDP-glucose 4-epimerase	95	99
<i>galE2</i>	1013	UDP-glucose 4-epimerase	pf01370: NAD dependent epimerase/dehydratase family; COG1087: UDP-glucose 4-epimerase	99	A
<i>galK</i>	1196	Galactokinase	pf00288: GHMP kinases N-terminal domain; pf08544: GHMP kinases C-terminal; COG0153: Galactokinase	97	99
<i>galT1</i>	1247	Galactose-1-phosphate uridylyltransferase	pf02744: Galactose-1-phosphate uridylyltransferase, C-terminal domain; pf01087: Galactose-1-phosphate uridylyltransferase, N-terminal domain; COG1085: Galactose-1-phosphate uridylyltransferase	93	96
<i>galT2</i>	1547	Galactose-1-phosphate uridylyltransferase	pf02744: Galactose-1-phosphate uridylyltransferase, C-terminal domain; pf01087: Galactose-1-phosphate uridylyltransferase, N-terminal domain; COG1085: Galactose-1-phosphate uridylyltransferase	96	99
<i>galU</i>	1544	UTP-glucose-1-phosphate uridylyltransferase	pf01704: UTP-glucose-1-phosphate uridylyltransferase; COG4284: UDP-glucose pyrophosphorylase	97	97
<i>rmlA</i>	862	dTDP-glucose pyrophosphorylase	pf00483: Nucleotidyl transferase; COG1209: dTDP-glucose pyrophosphorylase	79	99
<i>rmlB1</i>	1194	dTDP-glucose 4,6-dehydratase	pf01370: NAD-dependent epimerase/dehydratase family; COG1088: dTDP-D-glucose 4,6-dehydratase	74	89
<i>rmlCD</i>	1451	Possible fused dTDP-4-keto-L-rhamnose reductase and dTDP-4-keto-6-deoxyglucose 3,5-epimerase	pf00908: dTDP-4-dehydrorhamnose 3,5-epimerase; pf04321: RmlD substrate-binding domain; COG1898: dTDP-4-dehydrorhamnose 3,5-epimerase; COG1091: dTDP-4-dehydrorhamnose reductase	80	97

**B. longum* subsp. *longum* NCC2705 (GenBank accession no. AE014295).†*B. longum* subsp. *longum* DJO10A (GenBank accession no. CP000605).

and *galE* genes from the Leloir pathway, the *galU* gene involved in UDP-glucose biosynthesis and the *rml* genes required for dTDP-rhamnose biosynthesis. Transcription levels of the *gal* genes were influenced by the sugar source, depending on the growth phase (Table 3). Compared to glucose, when galactose was catabolized transcription of the Leloir pathway genes was higher at 6 h. Lactose fermentation led to an increase in transcription for *galK*, *galT1* and *galE2*. When fructose was fermented, compared to the glucose fermentation, only *galE1* transcription was higher. In the late exponential phase (10 h), the expression of epimerization and galactokinase genes (*galE1*, *galE2* and *galK*) was influenced by sugars (Table 3). Galactose led to increased transcription of the *galE2* gene. At 10 h, *galK* transcription was detected only in galactose ($C_t = 33.71 \pm 0.95$) and in lactose fermentations ($C_t = 38.43 \pm 1.35$), not when glucose or fructose were fermented. In the absence of expression in the glucose control condition, no expression ratio could be calculated (absent from Table 3). Even with the overall decline in metabolism through stationary phase (24 h), differences in transcription of Leloir pathway genes were observed

between sugar substrates (Table 3). Galactose fermentation was correlated with higher expression of *galT1*, *galT2* and *galE2*, but *galE1* transcription was comparable to that in glucose fermentation. At 24 h, *galK* transcription was detected at a very low level only in galactose ($C_t = 38.20 \pm 1.25$) and in lactose fermentations ($C_t = 37.79 \pm 0.80$). The *galU* gene encoding UTP-glucose-1-phosphate uridylyltransferase generates UDP-glucose from glucose 1-phosphate, linking glucose metabolism to the Leloir pathway. The *galU* transcription level was mostly stable among sugar sources for exponential, late-exponential and stationary phases, except for galactose fermentation, where lower transcription was found at 24 h.

In the exponential phase (6 h), expression of dTDP-rhamnose biosynthesis genes *rmlA*, *rmlB1* and *rmlCD* was significantly higher in fructose and lactose compared to glucose fermentation (Table 3). With galactose, only *rmlCD* had significantly higher expression than in glucose at 6 h. At later growth stages, *rmlA* transcription did not vary significantly compared to glucose. No transcription of *rmlB1* was detected in stationary phase for any of the sugar

Fig. 3. Growth profiles of *B. longum* subsp. *longum* CRC 002 in MRSCG2 (a), MRSCGA2 (b), MRSCF2 (c) and MRSL2 (d) at 37 °C. Viable cell count ($\bullet$, solid lines), pH ($\blacktriangle$; dotted lines) and titratable acidity in % of equivalent lactic acid ($\blacklozenge$; dashed lines). Error bars: standard error of four independent experiments.

sources. In stationary phase, *rmlCD* gene expression was significantly lower in lactose and galactose compared to glucose.

Transcription of the *wblE* gene in the exponential phase (6 h) was higher in fructose and lactose fermentations, compared to glucose fermentation (Table 3). In contrast, at 10 h, *wblE* transcription was lower for all sugars compared to glucose and no transcription was detected for any of the sugar substrates at 24 h (Table 3).

DISCUSSION

The EPS-producing bifidobacteria may influence the rheological behaviour of fermented food products, but they may also affect bacterial adhesion properties of some indigenous members of the human microbiota when products are ingested (Ruas-Madiedo *et al.*, 2006). The human intestinal ecosystem has been screened for EPS+ strains, and EPS+ bifidobacteria found were *B. pseudocatenulatum*, *B. longum*, *B. animalis* and *B. adolescentis* (Ruas-Madiedo *et al.*, 2007). However, little is known about EPS biosynthesis by *Bifidobacterium* spp., and no information about *eps* operons was available prior to this study. This study presents a potentially new EPS isolated from *B. longum* subsp. *longum* CRC 002 with a proposed repeating unit composition of 2 galactose:3 glucose, which is different from other excreted EPS characterized from *B. longum* (Abbad Andaloussi *et al.*, 1995; Roberts *et al.*, 1995; Salazar *et al.*, 2009) as well as from *B. pseudocatenulatum* (Salazar *et al.*, 2009). The EPS composition among *B. longum* subsp. *longum* strains seems diverse, which agrees with the strain variation found within other genera such as *Streptococcus*, *Lactococcus* and *Lactobacillus* (Broadbent *et al.*, 2003; De Vuyst & Degeest, 1999; Marshall *et al.*, 2001). Although strain CRC 002 might produce multiple EPS polymers, the extraction procedure recovers all EPS present in the culture supernatant, and the compositional analysis did not show the presence of rhamnose. This result points towards a secreted heteropolysaccharide; further studies are needed to determine the structure of the repeat unit. Although not present in the EPS, the rhamnose precursor may be used in cell wall polysaccharide biosynthesis, shunting glucose away from glycolysis and from production of other EPS precursor sugars.

The presence of genes encoding specific glycosyltransferases has a direct impact on the repeating units of the polymers, and monomer composition diversity may suggest different EPS-specific genes for *B. longum* subsp. *longum* strains (Jolly & Stingle, 2001; van Kranenburg *et al.*, 1999). Potential genes were identified from the CRC 002 strain in order to correlate gene expression with EPS production. The hybrid primers used to detect the pGTF were originally applied to *Lactobacillus* and *Streptococcus* species by our group (Provencher *et al.*, 2003). As the pGTF was successfully amplified from *B. longum* subsp. *longum* CRC 002 and ATCC 15707, this indicates that

Table 3. Relative transcription ratios of 10 *B. longum* subsp. *longum* CRC 002 genes when fructose, lactose or galactose were fermented versus the control fermentation with glucose in MRS broth

Transcription ratios were calculated using the relative expression software tool (REST2005). The values shown are means ($n=4$), where probability was calculated by the pair-wise fixed reallocation randomization test (50 000 permutations) between samples and control groups (* $P<0.05$; † $P<0.01$; ‡ $P<0.001$). The geometric means of control genes within each time point were used to normalize the Q-RT-PCR data as described in the supplementary material. ND, Not detected.

Gene	Fructose			Lactose			Galactose		
	6 h	10 h	24 h	6 h	10 h	24 h	6 h	10 h	24 h
<i>galE1</i>	8.87‡	10.96†	0.39†	0.35	0.50	0.26*	3.18‡	0.29	0.90
<i>galE2</i>	1.76	0.33*	1.18	4.95‡	1.50	0.78	11.33‡	14.91†	3.81†
<i>galK</i>	1.27	ND	ND	4.10‡	ND	ND	6.29‡	ND	ND
<i>galT1</i>	1.44	1.65	2.00*	2.78*	0.52	2.03*	4.44*	2.17	2.43†
<i>galT2</i>	0.98	1.10	1.11	1.99	0.63	1.82	9.64‡	2.33	1.82†
<i>galU</i>	1.00	0.69	0.75	1.46	1.39	0.57	2.81	1.30	0.23*
<i>rmlA</i>	3.96‡	0.53	0.81	6.72‡	0.55	1.65	2.63‡	1.33	0.80
<i>rmlB1</i>	2.39*	0.86	ND	1.37	0.56	ND	1.31	0.28*	ND
<i>rmlCD</i>	5.76‡	2.54†	1.47	0.34*	3.32	0.26*	5.12†	0.61	0.03‡
<i>wblE</i>	6.14‡	0.09‡	ND	9.29‡	0.10‡	ND	1.61	0.02‡	ND

primers designed using CODEHOP with A+T-rich genomes can be applied to new genera with very different mol% G+C content. Although these primers detect a pGTF gene in specific strains, not all EPS-producing strains may have the same type of gene. In fact, these primers detected a similar gene in only 7% of lactobacilli and bifidobacteria strains tested originating from human gastrointestinal microbiota (Ruas-Madiedo *et al.*, 2007). Diversity in EPS biosynthetic mechanisms and sequences among bifidobacteria would account for this.

All the functions necessary for heteropolysaccharide biosynthesis were found flanking the pGTF. Seven predicted gene products could potentially be involved in EPS production, with putative biological functions covering biosynthesis of the repeating unit (WblB, WblC and WblE), transport (Wzx), polymerization (WblA) and chain length determination (WblD, Wzb). The EPS biosynthesis region from CRC 002 contains four genes (*wblA*, *wblB*, *wblC* and *wzx*) with no homologues in two sequenced *B. longum* subsp. *longum* genomes (Schell *et al.*, 2002), but present in the genome sequence of *B. longum* subsp. *infantis* CCUG 52486. In this respect, recent comparison of the CRC 002 genome with that of NCC2705 by suppressive subtractive hybridization (SSH) revealed five glycosyltransferase genes in the CRC 002 strain that were not found in NCC2705 (Delcenserie *et al.*, 2008). One of these is identical to *wblC* identified in the present study, while *wblA* was also identified by SSH in the previous study.

The quantity of EPS produced by strain CRC 002 differed between sugar substrates. Even without pH control, EPS production exceeded 1 g l^{-1} in lactose fermentations. This strain shows high productivity compared to other bifidobacteria and many, if not most, lactic acid bacteria that produce heteropolymers (Cerning, 1995). This may be

related to the higher ATP production made possible by acetate production through the bifidus shunt (Palframan *et al.*, 2003). Although cell growth was not significantly higher, lactose seems to be the better sugar source for strain CRC 002 to produce EPS, as found previously for strain BB-79 (Roberts *et al.*, 1995). Lactose is preferred over glucose by *B. longum* subsp. *longum* NCC2705, as suggested by transcriptional repression of *glpP*, a glucose uptake gene (Parche *et al.*, 2007). As a disaccharide, lactose provides glucose for glycolysis as well as galactose that can be shunted directly to the Leloir pathway for sugar nucleotide biosynthesis. In addition, there are at least three permease systems in *B. longum* subsp. *longum* NCC2705 that are active for lactose uptake, while glucose is transported by two systems, of which the phosphotransferase is transcribed at a low level (Parche *et al.*, 2007). In order to determine whether the differences in EPS production between cultures of *B. longum* subsp. *longum* CRC 002 grown on different sugars could be correlated with the expression of genes involved in glycosyltransferase activity or precursor biosynthesis, differential gene transcription was investigated by Q-RT-PCR.

In *Lactobacillus delbrueckii* subsp. *bulgaricus*, higher EPS production was associated with higher levels of UDP-glucose pyrophosphorylase (GalU) and UDP-glucose epimerase (GalE) enzyme activities at higher growth rates (Welman *et al.*, 2006). In the present study, the transcription level of *galU* was not correlated with changes in EPS production in *B. longum* subsp. *longum* strain CRC 002, so glucose may not contribute to increasing the UDP-glucose precursor pool for EPS production. Genes of the Leloir pathway (*galK*, *galE1*, *galE2*, *galT1* and *galT2*), however, were induced by galactose and lactose during exponential growth, which contributes directly to increasing UDP-galactose and then to UDP-glucose formation,

thus increasing the pool of precursors for EPS production. Interestingly, the GalE and GalT enzymes are each encoded by two different genes, and sugar source does not appear to have the same effect on their transcription. This suggests different transcriptional regulation mechanisms for these duplicate genes encoding the same function. Moreover, even though rhamnose was not present in the EPS produced by *B. longum* subsp. *longum* CRC 002, dTDP-rhamnose may be needed as a sugar precursor in cell wall polysaccharide biosynthesis (Nagaoka *et al.*, 1995), and shunt glucose away from glycolysis. In fact, *rml* genes were differentially expressed depending on the sugar source and growth phase.

To increase EPS production, metabolic engineering has been attempted to overcome regulation in the basal metabolic reactions involved in sugar nucleotide biosynthesis in *Lactococcus lactis* and *Streptococcus thermophilus* (Boels *et al.*, 2001a, 2003a; Levander & Rådström, 2001; Levander *et al.*, 2002; Looijesteijn *et al.*, 1999). EPS yields have been enhanced in *S. thermophilus* by overexpressing genes coding for the Leloir enzymes (Levander *et al.*, 2002; Svensson *et al.*, 2005). EPS biosynthesis pathways and regulation mechanisms are generally complex, and enhancement of EPS production when metabolizing lactose may not be explained only by sugar nucleotide biosynthesis. Hypothetically, the galactose moiety from lactose hydrolysis, which induced gene transcription of the Leloir pathway, can be seen as a bypass from the stable non-inducible *galU* gene. In *L. lactis*, EPS production increased fourfold when the *eps* gene cluster was overexpressed by cloning into a high-copy-number vector (Boels *et al.*, 2003b). The overexpression of EPS-specific genes in *B. longum* subsp. *longum* CRC 002 also seems to have an important impact. In fact, the transcription of the CRC 002 *wblE* pGTF was upregulated almost 10-fold in lactose over glucose in the exponential phase. Thus, the pGTF gene *wblE* can be used as an indicator of EPS gene expression.

In summary, this study reports the identification, sequence and expression of genes predicted to be involved in sugar nucleotide production and EPS biosynthesis by *B. longum* subsp. *longum* CRC 002. We demonstrate the impact of sugar source on gene expression as a measure of metabolic activity. Thus, genes located in multiple transcriptional units were targeted, focusing on the pGTF as well as sugar nucleotide precursors for glucose, galactose and rhamnose. *B. longum* subsp. *longum* CRC 002 produces a high level of a potentially novel EPS consisting of glucose and galactose. Compared to glucose fermentations, when this strain catabolizes lactose, higher EPS production is associated with higher expression of the *wblE* gene and sugar nucleotide genes in the exponential phase, emphasizing the pertinence of targeting the pGTF as a key enzyme in the biosynthetic mechanism.

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