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Ribose utilization by the human commensal *Bifidobacterium breve* UCC2003

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Summary

Growth of *Bifidobacterium breve* UCC2003 on ribose leads to the transcriptional induction of the *rbsACBDK* gene cluster. Generation and phenotypic analysis of an *rbsA* insertion mutant established that the *rbs* gene cluster is essential for ribose utilization, and that its transcription is likely regulated by a LacI-type regulator encoded by *rbsR*, located immediately upstream of *rbsA*. Gel mobility shift assays using purified $RbsR_{His}$ indicate that the promoter upstream of *rbsABCDK* is negatively controlled by $RbsR_{His}$ binding to an 18 bp inverted repeat and that $RbsR_{His}$ binding activity is modulated by D-ribose. The *rbsK* gene of the *rbs* operon of *B. breve* UCC2003 was shown to specify a ribokinase (EC 2.7.1.15), which specifically directs its phosphorylating activity towards D-ribose, converting this pentose sugar to ribose-5-phosphate.

Introduction

Bifidobacteria are Gram-positive, pleomorphic, anaerobic bacteria, representing one of the dominant components of the intestinal microbiota of humans and other mammals (Picard *et al.*, 2005; Turroni *et al.*, 2008). Members of this genus are believed to play a positive role in host health through protection against infectious diseases, their beneficial influence on the immune system and active role in the maintenance of gut function (Tuohy *et al.*, 2003; Guarner, 2006; Parracho *et al.*, 2007; Turroni *et al.*, 2009). Their reported beneficial effects furthermore include prevention of diarrhoea, symptom alleviation of inflammatory bowel disease and irritable bowel syndrome, reduction of the risk of colorectal cancer and easing lactose maldigestion (Tuohy *et al.*, 2003; Leahy *et al.*, 2005; Gibson, 2008; de Vrese and Schrezenmeir, 2008).

Various carbohydrates have been shown or are claimed to selectively stimulate the growth and/or activity of (beneficial) (bifido)bacteria, a property for which the term prebiotic was coined (Gibson, 2008; Macfarlane *et al.*, 2008; Ramirez-Farias *et al.*, 2008). A prebiotic was originally defined as 'a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health' (Gibson *et al.*, 2004). Fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) are the most commonly used prebiotics on the market in Europe and Japan. Soybean oligosaccharides (SOS), isomalto-oligosaccharides (IMO) and xylo-oligosaccharides (XOS) represent promising prebiotic compounds that have not yet been widely marketed (Gibson, 2008). Interestingly, from available genome sequences it has become clear that intestinal bifidobacteria dedicate a significant portion of their coding capacity (approximately 8% of all annotated genes) to the metabolism of a wide variety of carbohydrates (Schell *et al.*, 2002; Ventura *et al.*, 2007a,b). Over 50 different bifidobacterial carbohydrases have been mentioned in the literature to date (recently reviewed by van den Broek *et al.*, 2008). Using *Bifidobacterium breve* UCC2003 as a model to study bifidobacterial carbohydrate metabolism, we previously characterized an operon encoding a β-fructofuranosidase (Ryan *et al.*, 2005), an extracellular amylopullulanase which hydrolyses α-1,4 and α-1,6 glucosidic linkages in starch and related polysaccharides (Ryan *et al.*, 2006; O'Connell-Motherway *et al.*, 2008a), a *galA* gene involved in the degradation of potato galactan (O'Connell-Motherway *et al.*, 2008b) and two novel α-glucosidases exhibiting hydrolytic activities towards panose, isomaltose, isomaltotriose and trehalose (Pokusaeva *et al.*, 2009). Moreover, a phosphoenolpyruvate phosphotransferase (PEP-PTS) system involved in fructose metabolism was identified and studied in this bacterium (Maze *et al.*, 2007).

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D-ribose, a component of DNA (present as deoxyribose), RNA and many cofactors, may be found in the human gut (Walker *et al.*, 2006) and therefore could represent a carbon source for components of the gastrointestinal microbiota. In order to expand our knowledge on bifidobacterial carbohydrate metabolism, we characterized ribose utilization in *B. breve* UCC2003. The genetic elements involved in ribose uptake from the environment and its subsequent phosphorylation have been described previously for several bacteria, including *Escherichia coli*, *Bacillus subtilis*, *Lactobacillus sakei* and *Corynebacterium glutamicum* (Lopilato *et al.*, 1984; Mauzy and Hermodson, 1992; Woodson and Devine, 1994; Sigrell *et al.*, 1998; Stentz and Zagorec, 1999; Tourneux *et al.*, 2000; Chuvikovsky *et al.*, 2006; Ogbunude *et al.*, 2007; Nentwich *et al.*, 2009). In the current study, we describe the identification of the ribose utilization (*rbs*) operon in *B. breve* UCC2003. We present the biochemical characterization of the *rbsK* gene, whose protein product is responsible for the phosphorylation of D-ribose. Furthermore, we present data that imply the LacI-type regulator RbsR in the regulation of the *rbs* promoter in a D-ribose-dependent manner.

Results

Growth of Bifidobacterium *strains on glucose and/or ribose*

In order to investigate if bifidobacteria are capable of ribose metabolism, growth in modified Rogosa medium (mMRS) supplemented with glucose or ribose was assessed for 36 different bifidobacterial strains (Table S1, in Supporting information), representing 11 bifidobacterial species, by measuring the OD_{600} following 24 h of anaerobic growth at 37°C (Fig. 1). All bifidobacterial strains grew well on glucose, reaching OD_{600} values in excess of 1. In contrast, just 18 out of the 36 strains tested were able to reach an OD_{600} higher than 1.0 when grown on ribose as the sole carbon source, seven of which belong to the *B. breve* species, including *B. breve* UCC2003. Eight strains were shown to grow rather poorly on ribose with final OD_{600} values ranging from 0.4 to 0.9, while the remaining eleven bifidobacterial strains could not or very poorly metabolize ribose ($OD_{600} < 0.4$). Only particular bifidobacterial strains are thus capable of utilizing ribose, which means that this sugar can be considered as a selective growth substrate for such strains.

Genome response of B. breve *UCC2003 to growth on ribose*

In order to investigate differences in global gene expression upon growth of *B. breve* UCC2003 on ribose, or a combination of ribose and glucose, as compared with glucose, DNA microarray experiments were performed. Total RNA was isolated from *B. breve* UCC2003 cultures grown on ribose, a combination of ribose and glucose, or glucose (see *Experimental procedures* in *Supporting information*).

Analysis of the DNA microarray data obtained from two independent biological replicates revealed that the expression of the adjacent *rbsA*, *rbsC*, *rbsB*, *rbsD* and *rbsK* genes was significantly upregulated (fold change > 3.0, $P < 0.001$) in *B. breve* UCC2003 cultures grown on ribose or a combination of ribose and glucose, whereas *rbsR* was downregulated, relative to cultures grown on glucose (Table 1). These results implicate the *rbsRACBDK* gene cluster in ribose metabolism in *B. breve* UCC2003. Furthermore, various genes encoding components of the PEP-PTS system (Maze *et al.*, 2007) and genes encoding a number of carbohydrate ABC transport systems were significantly downregulated when *B. breve* UCC2003 was grown in ribose (results not shown).

To confirm the microarray results, quantitative RT-PCR (qRT-PCR) analysis was performed using primer pairs representing individual genes of the *rbs* gene cluster

Table 1. Effect of D-ribose on the transcriptome of *B. breve* UCC2003.

Locus tag_gene	Putative function	Ribose[a]	Ribose + glucose[a]	qRT-PCR[b]
Bbr_1415_rbsK	Ribokinase	**8.70**	**3.30**	**36.30**
Bbr_1416_rbsD	Ribose mutarotase	**6.55**	**3.54**	**31.88**
Bbr_1417_rbsB	D-ribose-binding protein	**8.09**	**4.82**	**21.20**
Bbr_1418_rbsC	Ribose transport system permease protein	**12.15**	**4.33**	**22.30**
Bbr_1419_rbsA	Ribose transport system ATP-binding protein	**11.37**	**4.64**	**18.24**
Bbr_1420_rbsR	Transcriptional regulator, LacI family	–1.16	**–1.25**	**–3.09**

a. Genes for which transcription was changed when test cultures were grown on ribose or combination of ribose and glucose and reference cultures were grown on glucose. The numbers represent the expression ratios of ribose versus glucose or a combination of ribose and glucose versus glucose-grown cells. Positive values indicate upregulation; negative values indicate downregulation. Expression ratios presented in bold have a Bayesian *P*-value < 0.001 according to the Cyber *t*-test (Long *et al.*, 2001).

b. Expression ratios of selected genes quantified by qRT-PCR. cDNA templates were derived from RNA samples of *B. breve* UCC2003 culture grown on ribose or glucose as a negative control. A $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression (Livak and Schmittgen, 2001). Expression ratios presented in bold have standard deviation values < 0.12 and a Mann–Whitney *P*-value < 0.0022 according to the *t*-test (Livak and Schmittgen, 2001).

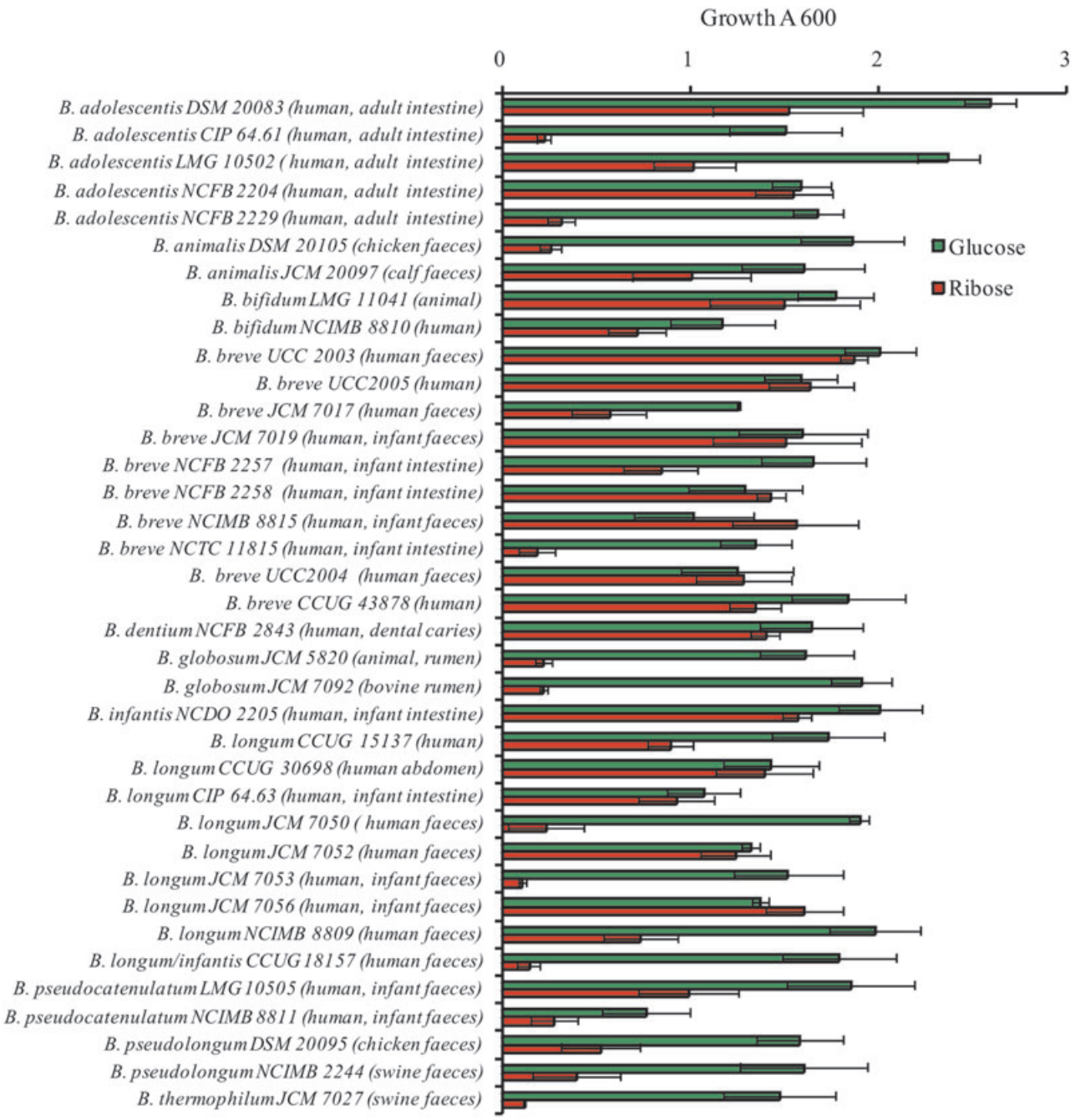

Fig. 1. Final optical density (OD_{600}) values obtained following 24 h growth of 36 different bifidobacterial strains in modified MRS containing 0.01 g ml^{-1} glucose or ribose as the sole carbon source. The results are mean values obtained from three separate experiments.

(Table S2 in Supporting information). cDNA templates were derived from RNA isolated from *B. breve* UCC2003 following growth on ribose, a combination of ribose and glucose, or glucose. As expected, the *rbsACBDK* genes were shown to be highly upregulated, while the *rbsR* gene was downregulated, consistent with the obtained microarrays results (Table 1).

Genetic organization of the putative ribose utilization operon

Our presumption, based on the microarray analysis, that the *rbs* gene cluster is involved in ribose utilization was supported by the high level of sequence similarity between *rbsRACBDK* and ribose-specific metabolic

genes from other bacteria. The genetic organization of the *rbs* gene cluster on the chromosome of *B. breve* UCC2003 and its comparison with similar clusters found in other bacteria are schematically displayed in Fig. 2. Due to its adjacent location (Fig. 2) and similarity to LacI-type transcriptional repressors, the protein product of the *rbsR* gene was suspected to represent the regulator of the *rbsACBDK* operon of *B. breve* UCC2003. The *rbsA*, *rbsC* and *rbsB* genes, which specify the presumptive ribose uptake system, encode putative ATP-binding, ribose permease and ribose-binding proteins respectively. A putative ribose mutarotase, which is an enzyme involved in catalysing the conversion of the β-pyran form into β-furan of ribose (Kim *et al.*, 2004), and a putative ribokinase are encoded by the *rbsD* and *rbsK* genes respectively. The gene order of the *B. breve* UCC2003 *rbs* gene cluster differs from *rbs* clusters that have previously been characterized from *E. coli* (*rbsDACBKR*), *B. subtilis* (*rbsRK-DACB*) and *C. glutamicum* (*rbsRACBD..rbsK1..rbsK2*) (Lopilato *et al.*, 1984; Woodson and Devine, 1994; Nentwich *et al.*, 2009). Furthermore, in some species, for example *L. sakei*, ribose uptake is not performed by an ABC transporter but by a secondary transport system (encoded by *rbsU* in *L. sakei*) (Stentz and Zagorec, 1999). Comparative genome analysis showed that the *B. breve* UCC2003 *rbs* operon is most similar to the similarly organized putative *rbs* gene cluster in the *Bifidobacterium dentium* ATCC27678 genome (Fig. 2). A predicted ribokinase-encoding gene is present in the genomes of the three sequenced *Bifidobacterium longum* strains, *B. breve* DSM20213 (also known as ATCC15700) as well as *Bifidobacterium adolescentis* DSM20083 (also known as ATCC15703), but the ABC transporter, ribose mutarotase and repressor-encoding genes (except for that of *B. longum* NCC2705) appear to be absent from the immediate vicinity of each of these *rbsK* genes. Remarkably, the ability of *B. adolescentis* DSM20083 to metabolize ribose as its sole carbon source indicates that it possesses a ribose uptake system, which is significantly different from that encoded by the *B. breve* UCC2003 genome.

Prevalence of rbs *genes in* B. breve *genomes*

In order to investigate whether homologues of the *rbs* genes are present in other *B. breve* strains, comparative genome hybridization analysis was performed using microarrays based on the *B. breve* UCC2003 genome and genomic DNA from eight *B. breve* strains (Table 2). *Bifidobacterium breve* UCC2005, NCFB2258, NCIMB8815 and UCC2004 were shown to possess homologous DNA representing all genes of the *rbs* cluster. The presumed presence of the *rbs* gene cluster in these strains correlates with their ability to grow on ribose as a sole carbon source (Fig. 1). *Bifidobacterium breve* JCM7017 and JCM7019 appear to harbour only a homologue of the *rbsC* gene, while the *B. breve* NCFB2257 and NCTC11815 genomes did not seem to contain sequences with significant identity to any of the *rbs* genes (Table 2). The CGH analysis is in agreement with growth profiles of *B. breve* JCM7017, NCFB2257 and NCTC11815, which were shown to grow very poorly on ribose. In contrast, *B. breve* JCM7019 is very well capable of metabolizing ribose, suggesting that this strain may have an alternative, non-homologous system for ribose utilization.

Complementation of ribose metabolism

In order to provide further evidence for the presumed involvement of the *B. breve* UCC2003 *rbs* operon in ribose metabolism we constructed a pWSK29-derivative plasmid, designated pWRbsK, which harbours the *rbsK* gene of *rbs* operon from *B. breve* UCC2003. This construct and the pWSK29 negative control were introduced into *E. coli* JW3731-1, which is a mutant strain that carries a deletion in the ribokinase-encoding gene, making it unable to grow in a medium that contains

Table 2. Prevalence of *rbs* genes in *B. breve* genomes.

	B. breve strain number							
Gene	UCC2005	JCM7017	JCM7019	NCFB2257	NCFB2258	NCIMB8815	NCTC11815	UCC2004
rbsR	+	–	–	–	+	+	–	+
rbsA	+	–	–	–	+	+	–	+
rbsB	+	–	–	–	+	+	–	+
rbsC	+	+	+	–	+	+	–	+
rbsD	+	–	–	–	+	+	–	+
rbsK	+	–	–	–	+	+	–	+

The plus sign (+) indicates the presence of the gene; the minus sign (–) the absence of the gene. Genes were considered absent when the fold ratio was < 0.2 and $P < 0.001$.

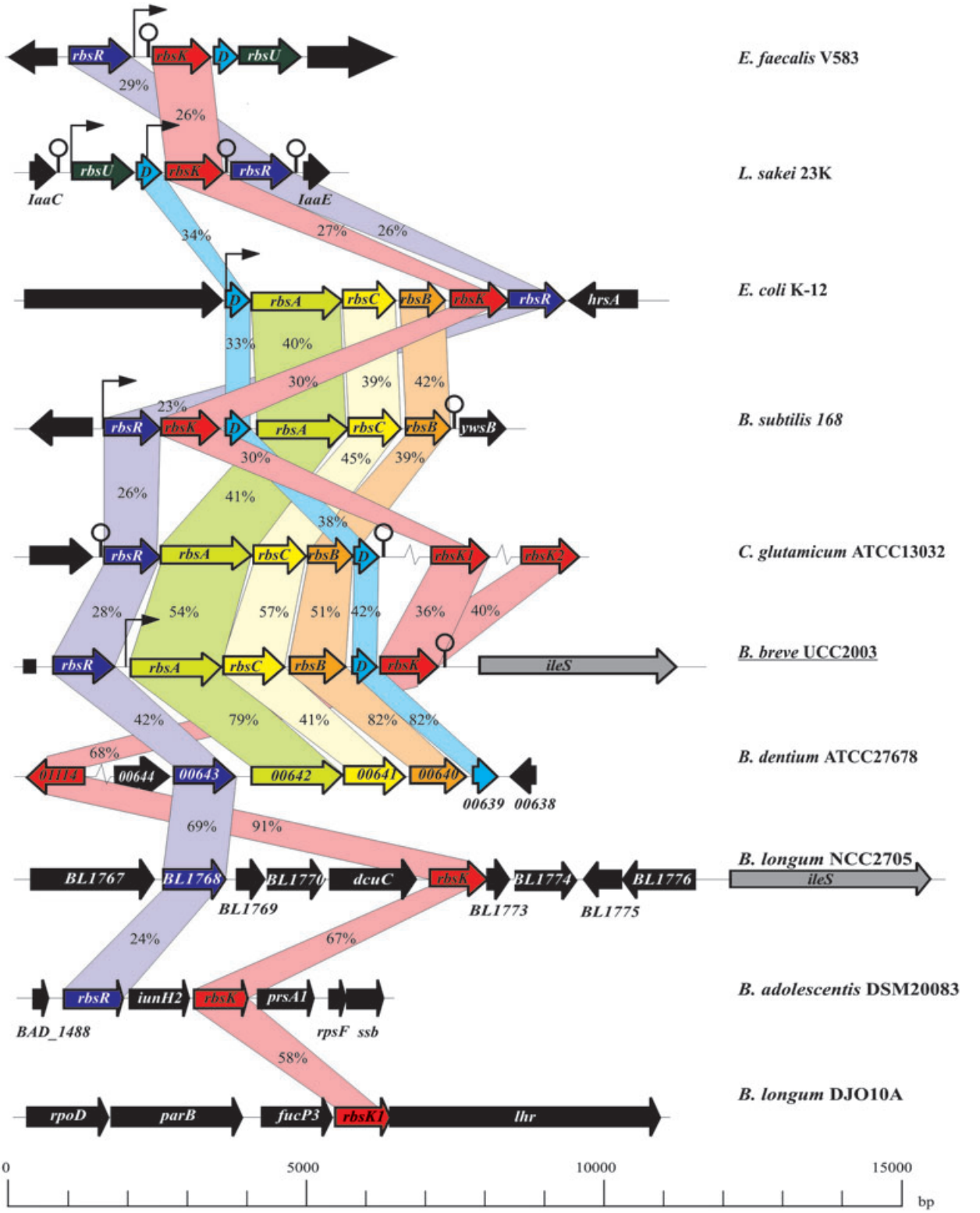

Fig. 2. Comparison of the *rbs* locus of *B. breve* UCC2003 with corresponding (putative or proven) ribose utilization loci from other bacteria. Each solid arrow indicates an open reading frame (ORF). The lengths of the arrows are proportional to the length of the predicted ORF and the gene locus name, which is indicative of its putative function, is indicated within the arrow. Orthologues are marked with the same colour while the amino acid identity of each predicted protein is indicated as a percentage relative to its equivalent protein encoded by *B. breve* UCC2003. The bent arrow indicates putative or proven promoter region; lollipop sign designates putative or proven *rho*-independent terminator region.

ribose as its sole carbon source. The resultant pWRbsK-containing *E. coli* JW3731-1 strain, in contrast to its pWSK29-containing equivalent, was shown to complement the growth defect of *E. coli* JW3731-1 on a medium containing ribose as the sole carbon and energy source (Fig. S1 in *Supporting information*). These results demonstrate that the *rbsK*-encoding gene from *B. breve* UCC2003 is responsible for phosphorylation of ribose.

Disruption of the rbsA *gene in* B. breve *UCC2003*

In order to establish if disruption of the *rbsACBK* operon in *B. breve* UCC2003 would result in loss of this strain's ability to metabolize ribose, an *rbsA* insertion mutant was generated (Fig. S2A in *Supporting information*). To verify the expected ribose-negative phenotype of the *B. breve* UCC2003::rbsA insertion mutant strain, both the wild type and the insertion mutant strain were analysed for their ability to grow in mMRS supplemented with ribose or glucose (positive control) as the sole carbon source (Fig. S2B in *Supporting information*).

As expected, and in contrast to the wild type, the *B. breve* UCC2003::rbsA insertion mutant was shown to be incapable of growth on ribose as the sole carbon source. Although the *rbsA* disruption in *B. breve* UCC2003::rbsA is likely to have a polar effect on the transcription of the downstream genes of the *rbs* operon, it nevertheless demonstrates that (elements of) the *rbs* gene cluster is uniquely necessary for ribose metabolism in *B. breve* UCC2003.

Transcriptional analysis of the rbs *gene cluster*

In *B. breve* UCC2003, the *rbsA*, *rbsC*, *rbsB*, *rbsD* and *rbsK* genes (Fig. 3B) are presumed to be expressed as a single transcript, based on their similar expression patterns as determined from microarray analysis and RT-PCR experiments, which showed that amplification products were generated from cDNA (generated from total RNA of ribose-grown *B. breve* UCC2003) using various primer combinations that spanned the individual genes of the *rbs* gene cluster (data not shown). Furthermore, the only predicted Rho-independent terminator structure in the DNA region that harbours the very closely organized *rbsACBDK* genes is present downstream of the *rbsK* gene (Fig. 3B). The transcriptional start site of the presumed *rbsABCDK* operon was determined by primer extension analysis (see *Experimental procedures* in *Supporting information*) and it was shown to be located 125 bp upstream of the predicted *rbsA* start codon (Fig. 3A, I and II) and 7 bp downstream of sequences resembling consensus −10 and −35 sequences of a vegetative promoter.

Determination of $RbsR_{His}$ DNA binding site

The helix–turn–helix motif of the N-terminus of RbsR is characteristic for members of the LacI-type superfamily of transcriptional factors (Nguyen and Saier, 1995), which are usually responsible for transcriptional regulation of genes involved in carbohydrate metabolism (Perez-Rueda and Collado-Vides, 2000). In order to determine if RbsR is capable of direct interaction with the *rbs* promoter, we first cloned the *rbsR* gene in the nisin-inducible vector pNZ8048 with the introduction of a His-tag-encoding sequence to facilitate purification (see *Experimental procedures* in *Supporting information*). The purified $RbsR_{His}$ protein was then used to perform electrophoretic mobility shift assays (EMSAs) (Fig. 3C), which clearly demonstrated that this protein was able to form a complex with IRD800-labelled DNA fragments encompassing the *rbs*ABCDK promoter region (Fig. 3B and C, I). The results showed that a 56 bp region, which was commonly present in DNA fragments R1, R2 and R3, contained a presumed RbsR-specific operator sequence. In support of this notion, we showed that fragments R4 and R5, which do not contain this RbsR DNA-binding sequence, were not bound by $RbsR_{His}$ (data not shown). These results were further validated by an EMSA performed with a shorter, 56 bp fragment, R6, which contained this operator sequence, and which was shown to be bound by $RbsR_{His}$ (results are not shown). Introduction of two point mutations in the putative RbsR-binding motif (a G→T and a C→T mutation in positions +7 and +22, respectively, relative to the transcription start site which was taken as +1, see Fig. 3A, I), corresponding to positions that were previously shown to play an important role in the binding affinity of other LacI-type regulatory proteins (Kim and Chambliss, 1997), was shown to prevent binding of $RbsR_{His}$ (data not shown). As with other LacI-type regulators, it was assumed that the RbsR binding activity will depend on the presence or absence of a particular sugar ligand, which would be identical or related to the carbohydrate that induces the expression of the *rbs* operon (Weickert and Adhya, 1992). In order to identify the RbsR ligand molecule, EMSAs were performed upon adding (10 mM final concentration) D-ribose or ribose-5-phosphate to the binding reaction. The results obtained clearly showed that the binding ability of $RbsR_{His}$ for the R1 fragment decreased about fourfold in the presence of D-ribose (approximately 50% of the R1 fragment was bound in the presence of 2.5 nM of protein), while ribose-5-phosphate did not affect $RbsR_{His}$ binding to the proposed operator sequence (0.625 nM of protein was required for an approximate 50% shift of the R1 target) (Fig. 3C, II and III). EMSAs of other intergenic regions within *rbs* operon and its surroundings did not result in any shift (data not shown) suggesting the presence of

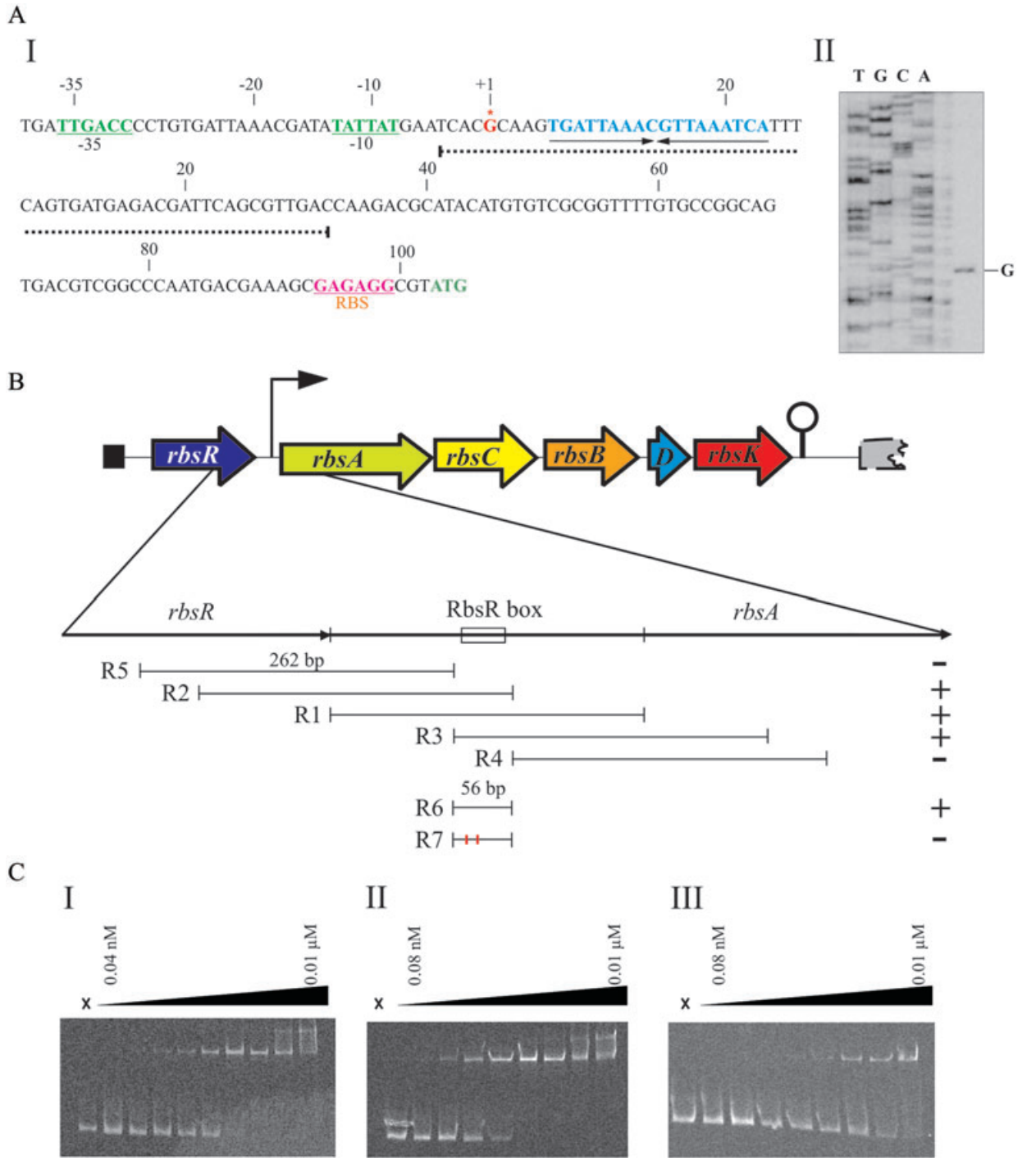

Fig. 3. A. Schematic representation of the *B. breve* UCC2003 *rbs* promoter region (I). Boldface type and underlining indicate the −10 and −35 hexamers as deduced from the primer extension results (II) and ribosomal binding site (RBS); the transcriptional start site (TSS) is indicated by an asterisk; arrows under sequence in bold indicate the inverted repeat sequence that represents the RbsR binding sequence. The R6 fragment (see legend to B) is indicated as a broken thick black line underneath the relevant sequence.
B. Representation of the *B. breve* UCC2003 *rbs* operon and DNA fragments used in electrophoretic mobility shift assays (EMSAs). Plus and minus signs indicate whether or not RbsR was able to bind to the particular DNA fragment respectively.
C. EMSA showing $RbsR_{His}$ interaction with DNA fragments encompassing fragment R1 (I); R1 fragment with the addition of D-ribose-5-phosphate (10 µM) (II); or R1 fragment upon addition of D-ribose (10 µM) (III). In each panel lane X represents a binding reaction to which no protein was added, while the remaining lanes represent binding reactions with the R1 DNA probe incubated with increasing amounts of $RbsR_{His}$ [concentrations ranging from 0.04 nM (for I) (or 0.08 nM for II and III) to 0.01 µM]. Each successive lane, from left to right, corresponds to a doubling in the concentration of $RbsR_{His}$.

a single operator sequence that is regulated by RbsR in *B. breve* UCC2003.

Identification and characterization of RbsK sequence and purified $RbsK_{His}$ from B. breve *UCC2003*

The *rbsK* gene is 1002 bp in length and encodes a protein of 333 amino acids (molecular weight ~34.4 kDa). BLASTP and multiple sequence alignments showed that RbsK is similar to putative and characterized ribokinases encoded by various different microorganisms, with the highest level of identity – 95%, to a hypothetical protein encoded by *B. breve* DSM20213, and 91% and 90% identity to putative PfkB family sugar kinases from *B. longum* NCC2705 and *B. longum* ssp. *infantis* CCUG52486 respectively (Table S3 in *Supporting information*). Overall, the deduced RbsK protein from *B. breve* UCC2003 exhibits varying levels of similarity (ranging from 53% to 69% identity) to 16 putative ribokinases, encoded by 13 different bifidobacterial genomes and all belonging to the ribokinase family (EC number 2.7.1.15). CLUSTAL W alignment of putative or proven RbsK enzymes from a variety of different bacteria revealed conserved amino acids known to be involved in substrate and ATP binding (see Fig. S3 in *Supporting information*) (Sigrell *et al.*, 1998).

In order to characterize the *B. breve* UCC2003 RbsK ribokinase, we purified and biochemically characterized a His-tagged version of this protein, designated $RbsK_{His}$ (see *Experimental procedures*). The molecular mass of $RbsK_{His}$ was estimated by SDS-PAGE to be approximately 38 kDa (see Fig. S4 in *Supporting information*), which is in good agreement with the deduced molecular mass of 36.2 kDa.

Using ribose and ATP as substrates the expected ribokinase activity of the *rbsK* gene product was confirmed by NMR (see *Experimental procedures*), which also allowed characterization of the phosphorylated product of the bifidobacterial ribokinase. The obtained ^{1}H-NMR spectrum of the reaction displayed two signals (5.43 and 5.27 ppm, Fig. 4A), in the region where the anomeric protons of sugars typically appear. These two resonances partially overlap those of ribose at 5.42 and 5.29 ppm corresponding to the α-furanosic and β-furanosic conformations, respectively (data not shown), whereas no resonances corresponding to the pyranosic forms were detected (β-pyranoribose, 4.88 ppm; α-pyranoribose, 4.82 ppm). The proton-decoupled ^{31}P-NMR spectrum showed two partially overlapping resonances (4.49 and 4.47 ppm, Fig. 4B, magnification) in the phosphomonoester region. These resonances appeared as triplets in a proton-coupled ^{31}P-NMR spectrum, indicating that the phosphate group is in the vicinity of two protons as in carbon 5 (CH_2). Altogether, these data led us conclude that the product of the ribokinase reaction was ribose-5-phosphate, which was confirmed by comparing ^{1}H- and ^{31}P-spectra with those derived from commercially obtained ribose-5-phosphate.

In order to investigate substrate specificity of the recombinant $RbsK_{His}$ from *B. breve* UCC2003 possible phosphorylated products were analysed by ^{31}P-NMR and/or by monitoring ATP hydrolysis using an ATP Bioluminoscent assay kit (see *Experimental procedures* for details). The *B. breve* UCC2003 ribokinase was shown to specifically phosphorylate D-(-)-ribose, but failed to phosphorylate other tested pentoses, hexoses or more complex carbon sources under the conditions used in this study (see Table S4 in *Supporting information*).

Interestingly, purified $RbsK_{His}$ enzyme was shown to be active at a rather broad pH spectrum, retaining 100% relative activity at pH 5.5–8.0 (results not shown).

Kinetic parameters (V_{max} and K_m) for ribose and ATP were determined using ^{31}P-NMR spectroscopy (see *Experimental procedures* for details). The ribose saturation curve of $RbsK_{His}$ showed a hyperbolic kinetic response. The K_m and V_{max} values were determined to be 2.9 ± 0.4 mM and 231.6 ± 12 μmol min^{-1} mg^{-1} respectively. Curiously, the ATP saturation curve showed a sigmoidal profile. The deduced K_m for ATP was 5.9 ± 0.2 mM, while the Hill coefficient was 1.4.

Discussion

D-ribose is one of the most abundant carbohydrates found in nature. More specifically, it is thought to be present in the intestinal tract as it is a component of RNA which is one of the dominant macromolecules in every living cell being released following cell death and subsequent degradation (Walker *et al.*, 2006). It has been previously shown that, among others (for a review see Rediers *et al.*, 2005), *E. coli* genes responsible for ribose transport are specifically expressed in the gastrointestinal tract (GIT) of gnotobiotic mice (Alpert *et al.*, 2009). Furthermore, the genes predicted to encode ribose permease (*rbsC*) and ribokinase (*rbsK3*) of *Lactobacillus plantarum* have been shown to be induced in GIT of BALB/c mice (Bron *et al.*, 2004), while transcription of *rbsR*, which regulates ribose utilization, was detected *in vivo* in *Klebsiella pneumoniae* (Lai *et al.*, 2001) and *Salmonella enterica* serovar *typhi* (Daigle *et al.*, 2001). These reports strongly suggest that ribose is a commonly accessible carbon source in the gut environment.

Within the above context it is important to understand how natural inhabitants of the human gut have adapted to this particular environment through their ability to utilize available carbon sources. In ribose-utilizing bifidobacteria it is expected that, once a ribose molecule is taken up and phosphorylated to ribose-5-phosphate, it can directly enter the 'bifid shunt' (de Vries *et al.*, 1967;

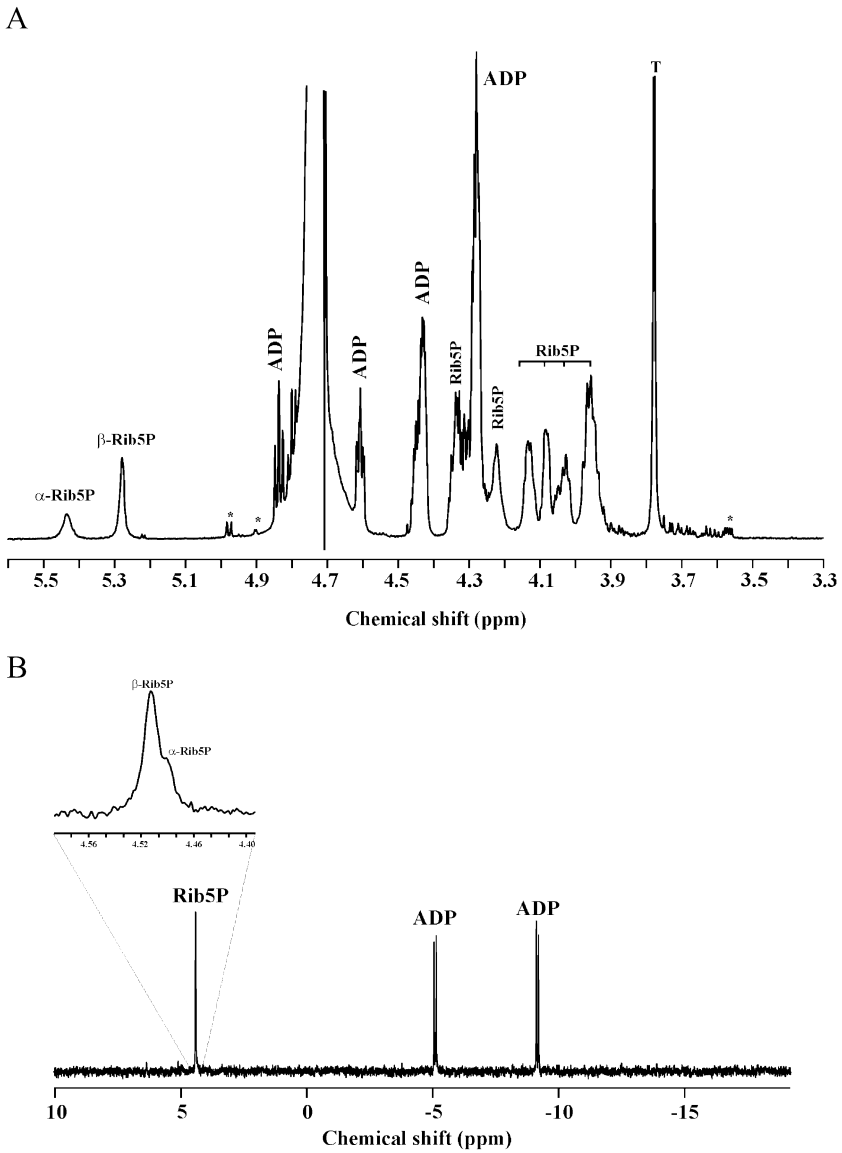

Fig. 4. ^{1}H-NMR (A) and ^{31}P-NMR (B) spectra of the ribokinase reaction. The sample was prepared in $^{2}H_2O$ as described in *Experimental procedures* and the spectra were acquired at 33°C. Proton and phosphorus spectra were processed with 0.1 and 1 Hz line broadenings. Rib5P, ribose-5-phosphate; the peaks indicated with an asterisk represent ribose. The inset (in B) shows magnification of the region between 4.49 and 4.47 ppm.

Sanchez *et al*., 2007), while it can also be used for the biosynthesis of nucleotides and histidine (Eriksen *et al*., 2000).

This report describes the characterization of the ribose utilization cluster, *rbsACBDK*, and its presumed regulator, encoded by *rbsR*, of *B. breve* UCC2003. The involvement of *rbsK* in ribose metabolism was confirmed by phenotypic complementation of an *rbsK*-negative *E. coli* mutant in a ribose-containing medium. The *B. breve* UCC2003::*rbsA* insertion mutant confirmed that the *rbs* operon described in this study is the only or at least the predominant system required for ribose metabolism in *B. breve* UCC2003.

Transcription of the *rbsACBDK* operon was shown to be repressed when *B. breve* UCC2003 was grown in modified Rogosa medium containing glucose, while growth in medium containing ribose or a combination of ribose and glucose caused clear transcriptional upregulation of this operon. However, the expression level of the genes of the *rbs* operon was decreased by approximately twofold (Table 1) when the culture was grown in a combination of glucose and ribose in mMRS (relative to the expression levels obtained from a culture grown in ribose only), thereby suggesting that expression of the *rbs* operon was downregulated to some degree by catabolite repression in the presence of a highly metabolizable sugar such as

glucose. PEP-PTS-regulated repression of ribose utilization has been observed in *L. sakei* (Stentz and Zagorec, 1999) and, although PEP-PTS-mediated carbon catabolite repression (CCR) system is common in bacteria such as *E. coli* and *B. subtilis* (for reviews see Deutscher *et al*., 1997; Gorke and Stulke, 2008), *B. breve* UCC2003 does not appear to encode the key regulators present in these paradigm systems (S.C. Leahy, M. O'Connell-Motherway, J.A. Moreno Muñoz, G.F. Fitzgerald, D.G. Higgins and D. van Sinderen, unpubl. data). In this context it is interesting to note that a glucose kinase from the high G+C Gram-positive bacterium *Streptomyces coelicolor* was shown to be involved in CCR (Gorke and Stulke, 2008), while *B. longum* NCC2705 exhibits a lactose-over-glucose preference representing an example of reverse CCR, the mechanism of which is as yet not fully understood (Parche *et al*., 2006). Our results suggest that *B. breve* UCC2003 possesses an unusually regulated CCR system thus warranting a more specific investigation to gain a better understanding of the mechanism involved.

The RbsR protein of *B. breve* UCC2003, which was shown to bind to the promoter region of the *rbsABCDK* operon, is presumed to act as an effective transcriptional repressor in the absence of the effector molecule, thereby preventing transcription. The RbsR-specific binding site was shown to be located five nucleotides downstream of the *rbs* operon transcriptional start site. So therefore binding of RbsR is expected to prevent the RNA polymerase to proceed across this region, thus blocking transcription of the *rbs* operon. The *B. breve* UCC2003 RbsR recognition sequence is similar to RbsR binding sites from *E. coli* K-12 (Mauzy and Hermodson, 1992), *B. subtilis* (Woodson and Devine, 1994) and *L. sakei* (Stentz and Zagorec, 1999) (data not shown). In addition, our RbsR binding results indicate that *rbs* transcription and therefore ribose metabolism are controlled by D-ribose, as the affinity of RbsR for its DNA target decreases, while the transcription of *rbsR* and therefore the assumed amount of RbsR are also reduced in the presence of this sugar. This observation is consistent with results from a previous study of RbsR regulation in *E. coli* (Mauzy and Hermodson, 1992). In contrast, the binding activity RbsR from *B. subtilis* and *C. glutamicum* does not appear to be affected by D-ribose (Strauch, 1995; Nentwich *et al*., 2009).

Comparative sequence analysis of *rbs* gene clusters from other bacteria showed that the genetic organization of the *B. breve* UCC2003 *rbs* operon is similar to those found in *E. coli* and *B. subtilis*. Interestingly, BLAST searches showed that most of the sequenced bifidobacterial genomes have at least one copy of an apparent *rbsK* homologue, although no complete *rbs* operon appears to be present in any of the currently available bifidobacterial genomes (Fig. 2). An exception to this observation is *B. dentium* ATCC27678, whose genome contains a similarly organized *rbs* operon, although the *rbsK* homologue appears to be disconnected from the rest of the gene cluster, similar to that of the recently described *rbs* operon of *C. glutamicum* (Nentwich *et al*., 2009). The results obtained from comparative sequence and genome hybridization analyses may indicate that *rbs* genes could have been lost in the chromosomes of some of the analysed strains that only retained parts of the *rbs* cluster. It could also mean that other bifidobacteria, such as *B. adolescentis* DSM20083, *B. breve* JCM7017, JCM7019 and NCFB2257, use a different ribose uptake system which has not yet been described or alternatively, these strains may encode a secondary transport-type protein for ribose uptake which is not homologous to the known RbsU protein from *L. sakei* (Stentz and Zagorec, 1999).

The ribokinase, encoded by *rbsK*, of *B. breve* UCC2003 was shown to preferentially phosphorylate D-ribose. The presence of ribokinase activity in bifidobacteria has not previously been reported, but this enzyme has been well studied in *E. coli*, *Leishmania major* and *S. enterica* (Tourneux *et al*., 2000; Chuvikovsky *et al*., 2006; Ogbunude *et al*., 2007). Our results demonstrate that the phosphorylation product of $RbsK_{His}$ from *B. breve* UCC2003 is D-ribose-5-phosphate (Fig. 4) and that D-ribose represents the only phosphorylation substrate (see Table S4 in *Supporting information*). This is consistent with a previous report on RbsK from *L. major* (Ogbunude *et al*., 2007), but contrasts with characterized ribokinases from *E. coli* and *S. enterica* serovar *typhi*, which were shown to have broader specificity (Chuvikovsky *et al*., 2006). Differences in primary structure between these enzymes may explain their different substrate preferences and kinetic parameters.

Previously, *B. breve* UCC2003 was shown to metabolize a number of different carbon sources (Ryan *et al*., 2005; 2006; O'Connell-Motherway *et al*., 2008a,b; Pokusaeva *et al*., 2009). The results presented here provide the first in-depth characterization of pentose metabolism in bifidobacteria and extends our understanding of carbohydrate utilization by our model bifidobacterial strain *B. breve* UCC2003, showing its adaptation to the available carbon sources present in the human and animal intestine.

Experimental procedures

The description of the experimental procedures can be found in Appendix S1 in *Supporting information*.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Growth of *E. coli* mutants complemented with a cloned fragment of the *rbsK* gene of *B. breve* UCC2003 on M9 agar plates supplemented with 0.2% ribose.

Fig. S2. A. A schematic representation of the relevant *rbs* regions of the *B. breve* UCC2003 and *B. breve* UCC2003::rbsA chromosomes. Chromosomal DNA is represented by a thin line, the *rbsA* gene is represented by a thick black arrow, the internal *rbsA* fragment used for homologous recombination to obtain the insertion mutant is indicated by a solid grey line. Segments of the integrated plasmid are indicated in solid red (*tetW* gene), solid (*lacZ* gene) and boxed green and solid blue (*emr* gene) lines.

B. Growth profile of wild-type *B. breve* UCC2003 and *B. breve* UCC2003::rbsA insertion mutant in mMRS supplemented with glucose and ribose. The results of the growth experiments are mean values obtained from four separate experiments.

Fig. S3. Alignment of seven putative or proven ribokinase sequences from different bacterial strains, using the program CLUSTAL W (Thompson *et al.*, 1997). First lane labelled as RbsK UCC2003 corresponds to a ribokinase encoded by *B. breve* UCC2003. Other labels indicate locus tags of genes that encode ribokinases from different bacteria (for details see Table S3). Residues that are known to interact with ribose or ATP/ADP are marked **R** or **A**, indicating a hydrogen bond with ribose or ADP respectively. **r** or **a** indicate an indirect hydrogen bond via one water molecule, and **H** and **h**, residues that are involved in van der Waals contacts with ribose or ADP respectively (Sigrell *et al.*, 1998). The first underlined

sequence represents a motif that is specifically involved in interactions between the ribokinase and the ribose substrate. The second underlined motif includes part of the binding site for the adenine ring. Conserved residues are shaded based on 50% similarity significance.

Fig. S4. SDS-PAGE analysis of $RbsK_{His}$ purification. Lane 1, molecular weight markers as indicated in the left-hand margin of each panel; lane 2, crude cell extract of induced *L. lactis* NZ9000 culture harbouring pNZ8048; lane 3, crude cell extracts of *L. lactis* NZ9000 culture harbouring pNZrbsK following induction with nisin; lane 4, flow-through; lanes 5–7, wash fractions; lanes 8–12, eluted fractions of purified $RbsK_{His}$ protein.

Table S1. Bacterial strains and plasmids used in this study.

Table S2. Oligonucleotide primers used in this study.

Table S3. Similarity levels of RbsK from *B. breve* UCC2003 to annotated ribokinases from other bacteria.

Table S4. Substrate specificity of purified $RbsK_{His}$ from *B. breve* UCC2003.

Appendix S1. Experimental procedures.

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