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Insights into teichoic acid biosynthesis by *Bifidobacterium bifidum* PRL2010

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ABSTRACT

Bifidobacteria are colonizers of the human gut, where they are interacting with their host as well as with other members of the intestinal microbiota. Teichoic acids (TAs) have previously been shown to play an important role in modulating microbe–host interactions in the human gut. However, so far, there is a paucity of information regarding the presence of TAs in the cell envelope of bifidobacteria. *In silico* analyses targeting the chromosomes of all 48 (sub)species that currently represent the genus *Bifidobacterium* revealed the presence of genes responsible for TA biosynthesis, suggesting that bifidobacteria contain both wall TAs and lipoteichoic acids. Transcriptome analyses of the infant gut commensal *Bifidobacterium bifidum* PRL2010 highlighted that the transcription of the presumptive TA biosynthetic loci is modulated in response to environmental conditions reflecting those of the human gut. Furthermore, chemical characterization of TAs produced by PRL2010 indicates the presence of lipoteichoic acids.

Keywords: genomics; functional genomics; microbiota; bifidobacteria; host–microbe interactions

INTRODUCTION

The cytoplasmic membrane of Gram-positive bacteria is covered by an envelope consisting of a thick layer of peptidoglycan (PG) (Vollmer, Blanot and de Pedro 2008) and several other molecules including teichoic acids (TAs), polysaccharides and extracellular proteins (Archibald *et al.* 1961; Ward 1981).

Most Gram-positive bacteria produce two distinct types of TAs, (i) the wall teichoic acids (WTAs), which are covalently coupled to PG, and (ii) lipoteichoic acids (LTAs) that are anchored to the cell membrane (Morath, von Aulock and Hartung 2005; Weidenmaier and Peschel 2008).

Common types of WTAs consist of glycerolphosphate (Gro-P) or ribitolphosphate (Rbo-P) subunits that are connected by phosphodiester bonds (Weidenmaier and Peschel 2008), and that represent the most abundant PG-linked glycopolymers of the bacterial cell (Over *et al.* 2011). Nonetheless, they display substantial diversity due to the presence of structural variations in their backbone, i.e. six different backbones are found in WTAs of *Lactobacillus plantarum* (Tomita *et al.* 2012), and due to the presence of D-alanine or monosaccharide substituents attached to the repeating monomers (Grangette *et al.* 2005; Brown, Santa Maria and Walker 2013).

LTAs are chemically similar to WTAs since they are composed of polyGro-P (PGP) polymers that are often substituted

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with the same molecules (Neuhaus and Baddiley 2003). However, rather than being attached to PG, LTAs are linked to membrane through a glycolipid anchor (Morath, von Aulock and Hartung 2005). TA biosynthetic pathways in lactobacilli have been characterized using mutagenic approaches (Brown, Santa Maria and Walker 2013). TAs exert key roles in host–microbe interactions, such as facilitating adhesion to human intestinal epithelial cells (Granato *et al.* 1999), and in host immune response activation due to subtle structure diversity of these molecules (Wells 2011; Percy and Grundling 2014), although some studies have indicated that there is no direct role of WTAs in immune signaling (Travassos *et al.* 2004; Rockel, Hartung and Hermann 2011; Bron *et al.* 2012).

TAs have been widely studied in various members of the *Firmicutes* taxon, e.g. *Bacillus subtilis*, *Staphylococcus aureus* and *Listeria monocytogenes* (Lambert, Coley and Baddiley 1977; Fischer 1994; Sutcliffe 1994). In contrast, the cell envelopes of members of the *Actinobacteria* taxon typically contain lipoglycans, such as lipomannans and lipoarabinomannans (Sutcliffe 1994; Nigou, Gilleron and Puzo 2003; Gilleron *et al.* 2005). However, LTAs have also been reported to be present in representatives of two genera of *Actinobacteria* including *Thermobifida fusca* and *Streptomyces* spp. (Potekhina *et al.* 1983; Rahman, Cummings and Sutcliffe 2009; Rahman, Dover and Sutcliffe 2009).

Bifidobacteria represent one of the dominant bacterial groups found in the intestinal microbiota of infants (Turroni *et al.* 2012). Various cell envelope-associated (macro)molecules, such as capsular polysaccharides and fimbriae/pili produced by bifidobacteria, are considered to be pivotal in mediating host interactions (O'Connell Motherway *et al.* 2011; Fanning *et al.* 2012; Ventura *et al.* 2012; Turroni *et al.* 2013, 2014a,b). However, there is very little information available on the occurrence of TAs in members of the *Bifidobacterium* genus (Milani *et al.* 2014). In contrast, lipoglycan structures have been identified in *Bifidobacteriu bifidum*, *B. breve* and *B. longum* cells (Iwasaki *et al.* 1990).

In this work, we performed a genomic survey of genes putatively involved in TA biosynthesis in all 48 members of the genus *Bifidobacterium* and we investigated the expression of such TA loci of *B. bifidum* PRL2010 in response to various environmental conditions simulating those of the human gut. Furthermore, we chemically characterized the LTA produced by PRL2010 cells.

MATERIALS AND METHODS

Growth conditions

Bacteria were cultivated in an anaerobic atmosphere (2.99% H_2, 17.01% CO_2 and 80% N_2) in a chamber (Concept 400; Ruskin) at 37°C for 16–32 h in de Man-Rogosa-Sharpe (MRS; Scharlau Chemie, Spain) medium. MRS was supplemented with 0.05% (w/v) L-cysteine hydrochloride.

Cocultivation

Approximately 10^8 cells/mL of *B. bifidum* PRL2010 as a monoculture or in combination with *B. breve* 12L or *L. paracasei* LMG9192 were used to inoculate 6 mL of MRS and incubated for 16 h at 37°C under anaerobic conditions, until cells had reached late exponential phase. The suspensions were harvested and total RNA extraction was performed to analyze the expression of predicted TA biosynthesis loci by means of qRT-PCR analyses.

Bacterial viability was assessed during cocultivation. Briefly, 100 μl samples were collected from cocultures after 16 h of incubation and plated on MRS agar. After incubation colonies were randomly picked and subjected to PCR analyses using specific primer pairs in order to identify bacteria. In the case of *B. breve* 12L, we used 5′-CGAAGTTCCAGTTCACCAT-3′ and 5′-GTTCTTGGCGTTCCAGATGT-3′; while for *L. paracasei* LMG9192 we employed the primers 5′-AGCAGTAGGGAATCTTC-3′ and 5′-CATGGAGTTCCACTGTC-3′.

Table 1. Oligonucleotide primers used in this study.

Primer name	Targeted ORF/genes	Sequence (5′ – 3′)
BBPR_0072Fw	BBPR_0072	AATATCTGCTGCACGCATTG
BBPR_0072Rv	BBPR_0072	GAGATTCCTGAGCCACTTCG
BBPR_0081Fw	BBPR_0081	CGCAAGGTTCATTTCCCTAA
BBPR_0081Rv	BBPR_0081	GCAAAGAAGCCGAAGCATAC
BBPR_0082Fw	BBPR_0082	TGGTCACGCACAGTATGGAT
BBPR_0082Rv	BBPR_0082	CTATACCGGTCTGCCACGTT
BBPR_0698Fw	BBPR_0698	GCTCAACATCGTCTACTGCC
BBPR_0698Rv	BBPR_0698	GGCTCGGATAGTGCTGGAAT
BBPR_0699Fw	BBPR_0699	CGCATCTATTCCGAGGTGTT
BBPR_0699Rv	BBPR_0699	ATCTCCTCGCGGTATTCCTT
BBPR_0704Fw	BBPR_0704	GTGTTCCCGTGGTTCTTCAT
BBPR_0704Rv	BBPR_0704	GACAGCGTGTAGAACGTGGA
BBPR_0705Fw	BBPR_0705	GGACATCCTGGTGGTGGAC
BBPR_0705Rv	BBPR_0705	CTTTGCCCTTCTGGATGACG
BBPR_1314Fw	BBPR_1314	CCAGCCAGGATACGAACAAT
BBPR_1314Rv	BBPR_1314	CGACGAGTACACGCTGTTGT
Reference genes		
BBP_atpD for	*atpD*	CAGAGCCGATCAATGGACGTG
BBP_atpD rev	*atpD*	GTGCTGCTCGACCTCAAGCGTGAT
BBP_ldh for	*ldh*	CACCATGAACAGGAACAAAGTTG
BBPR_ldh rev	*ldh*	GAATGATCGATGAGTACGAGCTC

RNA isolation, reverse transcription, and qRT-PCR, RNA isolation, reverse transcription and qRT-PCR

Total RNA was isolated using a previously described method (Turroni *et al.* 2011), using the RNeasy mini kit (Qiagen). The quality and integrity of the RNA sample was checked by Multi-Tape Station (Agilent) analysis. Reverse transcription to cDNA was performed with the iScript Select cDNA synthesis kit following the supplier's instruction (Bio-Rad Laboratories). Gene transcription levels were analyzed in quantitative Reverse Transcription PCR (qRT-PCR) using SYBR Green Supermix (Bio-Rad) on a Bio-Rad CFX96 system according to the manufacturer's instructions (Bio-Rad). Primers are described in Table 1. qRT-PCR was carried out according to the following protocol: one cycle of 94°C for 2 min, followed by 39 cycles of 94°C for 5 s and 60°C for 30 s. Gene transcription was normalized relative to housekeeping genes as previously described (Turroni *et al.* 2011).

Colonization of mice with PRL2010 cells

All animals used in this study were cared for in compliance with guidelines established by the Italian Ministry of Health. All procedures were approved by the University of Parma, as executed by the Institutional Animal Care and Use Committee. *In vivo* experiments were carried out as described previously (Turroni *et al.* 2013). Animals were sacrificed by cervical dislocation and their individual gastrointestinal tracts were removed, immediately treated with RNA later and subsequently used for RNA extraction.

Statistical analyses

Statistical significance between means was analyzed using the unpaired Student *t*-test, with a threshold *P*-value of <0.05. The obtained values were expressed as the mean ± the standard error based on the results from three independent experiments. Statistical calculations were performed using the software program GraphPad (La Jolla, USA) Prism 5.

Chemical purification of LTAs and data analysis

Bifidobacterium bifidum PRL2010 cells from overnight cultures were recovered by centrifugation at 1200 × *g* and washed with fresh PBS. The obtained cell pellet was suspended in PBS and subjected to sonication (30 s ON/30 s OFF, 10 cycles at high power). The resulting bacterial suspension was mixed with an equal volume of *n*-butanol (Sigma) and stirred for 30 min at room temperature; phases were separated by centrifugation for 20 min at 13 000 × *g* at 4°C. The aqueous phase was recovered, subjected to freeze-drying, after which LTAs were purified by an established protocol employing hydrophobic interaction chromatography (HIC) (Morath, Geyer and Hartung 2001). Briefly, the lyophilized bacterial extract was resuspended in chromatography start buffer (15% *n*-propanol in 0.1 M ammonium acetate, pH 4.75), centrifuged at 45 000 × *g* for 15 min and sterilized by membrane filtration (0.2 μm) and then purified using an Octyl-Sepharose column (GE Healthcare Life Sciences, UK). Elution was conducted by linear gradient from start buffer to elution buffer (60% *n*-propanol in 0.1 M ammonium acetate, pH 4.75).

The obtained fractions were assessed by a molybdenum blue test (Schnitger *et al.* 1959) to detect phosphate-containing fractions, which were pooled, lyophilized and analyzed by NMR. Dried fractions were dissolved in D_2O (Goss Scientific Instruments) and an aliquot of 540 μl was mixed with 60 μl of 1.5 M potassium phosphate buffer in D_2O, pH = 7.4, 0.1% 3-(trimethylsilyl)-[2,2,3,3–$^2H_4$]propionic acid sodium salt (TSP) and 2 mM sodium azide. ^{1}H NMR spectra were acquired using a Bruker 600 MHz spectrometer (Bruker, Germany) at an operating ^{1}H frequency of 600.13 MHz at 300 K. One-dimensional Car-Purcel-Meiboom-Gill (CPMG) spectra was acquired using a spin-echo pulse sequence: [recycle delay-90°-(τ-180°-τ)$_n$-acquire free induction decay]. The spin relaxation delay (2n τ) used was 160 ms. A 90° pulse was adjusted to 10 μs. A total of 32 scans were collected into 64 k data points with a spectral width of 20 ppm. Automatic phasing, baseline correction and reference to TSP signal at δ^1H 0.00 were performed on the ^{1}H NMR CPMG spectra. The processed NMR spectral data were imported to MATLAB (R2012a, 7.14.0.739, MathWorks) and digitized into approximately 20 k data points with a resolution of 0.0005 ppm. The water peak region (δ^1H 4.74–4.85) was removed due to its disordered peak shape caused by water suppression. Probabilistic quotient normalization was applied to the remaining spectral data.

RESULTS AND DISCUSSION

In silico analysis of the WTA biosynthetic pathways in *B. bifidum* PRL2010

BlastP analyses of the *B. bifidum* PRL2010 genome for WTA genes allowed the identification of homologs of *tagB*, *tagF*, *tagG*, *tagH* and *tagO* of *B. subtilis* 168, the products of which were shown to share significant sequence similarity ranging from 23 to 40% (Fig. 1a). In *B. subtilis*, *tag* genes are involved in the biosynthesis of the major WTAs (Soldo, Lazarevic and Karamata 2002) (Fig. 2). Most of the identified homologous genes are located next to each other, encompassing two putative gene clusters that were predicted to be involved in TA biosynthesis (Fig. 1b). Size, sequence similarity and the localization of BBPR_0698 and BBPR_0699 suggested that these genes represent, respectively, the *B. bifidum tagB* and *tagF* homologs. BBPR_0698 and BBPR_0699 are placed nearby two predicted ABC transporter-encoding genes (BBPR_0704/BBPR_0705), which appear to be homologs of *tagG*/*tagH*, in a similar genetic constellation observed for the *tagF* and *tagB* genes of *B. subtilis* aand *S. aureus* (Xia and Peschel 2008).

A glycosyltransferase-encoding *tagO* homolog was also identified (BBPR_1392), located elsewhere on the genome of PRL2010, which is reminiscent of the situation in *B. subtilis*.

Notably, *in silico* analyses did not reveal the occurrence of any homologous of *tagA*, which encodes an *N*-acetylmannosaminyl transferase widely identified in bacteria (Swoboda *et al.* 2010). This finding is similar to what has been found for other WTAs from other *Actinobacteria* such as *Streptomyces coelicolor* A3(2) (Kleinschnitz *et al.* 2011). The absence of this gene might request alternative strategies such as the replacement of a disaccharide linker with a monosaccharide linker.

The presence of genes on the *B. bifidum* PRL2010 genome that are homologous to genes involved in WTA biosynthesis indicates that this strain possesses a similar metabolic pathway for the biosynthesis of PGP–WTA.

A second cluster predicted to encode enzymes involved in WTA biosynthesis was identified in the genome of PRL2010. This cluster encompassed genes homologous to *tagG* and *tagH* (corresponding to locus tags BBPR_0081 and BBPR_0082, respectively), a putative glycosyltransferase-encoding gene (BBPR_0072) and a gene encoding a predicted nucleotidyl transferase (BBPR_0078) (Fig. 1). The gene corresponding to BBPR_0072 is homologous to both the *ggaA* and *ggaB* genes of *B. subtilis* 168, predicted to be involved in the biosynthesis of a minor, galactosamine-containing TA (Freymond *et al.* 2006). Sequence similarity as well as the genetic organization of this genetic locus suggests that BBPR_0072 and BBPR_0078 represent the *B. bifidum ggaA/B* and *gtaB* genes. These findings support the possibility that this cluster is involved in the biosynthesis of a structurally different TA, resembling the *B. subtilis* minor TA.

Bioinformatics analyses were also performed focusing on several other blastp queries, including the *tarIJKL* locus of *L. plantarum* WCFS1 (Bron *et al.* 2012), as well as the WTA-encoding genes of the more closely related bacterium *St. coelicolor* A3(2). Interestingly, blastp analyses involving the *tarIJKL* of *L. plantarum* WCFS1 led to identification of some localized homology between this cluster (involved in Rbo-P WTA biosynthesis) and the here-identified WTA cluster 1 of PRL2010, probably due to homology of Gro-P and Rbo-P WTA-synthetizing enzymes. However, the WTA encoding genes of *St. coelicolor* A3 were homologous to *wta* cluster 1 of PRL2010.

In silico analysis of the LTA biosynthetic pathway in *B. bifidum* PRL2010

In order to identify the occurrence of a potential LTA biosynthetic pathway in *B. bifidum* PRL2010 genome, we screened its genome for the presence of genes known to be involved in the synthesis of LTA in *S. aureus* subsp. *aureus* Mu50 (*gtaB, ypfP, pgcA, ltaA* and *ltaS*) and *B. subtilis* 168 (*gtaB, ugtP, pgcA, yflE, yqgS, yvgJ* and *yfnI*).

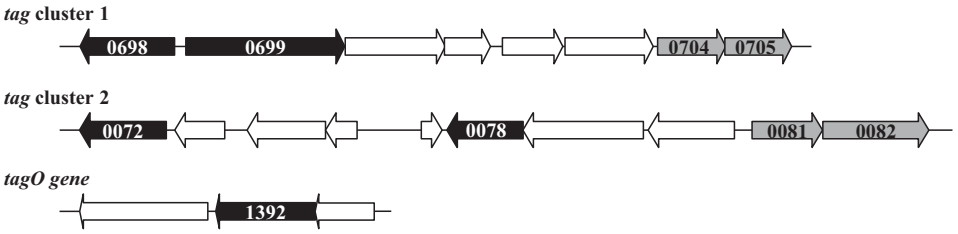
As displayed in Fig. 1c, homologs of all of these genes were identified in PRL2010 genome, with the exception of the

(a)

BBPR # name	identity to *tag* (%)	E value	putative function
0072	*ggaA* (45) *ggaB* (41)	3E-21 2E-19	glycosyltransferase involved in cell wall biogenesis
0073			hypothetical protein
0074			IS3520 family transposase
0075			IS6110 family transposase
0076			hypothetical protein
0077			IS1557 family transposase
0078	*gtaB* (28)	2E-21	RmlA glucose-1-phosphate thymidylyltransferase
0079			RmlC/RmlD dTDP-4-dehydrorhamnose 3,5-epimerase/ dTDP-4-dehydrorhamnose reductase
0080			RmlB dTDP-glucose 4,6-dehydratase
0081	*tagG* (23)	1E-11	Permease protein ABC transporter system for polysaccharides
0082	*tagH* (36)	1E-46	ATP-binding protein ABC transporter system for polysaccharides

BBPR # name	identity to *tag* (%)	E value	putative function
0698	*tagB* (25) *tagF* (23)	1E-27 2E-09	glycosyl transferase, group 2 family protein
0699	*tagB* (26) *tagF* (24)	4E-27 4E-22	teichoic acid biosynthesis protein B tagB
0700			hypothetical protein
0701			hypothetical protein
0702			2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
0703			NAD-dependent epimerase/dehydratase family protein
0704	*tagG* (28)	9E-25	sugar ABC transporter permease
0705	*tagH* (40)	8E-52	ABC transporter ATP-binding protein
1391			inosine-5'-monophosphate dehydrogenase
1392	*tagO* (32)	6E-45	undecaprenyl-phosphate-alpha-N-acetylglucosamine phosphotransferase
1393			Sua5/YciO/YrdC/YwlC family protein

(b)



(c)

Identity to BSU LTA (%)	E-value	BBPR # name	Identity to SAV LTA (%)	E-value	putative function
gtaB (28)	2E-21	BBPR_0078	*gtaB* (28)	2E-20	glucose-1-phosphate thymidylyltransferase
ugtP (26)	6E-09	BBPR_0556	*ypfP* (26)	5E-08	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase
pgcA (27)	1E-23	BBPR_1512	*pgcA* (24)	1E-15	phosphoglucomutase
yflE (27) *yfnI* (26) *yqgS* (24) *yvgJ* (24)	7E-07 4E-07 1E-05 2E-03	BBPR_1314	*ltaS* (24)	3,00E-05	phosphoglycerol transferase

(d)

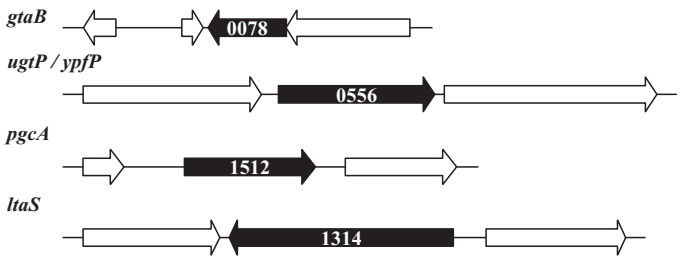


Figure 1. Gene clusters putatively involved in teichoic acids biosynthesis. In panel **a**, the predicted functions of the genes and their new designations in accordance with the coding of the *B. subtilis* homologs are listed. Similarities at amino acid level with *B. subtilis* 168 tag proteins involved in WTA biosynthesis are given. Panel **b** represents the genetic maps of the two *B. bifidum* PRL2010 gene clusters putatively involved in wall teichoic acid biosynthesis. Transferase genes are shown in black and ABC transporters depicted in gray. Panel **c** displays the putative function of the genes and their new designations in accordance with the naming of the *B. subtilis* and *S. aureus* homologs. Similarities to *B. subtilis* 168 or *S. aureus* subsp. *aureus* Mu50 proteins involved in LTA biosynthesis are given as percentages of identical amino acids over the core regions. Panel **d** shows the schematic representation of the *B. bifidum* PRL2010 genes putatively involved in LTA biosynthesis. Transferase genes are shown in black.

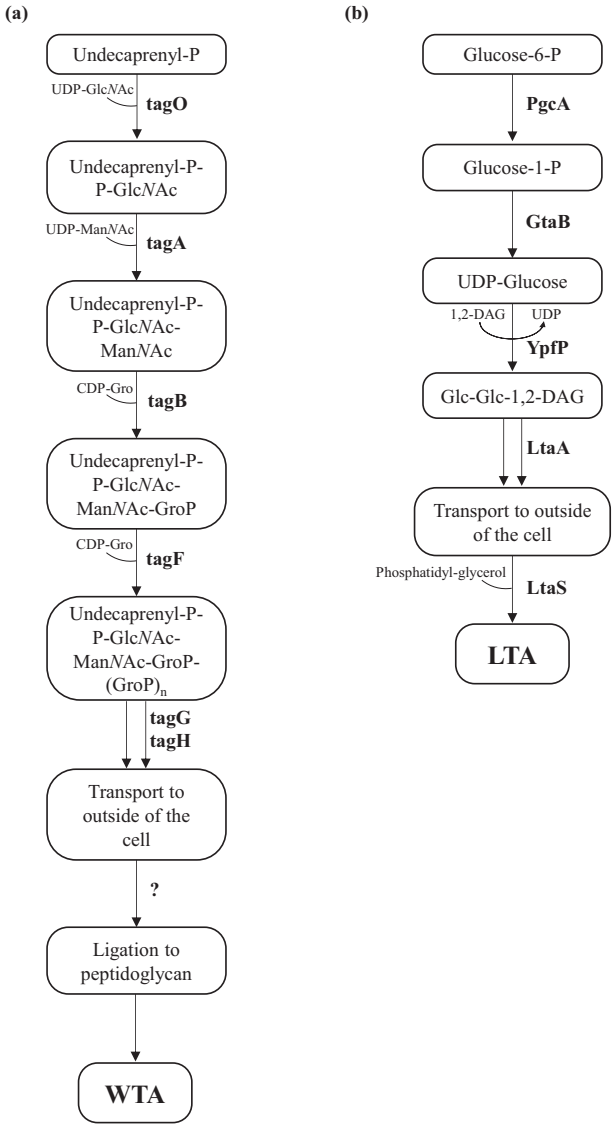


Figure 2. Schematic representation of WTA and LTA biosynthesis pathway. Panel **a** shows the pathway of biosynthesis of *B. subtilis* WTA: TagO transfers a GlcNAc phosphate to an undecaprenyl phosphate carrier (Soldo, Lazarevic and Karamata 2002), then TagA adds a ManNAc and TagB transfers one to three phosphoglycerol units to ManNAc (Neuhaus and Baddiley 2003; Swoboda *et al.* 2010). Finally, cytidylyltransferase TagF attach normally more than 30 glycerolphosphates and the TagGH ABC transporter system exports the WTA to the outside of the membrane (Schirner, Stone and Walker 2011), where it is coupled to PG by a so-far unknown mechanism. Panel **b** represents the biosynthetic pathway of LTA in *S. aureus*: the α-phophoglucomutase PgcA converts glucose-6-phosphate to glucose-1-phosphate that is activated by GtaB, leading to the production of UDP-Glc (Pooley, Paschoud and Karamata 1987; Lu and Kleckner 1994; Lazarevic *et al.* 2005; Grundling and Schneewind 2007). The glycosyltransferase YpfP transfers two glucose moieties from UDP-Glc to DAG, leading to the formation of the glycolipid Glc_2-DAG (Jorasch *et al.* 1998; Kiriukhin *et al.* 2001), which is transported outside the membrane by the LtaA 'flippase' (Grundling and Schneewind 2007). Finally, a single LTA synthase enzyme (LtaS) polymerizes the GroP to generate the PGP chain (Grundling and Schneewind 2007).

S. aureus ltaA gene, encoding for the 'flippase' involved in the translocation of the LTA precursors (Grundling and Schneewind 2007a,b). The product of this gene is an integral membrane protein, thus containing many hydrophobic residues that may be difficult to detect reliably by Blast searches. Notably, the PRL2010 *gtaB* homolog, BBPR_0078, encoding an α-glucose-1-phosphate uridyltransferase, is localized within the WTA cluster-2. The homologous *ltaS* of PRL2010 was identified as BBPR_1314. It is worth noticing that this gene is also homologous to four *B. subtilis ltaS* homologs, including the LTA primase-encoding gene *yvgJ* that is predicted to be involved in the production of the GroP-glycolypid intermediate, yet unable to polymerize the LTA chain (Wormann *et al.* 2011). In *S. aureus*, the latter reaction is catalyzed by the same LtaS, whose product presents a residual primase activity (Brown *et al.* 2010). Thus, the identified homology of BBPR_1314 with this gene suggests that PRL2010 LtaS also present both primase and polymerase activities.

In silico analyses of *B. bifidum* PRL2010 genome highlighted the absence of any *dlt* operon system, which is responsible for LTA modification (d-alanylation of the polyGro-P chain) (Neuhaus and Baddiley 2003). Interestingly, a *dlt* operon system has not been identified in any of the currently decoded genome sequences of *Actinobacteria*, indicating that the typical D-Alanine activation and transfer to PGP-LTA does not take place in this phylum (Rahman *et al.* 2009).

The DNA similarity values displayed by *B. bifidum* PRL2010 homologs with the currently known LTA biosynthetic genes of *S. aureus* or *B. subtilis* are rather low (below 30%), which may be explained by the distant phylogenetic relationships between these bacteria. Nonetheless, the occurrence of these homologous genes in PRL2010 genome strongly suggests that LTA is actually a component of the cell wall of this microorganism.

Prevalence of TA homologs across *B. bifidum* species and *Bifidobacterium* genus

In order to investigate the level of conservation of the genetic repertoire putatively involved in WTA and LTA biosynthesis within the *B. bifidum* taxon, we surveyed publicly available *B. bifidum* genome sequences (Duranti *et al.* 2015), as well as 48 chromosome sequences harboring the type strains of *Bifidobacterium* genus (Lugli *et al.* 2014; Milani *et al.* 2014). The obtained data showed that putative WTA-encoding gene cluster-1 (BBPR_0698–BBPR_0705) is highly conserved within the genomes of *B. bifidum* taxon but not in chromosome sequences of other (sub)species of the genus, thus clearly suggesting that this genetic cluster is *B. bifidum* species specific (Fig. 3).

In contrast, WTA gene cluster-2 and the predicted LTA loci are highly conserved in bifidobacterial genomes (Fig. 3).

These genetic data suggest that members of the *B. bifidum* species contain both the major and the minor WTA in their cell wall, as well as LTA. The genomes of members of the *Bifidobacterium* genus such as *B. pseudolongum* subsp. *pseudolongum* LMG 11571, *B. pseudolongum* subsp. *globosum* LMG 11569, *B. longum* subsp. *longum* LMG 13197 and *B. longum* subsp. *infantis* ATCC 15697 lack the major WTA gene cluster, although they seem to contain some homologous genes, scattered across their chromosomes (Fig. 3).

Transcriptional analysis of TAs loci

Transcriptome experiments based on qRT-PCR were performed upon cultivation of PRL2010 cells under different conditions, simulating those that such microorganisms may encounter in their ecological niche (Turroni *et al.* 2010). Specifically, PRL2010 cells were grown in liquid medium as well as on solid substrates, which have previously been shown to enhance the production of extracellular structures like pili, and thus the responsiveness of cells to environmental stimuli (Turroni *et al.* 2013, 2014a). In

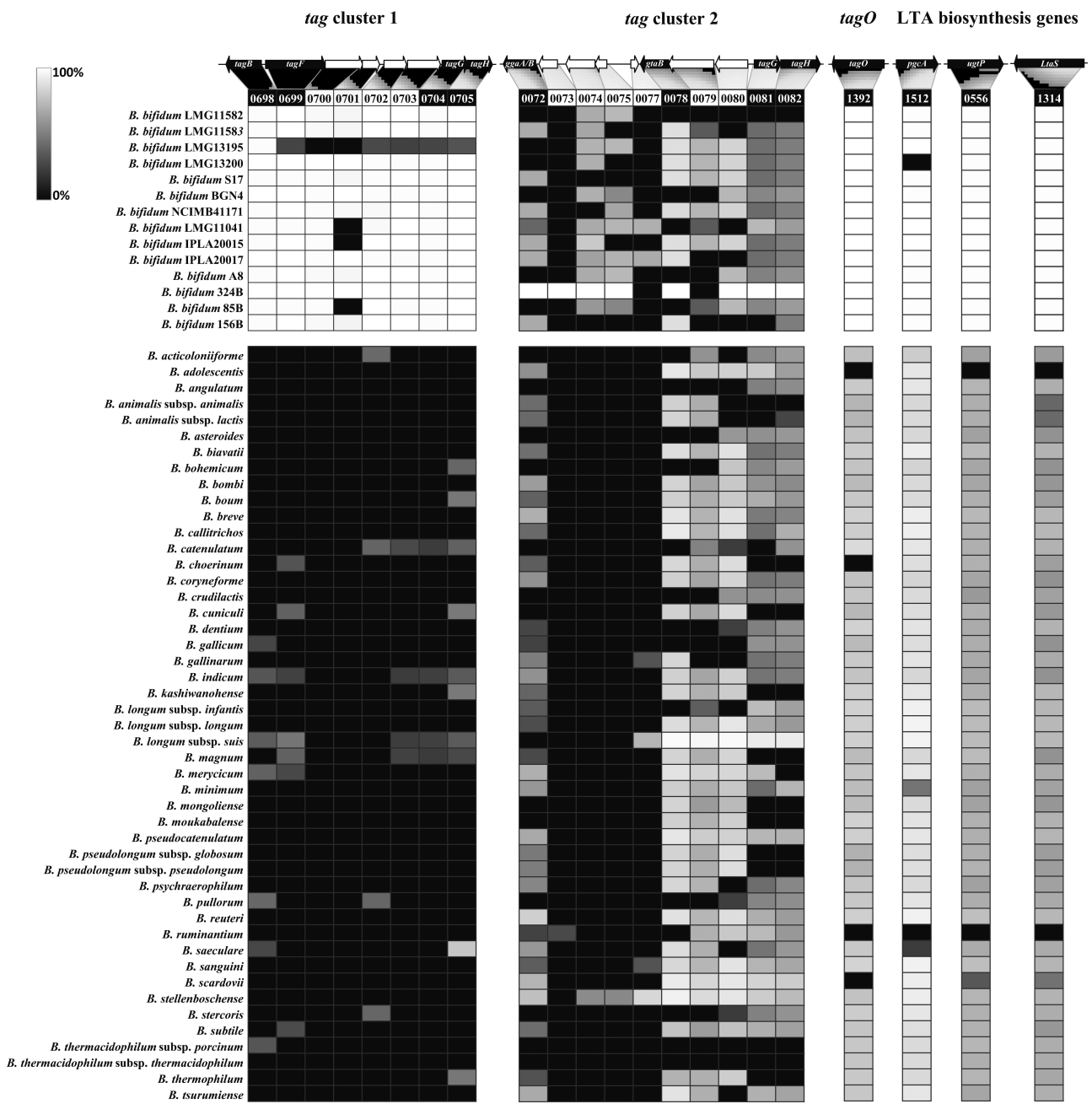


Figure 3. Prevalence of TA homologs across *B. bifidum* species and *Bifidobacterium* genus. Schematic comparative representation of the putative teichoic acid encoding genes of *B. bifidum* PRL2010 and of various other bifidobacterial strains by BLASTp analyses. Gradient of color according to homology percentage.

addition, qRT-PCR assays upon cocultivation of *B. bifidum* PRL2010 cells with other human gut commensals, i.e. *B. breve* 12L or *L. paracasei* LMG9192 cells, which have been highly responsive in terms of production of extracellular structures (Turroni *et al.* 2014a,b), were carried out. qRT-PCR experiments were also performed in order to evaluate the transcription of genes implicated in TA biosynthesis upon *in vivo* assays of colonization of BALBc mice with PRL2010 cells.

qRT-PCR analyses showed a higher level of expression of *wta* cluster-1 (BBPR_0698- BBPR_0705) when *B. bifidum* PRL2010 was cocultivated with *L. paracasei* (≥2.5-fold, $p \leq 0.024$) (Fig. 4b) and during *in vivo* colonization of mice with *B. bifidum* PRL2010 (≥3-fold, $p \leq 0.038$) (Fig. 4c), using as reference condition *B. bifidum* PRL2010 cultivated in MRS broth. In contrast, no significant differences were observed when PRL2010 cells were cultivated on agar plates, except for BBPR_0698 (Fig. 4a).

Analyses on *wta* cluster-2 (BBPR_0072-BBPR_0082) shown a significant increase in the expression (≥2-fold, $p \leq 0.029$) of all three genes encompassing the locus, when PRL2010 was grown on solid surfaces, i.e. MRS agar plates (Fig. 4a). However, no significant changes in expression levels were detected for the other tested conditions in comparison to the transcriptome obtained

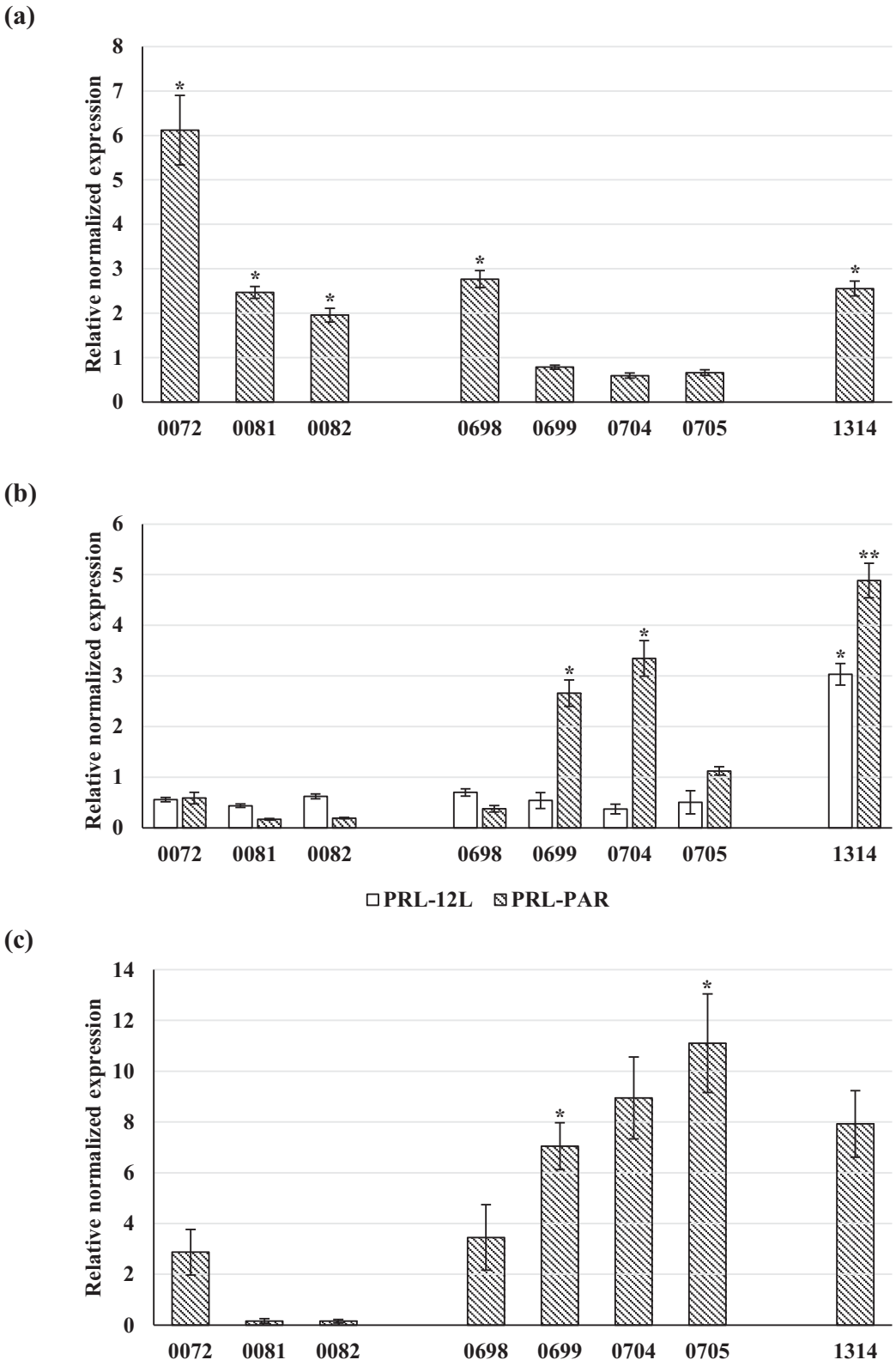


Figure 4. Evaluation of the transcription of PRL2010 TA loci. qRT-PCR analyses showing the relative transcription levels of teichoic acid-encoding genes from *B. bifidum* PRL2010 upon (panel **a)** *in vitro* cultivation on MRS agar plates, (panel **b)** *in vitro* cocultivation with *B. breve* 12L and *L. paracasei* LMG9192 and (panel **c)** *in vivo* colonization analyzed by quantitative real-time PCR assays. The reference condition used was *B. bifidum* PRL2010 cultivated in MRS broth. The *y*-axis indicates the fold induction of the investigated gene compared to the reference condition. Relative normalized expression is calculated as the relative quantity of transcription of a given gene of interest normalized to relative transcription quantity of the reference genes across samples. Asterisks indicate the level of statistical significance of the difference from control value according to an unpaired Student *t*-test (one asterisk, $P < 0.05$; two asterisks, $P < 0.001$).

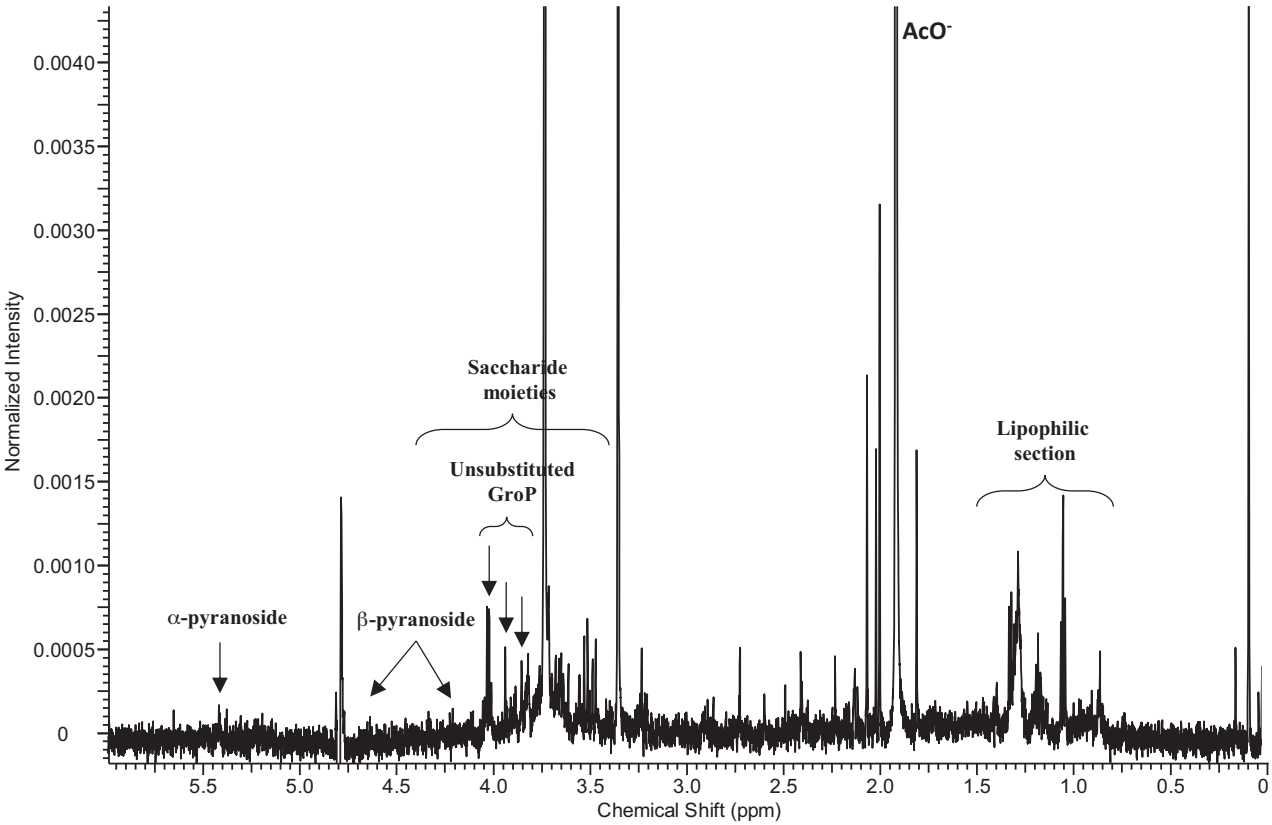


Figure 5. ^{1}H NMR spectrum (600 MHz, 300 K) with water suppression of LTA obtained after butanol extraction of PRL2010 cell wall. The ^{1}H NMR spectrum of LTA extracted from *S. aureus* was used as template (Morath *et al.* 2001).

from *B. bifidum* PRL2010 cultivated in MRS broth, which does not exclude that *wta* cluster-2 is constitutively expressed.

The expression of the *ltaS* PRL2010 homolog (BBPR_1314) was also evaluated. In both *in vitro* and *in vivo* conditions, qRT-PCR analysis showed an induction of this gene ranging from 3- to 8-fold ($P \leq 0.013$) with respect to the reference condition (Fig. 4). Overall, these results suggest a coexpression of BBPR_1314 with genes involved in TA biosynthesis, showing an upregulation of this gene during PRL2010 microbe–microbe and host–microbe interaction.

Bifidobacterium bifidum PRL2010 LTAs chemical characterization

In order to evaluate the chemical characterization of putative LTAs produced by *B. bifidum* PRL2010, we collected PRL2010 cells upon cultivation on MRS plates, which represents a growth condition that caused the highest level of *ltaS* transcription. A total of 56 fractions were obtained from HIC separation/purification on octyl-sepharose column, and 15 fractions were shown to contain phosphate as displayed by molybdenum blue test. These fractions were further chemically characterized by ^{1}H NMR analysis (Fig. 5), although structural investigations of LTA are challenging due to the high microheterogeneity of their fatty acid chains, the length of the repeating unit, the glycosylation and substitution pattern (Rahman *et al.* 2009).

Comparison with ^{1}H NMR spectra obtained from LTA isolated from *B. subtilis*, *S. aureus*, *St. pneumoniae* and *L. monocytogenes* and those from PRL2010 allowed the identification of signals corresponding to typical LTA features. In particular, the high-field region of the spectrum ranging among 0.8 to 1.5 ppm shows several resonances corresponding to the lipophilic section of LTA, while the very complicated pattern of signals ranging from 3.4 to 4.4 ppm is typical of the saccharide moieties of the molecule. Downfield from this crowded region of the spectrum are recognizable three doublets at $\delta = 5.4$, 4.6 and 4.2 ppm with coupling constant J of 3.5, 8 and 9 Hz, respectively. The chemical shift and the coupling constant of the former of these doublet are reasonably ascribed to the H1 proton of the α form of the pyranoside units, while the others to the corresponding β form. An unequivocal assignment of the nature of the pyranoside units of LTA (glucose or galactose or both) on the base of the sole chemical shift was not possible.

The spectrum showed also some peaks (δ_H 3.85, δ_H 3.94 and δ_H 4.02) putatively corresponding to unsubstituted glycerophosphate of the repeating unit of the molecule. Furthermore, no signals of D-alanine substitution of the Gro-P chain were detected, a finding that corroborated our genetic analyses.

The data obtained from chemical characterization trough HIC purification and ^{1}H NMR analysis did not allow the identification of a defined structure of the purified molecules; nonetheless, these data led to the identification of a molecule characterized by features that are typical of LTAs, thus supporting the hypothesis of the occurrence of LTAs in the *B. bifidum* PRL2010 cell wall.

CONCLUSIONS

This study provides the first detailed genetic characterization of TA biosynthesis in the genus *Bifidobacterium* with specific

emphasis on *B. bifidum* taxon. Our analysis of *B. bifidum* genes that encode the biosynthetic machinery for WTA and LTA biosynthesis revealed that compared to other bifidobacterial strains this taxon contains a species-specific WTA pathway, which may imply a taxon-specific phenotypic treat contributed by this *wta* locus. Future research activities will be carried out in order to better characterize WTA in *B. bifidum* PRL2010.

Moreover, the identification of *gtaB ypfP, pgcA* and *ltaS* homologs in the genomes of all type strains of the genus *Bifidobacterium* as well as other *B. bifidum* strains analyzed suggests the hypothesis that the cell wall of these bacteria, that typically contains lipoglycans, might also contain LTAs, which is also supported by chemical analysis of the structure of purified molecules.

The role of the identified TAs in *B. bifidum* PRL2010 is still not known. However, based on the transcriptomic data provided here, it is evident that their expression is enhanced when the PRL2010 cells are established in their ecological niche or in environmental conditions simulating the human gut. Thus, it is possible to envisage a functional role of the PRL2010 TAs structures supporting a microbe–microbe as well as host–microbe interaction in a similar fashion to what previously described for other gut commensals such as *Lactobacillus acidophilus* NCFM (Saber *et al.* 2011).

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