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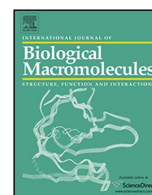

In silico mining and characterization of bifidobacterial lipoprotein with CHAP domain secreted in an aggregated form

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ABSTRACT

Bifidobacterium breve C50 secretes a lipoprotein associated with glucose, acting in an aggregating form (>600 kDa) as an agonist of TLR2/6. Similar lipoproteins were sought for in bifidobacteria. In silico, the closest homology was shown with a *Bifidobacterium longum* protein containing CHAP and lipobox domains. Two strains secreted aggregates whose peptides sequences aligned with the mined protein. C16:0 and C18:0 fatty acids detected in the aggregates further supported a lipoprotein structure. Glucose and mannose detected by gas chromatography were likely ligands of the lipoprotein. The binding of aggregates to galectin-1 indicated that hexosamines and galactose surrounded them. However, unlike *B. breve* C50, aggregate secreted by *B. longum* CBi0703 was unable to bind TLR2/6 likely because of a more hydrophobic structure. In gnotobiotic mice, the intake of *B. longum* aggregate induced, in splenic dendritic cells, the expression of genes involved in antigen presentation. A positive correlation between the number of dendritic cells and $CD4^+CD25^+$ cells was observed in mice receiving these aggregates. In conclusion, *B. longum* secretes a lipoprotein forming aggregates which may influence dendritic and $CD4^+CD25^+$ cell interactions independently of the TLR2/6 pathway.

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1. Introduction

Bifidobacteria have been shown to modulate the host innate immune system [1,2] possibly via an interaction with Toll-like Receptor 2 (TLR2) [3]. TLRs are evolutionarily conserved pattern recognition receptors (PRR) and represent primary triggers of the innate immunity. TLRs are constitutively expressed on monocytes, macrophages, dendritic cells (DCs) and intestinal epithelial cells (IECs) [4,5].

The interaction between bifidobacteria and TLRs can be mediated by extracellular components [6]. We recently described a complex structure secreted by *Bifidobacterium breve* C50 [7]. This structure could have a direct involvement in the interaction between bifidobacteria and TLRs. Hoarau et al. [8] showed that fermentation compounds of *B. breve* C50 were capable of inducing DC maturation and prolong DC survival via a TLR2 pathway. Prolonged DC survival was mediated by the Phosphatidylinositol-3 Kinase Signaling pathway [9]. In humans, BC is possibly involved in the modulation of the microbiota and the enhanced intestinal IgA response to poliovirus vaccination [10,11]. The high molecular weight extracellular components (>200 kDa) released by *B. breve*, acting as TLR2/6 agonists, were characterized as a complex aggregate made up of lipoprotein units associated with sugar [7]. The lipoprotein units contained a CHAP domain surrounded by oligosaccharides consisting of primarily glucose, but also galactose and hexosamines [7].

The CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain belongs to a large family of amidases

Abbreviations: BC, bifidobacterial compounds of high molecular weight; HM mice, descents of gnotobiotic mice associated with human healthy volunteer microbiota; RA mice, descents of gnotobiotic mice associated with human rheumatoid arthritis microbiota; CHAP domain, cysteine, histidine-dependent amidohydrolases/peptidases domain; GF, gel filtration.

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implicated in peptidoglycan hydrolysis. Most of these proteins are uncharacterized and could be multifunctional [12]. The cell-surface-exposed molecule Tga of *B. bifidum* MIMBb75 harbored CHAP and Lytic murein transglycosylase activities, however DCs activation and IL-2 production are induced through the sole CHAP domain [13]. Other protein members of the CHAP superfamily act as surface antigens, suggesting that their exposure at the cell surface is a common feature [14]. Furthermore, they are classified as putative glucan receptors [7].

The aim of this study was to decipher whether other bifidobacteria could secrete similar lipoproteins. Genomes of bifidobacteria were analyzed in silico and a lipobox was sought for in the sequences close to the lipoprotein of *B. breve*. A set of strains from the bifidobacterial species, exhibiting the closest sequence and a putative lipobox, were grown in a broth dedicated for the lipoprotein production. The high molecular weight compounds secreted by the best producing strain were then isolated by gel filtration chromatography and characterized by mass spectrometry and gas liquid chromatography. TLRs and galectin recognitions of the secreted compounds were also assayed. The whole protein sequence was deduced from DNA sequence. The protein sequence without its hydrophobic signal sequence and lipobox (and therefore without the thioacyl group) was cloned and expressed in *Escherichia coli*. Antibodies raised against the recombinant protein were used to detect the protein within the high molecular weight compounds.

At last, difference in TLRs recognition of either bifidobacterial compound could lead to disparate immune response in vivo. The microbiota, especially the intestinal bifidobacteria, has been shown to drive the immune response and to be directly implicated in the modulation of various host gene expression [15–18]. Besides, *B. breve* C50 high molecular weight compounds were shown to promote the intestinal bifidobacterial population [19,20]. Therefore to investigate the potential of the high molecular weight compounds secreted by either strain, gnotobiotic mice with low bifidobacteria counts were used. Dendritic cells, $CD4^+$ and $CD4^+CD25^+$ T lymphocytes were enumerated in spleen after intake of either bifidobacterial compounds. Furthermore the gene expression profile of splenic DCs from mice drinking high molecular weight compounds secreted by *B. longum* was analyzed and compared to the profiles of splenic DCs from control mice.

2. Experimental

2.1. In silico analysis

The protein sequence of *B. breve* C50 (UniProtKB no: A0A076FCY5) was used for Blast searches performed using the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov). Sequence analysis was executed using DNASTAR EditSeq, Map Draw and MegAlign. Alignments and motif searchers were performed using the EMBL-EBI web site (http://www.ebi.ac.uk/Tools/). Hydrophobicity plots were constructed using Expasy software (http://www.expasy.ch/cgi-bin/protscale.pl). Predict (http://www.ch.embnet.org/software/TMPRED_form.html) was used to calculate the transmembrane regions in the protein sequences. The lipoprotein signal peptide and the conserved pattern containing the "lipobox" characterizing the bacterial lipoprotein precursor were predicted in silico. The three bioinformatics tools used were: LipoP 1.0 server (http://www.cbs.dtu.dk/services/LipoP/), DOLOP (www.mrc-lmb.cam.ac.uk/genomes/dolop/) and PROSITE (www.expasy.org/prosite/).

2.2. Bacterial strains and culture conditions

The following *B. longum* strains used were used: the type strain (DSM 20088^T), PRSF B-028, PRSF B-085, PRSF B-086 (obtained in the framework of the EU project 'Biosafety Evaluation of Probiotic Lactic Acid Bacteria Used for Human Consumption'), CBi0701 and CBi0703 (from the collection of Bifinove, Lille, France) [21]. The strains were grown in Rosenow broth (Biorad, Marnes-la-Coquette, France) at 37 °C in anaerobic conditions [22], then adapted to lactose by three subculture in a 5 g/L lactose broth (15 g/L trypticase-peptone, 5 g/L lactose, 5 g/L NaCl, 3 g/L yeast extract, 2 g/L lactalbumin hydrolysate, 3 g/L KH_2PO_4, 3.84 g/L K_2HPO_4, 0.3 g/L ascorbic acid, 0.5 g/L $MgSO_4$ and 0.09 g/L $MnSO_4$). Production of the lipoproteins was carried out in the dedicated broth (lactose 70 g/L, hydrolyzed milk proteins 10 g/L and cysteine hydrochloride 0.03 g/L), previously described for producing *B. breve* C50 lipoprotein [7]. The inoculated broth was incubated at 37 °C under anaerobic conditions for 48 h.

2.3. Isolation of the bifidobacterial compounds

After fermentation in the production broth, the supernatant was collected by high-speed centrifugation, concentrated by means of ultrafiltration (100 kDa) and dialyzed on a 10-kDa membrane. The concentrated supernatants (SN) were lyophilized prior to being subjected to gel filtration chromatography using Superdex 200® column (Sigma–Aldrich, St. Quentin Fallavier, France).

2.4. Stability of the bifidobacterial compounds

The high molecular weight bifidobacterial compounds (BC) secreted by *B. longum* CBi0703 were isolated following gel filtration chromatography and lyophilized prior use. Protein contents were estimated using Lowry method [23].

BC from *B. longum* CBi0703 was subjected to SDS-PAGE (10%) prior and after incubation with the following surfactants: SDS (VWR International France), Nonidet P40 (Sigma–Aldrich), CHAPS (Sigma–Aldrich), Octyl glucoside (Sigma–Aldrich). Gels were stained using Coomassie blue and silver staining procedures.

Hydrolysis of BC from *B. longum* CBi0703 was carried out using HCl (0.6 M) at 100 °C and the released compounds were analyzed by GF chromatography and SDS-PAGE.

2.5. Enzyme linked immunosorbent type assays

The recombinant CHAP domain protein (=BLIF) from *B. longum* CBi0703 lipoprotein was produced in *E. coli* by Proteogenix (France). The cloned cDNA sequence (obtained as described in Section 2.8) started with the codon corresponding to amino acid number 20. The recombinant BLIF protein was purified using affinity column. Rabbit polyclonal antibodies were raised against the recombinant protein (Proteogenix). Lyophilized powders of the concentrated supernatant and of the gel filtration chromatography fraction were diluted in carbonate buffer (0.05 M, pH 9.6), then coated in a 96 well microplate (Sigma–Aldrich) and incubated overnight at 4 °C. The following day a series of 5 washes was performed using 10 mM PBS with 0.05% Tween 20, before adding the blocking solution (10 mM PBS, 0.5% Tween 20 and 2% BSA). This was followed by the addition of either anti-BLIF or Recombinant Mouse TLR2 or TLR6 Fc human chimera (R&D Systems Europe, Lille, France). Microplates were then incubated for 1 h at 37 °C. After another 5 washes, biotinylated monoclonal anti-human IgG against TLR2 or TLR6 Fc and biotinylated monoclonal anti-rabbit IgG against anti-BLIF antibodies (Sigma–Aldrich) were added and incubated for 1 h at 37 °C. After another series of 5 washes, streptavidin-peroxidase (Sigma–Aldrich) was added and incubated for 1 h at 37 °C. The revelation step was then performed using SIGMAFAST™ OPD (Sigma–Aldrich). The reaction was stopped by the addition of 50 µl H_2SO_4 (0.5 M) per well. The optical density (OD) was

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