FRUTOSE UPTAKE IN BIFIDOBACTERIUM LONGUM NCC2705 IS MEDIATED BY AN ABC TRANSPORTER

Xiao Wei1#, Yanhong Guo1,3#, Changlin Shao1,3#, Zhongke Sun1#, Daria Zhurina2, Dawei Liu1, Wei Liu1, Dayang Zou1, Zheng Jiang1, Xuesong Wang1, Jianguo Zhao1, Wei Shang2, Xuelian Li1, Xiangru Liao2, Liuyu Huang1*, Christian U. Riedel2*, Jing Yuan1*

1 Institute of Disease Control and Prevention, Academy of Military Medical Sciences, Beijing, China
2 Institute of Microbiology and Biotechnology, University of Ulm, Germany
3 School of Biotechnology, Jiangnan University, Wuxi, China
#: These authors contributed equally to this work.

Running title: A fructose-specific ABC transporter in Bifidobacterium longum NCC2705

To whom correspondence should be addressed: Prof. Jing Yuan, Institute of Disease Control and Prevention, Academy of Military Medical Sciences, No. 20 Dongda street, Fengtai District, Beijing, China, 100071; Tel: +86-10-66948434; Fax: +86-10-66948301; E-mail: yuanjing6216@163.com; Dr. Christian U. Riedel, Institute of Microbiology and Biotechnology, University of Ulm, Germany, D-89069 Ulm. Tel: +49-7315024853, Fax: +49-7315022719, E-mail: christian.riedel@uni-ulm.de; Prof. Liuyu Huang, Institute of Disease Control and Prevention, Academy of Military Medical Sciences; No. 20 Dongda street, Fengtai District, Beijing, China, 100071, Tel: +86-10-66948301; Fax: +86-10-66948301; E-mail: huangliuyuly@163.com

Key words: monosaccharide uptake, ABC Fru-transporter system, protein-protein interactions, specificity and reversibility, substrate binding

Background: Fructooligosaccharides as prebiotics are a preferred carbon source of bifidobacteria.

Results: A potential fructose-specific transporter was analyzed in B. longum NCC2705.

Conclusions: The identified transporter is a functional ABC transporter with high affinity for fructose and is regulated by its substrate.

Significance: This transporter represents that first fructose-specific sugar transporter identified in bifidobacteria and might display a slightly different conformations than previously published ABC transporters.

SUMMARY

Recently, a putative ABC transport system was identified in B. longum NCC2705 that is highly upregulated during growth on fructose as sole carbon source. Cloning and expression of the corresponding ORFs (bl0033-0036) results in efficient fructose uptake by bacteria. Sequence analysis reveals high similarity to typical ABC transport systems and suggests that these genes are organized as an operon. Expression of FruE is induced by fructose, ribose or xylose, and is able to bind these sugars with fructose being the preferred substrate. Our data suggest that BL0033-0036 constitute a high-affinity fructose-specific ABC transporter of B. longum NCC2705. We thus suggest to rename the coding genes to fruEKFG and the corresponding proteins to FruE (sugar binding protein), FruK (ATPase...
subunit), FruF and FruG (membrane permeases). Furthermore, protein-protein interactions between the components of the transporter complex were determined by GST pull-down and Western blot analysis. This revealed interactions between the membrane subunits FruF and FruG with FruE, which in vivo is located on the external side of the membrane, and with the cytoplasmatic ATPase FruK. This is in line with the proposed model for bacterial ABC sugar transporters.

Bifidobacteria are Gram-positive, high G+C content bacteria commonly present in human and animal intestines. The potential health-promoting or probiotic activities of bifidobacteria are well known and include reduction of symptoms of irritable bowel disease (1), stimulation of the immune response (2), and inhibition or competitive exclusion of pathogenic bacteria (3). Carbohydrates represent one of the major structural building blocks of all organisms. Colonization and survival of bifidobacteria in the large intestine are dependent on utilization and uptake of fermentable carbohydrates not absorbed or metabolized by the host. As saccharolytic organisms, bifidobacteria could utilize a wide range of catabolic pathways that confer a growth advantage over other bacteria (4, 5). Several non-digestible carbohydrates such as oligofructose, inulin, and raffinose, have been identified as bifidogenic compounds and are used as food additives (prebiotics) to selectively promote growth of bifidobacteria in the gut (5, 6).

Transport of sugars across the cytoplasmatic membrane is a prerequisite for the subsequent carbohydrate metabolism of bacteria. More than 8.5% of the predicted proteins encoded on the genome of \textit{B. longum} NCC2705 are involved in carbohydrate transport and metabolism, approximately 30% more than observed in other organisms (5). Bifidobacteria are reported to possess only one metabolic pathway for the fermentation of hexoses – the bifidus shunt. The first central intermediate of the bifidus shunt, fructose-6-phosphate, links this pathway to several other pathways including N-acetyl hexose fermentation, galactose catabolism, and peptidoglycan biosynthesis (7). Although \textit{B. longum} is able to grow on a wide variety of sugars as sole carbon source including glucose, fructose, and ribose, the specificity of the majority of transporter systems that feed these sugars into the catabolic pathways have not been defined to date.

Three energy-dependent sugar uptake mechanisms have been characterized in bacteria. The widely major facilitator superfamily (MFS) transporters are used for the uptake of galactose, xylose and lactose in \textit{E. coli} and operate by proton symport. A second system is the phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) for transporting glucose, fructose, mannose, and sucrose found in many Gram-negative bacteria. A third mechanism is the periplasmic binding protein-dependent ATP-binding cassette (ABC)-transporter which is ubiquitous membrane protein complexes that mediate the energy generated from ATP hydrolysis to uptake or export of a large variety of solutes across biological membranes (8, 9).

Most ABC transporters share a modular architecture containing two transmembrane domains or subunits (TMDs) and two cytosolic nucleotide-binding domains or subunits (NBDs), also known as ATP-binding cassettes. The substrate specificity is accomplished by the TMDs, which display basically no sequence homologies and feature varying numbers of transmembrane helices among different ABC transporters. NBDs with the ability to bind and hydrolyse ATP can provide the energy required for substrate translocation. In addition to the classical nucleotide-binding Walker A and B
motifs, each NBD also contains a conserved LSGG(N)QQ signature motif that is diagnostic of ABC ATPases (10). One of the best functionally characterized ABC transporters is the maltose uptake system (MalFGK2-E) of *E. coli* and *Salmonella typhimurium*, which is composed of a periplasmic maltose-binding protein (MBP; MalE), two integral membrane proteins (MalF and MalG), and two copies of the cytoplasmic ATP-binding cassette MalK. Thus, this system serves as a model for studying the molecular mechanism by which ABC importers exert their functions (11-14). In bacteria, the ABC superfamily transports a wide range of substrates, including a variety of monosaccharides such as arabinose or ribose as well as di- and tri-saccharides or higher oligosaccharides (15).

A total of 19 carbohydrate uptake systems were predicted from the genome sequence of *B. longum* NCC2705 in GenBank database provided by the National Center for Biotechnology Information, of which 13 are putative ABC transporters (5). Although carbohydrate utilization is critical to understanding bifidobacterial survival and colonization in the gut, only a few sugar transport systems in this genus have been characterized to date. A PEP:glucose PTS has been characterized in *B. breve* NCBF 2275. Activities of a lactose/proton symporter, a glucose/potassium symport, and an unsaturable galactose permease have been detected in *B. bifidum* DSM 20082 (7, 16). In *B. longum* NCC2705, glucose PTS activity and an inducible glucose/proton symport subject to lactose repression were detected (17, 18).

The transport of fructose in bacteria is usually performed by a fructose-specific PTS, which upon transport generates fructose-1-phosphate, or MFS transporters (9). Fructose-specific PTS systems show several features that are not found in the PTS of other sugars. While the PTS systems for other sugars use a common HPr to transfer phosphate between enzyme I and the sugar-specific enzyme II, the fructose-specific PTS has its own phosphate-carrier FPr (19).

Bioinformatics analysis of *B. longum* NCC2705 genome sequences revealed no fructose-specific PTS (5). We recently reported on a sugar-binding protein (BL0033) and ATP-binding protein (BL0034) which are part of a putative ABC transport system with similarity to the ribose-specific ABC transporter of *E. coli* (17) and are induced when bacteria are grown on fructose, ribose and xylose as sole carbon sources (20). We thus speculated that BL0033-0036 is an ABC transporter system involved in the uptake of fructose, ribose and/or xylose. This transport system could play an important role because fructose and fructooligosaccharides are preferred substrates of bifidobacteria. These fructose-containing sugars are components of human breast milk and frequently used as probiotics. It is presumed that they might be responsible for the early colonization of infants. In 2011, Fukuda *et al.* could show that *B. longum* NCC2075 and other bifidobacteria harbouring homologues of BL0033-0036 exert a protective effect against *E. coli* O157:H7 infections in mice while a mutant of *B. longum* NCC2075 and strains that did not encode homologues of BL0033-0036 had no protective effect (21). Moreover, compared to the mutant, *B. longum* NCC2075 was more efficient in the conversion of fructose to acetate in vitro and the amount of acetate in feces of mice treated with the wildtype strains was significantly higher (21). Both, the protective effect and efficient acetate conversion could be transferred to strains that did not encode homologues of BL0033-0036 by heterologous expression of the corresponding genes *bl0033*-*0036* (21).

We thus propose to rename the genes *bl0033*-*0036* into *fruE*KFG and the encoded proteins into FruE, FruK, FruF and FruG. Here, we report the molecular characterization of a
fructose-specific ABC transporter in *B. longum* NCC2705. In addition to the preferred substrate fructose, ribose and xylose might be also transported by this system albeit with lower affinity. GST pull down and Western blot experiments showed interactions of the different components of the ABC transporter. Identification and characterization of carbohydrate transporter systems of bifidobacteria will help to further understand the nutritional lifestyle and their contribution to the protective effects of this important group of intestinal bacteria.

**EXPERIMENTAL PROCEDURES**

**Strains, medium, and growth conditions**—Strains, plasmids and oligonucleotides used for this study are listed in Table 1. *B. longum* DCP-18, deficient in fructose-transport and fermentation (see Table S1), was used as host for homologous complementation with bl0033. To analyze expression of ABC transporter system, *B. longum* strain NCC2705 (kindly provided by Nestlé Research Center, Lausanne, Switzerland) was grown anaerobically at 37 °C in 400 mL of De Man-Rogosa-Sharpe (MRS) broth (22) containing 0.05 % L-cysteine or modified Garches medium (MGM) containing D-glucose, fructose, xylose, or D-ribose as the sole carbon source as described previously (20) for 8 h (early exponential phase), 12 h (midexponential phase), 16 h (end of exponential phase), and 24 h (stationary growth phase). In the concentration series experiments, *B. longum* NCC2705 cells were grown in MGM supplemented with either fructose, glucose, xylose or D-ribose at 1, 2, 3, or 4 g/l, respectively. Cultures were grown in 500 ml flask and anaerobic conditions were maintained by sparging the cultures with O₂-free N₂ gas (5 ml/min). For experiments, bacteria were harvested in mid-exponential phase at an *A*₆₀₀ of 0.9 corresponding to 1.5×10⁸ colony forming units/mL.

For testing specificity and reversibility of relative gene expression induced by fructose, cultures were grown in 400 ml MGM containing fructose or glucose for 6 h as described above. Then each bacterial culture was washed twice with pre-warmed PBS, and the cells were split in two and resuspended in 400 ml of fresh medium with either fructose or glucose as control at a final concentration of 2 g/l. The two cultures were then incubated further for 6 h.

*Escherichia coli* DH5α, Rosetta (DE3) and BL21-CodonPlus (DE3)-R-IL were used cloning and expression hosts. *E. coli* mutant LR2-177 was used for heterologous complementation experiments. *E. coli* strains were routinely grown at 37°C in Luria-Bertani (LB) medium with shaking. Ampicillin (Amp) was added to a final concentration of 100 µg/ml for the selection of transformed bacteria in LB medium.

**Sugar transport assays**—The radiolabeled sugars [U⁻¹⁴C] fructose (10.2 GBq/mmol) and [U⁻¹⁴C] glucose (10.9 GBq/mmol) were purchased from GE Healthcare. Sugar transport assays were performed as described (9). Cells grown in MGM medium were harvested at OD₆₀₀ of 0.5 to 0.7, washed twice in PBS, and resuspended in carbon-free MGM medium at a final OD of 0.5. Uptake was determined by rapid filtration through glass microfiber filters (Whatman GF/F) which were rinsed with 3 ml of MGM medium. The radioactivity remaining on the filters was detected with a liquid scintillation spectrometer (Beckman Instruments, Villepinte, France). For competition experiments, cold unlabeled fructose, ribose, xylose, mannose, galactose, and glucose were added at a final concentration of 4.5 mM into a 50 µM [U⁻¹⁴C] fructose solution for 1min before filtration. All assays were conducted in triplicate on two independent cultures at 37 °C with 1 ml of cell suspension and radioactive substrate (100,000 dpm) at final concentrations of 0.5 to 500 µM for 1 to 10 min.

Determination of *Kₘ* and *Vₘₐₓ* was carried out
in *B. longum* NCC2705, *E. coli* LR2-177 (pGEX-FruEKFG) and *B. longum* DCP-18 (pDG7-FruE). For heterologous expression of pGEX-FruEKFG was induced in *E. coli* LR2-177 (23) for one hour (OD$_{600}$ of 0.5) with 1 mM IPTG. Cells were harvested and washed twice with chilled PBS. Fructose uptake was conducted at 37°C as described above.

**Specific substrate binding of FruE**- GST fused FruE and GST alone were purified by affinity chromatography using glutathione-sepharose 4B beads (GE Healthcare). After washing with buffer B (10 mM Tris-HCl pH 8.0, 14 mM β-mercaptoethanol, 0.1 % Tween 20, 20 % (v/v) ethanol and 0.5 M NaCl), proteins were eluted with buffer C (10 mM reduced glutathione, 50 mM Tris-HCl pH 7.5). Fructose, ribose, xylose or glucose (final concentration 1 g/l) was separately added into 10 ml purified protein solution and incubated for 1 h at room temperature. The mixture was transferred into ultrafiltration tubes which can retain macromolecules. After centrifugation at 1000×g for 30 min, both retentate and filtrate were collected and subjected to capillary electrophoresis (P/ACE MDQ capillary electrophoresis system, Beckman Coulter, CA, USA). Furthermore, the capacity of BL0033 to bind substrates was assayed by an enzymatic test using a Glucose/Fructose Kit (BioSenTec Co, France) at different substrate concentrations and incubation times according to the manufacturers instructions. In all experiments non-fused GST was used as a negative control and purified protein as positive control.

**Preparation of whole cell protein extracts**- Preparation of whole cellular protein extracts was performed as described previously (24) with modifications. Briefly, the cells were centrifuged for 10 min at 8,000×g in a Sigma 3K12 centrifuge (Sigma, St. Louis, MO, USA), and washed 4 times with 40 ml ice-cold low-salt washing buffer (3 mM KCl, 1.5 mM KH$_2$PO$_4$, 68 mM NaCl, 9 mM NaH$_2$PO$_4$) (24). Bacterial pellets were resuspended in 5 ml of lysis buffer (7 M urea, 2M thiourea, 4% (w/v) CHAPS and 50 mM DTT) containing complete protease inhibitors (Roche Diagnostics, Mannheim, Germany) at 1.25 mM. The cells were disrupted by sonication for 5 minutes (cycles of 2 s of sonication followed by a 3 s rest) on ice with a Sonifier 750 (Branson Ultrasonics Corp., Danbury, CT, USA) set at 25 % duty cycle. After adding 2.5 mg of RNase A (Promega, Madison, WI) and 100 unit of RQ1 DNase (Promega), the bacterial lysate was incubated for 1 h at 15°C to solubilize proteins, and then centrifuged for 45 min at 20,000×g to pellet the insoluble components. The supernatants were collected and protein concentrations were measured using the PlusOne 2-D Quant Kit (GE Healthcare Life Sciences, Uppsala, Sweden). Samples were stored at -70°C in 1 mg aliquots until further use. Experiments were performed at least six times.

**Two-dimensional polyacrylamide gel electrophoresis**- Isoelectric focusing (IEF) was carried out as described previously (24) by loading 1 mg of protein onto pH gradient (IPG) strips in three pH ranges (pH 3-10, nonlinear/linear, 18 cm; pH 4-7 and pH 4.5-5.5, linear, 18 cm; Amersham Pharmacia Biotech, Sweden). IEF was conducted at 20 °C for 65,000 Vhrs in the IPGphor system (GE healthcare Life Sciences). For the second dimension, vertical slab SDS-PAGE (12.5 % gel) was performed using the Bio-Rad Protein II Xi apparatus (Bio-Rad laboratories, Hercules, CA, USA), with runs for about 4 h at 30 mA per gel. Following electrophoresis, gels were stained with Coomassie Brilliant Blue G-250 (Amresco, Solon, OH, USA) and then scanned with an ImageScanner (GE healthcare Life Sciences). Image analysis was carried out using ImageMaster 2D Platinum software (GE Healthcare Life Science). To facilitate the discrimination between true spots and artifacts, the spot detection parameters of the software were set to: smooth, 3; minimum area, 50; and
saliency, 6. The relative volume of each spot
was quantified by determining the spot intensity
in pixel units, and normalizing that value to the
sum of the intensities of all the spots in the gel.
Proteins were considered differentially
expressed if their relative intensity differed more
than 3-fold between the two conditions
compared. Each experiment was performed at
least three times.

In-gel protein digestion and MALDI-TOF/
TOF MS/MS and protein identification- The
Coomassie-stained protein spots were excised
and the protein digested as previously described
(24, 25). MALDI-TOF/TOF MS/MS
measurements were performed on a Bruker
Ultraflex III TOF/TOF-MS (Bruker Daltonics
GmbH, Bremen, Germany) equipped with a 337
nm wavelength nitrogen laser (model LSI 337I,
Bruker) working in reflectron mode. The mass
range of peptides detected (M/Z) was from 800
to 4500 Da. The MASCOT search engine uses
mass spectrometry data to identify proteins from
primary sequence databases as previously
described (25). Peptide mass fingerprinting
(PMF) searches were performed by using the
program MASCOT v2.2.06 (Matrix Science Ltd,
http://www.matrixscience.com) licensed
in-house (http://www.proteomics.cn) against the
publically available NCBI non-redundant
protein databases (NCBI n r v20100616,
11205216 sequences; 3821460163 residues). For
those proteins identified in the NCBI n r database,
the proteins of B. longum spp. were selected as
the best hits from the lists of homologue
proteins.

Nanospray ESI MS/MS- The peptide solution
collected after in-gel protein digestion was dried,
reconstituted in 30 μl of 0.1% TFA in 30%
ACN/water, and then desalted with ZipTip C18
pipette tips (Millipore, Bedford, MA, USA).
ESI-MS/MS was carried out with a hybrid
quadrupole orthogonal acceleration tandem mass
spectrometer (Q-TOF2; Micromass, Manchester,
UK). Glufibrinopeptide was used to calibrate the
instrument in the MS/MS mode, and internal
 calibration was carried out using enzyme
autolysis peaks. MS/MS peaks lists were created
by MaxEnt3 (MassLynx v3.5, Micromass,
Manchester, UK), and amino acid sequences
were interpreted manually using MassSeq
(Micromass, Manchester, UK). All MS/MS ion
database searches were performed using
MASCOT v2.2.06 on the freely accessible
internet website (http://www.matrixscience.com)
against protein databases of NCBI n r
(v20100430, 10927723 sequences; 3720794783
residues of all bacteria). We checked the
fragment ion intensity patterns as the peak
intensities should be matched by the search.

Gene expression analysis by semi-quantitative
RT-PCR- The relative abundance of mRNA of
genes of interest was analyzed by
semi-quantitative RT-PCR. Total RNAs
were extracted using the EPICENTRE MasterPure
RNA Purification kit (Epicentre Technologies,
Madison, Wis.) as recommended by the
manufacturer. The RNA concentrations were
determined by an ND-1000 Spectrophotometer
(Nanodrop Technologies) at 260 nm. Reverse
transcription was performed using the
Omniscript Reverse Transcription kit (Qiagen)
with 2 μg of total RNA as template. Primers
used for RT-PCR assays were designed to
generate PCR products of comparable sizes
(Table 1). The PCR amplification was performed
with the following conditions: 94°C for 5 min
initial denaturation followed by 25-30 cycles at
94°C for 20 s, 60°C for 30 s, and 72°C for 1 min;
and a final extension at 72°C for 5 min to
complete the reaction. In order to control for
RNA quantity, RT-PCR was performed
targeting the 16S rRNA as described elsewhere
(26). Negative control PCRs were performed
without primers, reverse transcriptase, or Taq
polymerase (Promega) to confirm the absence of
contaminating DNA in the RNA preparations.
PCR products were analyzed on a 1% agarose
gel and visualized by ethidium bromide staining.
Signal intensities were quantified using Quantity One quantitation software (Bio-Rad).

Cloning, expression and purification of the ABC transport system and antibody preparation:

Total cellular DNA was prepared from *B. longum* NCC2705 as previously described (24). Based on the gene sequence of the component of the identified ABC transporter system (*bl0033, bl0034, bl0035* and *bl0036*), primers were designed (Table 1) to amplify these genes by PCR.

The amplified PCR products were cloned into plasmids pET32a and/or pGEX-4T-1 or pDG7 (27) using *Bam*HI and *Xho*I restriction sites (underlined in the primer sequences in Table 1). All constructed expression plasmids were verified by DNA sequencing (AUGC Company, Beijing). Transformants were cultured in LB broth with Amp (100 mg/l) to an OD<sub>600</sub> of 0.6. Expression of the inserted gene was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.05 mM; GIBCO BRL), and the culture was grown for an additional 10 h at 16°C. To determine the subcellular localization of the expressed protein, the supernatant and pellet were analyzed on a 12% SDS-PAGE using a Mini-Protein II gel apparatus (Bio-Rad).

The GST-fusions of the two membrane permeases FruF and FruG were purified using a protocol for the preparation of insoluble proteins (28). *E. coli* Rosetta strains containing the pGEX-constructs harboring *fruF* or *fruG* were grown at 37°C in LB medium with 100 μg/ml of ampicillin to an OD<sub>600</sub> of 0.6, and induced with 0.5 mM of IPTG for 4h. Cells were harvested and washed twice with chilled STE (10mM Tris, pH8.0, 150 mM NaCl, 1 mM EDTA). The pellet was solubilized in ice-cold STE containing 100 μg/ml of lysozyme, and incubated on ice for 15 min. Just before sonication, add 5mM DTT, 1.25mM protease inhibitors (Roche Diagnostics, Mannheim, Germany), and 0.7% sarkosyl (Sigma). Mixed thoroughly for 5s, cells were sonicated on ice for 1 min. After centrifugation at 16,000×g for 20 min, the supernatant was transferred to a new Eppendorf tube, Triton X-114 was added to the desired final concentration (2%) from a 10% stock in STE, and the lysate was incubate at room temperature for 30 min.

GST fusion proteins were purified by Glutathione-sepharose 4B beads (GE healthcare) to bacterial lysates and incubation at room temperature for 1h. Beads were washed once with PBS and protein was eluted by GST elution butter (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0).

In the case of the His<sub>6</sub>-tagged constructs, the coding DNA fragments were amplified from *B. longum* NCC2705 genomic DNA and fused to an His<sub>6</sub>-tag in pET32a at the N-terminus. Polyhistidine has a high affinity for nickel-nitrilotriacetic acid (Ni-NTA) resin. The His<sub>6</sub>-tagged protein was purified batch-wise under native conditions using Ni-NTA agarose and 1 mg/ml total protein from bacterial pellets following the manufacturer’s instructions (Qiagen). The washing buffer contained 20 mM imidazole and the elution buffer contained 500 mM imidazole. The protein was concentrated using an Amicon Ultra-4 Centrifugal Filter Unit equipped with a 50 kDa cut-off membrane (Millipore, Billerica, MA, U.S.A.), transferred to 100 mM Tris-HCl buffer, pH 7.4, and stored on ice. Protein concentrations were determined by the Bradford method. All protein samples were subjected to SDS-PAGE and analyzed by Western immunoblotting as described below.

To generate polyclonal antibodies, purified His- tagged BL0033, BL0034, BL0035 and BL0036 proteins were injected subcutaneously into BALB/c female mice. Sera from the immunized mice were collected and purified using an Immobilized Protein A kit (Pierce) according to the manufacturer’s instructions.

Resistance of FruF and FruG to proteinase K was determined as described (29) by adding 100
mg/ml of proteinase K to *E. coli* spheroplasts expressing either FruF or FruG, and subsequent analysis by SDS-PAGE and Western blot using specific antibodies.

**Protein-protein interaction by GST-pull down and Western blotting**— In GST pull-down experiments, the GST-fusions were incubated with bacterial lysates of *E. coli* BL21 cells expressing different His6-tagged proteins in equimolar ratios for 2 h in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.5) at 4°C. Glutathione-sepharose 4B beads were added and the bound proteins were precipitated by centrifugation.

Total cell proteins or periplasmic proteins were separated by PAGE and transferred to nitrocellulose membrane (Hybond protein; 0.2-mm pore size) by electroblotting. Immunoblotting was performed using a 1/5000 dilution of a polyclonal serum raised against FruE or FruK of *B. longum* NCC2705. In direct binding assays, the adsorbates were washed with lysis buffer and then subjected to SDS-PAGE and immunoblot analysis with anti-His, anti-GST and anti-FruE or anti-FruK antibodies. In all GST pull-down and Western blotting experiments, GST-vector or His-vector was used as negative control.

**Bioinformatic analysis**— Proteins sequences were analyzed for conserved domains/motifs, trans-membrane helices etc. using the web-based InterProScan Sequence Search (http://www.ebi.ac.uk/Tools/pfa/iprscan; (30)) and the ScanProsite software of the Swiss Institute of Bioinformatics (http://prosite.expasy.org). The signal peptide of BL0033/FruE was verified using the SignalP server version 4.0 (http://www.cbs.dtu.dk/services/SignalP, (31))

**Statistical analysis**— Each experiment was performed at least three times. Statistical significance was determined using the Student’s *t*-test.

---

**RESULTS AND DISCUSSION**

**Fructose uptake activity in *B. longum* NCC2705** To analyze the parameters of fructose transport *B. longum* NCC2705 was grown in MGM medium with 1 g/l fructose as sole carbon source to mid exponential growth phase. Bacteria were then washed and uptake of fructose was measured using [U-14C] fructose concentrations ranging from 0.5 to 500 mM (data not shown). The apparent *Km* of fructose uptake by *B. longum* NCC2705 was found to be 8 ± 1 μM with a *V*<sub>max</sub> of 45 ± 5 nmol/min/mg of protein, indicating the presence of a high-affinity fructose transport system. Growth in presence of 2 g/l fructose did not significantly modify the *Km* but resulted in a doubling of the *V*<sub>max</sub> (96 ± 4 nmol/min/mg of protein), indicating that the substrate induces its own transport.

**Analysis of bl0033-0036 in fructose-negative strains** Analysis of the fermentation pattern of 45 *B. longum* strains isolated from human feces revealed that one of the isolates, *B. longum* DCP-18, was unable to grow on fructose as a sole carbon source (i.e. it is fructose-negative, Table S1). We thus performed a time course analysis of fructose uptake with a substrate concentration of 10 μM, which clearly demonstrated that *B. longum* DCP-18 is not able to transport fructose (Fig. 1A). Similar results were obtained for substrate concentrations up to 100 μM. In contrast, transport assays using glucose showed that the uptake of the sugar was not altered in *B. longum* DCP-18 (data not shown), which in agreement with the growth phenotype. These results clearly suggested that in *B. longum* NCC2705 (and other strains) fructose is transported by a unique high-affinity system, which is absent or inactivated in the fructose-negative strain *B. longum* DCP-18.

The presence of all four genes of the operon encoding our putative fructose-specific ABC transporter (*fruE-G*) was analyzed by PCR on isolated genomic DNA. All strains harboured homologues of *fruK*, *fruF*, and *fruG*. By contrast,
B. longum DCP-18 was the only strain that did not yield a PCR product for *fruE* (Table S1), suggesting that this strain lacks a functional *fruE* gene. In order to probe if this activity is related to the putative sugar binding protein FruE, *fruE* was cloned into pDG7 under the control of its native promotor and transformed into *B. longum* DCP-18. Interestingly, the recombinant strain *B. longum* DCP-18 (pDG7-FruE) was able to grow on fructose as sole carbon source and, more importantly, was able to transport fructose with almost the same efficiency as *B. longum* NCC2705 (Fig. 1A).

Additionally, the full length operon *fruE-G* was cloned into pGEX-4T-1 and the resulting plasmid transformed into *E. coli* LR2-177, a strain that is not able to transport fructose (23). This successfully complemented the fructose-negative phenotype of *E. coli* LR2-177 (Fig. 1B). Collectively these data strongly suggest, that *fruE-G* encode for a functional ABC transporter specific for fructose.

**Sequence analysis of the fructose transporter components** The putative ABC transporter is encoded by the four open reading frames *fruE-G* preceded by conserved ribosome-binding sites. Transcriptional coupling is very likely in the case of *fruK, fruF* and *fruG*, which are overlapping by two and three nucleotides, while *fruE* and *fruK* are separated by an intergenic region of 141bp. In silico analysis of this intergenic region between *fruE* and *fruK* did not detect putative transcriptional terminator structures in this intergenic region. This suggests that *fruEFGK* form an operon. The deduced amino acid sequence of the four gene products showed significant similarity to the *E. coli* proteins RbsB (FruE), RbsA (FruK) and RbsC (FruF and FruG) which form high-affinity ribose-specific ABC transporter (Fig. S1A-C).

A search for conserved domains/motifs revealed that FruE contains a conserved periplasmatic sugar binding domain (PFAM PF00532, amino acid residues 40-268) and a Gram-positive lipoprotein precursor signal peptide with a predicted cleavage site between amino acid residues G24 and S25 (Fig. S2A). The FruK protein contains a duplicate ATP-binding cassette domain (PFAM PF00005, residues 47-173 and 305-433) the ATP-binding and hydrolysis motifs Walker A (GXXGXGKS, residues 40-47) and Walker B (XiDEPT, residues 166-173 and 426-433) at the end of the ATP binding cassettes as well as the conserved signature motif (LSGGNQQ/RQ, residues 406-412), which are all specific features of the ATP-binding subunits of ABC transporters (Fig. S1B; (15, 32)).

Both, FruF and FruG display a ABC transporter permease domain (PFAM PF02653, FruF: residues 60-325; FruG: residues 55-326) and 7 and 8 transmebrane helices, respectively (Fig. S1C). Moreover, by aligning the amino acid sequence of FruF to other known membrane permeases ABC sugar transport systems identified in of Gram-positive bacteria, a conserved EAA loop was identified (Fig. S1D). The resistances of soluble FruF and FruG against proteinase K was tested and revealed that both proteins were resistant both at 37 and 42 °C as described previously for MalF of *E. coli* (29).

Thus, FruE, FruK, FruG, and FruF are the sugar-binding periplasmic protein, the ATP-binding cytoplasmic protein, and the integral membrane proteins (permease) of the putative ABC sugar transporter. We name this fructose transporter into FruEFGK according to the name of the maltose uptake system (MalEFGK) of *E. coli* and *Salmonella typhimurium*.

**Sugar substrate binding specificity of FruE** Based on the similarity to the ribose ABC transporter of *E. coli*, Parche et al. suggested that this system may transport ribose or ribose-containing oligosaccharides in *B. longum* NCC2705 (17). However, at the same time, the authors observed that this operon was induced in...
the presence of FOS, which is in contrast to the predicted substrate specificity (17). Recently, we identified FruE as one of the proteins that are induced when *B. longum* NCC2705 is grown in the presence of fructose, ribose and xylose (20). We thus analyzed, the substrate specificity of FruE in more detail.

To demonstrate FruE sugar-binding activity, a GST-FruE fusion protein was incubated with fructose, ribose, xylose or glucose and analyzed by capillary electrophoresis. Furthermore, the binding intensity of the substrate was assayed and unfused GST was used as a negative control in all experiments as described in the methods section. A specific peak of FruE-fructose, FruE-ribose, or FruE-xylose was observed at approximately 2 min retention time when GST-FruE was incubated with a 5 mM excess of fructose, ribose and xylose, respectively (Fig. 2A). This peak was absent when GST-FruE was incubated with glucose and GST alone suggesting that the binding of fructose, ribose, xylose by FruE was specific. However, the binding peaks were slightly weaker when GST-FruE was incubated with ribose and xylose compared to fructose. The binding intensity of FruE with fructose was 2.5 mmol (0.45 mg) fructose per gram of GST-FruE (Fig. 2B) as assayed quantitatively using the Glucose/Fructose Kit. Glucose was not bound by FruE under any condition tested (Fig. 2B).

Since binding of ribose and xylose to FruE suggests that these sugars might also transported by the FruEKFG system, we tested binding of fructose to FruE in the presence of competing ribose or xylose. The peak of FruE-fructose in the presence of an excess of ribose or xylose was similar to the FruE-fructose without the presence of other sugars (Fig. 2A). However, a slight reduction of the amount of fructose bound to FruE was observed in the presence of ribose or xylose as a competitor (Fig. 2B). We thus quantitatively assessed the inhibition of fructose binding activity of FruE by different sugars using [U-14C] fructose. Addition of cold ribose and xylose inhibited fructose binding activity of FruE by 52% and 46% respectively when using a 50-fold excess of competitor (Table 2).

Addition of glucose, galactose, and mannose as competitors at the same excess ratio only had minor effects on [U-14C] fructose binding. These results suggest that *B. longum* NCC2705 uses the FruEKFG ABC transport system for uptake of fructose, ribose and xylose with fructose as the preferred substrate.

**FruE is induced specifically and reversibly by fructose** FruE as a probable sugar binding protein of an ABC transporter system that mediates the first step of sugar uptake. To further investigate if the FruE expression is induced specifically and reversibly by its substrates, we compared its expression both at the protein and transcriptional levels in cells grown on fructose, ribose, xylose or glucose in early exponential phase (8 h), midexponential phase (12 h), end of exponential phase (16 h) and stationary phase (24 h). The different time points were determined by recording bacterial growth curves. To assess the protein abundance, 2D-PAGE was performed. Using our previously published proteome reference map of *B. longum* NCC2705 (25) the spots representing FruE were searched on the 2D gel (Fig. 3) and their relative intensities were quantified. To verify that these spots really represent FruE, the spots were then excised and protein was extracted and analyzed by MALDI-TOF/TOF MS/MS and ESI-MS/MS. This identified all spots non-ambiguously as FruE by several internal peptides (Table S2).

Clearly, expression of FruE was increased over time during growth on MGM containing fructose, ribose or xylose as sole carbon source while in glucose grown cells FruE is barely detectable (Fig. 3A upper panel). To corroborate these results, RT-PCR analysis was performed targeting the *fruE* mRNA. This confirmed the results obtained in 2D-PAGE analysis on the transcriptional level (Fig. 3A lower panel).
suggests that expression of FruE is either repressed in the presence of glucose or induced by fructose, ribose and xylose.

We then performed experiments with *B. longum* NCC2705 cells grown to mid-exponential growth phase in MGM supplemented with different concentrations of fructose, ribose and xylose (1, 2, 3, or 4 g/L). Interestingly, both protein and transcript levels of FruE increased with increasing fructose concentration, while no concentration-dependent increase was observed for ribose, xylose and glucose (Fig. 3B). This suggests that expression of FruE is induced in the presence of fructose in a dose-dependent manner.

Finally, we tested the reversibility of the FruE induction by fructose, ribose and xylose. *B. longum* NCC2705 cells cultured on glucose, fructose, ribose or xylose for 6 h were harvested, washed by PBS, split in two and resuspended in fresh medium with the previous or any of the other three substrates. The two cultures were then further incubated for 6 h and analyzed for FruE protein or *fruE* transcript levels by 2D-PAGE and RT-PCR. As observed in our previous experiments, expression of FruE was strongly induced when bacteria were grown in the presence of its substrate fructose, ribose and xylose but not on glucose. Furthermore, expression of FruE was greatly reduced both on the transcript and protein level when these inducers were withdrawn from the culture medium (Fig. 3C), indicating a reversible mechanism of regulation by these sugars.

**Protein-protein interactions of the FruEKFG ABC transporter complex** In most bacteria, typical ABC sugar transporters are composed of the following components: a secreted or membrane-anchored substrate-binding protein, a membrane-integral permease, and an ATPase subunit (9, 10, 15). Within the *fruE-G* operon, all genes required for a fully functional ABC transport system are present. Both the sugar-binding protein and the ATPase subunit must interact with the membrane-spanning permease(s) in order to transmit conformational changes evoked by hydrolysis of ATP and subsequently leading to import of the substrate. However, whether or not the proteins encoded by *fruE-G* constitute a functional ABC transporter has yet to be demonstrated.

Thus, we cloned and expressed glutathione-S-transferase (GST)-fusions of all proteins of the system. Additionally, His-tagged FruE and FruK proteins were obtained. All fusion proteins were found to be preferentially expressed in soluble form in the cytoplasm of the *E. coli* strain used for expression (Fig. 4A). All proteins were purified by standard affinity chromatography using their respective tags (Fig. 4A).

These proteins were then tested for interaction with each other using GST pull-down experiments in which GST-tagged proteins were incubated with cellular lysates of *E. coli* BL21 expressing His6-fusions of the respective interaction partner. Adsorbates were probed with anti-His, anti-GST, anti-FruE or anti-FruK antibodies. GST alone was used as negative control and purified protein as positive control. As expected both the sugar-binding protein FruE and the ATPase subunit FruK showed an interaction with the FruF and FruG (Fig. 4B).

**CONCLUSION**

The ability of the *Bifidobacterium* species to survive and persist in the competitive environment of the intestinal tract is correlated with their capacity to utilize fermentable carbohydrates not absorbed and metabolized by the host. Although bifidobacteria have been studied for over a century, the lack of genetic tools has prevented a comprehensive and coherent view of their biosynthetic capabilities. Until now, very little was known about fructose uptake in bifidobacteria. Basically, glucose transport in *B. longum* was characterized as an active nonphosphorylating process (16) We
recently identified BL0033 as a protein that is highly expressed in *B. longum* NCC2705 grown on fructose, ribose or xylose (20), which is part an operon with similarity to a ribose-specific ABC transporter of *E. coli* (17).

In this study, we have characterized the *fruE-G* operon and the functionality of the encoded proteins as an ABC sugar transporter. This system displays many of the characteristics of binding protein-dependent ABC transporters with a soluble periplasmic substrate-binding protein (BL0033/FruE), integral membrane proteins (permeases, BL0035/FruF and BL0036/FruG), and an energy-transducing ATP-binding cassette protein (BL0034/FruK). These proteins display the invariably conserved motifs found in bacterial ABC transporters.

The high homology of the components of the FruEKFG proteins of *B. longum* NCC2705 with the ribose ABC transporter of *E. coli* suggests a common origin. This hypothesis is strengthened by the (i) the presence of all classical components of an ABC transport system in the *fruE-G* locus and (ii) the apparent ability of the *B. longum* system to transport ribose and xylose, as shown by uptake and binding competition experiments.

To show that FruEKFG constitutes a functional fructose-specific ABC transporter, we screened a large number of *B. longum* strains for the inability to grow on fructose as sole carbon source. Out of 45 strains screened a single strain (*B. longum* DCP-18) exhibited a fructose-negative phenotype. In addition to the inability to ferment fructose, this strain also lacks a functional *fruE* gene and is deficient for fructose transport across the cytoplasmic membrane. Complementation of this strain with a plasmid based *fruE* gene restored fructose transport and fermentation. Similar results were obtained with *E. coli* LR2-177, a strain that can not transport fructose and thus does not grow on this sugar.

We further could show that FruE is a able to bind to fructose, ribose and xylose. By using competition assays we observed that fructose binding of FruE is inhibited only partially in the presence of an excess of ribose or xylose suggesting that FruE has affinity to all three sugars with a preference of fructose over the other two substrates.

Expression analysis of FruE by 2D SDS-PAGE and RT-PCR revealed that FruE is induced specifically and reversibly by fructose both in a time- and dose-dependent manner. Collectively, our data suggests that FruEFGK constitute a functional ABC transporter with high affinity to fructose. We thus propose to rename the genes *bl0033-0036* and the encoded proteins BL0033-0036 into *fruEKFG* and FruEKFG, respectively.

Furthermore, we found that both the sugar-binding protein FruE and the ATPase subunit FruK interacted with the two membrane permeases. Thus, the interactions observed support a conformation of the FruEKFG holocomplex in which FruE is located on the cell surface and interacts with the permeases. Intracellularly FruK would then be bound to the permeases and upon ATP hydrolysis evokes conformational changes leading to fructose import.

Most described fructose transporters belong to the PTS family. The only known fructose-specific ABC transporter was identified in *Sinorhizobium meliloti* (33). To our knowledge, the system described here is the first report on a fructose-specific ABC transport system in Gram-positive bacteria. Our analysis extends previous studies on the physiological characteristics and supports the hypothesis regarding the adaptation to the human gastrointestinal tract, as formulated by Schell and colleagues. More importantly, our data confirmed that the Fru-ABC transporter system could clearly serve to import fructose and with lower affinity of ribose and xylose. Our study on the transporter and metabolism of fructose and other sugars will hopefully facilitate and
stimulate further in-depth research to elucidate the nutritional lifestyle of *B. longum* NCC2705.

REFERENCES


**FOOTNOTES**

* We are indebted to Nestlé Research Center for kindly providing *B. longum* strain NCC2705 and helpful information. We are grateful to Feng Liu for technical assistance and helpful discussions. This work was supported by grants from the National Natural Science Foundation of China (no. 81071321), the Mega-projects of Science and Technology Research of China (No. 2009ZX10004-205), a grant from the National High Technology Research and Development Program of China (863 Program; grant no. 2007AA02Z118) and Christian U. Riedel is supported by the German Academic Exchange Service/Federal Ministry of Education and Research (grant D/09/04778).

**Abbreviations:** 2D PAGE: Two-dimensional polyacrylamide gel electrophoresis; ACN: acetonitrile; CAI: codon adaptation index; cDNA: complementary DNA; ESI: electrospray ionization; FOS: fructooligosaccharid; MS/MS: tandem mass spectrometry; PTM: post-translational modification; RT-PCR: reverse transcription-PCR; TFA: trifluoroacetic acid; WB: Western blot;

**TABLES**

<table>
<thead>
<tr>
<th>Strain, plasmid, or oligonucleotides</th>
<th>Relevant characterization or nucleotide sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference or purpose</td>
<td></td>
</tr>
</tbody>
</table>

14
<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. longum</em> NCC2705</td>
<td>Type strain, genome sequenced (5)</td>
</tr>
<tr>
<td><em>B. longum</em> DCP-18</td>
<td>Fructose-negative strain This study</td>
</tr>
<tr>
<td><em>E. coli</em> mutant LR2-177</td>
<td><strong>galP manA nagE glcA fruA ptsI glk</strong> + mak (deficient for mannofructokinase activity)</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>hsdR17 (rK mK-) recA1 endA1 gyrA96 thi-1 relA1 Commercial strain</td>
</tr>
<tr>
<td><em>E. coli</em> BL21-CondonPlus(DE3)-R-IL</td>
<td>dcm hsdS8 (rK mK-) gal(λDE3[lacI lacUV5-T7 geneI ind1 Sam7 nin5]) Commercial strain</td>
</tr>
<tr>
<td><em>E. coli</em> Rosetta (DE3 )</td>
<td>BL21 derivatives designed to alleviate codon bias when expressing heterologous proteins in <em>E. coli</em> Commercial strain</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET-32a</td>
<td>Amp r lacI; expression vector; 5.4kb Commercial plasmid</td>
</tr>
<tr>
<td>pGEX-4T-1</td>
<td>Amp r lacI; expression vector; 4.9kb Commercial plasmid</td>
</tr>
<tr>
<td>pET32a-3</td>
<td>pET-32a containing the gene fruE This study</td>
</tr>
<tr>
<td>pET32a-4</td>
<td>pET-32a containing the gene fruK This study</td>
</tr>
<tr>
<td>pGEX-4T-1-3</td>
<td>pGEX-4T-1 containing the gene fruE This study</td>
</tr>
<tr>
<td>pGEX-4T-1-5</td>
<td>pGEX-4T-1 containing the gene fruF This study</td>
</tr>
<tr>
<td>pGEX-4T-1-6</td>
<td>pGEX-4T-1 containing the gene fruG This study</td>
</tr>
<tr>
<td>pGEX-FruEKFG</td>
<td>pGEX-4T-1 containing the full fruEKFG operon This study</td>
</tr>
<tr>
<td>pDG7</td>
<td><em>E. coli</em>-<em>Bifidobacterium</em> shuttle vector (27)</td>
</tr>
<tr>
<td>pDG7-FruE</td>
<td>pDG7 vector containing the fruE gene This study</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oligonucleotides for reverse transcription PCR, gene cloning and expression</th>
<th>Cloning of</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SF</td>
<td>TCCAGTTGATCGCATGGTC RT-PCR</td>
</tr>
<tr>
<td>16SR</td>
<td>GGGAAGCCGTATCTCTACGA</td>
</tr>
<tr>
<td>fruE-F</td>
<td>AGGTGGCAGCTCGGAATCC</td>
</tr>
<tr>
<td>fruE-R</td>
<td>GCAGCGTCAAGGTCTTGG</td>
</tr>
<tr>
<td>fruE-F’</td>
<td>GGCGCGCATCCATGAAAGATGGGAAGAAGGC</td>
</tr>
<tr>
<td>fruE-R’</td>
<td>ATTACCGCTCGAG TCGTAGTGGCGCGGTGTTT</td>
</tr>
<tr>
<td>Ebl33F</td>
<td>CGGAATTCTCCTGGTGCCGCTAGTAGGTTT</td>
</tr>
<tr>
<td>Ebl33R</td>
<td>CCCAAGCTTTCAGTAGGCGCGGTGTTT</td>
</tr>
<tr>
<td>fruK-F</td>
<td>GCAGCGCATCC ATGACAGATAAACCC</td>
</tr>
<tr>
<td>fruK-R</td>
<td>TACCGCTCGAG TCAAGATGATGGTTT</td>
</tr>
<tr>
<td>fruF-F</td>
<td>CGGATCCATGACAAACAGCTACCGGAA</td>
</tr>
<tr>
<td>fruF-R</td>
<td>CCGCTCGAGCTATTTTTAATCTCCGCGG</td>
</tr>
<tr>
<td>fruG-F</td>
<td>GCAGCGATCCATGGCAGTAAGGCAAAAGC Cloning of</td>
</tr>
</tbody>
</table>

---

By guest on October 5, 2017 http://www.jbc.org/ Downloaded from
Primer specific to sequences within 16S rDNA of \textit{B. longum} and designed to produce a cDNA of 831 bp.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Mean inhibition of uptake (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negtive control</td>
<td>0</td>
</tr>
<tr>
<td>D-glucose</td>
<td>12</td>
</tr>
<tr>
<td>D-mannose</td>
<td>10</td>
</tr>
<tr>
<td>D-galactose</td>
<td>12</td>
</tr>
<tr>
<td>D-ribose</td>
<td>52</td>
</tr>
<tr>
<td>D-xylose</td>
<td>46</td>
</tr>
<tr>
<td>D-fructose</td>
<td>99</td>
</tr>
</tbody>
</table>

$^a$ The results are expressed as percent inhibition of fructose uptake and are means of five measurements from two independent experiments, with a variation of less than 5%. Uptake was realized with \([U-^{14}\text{C}]\) fructose at 50 \(\mu\text{M}\) and 50-fold excess of unlabeled competitors. The control value was 26 nmol of fructose transported/min/mg of protein.

FIGURE LEGENDS

FIGURE 1. Fructose uptake activity in \textit{B. longum} and \textit{E. coli} strains, binding assays of FruE with different substrates and quantitative assay of FruE sugar binding. A: Fructose uptake activity in \textit{B. longum} NCC2705 wild-type (♥), DCP-18 (▲) and DCP-18/pDG7-FruE (■). Bacteria were grown in MGM medium with glucose (1 g/l) as sole carbon source, harvested at an OD$_{600}$ of 0.5, and assayed for uptake of \([U-^{14}\text{C}]\) fructose which was added to the assay at a final concentration of 10 mM. (B) Fructose uptake of \textit{E. coli} LR2-177 (▲) and LR2-177/pGEX-FruEKFG (■). \textit{E. coli} strains were grown in LB medium with glucose (1 g/l) and expression of FruEKFG was induced with IPTG 0.05 mM. All values are mean of three independent cultures standard error bars.

FIGURE 2. Sugar binding of GST-FruE. (A) Specific sugar-binding of purified GST-FruE with glucose, fructose, ribose and xylose measured by capillary electrophoresis. Peaks corresponding to FruE, FruE+glucose, FruE+fructose, FruE+ribose, FruE+xylose, FruE+fructose and FruEFGK+fructose with the excess of ribose or xylose are indicated. (B): Quantitative assay of glucose (blue bars) and fructose (red bars) bound to purified recombinant GST-FruE protein. 5 \(\mu\text{M}\) glucose or fructose were used in all assays. To test accuracy of the assay 5 \(\mu\text{M}\) glucose or fructose were assayed without any protein. GST alone was not bound by any of the two sugars. For the competitive binding, 5 \(\mu\text{M}\) ribose or xylose were added to the assays additionally to fructose/glucose.

FIGURE 3. Specificity and reversibility of induction of FruE by fructose, ribose and xylose. A: Spots corresponding to FruE in 2D proteome maps (upper panel) and the RT-PCR-amplified fragments of \textit{fruE} and 16 S rDNA (lower panel) of \textit{B. longum} NCC2705 grown for 8, 12, 16 h, and 24h in MGM medium with fructose, ribose, xylose or glucose as sole carbon source. B: 2D proteome maps zoomed in on the FruE spots and RT-PCR targeting \textit{fruE} of \textit{B. longum} NCC2705 grown to mid-exponential growth phase in MGM medium with 1, 2, 3, or 4 g/liter of fructose,
ribose or xylose, respectively). C: FruE spots on 2D proteome maps and RT-PCR targeting fruE of B. longum NCC2705 grown on MGM containing any of the substrates for 6 h and subsequently changed to a medium containing another sugar.

**FIGURE 4.** SDS-PAGE and GST pull-down assays to analyze protein-protein interactions of the FruEKFG ABC transporter subunits. A: Coomassie-stained SDS-PAGE of crude extracts (lanes 1, 3, 5, 7, and 9; 30 µg of protein were loaded per sample) and purified proteins (lanes 2, 4, 6, 8, and 10; 5-10 µg of purified protein was loaded per sample) of IPTG-induced E. coli BL21(DE3) containing pET32a-FruE (lanes 1 and 2), pET32a-FruK (lanes 3 and 4), pGEX-4T-1-FruF (lanes 5 and 6), pGEX-4T-1-FruG (lanes 7 and 8), and pGEX-4T-1-FruE (lanes 9 and 10); M: molecular weight marker. Proteins were purified by Ni²⁺ affinity column (His-FruE and His-FruK) or GST beads (GST-FruF, GST-FruG, and GST-FruE). B: GST pull-down assays probing interactions between FruE or FruK with the membrane permases FruF and FruG. For pull-down, 25 µg of GST-fusion protein was incubated with 5 µL Glutathione-sepharose 4B beads for 2 h in PBS at 4°C. Then 200 µl lysate containing a total of 25 µg protein of an E. coli BL21 strain expressing the respective His₆-tagged protein were added and bound proteins were precipitated by centrifugation. Lane 1: GST+His-FruE (negative control); lane 2: GST-FruF+His-FruE; lane 3: His-FruE (positive control); lane 4: GST+His-FruK (negative control); lane 5: GST-FruF+His-FruK; lane 6: His-FruK (positive control); lane 7: GST+His-FruE (negative control); lane 8: GST-FruG+His-FruE; lane 9: His-FruE (positive control); lane 10: GST+His-FruK (negative control); lane 11: GST-FruF+His-FruK; Lane 12: His-FruK (positive control).

**FIGURES**

![Figure 1](source_url)
Figure 3
Figure 4

A MW

95KD--
72KD--
55KD--
43KD--
34KD--

B

GST  +  -  -  +  -  +  -  +  -  -  -  
GST-FruF -  +  -  -  +  -  -  -  -  -  -  -  
GST-FruG -  -  -  -  -  -  +  -  -  +  -  
His-FruE +  +  +  -  -  +  +  +  -  -  -  
His-FruK -  -  -  +  +  -  -  -  +  +  +  

IB: GST

IB: GST

IB: His

IB: anti-FruE

IB: anti-FruK

IB: GST-FruF

IB: GST-FruG

IB: GST
Fructose uptake in bifidobacterium longum NCC2705 is mediated by an ABC transporter

Xiao Wei, Yanhong Guo, Changlin Shao, Zhongke Sun, Daria Zhurina, Dawei Liu, Wei Liu, Dayang Zou, Zheng Jiang, Xuesong Wang, Jiangli Zhao, Wei Shang, Xuelian Li, Xiangru Liao, Liuyu Huang, Christian U. Riedel and Jing Yuan

J. Biol. Chem. published online November 18, 2011 originally published online November 18, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.266213

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/11/18/M111.266213.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2011/12/28/jbc.M111.266213.full.html#ref-list-1