Identification of surface-associated proteins of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603 by enzymatic shaving

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ABSTRACT

Bifidobacteria are commensal microorganisms of the human and animal intestinal tract, and their surface proteins can mediate bacterial communication and chemical sensing in the environment, as well as facilitate interactions between bacteria and the host. However, a systematic study of the outer surface-associated proteome of bifidobacteria has not been undertaken. In the present study, the proteins located on the surface of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603 were systematically identified by a nongel proteomic approach, which consisted of the shaving of the bacterial surface with trypsin and an analysis of the released peptides by liquid chromatography-tandem mass spectrometry. A total of 105 surface-associated proteins were found, of which 15 proteins could potentially be involved in adhesion and interactions between bifidobacteria and the host. The proteins related to adhesion and interaction between bacteria and the host include pilus structure proteins (Fim A, Fim B), 10 moonlighting proteins, an NLP/P60 family protein, an immunogenic secreted protein, and a putative sugar-binding secreted protein. The results provide the basis for future studies on the molecular mechanisms of the interactions between bifidobacteria and the host.

Key words: probiotics, surface proteins, shotgun proteomics, interaction between probiotics and host

INTRODUCTION

Probiotics are defined as live microorganisms that confer a health benefit on the host when consumed in adequate amounts. Health benefits conferred by probiotics may include modulating intestinal microflora (Marteau et al., 2002; Ouwehand et al., 2002), suppress-

ing pathogens (Shah, 2000; Shiby and Mishra, 2013), alleviating diarrhea, improving lactose metabolism (Jiang et al., 1996; Martini et al., 1991), antimutagenic and anticancer properties, reducing the level of serum cholesterol, treating inflammatory bowel disease, and stimulating the immune system. Species of the *Lactobacillus* and *Bifidobacterium* genera are mainly considered probiotics. Bifidobacteria are commensal microorganisms of the human and animal intestinal tract, which are potential probiotic microorganisms that contribute to the health of the host.

To exert their functions, probiotic strains must be able to adhere to and colonize the host's intestinal tract (González-Rodríguez et al., 2013; Wang et al., 2013). Therefore, one important criterion for probiotic characterization is the ability to adhere to epithelial cells of the intestinal tract. Adhesion to the gut is believed to be mediated by adhesive factors, including lipoteichoic acids, carbohydrates, and proteins located on the outer surface of the cell wall (Sanchez et al., 2010). Surface proteins may interact with the host and mediate bacterial communication and chemical sensing in the environment, as well as immunomodulation of the host (Konstantinov et al., 2008; Le Maréchal et al., 2015). The functions of some surface proteins have been reviewed recently (Sánchez et al., 2010, 2013; González-Rodríguez et al., 2013).

Proteomics techniques can identify a large number of proteins in complex samples. Nevertheless, the identification of the bacterial surface proteome is a challenging task because of the difficulty of obtaining samples with almost no contamination of cytoplasmic proteins. Moreover, first-generation proteomics—separation of proteins by 2-dimensional electrophoresis and identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry—cannot effectively detect bacterial surface proteins and membrane proteins because these proteins are normally hydrophobic and low abundance. Compared with gel-based approaches, gel-free second-generation proteomics have been successful in identifying microbial surface proteins,

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including the surface shaving method, which is shaving intact living cells by proteases (Insenser et al., 2010). After this approach was first applied to the pathogen *Streptococcus pyogenes* (Rodríguez-Ortega et al., 2006), it has become a common approach for a fast and reliable identification of surface proteins. This shaving strategy has already been applied to a wide variety of microorganisms, including gram-positive bacteria, such as *Streptococcus pneumoniae* (Olaya-Abril et al., 2013), *Enterococcus faecalis* V583 (Böhle et al., 2011) and *Lactococcus lactis* (Meyrand et al., 2013b), and gram-negative bacteria, such as *Listeria monocytogenes* (Zhang et al., 2013), *Escherichia coli* (Walters and Mobley, 2009), and *Salmonella enterica* (Van Gerven et al., 2009). This method has also been used in eukaryotes, such as *Saccharomyces cerevisiae* (Braconi et al., 2011), *Candida albicans* (Vialás et al., 2012), and *Schistosoma mansoni* (Castro-Borges et al., 2011). Guglielmetti et al. (2008) identified the outer surface lipoprotein, BopA, of *Bifidobacterium bifidum* MIMBb75 by proteinase K and lithium chloride treatments, which plays a key role in bifidobacterial cell adhesion to Caco-2 cells (Guglielmetti et al., 2008). However, a systematic study of the surface-associated proteome of *Bifidobacterium* has not been carried out by shaving intact living bacterial cells.

Intracellular proteins may be released from cell lysis after trypsin treatment, and the surface-associated proteins identified would be contaminated by intracellular proteins (Bohle et al., 2011). Therefore, the assessment of bacterial cell viability and membrane integrity after trypsin treatment is vital for successful identification (Solis et al., 2010). The plate counting method is not a reliable method to evaluate bacterial cell viability and membrane integrity because some bacteria are viable and integral but not cultivable (Berney et al., 2008; Hammes et al., 2011). Quantitative real-time PCR (qPCR) combined with propidium monoazide (PMA) can accurately assess viability and integrity of bacterial cells compared with the plate counting method (Fujimoto et al., 2011; Villarreal et al., 2013).

Given the potential role of proteins located on the outer surface of *Bifidobacterium* in bifidobacterial adhesion to host, *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603, which adheres to Caco-2 intestinal epithelial cells, was chosen in the present study (Wang et al., 2013). Then, the surface proteins of this strain were systematically identified by surface shaving with trypsin and MS identification. The PMA-qPCR combined with electron microscopy was established as a method for observing bacterial cell viability and membrane integrity. The results will provide the basis for studies of the molecular mechanisms of interaction between *Bifidobacterium* and the host.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bifidobacterium animalis ssp. *lactis* KLDS 2.0603 (KLDS, Key Lab of Dairy Science, Ministry of Education, Northeast Agricultural University, China), which was isolated from adult feces, was used in this study. The strain was cultured in modified de Man, Rogosa, and Sharpe broth containing 0.05% (wt/vol) cysteine-hydrochloride at 37°C in an anaerobic chamber (Thermo Fisher Scientific, Nepean, ON, Canada) as previously described (Zhai et al., 2013).

Surface Digestion of Live Cells

Fifty milliliters of bacterial culture in midexponential growth phase (corresponding to approximately 108 cells) was removed and centrifuged at $5,000 \times g$ for 10 min at 4°C and then washed 3 times with PBS. Cells were resuspended in 1.0 mL of PBS containing 30% sucrose. Shaving reactions were carried out with addition of 20 µg of trypsin (Promega, Madison, WI) or no addition of trypsin (control samples). The samples were incubated for 2 h at 37°C with shaking at 150 rpm. The digestion mixtures were centrifuged at $3,500 \times g$ for 10 min at 4°C, and the supernatants (containing proteins and the peptides) were filtered using 0.22-µm filters (Millipore, Billerica, MA). An additional 2 µg of trypsin was added to the supernatants, and the mixture was incubated for 24 h at 37°C with agitation at 150 rpm. The overnight trypsin digestion of the supernatants was stopped by adding formic acid to a final concentration of 0.1% (vol/vol). Prior to nanoLC-MS/MS analysis, peptides were concentrated and purified using C18 Dynabeads (Invitrogen, Waltham, MA) as described by Rappsilber et al. (2003). Three independent biological replicates were carried out for each treatment.

Determination of Cell Viability After Surface Digestion

Total viable counts of *B. animalis* KLDS 2.0603 were determined by a pour plate method using de Man, Rogosa, and Sharpe agar after serial 10-fold dilution in maximum recovery diluent solution. Plates were incubated anaerobically at 37°C for 48 h, and the colony-forming units were determined. Cell viability was expressed as the colony-forming units per milliliter before and after treatment with the enzyme (1, 2, and 4 h). Evaluation of bacterial viability by qPCR before and after enzyme treatment was performed as described by Desfossés-Foucault et al. (2012) with modification. This method consists of 4 steps, as follows.

(1) PMA Treatment. Propidium monoazide (Bio-tium Inc., Hayward, CA) stock solution (20 mM) was prepared by dissolving PMA in 20% dimethyl sulfoxide and stored at -20°C in the dark. Cell shaving samples were treated with 50 μM PMA and were incubated at 20°C for 5 min in the dark. Then, the mixture was photoactivated for 2 min at a distance of approximately 20 cm from 500-W halogen light sources as described by Fujimoto et al. (2011). The PMA-treated bacterial cells were stored at -20°C until DNA extraction was performed.

(2) DNA Extraction. Total genomic DNA from samples was obtained using a TIANamp Bacteria DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The DNA samples were stored at -20°C .

(3) qPCR Amplification. Primers for the *B. animalis* KLDS 2.0603 16S rRNA gene were designed for viable bacteria quantification (forward primer Bif-F: 5'-TCTGGCTCAGGATGAACGC-3'; reverse primer Bif-R: 5'-CACCGTTACACCGGAATTC-3').

(4) Standard Curves. Serial dilutions (from 10^9 – 10^4 cfu/mL) of bacterial cells were spiked and treated with PMA. The DNA was then extracted following the same protocol as the control samples (without treatment of trypsin). When the standard curves were made, the DNA from the highest bacterial concentration was serially diluted in nuclease-free water. The PCR amplifications were carried out in triplicate, and cycle threshold results were then plotted against the corresponding microbiological count (cfu/mL).

SDS-PAGE Analysis

To visualize proteins or protein fragments that were resistant to trypsin, 20 μL of the supernatant obtained from the 24-h trypsinization was loaded on 10% Nu-PAGE Novex Bis-Tris gels (Invitrogen) for SDS-PAGE analysis. The gels were stained using SilverSNAP Stain (Pierce, Rockford, IL) following the manufacturer's procedure.

Observation of the Surface Characteristics of Bacterial Cells

The bacterial cell morphology of *B. animalis* KLDS 2.0603 and the integrity of the bacterial cells were observed by scanning electron microscopy. Bacterial cell pellets were harvested by centrifugation ($5,000 \times g$, 10 min). The pellets were washed twice with cold PBS (pH 7.2) and then resuspended in shaving buffer. *Bifidobacterium animalis* KLDS 2.0603 was treated with trypsin and incubated for 1 h or 2 h at 37°C . Bacterial

cell suspensions were centrifuged at $5,000 \times g$ for 10 min and the supernatant was discarded. The cell pellets were resuspended in NaCl solution (0.9%, wt/vol) and fixed for 2 h in 2% (wt/vol) glutaraldehyde solution. The ultrastructure of the bacterial cells was examined with scanning electron microscopy. Cells not treated with trypsin were processed the same way (control).

Processing the samples for transmission electron microscopy was carried out as reported by Wang et al. (2013). After fixation, *B. animalis* KLDS 2.0603 cells were washed twice with PBS (pH 7.2). Control and treated cells were then spotted onto a vegetable parchment and adsorbed by the film with a copper screen. Bacterial cells of *B. animalis* KLDS 2.0603 adhering to the film were stained with 3% phosphotungstic acid and examined microscopically (magnification, $\times 40,000$).

LC-Electrospray Ionization MS/MS Analysis

Experiments were performed on a Q Exactive mass spectrometer that was coupled to an EASY-nLC system (Thermo Fisher Scientific). Six microliters of each fraction was injected for nanoLC-MS/MS analysis. The peptide mixture (5 μg) was loaded onto the C18-reversed phase column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) at a flow rate of 250 nL/min controlled by IntelliFlow technology (Thermo Fisher Scientific, Waltham, MA) for 140 min. Mass spectrometry data were acquired using a data-dependent top 10 precursor ions method, dynamically choosing the most abundant precursor ions from the survey scan (300–1,800 m/z) for high-energy collision-induced dissociation fragmentation. Determination of the target value is based on predictive automatic gain control. The dynamic exclusion duration was 60 s. Survey scans were acquired at a resolution of 70,000 at m/z 200, and the resolution was set to 17,500 at m/z 200 for high-energy collision-induced dissociation spectra. The normalized collision energy was 30 eV, and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum-fill time, was defined as 0.1%.

Sequence Database Searching and Data Analysis

The MS/MS spectra were searched using the MAS-COT engine (version 2.2; Matrix Science, London, UK) against the UniProt *Bifidobacterium* database (released January 2014; 117795 sequences, <http://www.uniprot.org/>). The parameters used for protein identification were as follows: peptide mass tolerance at 20 ppm, MS/

MS tolerance at 0.1 Da, trypsin with the number of missed cleavage up to 2, fixed modification: carbamidomethyl (C), variable modification: oxidation (M).

Bioinformatic Analysis of the Identified Proteins

Subcellular localization predictions of all proteins identified were carried out as described (Olaya-Abril et al., 2012; Cao and Bazemore-Walker, 2014). Briefly, we used the web-based PSORTb v.3.0 software (<http://www.psort.org/psortb/>) to predict subcellular localization of identified proteins. Other software programs were also used to analyze sequence features of these proteins: TMHMM-2.0 (Krogh et al., 2001) for searching transmembrane helices; SignalP 4.0 (Bendtsen et al., 2004b) for identifying type-I signal peptides; and LipoP (Juncker et al., 2003) for identifying type-II signal peptides, which lipoproteins possess. Prediction of nonclassically secreted proteins was performed using the SecretomeP 2.0 Server (Bendtsen et al., 2004a). Subcellular locations of identified proteins were compared with those of the KLDS 2.0603 genome. The whole genome sequencing and annotating of *B. animalis* KLDS 2.0603 has been performed previously in our laboratory and deposited in the NCBI database (accession number CP007522.1). Protein sequence alignments of the proteins identified by MS were carried out using BLASTP from the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against the nonredundant protein sequences (nr) database. All identified proteins were classified based on gene ontology (GO) categories using the Web Gene Ontology Annotation Plot (WEGO; Ye et al., 2006). All identified proteins were further categorized into 3 functional groups: molecular function, cellular components, and biological processes (<http://wego.genomics.org.cn/cgi-bin/wego/index.pl>).

RESULTS

Survival of *B. animalis* KLDS 2.0603 Before and After Trypsin Treatment

Viability of *B. animalis* KLDS 2.0603 cells was evaluated before and after trypsin treatment (1, 2, and 4 h) using 3 methodologies (plate counting, qPCR, and PMA-qPCR; Table 1). Viability of the bacterial cells was influenced by the method employed and time of trypsin treatment. Higher cell counts were always obtained by the qPCR method compared with the results from the plate counting and the PMA-qPCR techniques because the qPCR method was unable to distinguish live cells from dead cells. Likewise, results obtained by plate counting were lower than those obtained by the

other 2 methods after 2 h of trypsin treatment. Viable bifidobacterial counts dropped significantly after 4 h of trypsin treatment using the PMA-qPCR method (from 6.43×10^9 cfu/mL) and the plate counting method (from 4.89×10^9 cfu/mL). Therefore, 2 h of trypsin treatment was chosen for surface digestion of live cells.

Surface Examination of Bifidobacterial Cells Subjected to Trypsin Shaving

Scanning electron microscopy was used to observe the morphology of bacterial cells subjected to trypsin shaving, and transmission electron microscopy was used to assess the integrity of the cytoplasmic membrane. When the cells were subjected to trypsin treatment (1 and 2 h), bacterial cells still maintained an intact morphology (Figure 1E and F), and the cytoplasmic membrane was clearly observed and integral (Figure 1B and C), which suggested that bacterial cells were not lysed after enzymatic shaving even after 2 h of treatment, likely because of the strong cell wall (Figure 1C and F).

Identification of Outer Surface-Associated Proteins

The extracted surface-associated proteins were analyzed by SDS-PAGE (Figure 2). Similarities between different samples treated by trypsin were high according to the protein bands, as shown in Figure 2. A total of 105 surface-associated proteins were identified (Table 2). The subcellular locations of the identified proteins were predicted by PSORT, SignalP 4.0, LipoP, SecretomeP 2.0, and TMHMM-2.0 and are shown in Table 3 compared with results of the genome of *B. animalis* ssp. *lactis* KLDS 2.0603. Most identified proteins did not possess traditional secretion signals or recognizable anchoring motifs. Sixteen proteins had a signal peptide sequence, as shown in Table 4, and 15 proteins out of 105 were potentially related to adhesion and interaction between the bacteria and host, based on genome analysis and previous experimental evidence (references in Table 2).

Biological Functional Analysis of Cell Surface Proteins

Gene ontology annotation of 76 proteins from the 105 surface-associated proteins was available. Based on the GO hierarchy obtained by WEGO, proteins were classified into cellular components, molecular functions, and biological processes (Figure 3). The cellular components ontology of proteins refers to the location in the cell where proteins are active (Ashburner et al., 2000). Of

Table 1. Survival of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603 after trypsin digestion of 1 or 2 h ($\pm$ SD)

Digestion time	Method of analysis ($\times 10^9$ cfu/mL)		
	Plate counting	Quantitative real-time PCR (qPCR)	qPCR with propidium monoazide
0 h (control)	8.70 $\pm$ 1.20	9.84 $\pm$ 0.80	9.10 $\pm$ 0.65
1 h	7.03 $\pm$ 0.78	8.29 $\pm$ 0.36	8.01 $\pm$ 0.46
2 h	4.89 $\pm$ 0.34	7.20 $\pm$ 0.43	6.43 $\pm$ 0.42
4 h	0.37 $\pm$ 0.13	2.96 $\pm$ 1.28	1.01 $\pm$ 0.78

these proteins, 46 were in the cell (GO:0005623) and 21 were on the ribosome (GO:0015935, GO:0005840), with the remainder located in the membrane (GO:001602), extracellular region (GO:0005576), and macromolecular complexes (GO:0043234, GO:0000015).

We used 8 subcategories for the molecular function ontology, but binding activity, catalytic activity, structural molecules, and transporters are the 4 main molecular function categories. Most of the assigned binding activity was protein binding, RNA binding, and nucleotide binding, and most proteins had catalytic

activity, which is related to carbohydrate metabolism (e.g., acetate kinase, enolase, and transaldolase). Six proteins contained ABC transporter domains for metabolism and transport of sugars and AA.

In the biological process category, a large proportion of proteins was related to cellular and metabolic processes. Most of the cellular and metabolic processes were related to carbohydrate, nucleotide, and AA metabolism, which may play an important role in nutrition for bifidobacteria during adaptation to the intestinal tract environment.

Figure 1. Transmission (A, B, C) and scanning (D, E, F) electron microscopy micrographs of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603 at 0 (A, D; control), 1 (B, E), and 2 h (C, F) of treatment with 20 μ g of trypsin.

DISCUSSION

Optimization of Bacterial Treatment for Surface Shaving Identification

Before surface shaving was carried out, the effect of the trypsin treatment on the integrity of bifidobacterial cells was assessed. We assessed the effect of incubation time (30 min to 4 h) on bacterial viability and bacterial integrity in hypertonic buffer, which consists of PBS/30% sucrose pH 7.4 containing 20 μ g of trypsin in 50-mL cultures, using 3 methods (plate counting, qPCR, and PMA-qPCR). According to previous reports, 4 h of treatment was the longest time used (Severin et al., 2007), and 2 h of treatment was commonly used (Bohle et al., 2011). Although an incubation time of 2 h led to a significant reduction in the counts obtained by plate counting, the cell membrane and morphology of most

cells were intact according to the PMA-qPCR results (Table 1) and transmission electron microscopy and scanning electron microscopy (Figure 1). The qPCR method is unable to distinguish between live and dead cells (Fujimoto et al., 2011; Villarreal et al., 2013); however, PMA-qPCR enables the amplification of DNA from viable but not dead bacterial cells (Fujimoto et al., 2011; Villarreal et al., 2013). Compared with the plate counting method, PMA-qPCR accurately assesses integrity of bacterial cells, especially for bacteria that are viable and integral but not culturable (Villarreal et al., 2013). The long incubation time (4 h) led to an obvious decrease in viability and would likely result in an increase in the fraction of cytoplasmic proteins. However, the lysis of bacterial cells after the enzymatic treatment for 2 h was low. Therefore, 2 h was chosen for surface shaving identification. This shaving method was also used in another study (Benachour et al., 2009).

Figure 2. Sodium dodecyl sulfate-PAGE analysis of the supernatants obtained from intact cells treated with 20 μ g of trypsin for 0.5, 1, and 2 h. The group with no enzymatic treatment was included as a control.

Bioinformatics Analysis of Surface-Associated Proteins

The subcellular localization of all 105 identified proteins was analyzed using a variety of bioinformatic tools (PSORTb, SignalP 4.0, LipoP1.0, SecretomeP 2.0, and TMHMM). The results are shown in Table 2 and are discussed in each section.

Signal Peptide-Containing Proteins

Bacterial signal peptides include 4 major classes: secretory signal peptides (cleaved by signal peptidase I, SPase I), twin-arginine signal peptides (cleaved by SPase I), possible lipoprotein precursors (cleaved by signal peptidase II, SPase II), and type IV pilin signal peptides. Overall, we identified 16 proteins with a signal peptide, which accounted for 16% of all proteins identified (Table 4). These results showed that most proteins did not have conventional export pathways. Twelve of 16 proteins had secretory signal peptides cleaved by SPase I, whereas the other 4 proteins (solute-binding protein of the ABC transporter for peptides, ABC transporter substrate-binding protein, ABC transporter solute-binding protein, and dipeptide-binding ABC transporter periplasmic substrate-binding component) had signal peptidase SPase II cleavage sites. However, one protein of these proteins, FimB (fimbrial subunit), contained a C-terminal LPXTG motif, which was cell wall anchored (Table 4).

Cytoplasmic Proteins

Eighty-nine proteins out of the 105 identified proteins lack signal peptide sequences according to the prediction by PSORTb v.3.0. Fifty proteins out of 89 proteins were predicted to be cytoplasmic proteins, and 14 proteins did not possess exact subcellular locations because they lacked obvious sequence features. All these proteins were predicted to be nonclassical secretion proteins by SecretomeP, and the results also suggested that these proteins were not secreted by nonclassical secretion pathways.

Identification of cytoplasmic proteins on the bacterial surface and extracellular locations is common. For example, at least 20 cytoplasmic proteins were found in the surface proteome of *Enterococcus faecalis* (Benachour et al., 2009). In our study, many of the cytoplasmic proteins identified were highly abundant, including ribosomal proteins (Rbp), elongation factors (EF-T), enolase, transaldolase, GADPH, bile salt hydrolase and chaperones (GroES, GroEL, DnaK, and others; Table 2). The occurrence of cytoplasmic proteins on

the surface of the cells may be due to 3 reasons. First, these cytoplasmic proteins may come from cell lysis. Although we tried to control cell lysis when surface shaving was carried out, cell lysis still exists to some extent. Some strategies, including harvesting cell samples from logarithmic cultures, have been used to maximize the number of identifiable surface proteins without contamination of cytoplasmic proteins, and shaving experiments were also conducted under hypertonic conditions with sucrose, which decreases the release of cytoplasmic proteins (Espino et al., 2015). We also determined the bacterial cell viability and integrity using both PMA-qPCR and electron microscopy, and the results indicated that 2 h of trypsin treatment did not result in much bacterial cell lysis. Moreover, intracellular proteins were released due to cell lysis during culturing before trypsin treatment. Second, some cytoplasmic proteins are present at the cell surface, but their secretion pathways are unknown. For example some cytoplasmic proteins possess extracellular functions. Thus, these proteins are called moonlighting proteins. Some extracellular moonlighting proteins (EF-Tu, DnaK, enolase, and GAPDH) have adhesive roles. Third, when these proteins are trapped in membrane-bound vesicle structures, they can be released to the surface by shedding (Olaya-Abril et al., 2014).

Extracellular Proteins

The proteins that contain sequences with SPaseI cleavage sites but did not have cell wall-binding domains were classified as extracellular proteins. Eight extracellular proteins were identified and accounted for 53.3% of extracellular proteins in the *B. animalis* KLDS 2.0603 genome (Table 3), which was high compared with a previous study (Bohle et al., 2011). Among 8 extracellular proteins, 3 proteins were uncharacterized proteins whose functions were unknown. Two surface antigen proteins (NLP/P60 family protein, immunogenic secreted protein) were involved in triggering the immune response (Lei et al., 2000; Yan et al., 2007a). One protein (cell wall-associated hydrolase) was related to biogenesis of the cell wall and membrane (Sánchez et al., 2008). The other 2 proteins (one sugar-binding secreted protein and one fibro-slime domain protein) have similar functions.

Proteins on the Surface

Proteins on the bacterial surface theoretically consist of cell wall- and cell membrane-anchored proteins. Among the 105 proteins, 4 proteins contain the LPXTG cell wall-anchored motif, and 23 were nonsecreted and

Table 2. Identified proteins on the surface of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603 using enzymatic shaving

No.	Protein	UniProt number	Subcellular location prediction	Host-interacting functions	Unique peptide count ¹							Reference
					(0 h) Control			2 h				
					E1	E2	E3	E1	E2	E3		
1	Maltose/maltodextrin ABC transporter, substrate-binding periplasmic protein MalE	tr I6PXR6	Cytoplasmic membrane		4	3	3	3	11	2		
2	Fimbrial subunit FimA	tr S0A7E7	Cytoplasmic membrane 2 internal helices	Formation of pili, which serve as adhesive factors that facilitate attachment to host cells	1	2		1	8	10	Kankainen et al. (2009), Kline et al. (2010), Schell et al. (2002) ²	
3	Fimbrial subunit FimB	tr G2STW5	Cell wall LPXTG proteins	Composed of pili, which serve as adhesive factors that facilitate attachment to host cells				3	8	4	O'Connell Motherway et al. (2011) ²	
4	Putative uncharacterized protein	tr C6A833	Cytoplasmic membrane 1 internal helix		2	2		4	7	8		
5	ABC transporter solute-binding protein	tr G2SUB9	Unknown		4	1		4	6	4		
6	Bile salt hydrolase	tr G0YYC2	Unknown	Binding of human plasminogen	1	1	1	1	5	6	Candela et al. (2007) ²	
7	30S ribosomal protein S16	tr D3R3G9	Cytoplasmic					2	4	2		
8	Thioredoxin	tr D5TH8	Cytoplasmic		2	3	5	6	4	4		
9	50S ribosomal protein L30	tr H0KJF5	Cytoplasmic					1	4	4		
10	Putative uncharacterized protein	tr C6AHD2	Cytoplasmic membrane		2	2		2	4	4		
11	Ribonuclease G	tr I6PVU4	Cytoplasmic		2	2			4	2		
12	30S ribosomal protein S5	tr I6PVX9	Cytoplasmic						3	2		
13	NLP/P60 family protein	tr I6PYA0	Extracellular	Invasion-associated protein				1	3	2		
14	Immunogenic secreted protein	tr D3R499	Cytoplasmic membrane 1 internal helix	Bind to antibodies in human serum				5	3	8	Yan et al. (2007b)	
15	Enolase	tr S0A602	Cytoplasmic	Bind to epithelial cells and extracellular matrix proteins				1	3	2	Candela et al. (2009) ²	
16	Glyceraldehyde-3-phosphate dehydrogenase	tr G2SSA6	Cytoplasmic		1	5		3	3	3	Glenting et al. (2013)	
17	ABC transporter amino acid-binding protein	tr D3R4A1	Cytoplasmic Membrane 1 internal helix					2	3	3		
18	Probable phosphotetolase	tr I6PPJ6	Unknown						3	1		
19	Possible solute-binding protein of ABC transporter	tr B2ECD6	Cytoplasmic membrane		1		16	7	6	4		
20	Formyl-coenzyme A transferase	tr I6PTN4	Cytoplasmic						2	2		
21	30S ribosomal protein S2	tr U3Q2M5	Cytoplasmic					3	2	1		
22	30S ribosomal protein S5	tr B2E9V2	Cytoplasmic					2	3	2		
23	Transaldolase	tr C6A7V7	Cytoplasmic	Mucin adhesion and aggregation	1	2	1	3	2	5	González-Rodríguez et al. (2012) ²	
24	Protein translocase subunit SecA	tr C6A8L8	Cytoplasmic					2	2	3		

Continued

Table 2 (Continued). Identified proteins on the surface of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603 using enzymatic shaving

No.	Protein	UniProt number	Subcellular location prediction	Host-interacting functions	Unique peptide count ¹							Reference
					(0 h) Control			2 h				
					E1	E2	E3	E1	E2	E3		
25	30S ribosomal protein S13	tr D3R3V3	Cytoplasmic				2	5	1			
26	50S ribosomal protein L7/L12	tr H0KJA9	Cytoplasmic		1	1	1	2	2	2		
27	Transcription termination factor Rho	tr H0KM98	Cytoplasmic					2	2	2		
28	Uncharacterized protein	tr S0AAB1	Unknown		1	1		1	2	1		
29	Uncharacterized protein	tr H0KMJ9	Cytoplasmic					2	2	2		
30	Acetate kinase	tr I6PL28	Cytoplasmic					2	1	2		
31	Uncharacterized protein	tr I6PP22	Cell wall					2	2	2		
32	Phosphoglycerate kinase	tr E4R2V8	Cytoplasmic					2	2	3		
33	Membrane carboxypeptidase	tr I1WBA0	Cytoplasmic membrane		2	5	5	1	2	2		
34	Fibro-slime domain protein	tr B6XSC0	Extracellular Signal peptide					2	2	1		
35	Membrane protein with transport function	tr C6AGJ3	Cytoplasmic membrane					2	2	3		
36	Uncharacterized protein	tr H0KIM1	12 internal helices Extracellular					2	2	1		
37	30S ribosomal protein S18	tr U2CTY4	Signal peptide									
38	Maltose/maltodextrin transport ATP-binding protein MalK	tr I6PX99	Cytoplasmic		1	1		1	2	4		
39	Fatty acid synthase Fas	tr S0A782	Cytoplasmic membrane					2	2			
40	30S ribosomal protein S1	tr B2E8J4	Cytoplasmic					3	4	4		
41	DNA-binding protein HU	tr D3R4J2	Cytoplasmic					1	2	6		
42	Phosphoenolpyruvate carboxylase	tr H0KIR8	Cytoplasmic					1	1			
43	Formate acetyltransferase	tr S0A9R8	Cytoplasmic		1	1		3	2	1		
44	Uncharacterized protein	tr S2ZNG0	Cytoplasmic					1	1	2		
45	50S ribosomal protein L33	tr C6AH57	Cytoplasmic		1	3		1	3	1		
46	Hypothetical cytosolic protein	tr D3R5Y5	Cytoplasmic					1	1	1		
47	GroEL	tr E5RLX8	Cytoplasmic	Interactions with the host, adhesion				1	4	3		Bergonzelli et al. (2006)
48	50S ribosomal protein L18	tr G2SUM1	Cytoplasmic					1	1	2		
49	Inositol-1-phosphate synthase	tr I6PY15	Cytoplasmic membrane					1	1	1		
50	30S ribosomal protein S7	tr D3R4G9	Unknown					1	1	1		
51	Methylenetetrahydrofolate reductase	tr C4FEQ6	Cytoplasmic					2	1	2		
52	Uncharacterized protein	tr C6A9G2	Unknown					1	1	1		
53	50S ribosomal protein L25	tr C6AIM0	Cytoplasmic					1	1	2		
54	Uncharacterized protein	tr D5TJ75	Cytoplasmic membrane					1	1			
55	Aspartate—tRNA (Asp/Asn) ligase	tr E3EPX0	Cytoplasmic					1	1			
56	Uncharacterized protein	tr B2EBV4	Unknown					1	1	1		
57	Uncharacterized protein	tr B8DTF7	Unknown					1	2			
58	Uncharacterized protein	tr B8DV69	Cytoplasmic membrane		1	1		1	2	1		
59	Dipeptide-binding protein	tr D3R4B1	Cell wall		2	1	2	15	5	2		
60	Copper-exporting ATPase	tr D3R5N5	Cytoplasmic membrane					1	2	1		Continued

Continued

Table 2 (Continued). Identified proteins on the surface of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603 using enzymatic shaving

No.	Protein	UniProt number	Subcellular location prediction	Host-interacting functions	Unique peptide count [†]						Reference
					(0 h) Control			2 h			
					E1	E2	E3	E1	E2	E3	
61	50S ribosomal protein L6	tr D5TFU4	Cytoplasmic				3	1	3		
62	50S ribosomal protein L20	tr G2SSA2	Cytoplasmic					1	2		
63	DO serine protease	tr H0KIU7	Unknown					2		1	
64	50S ribosomal protein L32	tr H0KJ41	Cytoplasmic		1	1		1	1		
65	Ribosome-recycling factor (RRF)	tr H0KKH1	Cytoplasmic					1	2	1	
66	Thioredoxin peroxidase	tr H0KKS8	Cytoplasmic								
67	50S ribosomal protein L2	tr H3KZ40	Cytoplasmic		1	1	2	5	3	5	
68	GroES	tr I1W8M0	Cytoplasmic					10	1	3	
69	Uncharacterized protein	tr I6PIM9	Cytoplasmic					1	2		
70	Uncharacterized protein	tr J6PIM9	Unknown		2	1		1	2	1	
71	Probable solute-binding protein of ABC transporter for peptides	tr S0A9V0	Unknown		3	1	3	2	5	4	
		tr A1A2E2	Cell wall					1	1	2	
72	Uncharacterized protein	tr B2E990	Cytoplasmic								
73	50S ribosomal protein L22	tr B2E9U0	Cytoplasmic					1	1	1	
74	Sigma 54 modulation protein/ribosomal protein S30EA	tr B8DT99	Cytoplasmic					6	1	2	
								2	1	2	
75	ABC transporter substrate-binding protein	tr C0BQZ3	Extracellular								
								1	6	1	
76	Family 31 glucosidase	tr C0BRN0	Cytoplasmic								
77	Putative sugar-binding secreted protein	tr C6AFZ9	Extracellular					1	1		
								8	1	11	
78	Myo-inositol-1-phosphate synthase	tr C6AGS2	Cytoplasmic								
								1	3	1	
79	50S ribosomal protein L28	tr C6AGW0	Cytoplasmic membrane								
80	Uncharacterized protein	tr D3R4R7	Cytoplasmic membrane		1	2		2	1	2	
81	Elongation factor Ts	tr D5TGV8	Cytoplasmic		1	1		1	1		
				Mediates the attachment of <i>Lactobacillus</i> to human intestinal cells and mucins				3	1	1	
										Granato et al. (2004)	
82	Type I restriction-modification system specificity determinant	tr F8AU31	Cytoplasmic					1	1		
83	Phosphoglucosamine mutase	tr F9Y2A9	Cytoplasmic								
				Binding of human plasminogen to <i>Bifidobacterium</i>				2	1		
										Candela et al. (2007) ²	
84	Uncharacterized protein	tr G0H6D9	Extracellular					1(1)	1(1)		
85	Cell division protein FtsZ	tr G2SSJ0	Cytoplasmic					2	1	3	
86	Oligopeptide ABC transporter periplasmic protein	tr H0KMH9	Cytoplasmic membrane					2	1	6	
87	Alpha-1,4 glucan phosphorylase	tr I1W7S4	Cytoplasmic								
								1	1		
88	50S ribosomal protein L29	tr I1W8P6	Cytoplasmic								
89	Uncharacterized protein	tr I1W9F3	Unknown		1	1		1	1		
								1	1	2	

Continued

Table 2 (Continued). Identified proteins on the surface of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603 using enzymatic shaving

No.	Protein	UniProt number	Subcellular location prediction	Host-interacting functions	Unique peptide count ¹						Reference
					(0 h) Control			2 h			
					E1	E2	E3	E1	E2	E3	
90	1-acyl-sn-glycerol-3-phosphate acyltransferase	tr I6PIQ0	Cytoplasmic				1	2			
91	DNA topoisomerase 1	tr I6PWL3	Cytoplasmic				1	2			
92	Uncharacterized protein	tr M4REW4	Cytoplasmic membrane				1	2			
93	50S ribosomal protein L13	tr S0A437	Unknown	Protective anti-inflammatory role				1	1	1	Basu et al. (2014)
94	50S ribosomal protein L15	tr S0A4F2	Unknown				1	1	2		
95	RNA polymerase sigma factor SigA	tr S0A949	Cytoplasmic					1	1	1	
96	Two-component system response regulator	tr G2SW73	Cytoplasmic					1	1	1	
97	ABC transporter amino acid-binding protein	tr G0H9G7	Cytoplasmic membrane					1	3		
98	Putative uncharacterized protein	tr H0KIM1	Extracellular				1	2	1		
99	Chaperone protein DnaK	tr I6PRT9	Signal peptide					3	1		Candela et al. (2010) ²
100	Glycine cleavage system H protein	tr D3R6M1	Cytoplasmic					1	2		
101	Response regulator	tr Q5YBW6	Cytoplasmic					1	2		
102	Cell wall-associated hydrolase	tr C6AHE4	Extracellular				1	3	2		
103	Cation diffusion facilitator family transporter	tr C4FFY8	Cytoplasmic membrane					2	1		
104	Phosphate-binding protein PstS	tr G0H8M3	Cytoplasmic membrane				2		1		
105	Dipeptide-binding ABC transporter, periplasmic substrate-binding component	tr I6PPZ6	Cytoplasmic membrane				1	1	1		

¹Letters E1, E2, E3 denote 3 independent experiments, and only those proteins that were identified in at least 2 independent experiments are listed in Table 2.²These references have previous experimental evidence from *Bifidobacterium*. Other references refer to evidence from other bacteria.

Table 3. Summary of subcellular locations of the identified proteins and genome of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603 predicted by PSORT, SignalP 4.0, LipoP, SecretomeP 2.0, and TMHMM-2.0 (CBS Prediction Servers, <http://www.cbs.dtu.dk/services/>)

Predicted localization	Number of identified proteins	Number in the <i>B. animalis</i> KLDS 2.0603 genome	Identified (%)
Cytoplasmic	56	922	6.07
Cytoplasmic membrane	23	448	5.13
Extracellular	8	15	53.3
Cell wall associated	4	7	57.1
Signal peptide-containing	16	57	28.1
Lipoprotein	19	56	33.9
LPXTG	1	17	5.8
Unknown	14	289	4.8

noncytoplasmic proteins (Table 3) that were classified as integral membrane proteins. Among 4 cell wall proteins, 3 proteins had functional annotations and 1 protein was uncharacterized. Among 3 proteins with functional annotations, 1 protein was involved in pilus biogenesis (fimbrial subunit FimB), which has usually been detected in bacterial surface proteome analyses (Meyrand et al., 2013a; Espino et al., 2015), and the other 2 proteins were related to solute binding (Gilad et al., 2011). We identified only 0.5% of the predicted transmembrane proteins in the *B. animalis* KLDS 2.0603 genome (Table 3). Such a low percentage of identified transmembrane proteins is common and similar to the results obtained in another study (Bohle et al., 2011). This is due to 2 reasons: first, expression of these proteins is kept to low levels, and second, the treatment with trypsin is unlikely to penetrate the thick cell wall. Therefore, these proteins could not be identified. More peptides matched the 3 identified transmembrane proteins (MalE, FimA, FimB) than those matching the other 23 transmembrane proteins (see Table 2).

The 19 transmembrane proteins are lipoproteins, representing 31% of the lipoproteins putatively encoded by the *B. animalis* KLDS 2.0603 genome (Table 3); six lipoproteins possess similar functions. Of 10 lipoproteins with predicted functions, 6 are related to the ABC transporter system involved in AA or carbohydrate transport and metabolism. Five transmembrane proteins are uncharacterized proteins according to predictions. Two proteins (FimA, FimB) are involved in pilus assembly, which is important in adhesion and host colonization (Danne and Dramsi, 2012). FimB contain a putative LPXTG anchor motif, which has adhesive functions (Danne and Dramsi, 2012).

Proteins Related to Adhesion and Host Interactions

Adhesion of bifidobacteria to human intestinal cells or extracellular matrix proteins is a vital step for in-

testinal colonization. *Bifidobacterium* have been shown not only to adhere to a variety of epithelial cells in vitro but also to inhibit adhesion of pathogenic enterobacteria. Some components of bifidobacteria act as adhesive factors, including lipoteichoic acids (Op den Camp et al., 1985), carbohydrates (Fanning et al., 2012a,b), extracellular proteins (González-Rodríguez et al., 2012), and surface proteins located on the outer surface of the cell wall. Bifidobacterial surface proteins play an important role in adhesion and colonization, as they directly interact with mucosal cells, such as epithelial and immune cells (Lebeer et al., 2010). However, until now, a comprehensive study of the outer surface-associated proteome of bifidobacteria has not been carried out.

The global profile of the bifidobacterial surface proteome was available by shaving living cells with enzyme in this study. A total of 105 surface-associated proteins were found, and 15 proteins were potentially related to adhesion and interactions between the bacteria and host according to previous experimental evidence (Table 2, Figure 4.). The proteins related to adhesion and interactions between bacteria and host include pilus proteins (Fim A, Fim B), 10 moonlighting proteins, a NLP/P60 family protein, an immunogenic secreted protein, and a putative sugar-binding secreted protein (Table 2, Figure 4.). The potential functions include binding of plasminogen, formation of pili, adhesion to collagen, adhesion to mucin and intestinal cells, a protective anti-inflammatory role, and stimulation of immune reactions (Table 2).

Bacterial pili can serve as adhesive factors, which mediate interaction with host cells (Mandlik et al., 2008; Danne and Dramsi, 2012). Pili are composed of 2 pilus subunits (Fim A, Fim B), which contain a sortase recognition motif, sortase, and a hypothetical protein (Gilad et al., 2011). The adhesive role of pili were revealed in *Lactobacillus rhamnosus* GG (Kankainen et al., 2009) and *Bifidobacterium breve* by immunogold electron micrograph. Those 2 studies showed that binding to mu-

Table 4. Identified proteins that contain signal peptide sequences in the surface proteome of *Bifidobacterium animalis* ssp. *lactis* KILDS 2.0603

No.	Protein	UniProt	Species	Signal peptide ¹
1	Fimbrial subunit FimA	tr S0A7E7	SPase I	MNLKRVFAGVAAATAALGTLAIGAAATANAAPLTGTESTITINEALEGQTFQAYQLATFTDATGANGVIND
2	Fimbrial subunit FimB	tr G2STW5	SPase I	MKRLKHSQAQSGADHAGRKGRPLRSVLA SLCAVAMSLGMA SSVAAFAADDRQPAATADPQAATASAGN
3	NLP/P60 family protein	tr J6PYA0	SPase I	MKRSISIATAVVAA SLAFTSIAPAATAADSQSGSNVYV
4	Fibro-slime domain protein	tr B6XSC0	SPase I	MRHTPIQQAQKPERSEAEIVAVIVAAAMLGGMGYATTSSA MAADAKQTTEQQA GISLGLYDYNRYTIND
5	Inositol-1-phosphate synthase	tr J6PY15	SPase I	MVYQARTVSSSSSPRAAHKASTSHRAPKRGQNAPKGP KKRTRNRKKGGLWVKI
6	50S ribosomal protein L32	tr H0KJ41	SPase I	MALPKYKMSRANTRTRRSTWKA KAAQTITPCNGGAPTTLAHVACPSG
7	Uncharacterized protein	tr S0A9V0	SPase I	ISTAMASVGLVAGVVATQSMYSRAEAQPTPSATAYSITESNHAASRG
8	Probable solute-binding protein of ABC transporter for peptides	tr A1A2E2	SPase II	MNRKKVIAALASVAALASLAACGGV KDGGAATGNTTIVGT TTDKITS LDPAGSYD
9	ABC transporter for peptides binding protein	tr C0BQZ3	SPase II	MISLRKLAAGSLAAVLA FFSMAACGSDSDSTDSSADSTSGKAVKVDEKSAKATSLAD
10	Putative sugar-binding secreted protein	tr C6AFZ9	SPase I	MKIEWKKA VGLAAVAAMIVPMAACGANGGDSNAADGSKPSETQSANLTVVWGPSEDSEW
11	Oligopeptide ABC transporter periplasmic protein	tr H0KMH9	SPase I	MKKKTLGLIAGICSLGMMMLSLSACGSNGGSSAKGSGDNIITAYNSEPQAN
12	ABC transporter solute-binding protein	tr G2SUB9	SPase II	MNRTVKATVGLIVAIAAMSLTGLAACGGSSADDGKGKVVYFLNFPETADQWVALAKKYTDKT
13	Maltose/maltodextrin ABC transporter, substrate-binding periplasmic protein	tr J6PXR6	SPase I	MKIEWKKA VGLAAVAAMIVPMAACGANGGDSNAADGSKPSETQSANLTVVWGPSEDSEWLKDMEEAFGK
14	ABC transporter amino acid-binding protein	tr G0H9G7	SPase I	KKVAAAFALGAAVILGSAACGTTDETQTAAKDSQNTSIVDSVKKDDKIAAMLPOKIAESG
15	Phosphate-binding protein PstS	tr G0H8M3	SPase I	KGNHMRNLARRVLAALCGMAFIATTTACGDNTPDANRPVANASSISGSFAGAG
16	Dipeptide-binding ABC transporter, periplasmic substrate-binding component	tr J6PPZ6	SPase II	SAHVTA AVASIAALGMILLAGCGTSGGTSPSAQGSSTATTSVITVNNYEPSANL

¹Sequences marked in bold are the signal peptide sequence of the identified proteins.

Figure 3. Gene ontology (GO) categories of surface-associated proteins of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603. The identified proteins were classified into cellular components, molecular functions, and biological processes by the Web Gene Ontology Annotation Plot (WEGO) according to their GO characteristics. Color version available online.

cus depended strongly on the expression of one of the pilus subunits. Some bifidobacterial genome sequences harbor clusters of genes encoding pili (Kankainen et al., 2009; Foroni et al., 2011; O'Connell Motherway et al., 2011). In the present study, 2 pilus subunit proteins (FimA and Fim B) were identified from bifidobacterial surface by gel-free shotgun proteomics (Table 2, Figure 4).

A second group of adhesive proteins were moonlighting proteins, which apparently lack secretion signals or binding domains. However, these moonlighting proteins have adhesive functions when on the bacterial surface in addition to their primary role in bacterial metabolism in cytoplasm. This group of proteins usually includes glycolytic and other related enzymes (enolase, transaldolase, bile salt hydrolase, phosphoglucosamine

mutase, GAPDH), housekeeping proteins (EF-T), molecular chaperones (GroEL, GroES, DnaK), and ribosomal proteins (50S ribosomal protein L13). Once these moonlighting proteins are exposed to the bacterial surface, they will develop adhesive functions. For this reason, they are described as moonlighting proteins.

CONCLUSIONS

Bifidobacteria usually exert their beneficial effects through adhesion or direct interaction with host cells (mainly enterocytes and immune cells) via surface-associated proteins or other factors, triggering downstream responses. The global profile of bifidobacterial surface-associated proteins was obtained by the shaving approach, which has already been shown to be a fast and

reliable way to identify surface-associated proteins. A total of 105 surface-associated proteins were found, and 15 proteins could potentially be involved in adhesion and interactions between bacteria and the host in the intestinal tract. The proteins related to adhesion and interactions include pilus structure proteins (Fim A,

Fim B), 10 moonlighting proteins, an NLP/P60 family protein, an immunogenic secreted protein, and a putative sugar-binding secreted protein. Further research will be needed to determine their exact function, especially for proteins with unknown functions that may potentially be involved in the adhesion of bifidobacteria

Figure 4. Overview of surface-associated proteins of *Bifidobacterium animalis* ssp. *lactis* KLDS 2.0603, which illustrates classification of subcellular locations (SCL) of these proteins and summarizes proteins related to adhesion and interaction with host. CM = cell membrane; CW = cell wall; CE = extracellular; PGMU = phosphoglucosamine mutase; BSH = bile salt hydrolase; SP proteins = proteins containing signal peptide. Proteins marked in gray (blue) are potentially involved in interactions between bifidobacteria and the host. Color version available online.

to the intestinal cells. These results provide the basis for future studies on relevant adhesive biomarkers and detailed analyses of the mechanisms of probiotics.

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