Integrated Transcriptomic and Proteomic Analysis of the Bile Stress Response in a Centenarian-originated Probiotic *Bifidobacterium longum* BBMN68§

Haoran An‡, François P. Douillard§, Guohong Wang‡, Zhengyuan Zhai‡, Jin Yang¶, Shuhui Song¶, Jianyun Cui‡, Fazheng Ren‡, Yunbo Luo‡, Bing Zhang¶, and Yanling Hao‡

Bifidobacteria are natural inhabitants of the human gastrointestinal tract and well known for their health-promoting effects. Tolerance to bile stress is crucial for bifidobacteria to survive in the colon and to exert their beneficial actions. In this work, RNA-Seq transcriptomic analysis complemented with proteomic analysis was used to investigate the cellular response to bile in *Bifidobacterium longum* BBMN68. The transcript levels of 236 genes were significantly changed (≥ threefold, \( p < 0.001 \)) and 44 proteins were differentially abundant (≥1.6-fold, \( p < 0.01 \)) in *B. longum* BBMN68 when exposed to 0.75 g l\(^{-1}\) ox-bile. The hemolysin-like protein and bile efflux systems were significantly over produced, which might prevent bile adsorption and exclude bile, respectively. The cell membrane composition was modified probably by an increase of cyclopropane fatty acid and a decrease of transmembrane proteins, resulting in a cell membrane more impermeable to bile salts. Our hypothesis was later confirmed by surface hydrophobic assay. The transcription of genes related to xylose utilization and bifid shunt were up-regulated, which increased the production of ATP and reducing equivalents to cope with bile-induced damages in a xylan-rich colon environment. Bile salts signal the *B. longum* BBMN68 to gut entrance and enhance the expression of esterase and sortase associated with adhesion and colonization in intestinal tract, which was supported by a fivefold increased adhesion ability to HT-29 cells by BBMN68 upon bile exposure. Notably, bacterial one-hybrid and EMSA assay revealed that the two-component system *senX3-regX3* controlled the expression of *pstS* in bifidobacteria and the role of this target gene in bile resistance was further verified by heterologous expression in *Lactococcus lactis*. Taken altogether, this study established a model for global response mechanisms in *B. longum* to bile. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.M114.039156, 2558–2572, 2014.

Bifidobacteria are common inhabitants of the human gastrointestinal tract (GIT)\(^1\), in which they generally persist at concentrations of 10\(^9\) to 10\(^{11}\) cells per gram of feces, constituting up to 91% of the gut microbiota in breast-fed infants (1). Although bifidobacteria account for only 3%-5% of the total fecal flora in adults (2), their presence has been associated with health-promoting effects, such as balancing of the intestinal microbiota in treatment of diarrhea and immunomodulation (3) and reducing serum cholesterol level (4). Some bifidobacteria are marketed as probiotics, and several *Bifidobacterium* strains have been used in functional foods, especially fermented dairy products (5, 6). Following consumption, probiotic bacteria are exposed to various physico-chemical stresses, such as low pH in the stomach or bile salts in the intestine. Typically, bifidobacteria colonize the lower GIT, where bile salts have a concentration of nearly 5 mM (7). Bile salts are detergent-like biological compounds with strong antimicrobial activities that disrupt the lipid bilayer structure of cellular membranes, induce protein misfolding and cause ox-
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Many studies have been performed to explore the bile resistance factors in bifidobacteria. On one hand, it is generally considered that bile salts hydrolases (BSHS) contribute to bile tolerance in bifidobacteria by decreasing toxicity of conjugated bile salts (9, 10). On the other hand, bile efflux transporters provide protection to bile stress in bifidobacteria, such as Ctr and BetA in *B. longum* (11, 12) and Bbr_0838 in *B. breve* strains (13). In addition, the F$_{1}$F$_{0}$-ATPase was suggested to be involved in bile resistance by inducing proton pumping and increasing the intracellular ATP reserve in *B. animalis* (14). In relation to bifidobacterial adaptation to bile, several studies have shown changes in the cell envelope including fatty acids composition and membrane proteins, resulting in decreased membrane permeability to bile salts (15, 16). Furthermore, two-dimensional electrophoresis (2-DE) proteomic analyses of *B. longum* NCIMB 8809 and *B. animalis* subsp. *lactis* IPLA 4549 under bile stress conditions showed that differentially expressed proteins participate in various biological processes, such as general stress response, carbohydrate, amino acid and nucleotide metabolism, and transcription and translation (17, 18). However, different mechanisms existed between these two bacterial species, for example, the carbon catabolic pathway is mainly rerouted to lactic acid production in strain NCIMB 8809, while it is displaced toward the acetate and an additional formate branch in strain IPLA 4549 (17, 18). Microarray approach also revealed the transcription level of a group of transporters was significantly up-regulated as a response to bile stress in *B. breve* UCC2003 (19). However, the comprehensive mechanisms of response to bile have not yet been established in bifidobacteria.

*B. longum* BBMN68 was isolated from a healthy centenarian in the Bama County of the Guangxi Zhuang Autonomous Region in China, which is famous for having a population with a high life-expectancy. Previous study showed that the proportion of bifidobacteria could reach up to 9.59% in the feces from the 90–109 years old population in a Bama suburb using real-time PCR and denaturing gradient gel electrophoresis (20, 21). Further study in our research group indicated that several remarkable characteristics of strain BBMN68 at the genome level, such as a higher abundance of genes associated with carbohydrate transport–metabolism category and two genes encoding bacteriocin, may be beneficial to the long-term colonization of BBMN68 in the human GIT (22). Furthermore, BBMN68 may enhance both innate and acquired immunity and improve intestinal function in mice (23, 24).

The probiotic potential of BBMN68 legitimates the need to further explore the biological functions of this strain, such as bile stress response.

Nowadays, the Next-Generation Sequencing (NGS) technology, *i.e.* RNA-Seq, is a powerful tool for transcriptomics profiling (25, 26). Compared with microarray methods, RNA-Seq provides higher efficiency and sensitivity as it can produce more in-depth information, such as low-abundant transcripts (27). In addition, 2-DE has been widely used to investigate the proteome of lactic acid bacteria and bifidobacteria under bile stress, providing insights into how bacteria respond and tolerate to bile stress, *i.e.* central metabolisms variation in the cytoplasm (17, 18, 28–33). In the present study, RNA-Seq transcriptomics combined with 2-DE proteomic approach was performed to analyze the bile stress response and resistance mechanisms in *B. longum* BBMN68.

To the best of our knowledge, this work represents the first combined functional genomic and proteomic analysis of bile response mechanisms in bifidobacteria.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—*B. longum* subsp. *longum* BBMN68 was cultured on MRS agar plate supplemented with 0.05% (w/v) l-cysteine (MRSc) at 37 °C anaerobically (5% CO$_{2}$, 5% H$_{2}$ and 90% N$_{2}$). For the present study, *B. longum* was also grown in 200 ml MRSc broth supplemented with the different bile salts concentrations: 0, 0.6, 0.75, 0.9, and 1.2 g 1$^{-1}$ (ox-bile, Sigma, St Louis, MO), respectively. The supplemented MRSc was inoculated at 1% (v/v) with overnight cultures and then incubated anaerobically and monitored by spectrophotometry at 600 nm. Cultures of 200 ml were grown to mid-exponential phase (OD$_{600}$ of 0.6), ∼2–3×10$^{7}$ CFU ml$^{-1}$ and cells were harvested for both transcriptomics and proteomics experiments. *Escherichia coli* strains were grown aerobically at 37 °C in 2×YT medium with shaking at 250 rpm. *L. lactis* was grown at 30 °C in M17 broth supplemented with 0.5% (w/v) glucose (GM17). When required, media were supplemented with the relevant antibiotics at the following concentrations: 25 μg ml$^{-1}$ kanamycin, 100 μg ml$^{-1}$ carbenicillin, and 10 μg ml$^{-1}$ tetracycline for *E. coli*, and 10 μg ml$^{-1}$ chloramphenicol for *L. lactis*.

**RNA Isolation and Purification**—Total RNA isolation was carried out using TRIzol reagent (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. Five milliliters of culture were centrifuged at 12,000×g for 10 min at 4 °C, and subsequently resuspended in 2 ml of TRIzol reagent. The mixture was vortexed for 1 min and incubated for 3 min at room temperature (RT). Unbroken cells and cell debris were removed by centrifugation at 12,000×g for 10 min at 4 °C. The supernatant was added with 400 μl of chloroform and mixed for 15 s. After incubation for 15 min at RT, the mixture was separated by centrifugation at 12,000×g for 15 min at 4 °C. The aqueous phase was mixed with 1 ml of isopropanol and placed for 10 min at RT for precipitation of total RNA. After centrifugation at 12,000×g for 10 min at 4 °C, the precipitate was washed by 1 ml of 75% ethanol and dissolved with 50 μl of RNase-free H$_{2}$O. Total RNA was purified using a RiboMinus Kit (Invitrogen) to eliminate ribosomal RNA, and then stored at −70 °C for further use.

**RNA-Seq**—The RNA-Seq library was constructed using a total RNA-Seq Kit (Applied Biosystems, Foster City, CA) for SOLID method according to manufacturer’s introductions. Briefly, 1 μg of purified RNA was digested by 1 μl of RNase III (Applied Biosystems) at 37 °C for 10 min, and the reaction was terminated by incubation at 65 °C for 20 min. Digested RNA was cleaned up by a RiboMinus Concentration Module (Invitrogen) and ligated with adaptor mix ( Applied Biosystems) at 65 °C for 10 min and subsequently at 16 °C for 5 min. The ligation reagents were added with the hybridization mix (Applied Biosystems) and incubated at 16 °C for 16 h. Complementary DNA (cDNA) was synthesized by incubation with RT Master Mix (Applied
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Biosystems) at 42 °C for 30 min. The MinElute PCR Purification Kit (Qiagen, Valencia, CA) was used to purify the cDNA preparation. cDNA was resolved by a NuPAGE Novex 4–12% Bis-Tris Gel (Invitrogen), and fragments in the size of 150–200 bp were selected for amplification with 15 emulsion PCR cycles and cleaned up with a PureLink PCR Micro Kit (Invitrogen). Then the cDNA library was sequenced using a SOLiD 4.0 sequencer (Applied Biosystems).

**Data Processing—** All sequenced reads were aligned to the genome of *B. longum* BBMN68 with accession number CP002286 (22) using SOLiD Corona Lite software (version 4.2, Applied Biosystems) available at http://waprna.big.ac.cn. We used a recursive strategy to improve the usable sequenced reads information (34). That is, 50mers reads were firstly mapped to the genome with ≤5 color-space mismatches and unmapped reads were progressively trimmed off five bases from the 3′ end once, then the trimmed reads were mapped to the reference genome again until a match was found or unless the read had been trimmed by ≤30 bases. All those uniquely mapped reads were used to calculate the RPMK values (reads per kilobase of exon per million mapped sequenced reads) of genes, which indicated the normalized gene expression level. Differentially expressed genes (DEGs) from different samples were identified according to an R package DESeq2 (http://waprna.big.ac.cn/maseq/function/deseq.jsp) (55). The analyzed transcritpomic data has been submitted to the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE46446.

**2-DE and Image Analysis—** Intracellular proteins were extracted as previously described (36). Protein samples were quantified using the Bradford method (37) and 2-DE were performed as previously described (36). Briefly, 500 μg of proteins were diluted into 450 μl isoelectric focusing (IEF) buffer containing 8 m urea, 1.5% CHAPS, 0.2% DTT, 0.5% pH 4 to 7 ICG buffer, and moderate bromphenol blue. The diluted samples were used to rehydrate 24-cm pH 4 to 7 linear IPG strips (GE Healthcare, Uppsala, Sweden) for 12 h. First-dimension IEF was performed using an Ettan ITPhor II System (GE Healthcare) for a total of 100 kVh at 20 °C. Focused IPG strips were subsequently equilibrated for 2 × 15 min in a buffer (6 m urea, 30% glycerol [v/v], 2% SDS [w/v], 50 mM Tris-HCl, pH 8.8) supplemented with 1% DTT in the first step and with 4% iodoacetamide (Sigma) in the second one. The second dimension was performed using an Ettan DALTsix System (GE Healthcare), and proteins were resolved at 40 mA per gel for 5 h at 25 °C. The gels were stained with Coomassie Brilliant Blue G-250 (Amresco, Solon, OH) as previously described (38). Spot detection and volume quantitation were carried out with Image Master 2D Platinum software (version 5.0, GE Healthcare).

**Protein Identification—** The selected protein spots were cut out, and in-gel digestion using 0.05 μg/μl trypsin was performed as previously described (40). For protein identification, peptide extracts from digested proteins were redissolved in 1 μl of 0.5% trifluoroacetic acid (TFA), which was mixed with 1 μl of matrix (4-hydroxy-cyano-cinnamic acid in 30% acetonitrile and 0.1% TFA) before spotting on the target plate. MALDI-TOF mass spectrometry and tandem TOF/TOF mass spectrometry were carried out on a 4800 Proteomics Analyzer (Applied Biosystems). In the MS mode, the generated ions were accelerated at the source (20 kV) and separated in the first TOF tube. In the MS/MS mode, the parent ion was focused into the gas cell and fragmented using CID. Combined mass and mass/mass spectra were generated through the GPS Explorer software (version 3.6, Applied Biosystems) and used to interrogate the NCBI database (NCBI nr 2011.04.09; 13,655,082 sequences) using the Mascot database search algorithms (version 2.1, Matrix Science, Boston, MA). The search criteria were as follows: taxonomy of entries “Bacteria,” trypsin digestion with one missed cleavage allowed, fixed modification of cysteine carbamidomethylation, and variable modification of methionine oxidation. Peptide tolerance and MS/MS tolerance were both 0.2 Da. All of the automatic data analysis and database searching were fulfilled by the GPS Explorer software (version 3.6, Applied Biosystems). Protein scores > 64 were considered to be significant (p < 0.05). For unambiguous identification of proteins, more than five peptides must be matched and the sequence coverage must be greater than 15% (see supplemental Data S1).

**Hydropobicity, Autoaggregation, and Adhesion Assay—** *B. longum* BBMN68 cells grown for 16–20 h (stationary phase) were harvested and resuspended in phosphate buffered saline (PBS, pH 7.4) and adjusted to an OD600 of 1.0. For hydrophobicity assay, 1 ml of cell suspension was added to 1 ml xylene and vortexed for 2 min. The OD600 was measured and cell surface hydrophobicity was calculated as [(1-ODaqueous phase/ODinitial) × 100%]. To test autoaggregation of bifidobacterial cells, 2 ml of cell suspension was placed in each tube and incubated anaerobically at 37 °C. Every two hours, 1 ml of the upper suspension was gently transferred and the OD600 was measured. Autoaggregation was expressed as [(1-ODupper suspension/ODinitial) × 100%].

Human colon adenocarcinoma cell line HT29 was selected for bifidobacterial adhesion assay. Prior to adhesion, HT29 cells were routinely grown in Dulbecco’s High Glucose Modified Eagles Medium (DMEM, HyClone, Thermo Scientific, Rockford, IL) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Sigma) in a humidified 5% CO2 balanced air incubator at 37 °C. Cells were subcultured every 2–3 days. For adhesion assay, cells were seeded at a density of 1 × 105 per well into 24-well plate and grown to ~90% confluence (~6 days after seeding). Bifidobacterial cultures grown for 24 h were harvested and washed twice with PBS, and resuspended in DMEM without antibiotics at a concentration of ~1010 CFU ml−1. Eukaryotic cells were washed twice with PBS and aliquots of 1 ml bifidobacterial suspension were added to wells. The plate was incubated for 1 h at 37 °C with 5% CO2. Afterward, the wells were gently washed three times with PBS to remove nonattached bacteria. 0.25% Trypsin (Sigma) was added to release the cells and bacterial counts were determined on MRSc plates. Adhesion ability was expressed as the percentage of bacteria adhered with respect to the bacteria added into the well.

**B1H Method and Motif Searches—** To determine the DNA-binding specificity of the response regulator RegX3, bacterial one-hybrid analysis was performed as previously described with slight modifications (41). The library oligonucleotide (Library-F and -R) and primers (RegX3-F and -R) for amplifying regX3 gene were listed in supplemental Table S1. The insert library DNA and vector pH3U3 were ligated after EcoRI and NotI digestion, resulting in the prey plasmid, which was then introduced into E. coli DH5α to generate a raw binding site library. Plasmids isolated from this prey library were electroporated into E. coli US0 followed by counter-selection with 2.5 mg S-FOA, resulting in a pre-purified library composed of ~5 × 107 transformants. The regX3 gene was amplified by PCR and inserted into the Kan/Xbal sites of the bait plasmid pB1H2ω2, and the recombinant plasmid, designated as pB1H2-regX3 was transformed into E. coli US0. To confirm the expression of omega-RegX3 fusion protein, Western blotting was carried out with anti-FLAG M2 antibody (Sigma). Then, the plasmids from binding site library were introduced into E. coli US0 harboring pB1H2-regX3, and a two-step selection procedure was used to isolate preys containing recognition sequences for RegX3. 10 mg 3-AT and 3.75 mg 5-FOA were respectively employed for the first and second step. At least 10 selected “preys” were sequenced, and the 18 bp randomized cassettes were submitted to MEME algorithm (42) to identify overrepresented motif. DNA sequences encompassing 250 bp segments upstream of the putative translation start site of the genes that were up-regulated at the transcription level in bile stress condition were analyzed by Target Explorer (43) software tools for detecting target genes of RegX3.
RESULTS AND DISCUSSION

Global Transcriptomic and Proteomic Analysis of the Bile Stress Response in B. longum BBMN68—B. longum BBMN68 was grown in batch cultures with different ox-bile concentrations ranging from 0.6 to 1.2 g l⁻¹. Our result showed that 1.2 g l⁻¹ ox-bile inhibited the growth of BBMN68, whereas the growth rate was approximately reduced by 50% at 0.75 g l⁻¹ bile (supplemental Fig. S1). Therefore, bile concentration of 0.75 g l⁻¹ was chosen to further study the bile stress response in BBMN68.

The next generation sequencer SOLiD platform was used to investigate the transcription level changes in BBMN68 in the presence of bile salts. A total of 19,002,680 and 16,275,706 unique-mapping-reads were obtained when growing BBMN68 in the presence or not of bile salts, respectively. After filtering, the number of effective reads that were mapped to the genome of BBMN68 was 15,095,199 and 12,893,121, respectively. Further analysis showed that 1849 and 1851 out of the whole 1878 genes in the genome were covered under bile stress conditions and the control, respectively (supplemental Fig. S2). Candidate genes involved in bile stress response were chosen according to the previously described criteria with some modifications (44): 1) more than 20 unique-mapping-reads, 2) more than threefold change after normalization, and 3) statistically significant level (p < 0.001). Finally, the transcription of 236 genes was detected to be associated with bile stress, including 76 genes up- and 160 genes down-regulated (supplemental Fig. S2, supplemental Table S2). Their putative functions were classified in different categories grouped by gene ontology (Fig. 1). In addition, 2-DE method was further performed to identify the differentially expressed proteins in BBMN68 between under bile stress conditions and the control. The intensity of 57 spots was significantly changed by a factor of ≥ 1.6-fold (p < 0.01), and these spots were subjected to mass spectrometry for identification. Finally, 44 spots were successfully identified (Fig. 2) and the protein functions were predicted (Table I). Among these proteins, 24 proteins were up-regulated and 20 proteins were down-regulated. Besides, four spots were detected only under bile stress conditions.

Although transcriptomic analysis identified 236 differentially expressed genes, only 57 proteins were detected to be differentially produced. A total of 15 genes were regulated at both transcription (≥ 2-fold) and translation (≥ 1.6-fold) levels, i.e. 10 up-regulated genes and five down-regulated genes. These results illustrate a low correlation between transcriptomic and proteomic data of the bile stress response in BBMN68. Such observation constitutes another example among others where integrative “omics”-approaches proved to be a real challenge (33, 45, 46). In this study, the RNA-Seq
was employed to investigate the expression profile of the whole genes at the transcription level under bile stress in BBMN68. However, the 2-DE focused on the change of cytoplasmic soluble proteins with pI of 4 to 7. The difference of coverage between these two methods may explain the low correlation between transcriptome and proteome. In addition, RNA secondary structure, Shine Dalgarno sequence differences, regulatory proteins and sRNAs, codon bias, and ribosome occupancy constitute many regulatory points that may influence the protein production (47), therefore resulting in differences between mRNA level and protein abundance of some genes. Moreover, targeted degradation of individual proteins in response to bile salts (48) may also cause the variation in protein abundances, impacting on the correlation between transcriptome and proteome in BBMN68 during the bile stress response.

In conclusion, the SOLID combined with the 2-DE analyses showed that 236 genes and 44 proteins were affected by ox bile and could be related with the response and resistance of B. longum BBMN68 to bile stress, which were discussed in details as follows.

Genes Involved in Bile Resistance in BBMN68 — In the presence of ox-bile, the transcription or translation of eight genes related with bile resistance were significantly up-regulated in BBMN68. BBMN68 (hereafter as BN) _664, encoding a permease of the major facilitator superfamily (MFS), was the most highly up-regulated gene at the mRNA level in BBMN68 in response to bile (84.50-fold up-regulated). BN_664 shows 99% identity with BL0920 in B. longum NCC2705, which encodes a bile-inducible efflux transporter BetA conferring bile resistance (12). Meanwhile ox-bile also increased the transcription of another gene encoding MFS permease (BN_1049), which could play a similar role with BetA. In addition, the transcription level of BN_1434-1433 encoding an ABC-2 type transporter was respectively 3.59- and 4.73-fold up-regulated under bile stress conditions. Among ABC-2 type transporters, Drr and Evr have been reported to pump toxic compounds, such as daunorubicin and doxorubicin in Streptomyces lucereus (49) and viologen in Synechocystis (50). These results suggested that this transporter may possibly perform similar function of bile exclusion in BBMN68 in response to bile stress.

Remarkably, the transcription and translation of a gene encoding BSH (BN_536), which catalyzes deconjugation of glycine- or taurine-linked bile acids, was 3.05-fold and 3.44-fold up-regulated, respectively. Deconjugation of bile salts may play an important role in the bile tolerance because of the detoxification properties. Therefore, BSH activity may be a desirable feature of a probiotic, as it can increase its survival ability in gut conditions (51). However, BSH was not up-regulated in B. animalis sp. lactis IPLA 4549 (18), B. animalis sp. lactis BB-12 (52) and B. longum NCIMB 8809 (17) in response to bile, supporting the idea that different bile salts hydrolases, even within the same species, are differentially regulated in response to bile. The transcriptions of BN_167 and BN_1224, encoding hemolysin-like protein (HLP) and calcineurin-like phosphoesterase, were 10.08- and 4.23-fold up-regulated upon bile exposure, respectively. In cyanobacterium Synechocystis, HLP is located on the cell surface and functions as a barrier against the adsorption of toxic compounds (53). Another work from our research group indicated a two- to threefold increased survival of the recombinant L. lactis NZ9000 strain harboring BN_167 exposed to tauro-conjugated bile salts (TCA and TDCA) (manuscript in preparation), suggesting that HLP really conferred resistance to bile in BBMN68, especially tauro-conjugated bile salts. In addition, complete genome analysis of the most radiation-resistant bacteria Deinococcaceae reflected expansion of a calcineurin-like phosphoesterase, which appear to be related with stress resistance possibly through decomposing damage products under stress conditions (54).

Taken altogether, we proposed that these differentially expressed genes are associated with bile resistance in BBMN68.
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| Category | Spota | Gene | Gene ID | Functionb | Mass | pl | Score | Coverage | Fold change |
|----------|-------|------|--------|-----------|------|----|-------|----------|-------------|
| Stress response | 13 | cbaH | BBMN68_536 | Conjugated bile acid hydrolase | 35.0 | 4.7 | 449 | 52 | 3.44 | 3.05 |
| Carbohydrate metabolism | 16 | groES | BBMN68_1589 | Co-chaperonin HSP10 | 10.6 | 5.1 | 260 | 67 | 2.66 | 2.65 |
| | 21 | uspA1 | BBMN68_51 | Universal stress protein | 34.4 | 5.2 | 333 | 72 | 2.06 | - |
| Carbohydrate metabolism | 1 | zwf | BBMN68_1185 | Glucose-6-phosphate-1-dehydrogenase | 57.1 | 6.2 | 325 | 57 | +0.1 | 3.12 |
| | 4 | rbsK2 | BBMN68_1094 | Ribokinase family sugar kinase | 35.9 | 5.7 | 160 | 49 | ++ | 3.23 |
| | 6 | gapA | BBMN68_254 | Glyceraldehyde-3-phosphate dehydrogenase, type I | 37.8 | 5.2 | 469 | 63 | 12.6 | - |
| | 8 | lpdA | BBMN68_825 | Dihydrolipoamide dehydrogenase | 52.1 | 5.6 | 210 | 58 | 6.17 | 2.55 |
| | 9 | xfp | BBMN68_708 | Xylose-5-phosphate/fructose-6-phosphate phosphoketolase | 92.5 | 5.1 | 163 | 27 | 5.95 | - |
| Amino acid metabolism | 11 | dhaT | BBMN68_1706 | 1,3-propanediol dehydrogenase/lactaldehyde reductase | 40.6 | 4.8 | 561 | 69 | 5.56 | - |
| | 12 | putA2 | BBMN68_1612 | NAD-dependent aldehyde dehydrogenase | 98.8 | 5.9 | 226 | 28 | 5.37 | 4.15 |
| | 15 | gicC | BBMN68_806 | ADP-glucose pyrophosphorylase | 45.8 | 5.5 | 253 | 60 | 2.61 | - |
| | 18 | gpmA | BBMN68_1687 | Phosphoglycerate mutase I | 28.7 | 5.8 | 334 | 87 | 2.25 | 2.41 |
| | 23 | pgk | BBMN68_399 | 3-phosphoglycerate kinase | 41.8 | 4.9 | 390 | 63 | 1.81 | - |
| | 24 | ackA | BBMN68_728 | Acetate kinase | 44.2 | 5.5 | 389 | 64 | 1.71 | - |
| | 39 | tasA | BBMN68_1072 | Aldo/keto reductase | 31.7 | 5.0 | 360 | 64 | -2.18 | - |
| | 42 | psm | BBMN68_1663 | Phosphoglucomutase | 60.2 | 4.9 | 686 | 59 | -3.01 | - |
| Nucleotide metabolism | 26 | mmT3 | BBMN68_917 | Cystathionine β-lyases/cystathionine γ-synthases | 41.9 | 5.3 | 511 | 56 | -2.00 | - |
| | 40 | hisG | BBMN68_446 | ATP phosphoribosyltransferase | 30.9 | 5.7 | 121 | 52 | -2.34 | - |
| | 41 | asd | BBMN68_1227 | Aspartate-semialdehyde dehydrogenase | 40.3 | 5.7 | 449 | 74 | -2.67 | - |
| | 10 | purA | BBMN68_1278 | Adenylosuccinate synthase | 46.4 | 5.4 | 93 | 37 | 5.69 | - |
| | 19 | purH | BBMN68_424 | AICAR transformylase/IMP cyclohydrolase | 58.4 | 5.5 | 114 | 32 | 2.20 | - |
| | 27 | prsA2 | BBMN68_1158 | Phosphoribosylpyrophosphate synthetase | 36.7 | 5.3 | 200 | 43 | -1.69 | - |
| | 28 | argL | BBMN68_809 | Argininosuccinate synthase | 45.5 | 5.1 | 256 | 52 | -1.72 | - |
| | 30 | ilvC1 | BBMN68_1262 | Keto-acid reductoisomerase | 38.5 | 5.1 | 641 | 67 | -1.93 | -3.81 |
| | 33 | metC3 | BBMN68_917 | Cystathionine β-lyases/cystathionine γ-synthases | 41.9 | 5.3 | 511 | 56 | -2.00 | - |
| | 29 | purA | BBMN68_1360 | Aspartate-semialdehyde dehydrogenase | 40.3 | 5.7 | 449 | 74 | -2.67 | - |
| | 31 | purP | BBMN68_870 | Phosphoribosylaminomimidazole (AIR) synthetase | 36.0 | 4.6 | 119 | 52 | -3.40 | - |
| Coenzyme metabolism | 20 | nppY | BBMN68_988 | NAD⁺ diphosphatase | 45.3 | 5.4 | 182 | 54 | 2.13 | 1.96 |
| | 32 | mdaB | BBMN68_1782 | Putative NADPH-quinone reductase | 19.3 | 4.6 | 76 | 44 | -1.96 | - |
| Signal transduction | 22 | luxS | BBMN68_914 | LuxS protein for autoinducer Al2 synthesis | 18.5 | 5.5 | 120 | 71 | 2.03 | 3.99 |
| Transcription | 34 | omtA | BBMN68_1756 | Oligobenzoate | 24.3 | 5.0 | 137 | 51 | -2.01 | 3.14 |
| | 36 | gatA | BBMN68_764 | Transcription elongation factor | 17.1 | 4.8 | 126 | 71 | -2.12 | 3.22 |
| Translation | 3 | rpsB | BBMN68_371 | 30S ribosomal protein S2 | 30.8 | 5.3 | 536 | 86 | ++ | - |
| | 7 | rpsO | BBMN68_307 | Ribosome-associated protein Y | 24.7 | 5.7 | 288 | 48 | 7.77 | 2.23 |
| | 38 | rlpA1 | BBMN68_765 | PKB-type peptidyl-prolyl cis-trans isomerase | 14.4 | 4.9 | 191 | 74 | -2.16 | - |
| Cell wall biogenesis | 44 | ppiB | BBMN68_332 | Peptidyl-prolyl cis-trans isomerase | 19.5 | 4.8 | 69 | 49 | -3.92 | 2.01 |
| Unknown | 14 | murC | BBMN68_210 | UDP-N-acetylmuramate-alanine ligase | 52.6 | 5.4 | 204 | 51 | 3.32 | - |
| | 5 | BBMN68_416 | Hypothetical protein | 26.9 | 4.6 | 343 | 75 | 13.3 | - |
| | 31 | BBMN68_912 | Hypothetical protein | 49.1 | 5.8 | 70 | 35 | -1.98 | -2.22 |

a Spot numbers refer to the proteins labeled in Fig. 2.
b Functions were assigned from the KEGG pathways for *B. longum* BBMN68.
c Proteomic fold change.
d Transcriptional fold change.
* The corresponding transcription was not influenced by bile.
* Proteins were detected only in the presence of bile.

mainly by bile efflux, bile salts decomposition and prevented bile adsorption.

**Bile Stress Induces General Stress Response in BBMN68** — Many molecular chaperones and proteases related to the general stress response were up-regulated in BBMN68 in response to bile. The transcription of *groEL* (**BN_44**) was 3.25-fold up-regulated upon exposure to bile salts, and the expression of *groES* (**BN_1589**) was 2.65-fold and 2.66-fold up-regulated at the mRNA and protein level, respectively. GroEL/ES complex is required for the proper folding of proteins and frequently involved in common stress response in bacteria (17, 45, 55–57). At the transcription level, **BN_1305**
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encoding HSP20 chaperone lbpA was 4.81-fold up-regulated upon bile exposure. In *B. breve*, the transcription of *hsp20* is strongly up-regulated by heat shock and osmotic shock (58). HSP20 chaperone belongs to the small heat shock protein (SHSP) which is important for the prevention of irreversible denaturation of heat-damaged protein (59). In addition, the transcription of four genes coding for proteolyis functions were up-regulated: trypsin-like serine protease DegQ (*BN* 1289, 3.50-fold), proteasome assembly chaperone (*BN* 413, 8.17-fold), dipeptidase (*BN* 1184, 3.82-fold) and endopeptidase (*BN* 1763, 3.09-fold). DegQ has been proved to be a pH-sensitive protease that controls protein quality under mild and severe stress conditions (60). These proteasome and peptidase play a major role in degradation of damaged proteins and reutilization.

*BN* 86 and *BN* 1435 encoding nitroreductase were 4.46- and 7.03-fold up-regulated at the mRNA level upon bile salts exposure. Nitroreductase is related with oxidative stress response by regulating the activities of antioxidant enzymes in *Saccharomyces cerevisiae* (61), and also protects *L. lactis* against oxidative stress (62). In addition, the transcription of a gene encoding cystathionine gamma-lyase (*BN* 1590) was 3.22-fold up-regulated in response to bile salts. This enzyme breaks down cystathionine into cysteine and 2-oxobutanoate, accompanying with the formation of H$_2$S or NH$_3$. Recent research has shown that H$_2$S could mitigate oxidative stress imposed by antibiotics in bacteria (63). Meanwhile, cystathionine gamma-lyase is a component of cysteine-mediated oxidative defense in *L. reuteri* (64). Moreover, NH$_3$ could capture cytoplasmic protons, thus contributing to acid stress tolerance of the host strain (65).

Other general stress-related genes up-regulated in *B. longum* in response to bile were *BN* 1764 (HdeD protein, 5.74-fold at the mRNA level), *BN* 138 (DNA helicase, 5.04-fold at the mRNA level), and *BN* 51 (universal stress protein, 2.06-fold at the protein level). It has been demonstrated that HdeD is a component of the H-NS-dependent regulatory cascade of acid stress defense in *E. coli* (66), also involved in general stress resistance (67). DNA helicase is essential in nucleic acid metabolism involving in DNA repair, replication, recombination, and RNA processes (68). Under stress conditions, DNA helicase mainly performs repair function and deals with DNA damages caused by bile. On the other hand, a DNA-damage inducible protein D (*BN* 1457) was down-regulated at the transcript level, reflecting high DNA quality control under bile stress. In *B. longum*, these genes play an important role in coping with other stress effects including protein misfolding, oxidative stress, low pH, and DNA damage caused by bile salts.

*Bile Stress Accelerates the Xylose Catabolism and Enhances the Inhibition of Polysaccharides Utilization by Glucose*—In *B. longum* NCIMB 8809 and *B. animalis* IPLA 4549, bile stress stimulated the carbohydrate catabolism, typically in the pentose phosphate pathway so-called bifid shunt (17, 18). In this study, most enzymes involved in bifid shunt were 3.02–4.15-fold up-regulated at the mRNA level (supplemental Table S2) and 1.71–12.6-fold overexpressed at the protein level (Table I), respectively, demonstrating that the bifid shunt was also enhanced in strain BBMN68 in response to bile. Meanwhile, three enzymes providing substrates for the bifid shunt were up-regulated under bile stress conditions. Glucose-6-phosphate 1-dehydrogenase (*Zwf*) and ribokinase (RbsK2) were only detected on the 2-DE gels of cultures treated by bile. *Zwf* catalyzes β-glucose-6-P to form glucono-1,5-lactone-6-P and RbsK2 catalyzes the reaction ribose + ATP → ribose-5-P. The two products can be transferred to xylose-5-P, which feeds the key enzyme xylose-5-phosphate/fructose-6-phosphate phosphoketolase (*Xfp*) in bifid shunt. Remarkably, the transcription of *BN* 1736 encoding xylose isomerase (*XylA*) was highly up-regulated (26.9-fold). *XylA* catalyzes the interconversion of xylose and xylolose, through which the xylose enters into the glycolysis pathway in *B. longum*. In colon, xylan is abundant from plant food, which cannot be digested by the host, while it can be decomposed into xylose by gut microbes and then be used by *B. longum*.

In the presence of bile, phosphoglucuronatase (Pgm) was among the most highly repressed protein (3.01-fold down) in *B. longum*. Pgm catalyzes the interconversion of glucose-1-P and glucose-6-P, the second step of starch and glycogen catabolism. Nine other genes involved in hydrolysis of oligo/polysaccharides were also down-regulated at the transcription level under bile stress conditions. In addition, six of the nine genes were transcribed at low levels even in sample untreated by bile salts (< 1000 mapping reads) (Table II). These results suggested that the expression of genes for polysaccharides utilization may be inhibited by glucose from the medium and such inhibition was more remarkable upon exposure to bile salts. Because glucose is more efficient for energy production, this superiority is beneficial for *B. longum* to cope with the bile stress, because ATP and reducing equivalents are essential to protect against bile stress, through pumping-out bile (12), repairing damaged protein and DNA (17), and regulating the internal pH (14).

**Effect of Bile Stress on Amino Acids, Nucleotides, and Fatty Acids Metabolism Processes**—Three enzymes related with aromatic amino acids synthesis were up-regulated in *B. longum* in response to bile salts. The transcription of *BN* 1136 encoding chorismate mutase was 3.98-fold up-regulated. It catalyzes the reaction chorismate → prephenate, which is the precursor of phenylalanine and tyrosine (69). *BN* 1775 and *BN* 1360 encode the same enzyme aspartate/tyrosine/ aromatic aminotransferases, which were 3.90-fold and 2.62-fold up-regulated at the transcription and translation level, respectively. These results suggested that aromatic amino acids were more abundant when *B. longum* was grown in MRSc with bile. Hydrophobic amino acids, such as aromatic amino acids and branched-chain amino acids (BCAAs) could protect proteins against attack of bile by building hy-
while, the productions of BN_529 and BN_533 were also less abundant in proteome, indicating that bile stress inhibited the synthesis of pyrimidine in BBMN68 in response to bile. A schematic representation of the nucleotides metabolism regulated by bile is shown in Fig. 3.

BN_1705, encoding cyclopropane fatty acid (CFA) synthase, was 6.52-fold up-regulated at the transcription level upon bile exposure. CFA synthase catalyzes the cyclization of phospholipid olefinic fatty acid, producing phospholipid CFA that could modify the viscosity and permeability of cell membrane. CFAs have been previously proved to protect bacteria from stress environments, such as acid stress (74) and rifaximin (75) by decreasing membrane permeability. Moreover, CFAs is also involved in resistance to cold, osmotic and ethanol in lactic acid bacteria (76–80). On the other hand, the fatty acid biosynthesis operon (BN_1556–1558) was down-regulated, indicating a general decrease in fatty acid synthesis process in response to bile stress, which has been suggested in some lactobacilli (29, 31, 33, 81), Enterococcus faecalis (82) and B. animalis (18, 52).

Effect of Bile Stress on Transmembrane Transport in BBMN68—Transcriptomic analysis revealed that 61 annotated genes encoding transmembrane transporters were regulated in BBMN68 under bile stress conditions (supplemental Table S3). Besides three presumed bile efflux transporters mentioned above (BN_664, 1049, 1434–1433), only five transporters were more abundant, including three monosaccharide transporters or its components, encoded by BN_1738 (simple sugar ABC-type transporter permease component, 6.63-fold), BN_1664 (MFS glucose/H⁺ symporter, 3.84-fold), and BN_1724 (pentose ABC-type transporter ATPase component, 3.32-fold), respectively. The enhanced monosaccharide transport corresponds to the observed acceleration in glycolysis using glucose and xylose as substrates in BBMN68 upon bile exposure. In addition, the transcription of BN_1101, encoding a small-conductance mechanosensitive channel (MscS), was 3.10-fold up-regulated by bile. Mechanosensitive
channels of large (MscL) and small conductance respond to the change in membrane tension and allow the passage of water and ions, preventing cell lysis (83, 84). Because bile is considered to cause membrane damage and permeability disorder leading to increased osmolality (48), increased expression of MscS may confer protection to BBMN68 from raising turgor pressure and cell lysis.

On the other side, most transporter genes were down-regulated when BBMN68 was grown in bile. Remarkably, 19 genes encoding components of eight transporters for oligo/polysaccharides utilization were strongly down-regulated (3.41- to 67.41-fold down), including six ABC-type transporters, one MFS transporters and one symporter. These results were corresponding to the repressed oligo/polysaccharides metabolisms in BBMN68 under bile stress. The repression of transporters specific for oligosaccharides has been detected by a microarray transcriptomic study in \textit{L. plantarum} in response to bile (85). Meanwhile, the transcription of genes involved in transport of amino acids, peptides, inorganic ions, and other unknown substrates were also down-regulated at different levels from 3.00- to 10.14-fold by bile. These transporters are transmembrane proteins, and the down-regulated expression could result in a more hydrophobic cell surface in BBMN68, which was verified through the cell surface hydrophobicity assay. The result showed that the surface hydrophobicity was increased from 1.4% to 17.7% in BBMN68 upon bile exposure (supplemental Fig. S3). It has been suggested that bile primarily exerts its antimicrobial effects on cell membranes by causing membrane damages (48). And we hypothesized that transmembrane proteins may be the pathway through which conjugated bile acids enter into cytoplasm because of the hydrophobicity of the bilayer lipid skeleton. In this case, bifidobacteria could decrease the protein proportion of cell membrane and enhance surface hydrophobicity, thereby protecting themselves from invasion of bile salts.

Bile Salts Promote Adhesion and Adaptation of BBMN68 in the GIT—In BBMN68, the transcription of \textit{BN_119} was 7.42-fold up-regulated under bile stress conditions. \textit{BN_119} encodes an esterase that has been considered as a relevant factor with adaptation to the gastrointestinal tract, through decomposing and reorganizing peptidoglycans in cell wall (81, 86). The bile-resistant mutants of \textit{Salmonella enteria} showed alteration of the cell wall lipopolysaccharide (87), which may be an adaptation mechanism to the enteric bile stress. So it was speculated that cell wall construction realignment of BBMN68 in response to bile salts could improve adaptation in the GIT. In addition, bile induced the transcription of \textit{BN_1395} (4.77-fold), encoding a housekeeping sortase related with anchoring of pili to the cell wall in Gram-positive bacteria (88, 89). Genomic analysis of \textit{B. longum} NCC2705 suggested \textit{B. longum} produced fimbriae for attachment in the GIT (90). To further investigate the influence of bile salts on the colonization and persistence of BBMN68 in the gut, the adhesion experiments and autoaggregation were performed in this study. As expected, the adhesion ability was increased by fivefold when BBMN68 grown with bile (Fig. 4A). Meanwhile, the autoaggregation degree was decreased from 52.67% to 38.10% after 8h incubation (Fig. 4B). In this case, once BBMN68 enters into the intestinal tract, the bifidobacterial cells tend to disaggregate because of enteric bile salts, which benefits subsequently adhere to the epithelium. Taken to-

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**Fig. 3.** Nucleotides metabolism influenced by bile stress in \textit{B. longum} BBMN68. A, An operon composed of seven genes related with pyrimidine metabolism was repressed in the presence of bile. Changes in transcription level are represented as log2 intensity ratio values shown with the gene names. P and T indicate putative promoter and terminator, respectively. B, Schematic representation of the regulated pathways in nucleotides metabolism. Red and blue gene names indicated up- and down-regulation in the presence of bile.
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**Fig. 4.** Effect of bile on adhesion to HT-29 cells (A), and autoaggregation (B), of *B. longum* BBMN68. Values are mean ± standard deviation of one representative of two independent experiments performed in triplicate. Abbreviations: CK, BBMN68 grown without bile; OG, BBMN68 grown in the presence of bile.

Together, bile could act as a gut signal and facilitate the adhesion and colonization of BBMN68 in the host.

The transcription and translation of *luxS* (*BN_914*) encoding S-ribosylhomocysteine lyase for autoinducer-2 (Al-2) synthesis were respectively up-regulated by 3.99- and 2.03-fold, which indicated that bile also stimulates the communication between BBMN68 and other enterobacteria. Autoinducers are signal molecules that activate the quorum sensing in bacteria, and Al-2 acts as a signal which is used for interspecies cell-cell communication in both Gram-negative and Gram-positive bacterial species (91). The previous study has been proposed that *B. longum* NCC2705 may use Al-2 to recognize its presence within a host and enhance the expression of a series of metabolic genes required for fast propagation. Meanwhile, the secreted lactic acid and acetic acids would prevent the colonization of pathogens (92). Finally, the transcription of an *IS1595* family insertion sequence (*BN_1164-1166*) from *Streptococcus agalactiae* was up-regulated (3.37–4.60-fold) in BBMN68 in response to bile. Aminoglycoside nucleotidyltransferase (*BN_1166*), which decomposes aminoglycosidic antibiotics, may confer resistance to the corresponding antibiotics produced by other enterobacteria or derived from diet and therapies medicine. In addition, this *IS1595* family insertion sequence was only found in BBMN68 genome, which contributed to the long-term colonization of this strain in the gut of healthy centenarian.

**Transcription and Translation of BBMN68 were Influenced by Bile**—In the presence of bile, many transcription regulatory genes were differently expressed at the mRNA level, including three two-component systems (TCSs) and 10 transcription factors (TFs). The transcription of a gene encoding an extra-cytoplasmic function (ECF) sigma factor RpoE (*BN_247*), which belongs to the *σ^70^* family (93), was 4.50-fold up-regulated in BBMN68 in response to bile salts stress. Upon receiving environment stimulus, ECF σ factor is released and binds to RNA polymerase to stimulate transcription of a specific group of genes (94). RpoE has been determined as an important regulator in diverse stress response, such as heat shock in *E. coli* (95), acid adaptation in *Streptococcus mutans* (96), and oxidative stress in many bacteria (97). In this study, it was for the first time reported that RpoE was involved in resistance to bile stress in bifidobacteria. Moreover, there is no clear function for the other regulatory genes except TCS *senX3-regX3*. But their role cannot be disregarded and the regulatory network controlling bile stress response should be deeply investigated, which may be a basis to further comprehend and examine the bile stress response in bifidobacteria.

In the aspect of translation process, ribosome-associated protein Y (*BN_307*) was 7.77-fold up-regulated in BBMN68 upon bile exposure. Ribosome-associated proteins are key factors that promote the folding pathways of newly synthesized proteins (98). The expression of two genes encoding putative elongation factor (*BN_118*) and elongation factor Ts (*BN_372*) were 5.12-fold down-regulated at the transcription level and 1.73-fold down-regulated at the protein level, indicating a slower translation rate in BBMN68 under bile stress. The transcription of *relBE* operon (*BN_287–288*) was 3.99-fold and 3.70-fold down-regulated under bile stress conditions, respectively. *relA* (*BN_296*), encoding GTP-pyrophosphokinase involved in (p)ppGpp formation (99), was 4.02-fold up-regulated at the transcription level. The *relBE* promoter is repressed by its product RelB, and RelE functions as a corepressor (100). The decreased mRNA level of *relBE* suggested an accumulated amount of RelBE, which belong to toxin-antitoxin (TA) complex family. The toxin RelE can be activated by protease Lon through the polyphosphate (PolyP)-dependent signal pathway initiated by ppGpp, subsequently cleaves mRNA at the ribosomal A-site in translation process, and thus reduces the global level of translation under nutritional stress (101). These results implied that BBMN68 may employ the RelBE system to control the translation process under bile stress conditions.

The *TCS senX3-regX3* Played an Important Role in Bile Stress Response in BBMN68—In BBMN68, bile salts induced the transcription of *BN_1080* (5.55-fold) encoding a sensor
Inorganic phosphate (Pi) acquisition, regulated (102) and controls the expression of genes involved in the presence of 0.75 g l⁻¹ nisin induction; lane 3 and 4, purified products of NZCK and NZregX3-His₆. D, EMSA showed the specific interaction of RegX3 and predicted binding sites upstream pstS. Binding reactions consisted of: (lane 1) 20 fmol labeled probe alone, (lane 2) 20 fmol labeled probe and 3.5 μg RegX-His₆, (lane 3) 20 fmol labeled probe, 4 pmol unlabeled probe, and 3.5 μg RegX-His₆. Abbreviations: M, marker; B, binding complex; F, free DNA.

histidine kinase, and the downstream BN_1079 encoding a response regulator was also up-regulated by 2.66-fold upon bile exposure. The two genes composed a TCS, which has been known as senX3-regX3 system in Mycobacterium tuberculosis. In M. tuberculosis, senX3-regX3 is positively auto-regulated (102) and controls the expression of genes involved in inorganic phosphate (P) acquisition, i.e. phoA and pstS (103). However, its role in bile stress response has not been reported in bacteria.

In this study, the regulatory target genes of response regulator RegX3 were predicted by the B1H method. The recombinant bait vector pB1H2-regX3 was introduced into E. coli US0, and whole-cell lysates were prepared and subjected to Western blotting using anti-Flag antibody. A ~ 40 kDa band was observed on the membrane, indicating the expression of RegX3 as a carboxy-terminal fusion to the ω-subunit of RNA polymerase (Fig. 5A). The DNA-binding sequence for RegX3 was identified by a two-step selection resulting in 13 sequences showed a 7-bp motif (GARRACY, where r = G/A and Y = C/T, E-value = 1.7e⁻⁸) in MEME analysis (Fig. 5B). Target Explorer analysis (cut-off score 5.00) predicted that RegX3 controlled the transcription of pstS (BN_1078), putA2 (BN_1612), hdeoD (BN_1764), and six other genes (supplemental Table S4). Among these genes, pstS has been mentioned to confer resistance to some toxic compounds, such as penicillin on Streptococcus pneumonia (104) and sodium benzoate on E. coli (105). Interestingly, in the genome of bifidobacteria, senX3-regX3 is followed by a gene cluster pstSCAB, encoding the high-affinity phosphate transporter Pst. And the senX3-regX3 operon and pstS were simultaneously up-regulated at the transcription level, implying that the senX3-regX3 system enhanced the expression of pstS and then conferred bile resistance on BBMN68.

To verify this hypothesis, the interaction between RegX3 and the upstream sequence of pstS was further verified by EMSA. SDS-PAGE revealed a single band with an expected molecular mass of ~ 27 kDa, suggesting that the recombinant RegX3-His₆ has been successfully expressed and purified (Fig. 5C). The EMSA result indicated that the purified RegX3-His₆ bounded to biotin-labeled ptsS and retarded the mobility (Fig. 5D). Assays were further performed using unlabeled probes as a specific competitor the biding site. This competitor abolished the specific shift and suggested the specific binding of RegX3His to the probe ptsS. Furthermore, the recombinant strain L. lactis NZpstS showed 31.4-fold enhanced resistance to 1.0% ox-bile compared with the control L. lactis NZCK (supplemental Fig. S4). Based on these results, we suggest that the senX3-regX3 system senses the bile stress signal in the gut and promotes the expression of pstS to maintain a high-level of Pᵢ uptake in the sterile environment. The accumulated Pᵢ cooperates with the enhanced glycolysis process to produce more ATP to confer bile resistance phenotype.

Concluding Remarks—In this study, we explored the bile stress response of a potential probiotic strain B. longum BBMN68 using an RNA-Seq transcriptome profiling complemented with a 2-DE proteomic analysis. Nearly 300 genes were differentially expressed at either mRNA or protein levels in the presence of 0.75 g l⁻¹ ox-bile. The reported changes in gene expression appeared to be associated with pathways contributing to cope with bile stress in BBMN68. First, HLP was located on the cell surface and functioned as a barrier against the adsorption of bile salts; the cell membrane composition was modified by increased CFAs and decreased transmembrane transporters, which prevent or at least reduce the influx of bile salts to the bacterial cell. When bile salts entered into cytoplasm, overexpressed BSH played a central role by catalyzing the deconjugation of glycine- or taurine-linked bile acids. The up-regulated bile efflux transporters would also pump the bile salts out from the cell. Furthermore, various genes related to general stress response, such as genes encoding DegQ, nitroreductase, and HdeoD, were induced to protect cell against bile damages. On the other
hand, central metabolism processes were modulated as an adaptation mechanism to bile stress. In order to effectively cope with bile stress for BBMN68 in the xylan-rich colon environment, xylose utilization and bifid shunt pathways were enhanced for producing more reducing equivalents and energies. Nucleotide metabolisms except ATP synthesis and global fatty acid biosynthesis were reduced in BBMN68 in response to bile salts. In addition, RelBE system was employed to slow down translation process under bile stress conditions. And bile functions as a gut signal and promotes interactions of BBMN68 with the host and other enterobacteria. For example, bile-induced abundance of sortases related with anchoring of pili in Gram-positive bacteria facilitated the adhesion and colonization of BBMN68 in the gut. BBMN68 was supposed to perceive the presence of competitors using the up-regulated LuxS/AI-2 system and then annihilate AI-2 produced by other bacteria, resulting in rapid propagation of BBMN68 and inhibition of pathogens’ growth. Finally, 15 regulatory genes were differentially expressed in BBMN68 under bile stress conditions. Among those, an ECF sigma factor RpoE was revealed to involve in resistance to bile salts probably by stimulating the transcription of a specific group of genes related with diverse stress response. And the senX3-regX3 system was suggested to sense the bile salts signal and promote the transcription of pstS leading to accelerated Pi uptake for producing more ATP. Our present findings allowed us to propose a bile stress response mechanism model in B. longum BBMN68 and even in bifidobacteria (Fig. 6).

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**S** This article contains supplemental Data S1, supplemental Tables S1–S4, and supplemental Fig. S1–S4.
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