Journal of Bioscience and Bioengineering
VOL. 112 No. 5, 451 – 457, 2011

www.elsevier.com/locate/jbiosc

Comparison of *Bifidobacterium breve* strain Yakult transcriptomes in germ-free mice with those in fecal cultures

Eiji Ishikawa,* Tatsuichiro Shima, Kazunori Suda, Yukio Shirasawa, Takashi Sato, and Yoshinori Umesaki

Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi-shi, Tokyo 186–8650, Japan

Received 11 May 2011; accepted 19 July 2011
Available online 8 September 2011

Bifidobacteria are beneficial to human health, but the mechanism remains unknown. We employed oligonucleotide microarrays to identify the *Bifidobacterium breve* strain Yakult (BbrY) genes up-regulated specifically in mouse intestine. Based on BbrY transcriptional responses in germ-free mice and in fecal cultures, *k*-means clustering picked up 93 genes that were up-regulated in the mouse intestine and thereafter Venn analysis to exclude genes that were up-regulated in both the mouse intestine and the fecal culture classified 45 genes as up-regulated specifically in the mouse intestine. Most of those genes are involved in sugar transport or sugar liberation, although the functions of several genes are unknown. Most of these genes are clustered on the BbrY genome and appear to be organized into operons. Expressions of several genes were further investigated by real time PCR, revealing that their expression profiles were identical in the mouse cecum and colon. The up-regulation of genes involved in sugar liberalization and uptake suggests that BbrY could possibly maintain energy homeostasis inside the mouse intestine, which contains low quantities of readily fermentable sugars.

[**Key words:** Fecal culture; Glycosidases; Monoassociated mice; Mucosal crosstalk; Sugar transport]

The human gut is populated with as many as 100 trillion microbes, creating a complex ecosystem (i.e., gut microbiota). Intestinal microbes metabolize dietary components remaining after digestion and absorption (1); they also metabolize sugar derived from host mucin to produce short-chain fatty acids (SCFAs) that are utilized for energy source by the epithelial cells of the large intestine (2). The composition of the gut microbiota depends on diet, medications, and age of the host, and thereafter appear to play an important role in host homeostasis. Microbes comprising the microbiota vary depending upon the region of the intestine: because the conditions in the large intestine are anaerobic and weakly acidic, the large intestine is occupied by obligate anaerobes such as bacteroides, bifidobacteria, and clostridia, so that the population of each bacterium can reach a density of as many as 10^9 to 10^{11} cells g^{-1}(3).

Bifidobacteria, a major bacterial group in the human large intestine, bestow health benefits on humans, but their numbers decrease upon weaning and aging (4,5). So far, bifidobacteria have been utilized as probiotics (6). Intake of daily products containing bifidobacteria improves intestinal disorders such as intractable diarrhea (7), inflammatory bowel diseases (8,9), and weight gain in premature infants (10), and also prevents *Campylobacter enteritis* infection (11). Today, bifidobacteria are economically important because they are added in high numbers as live bacteria to numerous food preparations, with various health-related claims (12). Although some beneficial properties of bifidobacteria are documented in clinical trials as mentioned earlier, how they interact with their host remains largely unknown. We hypothesize that the beneficial effects of bifidobacteria require biological interactions (*viz.* mucosal crosstalk) with the intestinal epithelium, and assume that bifidobacteria will induce the genes involved in mucosal crosstalk within the gastrointestinal (GI) tract. Mucosal crosstalk has been first reported on a commensal bacterium, *Bacteroides thetaiotaomicron* (13), and thereafter studied mainly on expression analysis of epithelial cells (14). However, the term "mucosal crosstalk" is literally not a unidirectional communication but bidirectional; analysis of bacterial gene expression in the intestine is required for the study of mucosal crosstalk. Recent microbial genome analyses have enabled us to explore microbial host specificity and mucosal crosstalk with the host (15). The first bifidobacterial genome sequenced was that of *Bifidobacterium longum* NCC2705 (16,17). Nowadays several genomes of bifidobacteria are available and the microarrays designed to analyze bifidobacterial transcriptomes on various conditions (18,19). We have also sequenced the *Bifidobacterium breve* strain Yakult (BbrY) genome (20) and have designed a microarray to analyze its transcriptomes under various conditions, especially those within the GI tract.

In this study, we aimed to identify the BbrY genes that were up-regulated (i) in the intestine and (ii) in fecal cultures imitating intestinal conditions. Excluding the genes that are up-regulated in both the intestine and the fecal culture allows us to determine the genes implicated in mucosal crosstalk, because these genes should be up-regulated specifically in the mouse intestine in accordance with bidirectional communication postulating that nutritional conditions

* Corresponding author. Tel.: +81 42 577 8984; fax: +81 42 577 3020.
E-mail address: eiji-ishikawa@yakult.co.jp (E. Ishikawa).

1389-1723/$ - see front matter © 2011, The Society for Biotechnology, Japan. All rights reserved.
doi:10.1016/j.jbiosc.2011.07.016

inside intestine were precisely mimicked by fecal cultures. This is the first report to describe *B. breve* transcriptomes *in vivo* and *in vitro*, and to present hints about the molecular mechanisms of host–bacterial symbiosis.

MATERIALS AND METHODS

Animals and BbrY administration Germ-free (GF) BALB/c mice (female, 15 weeks old, n = 4 in each group) from our institute were fed on a solid FR-2 diet (Funabashi Farms, Chiba, Japan) comprising fishmeals, soybeans, corn, flour, bran, skim milk, soybean oil, vitamin mixture, and mineral mixture, which was treated with gamma-irradiation and used for all animal experiments. BbrY was anaerobically cultivated in modified ILS (m-ILS) medium comprising 10 g trypticase peptone (Difco Laboratories, Detroit, MI, USA), 5 g yeast extract (Difco Laboratories), 3 g tryptose (Difco Laboratories), 10 g lactose, 3 g KH_2PO_4, 3 g K_2HPO_4, 2 g tri-ammonium citrate, 1 mL pyruvate, 0.3 g cysteine hydrochloride, 1 mL Tween 80 (Sigma, St. Louis, MO, USA), 0.575 g $MgSO_4 \cdot 7H_2O$, 0.12 g $MnSO_4 \cdot 4H_2O$, and 0.034 g $FeSO_4 \cdot 7H_2O$ in 1.0 L distilled water (pH 6.8). The cells cultivated until late exponential growth were then washed and resuspended in phosphate-buffered saline. Thereafter, we orally inoculated each GF mouse with 10^9 CFU of BbrY; the GF mice thus monoassociated with BbrY are abbreviated hereafter as BbrY-mice. All experimental procedures were approved by Yakult Central Institute's ethical committee for animal experiments.

Bacterial enumeration Contents of the cecum and colon were sampled immediately after the mice were killed. Serial dilutions of the intestinal contents of BbrY-mice were plated on agar plates of m-ILS medium and cultivated at 37°C. Preparation of the dilutions and cultivation were carried out anaerobically.

Fecal culture Feces collected from GF mice were suspended in phosphate buffer (100 mM, pH 6.8) at a volume-to-weight ratio of 5 mL buffer to 1 g feces, and the fecal suspensions were filtrated with gauze to remove large suspended solids. The filtrate was dispensed into vials and bubbled with O_2-free nitrogen gas. The vials were then sealed with a butyl rubber stopper and autoclaved (121°C, 15 min). This fecal medium was inoculated by syringe with 5% (v/v) of the late exponential m-ILS culture and incubated at 37°C over night to give the fecal pre-culture. Another fecal medium was inoculated by syringe with 5% (v/v) of the fecal pre-culture to minimize carryover of m-ILS medium.

Preparation of bacterial RNA Intestinal contents or fecal culture were mixed with twice volume of RNA protect Bacteria Reagent (Qiagen, Heidelberg, Germany) and centrifuged (20,000 × *g*, 4°C, 5 min). Pellets of fecal culture or intestinal contents were suspended in 1.0 mL of Tris–EDTA buffer (Tris, 50 mM; EDTA, 10 mM; pH 8.0) that contained 10% (w/v) of sucrose; thereafter, 100 μL each of N-acetyl-muramidase (0.5 mg mL^{-1}) (Seikagaku-kogyo, Tokyo, Japan) and lysozyme (50 mg mL^{-1}) (Sigma) was added to the suspensions to make protoplasts of the BbrY cells after a 10 min incubation at 37°C. The enzyme-treated suspensions were centrifuged at low speed for 1 min at 200 × *g* and 4°C to remove any insoluble materials that might inhibit bacterial RNA extraction. Discarding the precipitated impurities, the bacterial suspensions were transferred into new micro tubes and subjected to high-speed centrifugation (20,000 × *g*, 4°C, 5 min). The low-speed centrifugation removal step is unnecessary for m-ILS cultures, because m-ILS medium contains no insoluble materials. RNA was extracted from the precipitated BbrY spheroplasts by using RiboPure-Bacteria (Ambion, Austin, TX, USA). The decomposition and concentration of the bacterial RNA were checked by a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) and RNA 6000 Series II Nano kit (Agilent Technologies).

Microarray analysis Custom microarrays (Takara Bio, Shiga, Japan) based on the genome sequence of BbrY were equipped with 60mer probes that specifically detect each transcript of the BbrY genes. RNAs extracted from different samples (e.g., m-ILS culture, intestinal content, fecal culture) were labeled with cyanine 3-dUTP (Cy3) or cyanine 5-dUTP (Cy5) by using an RNA fluorescence labeling core kit (M-MLV version) v2.0 (Takara Bio). The Cy3- and Cy5-labeled RNAs were mixed and competitively hybridized onto the BbrY microarrays. Unless specified, RNA extracted from the exponential m-ILS culture and labeled with Cy3 was used as the reference for inter-microarray comparison, because RNA from the exponential m-ILS culture should be the most uniform and abundant throughout this study. After washing the BbrY microarrays according to manufacturer's protocol, a DNA Microarray Scanner (Agilent Technologies) was used to detect dual channel signals (Cy3 and Cy5 fluorescence), and the microarray images were analyzed by GenePix Pro 6.0 (Molecular Device, Sunnyvale, CA, USA). Clustering analyses were performed with Acuity 4.0 (Molecular Device) and Venn analyses were performed with SilicoCyte (SilicoCyte, Chesterfield, MO, USA). Statistical analysis was performed by the Benferroni one-sample significance test with Acuity 4.0 (Molecular Device).

Real-time polymerase chain reaction (PCR) Complementary DNA was individually synthesized from RNA preparations using a random nonamer, RNase inhibitor (Toyobo, Osaka, Japan), and RevertraAce (Toyobo). Real-time PCR was performed with an ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master Mix (Toyobo). The intensity of the expression of each gene was normalized against that of a putative housekeeping gene encoding ribosomal S1 protein (CDS0788) (S1). The specificity of gene amplification was determined from the dissociation curve according to manufacturer's instructions. Quantification values are expressed as the change relative to the exponential m-ILS culture used as the reference. Real-time PCR quantifications were performed with the following primers: 5′-TCAACTTCGGCGCTTTCG-3′ and 5′-GACAGCTCGGAAACGTGGAT-3′ for S1; 5′-CCCAGGTCTCTTGGAACTTCAC-3′ and 5′-GGGCCTGACCGATCTTGTT-3′ for ABC transporter periplasmic component (CDS1940) (ABC_PC); 5′-CATCGGCTACGTGTT-CAAGTACA-3′ and 5′-CCGAGTGCCTTGCCAAGT-3′ for ABC transporter membrane spanning permease (CDS1960) (ABC_MSP); 5′-ACGCTGGTTACGGTACTGAACA-3′ and 5′-GCAACGTTCACATCCGTCTTC-3′ for phosphotransferase system β-glucoside-specific enzyme IIABC component (CDS1686) (PTS); 5′-TGAGCTGCCTGATGCAAAAG-3′ and 5′-CAGGCACAGCATGGAAAGC-3′ for β-glucosidase (CDS1750) (BGL); 5′-TTGGGATGCGTGGGAGAT-3′ and 5′-TTGATCCGGTCATGGCATT-3′ for α-mannosidase (CDS1205) (AMN); 5′-TTCGTCAGCTGCGAGTACATG-3′ and 5′-GCCGGAG-TAGCGCTCGTA-3′ for β-galactosidase (CDS1640) (BGA).

Search for homologues on other bifidobacteria We used BLASTP to survey the *Bifidobacterium longum* NCC2705 and *B. adolescentis* ATCC15703 (21) genomes deposited in the GIB microbial genome sequence database (http://gib.genes.nig.ac.jp/) for any similarities to the deduced amino acid sequences in the BbrY protein coding region. Search criteria were 30% identity and 50% similarity based on amino acid sequence.

RESULTS

BbrY population in mouse intestine After administering 10^9 CFU of BbrY to GF mice, the live cell counts in the feces reached approximately 10^{10} cells per gram by day 3 on which BbrY population in feces reached a plateau, and increased slightly by day 28 (Fig. 1A). Day 28 was employed as late stage on colonization because the intestinal contents of early stage were presumably washed out.

Up-regulated genes in mouse intestine To examine expression profiles in the mouse intestine, contents of the cecum on days 3 and 28 after BbrY administration were analyzed by microarrays. Scatter plots revealed that sugar ATP-binding cassette (ABC) transporters and acetate production via pyruvate (AVP) were up-regulated on day 3 (Fig. S1, Table S1). In addition to those genes, phosphotransferase system (PTS) and glycosidases were up-regulated on day 28 in the cecum (Figs. S2, S3 and Table S1). Although genes down-regulated in mouse cecum were investigated, the numbers of such genes were few (Figs. S1, S2). Such genes are putatively up-regulated in m-ILS culture but not down-regulated in mouse intestine. Therefore we focused on up-regulated genes in this study. As the amount of bacterial RNA from intestinal contents of each mouse was so limited that we would rather have tested various combinations of interests (e.g., day 3 vs. day 28; cecum vs. colon) than have done biological replicates for statistical analysis. Therefore statistical analysis was unavailable except for data on day-28 mouse ceca. However, microarray data of intestinal BbrY were very reproducible among mice.

To compensate for a lack of sufficient sampling points for clustering on dual-channel microarrays, we employed a loop design analysis (22) for the *in-vivo* experiments (Fig. 1B). The genes that were notably up-regulated on the scatter plots converged to form a compact cluster, and we assumed that several of these genes would be involved in mucosal crosstalk between BbrY and epithelial cells (Fig. 1B). The *k*-means clustering picked up most of the prominent genes on the hierarchical clustering and scatter plots to present 93 genes potentially involved in mucosal crosstalk (Table S1); statistical analysis revealed that most of 93 genes were significantly ($p < 0.05$) up-regulated in mouse ceca on day 28, suggesting that clustering method we used is reliable. Because most of the genes up-regulated in the intestine were unidirectionaly clustered on the BbrY genome, they appeared to be organized into operons.

BbrY RNAs from cecal and colonic contents on day 28 were hybridized competitively onto the same microarray; almost all spots were plotted along the diagonal line (Fig. S4), suggesting that the expression profiles of BbrY were almost the same in the cecum as in the colon.

Up-regulated genes in fecal cultures Because we suspected that the 93 candidate genes (Table S1) also included those responding