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Differences in acid tolerance between *Bifidobacterium breve* BB8 and its acid-resistant derivative *B. breve* BB8dpH, revealed by RNA-sequencing and physiological analysis

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ABSTRACT

Bifidobacteria are common inhabitants of the human gastrointestinal tract, and their application has increased dramatically in recent years due to their health-promoting effects. The ability of bifidobacteria to tolerate acidic environments is particularly important for their function as probiotics because they encounter such environments in food products and during passage through the gastrointestinal tract. In this study, we generated a derivative, *Bifidobacterium breve* BB8dpH, which displayed a stable, acid-resistant phenotype. To investigate the possible reasons for the higher acid tolerance of *B. breve* BB8dpH, as compared with its parental strain *B. breve* BB8, a combined transcriptome and physiological approach was used to characterize differences between the two strains. An analysis of the transcriptome by RNA-sequencing indicated that the expression of 121 genes was increased by more than 2-fold, while the expression of 146 genes was reduced more than 2-fold, in *B. breve* BB8dpH. Validation of the RNA-sequencing data using real-time quantitative PCR analysis demonstrated that the RNA-sequencing results were highly reliable. The comparison analysis, based on differentially expressed genes, suggested that the acid tolerance of *B. breve* BB8dpH was enhanced by regulating the expression of genes involved in carbohydrate transport and metabolism, energy production, synthesis of cell envelope components (peptidoglycan and exopolysaccharide), synthesis and transport of glutamate and glutamine, and histidine synthesis. Furthermore, an analysis of physiological data showed that *B. breve* BB8dpH displayed higher production of exopolysaccharide and lower H^+-ATPase activity than *B. breve* BB8. The results presented here will improve our understanding of acid tolerance in bifidobacteria, and they will lead to the development of new strategies to enhance the acid tolerance of bifidobacterial strains.

1. Introduction

Bifidobacteria are common inhabitants of the human gastrointestinal tract, constituting up to 91% of the gut microbiota in breast-fed infants, and 6.9% of the gut microbiota in adults [1,2]. Several species in the genus *Bifidobacterium* are considered probiotics, and their presence has been associated with health-promoting effects. Therefore, some bifidobacteria have been used in functional foods, especially fermented dairy products [3]. As probiotics, it is generally believed that they must survive passage through the gastrointestinal tract and reach the distal part of the intestine in sufficient numbers (approximately 10^6–10^8 CFU/g) to exhibit their beneficial effects [4]. However, bifidobacteria are usually exposed to various acidic environments (e.g., the low pH of fermented dairy products in which bifidobacteria are added as probiotics, and the low pH of the stomach), which reduce their viability, thereby resulting in less than the recommended sufficient numbers reaching the intestine. Consequently, acid tolerance is recognized as a desirable property of potential probiotic bifidobacteria [5]. In addition, most bifidobacteria have a weak acid tolerance [6], which limits their application in probiotic products.

As part of a strategy to improve the acid tolerance of bifidobacteria, it is necessary to fully understand their acid tolerance mechanisms. To survive in acidic environments, several response mechanisms are employed by bifidobacteria, including the

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maintenance of pH homeostasis by H^+-ATPase, the production of NH_3, and the regulation of global signaling systems and the general stress response [7–10]. These studies mainly focused on the responses of bifidobacteria to acid stress or adaptation. Nevertheless, it is not clear why different strains, including acid-resistant derivatives and their parental strains, have different acid tolerance levels.

Currently, next-generation sequencing technology, e.g., RNA-sequencing (RNA-seq), is a powerful tool for transcriptome profiling [11,12]. Compared with microarray methods, RNA-seq provides higher efficiency and sensitivity, and can quantify low-abundance transcripts [13]. Moreover, in terms of accuracy and precision, RNA-seq is comparable to real-time quantitative PCR (RT-PCR) [14]. In the present study, a comparative analysis between *B. breve* BB8 and its acid-resistant derivative BB8dpH was conducted, based on transcriptome and physiological data, to explore the reasons for their different acid tolerance levels.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study were *B. breve* BB8 (GenBank Accession No. HM068368) and its acid-resistant derivative BB8dpH (China General Microbiological Culture Collection Center (CGMCC) Accession No. 8370). The d in BB8dpH stands for derivative. Strains were grown under anaerobic conditions, at 37 °C, in batch cultures in de Man-Rogosa-Sharpe (MRS) [15] supplemented with 0.05% (w/v) L-cysteine (MRSC, initial pH 6.5). Stationary growth phase cells were obtained after 16 h incubation, harvested by centrifugation, and then used for all subsequent experiments. After 16 h of growth, both strains grew to similar densities (approximately 9.3 log CFU/mL) and reduced the pH of the medium to 5.2. To compare the growth rate of strains *B. breve* BB8 and its acid-resistant derivative BB8dpH, cultures of each strain were transferred to fresh batch MRSC medium by 1% inocula, respectively, and incubated anaerobically at 37 °C. Cell growth was measured spectrophotometrically at 600 nm.

2.2. Isolation of the acid-resistant derivative

Cells from an overnight culture of strain *B. breve* BB8, previously subcultured three times in standard conditions, were washed in phosphate-buffered saline containing 0.05% (w/v) L-cysteine (pH 7.4) and then transferred to fresh MRSC medium adjusted to pH 3.2 with 6 N HCl. These cultures were incubated at 37 °C for 16 h, and then cells resistant to acidic conditions were recovered by plating on MRSC agar (pH 6.5), followed by incubation at 37 °C for 3–4 days. The acid-resistant derivative *B. breve* BB8dpH was obtained after repeating this process many times.

2.3. Molecular identification of the acid-resistant derivative

Genomic DNA of the acid-resistant derivative was extracted according to the method of Ausubel et al. [16], which was slightly modified by adding a cell disruption step with a Fast prep instrument (Thermo Fisher Scientific, USA) prior to the extraction procedure. Primers (5′-ATAATGCGGCCGCACGGGCGGTGTGTRC-3′ and 5′-TAATAGCGGCCGCAGCMGCCGCGGTAATWC-3′) were used to amplify the 16S rRNA partial gene (900-bp fragment length) as previously described [17]. The PCR products were purified with TaKaRa DNA fragment Purification Kit (TaKaRa Biotechnology, Dalian, China) and sequenced on an ABI-Prism 3730 automated sequencer (PE Applied Biosystems. USA). Identification of the strain was accomplished by analyzing the 16S rRNA gene sequences using RDP classifier software (version 2.2) in the GenBank database (http://www.ncbi.nlm.nih.gov/blast).

2.4. Acid tolerance assays

Stationary growth phase cells were harvested by centrifugation (7600 × g, 10 min), washed twice with phosphate-buffered saline with 0.05% (w/v) L-cysteine (pH 7.4), and re-suspended in fresh MRSC medium (adjusted to pH 3.2 with 6 N HCl). Aliquots of cell suspension were incubated at 37 °C under anaerobic conditions, and samples were withdrawn at different times, serially diluted and plated on MRSC agar (pH 6.5) plates. Plates were incubated at 37 °C for 48–72 h under anaerobic conditions, after which colony forming units (CFU) were enumerated. The experiments were performed in triplicate.

2.5. Stability evaluation of acid-resistant phenotype of the derivative

The stability of acid-resistant phenotype of the derivative *B. breve* BB8dpH was determined according to the following procedure. Briefly, cultures of strains BB8dpH and BB8 were transferred daily into fresh batch MRSC medium (initial pH 6.5) for 20 consecutive days by 1% inocula at each transfer, respectively. After each transfer, the cultures were incubated at 37 °C for 16 h anaerobically, collected and then used to evaluate their survival in acidic conditions (pH 3.2 for 4 h) as described above. The parental strain *B. breve* BB8 was included as a control.

2.6. Sample preparation and RNA isolation

Stationary growth phase cells were collected by centrifugation (10,000 × *g* for 10 min at 4 °C), and washed twice with phosphate-buffered saline containing 0.05% (w/v) L-cysteine (pH 7.4). The cell pellets were immediately ground into a fine powder in the presence of liquid nitrogen, and then total cellular RNA was isolated from each of the cell samples using the Easy Pure RNA Kit (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. RNA quality was characterized initially on an agarose gel and NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the further assessed by RIN (RNA Integrity Number) value (>8.0) using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA).

2.7. RNA-seq and subsequent data analysis

Sequencing libraries were prepared using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads (Life technologies, CA, USA). Fragmentation of the remaining RNA was carried out using divalent cation fragmentation buffer (New England Biolabs, Ipswich, MA, USA). First-strand cDNA was synthesized using random hexamer primers. Second-strand cDNA was synthesized using dNTPs mixture containing dUTP, DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities and enzymes were removed. After adenylation of 3′ ends of blunt-end DNA fragments, NEBNext adaptor oligonucleotides were ligated to cDNA fragments. In order to select cDNA fragments of preferentially 200-bp in length, the library fragments were purified with AMPure XP beads system (Beckman Coulter, Beverly, USA). The index adaptors were introduced by PCR using NEB Universal PCR Primer and index adaptor primer. The second-strand cDNA containing dUTP was digested with USER enzyme (New England