Short communication: Modulation of fatty acid metabolism improves oxygen tolerance of *Bifidobacterium animalis* ssp. *lactis* Probio-M8

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ABSTRACT

Bifidobacterium animalis ssp. *lactis* Probio-M8 is a potential probiotic strain that was isolated from human milk. Previously, we obtained an oxygen-resistant variant (Probio-M8o) of Probio-M8 by an adaptive evolution strategy. In the present study, a comparative transcriptomic analysis of Probio-M8o and Probio-M8 was carried out to reveal the cellular mechanism of the oxygen-resistant phenotype. Using RNA-seq, 210 and 217 differentially expressed genes were identified in Probio-M8o compared with Probio-M8 after oxygen exposure for 30 and 60 min, respectively. The oxygen treatment upregulated a set of genes that encoded proteins responsible for fatty acid biosynthesis. This observation was in good agreement with the composition change in fatty acids at the biochemical level. Our study showed that the oxygen-resistant phenotype could be related to adaptation of fatty acid metabolism.

Key words: *Bifidobacterium animalis* ssp. *lactis* Probio-M8, transcriptomic analysis, oxidative stress, adaptive evolution, fatty acids

Short Communication

Bifidobacterium animalis ssp. *lactis* is one of the *Bifidobacterium* species that has been widely recognized as a probiotic. Several strains of this species, such as *B. animalis* ssp. *lactis* BB12 and *B. animalis* ssp. *lactis* V9, are beneficial to human health and are implemented in commercial dairy products (Taipale et al., 2016; Zhang et al., 2019). Probiotics need to survive the harsh conditions in the host gastrointestinal tract, so they are anticipated to have high tolerance to low pH, bile salt, and oxygen (Garrigues et al., 2010; Sun et al., 2010). Of particular importance is the capacity of resistance to oxygen stress, as it is present throughout the process of functional food production (Ruiz et al., 2012).

To improve the oxygen tolerance of *Bifidobacterium*, 2 different strategies can be used. One strategy is homologous expression of genes that can help the strain to cope with oxidative stress (Zuo et al., 2014). The other strategy is via adaptive evolution under oxidative condition, so that adapted strains with enhanced oxygen tolerance will be generated through natural selection (Bachmann et al., 2015). The latter approach has been extensively applied to create a starter culture of desired properties to be used in industrial food fermentation (Cao et al., 2020). Such a strategy was also successful in strengthening the oxygen tolerance of *Bifidobacterium*, suggesting that the oxygen resistance of bifidobacteria could be modulated genetically (Ladero and Sanchez, 2017). Although remarkable changes might occur in cellular fatty acid (FA) profiles of bifidobacteria under a partially aerobic condition (Jun-Bae et al., 2001), the molecular mechanism behind the enhanced oxygen-tolerant phenotype remains largely unknown.

Bifidobacterium animalis ssp. *lactis* Probio-M8 is a probiotic strain that was isolated from human milk (Wang et al., 2020). Previously, we obtained an oxygen-resistant variant (Probio-M8o) by adaptive evolution under oxidative stress (Wang et al., 2020). In the present study, comparative transcriptomic analysis was performed on Probio-M8 and Probio-M8o to elucidate the adaptive molecular mechanism of the resistant phenotype. Moreover, the genome of Probio-M8 was decoded to decipher the transcriptomic data.

The wild-type strain Probio-M8 and its mutant Probio-M8o were obtained from the Collection Centre of Lactic Acid Bacteria at the Inner Mongolia Agricultural University of China. Bacteria were propagated in de Man, Rogosa, and Sharpe broth. Genomic DNA of these strains was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Genome sequencing of Probio-M8 and Probio-M8o were performed on PacBio RS II (Pacific Biosciences, Menlo Park, CA) and Illumina Hiseq (Illumina Inc., San Diego, CA) sequencers (Zhang et al., 2015; Wang et al., 2017). De novo assembly of the Probio-M8 genome was based on the standard hierarchical genome assembly

Received December 12, 2019.

Accepted April 12, 2020.

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Table 1. General information of primers used for real-time quantitative PCR validation

Gene locus	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
M8PIadj_0329	GGCGGATTCCTCTTCGGATTTCGTG	CGGGTGCATATCGTGCTTGGTGC
M8PIadj_0330	GAGGACCCGAAGACGAACCTGACCC	TTCGACTACGCCGGAATCCACCC
M8PIadj_0331	ACAGGACTTCTCGGTGCGTGGTGG	GGAAGATGTTGCCGTATTTCGGTGAGC
M8PIadj_0334	AAGTGGGCGGCGAAGGAGTCTGT	CCGTGTATGGGCGCTCGGTGATT
M8PIadj_0178	CCGACACGACGATCTACACCTATAACA	AGATTCGCATTTCTCGCCTTTTG
<i>atpD</i>	CGGCGAGGAAGACAAGACCA	TCTCCTCCGACGACGCTAG

process (Zhang et al., 2015). Genome annotations and identification of single nucleotide variations (SNV) were performed according to previous studies (Sun et al., 2010; Wang et al., 2017).

Cultures of Probio-M80 and Probio-M8 were exposed to 9% O₂ for 30 and 60 min, respectively (Xiao et al., 2011). Bacterial RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The RNA sequencing libraries of a total of 12 samples were prepared, and sequencing was performed on an Illumina HiSeq platform. Briefly, the RNA sequencing libraries were prepared from 2 µg of total RNA using the TruSeq Kit (Illumina Inc.). Ribosomal RNA was removed using the Ribo-Zero rRNA Removal Kit (Illumina Inc.). The average size of inserts from the RNA sequencing libraries was approximately 200 to 300 bp.

Raw data were filtered using Cutadapt (v1.15; <https://cutadapt.readthedocs.io/en/stable/>). The reference genome index was built by Bowtie2 (v2.2.6), and the filtered reads were subsequently mapped to the reference genome (<http://bowtie-bio.sourceforge.net/index.shtml>). The gene read count value determined by HTSeq (v0.9.1; <https://htseq.readthedocs.io/en/master/>) was assigned as the original expression level of the gene. DESeq (v1.30.0; <http://www.bioconductor.org/packages/release/bioc/html/DESeq.html>) was used to identify differentially expressed genes (DEG); transcripts with log₂[fold change] > 1 and *P*-value < 0.05 were considered as DEG. Volcano plots of DEG were generated using the R package (R Foundation for Statistical Computing, Vienna, Austria). Gene Ontology (GO, <http://geneontology.org/>) and Kyoto Encyclopedia of Genes and Genomes (<http://www.kegg.jp/>) enrichment analyses were performed using the Fischer's exact test; GO terms and pathways with a *P*-value ≤ 0.05 were considered significantly enriched.

The same RNA samples from RNA-seq analysis was used for real-time quantitative PCR (RT-qPCR) validation. Primers were designed using the Primer 5 software and were listed in Table 1. Reverse transcription was performed using a HiScript II Q RT SuperMix for qPCR kit (Vazyme Biotech Co. Ltd., Nanjing, China).

The RT-qPCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co. Ltd.) on an ABI StepOne Plus RT-qPCR System (Applied Biosystems, Carlsbad, CA). The RT-qPCR was conducted in a final volume of 20 µL. The thermal cycling profile was as follows: initial denaturation at 95°C for 30s, followed by 40 cycles of amplification at 95°C for 10 s, and at 60°C for 30 s. The reference gene, *atpD*, was used as an endogenous control and to normalize the expression of each individual gene (Feroni et al., 2011). The RNA relative quantitation was calculated using the 2^{−ΔΔC_t} method (Livak and Schmittgen, 2001).

To facilitate downstream analysis of the RNA-seq data, Probio-M8 and Probio-M80 were subjected to genome sequencing. Their genomes were deposited in the National Center for Biotechnology Information under accession numbers CP047190 (<https://www.ncbi.nlm.nih.gov/nuccore/CP047190>) and PRJNA624000 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA624000&utm_source=gquery&utm_medium=search). The Probio-M8 genome consists of a circular chromosome of 1,937,197 bp, with an average G+C content of 60.49%. The chromosome has 1,625 coding sequences, 52 transfer RNA, and 3 RNA operons. Comparative genomic analysis together with 16 publicly available *B. animalis* ssp. *lactis* complete genomes identified 75 unique genes in Probio-M8. Sixty-three of these unique genes encode proteins of unknown function, reflecting specific adaptations of this strain to human milk, its original habitat (unpublished data). In the genome of Probio-M80, 8 SNV were identified when compared with Probio-M8. These SNV include 1 synonymous and 6 nonsynonymous mutations.

To elucidate the mechanism of oxygen tolerance of the adapted strain Probio-M80, transcriptional analysis was performed on both Probio-M8 and Probio-M80 subject to exposure to 9% O₂ for 30 and 60 min. The RNA-seq data were deposited in the National Center for Biotechnology Information Short Read Archive database under accession numbers SRR10989859–SRR10989870. The results of RNA-seq revealed 210 (59 upregulated genes and 151 downregulated genes) and 217 (80 upregulated genes and 137 downregulated

Figure 1. Differentially expressed genes (DEG) of Probio-M8o compared with strain Probio-M8 after O_2 treatment for 30 min (a) and 60 min (b). Blue dots represent downregulated DEG; red dots represent upregulated DEG.

genes) DEG in Probio-M8o compared with Probio-M8 after exposure to O_2 for 30 min (Figure 1a) and 60 min (Figure 1b), respectively.

The 30-min O_2 treatment significantly enriched metabolic pathways related to ribosome, pyruvate, and galactose, whereas the 60-min O_2 treatment significantly enriched metabolic pathways of the cell cycle. Interestingly, the 25 ribosome metabolism-related genes enriched by the 30-min O_2 treatment were distributed to 14 GO terms, whereas no GO term was found to be enriched subject to 60 min of O_2 treatment.

To validate the expression data generated by RNA-seq technique, RT-qPCR was performed on 4 representative genes. These genes code for a biotin biosynthesis protein BioY (M8PIadj_0329), a carboxylate-amine ligase (M8PIadj_0330), a methylmalonyl-CoA carboxyltransferase (M8PIadj_0331), and an acyl carrier protein (ACP) synthase (M8PIadj_0334).

The RT-qPCR results were largely consistent with that of the RNA-seq (Figure 2). Results of RT-qPCR showed that 3 of the selected genes (M8PIadj_0330, M8PIadj_0331, and M8PIadj_0334) significantly up-

Figure 2. Comparison of expression levels of 4 selected genes by RNA-seq and real-time quantitative PCR (RT-qPCR) after 30 min (a) and 60 min (b) of O_2 treatment.

regulated after 30 or 60 min of O₂ treatment. However, based on the RT-qPCR results, the gene M8PIadj_0329 was only slightly upregulated after 30 min but not 60 min of O₂ treatment, which was below the cutoff of DEG selection.

Fatty acids are essential components of the cell envelope, which plays crucial roles for bacteria when combating oxidative stress. Alterations in the cellular FA composition and structure in response to stress are therefore widely studied. In *Bifidobacterium adolescentis*, the kinetics of oxygen toxicity varied with changes in the FA composition (Shin and Park, 1998). A rapid profile shift from the content of C_{14:0} and C_{12:0} to C_{18:0} FA could disturb the homeostasis of the cell membrane in *Bifidobacterium longum* (Jun-Bae et al., 2001). A similar phenomenon was also observed in our previous study that found a significant increase in the ratio of C_{18:1} and C_{18:2} FA in the oxygen-tolerant mutant of *B. animalis* (Probio-M8o) in comparison with its wild-type, Probio-M8 (Wang et al., 2020).

Consistently, the transcriptomic data in the current study showed consecutive upregulation of a set of FA metabolism-associated genes in Probio-M8o, including an AMP-binding protein (M8PIadj_1424), a carboxylate-amine ligase (M8PIadj_0330), a methylmalonyl-CoA carboxyltransferase (M8PIadj_0331), an ACP S-malonyltransferase (M8PIadj_0333), and an ACP synthase (M8PIadj_0334) in response to exposure to 9% O₂ for 30 and 60 min. During the process of FA synthesis, the ACP bind fatty acyl intermediates via a phosphopantetheine linker (Chan and Vogel, 2010). These results suggest that modulation of FA metabolism was the mechanism behind the oxygen-tolerant phenotype.

After exposure to oxygen, Probio-M8o showed upregulation in the expression of the FtsW (M8PIadj_1220) and FtsQ (M8PIadj_1217). A dynamic balance between the rates of cell division and death determines the viability and growth of a cell population. The bacterial division process is mediated by a group of proteins that drive the coordinated invagination of cell membranes along with septal peptidoglycan synthesis (Bramkamp and van Baarle, 2009). Among them, the integral membrane protein (FtsW) is required for the septal peptidoglycan synthesis (Karimova et al., 2012), whereas the protein FtsQ involves in the cell division process. Although these proteins are nonessential for the viability of cells under normal growth conditions (de Boer, 2010), it might be of biological significance for the Probio-M8o cells to adapt to the oxygen environment.

To sustain propagation under oxidative stress, it might be even more important for the bacteria to acquire adequate nutrients from the growth environment.

As expected, abundant genes involved in carbohydrate and AA metabolism were upregulated, such as genes coding key proteins in the metabolism of galactose (M8PIadj_1550) and starch (M8PIadj_0965). Interestingly, a gene that functions in metabolism of branched-chain AA (M8PIadj_0178) also showed increased expression. In *Bifidobacterium bifidum*, AA metabolism might play a significant role in countering oxidative stress (Ferrario et al., 2015).

To attenuate oxygen toxicity and ensure cell survival, timely transcriptional changes to protein modifications are necessary to activate the synthesis of defense proteins (Zhu and Dai, 2019). The translational capacity is then controlled by the ribosomal proteins (r-proteins), which are important components of the translational machinery. Our study found that several r-protein-coding genes were repressed in Probio-M8o. Such a response might help slow down the translational elongation process and subsequently influence the bacterial viability. As cell growth and possibly other cellular processes were retarded by the oxidative stress, r-protein synthesis might be reduced subsequently due to a smaller demand for cellular proteins (Browne and Dowds, 2002).

Using transcriptomic techniques, we revealed genetic clues behind the development of the oxygen-tolerant phenotype of a *B. animalis* ssp. *lactis* mutant in comparison with its wild-type. Our results provide insights into understanding of the physiological behavior of *B. lactis* in response to oxidative stress, which would promote its future industrialized production. However, the exact role of the overexpressed genes in oxidative stress protection still needs to be further elucidated.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (31720103911 and 31922071, Beijing, China) and Inner Mongolia Science & Technology Major Projects (ZDZX2018018, Huhhot, China). All authors have declared that they do not have any conflicts of interest in publishing this research.

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