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Genomic resequencing combined with quantitative proteomic analyses elucidate the survival mechanisms of *Lactobacillus plantarum* P-8 in a long-term glucose-limited experiment

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ABSTRACT

Lactobacillus plantarum, commonly isolated from plant material, is widely used to produce various types of fermented foods. However, nutrient-limiting conditions are often encountered during industrial applications. The present study aimed to investigate the response of *L. plantarum* P-8 to glucose-limited conditions in a long-term experiment. Genotypic and proteomic changes in *L. plantarum* P-8 were monitored over 3 years in glucose-limited and glucose-normal media using whole-genome resequencing and tandem mass tag-based quantitative proteomic analysis. Results showed that *L. plantarum* employed numerous survival mechanisms, including alteration of the cell envelope, activation of the PTS system, accumulation and consumption of amino acids, increase in the metabolism of carbohydrates (via glycolysis, citric acid cycle, and pyruvate metabolism), and increase in the production of ATP in response to glucose starvation. This study demonstrates the feasibility of experimental evolution of *L. plantarum* P-8, while whole-genome resequencing of adapted isolates provided clues toward bacterial functions involved and a deeper mechanistic understanding of the adaptive response of *L. plantarum* to glucose-limited conditions.

Significance: We have conducted a 3-year experiment monitoring genotypic and proteomic changes in *Lactobacillus plantarum* P-8 in glucose-limited and glucose-normal media. Whole-genome resequencing and tandem mass tag-based quantitative proteomics were performed for analyzing genomic evolution of *L. plantarum* P-8 in glucose-limited and glucose-normal conditions. In addition, differential expressed proteins in all generations between these two conditions were identified and functions of these proteins specific to L group were predicted. *L. plantarum* employed numerous survival mechanisms, including alteration of the cell envelope, activation of the PTS system, accumulation and consumption of amino acids, increase in the metabolism of carbohydrates (glycolysis, citric acid cycle, and pyruvate metabolism), and increase in the production of ATP in response to glucose starvation.

1. Introduction

Lactobacillus plantarum is an enormously versatile lactic acid bacteria isolated from numerous habitats, such as the gastrointestinal tract of humans, poultry, and insects as well as from food materials, including fish, meat, vegetables, and fermented or raw dairy products [1]. *L. plantarum* strains have been marketed as probiotics [2], and their potential beneficial effects on human and animal health have been recently reported as being able to promote juvenile growth in *drosophila* and mice in the presence of nutritional challenges [3]. The remarkable adaptability, wide industrial utility, and potent impact on animal physiology have made *L. plantarum* of significant interest to the scientific community. They can also be used as a food preservative because they can produce antimicrobial agents, such as organic acids and bacteriocins [4]. Bioactive peptides, which are nutritional relevant peptides, can be derived from protein cleavages or from the food microbiota [5]. Multifunctional bioactive peptides, particularly those produced by specific *L. plantarum* strains, have been shown to possess valuable potential as dietary bioactive compounds for the development of biotechnological products for the nutraceutical industry [6]. However, *L. plantarum* have complex nutritional requirements and they often encounter various nutrient-limiting and other stressful conditions during industrial applications. In particular, poor nutritional conditions induce the stationary phase, low viability, slow growth, low cell

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densities, and slight accumulation of end products [7].

Several studies have depicted the genetic diversity of *L. plantarum* strains through different genotypic approaches, such as amplified fragment length polymorphism, random-amplified polymorphic DNA as well as microarray-based comparative genomic hybridization analyses [1,8–11]. These approaches have allowed investigation of *L. plantarum* differentiation at inter- and intra-species levels as well as elucidation of a potential link between their genome and niche adaptation or fitness. Previous studies have demonstrated that bacterial adaptation to a new habitat almost always accompanied with changes in the bacterial genome and gene regulation [1,10,12,13]. *L. plantarum* is a generalist species that encompasses phenotypically and genotypically diverse strains and microarray-based comparative genomic hybridization studies performed on this species clearly indicate its high genomic diversity [3,14].

The popularization of next–generation sequencing technology has made it possible to characterize genomic evolution in bacteria [15]. Moreover, in recent years, mechanisms underlying adaptive responses of *L. plantarum* toward nutrient stress are numerous and have generated much interest. The most investigated stress factors are carbon (glucose), phosphate, and nitrogen sources [16,17]. For example, Teusink et al. tried to understand the adaptive evolution of *L. plantarum* for optimizing its yield when grown under limited carbon condition [18]. Reverón et al. identified marked induction of genes with roles in gastrointestinal survival as well as repression of genes coding for ATP–binding cassette (ABC)–type transporters and genes involved in the control of intracellular ammonia levels in the adaptation of *L. plantarum* to gallic acid [19]. Furthermore, Li et al. identified genes that encode proteins related to genetic adaptation, including proteolytic enzyme systems, amino acid biosynthesis, stress responses, CRISPR adaptive immunity, and bacteriocin biosynthesis [20].

L. plantarum P-8 was originally isolated from traditional fermented cow milk in Inner Mongolia, China [21]. Recently, there are significant advances in the understanding of the adaptation of some *L. plantarum* strains [22,23]. However, it is not clear whether findings, such as the number and impact of mutations, alterations at the protein level are the same for adaptive evolution of *L. plantarum* P-8. The stress response and survival capacity of *L. plantarum* P-8 have not to our knowledge been investigated under glucose-limited conditions, particularly the genomic and proteomic differences between strains from glucose-limited and those from glucose-normal conditions. Comparative genomic analysis of multiple strains might provide insights into functional and evolutionary processes of the genome of *L. plantarum*. The present study investigated the genotypic and proteomic changes in *L. plantarum* P-8 in response to long-term (3 years) glucose limitation using whole-genome resequencing and tandem mass tag (TMT)-based quantitative proteomic analysis. The genome of *L. plantarum* P-8 was previously sequenced and was observed to comprise a circular 3,033,693-bp chromosome and six plasmids [24]; this information was used as a reference for identifying genotypic mutations. Our findings will be helpful in multilevel system regulation studies and will provide a deeper mechanistic understanding of the adaptive response of *L. plantarum* P-8 to glucose limitation. This study also provides clues for future examination of stress-linked adaptive response in other *L. plantarum* strains.

2. Materials and methods

2.1. Strains and culture conditions

Ancestral and CHL strains of *L. plantarum* were obtained from the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education (Huhhot, China). For propagation, *L. plantarum* P-8 strains were anaerobically grown at 37 °C in de Man-Rogosa-Sharpe (MRS) broth.

2.2. Experimental evolution

Laboratory evolution experiments were designed based on a standard protocol reported previously [15]. Briefly, original stock of *L. plantarum* P-8 was subcultured twice in MRS broth, streaked onto MRS agar plates and subsequently, cultivated at 37 °C for 72 h. Three colonies were randomly selected and inoculated into glucose-normal [2% (v/v); N group] and glucose-limited [0.02% (v/v); L group] MRS broth under anaerobic environment. Then, cultures were propagated by transferring 1% (v/v) of each culture into fresh medium every 24 h. Within a cycle, the number of generations was estimated from the initial and final population size [15]. Throughout the experiments, cultures were sampled at every 1000 (1K) generation for further tests, and bacterial stocks were also prepared and frozen at 1K-generation intervals. Growth curves of *L. plantarum* P-8 in N and L media were constructed based on the number of optical density (OD_{600}) and viable counts every 2 h between 0 and 30 h of fermentation. All analyses were performed in triplicate.

2.3. Genomic resequencing and alignment

Genomic DNA isolated from the eight generations (we refer to the generations as 1K, 2K, 3K, 4K, 5K, 6K, 7K, and 8K) of *L. plantarum* P-8 from both N and L groups were subjected to genomic resequencing using an Illumina TruSeq™ DNA Sample Prep Kit [25], in accordance with the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). Genomic DNA isolated from generations 1K, 2K, and 3K of *L. plantarum* P-8 from L and N groups were sequenced using an Illumina MiSeq™ platform [26], whereas those isolated from the other generations were sequenced on an Illumina HiSeq™ platform [27] as per methods described in previous studies [28,29]. Briefly, DNA (amount ≥ 3 μg; concentration ≥ 100 ng/μL) of each generation was extracted using the hexadecyl trimethyl ammonium bromide (CTAB) extraction protocol as described previously [30]. The DNA quality was assessed using 1% agarose gel electrophoresis. Then, genomic DNA was randomly sonicated into 400–500 bp fragments using Covaris M220 (ThermoFisher Scientific, San Diego, CA, USA). DNA fragments of the desired length were gel-purified, ligated to adapters, and resequenced. Raw reads were first filtered to obtain clean reads after removing wrong bases and low quality reads, i.e., 1) reads with a quality score (Q-value) of < 10 at the two ends of the reads, 2) those with < 80% sequences of with Q-value of > 20, or 3) those with read length of < 50 nt. The quality control process was conducted using the FASTX toolkit and PRINSEQ lite tools [31]. Raw and clean reads were further counted on Q20 and read length for evaluating the validity and reliability of sequencing. All clean reads were aligned to the reference sequences of *L. plantarum* P-8 using Burrows-Wheeler Aligner software [32].

2.4. SNP and Indel calling and annotation

SNP and Indel mutations were detected using a GATK unified genotype [33]. The GATK unified genotype is a publicly available variant caller that combines numerous mutation detection methods and can be used for detecting mutations within a single sample or population. A Bayesian maximum likelihood model was used for estimating the genotype and allele frequency. Finally, each mutation site and the genotype of each sample were given an accurate posterior probability. SNPs and Indels were also termed as Samtools software, and the results were integrated into the Select Variants program in GATK to screen for reliable SNPs and Indels for further analysis [34]. SNPs and Indels were further filtered using the Variant Filtration program in GATK according to the default criteria. SNPs and Indels were annotated to the reference genome NC_021224.2 in the National Center for Biotechnology Information (NCBI) genome database using the SnpEff tool [35].