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Whole-genome sequencing reveals the mechanisms for evolution of streptomycin resistance in *Lactobacillus plantarum*

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ABSTRACT

In this research, we investigated the evolution of streptomycin resistance in *Lactobacillus plantarum* ATCC14917, which was passaged in medium containing a gradually increasing concentration of streptomycin. After 25 d, the minimum inhibitory concentration (MIC) of *L. plantarum* ATCC14917 had reached 131,072 μg/mL, which was 8,192-fold higher than the MIC of the original parent isolate. The highly resistant *L. plantarum* ATCC14917 isolate was then passaged in antibiotic-free medium to determine the stability of resistance. The MIC value of the *L. plantarum* ATCC14917 isolate decreased to 2,048 μg/mL after 35 d but remained constant thereafter, indicating that resistance was irreversible even in the absence of selection pressure. Whole-genome sequencing of parent isolates, control isolates, and isolates following passage was used to study the resistance mechanism of *L. plantarum* ATCC14917 to streptomycin and adaptation in the presence and absence of selection pressure. Five mutated genes (single nucleotide polymorphisms and structural variants) were verified in highly resistant *L. plantarum* ATCC14917 isolates, which were related to ribosomal protein S12, LPXTG-motif cell wall anchor domain protein, LrgA family protein, Ser/Thr phosphatase family protein, and a hypothetical protein that may correlate with resistance to streptomycin. After passage in streptomycin-free medium, only the mutant gene encoding ribosomal protein S12 remained; the other 4 mutant genes had reverted to the wild type as found in the parent isolate. Although the MIC value of *L. plantarum* ATCC14917 was reduced in the absence of selection pressure, it remained 128-fold higher than the MIC value of the parent isolate, indicating that ribosomal protein S12 may play an important role in streptomycin resistance. Using the mobile elements database, we demonstrated that streptomycin resistance–related genes in *L. plantarum* ATCC14917 were not located on mobile elements. This research offers a way of combining laboratory evolution techniques and whole-genome sequencing for evaluating antibiotic resistance in probiotics.

Key words: *Lactobacillus plantarum*, antibiotic resistance, minimum inhibitory concentration, experimental evolution, whole-genome sequencing

INTRODUCTION

Probiotics are live cultures of microbes that, when consumed in food or supplements, alter the intestinal microbial balance resulting in improved health and growth (Sánchez et al., 2017). Probiotics used in this way are generally recognized as safe (GRAS; Monahan, 2011). However, the Food and Agriculture Organization of the United Nations and the World Health Organization have produced guidelines for the safety evaluation of probiotics, which must be followed before any probiotic can be used commercially in food, including assessments for antibiotic resistance (FAO-WHO, 2002).

Globally, antimicrobial resistance is a growing problem that poses a threat to human and animal health (Laxminarayan et al., 2016). With wider use of probiotics, their antibiotic resistance status requires attention. Currently, relatively few standard methods exist for the evaluation of antibiotic resistance in probiotics. Although protocols are available for detection of antibiotic resistance in lactic acid bacteria, standards are scarce in antibiotic resistance gene assessment. Protocols include Clinical and Laboratory Standards Institute (CLSI) M45-P antimicrobial dilution disk susceptibility testing, which involves determining the MIC of a few genera of lactic acid bacteria and antibiotics (CLSI, 2006). The comprehensive standards of International Organization for Standardization (ISO) and the International Dairy Federation (IDF) include norms for the MIC detection methods for lactic acid bacteria (ISO10932/IDF223) that define all the parameters,

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including the medium to use, cultivation conditions, isolates to include for quality control, and so on (ISO-IDF, 2010). Furthermore, EFSA (2012) defines cut-off values for resistance in lactic acid bacteria to different antibiotics. When the MIC of the probiotic is higher than the defined breakpoint value, then any antibiotic-resistant gene present should be identified before the probiotic can be used safely in foods. However, there are no standards for the detection and identification of antibiotic resistance genes or their transferability between different isolates and species. This is because resistance genes are numerous, making it difficult to standardize methods, and their detection requires reference isolates and gene sequences, which are few or incomplete.

The potential to use combinations of probiotics and antibiotics is a curative strategy (Govender et al., 2016), but it remains controversial. Probiotics with low-level resistance may not be effective in combination with antibiotics if they are inhibited by the antibiotics, whereas probiotics with high-level resistance may represent a safety issue due to the potential for transfer of antibiotic resistance genes from the probiotic to co-occurring pathogens. Research is required to solve the therapeutic issues that currently limit the combined use of antibiotics and probiotics. In particular, more information is required on the identity and frequency of antibiotic resistance genes in probiotics with high-level resistance and the likelihood that these genes might transfer to other species.

According to current standards (EFSA, 2012), probiotics in foods should be susceptible to antibiotics. Therefore, experimental laboratory evolution studies are the most effective way to obtain isolates with high-level resistance and to study bacterial adaptation trajectory in the environment (Lukačišinová and Bollenbach, 2017). Bacteria can be grown experimentally under continuous antibiotic pressure to encourage the selection of resistant genotypes adapted to the environment. In such experiments, one or more influencing factors can be established and applied continuously or intermittently. By detecting phenotypic changes over time, the influence of particular variables on the adaptation process can be studied over evolutionary time. In such a highly controlled laboratory experiment, other variables can be minimized effectively. Experimentally passaged isolates can be sampled at different time points and compared with the original isolate grown in the absence of selection pressure to study the relationship between genotype and their regulated phenotype. In this way, the potential mechanisms of adaptation in bacteria under selection pressure can be elucidated. These types of studies have been used widely to shed light on mechanisms of adaptation for antibiotic resistance in pathogenic bacteria under sustained drug selection (Baym et al., 2016; Cairns et al., 2017; Levin-Reisman et al., 2017). However, they have only rarely been used in the study of probiotics, especially antibiotic resistance adaptation in probiotics. A recent publication reported the antibiotic resistance and genomic changes of the probiotic strain *Lactobacillus casei* Zhang under long-term (10 mo) amoxicillin or gentamicin pressure, and showed that *Lb. casei* Zhang has high genome stability under antibiotic selection forces; this research is a good reference for study on genome adaptive evolution in *Lactobacillus* under long-term antibiotic stress (Wang et al., 2017).

Lactobacillus spp. are important probiotics and commonly found in dairy products, as well as in the gastrointestinal tract, where they contribute to the regulation and improvement of the intestinal flora of humans and promote the absorption of nutrients (Stefanovic et al., 2017). In our previous research, we selected for high streptomycin resistance in *Lactobacillus plantarum* IMAU60045 using a laboratory evolution approach; the MIC value was 1,024-fold higher in the adapted isolate than the original isolate. Using quantitative PCR on 2 streptomycin-related genes [*ant*(*6*) and *aadA*], we were able to make preliminary studies on the potential mechanism for adaptation but we failed to identify specific mutations at the whole-genome level (Shao et al., 2015).

Advances in next-generation sequencing technologies allow whole-genome sequencing and make it possible to trace the emergence of variants in a population and analyze genomic differences between individuals within a population under different selection pressures or environments (Barrick and Lenski, 2013; Tenaillon et al., 2016; Lenski, 2017). Based on the whole genome of a reference isolate, subsequent isolates developed under different stressful environments can be sequenced and their sequences compared with the original reference genome to identify mutations. In this study, we investigated streptomycin resistance mechanisms in a probiotic strain, *L. plantarum* ATCC14917 (Wang et al., 2016; Tomaro-Duchesneau et al., 2014). Isolates with high-level resistance were produced using laboratory evolution techniques under gradual and incrementally increasing concentrations of streptomycin. The adaptation and resistance mechanisms of *L. plantarum* ATCC14917 were further studied by whole-genome sequencing of the adapted isolates compared with the original isolate. Then, the mobile element database was used to analyze the potentially transferability of the mutant genes to determine the safety of isolates with high-level streptomycin resistance, which provides the method for evaluating antibiotic resistance in probiotics.