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Comparative proteomic analysis of a potentially probiotic *Lactobacillus pentosus* MP-10 for the identification of key proteins involved in antibiotic resistance and biocide tolerance

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ABSTRACT

Probiotic bacterial cultures require resistance mechanisms to avoid stress-related responses under challenging environmental conditions; however, understanding these traits is required to discern their utility in fermentative food preparations, versus clinical and agricultural risk. Here, we compared the proteomic responses of *Lactobacillus pentosus* MP-10, a potentially probiotic lactic acid bacteria isolated from brines of naturally fermented Aloreña green table olives, exposed to sub-lethal concentrations of antibiotics (amoxicillin, chloramphenicol and tetracycline) and biocides (benzalkonium chloride and triclosan). Several genes became differentially expressed depending on antimicrobial exposure, such as the up-regulation of protein synthesis, and the down-regulation of carbohydrate metabolism and energy production. The antimicrobials appeared to have altered *Lb. pentosus* MP-10 physiology to achieve a gain of cellular energy for survival. For example, biocide-adapted *Lb. pentosus* MP-10 exhibited a down-regulated phosphocarrier protein HPr and an unexpressed oxidoreductase. However, protein synthesis was over-expressed in antibiotic- and biocide-adapted cells (ribosomal proteins and glutamyl-tRNA synthetase), possibly to compensate for damaged proteins targeted by antimicrobials. Furthermore, stress proteins, such as NADH peroxidase (Npx) and a small heat shock protein, were only over-expressed in antibiotic-adapted *Lb. pentosus* MP-10. Results showed that adaptation to sub-lethal concentrations of antimicrobials could be a good way to achieve desirable robustness of the probiotic *Lb. pentosus* MP-10 to various environmental and gastrointestinal conditions (e.g., acid and bile stresses).

1. Introduction

Lactobacillus pentosus is the most prevalent species of lactic acid bacteria (LAB) found in naturally-fermented Aloreña table olives (Abriouel et al., 2011, 2012) and Spanish-style green fermented olives (Maldonado-Barragán et al., 2011). Furthermore, these versatile bacteria have been detected in various environmental niches such as plant materials, silage, fermented foods (dairy, vegetable and meat), as well as the oral cavities, gastrointestinal tracts (GIT), and vaginas of humans and animals (Anukam et al., 2013; Okada et al., 1986; Tajabadi et al., 2011; Todorov and Dicks, 2004). Due to their wide distribution and beneficial effects, special and deserved attention was recently given to the application of lactobacilli, especially of vegetable origin, as a starter culture in different fermentations (Rodríguez-Gómez et al., 2014; Ruiz-Barba and Jiménez-Díaz, 2012), as a probiotic in silage (EFSA, 2011), dairy (Anukam and Olise, 2012) and fermented olives (Rodríguez-Gómez et al., 2014), as they provide bio-therapeutic benefits via bacterial pathogen inhibition and an improved immune system. More specifically, *Lb. pentosus* MP-10 isolated from brine of naturally fermented Aloreña olives (Abriouel et al., 2011, 2012) could be used as a probiotic strain due to their ability to inhibit pathogenic bacteria and tolerate low pH (1.5) and bile salts (3%) in the gastrointestinal environment.

Besides the technological and health-promoting effects shown by lactobacilli with probiotic properties, such as production of antimicrobial substances and survival in gastrointestinal tracts, other requirements should be proven to justify their utility. The most important selection criteria for bacterial strains intended for use as probiotics include: 1) intrinsic resistance to antibiotics of human and veterinary importance and 2) lack of transferable resistance genes to avoid the risk of horizontal gene transfer to other bacteria in the food chain and environment

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(EFSA, 2008; EFSA Panel on Biological Hazards, 2010). As such, many studies have focused on genotypic methods to highlight the presence or absence of antimicrobial resistance determinants (e.g., Bautista-Gallego et al., 2013; Duran and Marshall, 2005; EFSA, 2012; Franz et al., 1999; Zhang et al., 2009). However, several aspects of bacterial fitness, which develop tolerance or resistance to different antimicrobials used in a clinical setting or disinfection, remain unexplored. Bacterial adaptation to antimicrobials, which was referred by Maisonneuve and Gerdes (2014) as "bacterial persisters," is the intermediary stage that links between sensitive and resistant phenotypes. Thus, more attention should be provided to the potential for bacterial adaptation, such as further induction of cross-resistance to other treatments and modifications in colonization or virulence (Dubois-Brissonnet, 2012). Detecting the mechanisms adopted by different bacteria to resist different drugs in various environmental niches remains important. In this respect, several studies report that physiological modifications occur during adaptation such as differential protein expression, which seems to be concomitant to increased tolerance (Dubois-Brissonnet, 2012) and cross-resistance to other environmental stressors (Karatzas et al., 2007, 2008).

In the last decade, proteomics has been used to study bacterial physiological responses to different stressors; this has progressed significantly with the availability of whole-genome sequences, progress in mass spectrometry and bioinformatics. Proteomics, as a key in the post genomic era, provides useful data to identify new diagnostic markers and therapeutic targets in diseases. Recently, genomic and proteomic analyses of *Lactobacillus* genus have rapidly expanded, especially with *Lb. pentosus* having one of the largest genomes known among LAB (Abriouel et al., 2011; Maldonado-Barragán et al., 2011); however, little is known about the mechanisms adopted by *Lb. pentosus* to tolerate or resist several stressors. This information should be of great concern since the knowledge of these mechanisms could be exploited to improve the functionality of probiotic starter strains and, thus, their health promoting benefits.

The present study aimed to determine the phenotypic and genotypic antimicrobial-resistance profiles of *Lb. pentosus* MP-10 and the selected mechanisms, by which these bacteria adapt under different antimicrobial stress. We compared the proteomic profiles of this strain induced by different antimicrobials (antibiotics or biocides), each with a distinct mechanism of action. The comparative analysis provides valuable knowledge and a broad overview of the key proteins involved in antibiotic and biocide tolerance.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Lb. pentosus MP-10, isolated from naturally-fermented Aloreña green table olives (Abriouel et al., 2011, 2012), was routinely cultured at 30 °C in Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) or agar under aerobic conditions for 24–48 h. The strain was stored long-term in 20% glycerol at −80 °C.

2.2. Antimicrobial agents

The antimicrobial agents used in this study were clinically relevant antibiotics: amoxicillin "AMX", ampicillin "AMP", cefuroxime "CFX", chloramphenicol "CMP", ciprofloxacin "CIP", clindamycin "CLI", erythromycin "ERY", gentamicin "GEN", kanamycin "KAN", streptomycin "STR", sulfamethoxazole/trimethoprim "SMZ/TMP", teicoplanin "TC", trimethoprim "TMP", tetracycline "TET" and vancomycin "Van"; and biocides commonly used in the food industry: benzalkonium chloride "BC" and triclosan "TC". All antibiotics and benzalkonium chloride were purchased from Sigma Aldrich (Madrid, Spain); however, triclosan was obtained from Fluka (Madrid, Spain).

2.3. Phenotypic and genotypic antibiotic testing

2.3.1. Antibiotic susceptibility testing and MIC determination

The MICs of the above-mentioned antibiotics were determined for *Lb. pentosus* MP-10 as described by Casado Muñoz et al. (2014) in LSM broth [a mixture of 90% IST broth (Oxoid, Madrid, Spain) and 10% MRS broth (Fluka, Madrid, Spain)] (Klare et al., 2005) according to the ISO 10932/IDF 233 standard (International Organization for Standardization, 2010).

2.3.2. PCR detection of antibiotic resistance genes

PCR amplifications of well-known gene determinants associated with resistance to β-lactam antibiotics (*bla* and *blaZ*, the β-lactamase genes), sulfonamides (*dfrA* and *dfrD*) and glycopeptides (*vanA*, *vanB*, *vanC* and *vanE*) were performed using conditions described elsewhere (Dutka-Malen et al., 1995; Fines et al., 1999; Hummel et al., 2007; Liu et al., 2009; Martineau et al., 2000; Miele et al., 1995). Furthermore, PCR of genes mediating antibiotic resistance through other mechanisms, such as efflux pumps (*mdfA*, *norE*, *acrA*, *acrB*, *tolC*, *mepA*, *norA*, *norC*, *mefA* and *mdeA*), were also performed in the present study. Template DNA for PCR reactions were prepared as reported in Jensen et al. (1998).

2.4. Tolerance induction

Tolerance to antibiotics or biocides was assessed by investigating the ability of *Lb. pentosus* MP-10 to grow in the presence of sub-lethal concentrations of the corresponding antimicrobials, to which the strain was originally sensitive (amoxicillin, chloramphenicol, tetracycline, benzalkonium chloride and triclosan). Tolerant phenotypes were developed by increasing the concentrations of different antimicrobials as described by Casado Muñoz et al. (unpublished data). Briefly, antimicrobial tolerance in *Lb. pentosus* MP-10 was induced by exposure to triclosan (1 μg/ml), benzalkonium chloride (1 μg/ml), cholamphenicol (5 μg/ml), tetracycline (10 μg/ml) or amoxicillin (0.1 μg/ml) at 30 °C for 48 h; cells were then harvested by centrifugation (Casado Muñoz et al., unpublished data). All *Lb. pentosus* isolates were stored in 20% glycerol at −80 °C until use. Isolates were streaked onto MRS-agar; a single colony was selected and subsequently used to inoculate MRS-broth for 24 h at 30 °C. The resulting culture was used to inoculate fresh MRS-broth at a dilution of 1:100. Cultures (both induced and non-induced controls) were harvested at mid-logarithmic growth phase ($OD_{600\ nm} = 0.6$).

2.5. Whole cell protein extraction

The cell pellets obtained, as described above, from isogenic mutants were resuspended in 2 ml of PBS and dispersed into liquid nitrogen with a 200-μl micropipette to obtain cryobeads. Whole-cell protein extraction was done as described by Caballero Gómez et al. (2013). The bacterial beads were ground in liquid nitrogen using a cryogenic grinder (6870 Freezer/Mill, SpexCertiPrep, Stanmore, UK) with three steps of 3 min at a rate of 24 impacts/s. The samples were centrifuged at 5000 × *g* for 5 min (at 4 °C), and the resultant supernatants were filtered through a 0.45-μm pore size filter (Chromafil PET; Macherey-Nagel, Düren, Germany). Proteins were extracted from the filtered supernatants with TRIzol reagent (Euromedex, Souffelweyersheim, France) as previously described (Izquierdo et al., 2009). Protein concentrations were determined using the Bradford protein assay (Bio-Rad) according to the manufacturer's instructions.

2.6. 2-D gel electrophoresis

Protein extracts (150 μg) were loaded onto 17-cm strips with a pH range of 3 to 10 (Bio-Rad), focused for 60,000 V h, and then separated on a 12% SDS-polyacrylamide gel as reported previously (Izquierdo