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A genetic diversity study of antifungal *Lactobacillus plantarum* isolates

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ABSTRACT

Lactobacillus plantarum is a lactic acid bacterium commonly found on fruits and vegetables and also used in a variety of food fermentations. Strains from this species are also regularly reported as having antifungal or probiotic activity. Genotyping methods can be used to differentiate strains of the same species thus determining if strains are related or not. However for *L. plantarum*, the currently used methods have limitations including DNA band profile interpretation difficulty and cost. In this study, a new genotyping method based on multi-locus variable number tandem repeat analysis (MLVA) was developed and compared to a previously reported randomly amplified polymorphic DNA-PCR (RAPD-PCR) method for *L. plantarum*. With a selection of 13 antifungal strains of *L. plantarum* isolated from heterogeneous sources (cheese, silage, sauerkraut, vegetables and a probiotic product), RAPD-PCR revealed 9 different profiles resulting in a Hunter-Gaston discrimination index (*D*-value) of 0.94. The new MLVA method which compares the lengths of 4 repetitive regions within LPXTG motif-containing surface protein genes differentiated the 13 *L. plantarum* strains into 10 different subtypes leading to a *D*-value of 0.95. Interestingly 11 additional *L. plantarum* isolates obtained in a previous study during a screen for antifungal activity against the common cheese spoilage mould *Penicillium commune* all possessed the same RAPD-PCR and MLVA profile as each other and the commercial probiotic strain *L. plantarum* 299v. This study demonstrates that the new MLVA method can be used to simply and inexpensively differentiate *L. plantarum* strains and provide information regarding strain relatedness and thus potential insight into strain properties.

1. Introduction

Lactobacillus spp. are members of the lactic acid bacteria (LAB) group, are commonly found in the environment and raw foods and have 'generally recognized as safe' (GRAS) status. They are key components in a variety of fermentations whereby their production of organic acids and other antimicrobial compounds enhance the quality and safety of foods (Reis, Paula, Casarotti, & Penna, 2012). *Lactobacillus plantarum* is one species of particular interest as it is used in dairy, vegetable and meat fermentations, in the conversion of grass to silage and some strains are marketed as commercial probiotics with health promoting properties (de Vries, Vaughan, Kleerebezem, & de Vos, 2006). In studies where large LAB collections were screened for antifungal activity, *L. plantarum* was frequently isolated (Cheong et al., 2014; Crowley, Mahony, & van Sinderen, 2013a; Magnusson, Strom, Roos, Sjogren, & Schnurer, 2003). Antifungal activity of *L. plantarum* strains has been shown to be due to organic acids, phenyllactic acid, cyclic dipeptides and fatty acids (Crowley, Mahony, & van Sinderen, 2013b), however the genetic basis for the production of these compounds has not been characterised at present.

Genotyping or subtyping methods can provide information about the relatedness of strains within a species. For *L. plantarum*, the most commonly used subtyping method has been random amplification of polymorphic DNA (RAPD) PCR (Di Cagno et al., 2010; Johansson, Quednau, Molin, & Ahrne, 1995; Rossetti & Giraffa, 2005). Other subtyping methods such as amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) have been used to characterise *L. plantarum* and to differentiate it from closely related species (Johansson, Molin, Pettersson, Uhlen, & Ahrne, 1995; Torriani et al., 2001). Two multilocus sequence typing (MLST) schemes for *L. plantarum* have been developed (Xu et al., 2015; de Las Rivas, Marcobal, & Munoz, 2006). However MLST can be costly, involving sequencing

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of 6–8 housekeeping gene loci for each strain. These MLST methods have identified up to 73 subgroups within *L. plantarum* suggesting the species is genetically heterogeneous and may have variation in phenotypes such as antifungal activity.

Although the above genotyping methods have provided valuable information on the *L. plantarum* species, they have limitations including discriminatory power, difficulties in band profile interpretation or cost. Multilocus variable number tandem repeat analysis (MLVA) is a subtyping method which involves analysis of repetitive regions which can be identified from whole genome sequences using online programs such as Tandem Repeat Finder (TRF) (Benson, 1999). Repeat regions undergo rapid evolution due to slip-strand mispairing leading to good discriminatory power (Keim et al., 2004). DNA fragments are amplified and their lengths can be compared using capillary electrophoresis, high resolution melt analysis (HRMA) or more simply with agarose gel electrophoresis. With respect to foodborne bacteria, MLVA has been successfully used to differentiate strains within *Lactobacillus casei* (Diancourt et al., 2007), *Bifidobacterium longum* (Matamoros, Savard, & Roy, 2011), *Clostridium tyrobutyricum* (Nishihara et al., 2014), *Geobacillus* spp. (Seale et al., 2012; Krilaviciute & Kuisiene, 2013), *Bacillus licheniformis* (Dhakal et al., 2013), *Staphylococcus aureus* (Schouls et al., 2009), *Listeria monocytogenes* (Chen, Li, Saleh-Lakha, Allen, & Odumeru, 2011) and *Salmonella enterica* (Kruy, van Cuyck, & Koeck, 2011).

In this report, a new MLVA method was developed and compared to an established RAPD-PCR method for genotyping *L. plantarum* strains from a variety of sources. Both gave congruent results and showed that antifungal *L. plantarum* strains are genetically heterogeneous. In addition, the new MLVA method proved to be more discriminatory and easier to interpret than the RAPD-PCR method.

2. Materials and methods

2.1. Bacteria, mould and their growth conditions

Eleven *L. plantarum* isolates from a variety of sources, one commercial probiotic strain *L. plantarum* 299v, 12 previously identified antifungal strains and 2 non-antifungal control isolates *Weissella soli* and *Lactococcus lactis* (Table 1) were collected from previous research (Cheong et al., 2014). All bacterial isolates were cultured using de Man Rogosa Sharpe (MRS; Oxoid) media containing 1.5% agar and were incubated at 30 °C for 24 h under anaerobic conditions (AnaeroGen system; Oxoid). Strains were grown in MRS broth and incubated at 30 °C overnight under non-shaking conditions. Bacteria and mould were frozen in 40% glycerol at −80 °C for long-term storage. *Penicillium commune* FRR 4117 was plated out on malt extract agar (MEA; Difco) from a frozen stock, and incubated at room temperature (~23 °C) for 7 days.

2.2. Antifungal activity testing

All *L. plantarum* strains were tested against *Penicillium commune* FRR 4117 on MEA plates using a method similar to that described previously (Cheong et al., 2014). *P. commune* spores were harvested from MEA plates using 0.2% (w/v) peptone water (Oxoid), and the concentration was adjusted to an OD_{600} of 0.5 (~1×10^6 mould spores/ml). The *P. commune* spore suspension (130 μl) was added to 8 ml of soft MEA (0.7% agar) and was poured onto an MRS agar base (15 ml). *L. plantarum* strains (5 μl) from pure frozen stocks were spotted onto the fully dried *P. commune*-containing plates. Plates were first incubated anaerobically at 30 °C for 24 h to allow for initial growth of *L. plantarum* and then incubated aerobically at room temperature (~23 °C) for 3 days. Inhibition of *P. commune* growth was seen when the mould did not grow to the edge of the bacterial colony. Non-antifungal isolates *W. soli* 33 and *L. lactis* 38 (Cheong et al., 2014) were included as negative controls in the test and *P. commune* was able to grow over their colonies.

2.3. RAPD-PCR amplification and gel electrophoresis

The RAPD-PCR method used was adapted from that described previously (Di Cagno et al., 2010) using the M13 primer (5′-GAGGGTGGCGGTTCT-3′) for amplification. The GenElute™ Bacterial Genomic DNA Kit (Sigma) was used to extract bacterial DNA from 1.5 ml of overnight broth culture using the manufacturer's instructions, with 200 μl elution buffer used. A 25 μl total reaction volume for each sample contained 12.5 μl of GoTaq® Green 2x Master Mix (Promega), 2 μl of primer M13 (final concentration 6 μM), 9.5 μl water and 1 μl of undiluted bacterial DNA template. Negative controls where water replaced the template DNA were included in all runs. Thirty amplification cycles: 94 °C/60 s, 42 °C/20 s and 72 °C/2 min were carried out after an initial 94 °C/2 min denaturation. A final extension step of 72 °C/10 min was performed. The PCR products were resolved using a 1.5% agarose gel in TAE buffer and stained using SYBR® Safe (Invitrogen). A DNA marker was included in each gel (1 kb plus ladder, Invitrogen). Gels were run at 100 V for 2 h (Mini-300 unit; Major Science) and the banding profiles were captured under UV light (Smart View Pro 1100 Imager System; Major Science). Groupings were identified and the Hunter-Gaston discriminatory index (*D*-value) was determined (Hunter & Gaston, 1988).

2.4. MLVA development

The TRF program (Benson, 1999) was used to identify the DNA repeat regions in *L. plantarum* WCFS1 (GenBank accession: NC_004567.2). Eighty five repeat regions were located and 23 loci were chosen based on having a repeat size of ≥20bp and a repeat copy number of ≥2 as adopted previously (Seale et al., 2012). Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primers flanking the repeat regions (Ye et al., 2012). Two hundred bp of flanking DNA and the repeat region were analysed by Primer-BLAST and 46 primers for the 23 repeat loci were obtained. Four isolates (278, 291, 1299 and 299v; Table 1) were used to test the 23 sets of primers. The thermal cycling conditions were: 95 °C/2 min, followed by 30 cycles of 95 °C/15 s, 63 °C/30 s, 72 °C/2 min followed by a final extension step of 72 °C/2 min. The PCR products were resolved using a 2.5% agarose gel with conditions as described above. Four primers which generated PCR products with the greatest variation in length using the 4 tested strains were named variable number tandem repeat 1 (VNTR1), VNTR2, VNTR3 and VNTR4 (Table 2) and were used to amplify DNA from all strains. Based on different sizes of the PCR products, bands were scored from 1 to 6, with 1 being the largest PCR fragment.

3. Results

3.1. RAPD-PCR analysis of antifungal L. plantarum isolates from a variety of sources

In previous work, antifungal *L. plantarum* strains were identified in a screen of isolates from fruits and vegetables (Cheong et al., 2014). Testing of additional food and probiotic *L. plantarum* strains listed in Table 1 for antifungal activity against *P. commune* was carried out. It was found that all *L. plantarum* isolates inhibited *P. commune* growth similar to that shown for strains 299v, ASCC 3005 and 885 (Fig. 1). To explore the genetic relatedness of antifungal *L. plantarum* strains, a previously described RAPD-PCR