Veterinary Microbiology 167 (2013) 592–599

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Characterization of *Lactococcus lactis* isolates from bovine mastitis

Carme Plumed-Ferrer *, Kaisa Uusikylä, Jenni Korhonen, Atte von Wright

Food Biotechnology, Institute of Public Health and Clinical Nutrition, University of Eastern Finland, P.O. Box 1627, FI-70210 Kuopio, Finland

ARTICLE INFO

Article history:
Received 21 May 2013
Received in revised form 4 September 2013
Accepted 6 September 2013

Keywords:
Lactococcus lactis
Bovine mastitis
Starter strains

ABSTRACT

Lactococcus lactis is a widely used mesophilic dairy starter and has been included in the Qualified Presumption of Safety (QPS) list of the European Food Safety Authority. However, it is increasingly found as the cause of human or animal infections, such as bovine mastitis, probably due to the improvement of the identification of the infective microorganisms. Since there are some grounds to suspect that at least certain variants of *L. lactis* may cause animal or human diseases, it is important to properly identify the differences between the strains associated with infections and the safe starter strains. Bovine mastitis isolates and dairy starter strains were genotypically characterized and clustered by the 16S rRNA gene sequence and RAPD-PCR fingerprint patterns, and phenotypically characterized by their tolerance to different stress conditions typically found in the intestinal tract of mammals, the carbohydrate- and antibiotic resistance profile, as well as the *in vitro* adhesion capacity to udder epithelial cells. Genotypically, there were no differences between the mastitis isolates and the dairy starter strains. However, there were clear phenotypic distinctions between mastitis isolates and typical starter strains, the former showing an increased tolerance to temperature, lysozyme, bile salts, pH and antibiotics, as well as improved carbohydrate fermentation capacity, and *in vitro* adhesion to udder epithelial cells. Although these differences might not be considered as actual virulence factors, they improve the ability of the strain to survive in the body of homeothermic animals and, interestingly, are also typical properties associated with potential probiotic strains.

1. Introduction

L. lactis and its subspecies *lactis* and *cremoris* are widely used mesophilic dairy starters in the production of several types of cheeses, fermented milks and plant materials (see von Wright, 2012 for a review), and have been included in the Qualified Presumption of Safety (QPS) list of the European Food Safety Authority (Leuschner et al., 2010).

In the past, only the reported opportunistic infections associated with the genus *Lactococcus* were rare (Collins et al., 1983; Teixeira et al., 1996). Nowadays, *Lactococcus garvieae*, another species of *Lactococcus* and closely related to *L. lactis*, is classified as an emerging pathogen (Morita et al., 2011 causing infections in fish (Vendrell et al., 2006), bovine mastitis (Collins et al., 1983; Teixeira et al., 1996) and has been associated with human clinical cases (Reimundo et al., 2011). Recently, also *L. lactis* has been isolated from diseased fish (Wang et al., 2008; Chen et al., 2012; Perez et al., 2011), bovine mastitis cases (Devriese et al., 1999; Fortin et al., 2003; Haguingan et al., 2010; Wyder et al., 2011; Romero et al., 2011), bird infections (Goyache et al., 2001) and numerous human clinical infections (for review, Mofredj et al., 2007; Uchida et al., 2011). Moreover, the difficulties to distinguish lactococci from either streptococci or enterococci may have caused a misidentification of many earlier clinical isolates, and the number of infections caused by *L. lactis* can be considerably higher than the published reports indicate (Uchida et al., 2011).

* Corresponding author. Tel.: +358 403553573; fax: +358 16173322.
E-mail address: Carme.Plumed@uef.fi (C. Plumed-Ferrer).

0378-1135/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetmic.2013.09.011

Mastitis is considered as the most frequent and costly disease in dairy cows worldwide (Wyder et al., 2011). The standard mastitis control practices have successfully reduced the prevalence of contagious pathogens such as *Streptococcus agalactiae* and *Staphylococcus aureus*, but also opened a window of opportunity for the environmental and opportunistic bacteria to become dominant, even in well-managed dairy herds (Nam et al., 2010; Petrovski et al., 2011). Mastitis bacterial isolates are mainly identified using protocols based on conventional culturing and biochemical characteristics (NMC, 1999), and the differentiation between some groups of bacteria (especially gram-positive, catalase-negative cocci), such as *Lactococcus, Streptococcus* and *Enterococcus*, might have not been accurate (Fortin et al., 2003).

Since there are some grounds to suspect that at least certain variants of *L. lactis* may cause animal or human diseases, it is especially important to properly study the differences between the strains associated with infections and the safe starter strains and to differentiate pathogenic from non-pathogenic strains. The aim of this work is to characterize *L. lactis* strains of different origins and specifically to compare mastitis isolates and dairy starter strains for both, genotypic and phenotypic characteristics.

2. Materials and methods

2.1. Strains, media and culture conditions

The strains/isolates used in this study are listed in the supplementary material 1. Of the altogether 27 *L. lactis* isolates from clinical/subclinical mastitis cases originated in different herds, 23 were isolated and characterized by the Finnish Food Safety Authority (Evira) and the rest by Valio Oy (Helsinki, Finland). The isolates were gram-positive, catalase-negative, esculin-positive, penicillin-susceptible, originated from the milk of quarters with high somatic cell counts ($SCC > 3 \times 10^6$ cells/ml), were monocultures or the main isolate, and were identified as *Lactococcus lactis/Enterococcus faecium* using API Strep (bioMérieux, Marcy l'Etoile, France) (Pitkala et al., 2004, for the origin of the isolates). For the comparison, seven bulk starter strains from Valio Oy and one commercial Direct Vat Set (DVS) starter strain were also included in the study, as well as a *L. garvieae* 20684 strain isolated from a mastitis case (Collins et al., 1983). All strains were routinely grown in M17 broth (Oxoid Ltd., Hampshire, United Kingdom) at 30 °C.

2.2. Phenotypic characterization and antibiotic resistance

The isolates and starter strains were examined for phenotypic properties including colony morphology and pigmentation, production of oxidase and catalase, Gram staining, and cellular morphology. The temperature tolerance at 37 °C was also monitored. Moreover, the carbohydrate fermentation profile of the isolates and starter strains was studied using API 50 CHL assay (bioMérieux) according to the manufacturer's instructions.

The minimum inhibitory concentrations (MICs) of nine antibiotics (gentamicin, kanamycin, streptomycin, erythromycin, chloramphenicol, tetracycline, ampicillin, neomycin and penicillin) were determined according to ISO 10932:2010 standard except that the agar dilution method, as described by Korhonen et al. (2007), was used instead of broth microdilution, and aerobic conditions were applied. Epidemiological cut-off values were defined according to the committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org).

2.3. Molecular identification of isolates and phylogenetic analysis

The identities of the mastitis isolates and starter strains were verified by molecular methods, including partial amplification of 16S rRNA gene, sequencing and sequence comparison. For this, total genomic DNA from overnight cultures was isolated using a DNA extraction kit (NucleoSpin Tissue, Macherey-Nagel, Düren, Germany). Genomic DNA quality and concentrations were determined with a nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and 1% agarose gels. A fragment of approx. 650 bp of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers 685r (5′-TCTACGCATTTCACCGCTAC-3′) and H121 (5′-GAGTTTGATCCTGGCTCAGGA-3′) or 27F (5′-AGAGTTTGATCCTGGCTCAG-3′). PCR reaction was performed in a 50-μl total volume containing 1 unit of GoTaq DNA polymerase (Promega, Mannheim, Germany), 10 pmol of each primer, 200 μM of each dNTP, 3 mM $MgCl_2$ and 50 ng of genomic DNA. PCR amplifications were performed using Biometria T3 thermocycler (Thistle Scientific Ltd., Glasgow, UK). The PCR parameters were following: initial denaturation at 94 °C for 6 min; 30 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min; final extension at 72 °C for 10 min. Amplicons were purified using a PCR clean-up gel extraction kit (NucleoSpin Extract II, Macherey-Nagel) and sent for sequencing (LGC Genomics GmbH, Berlin, Germany). Homology searches were performed using GenBank and Ribosomal Database Project II databases. Phylogenetic and molecular evolutionary analyses were conducted by *MEGA* 5 software (Tamura et al., 2011) using the neighbor-joining method. Bootstraps values were obtained from 1000 replicates.

2.4. RAPD-PCR analysis

In order to further characterize the mastitis isolates, randomly amplified polymorphic DNA (RAPD-PCR) analysis was carried out using three different primers: P16 (5′-TCGCCAGCCA-3′), P17 (5-CAGACAAGCC-3′) and P2 (5′-GATCGGACGG-3′). PCR reaction was performed in 25-μl total volume containing 1 unit of GoTaq DNA polymerase (Promega), 10 pmol of primer, 200 μM of each dNTP, 3 mM $MgCl_2$ and 50 ng of genomic DNA. PCR amplifications were performed as follows: initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 24 °C for 2 min and 72 °C for 2 min; final extension at 72 °C for 5 min. Amplification products were electrophoretically separated in 1% agarose gels containing SYBR safe DNA gel staining (Life Technologies Ltd., Paisley, UK). Gels were visualized and photographed using the Gel Doc UV transilluminator 2000