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Development of a novel loop-mediated isothermal amplification assay for the detection of lipolytic *Pseudomonas fluorescens* in raw cow milk from north China

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ABSTRACT

Lipases secreted by psychrotrophic bacteria are known to be heat resistant and can remain active even after the thermal processing of milk products. Such enzymes are able to destabilize the quality of milk products by causing a rancid flavor. Rapid detection of a small amount of heat-resistant lipase-producing psychrotrophic bacteria is crucial for reducing their adverse effects on milk quality. In this study, we established and optimized a novel loop-mediated isothermal amplification (LAMP) assay for the detection of *Pseudomonas fluorescens* in raw cow milk, as the most frequently reported heat-resistant lipase-producing bacterial species. *Pseudomonas fluorescens*-specific DNA primers for LAMP were designed based on the lipase gene sequence. Reaction conditions of the LAMP assay were tested and optimized. The detection limit of the optimized LAMP assay was found to be lower than that of a conventional PCR-based method. In pure culture, the detection limit of the LAMP assay was found to be 4.8×10^1 cfu/reaction of the template DNA, whereas the detection limit of the PCR method was 4.8×10^2 cfu/reaction. Evaluation of the performance of the method in *P. fluorescens*-contaminated pasteurized cow milk revealed a detection limit of 7.4×10^1 cfu/reaction, which was 10^2 lower than that of the PCR-based method. If further developed, the LAMP assay could offer a favorable on-farm alternative to existing technologies for the detection of psychotrophic bacterial contamination of milk, enabling improved quality control of milk and milk products.

Key words: loop-mediated isothermal amplification, psychrotrophic bacteria, *Pseudomonas fluorescens*, raw cow milk

INTRODUCTION

Cold storage and transportation of raw cow milk creates favorable conditions for the growth of psychrotrophic bacteria (Baur et al., 2015). Psychrotrophic bacteria account for less than 10% of the total microbiota found in fresh raw cow milk, but dominate the microbial community during cold storage (Rasolofo et al., 2010; Weber et al., 2014). A large number of psychrotrophic bacteria have been identified as belonging to the genus *Pseudomonas* (Quigley et al., 2013; von Neubeck et al., 2015). These bacteria can grow to high numbers during refrigerated storage, and many can also produce heat-stable extracellular lipases (Dogan and Boor, 2003). Among the genus, *Pseudomonas fluorescens* exhibits the highest metabolic and lipolytic activity in refrigerated milk (Capodifoglio et al., 2016; Stuknytė et al., 2016). The lipolytic enzymes remain active, even after thermal processing steps that can destroy the bacteria themselves (Vithanage et al., 2016). The lipolytic enzymes hydrolyze triglycerides, causing changes in the flavor of the milk, turning it rancid, unclean, soapy, or bitter tasting, and leading to the product being rejected outright (Capodifoglio et al., 2016). The effects are even more of a problem in dairy products with a long shelf life, such as UHT milk (Decimo et al., 2014).

Because it is difficult to inactivate the enzymes secreted by psychotropic bacteria in affected milk using existing technologies, it is very important to develop more vigilant test methods for their detection in raw cow milk. The culture-dependent method for the detection of microorganisms in biological samples is both laborious and time consuming, and can thus result in a missed opportunity to control and improve the quality of raw cow milk. Culture-independent methods have been

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shown to be useful for the rapid and specific detection of microorganisms (Cho et al., 2014; Bosward et al., 2016; Cornelissen et al., 2016). As a nucleic acid-based amplification method, loop-mediated isothermal amplification (**LAMP**) has been developed for the rapid detection of a small amount of microorganisms. Four to 6 specific primers have been designed for use in LAMP, together with a strand-displacing Bst DNA polymerase that can amplify the target DNA by up to 10^9 copies per hour (Notomi et al., 2000). In addition, LAMP proceeds under isothermal conditions and does not require expensive and sophisticated instruments (Cornelissen et al., 2016). Positive LAMP reactions can be judged by the naked eye following DNA amplification with a thermal cycler and gel electrophoresis (Kumar and Mondal, 2015). Additionally, they can be judged by the formation of optic visible magnesium pyrophosphate (turbidity) or by fluorescence using DNA-intercalating dyes, the latter of which enables real-time monitoring of LAMP reactions to be performed by measuring the fluorescence.

In this study, a specific primer set based on the lipase gene sequence of *P. fluorescens* was designed and used to establish a fluorescence-based real-time LAMP assay, with the aim of developing a more specific approach that has a lower detection limit for the detection and quantification of *P. fluorescens* as a potent producer of heat-resistant lipase in raw cow milk.

MATERIALS AND METHODS

Strains and Cultures

Twenty-one different lipolytic *Pseudomonas* isolated strains from 6 different *Pseudomonas* species were identified in raw cow milk originating from north China in our previous study (Xin et al., 2017). These are listed, along with other standard strains that are commonly isolated from raw cow milk, in Table 1. All of these strains were used to evaluate the specificity of the newly developed LAMP assay. *Pseudomonas fluorescens* ATCC 13525 acted as the positive control in this evaluation. *Pseudomonas fluorescens* 38 was used for the optimization of the LAMP assay and to test the detection limit of the assay. The strains were cultured in 10 mL of LB medium for 16 h at 25°C (*Pseudomonas* spp.) or for 24 h at 37°C (non-*Pseudomonas* spp.).

Bacterial DNA Extraction

The DNA content of all strains listed in Table 1, cultured in pure culture, was extracted using the E. Z. N. A. Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA) for use in LAMP and PCR assays.

Primers for LAMP and PCR

The primer set for use in LAMP was designed based on the conserved gene sequence of the *P. fluorescens* lipase using Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html). The primer set included 2 outer primers (F3 and B3) and 2 inner primers (FIP and BIP). The primer sequences are shown in Table 2. The PCR was performed with primers F3 and B3. Primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

Optimization of LAMP Reaction Conditions

We initially adopted the conditions reported by Notomi et al. (2000) as the default LAMP reaction conditions. Thereafter, we optimized the reaction conditions by evaluating the effects of adjusting the following reaction variables on the visualized intensity of electrophoretic bands produced by gel electrophoresis of the amplified reaction products: Mg^{2+} concentration (0.0, 4.0, 6.0, 8.0, 10.0, and 12.0 mmol/L), deoxynucleotide triphosphate (**dNTP**) concentration (0.0, 0.4, 0.8, 1.2, 1.6, and 2.0 mmol/L), Bst DNA polymerase (New England Biolab, Ipswich, MA) concentration (0.0, 0.4, 0.8, 1.2, 1.6, and 2.0 μL), inner/outer primer ratio (1:1, 2:1, 4:1, 6:1, 8:1, and 10:1), reaction time (20, 30, 40, 50, 60, and 70 min), and temperature (60, 61, 62, 63, 64, and 65°C). After reaction completion, the reaction mixtures were inactivated at 80°C for 5 min. A negative control, consisting of an identical reaction mixture containing distilled water in place of the DNA template, was included in each reaction run. The LAMP reaction products were analyzed using 2.5% agarose gel electrophoresis at 100 V for 40 min.

A real-time LAMP reaction system was developed by replacing 0.3 μL of double-distilled H_2O with 0.3 μL of a 1:200 dilution of 10,000 × SYBR green I (Sigma-Aldrich Co., St. Louis, MO) in the optimized LAMP reaction system. Real-time LAMP reactions were conducted in an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) under the optimized reaction conditions, during which fluorescence of the SYBR Green I was measured every 45 s.

PCR Amplification Conditions

The PCR was carried out in a MyCycler thermal cycler (Bio-Rad, Hercules, CA). Reactions were carried out in a total volume of 25 μL containing 2 × PCR Mix, 20 pmol of each primer, 2 μL of template DNA, and distilled water. Amplification conditions were 94°C for 5 min, followed by 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. This sequence was repeated for 35 cycles.