Food Microbiology 68 (2017) 97–103

Contents lists available at ScienceDirect

Food Microbiology

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

Characterization of *Enterococcus durans* 152 bacteriocins and their inhibition of *Listeria monocytogenes* in ham

Lihui Du [a], Fang Liu [b], Ping Zhao [c], Tong Zhao [c, *], Michael P. Doyle [c]

[a] College of Food Science and Engineering, Collaborative Innovation Center for Modern Grain Circulation and Safety, Key Laboratory of Grains and Oils Quality Control and Processing, Nanjing University of Finance and Economics, Nanjing, 210023, China
[b] Institute of Agricultural Products Processing, Jiangsu Academy of Agricultural Sciences, Nanjing, 210014, China
[c] Center for Food Safety, College of Agriculture and Environmental Sciences, University of Georgia, 1109 Experiment Street, Griffin, GA, 30223, USA

ARTICLE INFO

Article history:
Received 16 May 2017
Received in revised form
6 July 2017
Accepted 7 July 2017
Available online 7 July 2017

Keywords:
Bacteriocins
Enterococcus durans
Listeria monocytogenes
Ready-to-eat meat

ABSTRACT

Listeria monocytogenes is a nonfastidious, widely occurring foodborne pathogen that is a major challenge to the food industry. *Enterococcus durans* 152, a confirmed *L. monocytogenes*-control microorganism, was isolated from floor drain samples from a food processing facility. In this study, the two bacteriocins produced by *E. durans* 152 were characterized and identified as Dur 152A (an enterocin L50A derivative with two amino acid substitutions of I→M) and enterocin L50B. The bacteriocins were then partially purified and evaluated for inhibitory activity to *L. monocytogenes* in deli ham. Results revealed that at 400 AU/ml, the bacteriocins prevented growth of listeria in deli ham for at least 10 weeks at 8 °C and at least 30 days at 15 °C. For comparison, 500 ppm Nisin controlled listeria growth for up to 6 weeks at 8 °C and up to 18 days at 15 °C. These findings reveal the potential for the bacteriocins of *E. durans* 152 to serve as anti-listerial agents in deli meat.

1. Introduction

Listeria monocytogenes is a resilient foodborne pathogen that causes human listeriosis, a relatively rare but serious illness in susceptible populations. Listeriosis can result in high hospitalization rates (>90%) and mortalities (20%–30%) in large outbreaks (Hernandez-Milian and Payeras-Cifre, 2014). *L. monocytogenes* occurs naturally in agricultural environments such as soil, manure and water (Leclercq, 2015), and has the ability to grow at refrigeration temperature and thrive in moist environments. Hence, controlling this pathogen in processing environments and food is a major challenge for the food industry in foods ranging from processed, ready-to-eat (RTE) meats to retail or restaurant fresh-cut produce (Ding et al., 2013; Jamali et al., 2013). The United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) reported in the 1990's the prevalence of *L. monocytogenes* as high as 7.69% on sliced ham and luncheon meats which was determined from a nationwide sampling program for ready-to-eat meats (Levine et al., 2001). Controlling the widely occurring psychrotrophic *L. monocytogenes* has been a continuing challenge for the food industry. Considerable research has been conducted to better understand the growth characteristics of *L. monocytogenes* (Vail et al., 2012; Curtis et al., 2016), as well as its response to antimicrobials (Kjos et al., 2010; Cotter et al., 2002) and the effectiveness of sanitizers on *L. monocytogenes* biofilms (Liu et al., 2017). Many RTE meat products rely on salt and curing agents for microbial safety; however, *L. monocytogenes* is able to grow in high salt concentrations and to form biofilms, which enhance the resistance of listeria to sanitizers, and thereby contribute to the persistence of the pathogen in RTE meat processing plants (Kyoui et al., 2016; Colagiorgi et al., 2016). Hence, there continues to be a need for the development of innovative effective preventive controls to enhance the safety of food, including minimally processed RTE meat products.

Lactic acid bacteria (LAB) can produce a variety of antimicrobials such as organic acids, hydrogen peroxide, antifungal peptides, and bacteriocins (El-Ziney et al., 2000; Magnusson and Schnürer, 2001). Of these, bacteriocins produced by "food-grade" LAB may have the potential to be applied as nontoxic food preservatives (Cotter et al., 2005). Considerable research has been conducted on bacteriocinogenic LAB cultures or bacteriocin-containing culture supernatant fluids as food preservatives (Deegan et al., 2006). LAB bacteriocins, such as Nisin and pediocin PA-1, have already been

* Corresponding author.
E-mail address: tongzhao@uga.edu (T. Zhao).

http://dx.doi.org/10.1016/j.fm.2017.07.002
0740-0020/

used as biopreservatives, and only nisin received approval to be used as food additive (Cleveland et al., 2001; Cotter et al., 2005). This is partially due to the slow progress on the characterization of novel bacteriocins with strong antimicrobial activities. In a previous study, *Enterococcus durans* (*E. durans*) 152 was isolated from a listeria-free floor drain sample of a food processor, and characterized. This strain produced undetermined antimicrobials (possibly bacteriocins) and can inhibit the growth of *Listeria* spp. in floor drains by competitive exclusion (Zhao et al., 2004, 2006), offering a promising biocontrol method for killing or suppressing the growth of *L. monocytogenes* in food processing facilities.

In this study, we characterized the bacteriocins produced by *E. durans* 152 and then examined the effectiveness of the bacteriocins in inhibiting the growth of *L. monocytogenes* on deli ham.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

All cultures were obtained from the University of Georgia Center for Food Safety culture collection, except for those specifically stated. *E. durans* 152 was previously isolated from a floor drain sample and identified by 16S rRNA sequencing (Zhao et al., 2004). The strain was grown in tryptic soy broth (TSB) (Difco, Franklin Lakes, NJ) at 37 °C for 16 h. *Escherichia coli* (*E. coli*) JM109 competent cells (Thermo Fisher, Tewksbury, Mass) was used as a transformation host. It was grown in Luria-Bertani (LB) (Difco) media at 37 °C for 16 h, with 50 μg/ml ampicillin (Sigma-Aldrich, St. Louis, MO) added when needed. For *Listeria* inoculation on the deli ham samples, a five-strain mixture of *L. monocytogenes* was prepared, including LM101 (serotype 4b, salami isolate), LM112 (serotype 4b, salami isolate), LM113 (serotype 4b, pepperoni isolate), H9666 (serotype 1/2c, human isolate), and ATCC 5779 (serotype 1/2c, cheese isolate). Each culture was individually transferred for at least three times in brain heart infusion broth (BHI) (Difco) at 37 °C for 16 h. The five-strain mixture was obtained by mixing equal volumes of overnight cultures from each strain. The mixture was serially diluted in 0.1% (w/v) peptone and this dilution, with a concentration of approximately 10^4 CFU/ml, was used for inoculation.

2.2. PCR analysis for the presence of known enterocins

E. durans 152 was first screened by PCR analysis of gene sequences for the presence of known enterocins. Primers were designed according to the published nucleic acid sequences or adapted from previous publications (Franz et al., 1999; Du et al., 2012a) (Table 1). PCR was performed on a Beckman thermocycler with Taq DNA polymerase (Invitrogen, Carlsbad, CA) using the conditions described previously (Du et al., 2012b). The PCR products were separated by electrophoresis on a 1% (w/v) agarose gel in TBE buffer.

2.3. Plasmid isolation and sequencing of the bacteriocin-encoding region

Overnight cultures were used for plasmid preparation. For *E. durans* 152, the plasmid DNA was prepared by a rapid mini-prep method (O'Sullivan and Klaenhammer, 1993). For *E. coli* JM109, a PureLink rapid purification preparation kit (Invitrogen) was used. Plasmid profiles were analyzed by agarose gel electrophoresis. Prepared *E. durans* 152 plasmid DNA was first digested by single enzymes of *Stu* I, *Hin*d III, *Sal* I, *Asc* I (Invitrogen) and *Spe* I (New England Biolabs, Ipswich, NE) to determine possible enzyme restriction sites and then was double digested by a combination of *Hin*d III and *Sal* I or *Hin*d III and *Spe* I. The digested fragments were ligated into the pGEM-T vector (Thermo Fisher) (Du et al., 2012a). The ligation mixture was transformed into *E. coli* JM109 competent cells and the transformants were spread onto LB agar plates containing 100 μg/ml ampicillin, 0.5 mM IPTG and 40 μg/ml X-Gal (Sigma-Aldrich) which were added to select for the white colonies. The positive clone was sequenced by Sequetech (Mountain View, CA, USA) using universal M13 forward and reverse primers. The primer walking strategy was used to sequence multiple reactions and based on the obtained sequences; primer 5 was designed to locate the bacteriocin region according to the plasmid sequence of pEF1 (Accession no: DQ198088.1).

2.4. Partial purification of E. durans 152 bacteriocins

Bacteriocins were partially purified from cell pellets. Briefly, *E. durans* 152 was inoculated at 1% (v/v) in TSB and grown at 37 °C for 24 h. The cells were collected by centrifugation at 8000×*g* for 30 min and resuspended in 70% isopropanol (pH 2.0), then the supernatant was collected by centrifugation at 8000×*g* for 30 min. The process was repeated and the supernatants were combined. The supernatant was evaporated on a Speedvac (Savant Instruments Inc., NY) to 1/10th of the initial volume and then freeze-dried by a customized dryer in Apex Lyo, Inc. (Country Club, Missouri, USA). For use, the freeze-dried powder was dissolved in ddH_2O before neutralization and the bacteriocin activity was determined by a two-fold dilution assay (Du et al., 2012b).

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation and identification of the active component

The partially purified bacteriocins were separated by SDS-PAGE using NuPAGE 12% Bis-Tris gel (Invitrogen) as previously reported (Du et al., 2012b). The electrophoresis was carried out at 200 V for 1 h with a SeeBlue Plus2 Pre-Stained protein marker (Invitrogen). The gel was fixed in 20% isopropanol, 10% acetic acid (100 mL, 2 × 45 min), and then the SDS was removed by washing the gel overnight in distilled water with agitation (20 rpm). The gel was then overlaid onto a BHI agar plate seeded with 0.5% (v/v) of newly transferred *L. monocytogenes* culture and incubated at 37 °C for 8 h to determine the active band. The partially purified bacteriocins were analyzed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) (Bruker Autoflex, core facilities in the University of Georgia). The MS spectra were acquired in linear mode.

2.6. Stability of the bacteriocins to proteinase K, heat and pH

The partially purified *E. durans* 152 bacteriocins were tested for sensitivity to proteinase K (Sigma-Aldrich, St. Louis, MO) by loading 5 μl of proteinase K to the edge of the spot where the bacteriocins were deposited on the *L. monocytogenes*-seeded BHI agar plate. The plate was held at 4 °C overnight before incubating at 37 °C. The partially purified bacteriocins and proteinase K individually were used as controls. Heat sensitivity of the bacteriocins was determined by incubating the partially purified bacterioicns at different temperatures ranging from 37 to 100 °C for 30 min or by autoclaving at 121 °C for 15 min. The inhibitory effect against *L. monocytogenes* was tested thereafter. The effect of pH was determined by adjusting the pH of the partially purified bacteriocins solutions between pH 2 and pH 11 with 3 M HCl or 1 M NaOH and incubating for at least 2 h at room temperature. The samples were then neutralized to pH 7.0 by 1 M lactic acid or 1 M NaOH before testing for antimicrobial activity.