Food Control 22 (2011) 1887–1892

Contents lists available at ScienceDirect

Food Control

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

Heterologous production of pediocin for the control of *Listeria monocytogenes* in dairy foods☆

J.A. Renye Jr.[a,*], G.A. Somkuti[a], J.I. Garabal[b], L. Du[c]

[a] Dairy and Functional Foods Research Unit, Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, ERRC-ARS-USDA, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA
[b] Agricultural Research Center of Mabegondo (CIAM), Xunta de Galicia, 15318 Abegondo, Spain
[c] College of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing, PR China

ARTICLE INFO

Article history:
Received 22 September 2010
Received in revised form
18 April 2011
Accepted 30 April 2011

Keywords:
Lactic acid bacteria
Pediocin
Listeria monocytogenes

ABSTRACT

Pediocin is an antimicrobial peptide naturally produced by Pediococci with the potential to serve as a food-grade preservative for controlling Listeria contamination. The use of Pediococci in dairy products is limited due to their inability to ferment lactose, thus lactic acid bacteria (LAB) have been considered as potential hosts for the heterologous production of pediocin. In this study the four gene operon (*papA-D*) required for pediocin production was cloned on the nisin-inducible expression vector pMSP3535H3. The resulting vector, pRSNPed2, was electrotransformed into *Streptococcus thermophilus*, *Lactococcus lactis* ssp. *lactis* and *Lactobacillus casei*. Transformants containing the properly constructed vector were identified by PCR analysis and shown to inhibit the growth of *Listeria monocytogenes* Scott A. *S. thermophilus* transformants were all shown to constitutively express pediocin; however, in *L. lactis* and *L. casei* both constitutive and inducer-dependent expression was observed. In all cases nisin-induction resulted in optimal pediocin production. Transformants from each LAB host were also shown to inhibit the growth of *L. monocytogenes* NR30, a nisin-resistant variant of *L. monocytogenes* Scott A. These results suggest pediocin has the potential to serve as a hurdle component along with nisin for prevention of Listeria contamination of foods.

Published by Elsevier Ltd.

1. Introduction

Listeria monocytogenes is a foodborne pathogen that causes the serious human illness listeriosis in susceptible individuals, which typically include the elderly, pregnant women and the immunocompromised (Francois et al., 2006; Lianou & Sofos, 2007). Although listeriosis is rare in comparison to other foodborne illnesses, it results in a higher percentage of hospitalization (>89%) or mortality (12–20%) among infected individuals (CDC, 2010; Mead et al., 1999). As a consequence, the United States has adopted zero-tolerance standards for ready-to-eat (RTE) foods commonly associated with listeriosis outbreaks such as RTE deli meats (Lianou & Sofos, 2007; Pradhan et al., 2010) and raw milk soft cheeses (MacDonald et al., 2005). Although pasteurization has been shown to kill *L. monocytogenes*, post-pasteurization contamination may occur and persist due to its ability to adapt to environmental stresses such as high salt concentrations, refrigeration (4 °C), and low pH (Bayles, 2004; Bolton & Frank, 1999; Lianou et al., 2006).

The ability of *L. monocytogenes* to survive under conditions established for the preservation of food suggests that additional methods must be implemented to prevent contamination. Bacteriocins are ribosomally synthesized antimicrobial peptides that have the potential to serve as safe and natural food preservatives when produced by food-grade lactic acid bacteria (LAB). These bacteria have been given the designation of Generally Regarded as Safe (GRAS) due to their extensive history of use in the food industry and consumption by humans (Cleveland, Montville, Nes, & Chikindas, 2001). Currently the lantibiotic nisin, naturally produced by *Lactococcus lactis* subsp. *lactis*, is the only bacteriocin approved for use as a food additive in the United States (Federal Resister, 1988). Nisin has been shown to inhibit the growth of *L. monocytogenes* in several fermented dairy products (Davies, Bevis, & Delves-Broughton, 1997; Ferreira & Lund,, 1996), however, nisin-resistant strains (Gravesen, Jydegaard Axelsen, Mendes da Silva, Hansen, & Knochel, 2002; Mazzotta & Montville, 1997) have been identified suggesting a need for additional

☆ Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

* Corresponding author. Tel.: +1 215 233 6517; fax: +1 215 233 6795.
E-mail address: john.renye@ars.usda.gov (J.A. Renye).

0956-7135/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.foodcont.2011.04.031

"food-grade" bacteriocins. Bacteriocins with antilisterial activity have been identified in dairy starter LAB, including *Streptococcus thermophilus* (Fontaine & Hols, 2008; Gilbreth & Somkuti, 2005; Marciset, Jeronimus-Stratingh, Mollet, & Poolman, 1997; Villani et al., 1995) and *L. lactis* (Akcelik, Tukel, Ozcengiz, & Akcelik, 2006; Elotmani, Revol-Junelles, Assobhei, & Millere, 2002; Ferchichi, Frere, Mabrouk, & Manai, 2001; Kim, Kim, Kim, & Kim, 2006; McAuliffe et al., 1998), and in non-starter LAB (NSLAB) including lactobacilli (Corr et al., 2007; Ennahar et al., 1996; Holck, Axelsson, Birkeland, Aukrust, & Blom, 1992; Miteva et al., 1998; Mueller, Carrasco, Tonarelli, & Simonetta, 2009; Tichaczek, Vogel, & Hammes, 1993) and enterococci (Franz, van Belkum, Holzapfel, Abriouel, & Galvez, 2007; Khan, Flint, & Yu, 2010; Renye, Somkuti, Paul, & Van Hekken, 2009).

Pediocin, a 44 amino acid class IIa bacteriocin (Ray, Johnson, & Ray, 1989), also has the potential to serve as a food-grade inhibitor of *L. monocytogenes*, but *Pediococcus* spp. are unable to ferment lactose and require specific co-culturing in order to allow for their use in fermented dairy products (Somkuti & Steinberg, 2010). As a consequence, the four gene pediocin operon (*pap*A-D) has been cloned downstream of endogenous promoters and shown to express in the dairy lactic acid bacteria *S. thermophilus* (Coderre & Somkuti, 1999; Somkuti & Steinberg, 2003) and *L. lactis* (Chikindas, Venema, Ledeboer, Venema, & Kok, 1995). Chimeras of pediocin PA-1 fused to the signal peptides of lactococcin A or enterocin P have also been expressed and secreted via the lactococcin A secretory apparatus or the sec-dependent machinery in *L. lactis* (Horn et al., 1998, 1999; Martin et al., 2007).

The nisin-controlled expression (NICE) system, based on the autoregulatory elements of the lactococcal gene cluster (de Ruyter, Kuipers, & de Vos, 1996; Kleerebezem, Beerthuyzen, Vaughan, de Vos, & Kuipers, 1997), has also been used to regulate expression of the chimeric lactococcin A-pediocin PA-1 peptide in *L. lactis* (Horn, Fernandez, Dodd, Gasson, & Rodriguez, 2004), and the intact pediocin operon in *S. thermophilus*, *L. lactis* and *Lactobacillus casei* (Renye & Somkuti, 2009). In the later study, the pMSP3535 single vector NICE system (Bryan, Bae, Kleerebezem, & Dunny, 2000) was used and shown to function less efficiently in *S. thermophilus* compared to the other two LAB hosts. In addition, *L. casei* was the only host shown to inhibit the growth of *L. monocytogenes* NR30, a nisin-resistant variant of the Scott A strain (Mazzotta & Montville, 1997).

In this study, the intact pediocin operon was cloned into pMSP3535H3 (Oddone, Mills & Block, 2009), a modified version of the original pMSP3535 vector. Initial modifications to the *nisA* promoter and the copy number control region resulted in the vector pMSP3535H2 (Kim & Mills, 2007), followed by insertion of the nisin immunity gene, *nisI*, resulting in the vector pMSP3535H3 (Oddone, Mills & Block, 2009). The resulting expression vector created in this study was used to test for increased pediocin expression in *S. thermophilus*, *L. lactis* and *L. casei*, and to see if the improved system would allow for the other two hosts to inhibit the growth of nisin-resistant *L. monocytogenes*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. thermophilus ST128 and *L. lactis* subsp. *lactis* ML3 were grown in tryptone-yeast extract-lactose (TYL) broth at 37 °C and 34 °C respectively (Somkuti & Steinberg, 1986). *Pediococcus acidilactici* F and *Lactbacillus casei* C2 were cultured in de Mann, Rogosa and Sharpe medium (MRS, Difco Laboratories, Detroit, MI, USA) at 37 °C. OmniMAX™ 2 T1 Phage-Resistant chemically competent *Escherichia coli* (Invitrogen Corporation, Carlsbad, CA) and *L. monocytogenes* Scott A and NR30 (Mazzotta & Montville, 1997) were grown in brain heart infusion broth (BHI) (Difco) at 37 °C under aerobic conditions. Genetic transformants of *S. thermophilus* ST128 and *L. lactis* subsp. *lactis* ML3 were propagated in TYL containing 15 μg ml^{-1} erythromycin (Em), and *L. casei* C2 transformants were grown in MRS containing 15 μg ml^{-1} Em. *E. coli* transformants were propagated in BHI containing 150 μg ml^{-1} Em.

2.2. Molecular cloning procedures

The vector pMSP3535H3 was constructed as described previously (Oddone, Mills, & Block, 2009) and provided by D. Mills (Univ. of California, Davis). The vector pUC18 was purchased from Life Technologies Inc. (Gaithersburg, MD, USA), and pPC418 was constructed according to a strategy previously described (Coderre & Somkuti, 1999). Restriction enzymes, Antarctic phosphatase, T4 DNA ligase and Taq DNA polymerase were from New England Biolabs (Beverly, MA, USA). Plasmids were isolated from *E. coli* using the QIAGEN Miniprep Kit (Valencia, CA) or the alkaline lysis method (Sambrook, Maniatis, & Fritsch, 1989) followed by CsCl/ethidium bromide ultracentrifugation (Stougaard & Molin, 1981). DNA was analyzed by gel electrophoresis in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) with 1% agarose. DNA fragments required for cloning were cleaned using Elutip minicolumns (Schleicher & Schuell, Keene, NH) prior to phosphatase treatment.

Transformation of OmniMAX™ 2 T1 Phage-Resistant chemically competent *E. coli* was carried out by a heat-shock method (Sambrook, Maniatis, & Fritsch, 1989), while *S. thermophilus* ST128, *L. lactis* subsp. *lactis* ML3 and *L. casei* C2 were electrotransformed by a standard protocol previously described (Somkuti & Steinberg, 1988). Transformants were initially screened for the presence of recombinant plasmid by PCR. PCR primer pairs designed (Integrated DNA Technologies, Coralville, IA, USA): nisAFwd (5′TACGGATCAGATCTAGTCTTA3′) and PedRev (5′GGCTGGCAATCTTGTTGT3′); and papCFwd (5′CCATATTAACC-AGGTGACTAC3′) and papCRev (5′GTGGCCTAGCCAATAGTACTG3′). PCR amplification protocol was as follows: 5 min at 95 °C; followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 74 °C for 1 min per 1 kb DNA; with a final extension at 74 °C for 5 min. DNA fragments amplified using the nisAFwd and PedRev primer set were analyzed by nucleic acid sequencing using an ABI PRISM 3730 (Perkin–Elmer, Wellesley, MA) DNA analyzer with ABI PRISM Big Dye terminator cycle sequencing reagent. Obtained sequences were analyzed using Sequencher 4.9 (Gene Codes Corp., Ann Arbor, MI).

2.3. Nisin induced expression of pediocin

Nisin (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 0.02 mol l^{-1} HCl and filter-sterilized (0.2 μm), at a stock solution of 25 μg ml^{-1}. *S. thermophilus* ST128 and *L. lactis* subsp. *lactis* ML3 transformants were grown overnight in TYL broth containing 15 μg ml^{-1} Em at 37 °C and 34 °C respectively. *L. casei* C2 transformants were grown overnight in MRS broth containing 15 μg ml^{-1} Em at 37 °C. Following overnight growth, the cells were pelleted and washed 3× in 0.1% peptone broth. The cultures were diluted 1.6-fold into fresh TYL containing various concentrations of nisin: 10 ng ml^{-1}, 50 ng ml^{-1}, and 100 ng ml^{-1}. Cells were incubated in the presence of nisin for up to 24 h. Cell-free supernatants (45 μl) were loaded into precast wells in BHI agar inoculated with an overnight culture of *L. monocytogenes* Scott A or NR30 (0.3%, v/v). Plates were allowed to equilibrate at 4 °C for 3h, incubated at 37 °C for up to 8 h, and examined for zones of inhibition. Well-diffusion assays were carried out in triplicate for each LAB host.

The amount of pediocin produced by LAB was determined as previously described (Coderre & Somkuti, 1999). Pediocin concentration was estimated by serial two-fold dilutions of cell-free