Food Control 60 (2016) 511–518

Contents lists available at ScienceDirect

Food Control

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

Isolation and characterization of a *Bacillus atrophaeus* strain and its potential use in food preservation

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ARTICLE INFO

Article history:
Received 3 April 2015
Received in revised form
14 August 2015
Accepted 25 August 2015
Available online 28 August 2015

Keywords:
Bacillus atrophaeus
Antimicrobials
Subtilosin
Food preservation

ABSTRACT

Microorganisms are widely distributed in food and contribute to food safety due to production of antagonistic substances. A new bacterial strain, OSY-7LA, was isolated from a Chinese delicacy food and exhibited strong antagonistic activity against *Listeria innocua*. It was identified as *Bacillus atrophaeus* by morphological, physiological, and biochemical properties and genetic relatedness. The culture supernatant has antimicrobial activities against the Gram-positive pathogens tested, namely, *Listeria monocytogenes*, *Bacillus cereus* and methicillin-resistant *Staphylococcus aureus*. The antimicrobial agents were harvested by solvent extraction and were purified by high performance liquid chromatography (HPLC). Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) and tandem mass spectrometry (MS/MS) were performed to identify these compounds. A protonated ion at m/z 3401.414 corresponded to the molecular mass of subtilosin, and the identity of the antimicrobial agent was confirmed by amplification of subtilosin gene (*sbo*) from isolate's genomic DNA. Sodiated ions at m/z 1030.553, 1044.642 and 1058.701 were identified as C13, C14 and C15 surfactins. LC/MS analysis proved the production of plipastatin by OSY-7LA. Supplement of crude extract of OSY-7LA supernatant in Vienna sausage that was inoculated with *L. innocua* showed 2-log reduction after 12 and 24 h. The new strain and related antimicrobials are potentially useful in food preservation.

1. Introduction

Foodborne pathogens have posed serious health challenges to human and led to big economic losses in food production and storage. Preservatives are commonly used to combat these pathogens and prevent food spoilage. Since the demand for chemical free food is increasing, alternatives to chemical preservatives are needed. Ideally, the alternatives are isolated from natural sources and are well-suited for food applications. Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins from bacteria (Jack, Tagg, & Ray, 1995). Bacteriocins have relatively narrow spectrum and are good candidates as food preservatives, shelf life-extenders and ingredients (Galvez, Abriouel, Lopez, & Ben Omar, 2007). The feasibility of applying bacteriocins in food has been widely evaluated. Lacticin 3147 has been used to control nonstarter lactic acid bacteria in cheese, and to inhibit *Listeria* strains and *Bacillus cereus* in different foods (Guinane, Cotter, Hill, & Ross, 2005). Pediocin PA-1/AcH significantly reduced the number of *Listeria monocytogenes* on meat surface when it was incorporated into a biocomposite packaging film (Woraprayote et al., 2013). The enterocin AS-48 producing strain inhibited growth of *B. cereus*, but had no effect on the starter when it was inoculated in the milk for cheese production (Galvez et al., 2007). However, nisin is the only commercially available bacteriocin for food applications (Perez, Zendo, & Sonomoto, 2014). Nisin is produced by *Lactococcus lactis* and the bacteriocin has been applied in food in more than fifty countries (Reunanen & Saris, 2003). It was reported to decrease the number of *L. monocytogenes* in cottage cheese and has been showed to be effective in many foods such as milk, fish and meat products

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http://dx.doi.org/10.1016/j.foodcont.2015.08.029
0956-7135/

(Ross, Morgan, & Hill, 2002). Nevertheless, resistance of pathogens (e.g., *L. monocytogenes*) to nisin has been reported (Crandall & Montville, 1998). Lack of efficacy of nisin against Gram-negative bacteria and low efficacy at neutral pH are some of the drawbacks that are limiting the application of nisin in food (He et al., 2007).

Subtilosin represents a new kind of lantibiotic due to the unique thioether bond formation between Cys and Phe (Kawulka et al., 2004). It exhibited good antimicrobial activity against *L. monocytogenes*, when used alone or in combination with curcumin (Amrouche, Sutyak Noll, Wang, Huang, & Chikindas, 2010). Shelburne et al. (2007) found that subtilosin was effective against both Gram-positive and Gram-negative bacteria, and it was active in a wide pH range from 2 to 10 (Sutyak, Wirawan, Aroutcheva, & Chikindas, 2008). These findings suggest that subtilosin is potentially a good substitute of nisin as food preservative. However, the application of subtilosin in food has not been reported.

The aim of this study was to screen food samples for bacterial strains and bacterioins with promising antimicrobial properties. A bacterial strain, designated as OSY-7LA, was isolated and found to produce the lantibiotic subtilosin. Production, purification and identification of subtilosin and its application in Vienna sausage were described.

2. Materials and methods

2.1. Strain screening

Food samples, including different kind of cheeses and vegetables, were purchased from local food stores (Columbus, OH) and were screened for isolates with antimicrobial activity. The screening procedure of He et al. (2007) was used with modifications. Briefly, samples were suspended in 0.1% peptone water and homogenized by a stomacher or a blender. The homogenates were serially diluted and passed through hydrophobic grid membranes with pore size of 0.45 μm (ISO-GRID; Neogen Corporation, Lansing, MI). The membranes were then placed onto tryptose agar and incubated at 30 °C for 48 h. The membranes carrying bacterial colonies were removed and saved, but the agar plates were overlaid with soft agar seeded with *Listeria innocua* ATCC 33090 or *Escherichia coli* K-12. The seeded agar plates were incubated at 37 °C overnight and inspected for signs of antimicrobial activity. Colonies on the membrane that produced inhibitory zones were transferred and streaked onto new tryptose agar plates. A sample of "Facai", a Chinese delicacy food (Gao, 1998), yielded a strain with potent antimicrobial activity against *L. innocua* and was given OSY-7LA designation.

2.2. Cultures and media

Tryptose agar was used to propagate the new isolate OSY-7LA. Indicator organisms and media used in this study are listed in Table 1. A stock of the new isolate was prepared by inoculation in tryptic soy broth and overnight incubation at 30 °C; incubated culture was mixed with sterile glycerol (final concentration 20%), and stored at −80 °C.

2.3. Phenotypic examination

Gram staining, spore staining and scanning electron microscopy were employed to examine the morphological properties of OSY-7LA. Sample preparation for scanning electron microscopy was done as described previously (Kaletunc, Lee, Alpas, & Bozoglu, 2004) with modifications. Briefly, the isolate OSY-7LA was grown on tryptose agar at 30 °C for three days. A single colony was suspended in phosphate buffer (0.05 mol/l, pH 7.0) and washed three times by centrifugation and resuspension in the same buffer. Cells were fixed in fixative (2.5% glutaraldehyde in 0.1 mol/l phosphate buffer with 0.1 mol/l sucrose, pH 7.4) at 4 °C for ~12 h and resuspended in phosphate buffer (0.05 mol/l, pH 7.0). The cell suspension was passed through a 0.22 μm membrane (Millipore Corp., Bedford, MA), and bacteria on the membrane were dehydrated using an ascending series of ethanol solutions. After dehydration by ethanol solutions, bacterial cells were chemically dried using an ascending series of hexamethyldisilazane (HMDS) in ethanol and the residual HMDS solution was evaporated. The bacteria were coated with a thin layer of gold-palladium in a Cressington 108 Sputter Coater (Ted Pella Inc., Redding, CA) and examined by a scanning electron microscope (SEM; NOVA NanoSEM 400, FEI, Hillsboro, OR) with accelerating voltage at 5 kV. The SEM observations were performed at The Ohio State University Campus Microscopy and Imaging Facility.

Table 1
Antimicrobial spectrum of OSY-7LA cultural supernatant.

Strains[a]	Media	Activity
Gram-positive bacteria		
Bacillus cereus ATCC 14579	TSBYE	+++
B. cereus ATCC 11778	TSBYE	+++
Enterococcus faecalis	TSBYE	++
Listeria innocua ATCC 33090	TSBYE	+++
L. monocytogenes Scott A	TSBYE	+++
Staphylococcus aureus ATCC 6538	TSBYE	++
S. aureus (methicillin-resistant)	TSBYE	++
Gram-negative bacteria		
Escherichia coli K-12	LB	-
E. coli O157:H7 EDL 933	LB	-
Salmonella enterica serovar Typhimurium	TSBYE	-
Yesinia enterocolitica	TSBYE	-
Fungus		
Rhizopus arrhizus	APCA	++

[a] Strains obtained from the culture collection of The Ohio State University food safety laboratory. TSBYE, Tryptic soy broth supplemented with 0.6% yeast extract; LB, Luria–Bertani medium; APCA, plate count agar containing chlortetracycline hydrochloride and chloramphenicol. +++, inhibitory zone diameter >20 mm; ++, inhibitory zone diameter between 10 mm and 20 mm; +, inhibitory zone diameter <10 mm; -, no inhibition.

2.4. Biochemical tests

A commercial biochemical test kit (API 50 CH strips and CHB medium, BioMerieux, Inc., Durham, NC) was used to characterize the carbohydrates fermentation pattern of the new isolate. The biochemical tests included catalase, nitrate reduction, formation of indole, deamination of phenylalanine, utilization of citrate and hydrolysis of starch and casein. The wells containing substrates were inoculated with the new isolate at appropriate density and were incubated at 30 °C for 24–48 h. Results were recorded and identification was done by referring to the database provided by the manufacturer.

2.5. Genetic relatedness by sequencing 16S rDNA

Genomic DNA of the new isolate was extracted using a commercial kit (DNeasy Blood & Tissue, QIAGEN, Valencia, CA). Universal primers specific for bacterial 16S rDNA were used in the polymerase chain reaction, PCR (Weisburg, Barns, Pelletier, & Lane, 1991). The PCR included incubation at 94 °C for 3 min, followed by 30 cycles, each including 1 min at 94 °C, 1 min at 52 °C, and 2 min at 72 °C. The final extension was performed at 72 °C for 10 min. The