International Journal of Food Microbiology 238 (2016) 165–171

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Inhibition of *Staphylococcus aureus* by antimicrobial biofilms formed by competitive exclusion microorganisms on stainless steel

Hyeri Son [a], Sunhyung Park [a], Larry R. Beuchat [b], Hoikyung Kim [c,*], Jee-Hoon Ryu [a,*]

[a] Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Republic of Korea
[b] Center for Food Safety and Department of Food Science and Technology, University of Georgia, 1109 Experiment Street, Griffin, GA 30223-1797, USA
[c] Department of Food and Nutrition, Wonkwang University, 460 Iksandae-ro, Iksan, Jeonbuk 54538, Republic of Korea

ARTICLE INFO

Article history:
Received 20 June 2016
Received in revised form 31 August 2016
Accepted 11 September 2016
Available online 13 September 2016

Keywords:
Competitive-exclusion microorganisms
Biofilm
Staphylococcus aureus
Stainless steel
Antimicrobial surface

ABSTRACT

The goal of this study was to develop a desiccation resistant antimicrobial surface using biofilm of competitive exclusion (CE) microorganism inhibitory to *Staphylococcus aureus*. We isolated 161 microorganisms from soils, foods, and food-contact surfaces that are inhibitory to *S. aureus*. Among them, three CE microorganisms (*Streptomyces spororaveus* strain Gaeunsan-18, *Bacillus safensis* strain Chamnamu-sup 5–25, and *Pseudomonas azotoformans* strain Lettuce-9) exhibiting strong antibacterial activity and high growth rates were selected for evaluation. These isolates formed biofilms within 24 h on stainless steel coupons (SSCs) immersed in Bennet's broth and tryptic soy broth at 25 °C. Cells in these biofilms showed significantly ($P \leq 0.05$) enhanced resistance to a desiccation (43% relative humidity [RH]) compared to those attached to SSCs but not in biofilms. The antimicrobial activities of biofilms formed by these isolates on SSCs against *S. aureus* at 25 °C and 43% RH were determined. Compared to SSCs lacking biofilms formed by CE microorganisms, populations of *S. aureus* on SSCs harboring CE biofilms were significantly lower ($P \leq 0.05$). Results indicate that persistent antimicrobial activity against *S. aureus* on stainless steel surfaces can be achieved by the presence of biofilms of CE microorganisms. This information will be useful when developing strategies to improve the microbiological safety of foods during storage, processing, and distribution by facilitating the development of effective antimicrobial food-contact surfaces.

1. Introduction

Staphylococcal intoxication is one of the most common foodborne diseases worldwide. It results from ingestion of foods containing staphylococcal enterotoxin produced mainly by *Staphylococcus aureus* (Lawley et al., 2008; Seo and Bohac, 2013). The most common symptoms of staphylococcal foodborne intoxication are nausea, vomiting, and abdominal cramping (Forsythe, 2010). In the United States, it is estimated that 60 outbreaks, 1502 illness, 67 hospitalization and 1 death occurred during 2011–2014 due to Staphylococcal food poisoning (Centers for Disease Control and Prevention, 2014). *S. aureus* is highly resistant to desiccation and is not uncommonly found on food-processing equipment and surrounding environmental surfaces, becoming a source of food contamination (Bennet and Monday, 2003; Chaibenjawong and Foster, 2011; Hennekinne et al., 2012; Landgraf and Destro, 2013). To prevent *S. aureus* intoxication, it is important to inhibit the growth of *S. aureus* on food-contact surfaces as well as on surfaces in the surrounding environment.

To inhibit the growth of hazardous microorganisms on food-contact surfaces and in food processing environments, biological methods which do not leave sanitizer residues, affect sensorial properties of foods, or cause corrosion of metals, have been examined. These methods typically involve the use of competitive exclusion (CE) microorganisms which have antagonistic activity against foodborne pathogens. The mechanisms of CE microorganisms to inactivate or prevent growth of pathogens are based on competition for attachment sites or nutrients, production of antimicrobial substances, or more rapid growth (Gálvez et al., 2012; Ukuku et al., 2015). Several studies focused on inactivation of *Listeria monocytogenes*, *Salmonella enterica*, and enteropathogenic *Escherichia coli* using CE microorganisms have been reported (Belák and Maráz, 2015; Leverentz et al., 2006; Liao, 2009). However, the inhibition of *S. aureus* by CE microorganisms on abiotic surfaces has not been reported.

Biofilms have been defined as sessile bacterial communities attached to a biological or abiotic surface, usually embedded in extracellular polymeric substances (EPSs) (Cloete et al., 2009; Costerton et al., 1999). Because EPSs act as physical barriers against environmental stresses, biofilm-embedded foodborne pathogens exhibit increased resistance to detrimental effects of stresses such as desiccation, antibiotics, and sanitizers (Kim et al., 2007; Olson et al., 2002; Robbins et al., 2005;

* Corresponding authors.
E-mail addresses: hoikyung@wku.ac.kr (H. Kim), escheri@korea.ac.kr (J.-H. Ryu).

http://dx.doi.org/10.1016/j.ijfoodmicro.2016.09.007
0168-1605/

Ryu and Beuchat, 2005a, 2005b). The increased resistance of hazardous microorganisms in biofilms to environmental stresses has therefore been a focus of research, with the aim of developing effective decontamination measures. In the study we report here, it was hypothesized that biofilm formation by CE microorganisms that inhibit *S. aureus* on an abiotic surface would be enhanced by their resistance to desiccation stress. If this hypothesis is true, antimicrobial biofilms formed by CE microorganisms on abiotic surfaces may have application to control the growth of *S. aureus*. For examples, the biofilms of CE microorganisms could be applied to the surfaces encountered during production of agricultural commodities, storage of raw and processed foods, production of processed foods, transportation and distribution of foods, etc.

The objective of this study was to develop a desiccation resistant antimicrobial surface using biofilm of CE microorganism inhibitory to *Staphylococcus aureus*. To achieve this goal, we isolated and identified CE microorganisms from soils, foods, and food-contact surfaces that were inhibitory to *S. aureus* and evaluated their ability to form biofilms. The desiccation resistance profiles of CE microorganisms in biofilms formed on stainless steel were examined and their antimicrobial activities against *S. aureus* were confirmed.

2. Materials and methods

2.1. Strains of S. aureus used

Five strains of *S. aureus* were used: ATCC 25923 (clinical isolate), KCTC 1928 (clinical isolate), ATCC 13565 (isolated from an outbreak associated with ham), ATCC 23235 (isolated from turkey salad), and ATCC 27664 (isolated from chicken tetrazzini). Cryopreserved *S. aureus* cells were activated in 10 mL of tryptic soy broth (TSB; BBL/Difco, Sparks, MD, USA) at 37 °C for 24 h. Each of the five activated strains was transferred to 10 mL of TSB at 37 °C at three consecutive 24 h intervals. A *S. aureus* cocktail (10 mL) was prepared by combining 2 mL of each of the five strains. The cocktail was centrifuged at 2002 ×*g* for 15 min at room temperature (22 ± 2 °C), supernatants were decanted, and the cells were resuspended in 10 mL of sterile 0.1% peptone water (PW). This procedure was repeated, and the suspensions were serially diluted in 0.1% PW to prepare *S. aureus* inocula (ca. 7 or 5 log CFU/mL).

2.2. Isolation of microorganisms from soil, foods, and food-contact surfaces

To screen for microorganisms with antimicrobial activity against *S. aureus*, isolates with different colony colors and morphologies were collected from soil, foods, and food-contact surfaces.

Soil samples were collected from 30 diverse locations, including mountains, lakes, and dams, in the Republic of Korea. Samples were collected as described by Kim et al. (2011). Briefly, samples were collected from a depth of 10 cm in the ground. Subsamples (1 g) were suspended in 10 mL of sterile distilled water (DW) and incubated at 25 °C for 1 to 3 h with shaking at 150 rpm. The supernatant (0.1 mL) was spread-plated on humic acid vitamin agar medium and incubated at 28 °C for 3 to 14 days. Cells from selected colonies were transferred using a loop to 10 mL of 20% glycerol in DW and stored at −80 °C.

Fresh produce (lettuce [*Lactuca savita* var. *crispa*], iceberg lettuce [*Lactuca savita* var. *capitata*], cabbage [*Brassica oleracea* var. *capitata*], red cabbage [*Brassica oleracea var. capitata*], Chinese cabbage [*Brassica rapa*], perilla leaf [*Perilla frutescens* var. *japonica*], spring greens [*Brassica campestris*], chicory [*Cichorium intybus*], and mustard greens [*Brassica juncea*]) and fishery products (fish [mackerel and yellow croaker], crustaceans [shrimp], and shellfish [mussel and ark shell]) were purchased from eight stores in traditional wholesale markets (Cheongnyang wholesale market and Noryangjin fisheries wholesale market, Seoul, Republic of Korea). Samples (10 g) were combined with 100 mL of sterile 0.1% PW and pummeled for 2 min using a stomacher (Interscience BagMixer® 400 W; Interscience, Saint Nom, France). The homogenate was spread-plated on tryptic soy agar (TSA; BBL/Difco) supplemented with 0.1% (w/v) sodium pyruvate (Kanto Chemical Co, Inc., Tokyo, Japan), and incubated at 25 °C for 48 h. Colonies with different colors and morphologies were streaked on TSA, followed by incubation at 25 °C for 24 h and storage at 4 °C.

To isolate microorganisms from food-contact surfaces, cutting boards and knives that had been used in two modern supermarkets and four stores in a traditional fisheries wholesale market located in Seoul were swabbed 100 times using a swab slightly moistened with sterile DW. The swabs were placed separately in 50 mL conical tubes containing 10 mL of sterile 0.1% PW, followed by vortexing for 2 min. Microorganisms were isolated using the methods described above for foods.

To evaluate isolates for antimicrobial activity against *S. aureus*, isolates from soil were streaked on Bennet's agar and incubated at 25 °C for 6 days. One to five colonies per plate were transferred to 50 mL conical tubes containing 10 mL of TSB and 0.5 g of sterile glass beads (1 mm, Glass beads 1, Glastechnique Mfg., Germany), followed by incubation at 25 °C for at least 3 days with shaking at 200 rpm. Isolates from foods or food-contact surfaces were inoculated in TSB at 25 °C and transferred three times at 24 h intervals before examining for CE activity against *S. aureus*.

2.3. Double layer assay to screen CE microorganisms for inhibitory activity against S. aureus

The ability of isolates from soil, foods, and food-contact surfaces to inhibit *S. aureus* was determined using a double-layer assay (Zhao et al., 2004). Suspensions (10 μL) of cells of isolates prepared as described above were spot-inoculated onto TSA plates (spot diameter ca. 7 mm; four isolates per plate). Plates were held in a laminar flow biosafety hood at room temperature (22 ± 2 °C) for 30 min and then incubated at 25 °C for 24 h. Molten TSA (10 mL) containing *S. aureus* (ca. 5 log CFU/mL) was poured onto the surface of TSA and the plates were dried in a laminar flow biosafety hood for 30 min followed by incubation at 37 °C for up to 24 h. The antimicrobial activity of isolates against *S. aureus* was assessed by measuring the diameters of zones of inhibition surrounding colonies.

2.4. Identification of CE microorganisms inhibitory to S. aureus

To identify the genus and species of isolates with strong antimicrobial activity against *S. aureus*, 16S rRNA sequence analysis was performed (http://www.macrogen.co.kr, Seoul, Korea). Sequences were analyzed using BLAST software available on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/, USA). Phylogenetic analysis of the 16S rRNA gene sequences of isolates was performed using Mega software version 5.05 with the neighbor-joining method. Bootstrap values are expressed as percentages of 1000 replications.

2.5. Biofilm formation by CE microorganisms on stainless steel

Stainless steel coupons (SSCs; Type 304, 5 × 2 cm, no. 4 finished) were washed using 15% phosphoric acid and 15% alkaline detergent as described by Nam et al. (2014). The washed SSCs were boiled in DW for 15 min, dried for 24 h, and autoclaved at 121 °C for 15 min.

Suspensions of CE microorganisms prepared as described above were centrifuged at 2002 ×*g* for 15 min at room temperature (22 ± 2 °C), and pelleted cells were resuspended in 10 mL of phosphate-buffered saline (PBS, pH 7.4). The procedure was repeated and cell suspensions were diluted in PBS to a density of ca. 5 log CFU/mL and deposited in a sterile sprayer (30 mL, code 46,322; Daiso, Seoul, Korea). To attach CE microorganisms on SSC surfaces, a sterile SSC was deposited in a polystyrene dish (60 mm diameter by 8 mm height) which had been placed in a Petri dish (90 mm diameter by 15 mm height; SPL, Seoul, Korea). Suspensions (ca. 1 mL) of CE microorganisms were