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Development of non-pathogenic bacterial biofilms on the surface of stainless steel which are inhibitory to *Salmonella enterica*

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ABSTRACT

Non-pathogenic bacterial biofilms were developed on the surface of stainless steel possessing desiccation tolerance and antimicrobial activity against *Salmonella enterica*. Three bacteria exhibiting strong antimicrobial activities against *S. enterica* were isolated from various soils, foods, and food-contact surfaces. Isolates were identified as *Pseudomonas extremorientalis* (strain Lettuce-28), *Paenibacillus peoriae* (strain Lettuce-7), and *Streptomyces cirratus* (strain Geumsan-207). These bacteria grew rapidly and formed biofilms within 24 h on the surface of stainless steel coupons (SSCs) immersed in laboratory media (tryptic soy broth or Bennet's broth) at 25 °C. Cells in biofilms had enhanced tolerance to desiccation (exposure to 43% atmospheric relative humidity [RH]) and retained antimicrobial activity against *S. enterica*. Populations of *S. enterica* deposited on SSCs containing biofilm formed by *Ps. extremorientalis* strain Lettuce-28, for example, decreased by > 2.5 log CFU/coupon within 24 h at 25 °C and 43% RH, while the number of cells inoculated on SSCs lacking biofilm decreased by 1.5 log CFU/coupon. Antimicrobial activities of the three antagonistic bacteria against *S. enterica* persisted in desiccated biofilms. This study provides insights to developing strategies to inactivate *Salmonella* and perhaps other foodborne pathogens on abiotic surfaces using non-pathogenic antagonistic bacteria.

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1. Introduction

Salmonella enterica is a major cause of foodborne illness (Mukhopadhyay and Ramaswamy, 2012; Paterson, 2006). Globally, nontyphoidal *Salmonella* is responsible for ca. 93.8 million illnesses and 155,000 deaths every year (Majowicz et al., 2010). In the United States, non-typhoidal *Salmonella* spp. are believed to cause ca. 1 million illnesses annually, accounting for 11% of all foodborne illnesses (Scallan et al., 2011). Non-typhoidal salmonellosis in immunocompetent individuals is usually characterized by self-limited enterocolitis with diarrhea, whereas in sensitive groups such as infants, young children, the elderly, and immunocompromised individuals, it may cause severe sequelae (Kariuki et al., 2015).

S. enterica may occasionally be present in raw food materials and transferred to processed foods by cross-contamination in food production and processing environments (Carrasco et al., 2012). *S. enterica* associated with poultry and poultry meat, for example, has been reported to originate from diverse environments, including hatcheries, transport tray liners, broiler houses, slaughterhouses, and carcass processing facilities (Heyndrickx et al., 2002; Wideman et al., 2015). Thus, methods to effectively eliminate *S. enterica* from food production and processing environments are urgently needed.

Several researchers have reported that antagonistic bacteria or their metabolites can be used to inhibit foodborne pathogens on abiotic surfaces in food production and processing environments. Antimicrobial activities of *Paenibacillus polymyxa* against *Cronobacter* spp. (Yang et al., 2013) and *Escherichia coli* O157:H7 (Kim et al., 2013) on stainless steel surfaces have been reported. Perez-Ibarreche et al. (2016) investigated the antimicrobial activity of bacteriocins produced by *Lactobacillus sakei* strain CRL1862 against *Listeria monocytogenes* on stainless steel and polytetrafluoroethylene surfaces. Son et al. (2016) inhibited *Staphylococcus aureus* on stainless steel surfaces using biofilms formed by antagonistic bacteria. Biological control strategies that use microorganisms and/or their metabolites are becoming more attractive because they can be regarded as health-friendly by consumers and may minimize

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negative effects on nutritional and sensorial properties of foods (Gálvez et al., 2010; Oliveira et al., 2015). Studies focused on inactivating *S. enterica* using antagonistic bacteria on abiotic surfaces have not been performed.

Biofilms are assemblages of microbial cells attached to and grown on biological or abiotic surfaces that are frequently enmeshed in extracellular polymeric substances (EPSs) (Costerton et al., 1995; Donlan, 2002; Hall-Stoodley et al., 2004; Kim et al., 2006). EPSs act as protective barriers for microbial cells in biofilms, enhancing their tolerance to environmental stresses such as desiccation, osmotic pressure, ultraviolet radiation, sanitizers, and antibiotic treatments (Donlan, 2002; Hall-Stoodley et al., 2004; Kim et al., 2006; Le Magrex-Debar et al., 2000; Ryu and Beuchat, 2005; van de Mortel et al., 2004). It is known that foodborne pathogens have increased tolerance to desiccation when present in biofilms (Steenackers et al., 2012). Researchers have therefore focused their attention on inactivating foodborne pathogens present in biofilms on abiotic surfaces in food production and processing environments. Conversely, few studies have investigated the survival of bacteria inhibitory to foodborne pathogens in dry environments.

The objective of this study was to develop a desiccation-tolerant antimicrobial biofilm on the surface of stainless steel that is inhibitory to *S. enterica*. To achieve this goal, three bacteria that exhibited strong antimicrobial activity against *S. enterica* were isolated and identified from diverse environments (soils, foods, and food-contact surfaces), and their abilities to form biofilms on the surface of stainless steel were evaluated. The enhanced tolerance of these antagonistic bacteria against desiccation resulted by the biofilm formation was determined. Finally, antimicrobial activities in biofilms formed by these isolates on stainless steel surfaces against *S. enterica* were confirmed.

2. Materials and methods

2.1. Strains of S. enterica used

Five serovars of *Salmonella enterica* subsp. *enterica* were used: *S.* Enteritidis, *S.* Hartford, *S.* Heidelberg, *S.* Newport, and *S.* Typhimurium. Cryopreserved *S. enterica* cells were activated in tryptic soy broth (TSB; BBL/Difco, Sparks, MD, USA) by incubating at 37 °C for 24 h, and each of the five activated serovars was then transferred to 10 mL of TSB three times at 24-h intervals, followed by incubation at 37 °C. A *S. enterica* cocktail (10 mL) was prepared by combining 24-h cultures of the five serovars (2 mL of each) and centrifuging at 2000×*g* for 15 min at room temperature (22 ± 2 °C). Supernatants were decanted and pelleted cells were resuspended in 10 mL of sterile 0.1% peptone water (PW) or TSB. The procedure was repeated, and the suspensions were serially diluted in 0.1% PW or TSB to prepare *S. enterica* inocula with two populations (ca. 7 or 5 log CFU/mL).

2.2. Isolation and identification of bacteria inhibitory to S. enterica

2.2.1. Isolation of bacteria inhibitory to S. enterica from soil, food, and food-contact surfaces

Bacteria were isolated from soils, foods, and food-contact surfaces using procedures described by Kim et al. (2011) and Son et al. (2016). In total, 1648 isolates from 133 soil samples (30 diverse locations in Republic of Korea) were streaked on Bennet's agar and incubated at 25 °C for 6 days. Cells from colonies were transferred into 50-mL conical tubes containing 10 mL of Bennet's broth and 5 g of sterile glass beads (1 mm, Glass beads 1; Glastechnique Mfg., Germany) and incubated at 25 °C for at least 3 days with shaking at 200 rpm. In total, 312 isolates from 17 food samples and 105 isolates from 15 food-contact surface samples were cultured in TSB at 25 °C and transferred three times at 24-h intervals before being used in experiments.

A double-layer assay (Zhao et al., 2004) was used to screen for isolates exhibiting strong antimicrobial activity against *S. enterica*. Suspensions (10 μL) of isolates prepared as described above were spot-inoculated on tryptic soy agar (TSA; BBL/Difco) plates (spot diameter ca. 7 mm; four isolates per plate). Plates were placed in a laminar flow biosafety hood at room temperature for 30 min, followed by incubating at 25 °C for 24 h. Molten TSA (10 mL) containing *S. enterica* (ca. 5 log CFU/mL) was poured onto the surface of spot-inoculated TSA, and the plates were held in a laminar flow biosafety hood for 30 min, followed by incubating at 37 °C for up to 24 h. The antimicrobial activity of isolates against *S. enterica* was evaluated by measuring the diameters of zones of inhibition surrounding the colonies formed by the test isolates.

Growth characteristics of the 15 bacteria with strong antimicrobial activities were determined by measuring populations in TSB (isolates from food) or Bennet's broth (isolates from soil) incubated at 25 °C for up to 120 h.

2.2.2. Identification of bacteria inhibitory to S. enterica

The genus and species of three isolates exhibiting antimicrobial activities against *S. enterica* with high growth abilities were determined by 16S rRNA sequence analysis (Macrogen, http://www.macrogen.co.kr, Seoul, South Korea). The 16S rRNA gene sequences were analyzed using BLAST software available online at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/, USA). The neighbor-joining phylogenetic tree (Saitou and Nei, 1987) was constructed by using distances corrected according to the Kimura two-parameter model (Kimura, 1980) with the Mega software version 5.05 (Tamura et al., 2011). Bootstrap values are expressed as percentages of 1000 replications.

2.3. Biofilm formation by antagonistic bacteria on stainless steel

Stainless steel coupons (SSCs; Type 304, 5 × 2 cm, no. 4 finish) were washed by sonicating in 15% (v/v) phosphoric acid solution (Daejung, Siheung, Republic of Korea) and 15% (v/v) alkaline detergent solution (Fs Pro-Chlor, Zep, Atlanta, GA, USA) at 70 °C for 20 min, as described by Nam et al. (2014). The sonicated SSCs were boiled in deionized water (DW) for 15 min, dried for 24 h, and autoclaved at 121 °C for 15 min.

Suspensions of three antagonistic (inhibitory to *S. enterica*) bacteria prepared as described above (section 2.2.1.) were centrifuged at 2000×*g* for 15 min at room temperature (22 ± 2 °C). The supernatant was decanted 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 give a population of ca. 5 log CFU/mL and deposited in a sterile sprayer (50 mL, code 46323; Daiso, Seoul, South Korea). To attach the antagonistic bacteria on SSCs, sterile SSCs were placed in polystyrene dishes (60 mm diameter, 8 mm high) and a suspension (ca. 1 mL) of test bacteria was applied by spraying perpendicularly to the surface of the SSC at a distance of 5 cm. Cells were dried on the surface of SSCs in a laminar flow biosafety hood at room temperature (22 ± 2 °C) for 1 h. A second set of SSCs with biofilms formed by attached antagonistic cells was prepared. SSCs with attached cells were placed in a polystyrene dish which was then placed in a Petri dish (90 mm diameter by 15 mm high; SPL, Seoul, South Korea). SSCs with attached *Pseudomonas extremorientalis* strain Lettuce-28 or *Paenibacillus peoriae* strain Lettuce-7 were immersed in 4 mL of TSB; SSCs with attached *Streptomyces cirratus* strain Geumsan-207 were immersed in 4 mL of Bennet's broth. The Petri dishes were sealed using Parafilm (Bemis, Neenah, WI, USA) and

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