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Isolation of indigenous bacteria from a cafeteria kitchen and their biofilm formation and disinfectant susceptibility

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ABSTRACT

Bacterial biofilm formation in foodservice facilities is a continuous cross-contamination risk through survival and persistence despite disinfectant treatments. In this study, we evaluated biofilm formation and disinfectant susceptibility of 178 strains obtained from a cafeteria kitchen and 70 foodborne pathogens and analyzed results by multivariate data analyses. A total of 23 areas in a cafeteria kitchen were selected for bacterial isolation and identification. The capacity for biofilm formation was tested using a crystal violet assay, and disinfectant susceptibility was examined using an agar well diffusion assay and resazurin reduction assay. The most frequently isolated genera were *Bacillus* (33%), *Acinetobacter* (17%), *Kocuria* (12%) and *Staphylococcus* (5%). The genus *Bacillus* showed the strongest capacity of the biofilm formation. The foodborne bacteria exhibited a wide range of susceptibility to disinfectants, such as sodium hypochlorite, hydrogen peroxide, benzalkonium chloride, lactic acid and citric acid. However, the susceptibilities changed after biofilm formation in a strain-dependent manner, and the relative resistance levels changed among the isolates. Overall, this study will be a great resource for selecting and using disinfectants in foodservice facility hygienic practices.

1. Introduction

Food hygiene control and safety management is essential and critical for protecting the public from foodborne illnesses. The HACCP program and other adequate programs have been globally accepted for systemically preventing and controlling food safety hazards (Grönholm, Wirtanen, Ahlgren, & Sjöberg, 1999; Notermans & Mead, 1996; Notermans, Gallhoff, Zwietering, & Mead, 1995). Despite efforts to minimize contamination, massive foodborne outbreaks occur throughout the world (Much, Pichler, Kasper, & Allerberger, 2009; Naimi et al., 2003). Such outbreaks can be due to inadequate use of cleaning and sanitizing practices as well as naturally occurring biofilms (Yang, Kendall, Medeiros, & Sofos, 2009).

Biofilm is a bacterial community that adheres to biotic or abiotic surfaces and produces exopolymeric substances to protect from the environmental stress including antibiotic treatment or disinfection (Rayner, Veeh, & Flood, 2004). Previous studies have confirmed the ubiquity of biofilm in household surfaces and suggest the importance of biofilm in household hygiene (Rayner et al., 2004), in various food processing plants (Srey, Jahid, & Ha, 2013) and even in fresh produce and meat (Jessen & Lammert, 2003). In phyllosphere microbiology, 30–80% of the total bacterial population in a plant is related to biofilm formation (Lindow & Brandl, 2003). Costerton et al. (1987) claimed that approximately 99% of all bacteria in nature exist as a biofilm and that most microorganisms can survive and contaminate biotic or abiotic surfaces. Further, 80% of bacterial infections in the USA are associated with biofilm (Costerton et al., 1987; Srey et al., 2013).

Bacterial biofilms in food ingredients or food handlers transfer bacteria to food contact surfaces, to other food handlers or the food processing environment, and contaminate the final food product (Pérez-Rodríguez, Valero, Carrasco, García, & Zurera, 2008). Because sessile bacteria can survive and persist in the environment, adequate and appropriate disinfectant use is necessary to minimize

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the risk of foodborne pathogen contamination (Gil, Selma, López-Gálvez, & Allende, 2009). Naturally acquired bacterial biofilms have been the main target of intervention technologies because they protect pathogenic bacteria and the sessile bacterial cells that were embedded in the biofilm were 10–1000 times more resistant to antibiotic treatment (Davies, 2003; Sanchez-Vizuete, Orgaz, Aymerich, Le Coq, & Briandet, 2015). Therefore, an increasing resistance to disinfectants is a critical issue in food safety (Meira, de Medeiros Barbosa, Alves Aguiar Athayde, de Siqueira-Júnior, & de Souza, 2012).

Until now, disinfectant susceptibility in bacterial biofilms was individually evaluated using select disinfectants. We isolated bacteria from a foodservice facility and evaluated their capacity for biofilm formation and disinfectant resistance/susceptibility compared with foodborne pathogens from our culture collection. We also compared the relationship between sessile and planktonic bacteria in response to the same disinfectants and compared the correlations among the bacterial strains and different disinfectants. This evaluation will provide a valid result through comparing bacteria under different antibiotic and surface-attached conditions.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Seventy foodborne pathogens including *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella* and *Staphylococcus aureus* were obtained from the culture collection of the Food Safety Research Team in the Korea Food Research Institute (Table S1). Bacteria was inoculated in Tryptic Soy Broth (Becton Dickinson Co, Franklin Lakes, New Jersey, USA) and incubated at 37 °C for 16–18 h in shaking incubator. The cultures were maintained in 15% glycerol at −80 °C until use.

2.2. Isolating microorganisms on food-contact surfaces

A cafeteria kitchen in a foodservice facility with an average daily attendance (ADA) greater than 250 people was used to isolate bacteria. Twenty-three different food-contact surfaces, including cooking utensils, kitchen appliances and the cooking area, were used to isolate background microflora, and the sampling was performed after daily cooking and cleaning (Fig. S1). Each surface (a total of 100 cm^2 area using one 10 × 10 cm or four 5 × 5 cm stainless steel frames depending on surface conditions) was swabbed 10 times in vertical, horizontal and diagonal directions, respectively, using Pipette swab plus (3 M, Minnesota, USA) in 10 mL of buffered peptone water (BPW). The researchers wore sterile latex gloves during collection to minimize cross-contamination from hands. Each sample was vortexed for 1 min to release the bacteria attached to the swab and plated onto plate count agar (PCA, BD) with the appropriate dilution and incubated at 37 °C for up to 48 h. The collected samples were further processed within two hours for quantification and isolation.

2.3. Isolation and identification

Bacteria were isolated from the colonies grown on PCA. Colony selection was performed based on the morphology of each colony and the food-contact surface area and 178 strains were isolated. Each colony was re-streaked on PCA and inoculated in tryptic soy broth (TSB, Merck & Co., Kenilworth, New Jersey, USA) at 37 °C for overnight. The overnight culture was mixed with 15% glycerol and kept at −70 °C until next use. In order to identify the isolates, the overnight culture was centrifuged at 9400 × *g* for 5 min and the pellet was used for the genomic DNA extraction using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and the DNA concentration was measured using NanoVue (GE Healthcare, Buckinghamshire, UK). The 16S rRNA gene of each DNA was amplified using 27 F (5′-GAGTTTGATCMTGGCTCAG-3′), and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers (Macrogen, Seoul, Korea). DNA templates, 10 pmol/μL of primers and PCR-grade water were prepared with Takara Ex Taq version 2.0 (Takara, Kusatsu, Japan) to a total of 25 μL for each reaction. The amplification was performed by following program; initial denaturation at 95 °C for 5 min, then 30 cycles of 1) denaturation at 94 °C for 1 min, 2) annealing at 55 °C for 30 s and 3) extension at 72 °C for 1 min, and the final elongation step at 72 °C for 7 min. The amplified PCR product was loaded on 0.8% agarose gel in TAE (40 mM Tris·HCl, 40 mM acetate, 1.0 mM EDTA) buffer stained with Staining STAR (DyneBio, Seongnam, Korea) and the amplification was confirmed with ultraviolet transilluminator using Gel DocTM EZ Imager (Bio-Rad, Richmond, CA, USA). The amplicon was further purified by QIAquick PCR purification kit (Qiagen). The sequencing was performed by Macrogen with 512F (5′–CCAGCAGCCGCGGTAAT-3′) for the downstream of 16S rRNA gene and 512R (5′-ATTACCGCGGCTGCTGG-3′) primers for the upstream of 16S rRNA gene. The sequencing results were analyzed with the EzTaxon (Kim et al., 2012) on the basis of 16 S rRNA sequence data (Table S2).

2.4. Biofilm formation on a microtiter plate-crystal violet assay

All stains from culture collection and kitchen isolates were inoculated in TSB and incubated for 16–18 h at 37 °C. The overnight culture was diluted to approximately 10^7 CFU/mL in TSB, and 200 μL of the culture was inoculated in a 96-well plate at 37 °C for 48 h to facilitate biofilm formation on the microtiter plate. After incubation, the culture medium was carefully removed and washed with PBS (phosphate-buffered saline, pH 7.0) once. After washing the microtiter plate with biofilm-forming cells, 200 μL of a 1% crystal violet (CV) solution (bioWORLD, Dublin, Ohio, USA) was added, and the microtiter plate was incubated for 30 min at room temperature. After washing with PBS 3 times, absolute ethanol 200 μL was added and incubated for 15 min at RT to destain the CV. From the destaining solution, 100 μL was transferred to a new 96-well plate, and the absorbance was measured at 595 nm using Infinite® 200 PRO NanoQuant (Tecan Group Ltd., Männedorf, Switzerland).

2.5. Disinfectant assay using non-biofilm bacteria

Twenty-eight strains of kitchen isolates and 18 strains from culture collection were selected for the following experiment based on the CV assay that resulted in strong biofilm forming ability (over the absorbance of 0.7 for the isolates from this study and 0.3 from the culture collection). The bactericidal activity of disinfectants was determined using the agar well diffusion method with Mueller Hinton II agar (BD). An overnight culture of the isolate was streaked using a cotton swab to prepare a lawn plate. A well was generated in the middle of each plate using a sterilized 8 mm cork borer (K-ACE, Jongro-gu, Seoul, Korea) and inoculated with 100 μL of a select disinfectant. The disinfectant concentrations were as follows: 2000 mg/L NaClO (Junsei, Tokyo, Japan), 500 mg/L benzalkonium chloride (BAC, Kukbo Science, Cheongju, Korea), 2000 mg/L H_2O_2 (Daejung, Siheung, Korea), 10% lactic acid (LA, Kanto, Tokyo, Japan), and 10% citric acid (CA, Junsei). The plates were then incubated at 37 °C for 18–24 h. The inhibition activity was measured based on the cleared zone.

2.6. Disinfectant assay using biofilm-forming bacteria

The bactericidal activity to disinfectants after biofilm formation