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*Full Length Research Paper*

# Assessment of antibiotic susceptibilities, genotypic characteristics and biofilm-forming abilities of *Staphylococcus aureus* and *Salmonella* Typhimurium

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**This study was designed to evaluate the antibiotic susceptibilities, genotypic characteristics and biofilm formation abilities of antibiotic-sensitive *Staphylococcus aureus* KACC 13236 ($SA^S$), multiple antibiotic-resistant *S. aureus* CCARM 3080 ($SA^R$), antibiotic-sensitive *Salmonella* Typhimurium KCCM 40253 ($ST^S$) and multiple antibiotic-resistant *Salmonella* Typhimurium CCARM 8009 ($ST^R$) cultured in various levels of pHs (4.0, 5.5 and 7.3) and temperatures (4, 20 and 37°C). The $SA^S$ biofilm cells were highly resistant to ampicillin, cephalothin, oxacillin and piperacillin ($MIC_{90}$ >256 µg/ml) compared with the $SA^S$ planktonic cells. The $ST^R$ strain was less susceptible to cephalothin, oxacillin and piperacillin than the $ST^S$. The $SA^R$ strain was positive for the staphylococcal enterotoxin genes (*sec*, *seg*, *sei*, *sel*, *sem*, *sen* and *seo*). The highest biofilm formation index (BFI) was 2.07 for the $SA^R$ biofilm cells cultured at 37°C and pH 5.5. The antibiotic-resistant pathogens showed multiple antibiotic resistance, genotypic heterogeneity and cross-protective responses to low pH in the form of biofilms. This study provides useful information for the comparison of antibiotic resistance patterns and biofilm formation abilities between antibiotic-sensitive and resistant pathogens in planktonic and biofilm cells.**

**Key words:** Antibiotic resistance, *Staphylococcus aureus*, *Salmonella* Typhimurium, biofilm formation, cross-protection.

## INTRODUCTION

Since 1929 when the first antibiotic, penicillin, was discovered, new classes of antibiotics have been developed and increasingly used to treat infectious diseases in human and promote growth in food-producing animals (Chander et al., 2007). The overuse and misuse of antibiotics, however, has led to a significant increase in the antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP), vancomycin-resistant *Enterococcus* spp. (VRE) and multidrug-resistant *Salmonella* Typhimurium (MRST) (Mason et al., 1992; Harwood et al., 2001; Kondoh et al., 2002; McLaughlin et al., 2006; Holmes and Jorgensen, 2008; Dahshan et al., 2010). Therefore, the emergence of antibiotic-resistant pathogens has become a serious worldwide concern because their infections reported as nosocomial outbreaks and sporadic infections are not limited to particular countries (Kotilainen et al., 2003). Moreover, the antibiotic-resistant pathogens can exist in the form of biofilms, leading to cross-resistances to other environmental stresses (Gilbert et al., 2002; Burmolle et

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**Abbreviations: $SA^S$,** *Staphylococcus aureus* KACC 13236; **$SA^R$,** multiple antibiotic-resistant *S. aureus* CCARM 3080; **$ST^S$,** antibiotic-sensitive *Salmonella typhimurium* KCCM 40253; **$ST^R$,** multiple antibiotic-resistant *Salmonella typhimurium* CCARM 8009.

al., 2006; McMahon et al., 2007; Telang et al., 2010).

In natural habitats, biofilm formation is an important survival strategy for bacteria that can be embedded within a self-produced extracellular polymeric matrix of polysaccharides, proteins, lipids and nucleic acids (Donlan and Costerton, 2002; Flemming and Wingender, 2010). Bacterial biofilms have received continued attention in the food-processing industry due to their increased resistance towards desiccation, heat, acid, preservatives and antibiotics (Chmielewski and Frank, 2003; Trachoo, 2007; Shi and Zhu, 2009; Van Houdt and Michiels, 2010). Over the last two decades, there have been many studies showing the ability of bacteria to form biofilms on biotic and abiotic surfaces (Hood and Zottola, 1997; Reid, 1999; Rode et al., 2007; Dongari-Bagtzoglou, 2008). However, relatively few studies have been focused on the biofilm-forming abilities of multiple antibiotic-resistant pathogens. Therefore, the objectives of this study were to evaluate the antibiotic susceptibilities and genotypic characteristics of planktonic and biofilm-associated pathogens and also to assess the biofilm-forming abilities of multiple antibiotic-resistant *S. aureus* and *S.* Typhimurium exposed to different pH and temperature conditions.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

Strains of *S. aureus* KACC 13236 and *Salmonella enterica* serovar Typhimurium KCCM 40253 were provided by the Korean Agricultural Culture Collection (KACC, Suwon, Korea) and the Korean Culture Center of Microorganism (KCCM, Seoul, Korea), respectively. Strains of *S. aureus* CCARM 3080 and *S. enterica* serovar Typhimurium CCARM 8009 were purchased from the Culture Collection of Antibiotic Resistant Microbes (CCARM, Seoul, Korea). All strains were cultivated in trypticase soy broth (TSB, BD, Becton, Dickinson and Co., Sparks, MD, USA) at 37°C for 20 h. After cultivation, cultures were centrifuged at 3,000 × *g* for 20 min at 4°C. The harvested cells were washed twice with 0.1% sterile buffered peptone water (BPW).

### Preparation of antibiotic stock solutions

Eight antibiotics, ampicillin, aztreonam, cefotaxime, cefoxitin, ceftazidime, cephalothin, oxacillin and piperacillin, were purchased from the Sigma Chemical Co. (St Louis, MO, USA). All antibiotic stock solutions were prepared at the concentrations of 256 and 51.2 µg/ml (aztreonam) in sterile distilled water and stored at -20°C prior to use.

### Preparation of biofilm cells

The diluted *S. aureus* KACC13236, *S*. Typhimurium KCCM 40253, *S. aureus* CCARM 3080 and *S*. Typhimurium CCARM 8009 cells (0.1 ml each) were transferred into 96-well flat-bottomed poly-styrene microtiter plates (BD Falcon, SanJose, CA, USA) at approximately $10^4$ CFU/ml in TSB and incubated at 37°C for 48 h under static conditions. Each well was rinsed three times with 0.1% sterile BPW to remove loosely attached cells. The biofilm cells were used for antibiotic susceptibility test.

### Antibiotic susceptibility assay

A broth microdilution method was used to determine the antibiotic susceptibility of planktonic and biofilm cells according to the Clinical and Laboratory Standards Institute (CLSI) procedure (CLSI, 2009).

The antibiotic stock solutions were serially diluted (1:2) from 51.2 to 0.05 µg/ml for aztreonam and 256 to 0.25 µg/ml for other antibiotics with TSB in 96-well plates. The inoculums of planktonic and biofilm indicator strains were approximately $10^6$ CFU/ml in each well. All inoculated plates were incubated for 20 h at 37°C. The total viable counts of planktonic and biofilm cells were determined using the pour plate method. The planktonic cell suspensions in each well of the 96-well plates were collected and serially (1:10) diluted with 0.1% BPW. For biofilm enumeration, the cotton swabs were cut into 2 pieces, moistened with 0.1% BPW and sterilized. The biofilm layers were collected by scraping the biofilm area with sterile cotton swabs. The swabs were immersed in 1 ml of 0.1% BPW and vigorously mixed to release the biofilm cells. The dilutions (0.1 ml each) of planktonic and biofilm cells were plated in duplicate on TSA. The agar plates were incubated at 37°C for 48 h. MICs (minimum inhibitory concentrations) were determined at the concentrations at which the initial cell numbers were reduced by 90%. The antibiotic dose-response curves based on the growth of planktonic and biofilm cells were analyzed to estimate the MICs using Nonlinear curve fitting function of Microcal Origin® 7.5 (Microcal Software Inc., Northampton, MA). The strains tested were defined as sensitive (S), intermediate (I) and resistant (R) bacteria based on the MIC values. The sensitive and resistant MIC breakpoints were less than 8 µg/ml and more than 32 µg/ml, respectively, for ampicillin, aztreonam, cefotaxime, cefoxitin, ceftazidime, cephalothin and oxacillin. The MIC breakpoints of piperacillin were less than 16 µg/ml (S) and more than 32 µg/ml (R) (Ayyagari and Gupta, 2009).

### RNA extraction

Each culture (0.5 ml) was mixed with 1 ml of RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) to stabilize RNA. The mixture was centrifuged at 5,000 × *g* for 10 min and used for RNA extraction according to the RNeasy mini protocol (Qiagen). The collected cells were homogenized with a buffer containing guanidine isothiocyanate (GITC) to disrupt the cells and incubated in a buffer containing lysozyme to lyse the cell wall. The lysates were mixed with ethanol to adjust binding conditions and loaded to an RNeasy mini column to isolate total RNA.

### Genotyping by reverse transcription-PCR

The synthesis of cDNA was performed according to the QuantiTect reverse transcription procedure (Qiagen). The RNA sample was mixed with a master mixture containing Quantiscript Reverse Transcriptase, Quantiscript RT buffer and RT primer mix. The reaction mixture was incubated at 42°C for 15 min and then subsequently, incubated at 95°C for 3 min to inactivate the quantiscript reverse transcriptase. The oligonucleotide primers (Tables 1 and 2) were synthesized by IDT (Integrated DNA Technologies Inc., coralville, IA, USA). The PCR mixture (20 µl) containing 10 µl of 2X QuantiTect SYBR Green PCR Master, each primer (60 pmol), cDNA sample (2 ng and water (6.8 µl) was amplified using an iCycler iQ™ system (Bio-Rad Laboratories, Hemel Hempstead, UK). The PCR mixture was denatured initially for 15 min at 95°C, followed by 45 cycles of 94°C for 15 s, 59°C for 20 s and 72°C for 15 s. The melt-curve analysis was carried out

**Table 1.** Primer sequences used in RT- PCR analysis for *S. aureus.*

| Gene | Molecular function | Primer sequence* | Size (bp) | $T_m$[†] (S/R[‡], °C) |
|---|---|---|---|---|
| *sea* | Enterotoxin A | F: CCTTTGGAAACGGTTAAAAC<br>R: CTCTGMACCTTYCCATCAAA | 128 | ND<br>ND |
| *seb* | Enterotoxin B | F: GGGTATTTGAAGATGGTAAAAATT<br>R: AGGCGAGTTGTTAAATTCATAGAGTT | 140 | ND<br>ND |
| *sec* | Enterotoxin C | F: TGTACTTRTAAGAGTTTATGAAAATA<br>R: TCCTAGCTTTTATGTCTAGTTCTTGAG | 104 | ND<br>72.43 |
| *sed* | Enterotoxin D | F: TCAATTTGTGGATAAATGGTGTAC<br>R: TTTCCTCCGAGAGTATCATTAT | 154 | ND<br>ND |
| *see* | Enterotoxin E | F: CCTATAGATAAAGTTAAAACAAGC<br>R: ACCGCCAAAGCTGTCTGAG | 116 | ND<br>ND |
| *seg* | Enterotoxin G | F: TTACAAAGCAAGACACTGGCTCA<br>R: TCCAGATTCAAAYGCAGAACMAT | 73 | ND<br>74.31 |
| *seh* | Enterotoxin H | F: TGAATGTCTATATGGAGGTACAAC<br>R: CTACCCAAACATTAGCACCAA | 80 | ND<br>ND |
| *sei* | Enterotoxin I | F: GGTAYCAATGATTTGATCTCAGAAT<br>R: GTATTGTCCTGATAAAGTGGCC | 147 | ND<br>73.68 |
| *sej* | Enterotoxin J | F: CTGCATGAAAACAATCAACTTTATG<br>R: GAACAACAGTTCTGATGCTATC | 79 | ND<br>ND |
| *sek* | Enterotoxin K | F: GTCACAGCTACTAACGAATATC<br>R: TAGTGCCGTTATGTCCATAAATG | 193 | ND<br>ND |
| *sel* | Enterotoxin L | F: TAGATTCGCCAAGAATAATACC<br>R: CTTTACCAGTATCATTGTGTCC | 176 | ND<br>73.68 |
| *sem* | Enterotoxin M | F: TCATATCGCAACCGCTGATGATG<br>R: TCAGCWGTTACTGTCGAATTAT | 150 | ND<br>75.08 |
| *sen* | Enterotoxin N | F: GATGAAGAGARAGTTATAGGCGT<br>R: ATGTTACCGGTATCTTTATTGTAT | 167 | ND<br>72.93 |
| *seo* | Enterotoxin O | F: GTGTAAGAAGTCAAGTGTAGAC<br>R: CAGCAGATWTTCCATCTAACC | 163 | ND<br>71.05 |
| *sep* | Enterotoxin P | F: GGAGCTAGACCTTCAGTCAAGA<br>R: ACCAGAAGAAGGGTGAAACTCA | 115 | ND<br>ND |
| *seq* | Enterotoxin Q | F: GGAATTACGTTGGCGAATCAA<br>R: TGATATCCATATTCCTGACC | 221 | ND<br>ND |
| *ser* | Enterotoxin R | F: TCCTATTCCTTATTCAGAATACA<br>R: GGGTATTCCAAACACATCTAAC | 102 | ND<br>ND |

immediately after amplification protocol with 0.4°C increments per 10 s for 85 cycles from 65 to 97°C. The PCR products were visualized and analyzed using the iQ5 PCR detection system (Bio-Rad Laboratories).

### Estimation of biofilm formation ability

Antibiotic-sensitive and resistant *S. aureus* and *S.* Typhimurium cells were cultured in TSB containing one-half MICs of antibiotics at different temperatures (4, 20 and 37°C) and pH 4.0, 5.5 and 7.3 adjusted by lactic acid. After 48 h incubation, the absorbance of planktonic cells was measured at 600 nm ($OD_{Planktonic}$). The total amount of biofilm cells was quantitatively measured by the crystal violet (CV) method. After removing the planktonic cells, the washed wells were air dried at 55°C for 1 h. The dried biofilm cells were stained with 1% CV solution at 37°C for 30 min, washed twice with sterile distilled water and then air-dried at 55°C for 1 h. The stained biofilm cells were destained with 95% ethanol and measured at 570 nm ($CV_{Biofilm}$). The negative control ($CV_{Control}$) was used to reduce the background staining from the CV-stained biofilm cells. The biofilm formation ability was expressed by biofilm formation index ($BFI=[CV_{Biofilm} - CV_{Control}]/OD_{Planktonic}$) (Niu and Gilbert, 2004).

### Statistical analysis

Data were analyzed using the statistical analysis system software (SAS). The general linear model (GLM) and least significant difference (LSD) procedures were used to determine significant mean differences among strains and culture conditions at $p < 0.05$.

**Table 2.** Primer sequences used in RT-PCR analysis for *S.* Typhimurium.

| Gene | Molecular function | Primer name and sequence* | Size (bp) | $T_m$† (S/R‡, ℃) |
|---|---|---|---|---|
| *acrA* | Multidrug efflux system | F: AAAACGGCAAAGCGAAGGT<br>R: GTACCGGACTGCGGGAATT | 64 | 80.02<br>80.02 |
| *acrB* | Multidrug efflux system | F: TGAAAAAAATGGAACCGTTCTTC<br>R: CGAACGGCGTGGTGTCA | 69 | 77.91<br>78.16 |
| *tolC* | Multidrug efflux system | F: GCCCGTGCGCAATATGAT<br>R: CCGCGTTATCCAGGTTGTTG | 67 | 80.14<br>80.22 |
| *tem-1* | β-lactamase | F: ATGAGTATTCAACATTTCCGTG<br>R: TTACCAATGCTTAATCAGTGAG | 861 | ND<br>ND |
| *ctx-m* | β-lactamase | F: TTTGCGATGTGCAGTACCAGTAA<br>R: CGATATCGTTGGTGGTGCCATA | 543 | ND<br>ND |
| *shv-1* | β-lactamase | F: ATGCGTTATATTCGCCTGTG<br>R: GTTAGCGTTGCCAGTGCTCG | 865 | ND<br>ND |
| *ompC* | Outer membrane protein C | F: TCGCAGCCTGCTGAACCAGAAC<br>R: ACGGGTTGCGTTATAGGTCTGAG | 244 | ND<br>ND |
| *ompD* | Outer membrane protein D | F: GCAACCGTACTGAAAGCCAGGG<br>R: GCCAAAGAAGTCAGTGTTACGGT | 239 | 83.94<br>83.95 |
| *ompF* | Outer membrane protein F | F: CGGAATTTATTGACGGCAGT<br>R: GAGATAAAAAAACAGGACCG | 1212 | ND<br>ND |
| *hilA* | Invasion gene activator | F: TATCGCAGTATGCGCCCTT<br>R: TCGTAATGGTCACCGGCAG | 50 | 80.17<br>80.68 |
| *fimA* | Major fimbrial subunit | F: TTGCGAGTCTGATGTTTGTCG<br>R: CACGCTCACCGGAGTAGGAT | 62 | 81.81<br>81.92 |
| *lpfE* | Fimbrial protein | F: GGTCAGTCGGGTCCGGA<br>R: GATTGCGCGTATGCCACA | 61 | 80.62<br>80.56 |
| *invA* | Invasion protein | F: ACAGTGCTCGTTTACGACCTGAAT<br>R: AGACGACTGGTACTGATCGATAAT | 454 | 79.40<br>79.41 |
| *stn* | Salmonella enterotoxin | F: GCCATGCTGTTCGATGAT<br>R: GTTACCGATAGCGGGAAAGG | 467 | 82.52<br>82.52 |

*F, forward; R, reverse; †ND, not detected; ‡S, antibiotic-sensitive strain; R, antibiotic-resistant strain.

**Table 3.** Minimum inhibitory concentrations ($MIC_{90}$, μg/ml)* of selected antibiotics against antibiotic-sensitive *S. aureus* ($SA^S$) and antibiotic-resistant *S. aureus* ($SA^R$).

| Antibiotic | $SA^S$ | | $SA^R$ | |
|---|---|---|---|---|
| | Planktonic | Biofilm | Planktonic | Biofilm |
| Ampicillin | 0.05 (S) | >256 (R) | 14.56 (I) | >256 (R) |
| Aztreonam | 35.92 (R) | >51.2 (R) | >51.2 (R) | >51.2 (R) |
| Cefotaxime | 0.14 (S) | 0.57 (S) | >256 (R) | >256 (R) |
| Cefoxitin | 0.46 (S) | 141.95 (R) | >256 (R) | >256 (R) |
| Ceftazidime | 3.51 (S) | 82.46 (R) | >256 (R) | >256 (R) |
| Cephalothin | 0.05 (S) | >256 (R) | 75.80 (R) | 69.57 (R) |
| Oxacillin | 0.05 (S) | >256 (R) | >256 (R) | >256 (R) |
| Piperacillin | 0.11 (S) | >256 (R) | 88.77 (R) | 150.95 (R) |

* S, Sensitive; I, intermediate; R, resistant.

**Table 4.** Minimum inhibitory concentrations ($MIC_{90}$, µg/ml)* of selected antibiotics against antibiotic-sensitive *S.* Typhimurium ($ST^S$) and antibiotic-resistant *S.* Typhimurium ($ST^R$).

| Antibiotic | $ST^S$ | | $ST^R$ | |
|---|---|---|---|---|
| | Planktonic | Biofilm | Planktonic | Biofilm |
| Ampicillin | 0.91 (S) | >256 (R) | 1.85 (S) | >256 (R) |
| Aztreonam | 0.04 (S) | 0.39 (S) | 0.04 (S) | 0.53 (S) |
| Cefotaxime | 0.05 (S) | 0.06 (S) | 0.25 (S) | 0.61 (S) |
| Cefoxitin | 3.58 (S) | 2.03 (S) | 3.09 (S) | 1.94 (S) |
| Ceftazidime | 0.44 (S) | 0.19 (S) | 0.54 (S) | 35.44 (R) |
| Cephalothin | 4.73 (S) | 9.33 (I) | 23.38 (I) | 152.38 (R) |
| Oxacillin | >256 (R) | >256 (R) | >256 (R) | >256 (R) |
| Piperacillin | 2.39 (S) | 1.98 (S) | >256 (R) | >256 (R) |

*S, Sensitive; I, intermediate; R, resistant.

## RESULTS

### Antibiotic susceptibilities of *S.* aureus planktonic and biofilm cells

The antibiotic susceptibility of biofilm-associated *S. aureus* cells were compared with the susceptibility of planktonic cell as shown in Table 3. For *S. aureus* KACC 13236, the biofilm cells were highly resistant to most antibiotics, except for cefotaxime, when compared with the planktonic cells. The most significant increases in resistance were observed in biofilm cells to ampicillin, cephalothin, oxacillin and piperacillin, showing the $MIC_{90}$ values of more than 256 µg/ml (Table 3). The resistance patterns of *S. aureus* CCARM 3080 strain were not significantly different between planktonic and biofilm cells,showing highly resistant towards all antibiotics used. The *S. aureus* CCARM 3080 was highly resistant to antibiotics in both planktonic and biofilm cells when compared with the *S. aureus* KACC 13236.

### Antibiotic susceptibilities of *S.* Typhimurium planktonic and biofilm cells

The antibiotic susceptibility profiles of planktonic and biofilm-associated *S.* Typhimurium cells are shown in Table 4. No significant susceptibility patterns between *S.* Typhimurium KCCM 40253 planktonic and biofilm cells were observed for all antibiotics with the exception of ampicillin. The $MIC_{90}$ values of ampicillin against the *S.* Typhimurium KCCM 40253 planktonic and biofilm cells were 0.91 and >256 µg/ml, respectively. The *S.* Typhimurium KCCM 40253 and CCARM 8009 in both planktonic and biofilm cultures showed the highest level of resistance to oxacillin, showing the $MIC_{90}$ values of more than 256 µg/ml. The $MIC_{90}$ value of ampicillin against the *S.* Typhimurium KCCM 40253 biofilm cells significantly increased from 0.91 (*S.* Typhimurium KCCM 40253 planktonic cells) to >256 µg/ml, indicating a decrease in susceptibility after biofilm formation. The $MIC_{90}$ values of ampicillin, ceftazidime and cephalothin against the *S.* Typhimurium CCARM 8009 biofilm cells significantly increased up to >256, 35.44 and152.38 µg/ml, respectively, when compared with those (1.85, 0.54 and 23.38 µg/ml) of *S.* Typhimurium KCCM 40253 planktonic cells.

### Genotypic characteristics of antibiotic-sensitive and resistant pathogens in planktonic and biofilm cultures

According to the antibiotic susceptibilities, *S. aureus* KACC13236, *S. aureus* CCARM 3080, *S.* Typhimurium KCCM 40253 and *S.* Typhimurium CCARM 8009 were assigned to relatively antibiotic-sensitive *S. aureus* ($SA^S$), multiple antibiotic-resistant *S. aureus* ($SA^R$), antibiotic-sensitive *S.* Typhimurium ($ST^S$) and multiple antibiotic-resistant *S.* Typhimurium ($ST^R$), respectively. The genotypic characteristics of $SA^S$, $SA^R$, $ST^S$ and $ST^R$ strains were evaluated by RT-PCR assay as shown in Table 5. The PCR results of $SA^S$ and $SA^R$ strains were positive for most reference genes (*ftsZ*, *gap*, *gyrB*, *hu*, *mdeA*, *norB*, *norC*, *pta*, *recA*, *rplD*, *rpoB* and *sodA*), with the exception of *tpi* gene. The staphylococcal enterotoxin (SE) genes (*sec*, *seg*, *sei*, *sel*, *sem*, *sen* and *seo*) were not detected in the $SA^S$, while the positive results for these SE genes were observed in the $SA^R$. In both $SA^S$ and $SA^R$ strains, *sea*, *seb*, *sed*, *see*, *seh*, *sej*, *sek*, *sep*, *seq*, *ser*, *seu*, *norA*, *mepA* and *blaZ* were not detected. There were no significant differences in genotype distribution between $ST^S$ and $ST^R$. The PCR products were positive for *acrA*, *acrB*, *fimA*, *hilA*, *invA*, *lpfE*, *ompD*, *stn* and *tolC* genes (Table 5), while *tem-1*, *ctx-m*, *shv-1*, *ompC* and *ompF* genes were not detected in both $ST^S$

**Table 5.** Distribution of reference and virulence genes among antibiotic-sensitive *S. aureus* ($SA^S$), antibiotic-resistant *S. aureus* ($SA^R$), antibiotic-sensitive *S.* Typhimurium ($ST^S$) and antibiotic-resistant *S.* Typhimurium ($ST^R$).

| Gene | PCR result* | | Gene | PCR result* | |
|---|---|---|---|---|---|
| | $SA^S$ | $SA^R$ | | $ST^S$ | $ST^R$ |
| *ftsZ* | + | + | *acrA* | + | + |
| *gap* | + | + | *acrB* | + | + |
| *gyrB* | + | + | *fimA* | + | + |
| *hu* | + | + | *hilA* | + | + |
| *mdeA* | + | + | *invA* | + | + |
| *norB* | + | + | *lpfE* | + | + |
| *norC* | + | + | *ompD* | + | + |
| *pta* | + | + | *stn* | + | + |
| *recA* | + | + | *tolC* | + | + |
| *rplD* | + | + | | | |
| *rpoB* | + | + | | | |
| *sec* | – | + | | | |
| *seg* | – | + | | | |
| *sei* | – | + | | | |
| *sel* | – | + | | | |
| *sem* | – | + | | | |
| *sen* | – | + | | | |
| *seo* | – | + | | | |
| *sodA* | + | + | | | |
| *tpi* | – | + | | | |

*+, Positive in specific PCR; –, negative in specific PCR.

and $ST^R$ strains.

### Biofilm-forming abilities of antibiotic-sensitive and resistant pathogens under different pH and temperature conditions

The abilities of $SA^S$, $SA^R$, $ST^S$ and $ST^R$ strains to form biofilms were evaluated in one-half MICs of antibiotics at different levels of pH (4.0, 5.5 or 7.3) and temperature (4, 20 or 37°C) (Figure 1). For all strains tested in this study, the biofilm cells were not detected under culture conditions (4°C +pH 7.3 and 37°C+pH 4.0). The highest biofilm formation indices (BFIs) of $SA^S$ and $SA^R$ were 1.59 and 2.07, respectively, at the culture condition of 37°C and pH 5.5, followed by 0.55 and 0.47 at the culture condition of 20°C and pH 7.3 (Figure 1a). At the culture condition of 37°C and pH 5.5 condition, the $SA^R$ showed significantly higher biofilm formation ability than the $SA^S$ ($p < 0.05$). Similar to the $SA^S$ and $SA^R$, the highest BFIs of $ST^S$ and $ST^R$ were 0.87 and 1.38, respectively, at the culture condition at 37°C C and pH 5.5 (Figure 1b). The $ST^R$ had the highest BFI at the culture condition at 37°C and pH 5.5 ($p < 0.05$). No significant differences in biofilm formation abilities were observed at other culture conditions ($p > 0.05$).

## DISCUSSION

This study demonstrates (1) the phenotypic and genotypic traits of antibiotic-resistant pathogens in planktonic and biofilm cultures and (2) the biofilm-forming abilities of multiple antibiotic-resistant pathogens grown at different pH and temperature conditions when compared with antibiotic-sensitive pathogens. Since the antibiotic resistance has emerged as a serious public health problem, understanding the physiogenetic characteristics of antibiotic-resistant pathogens is essential for preventing the development of multi-drug resistant pathogens under different environmental conditions and for developing a new type of antibiotic agents.

The $SA^S$ planktonic cells were more susceptible to ampicillin, cefotaxime, cefoxitin, ceftazidime, cephalothin, oxacillin and piperacillin than the $SA^S$ biofilm cells (Table 3). This observation is in good agreement with previous reports that the increased antibiotic resistance was observed in biofilm cells, which may be attributed to biofilm properties such as slow metabolic activity, low membrane permeability, high frequency of persisters and adaptive responses (Amorena et al., 1999; Costerton et al., 1999; Stewart, 2002). The $SA^R$ was more resistant towards all antibiotics than the $SA^S$. This result suggests that antibiotic-resistant strains are likely to acquire an

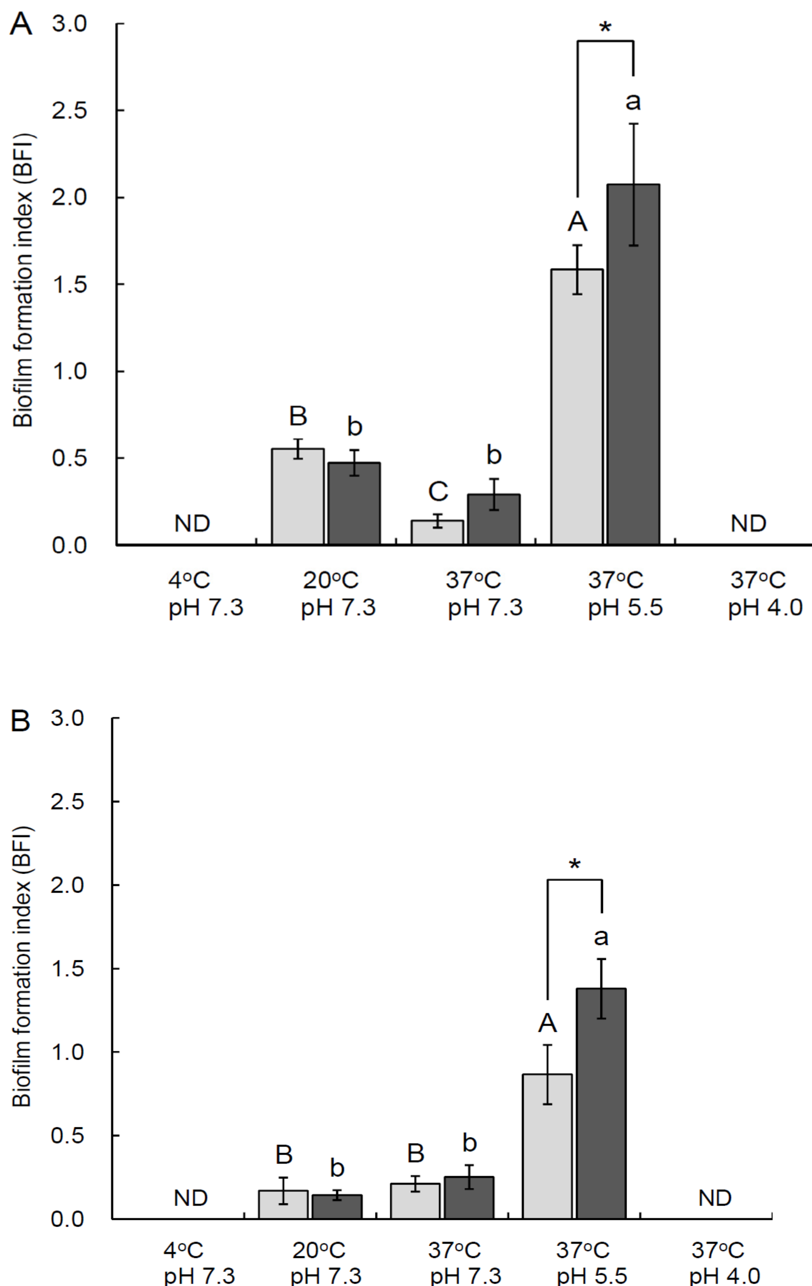


**Figure 1.** Specific biofilm formation indices of (**A**) antibiotic-sensitive *S. aureus* ($SA^S$, ■) and antibiotic-resistant *S. aureus* ($SA^R$, ■) and (B) antibiotic-sensitive *S.* Typhimurium ($ST^S$, ■) and antibiotic-resistant *S.* Typhimurium ($ST^R$, ■) cultured at different pH and temperature conditions for 48 h. ND and * indicate no detection of biofilm cells and significant difference at $p < 0.05$. Different letters (A-C and a-c) are significantly different within antibiotic-sensitive and antibiotic-resistant strains at $p < 0.05$. (n = 8).

increased resistant to antibiotics, leading to multidrug-resistant strains (Kwon et al., 2008; Petrelli et al., 2008). The antibiotic susceptibility patterns of *S.* Typhimurium were different from those observed in *S. aureus* (Table 4). The multidrug-resistance patterns were observed at low frequency in both $ST^S$ and $ST^R$.

Both $SA^S$ and $SA^R$ strains were positive for the genes, *ftsZ*, *pta*, *hu*, *gyrB*, *recA* and *rpoB*, which are well know reference genes in *S. aures* grown at various pHs and temperatures (Duquenne et al., 2010). The $SA^R$ was confirmed positive for the staphylococcal enterotoxin genes encoding SEC, SEG, SEI, SEL, SEM, SEN and SEO (Table 5). These extracellular staphylococcal enterotoxins (SEs) are the major causative agents of

staphylococcal food poisoning (SFP), bacteremias and endocarditis (Lowry, 1998; Derzelle et al., 2009). Theserotypes SEA to SEE are the most common enterotoxins responsible for SFP outbreaks. The expression of *sec* is regulated by SarA in *S. aureus* independent of *agr* operon. The gene *seg* coexists with *sei* within staphylococcal pathogenicity island (SaPI) (Becker et al., 2003). The production of SEs is mainly influenced by environmental factors such as temperature, water activity, pH, salt and oxygen. *S. aureus* exposed to different environmental stress conditions can induce cross-protection phenomenon (McMahon et al., 2007). No significant genotypic patterns were observed between $ST^S$ and $ST^R$ strains (Table 5). The $ST^S$ and $ST^R$ strains were positive for the genes encoding multidrug efflux proteins (*acrA*, *acrB* and *tolC*), outer membrane transportprotein (*ompD*), regulators of *Salmonella* pathogenicity island (SPI; *hilA*), invasion protein (*invA*), enterotoxin (*stn*) and fimbrial protein (*fimA* and *lpfE*) (Webber et al., 2009). Foodborne salmonellosis is caused by *S*. Typhimurium cells which invade the small intestine, colonize and then produce enterotoxins (Pfeifer et al., 1999). The genes *stn* and *invA* are mostly responsible for the production of enterotoxin and the penetration of the intestinal epithelium by *S*. Typhimurium (Chopra et al., 1994; Chopra et al., 1999; D'Souza et al., 2009).

No growth was observed when $SA^S$, $SA^R$, $ST^S$ and $ST^R$ strains were incubated at the culture conditions of 4℃+pH 7.3 (low temperature) and 37℃+pH 4.0 (lethal pH) (Figure 1). However, the highest BFIs were observed for $SA^R$ and $ST^R$ strains cultured at 37℃ and pH 5.5. This observation suggests that antibiotic resistance induced cross-protection against low pH and antibiotics (one-half MICs) during biofilm formation. At pH 5.5 and 37℃, the antibiotic-resistant strains ($SA^R$ and $ST^R$) formed stronger biofilms than the antibiotic-sensitive strains ($SA^S$ and $ST^S$). This result confirms previous reports that the biofilm formation ability was likely to be dependent on the antibiotic resistance profiles of bacterial strains (Kim and Wei, 2007; Kwon et al., 2008). In this study, the $SA^S$, $SA^R$, $ST^S$ and $ST^R$ planktonic cells were resistant to 0, 8 (ampicillin, aztreonam, cefotaxime, cefoxitin, ceftazidime, cephalothin, oxacillin and piperacillin), 1 (oxacillin) and 5 (ampicillin, ceftazidime, cephalothin, oxacillin and piperacillin) antibiotics, respectively. The biofilm formation ability of $SA^S$ strain was decreased at the culture condition of 37℃ and pH 7.3 compared with that at 20℃ and pH 7.3. This implies that the increased temperature could enhance the antibiotic activity, which might eventually contribute to the increased susceptibility of $SA^S$ to antibiotics (Russell, 2003).

In conclusion, the antibiotic-sensitive and resistant pathogens in planktonic and biofilm cultures exhibited significant differences in phenotypic and genotypic properties. The biofilms formed by antibiotic-resistant strains were more resistant to antibiotics than the planktonic cells, known as multidrug resistance. Different genotypic patterns were observed between antibiotic-sensitive and resistant strains. The antibiotic-resistant pathogens showed the highest biofilm-forming ability in low pH, known as cross-resistance. The biofilm formation by multidrug-resistant pathogens may increase the risk of severe infections related to food processing facilities and medical devices. This study would provide useful information on the epidemiologic study of antibiotic-resistant pathogen infections. Further study is needed to understand the virulence potential of antibiotic-resistant foodborne pathogenic biofilms under various environmental stress conditions.

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### REFERENCES

Amorena B, Gracia E, Monzon M, Leiva J, Oteiza C, Perez M, Alabart JL, Hernandez-Yago J (1999). Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed *in vitro*. J. Antimicrob. Chemother. 44: 43-55.

Ayyagari A, Gupta S (2009). Detection of antimicrobial resistance in common Gram-negative and Gram-positive bacteria encountered in infectious diseases-an update. ICMR Bull. 39: 1-20.

Becker K, Friedrich AW, Lubritz G, Weilert M, Peters G, von Eiff C (2003). Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. J. Clin. Microbiol. 41: 1434-1439.

Burmolle M, Webb JS, Rao D, Hansen LH, Sorensen SJ, Kjelleberg S (2006). Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic

Chander Y, Gupta SC, Goyal SM, Kumar K (2007). Antibiotics: Has the magic gone? J. Sci. Food Agric. 87: 739-742.

Chmielewski RAN, Frank JF (2003). Biofilm formation and control in food processing facilities. Compr. Rev. Food Sci. Food Saf. 2: 22-32.

Chopra AK, Huang JH, Xu X, Burden K, Niesel DW, Rosenbaum MW, Popov VL, Peterson JW (1999). Role of *Salmonella* enterotoxin in overall virulence of the organism. Microbial Pathogen, 27: 155-171.

Chopra AK, Xu XJ, Peterson JW (1994). *Salmonella typhimurium* enterotoxin epitopes shared among bacteria. FEMS Microbiol. Lett. 118: 237-242.

CLSI (2009). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M07-A8.

Costerton JW, Stewart PS, Greenberg EP (1999). Bacterial biofilms: a common cause of persistent infections. Science, 284: 1318-1322.

D'Souza DH, Critzer FJ, Golden DA (2009). Real-time reverse transcriptase polymerase chain reaction for the rapid detection of *Salmonella* using *invA* primers. Foodborne Pathog. Dis. 6: 1097-1106.

Dahshan H, Shahada F, Chuma T, Moriki H, Okamoto K (2010). Genetic analysis of multidrug-resistant *Salmonella enteric*a serovars Stanley and Typhimurium from cattle. Vet. Microbiol. 145: 76-83.

Derzelle S, Dilasser Fi, Duquenne M, Deperrois Vo (2009). Differential temporal expression of the staphylococcal enterotoxins genes during cell growth. Food Microbiol. 26: 896-904.

Dongari-Bagtzoglou A (2008). Mucosal biofilms: challenges and future directions. Expert Rev. Anti Infect. Ther. 6: 141-144.

Donlan RM, Costerton JW (2002). Biofimls: Survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15: 167-193.

Duquenne M, Fleurot I, Aigle M, Darrigo C, Borezee-Durant E, Derzelle S, Bouix M, Deperrois-Lafarge V, Delacroix-Buchet A (2010). Tool for quantification of staphylococcal enterotoxin gene expression in cheese. Appl. Environ. Microbiol. 76: 1367-1374.

Flemming H-C, Wingender J (2010). The biofilm matrix. Nat. Rev. Microbiol. 8: 623-633.

Gilbert P, Allison D, McBain A (2002). Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? J. Appl. Microbiol. 92: 98S-110S.

Harwood VJ, Brownell M, Perusek W, Whitlock JE (2001). Vancomycin-resistant *Enterococcus* spp. isolated from wastewater and chicken feces in the United States. Appl. Environ. Microbiol. 67: 4930-4933.

Holmes RL, Jorgensen JH (2008). Inhibitory activities of 11 antimicrobial agents and bactericidal activities of vancomycin and daptomycin against invasive methicillin-resistant *Staphylococcus aureus* isolates obtained from 1999 through 2006. Antimicrob. Agents Chemother. 52: 757-760.

Hood SK, Zottola EA (1997). Adherence to stainless steel by foodborne microorganisms during growth in model food systems. Int. J. Food Microbiol. 37: 145-153.

Kim SH, Wei CI (2007). Biofilm formation by multidrug-resistant *Salmonella enterica* serotype *typhimurium* phage type DT104 and other pathogens. J. Food Prot. 70: 22-29.

Kondoh K, Furuya D, Yagihashi A, Uehara N, Nakamura M, Kobayashi D, Tsuji N, Watanabe N (2002). Comparison of arbitrarily primed-polymerase chain reaction and pulse-field gel electrophoresis for characterizing methicillin-resistant *Staphylococcus aureus*. Lett. Appl. Microbiol. 35: 62-67.

Kotilainen P, Routamaa M, Peltonen R, Oksi J, Rintala E, Meurman O, Lehtonen O-P, Eerola E, Salmenlinna S, Vuopio-Varkila J, Rossi T (2003). Elimination of epidemic methicillin-resistant *Staphylococcus aureus* from a university hospital and district institutions, Finland. Emerg. Infet. Dis. 9: 169-175.

Kwon AS, Park GC, Ryu SY, Lim DH, Lim DY, Choi CH, Park Y, Lim Y (2008). Higher biofilm formation in multidrug-resistant clinical isolates of *Staphylococcus aureus*. Int. J. Antimicrob Agents, 32: 68-72.

Lowry FD (1998). *Staphylococcus aureus* infection. New Engl. J. Med. 339: 520-532.

Mason EO, Kaplan SL, Lamberth LB, Tillman J (1992). Increased rate of isolation of penicillin-resistant *Streptococcus pneumoniae* in a children's hospital and *in vitro* susceptibilities to antibiotics of potential therapeutic use. Antimicrob. Agents Chemother. 36: 1703-1707.

McLaughlin JB, Castrodale LJ, Gardner MJ, Ahmed R, Gessner BD (2006). Outbreak of multidrug-resistant *Salmonella* Typhimurium associated with ground beef served at a school potluck. J. Food Prot. 69: 666-670.

McMahon MAS, Xu J, Moore JE, Blair IS, McDowell DA (2007). Environmental stress and antibiotic resistance in food-related pathogens. Appl. Environ. Microbiol. 73: 211-217.

Niu C, Gilbert ES (2004). Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. Appl. Environ. Microbiol. 70: 6951-6956.

Petrelli D, Repetto A, D'Ercole S, Rombini S, Ripa S, Prenna M, Vitali LA (2008). Analysis of meticillin-susceptible and meticillin-resistant biofilm-forming *Staphylococcus aureus* from catheter infections isolated in a large Italian hospital. J. Med. Microbiol. 57: 364-372.

Pfeifer CG, Marcus SL, Steele-Mortimer O, Knodler LA, Finlay BB (1999). *Salmonella typhimurium* virulence genes are induced upon bacterial invasion into phagocytic and nonphagocytic cells. Infect. Immun. 67: 5690-5698.

Reid G (1999). Biofilms in infectious disease and on medical devices. Int. J. Antimicrob. Agent. 11: 223-226.

Rode TM, Langsrud S, Holck A, Møretrøa T (2007). Different patterns of biofilm formation in *Staphylococcus aureus* under food-related stress conditions. Int. J. Food Microbiol. 116: 372-383.

Russell AD (2003). Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. Lancet Infect. Dis. 3: 794-803.

Shi X, Zhu X (2009). Biofilm formation and food safety in food industries. Trends Food Sci. Technol. 20: 407-413.

Stewart PS (2002). Mechanisms of antibiotic resistance in bacterial biofilms. Int. J. Med. Microbiol. 292: 107-113.

Telang NV, Satpute MG, Niphadkar KB, Joshi SG (2010). An increased incidence of biofilm-producing multidrug-resistant methicillin-resistant *Staphylococcus aureus* in a tertiary care hospital from India: A 2-year study. Am. J. Infect. Control, 38: 165-166.

Trachoo N (2007). Biofilms and the food industry. Songklanakarin J. Sci. Technol. 25: 807-815.

Van Houdt R, Michiels C (2010). Biofilm formation and the food industry, a focus on the bacterial outer surface. J. Appl. Microbiol. 109: 1117-1131.

Webber MA, Bailey AM, Blair JMA, Morgan E, Stevens MP, Hinton JCD, Ivens A, Wain J, Piddock LJV (2009). The global consequence of disruption of the AcrAB-TolC efflux pump in *Salmonella enterica* includes reduced expression of SPI-1 and other attributes required to infect the host. J. Bacteriol. 191: 4276-4285.