Differential gene expression in planktonic and biofilm cells of multiple antibiotic-resistant Salmonella Typhimurium and Staphylococcus aureus

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biofilm; planktonic cell; antibiotic resistance; gene expression; Staphylococcus aureus; Salmonella Typhimurium.

Abstract
This study was designed to evaluate gene expression patterns of the planktonic and biofilm cells of Staphylococcus aureus and Salmonella Typhimurium in trypticase soy broth adjusted to pH 5.5 and pH 7.3. The planktonic and biofilm cells of multiple antibiotic-resistant S. aureus (S. aureusR) and S. Typhimurium (S. TyphimuriumR) were more resistant to β-lactams than those of antibiotic-susceptible S. aureus (S. aureusS) and S. Typhimurium (S. TyphimuriumS) at pH 5.5 and pH 7.3. The relative gene expression levels of norB, norC, and mdeA genes were increased by 7.0-, 4.7-, and 4.6-fold, respectively, in the biofilm cells of S. aureusS grown at pH 7.3, while norB, norC, mdeA, sec, seg, sei, sel, sem, sen, and seo genes were stable in the biofilm cells of S. aureusR. This study provides useful information for understanding gene expression patterns in the planktonic and biofilm cells of antibiotic-resistance pathogens exposed to acidic stress.

Introduction
Over the last decades, the prevalence of antibiotic-resistant bacterial infections has been rapidly increased because of the repeated and prolonged use of antibiotics, leading to a serious health problem worldwide (Wegener, 2003; Gootz, 2010). The emergence of antibiotic-resistant bacteria has become of great concern for public health, which widely appears as frequent outbreaks in recent years (Boonmar et al., 1998; Van et al., 2007). Therefore, prevention strategies for antibiotic resistance are essential to control the spread of antibiotic-resistant pathogens. However, the discovery and development of novel antibiotics has lagged behind the emergence of antibiotic-resistant pathogens because of the lengthy and expensive processes, requiring phases of clinical investigation trials to obtain approval, and the lack of information on the antibiotic resistance mechanisms (Yineyama & Katsumata, 2006). Therefore, understanding the molecular properties of strains that are antibiotic-resistant is vital for the treatment of diseases associated with antibiotic-resistant pathogens.

In the natural environments, most bacteria can form biofilms, embedded within a self-produced extracellular polymeric matrix consisting mainly of polysaccharide groups (Flemming & Wingender, 2010). The biofilm formation as a bacterial survival strategy leads to increased resistance to heat, acid, preservatives, and antibiotics (Stewart & William Costerton, 2001; Chmielewski & Frank, 2003; Van Houdt & Michiels, 2010). Bacterial infections can mainly occur after consumption of contaminated foods. The ingested bacteria are exposed to acidic stress and bile salt under oxygen-limited conditions during transit through the stomach, the small intestine, and the colon. These stress conditions can influence antibiotic resistance patterns, biofilm-forming abilities, and virulence properties (Riesenberg-Wilmes et al., 1996;
Gahan & Hill, 1999; Schobert & Tielen, 2010). Moreover, antibiotic-resistant bacteria can possibly reside in biofilms and lead to enhanced tolerance to adverse environmental conditions, causing serious infectious diseases (Gustafson et al., 2001; Langsrud et al., 2004; Ngwai et al., 2006; Kim & Wei, 2007). However, there is a lack of information on the biofilm-associated infections involved in altered virulence properties of antibiotic-resistant bacteria. Therefore, the objective of this study was to evaluate the gene expression patterns of biofilm and planktonic cells of antibiotic-resistant foodborne pathogens, *Salmonella Typhimurium* and *Staphylococcus aureus*, when exposed to acidic stress under anaerobic condition.

**Materials and methods**

**Bacterial strains and culture conditions**

Strains of *S. aureus* KACC13236 and *S. Typhimurium* KCCM 40253 were obtained from the Korean Agricultural Culture Collection (KACC, Suwon, Korea) and the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea), respectively. Strains of *S. aureus* CCARM 3080 and *S. Typhimurium* CCARM 8009 were purchased from the Culture Collection of Antibiotic Resistant Microbes (CCARM, Seoul, Korea). All strains were cultured in trypticase soy broth (TSB; BD, Becton, Dickinson and Co., Sparks, MD) at 37 °C for 20 h. The cultured cells were collected by centrifugation at 3000 g for 20 min at 4 °C, and then used to prepare biofilm cells for assays.

**Biofilm formation assay**

The biofilm formation was evaluated based on the ability of strains to adhere to the surface of polystyrene Petri dishes. The strains of *S. aureus* KACC13236, *S. Typhimurium* KCCM 40253, *S. aureus* CCARM 3080, and *S. Typhimurium* CCARM 8009 were inoculated at approximately 10⁶ CFU mL⁻¹ in TSB adjusted to a sub-lethal pH of 5.5 using 1 M HCl and TSB at pH 7.3 as the control. The inoculated strains were anaerobically cultured without mechanical agitation at 37 °C for 48 h in a GasPak anaerobic system (BBL, Cockeysville, MD) and centrifuged at 5000 g. The collected cells were serially diluted (1 : 10) with 0.1% sterile buffered peptone water (BPW). The collected cells were disrupted in a buffer containing guanidine isothiocyanate and lysozyme, mixed with ethanol to adjust proper binding conditions, and then loaded into an RNeasy mini column for RNA isolation.

**Antibiotic susceptible assay**

The antibiotic susceptibility of planktonic and biofilm cells was determined according to the Clinical Laboratory Standards Institute (CLSI) procedure (CLSI, 2009). The antibiotic stock solutions were prepared by dissolving them in sterile distilled water at concentrations of 256 μg mL⁻¹ (ampicillin, aztreonam, cefotaxime, cefoxitin, ceftazidime, cephalothin, oxacillin, and piperacillin) and serial dilution (1 : 2) with TSB (pH 7.3). The strains of *S. aureus* KACC13236, *S. aureus* CCARM 3080, *S. Typhimurium* KCCM 40253, and *S. Typhimurium* CCARM 8009 were anaerobically cultured in TSB at pH 5.5 and 7.3 to obtain planktonic and biofilm cells. In accordance with the CLSI procedure, the planktonic and biofilm cells grown in TSB at pH 5.5 and 7.3 were incubated in the diluted antibiotic solutions for 18 h at 37 °C to evaluate the susceptibility of cells to antibiotics. Minimum inhibitory concentrations (MICs) were determined at concentrations at which there was no visible growth. The susceptible (S), intermediate (I), and resistant (R) strains were defined based on MIC values of < 4 μg mL⁻¹, between 4 and 8 μg mL⁻¹, and more than 16 μg mL⁻¹, respectively (Hamilton-Miller & Shah, 1996).

**Microbiological analysis**

The numbers of planktonic and biofilm cells were estimated using the plate count method. For planktonic cell counts, the cell suspensions were collected and the remaining non-adherent cells were rinsed by flooding the plate surface with 10 mL of 0.1% sterile BPW. For biofilm cell counts, the attached cells were collected with a cell scraper (Thermo Scientific Nunc, Rochester, NY) and suspended by sonication at 20 kHz for 10 min in 20 mL of 0.1% sterile BPW. The collected cells were serially diluted (1 : 10) with 0.1% sterile BPW and the proper dilutions were plated on trypticase soy agar (TSA). The agar plates were incubated at 37 °C for 48 h for enumeration of planktonic and biofilm cells.

**RNA extraction**

Each planktonic or biofilm culture (0.5 mL) was mixed with 1 mL of RNaProtect Bacteria Reagent (Qiagen, Hilden, Germany) and centrifuged at 5000 g for 10 min. The collected cells were used for RNA extraction according to the RNeasy® Mini Handbook (Qiagen). The collected cells were disrupted in a buffer containing guanidine isothiocyanate and lysozyme, mixed with ethanol to adjust proper binding conditions, and then loaded into an RNeasy mini column for RNA isolation.

**RT-PCR amplification**

The cDNA was synthesized as described previously (Xu et al., 2010), according to the QuantiTect Reverse Transcription protocol (Qiagen). In brief, the RNA sample was mixed with a master mixture containing QuantiScript Reverse Transcriptase, QuantiScript RT Buffer, RT Primer
Mix and RNase-free water, incubated at 42 °C for 15 min, and then immediately incubated at 95 °C for 3 min to inactivate the QuantiScript Reverse Transcriptase. The custom-synthesized oligonucleotide primers using IDT (Integrated DNA Technologies Inc., Coralville, IA) were used in this study (Tables 1 and 2). The PCR mixture (20 μL) containing 2× QuantiTect SYBR Green PCR Master (10 μL), 60 pmol primer (0.6 μL), cDNA (2 μL), and RNase-free water (6.8 μL) was amplified using an iCycler iQ™ System (Bio-Rad Laboratories, Hemel Hempstead, UK) and 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 performed immediately after the 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 real-time PCR detection system (Bio-Rad Laboratories). The comparative Ct method (Livak & Schmittgen, 2001; Xu et al., 2010) was used to analyze the relative expression of targeted genes. The untreated cells were cultured anaerobically in TSB (pH 7.3) at 37 °C for 20 h.

### Statistical analysis

All experiments were conducted in duplicate for three replicates. Data were analyzed using 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 $P < 0.05$.

### Results

**Planktonic and biofilm growths of selected foodborne pathogens in different pH levels under anaerobic conditions**

The planktonic and biofilm cell growths of *S. aureus* KACC13236, *S. aureus* CCARM 3080, *S. Typhimurium* KCCM 40253, and *S. Typhimurium* CCARM 8009 were evaluated in TSB at pH 5.5 and 7.3 under anaerobic conditions (Table 3). At pH 5.5, the planktonic cell growths of antibiotic-resistant strains *S. aureus* KACC13236 and *S. Typhimurium* KCCM 40253 were inhibited during the 48-h incubation, showing a decrease in cell counts to 5.59 and 6.25 log CFU mL$^{-1}$, respectively. However, at pH 5.5 the planktonic cell growths of antibiotic-susceptible strains *S. aureus* KACC13236 and *S. Typhimurium* KCCM 40253 were inhibited during the 48-h incubation, showing a decrease in cell counts to 5.59 and 6.25 log CFU mL$^{-1}$, respectively. At pH 7.3, the planktonic cell populations of *S. Typhimurium* KCCM 40253, and *S. Typhimurium* CCARM 8009 increased to approximately 9 log CFU mL$^{-1}$.

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

| Gene | Function | Primer name and sequence | Size (bp) |
|------|----------|--------------------------|----------|
| sec | Enterotoxin | F: TGTACTTCTTAAGGTTTGTGAAT | 104 |
| C | | R: TCCTATCTTTTTGTCATCTTCG | |
| seg | Enterotoxin | F: TTCACAAGGCAAGACATGCTTCA | 73 |
| sei | Enterotoxin | F: GTTACAAGATTGATGCTACGAA | 147 |
| sel | Enterotoxin | F: TAGATGCGCAAGAAATATACC | 176 |
| sem | Enterotoxin | F: TCAATGCGCAACGGCTGATG | 150 |
| sen | Enterotoxin | F: GATAGAAGAGATGTATAAGGCT | 167 |
| seo | Enterotoxin | F: GTGTGAAAGAATCAAGTGAAC | 163 |
| norB | Efflux transporter protein | F: AGCCGGCCTGTACCTGCAC | 213 |
| norC | Efflux transporter protein | F: AATGAGGCTTACCGGACACAA | 216 |
| mdeA | Multidrug efflux system | F: GTTTATGCGATCGATGTTG | 155 |

F, forward; R, reverse.

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

| Gene | Molecular function | Primer name and sequence | Size (bp) |
|------|--------------------|--------------------------|----------|
| acrA | Multidrug efflux system | F: AAACCGGAAAGGCGAAGGT | 64 |
| | | R: GTACCGGATCCTGGGGAATT |  |
| acrB | Multidrug efflux system | F: TGAAGAAAAATGGAACGTTCTTC | 69 |
| | | R: CGAACCGCTGGTGTCA |  |
| tolC | Multidrug efflux system | F: GCCCGTGCAAGATATG | 67 |
| | | R: CCGCTTATCAGGTTG |  |
| ompD | Outer membrane protein D | F: GCCAAAGCCTGACAGGCGG | 239 |
| | | R: GCCAAAGAAGTACGTGTTACG |  |
| hiaA | Invasion gene activator | F: TATGGCAATGACGCTCC | 50 |
| | | R: TCGTAATGCGTACCAG |  |
| fimA | Major fimbrial subunit | F: TTGACGCTGTAAGTTGCG | 62 |
| | | R: CAGACCTACCGAGATG |  |
| lpfE | Fimbrial protein | F: GTCGACTTGGCTCGGA | 61 |
| | | R: GATGGCCGATGCAG |  |
| invA | Invasion protein | F: ACAGTGGTCGACCGCAA | 454 |
| | | R: AGACGGTCGACTGATCGAAAT |  |
| stn | *Salmonella enterotoxin* | F: GCCATGCTGCGATGAT | 467 |
| | | R: GTACCGGATAGGGGAAGG |  |

F, forward; R, reverse.
respectively, while the fewest biofilms were formed by CCARM 3080 in TBS at pH 5.5 and pH 7.3 after 48-h cultivation, were 8.26 and 8.32 log CFU mL\(^{-1}\) respectively.

Antibiotic susceptibility patterns of selected foodborne pathogens anaerobically grown at different pH levels

The gene expression patterns were evaluated in the antibiotic-susceptible (S. aureus\(^S\) and S. Typhimurium\(^S\)) and multiple antibiotic-resistant strains (S. aureus\(^R\) and S. Typhimurium\(^R\)) anaerobically cultured in TSB adjusted to pH 5.5 and 7.3 during the planktonic-to-biofilm transition for 48 h at 37 °C (Figs 1 and 2). The relative expression of norB, norC, mdeA, sec, seg, sei, sel, sem, sen, and seo genes was observed in the planktonic and biofilm cells of S. aureus\(^S\) and S. aureus\(^R\) (Fig. 1). The norB and mdeA genes were overexpressed at the planktonic cells of both S. aureus\(^S\) and S. aureus\(^R\) grown in TSB at pH 5.5 after 48-h incubation (Fig. 1a). The relative expression level of norC gene was increased 2.8-fold in S. aureus\(^S\). The relative gene expression levels of sel and sem were increased 5.0- and 3.0-fold, respectively, in the planktonic cells of S. aureus\(^S\) grown in TSB at pH 5.5. As shown in Fig. 1b, the relative gene expression of norC and mdeA was stabilized in the planktonic cells of both S. aureus\(^S\) and S. aureus\(^R\) grown in TSB at pH 7.3. The relative expression levels of norB, seg, and sei genes were increased 52.6-, 2.6-, and 5.9-fold, respectively, in the planktonic cells of S. aureus\(^S\) grown in TSB at pH 7.3. Unlike the planktonic cells, all genes were stable in the biofilm cells of S. aureus\(^R\) grown in TSB at pH 5.5 and pH 7.3, except for the sec gene in S. aureus\(^R\) biofilm cells formed in TSB at pH 5.5 (Fig. 1c,d). The relative gene expression levels of norB and mdeA were increased 1.9- and 2.0-fold, respectively, in the biofilm cells of S. aureus\(^S\) grown in TSB at pH 5.5 (Fig. 1c). The highest expression level (116.6-fold) was observed at the norB gene in the S. aureus\(^S\) biofilm cells grown in TSB at pH 5.5. As shown in Fig. 1d, the norB, norC, and mdeA genes were stable in the biofilm cells of both S. aureus\(^S\) and S. aureus\(^R\) grown in TSB at pH 7.3.

### Table 3. Viability of planktonic and biofilm cells grown at 37 °C for 48 h in TSB adjusted to pH 5.5 and 7.3

| Treatment | Strain* | 0 h (log CFU mL\(^{-1}\)) | 48 h (log CFU mL\(^{-1}\)) | 0 h (log CFU mL\(^{-1}\)) | 48 h (log CFU mL\(^{-1}\)) |
|-----------|---------|---------------------------|---------------------------|---------------------------|---------------------------|
| pH 5.5    | S. aureus\(^S\) | 6.51                      | 5.59                      | 6.51                      | 7.02                      |
|           | S. aureus\(^R\) | 6.62                      | 6.78                      | 6.62                      | 8.26                      |
|           | S. Typhimurium\(^S\) | 6.97                      | 6.25                      | 6.97                      | 5.48                      |
|           | S. Typhimurium\(^R\) | 6.92                      | 7.47                      | 6.92                      | 6.67                      |
| pH 7.3    | S. aureus\(^S\) | 6.54                      | 5.64                      | 6.54                      | 7.77                      |
|           | S. aureus\(^R\) | 6.64                      | 7.83                      | 6.64                      | 8.32                      |
|           | S. Typhimurium\(^S\) | 6.83                      | 8.96                      | 6.83                      | 7.88                      |
|           | S. Typhimurium\(^R\) | 6.84                      | 8.91                      | 6.84                      | 7.45                      |

n.d., not detected; S, antibiotic-sensitive; R, antibiotic-resistant.

*Staphylococcus aureus KACC13236, S. aureus\(^S\); S. aureus CCARM 3080, S. aureus\(^S\); Salmonella Typhimurium KCCM 40253, S. Typhimurium\(^S\); and S. Typhimurium CCARM 8009, S. Typhimurium\(^R\).

a–e Means with different subscripts within a column are significantly different at P < 0.05.
Table 4. MIC (μg mL⁻¹)* of selected antibiotics against *Staphylococcus aureus* strains grown in TSB adjusted to pH 5.5 and 7.3 at 37 °C

| Antibiotic | S. aureus KACC13236 |   | S. aureus CCARM 3080 |   |
|------------|---------------------|---|----------------------|---|
|            | Planktonic          | Biofilm | Planktonic          | Biofilm |
|            | pH 5.5 pH 7.3       | pH 5.5 pH 7.3 | pH 5.5 pH 7.3       | pH 5.5 pH 7.3 |
| Ampicillin | < 0.25 (S) < 0.25 (S) | < 0.25 (S) > 256 (R) | < 0.25 (S) > 256 (R) | 4 (S) 32 (R) |
| Aztreonam  | > 256 (R) > 256 (R)  | > 256 (R) > 256 (R) | > 256 (R) > 256 (R) | > 256 (R) > 256 (R) |
| Cefotaxime | < 0.25 (S) 2 (S)    | 2 (S) > 256 (R) | > 256 (R) > 256 (R) | 64 (R) > 256 (R) |
| Cefoxitin  | 0.5 (S) 4 (S)       | 2 (S) > 256 (R) | 64 (R) > 256 (R) | > 256 (R) > 256 (R) |
| Ceftazidime| 4 (S) 16 (I)        | 16 (I) > 256 (R) | > 256 (R) > 256 (R) | 64 (R) > 256 (R) |
| Cephalothin| < 0.25 (S) < 0.25 (S) | < 0.25 (S) > 256 (R) | 16 (I) 128 (R) | > 256 (R) > 256 (R) |
| Oxacillin  | < 0.25 (S) < 0.25 (S) | < 0.25 (S) > 256 (R) | 16 (I) > 256 (R) | > 256 (R) > 256 (R) |
| Piperacillin| < 0.25 (S) 1 (S) | 0.5 (S) > 256 (R) | 4 (S) 256 (R) | > 256 (R) > 256 (R) |

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

Table 5. MIC (μg mL⁻¹)* of selected antibiotics against *Salmonella* Typhimurium strains grown in TSB adjusted to pH 5.5 and 7.3 at 37 °C

| Antibiotic | S. Typhimurium KCCM 40253 |   | S. Typhimurium CCARM 8009 |   |
|------------|-----------------------------|---|--------------------------|---|
|            | Planktonic                  | Biofilm | Planktonic                  | Biofilm |
|            | pH 5.5 pH 7.3               | pH 5.5 pH 7.3 | pH 5.5 pH 7.3               | pH 5.5 pH 7.3 |
| Ampicillin | 4 (S) 2 (S)                 | > 256 (R) > 256 (R) | > 256 (R) > 256 (R) | > 256 (R) > 256 (R) |
| Aztreonam  | 1 (S) < 0.25 (S) 32 (I)    | 32 (I) > 256 (R) | < 0.25 (S) < 0.25 (S) 1 (S) | < 0.25 (S) < 2.5 (S) 2 (S) |
| Cefotaxime | 1 (S) 0.25 (S) 0.5 (S) 8 (S) | < 256 (R) > 256 (R) | 4 (S) 64 (R) | 16 (I) 64 (R) |
| Cefoxitin  | 8 (S) 32 (R) 8 (S) 128 (R) | 32 (R) > 256 (R) | 1 (S) 0.5 (S) 8 (S) | 64 (R) 64 (R) |
| Ceftazidime| 2 (S) 0.5 (S) 2 (S) 8 (S)  | 8 (S) > 256 (R) | 16 (I) 32 (R) | 256 (R) 256 (R) |
| Cephalothin| 8 (S) 16 (I) 16 (I) 32 (R) | 32 (R) > 256 (R) | 16 (I) 32 (R) | 256 (R) 256 (R) |
| Oxacillin  | > 256 (R) > 256 (R) > 256 (R) | > 256 (R) > 256 (R) | > 256 (R) > 256 (R) | > 256 (R) > 256 (R) |
| Piperacillin| 4 (S) 16 (I) 8 (S) > 256 (R) | > 256 (R) > 256 (R) | > 256 (R) > 256 (R) | > 256 (R) > 256 (R) |

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

The relative expression patterns of acrA, acrB, filmA, hilA, invA, lpfE, ompD, stn, and tolC genes were observed in the planktonic and biofilm cells of *S. Typhimurium* and *S. Typhimurium* (Fig. 2). The relative gene expression levels of hilA and lpfE were increased in the planktonic cells of both *S. Typhimurium* and *S. Typhimurium* grown in TSB at pH 5.5 after 48-h incubation (Fig. 2a). The highest expression level (46.4-fold) was observed at the lpfE gene in *S. Typhimurium* grown in TSB at pH 5.5. The relative gene expression levels were higher in *S. Typhimurium* than in *S. Typhimurium*. The relative expression levels of acrB and tolC genes were increased 1.8- and 1.5-fold, respectively, in *S. Typhimurium* (Fig. 2a). As shown in Fig. 2b, the relative gene expression levels of hilA and lpfE were increased more than fivefold in the planktonic cells of both *S. Typhimurium* and *S. Typhimurium* grown in TSB at pH 7.3 after 48-h incubation. The greatest changes in gene expression, 18.8- and 18.1-fold, were observed at the lpfE gene in *S. Typhimurium* and *S. Typhimurium*, respectively. The relative expression levels of acrB, filmA, invA, and tolC genes were increased 2.3-, 2.9-, 1.8-, and 1.4-fold, respectively, in *S. Typhimurium* grown in TSB at pH 7.3. Similar to the planktonic cells, the relative expression of lpfE gene was increased more than twofold in the biofilm cells of both *S. Typhimurium* and *S. Typhimurium* grown in TSB at pH 5.5 after 48-h incubation (Fig. 2c). The relative expression level of hilA gene was increased 1.1-fold in the biofilm cells of *S. Typhimurium* at pH 5.5. As shown in Fig. 2d, the acrA, acrB, lpfE, stn, and tolC genes were stable in the biofilm cells of both *S. Typhimurium* and *S. Typhimurium* grown in TSB at pH 7.3. The relative expression levels of all genes were increased in the biofilm cells of *S. Typhimurium* grown in TSB at pH 7.3, except for the ompD gene (Fig. 2d).

**Discussion**

This study describes the gene expression dynamics of planktonic and biofilm-associated foodborne pathogens with multiple antibiotic resistance profiles when grown at different acidic pH ranges under anaerobic conditions. As antibiotic resistance is one of the major public health problems worldwide, this study sheds light on new approaches to the understanding of virulence properties of antibiotic-resistant pathogens exposed to stress conditions.
The antibiotic-resistant strains *S. aureus* \(^R\) and *S. Typhimurium* \(^R\) grew well in TSB at pH 5.5 compared to the antibiotic-susceptible strains (Table 3), suggesting that the antibiotic-resistant strains can adapt better to acidic conditions than the antibiotic-susceptible strains can. The acid-adapted cells provide cross-protection against heat, pH, osmolarity, and antibiotics (Leyer & Johnson, 1993; Lee et al., 1994; Greenacre & Brocklehurst, 2006). The biofilm formation by antibiotic-susceptible strains (*S. aureus* \(^S\) and *S. Typhimurium* \(^S\)) was significantly inhibited by pH 5.5 compared to the antibiotic-resistant strains (*S. aureus* \(^R\) and *S. Typhimurium* \(^R\)) (Table 3). The results imply that acidic pH can negatively influence biofilm formation (Salsali et al., 2006). However, acid-adapted antibiotic-resistant bacteria can be more resistant to other environmental stresses (Leyer & Johnson, 1993; Lee et al., 1994; Greenacre & Brocklehurst, 2006; McKinney et al., 2009). The MIC values of biofilm cells of *S. aureus* KACC13236 grown in TSB at pH 5.5 and 7.3 were relatively greater for all antibiotics than the values for planktonic cells (Table 4), indicating that biofilm cells were significantly more resistant to antibiotics compared with the planktonic cells. The results are in good agreement with previous reports that biofilm formation was directly associated with the significant increase in antibiotic resistance of bacteria (Donlan & Costerton, 2002; Kim & Wei, 2007; Cho et al., 2008; Kwon et al., 2008). The antibiotic resistance of biofilm cells might be attributed to their structural and physiological properties, leading to the changes in membrane permeability and metabolic activity (Costerton et al., 1999; Donlan & Costerton, 2002; Stewart, 2002). Compared to pH 7.3, the planktonic and biofilm |

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**Fig. 1.** Relative gene expression in *Staphylococcus aureus* planktonic cells grown in TSB adjusted to pH 5.5 (a) and pH 7.3 (b) and *S. aureus* biofilm cells grown in TSB adjusted to pH 5.5 (c) and pH 7.3 (d). Means with different uppercase letters (A–E) within *S. aureus* KACC13236 (\(S. aureus^s\)) and lowercase letters (a–d) within *S. aureus* CCARM 3080 (\(S. aureus^r\)) are significantly different at \(P < 0.05\). Asterisk (*) indicates significant difference between *S. aureus* KACC13236 (\(S. aureus^s\)) and *S. aureus* CCARM 3080 (\(S. aureus^r\)) at \(P < 0.05\).
cells grown in TSB at pH 5.5 were highly susceptible to the antibiotics used in this study (Table 5). Acid stress can cause the changes in cellular membrane permeability, leading to increased susceptibility to antibiotics (Alakomi et al., 2000; Delcour, 2009).

The norB and mdeA genes were stable in S. aureus$^S$ and S. aureus$^R$ planktonic cells cultured at pH 5.5 (Fig. 1a). The enhanced resistance to multiple antibiotics is mediated by the relative gene expression associated with norB, norC, and mdeA genes in S. aureus (Huang et al., 2004; Truong-Bolduc et al., 2006; Ding et al., 2008). The gene expression stability of norB, norC, and mdeA in S. aureus planktonic cells may play an important role in antibiotic resistance under anaerobic conditions, resulting in an increased virulence in S. aureus exposed to the gastrointestinal tract. Staphylococcal enterotoxins, a family of pyrogenic toxin superantigen-carrying staphylococcal pathogenicity island, are the major causative agents of staphylococcal food poisoning (Lowry, 1998; Becker et al., 2003; Derzelle et al., 2009). The relative expression levels of norB, norC, mdeA, sec, seg, sei, sel, sem, sen, and seo genes were increased 23.9-, 7.7-, 2.8-, 3.4-, 4.5-, 6.6-, 16.4-, 36.4-, 6.3-, and 8.2-fold, respectively, in the biofilm cells of S. aureus$^R$ grown in TSB at pH 7.3 (Fig. 1d). The efflux pump and virulence-related gene expression may be changed during the biofilm formation by S. aureus$^R$. This confirms a previous report that the antibiotic resistance of biofilm cells contributed to the enhanced virulence (Rajesh & Vandana, 2009; Hoiby et al., 2010). The hilA and lipE genes were overexpressed in S. Typhimurium$^S$ and S. Typhimurium$^R$ planktonic cells cultured in TSB at pH 5.5 (Fig. 2a). This suggests that the adhesion

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**Fig. 2.** Relative gene expression in Salmonella Typhimurium planktonic cells grown in TSB adjusted to pH 5.5 (a) and pH 7.3 (b) and S. Typhimurium biofilm cells grown in TSB adjusted to pH 5.5 (c) and pH 7.3 (d). Means with different uppercase letters (A–E) within S. Typhimurium KCCM 40253 (■, S. Typhimurium$^S$) and lowercase letters (a–d) within S. Typhimurium CCARM 8009 (○, S. Typhimurium$^R$) are significantly different at $P < 0.05$. Asterisk (*) indicates significant difference between S. Typhimurium KCCM 40253 (S. Typhimurium$^S$) and S. Typhimurium CCARM 8009 (S. Typhimurium$^R$) at $P < 0.05$. **

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and invasion ability of S. Typhimurium can be enhanced under acid stress conditions (Chowdhury et al., 1996). The acrB and tolC genes were stable in S. Typhimurium\textsuperscript{R} grown in TSB at pH 5.5 (Fig. 2a). The AcrAB-TolC system is responsible for the increased antibiotic resistance, invasion ability, and virulence (Piddock, 2006; Nikaido et al., 2008; Pages & Amaral, 2009). Therefore, the observations imply that S. Typhimurium\textsuperscript{R} can effectively extrude antibiotics under acidic stress conditions. The AcrAB-TolC pump system can lead directly to multiple antibiotic resistance in bacteria (Piddock, 2006). Salmonella Typhimurium cells causing foodborne salmonellosis can invade the small intestine, which plays a role in bacterial pathogenicity (Pfeifer et al., 1999). The stn gene in S. Typhimurium is responsible for the production of enterotoxin (Chopra et al., 1994, 1999).

In conclusion, this study highlights the differential gene expression of the planktonic and biofilm cells of S. aureus (S. aureus\textsuperscript{R} and S. aureus\textsuperscript{R}) and S. Typhimurium (S. Typhimurium\textsuperscript{S} and S. Typhimurium\textsuperscript{R}) exposed to acidic stress under anaerobic conditions. The most significant findings in this study were that (1) the biofilm cells of multiple antibiotic-resistant S. aureus\textsuperscript{R} and S. Typhimurium\textsuperscript{R} were more resistant to acidic stress compared with the planktonic cells; (2) the biofilm-forming ability was increased in S. aureus\textsuperscript{R} and S. Typhimurium\textsuperscript{R} grown in TSB at pH 5.5 and 7.3; and (3) the relative expression of toxin-, virulence-, efflux pump-related genes in the biofilm of S. aureus\textsuperscript{R} and S. Typhimurium\textsuperscript{R} strains was distinct from that in the planktonic cells. The multiple antibiotic-resistant pathogens (S. aureus\textsuperscript{R} and S. Typhimurium\textsuperscript{R}) were more likely to form the biofilm, possibly leading to cross-protection against environmental stresses and enhanced pathogenesis. Further study is needed taking molecular approaches to elucidate the relationship between biofilm formation and the virulence potential of antibiotic-resistant foodborne pathogens exposed to various environmental stress conditions.

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