CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Strains and Growth Conditions

All bacterial strains used in this study and their relevant characteristics are described in Table 3.1 and 3.2. The bacteria were stored at -20°C in brain heart infusion (BHI; BD) broth containing 30% (v/v) glycerol. Unless otherwise specified, VISA stains were maintained on BHI agar containing 3 µg/ml of vancomycin (SIGMA). BHI broth was used for liquid cultures that were grown at 37°C with shaking at 200 rpm.

3.2 Obtaining of Laboratory-Derived Strains with Decreased Susceptibility to Vancomycin

Vancomycin sensitive *S. aureus* (VSSA) strains were used as starting cultures for generating laboratory-derived VISA strains. Culture was initiated by inoculating 3 to 5 colonies of each VSSA into BHI broth containing 0.5 µg/ml of vancomycin (SIGMA) and incubated at 37°C with shaking at 200 rpm for 18-20 h. Passage was performed daily by inoculating 40 µl of the overnight culture into 4 ml of fresh drug-containing BHI broth on increasing concentrations of vancomycin, until vancomycin MIC of the bacterial strains tested reached 7 µg/ml. Vancomycin resistance in *S. aureus* are defined according to the National Committee for Clinical Laboratory Standards (NCCLS), *S. aureus* for which MIC of vancomycin is ≤ 4 µg/ml are vancomycin-susceptible *S. aureus* (VSSA), strains with MIC of vancomycin is 8 to16 µg/ml are defined as vancomycin-intermediate *S. aureus* (VISA), and strains for which MIC of vancomycin is ≥ 32 µg/ml are vancomycin-resistant *S. aureus* (VRSA) (Loomba *et al*., 2010).

TABLE 3.1

VSSA and hVISA Strains Used in This Study

Bacterial Strains	Phenotypes	References
KY	VSSA	Clinical isolate
SS	VSSA	Clinical isolate
UH7	hVISA	Clinical isolate
UH9	hVISA	Clinical isolate
UH35	hVISA	Clinical isolate
Mu3	hVISA	ATCC 700698

TABLE 3.2

Laboratory-Derived Strains Used in This Study

Bacterial Strains	Phenotypes	References
KY-8	VSSA	This study
SS-8	VSSA	This study
UH7-8	hVISA	This study
UH9-8-8	hVISA	This study
UH35-8	hVISA	This study
Mu3-8	hVISA	This study
KY-8-1	VISA	This study
SS-8-1	VISA	This study
UH7-8-1	VISA	This study
UH9-8-1	VISA	This study
UH35-8-1	VISA	This study
Mu3-8-1	VISA	This study

3.3 Susceptibility Testing

The minimum inhibitory concentrations (MICs) of vancomycin were examined employing a microdilution method (Pfeltz *et al*., 2000). Ninety-six well microtiter plates containing two-fold serial dilutions of vancomycin (0 to 8 µg/ml) in BHI into which overnight cultures were added to

a final concentration of 5 x 10^5 CFU/ml. The plates were incubated at 37°C for 24 h, and the lowest concentration of vancomycin at which there was no visible growth was considered the MIC.

3.4 Population Analysis

Population analysis was performed as described by Pfeltz *et al*. (2000) and Trakulsomboon *et al*. (2001). Overnight cultures were adjusted in BHI broth to concentrations of 10^8 CFU/ml and then diluted to four dilutions by factors of 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7}. Three 10-µl droplets of each dilution were plated onto BHI agar plates containing various concentrations of vancomycin (0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 µg/ml). After incubation at 37°C for 48 h, the number of colonies grown on each plate were counted. The colony forming unit (CFU) of resistant cells was calculated and plotted on a semi-log scale forming colonies on a given concentration of vancomycin.

3.5 Whole Cell Autolysis Assay

The procedure was carried out as previously described (Hanaki *et al*., 1998; Utaida *et al*., 2006). Overnight cultures were transferred into 100 ml of tryptic soy broth (TSB; BD) and were grown to optical density (OD) at 600 nm of about 0.7 at 37°C with shaking at 200 rpm. Cells were harvested by centrifugation (10,000g, 4°C, 10 min) and washed twice with ice-cold 0.9% NaCl. The cells were resuspended in 50 ml of 0.01 M sodium phosphate buffer (Na_2HPO_4-NaH_2PO_4, pH 7.0) to initial OD_{600} of about 0.8. To examine effect of vancomycin on whole cell autolysis, the drug at concentration of one-half of the MICs was included in the assay buffer. The cell suspensions were incubated at 37°C with shaking at 200 rpm. Subsequent readings were taken every 30 min for 5 h. Autolysis was quantified as a percentage of the initial OD_{600} remaining at each sampling time point.

3.6 Zymographic Detection of Autolysins

3.6.1 Extraction of Crude Autolysins

Extraction of crude autolysins was performed as previously described by Koehl *et al*. (2004) and Utaida *et al*. (2006). Overnight cultures were inoculated into 100 ml of TSB medium with or without vancomycin at concentrations of one-half of the MICs and were grown to an

OD_{600} of 0.7 at 37°C with shaking at 200 rpm. The cells were harvested by centrifugation (10,000g, 4°C, 10 min), washed twice with ice-cold 0.9% NaCl, and resuspended in 1 ml of 0.01 M potasium phosphate buffer (K_2HPO_4-KH_2PO_4, pH 7.0). Autolysins were extracted by three freeze-thaw cycles (-20°C for 90 min and then 37°C for 10 min). The cells were removed by centrifugation (10,000g, 4°C, 10 min) and the supernatant was harvested.

3.6.2 Protein Concentration Determination

Protein concentrations were determined using Bio-Rad Protein Assay kit (BIO-RAD) with bovine serum albumin as a standard. The assay is based on Bradford dye-binding assay that involved a differential color change of acidic dye solution which responses to various concentrations of protein (Bradford, 1976). Crude protein extracts were resuspended in dye reagent solution containing phosphoric acid and measured the differential color change at OD_{595}. The protein concentration was calculated from the standard curve.

3.6.3 Autolysin Profiles by Zymography

For preparation of loading samples, 2 µg of crude extracted autolysins were boiled with 5x loading gel buffer containing bromophenol blue and sodium dodecyl sulfate (with 1/4 volume of the extracted protein). The samples were then loaded into each well of 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) containing 0.2% (w/v) lyophilized *Micrococcus luteus* cells (SIGMA). Following electrophoresis (150 V, 30 mA at room temperature), the gel was rinsed with deionized water and then incubated in renaturation buffer (25 mM Tris-HCl; pH 8.0, containing 1% (v/v) Triton X-100, and 10 mM $MgCl_2$) at 37°C overnight. The gel was rinsed and then stained with 1% (v/v) methylene blue in 0.01% (w/v) KOH for 30 min with gentle shaking and then destained in deionized water. Autolytic bands were observed as clear zones in the opaque gel.

3.7 Transmission Electron Microscopy

Samples were prepared for transmission electron microscopy as described by Mani *et al.* (1994) and Pfeltz *et al*. (2000). Overnight cultures were inoculated into 15 ml of BHI broth with or without vancomycin at concentrations of one-half of the MICs and were grown to an OD_{600} of 0.7 at 37°C with shaking at 200 rpm. Cell pellets were harvested in microfuges by

centrifugation (10,000g, 4°C, 5 min) and then resuspended in 2.5% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.4. After 24 h, cell pellets were centrifuged and mixed with 1% water agars and leaved them to solidify. The water agars were then cut into approximately 1-mm^3 pieces and fixed in glutaraldehyde for 30 min. The agar pieces were rinsed three times with sodium phosphate buffer and then postfixed in 1% osmium tetroxide in sodium phosphate buffer for 1 h. After that the agar pieces were rinsed with distilled water and then resuspened in 1% aqueous uranyl acetate for 1 h. Following dehydration in a graded series of ethyl alcohol and two changes in propylene oxide, the agar pieces were embedded in Epon 812 and thin sections were stained with uranyl acetate and lead citrate and then examined employing HITASHI H-700 transmission electron microscope. Cell wall morphologies were observed for thickness and roughness. Cut surfaces were measured for evaluation of cell wall thickness.