CHAPTER 5

CONCLUSION

Passaged cultures of five VSSA strains on increasing concentrations of vancomycin resulted in two groups of laboratory-derived strains with decreased susceptibility to vancomycin. First, bacterial strains with vancomycin MICs of 4 μg/ml and the resistant levels were in an intermediate range. The bacterial strains were further passaged and resulted in the second group for which vancomycin MICs were 7 μg/ml and was defined as intermediate resistance.

Both groups of the laboratory-derived strains were analyzed for heterogenous vancomycin resistance of subpopulations. The area under concentration curve (AUC) of population analysis profiles of each strain was calculated and AUC ratios of the test strains to that of the control strain were then determined. In the group of vancomycin MICs of 4 μg/ml, all laboratory-derived strains contained heterogeneous populations and were classified as hVISA. The laboratory-derived strains with vancomycin MICs of 7 μg/ml contained subpopulations that were resistant to vancomycin at concentrations of 5 μg/ml and higher. Moreover, their population profiles are similar to uniform of VISA in that 100% of population grew at 4 μg/ml of vancomycin and there were subpopulations of cells growing at 8 μg/ml of vancomycin or higher. Thus these derivatives were defined as VISA. These suggested that long-term exposure to vancomycin of hVISA favors the development to VISA.

To investigate autolytic characteristics of VISA, whole cell autolytic activites and autolysin profiles by zymographic analysis were performed. All hVISA derivatives but UH35-8 retained nearly all autolytic activities of their parental strains.

However, when vancomycin MICs of VISA derivatives reached 7μg/ml, more differences of whole cell autolytic activities between VISA derivatives and their parental strains were observed. All VISA derivatives examined had decreased whole cell autolytic activities comparing to those of their parental strains. These are consistent with previous reports (Pfeltz *et al*., 2000; Koehl *et al*., 2004; Utaida *et al*., 2006) in that VISA strains had reduced autolytic activities compared to that of vancomycin-susceptible strains.

The presence of vancomycin in the assay buffer resulted in a slight reduced autolytic activities of parental strains comparing to those with the absence of the drug. Similarly, two strains of VISA derivatives with the vancomycin MICs of 7 μg/ml (SS-8-1 and UH7-8-1) had slight reduced autolytic activities when the drug was presented. However, no differences of autolytic activities were observed for other three VISA derivatives with the MICs of 7 μg/ml. These results were similar to those of SS-8 and UH7-8 with the MICs of 4 μg/ml. Contrary, hVISA KY-8 with the MICs of 4 μg/ml had increased autolytic activity when vancomycin was presented. Inconsistent effects of vancomycin may due to differences in genetics of the organisms.

To investigate the correlation of autolytic enzymes and whole cell autolysis, extracted autolysins were analyzed by zymographic analyses. Autolysin profiles revealed that parental strains and hVISA derivatives expressed five autolytic bands that may corresponding to the unprocessed autolysin (bifunctional product of the *atl* gene;138 kDa), the intermediately processed autolysin (115 kDa), the autolysin processing intermediates at approximately bigger than 90 kDa, *N*-acetylmuramyl-L-alanine amidase at 62 kDa, and the endo-*β*-*N*-acetylglucosminidase (GL) (51 kDa). The hVISA strains with vancomycin MICs of 4 μg/ml including KY-8, SS-8, and UH9-8 had no difference of intensities of autolytic bands comparing to those of their parental strains. UH7-8 expressed slight increased intensities of autolytic bands. In contrast, intensities of autolytic bands of UH35-8 were less than those of its parental strain.

However, almost VISA derivatives with the MICs of 7 μg/ml showed reduced intensities of the autolytic bands comparing to those of their parental strains. This may due to reduced amounts or activities of the enzymes. The results are consistent with whole cell autolysis profiles in that VISA had reduced autolytic activities comparing to those of parental strains. However, the autolytic band intensities were not different between VISA UH35-8-1 and its parental strain UH35.

Vancomycin had various effects on autolysin profiles. Most parental strains showed almost cmparable intensities of the bands versus the band intensities observed in the absence of vancomycin. The group of hVISA derivatives with MICs of 4 μg/ml, slight reduced intensities of autolytic the bands were observed for UH9-8 and UH35-8 when vancomycin was presented. However, SS-8 had a slight increased intensities of the bands. Other two hVISA strains, KY-8,

and UH7-8, showed comparable intensities of autolytic bands versus the bands intensities observed for the bacteria grown in absence of vancomycin.

Inclusion of vancomycin in the media resulted in slight increased intensities of the autolytic bands of KY-8-1 and UH35-8-1 with vancomycin MICs of 7 μg/ml. While SS-8-1, UH7-8-1, and UH9-8-1 had no differences of the band intensities comparing to those of grown in the absence of the drug. However, the effect of vancomycin on autolysin profiles was inconsistent with whole cell autolysis profiles for some strains. The finding implied the complex regulation of autolytic activity.

Additionally, cell wall morphologies of VISA derivatives were examined. Thicker walls and rougher surfaces of VISA than those of parental strains were observed. These results are consistent with previous reports revealing that the thickened cell wall and roughened surface were common features of VISA (Pfeltz *et al*., 2000; Cui *et al*., 2006). These features may contribute to vancomycin resistance in that accumulation of cell wall materials blog the access of vancomycin to its target at the cytoplasmic membrane and prevent VISA or VRSA from cell wall degradation. However, UH9-8-1 had a slight thickened cell wall comparing to those of its parental strain, while, the roughed surface was not observed.

When vancomycin was included in the medium, the thickness and roughness of cell wall of all VISA derivatives and almost VSSA tested appeared to be not different between the bacteria grown in absence and presence of the drug. UH7 had a slight thickened cell wall in the presence of vancomycin. This may due to genetic background of this strain.

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APPENDIX

APPENDIXES

EQUIPMENTS AND CHEMICALS

1. Equipments

    1.1 Autoclave: Tomy ES-315

    1.2 Hot air oven: WTB BINDER

    1.3 Incubator shaker: Innova™ 4340

    1.4 Incubator: BINDER

    1.5 Larmina flow: Astec Microflow ABS 1200

    1.6 Mini-PROTEAN 3 Cell Electrophoresis: BIO-RAD

    1.7 pH meter: BECKMAN

    1.8 Refrigerated centrifuge: SORVALL RC50

    1.9 Refrigerated centrifuge: Jouan MR22

    1.10 Spectrophotometer: genesys 20

    1.11 Transmission electron microscope: HITASHI H-700

    1.12 Vortex mixer: Vortex-2 GENIE G-560E

    1.13 Water bath: ISOTEMP 210

2. Chemicals

    2.1 99.9% Acrylamide: BIO-RAD

    2.2 Agar: DIFCO

    2.3 Ammonium persulfate: USB Corporation

    2.4 Bis-N, N'-Methylene-bis-acrylamide: BIO-RAD

    2.5 Brain heart infusion: BD

    2.6 di-Potassium hydrogen phosphate: UNIVAR

    2.7 di-Sodium hydrogen phosphate: MERCK

    2.8 Glycerol: CARLO-ERBA

    2.9 Glycine: SIGMA

    2.10 Magnesium chloride: CARLO-ERBA

    2.11 2-Mercaptoethanol: SIGMA-ALDRICH

2.12 Methylene blue: Fluka

2.13 Potassium hydroxide: CARLO ERBA

2.14 Sodium dodecyl sulfate (SDS): CARLO-ERBA

2.15 Potassium dihydrogen phosphate: UNIVAR

2.16 Sodium dihydrogen phosphate: MERCK

2.17 Sodium chloride: CARLO ERBA

2.18 N, N, N', N'-Tetramethylethylenediamine (TEMED; USB Corporatio))

2.19 Tris-hydroxymethyl-methylamine (Tris-base): UNIVAR

2.20 Triton-X 100: CARLO-ERBA

2.21 Tryptic Soy Broth: BD

2.22 Vancomycin: SIGMA-ALDRICH

3. Preparation

3.1 Brain heart infusion (BHI) (1000 ml)

Suspended 37 g of the powder in 1000 ml of distilled water.

Sterilized by autoclaving at 121°C for 15 minutes.

3.2 Tryptic soy broth (TSB) (1000 ml)

. Suspended 30 g of the powder in 1000 ml of distilled water.

Sterilized by autoclaving at 121°C for 15 minutes.

3.3 0.1M Potassium phosphate buffer (K_2HPO_4-KH_2PO_4), pH 7.0 (500 ml)

Added 30.75 ml of 0.1 M di-Potassium hydrogen phosphate.

Added 19.25 ml of 0.1 M Potassium dihydrogen phosphate.

Adjusted the pH to 7.0 and added distilled water to 500 ml.

Sterilize by autoclaving at 121°C for 15 minutes.

3.4 0.1M Sodium phosphate buffer (Na_2HPO_4-NaH_2PO_4), pH 7.0 (500 ml)

Added 28.85 ml of 0.1 M di-Sodium hydrogen phosphate.

Added 21.15 ml of 0.1 M Sodium dihydrogen phosphate.

Adjusted the pH to 7.0 and added distilled water to 500 ml.

Sterilized by autoclaving at 121°C for 15 minutes.

3.5 1M Tris-HCl, pH 8.0 (500 ml)

Dissolved Tris-base 65.5 g in distilled water.

Adjusted the pH to 8.0 and added distilled water to 500 ml.

Sterilized by autoclaving at 121°C for 15 minutes.

3.6 Renaturation buffer (500 ml)

Added 3.25 ml of 1M Tris-HCl, pH 8.0.

Added 5 ml of Triton X-100.

Added 5 ml of 10mM Magnesium chloride.

Added distilled water to 500 ml.

3.7 Working solution for electrophoresis

3.7.1 30% (w/v) acrylamide solution (100 ml)

Dissolved 29.2 g of acrylamide in distilled water.

Dissolved 0.8 g of bis-acrylamide in distilled water.

Added distilled water to 100 ml.

3.7.2 4X separating gel buffer (100 ml)

Added 75 ml of 2M Tris-HCl, pH 8.8.

Added 4 ml of 10% (w/v) SDS.

Added distilled water to 100 ml.

3.7.3 4X stacking gel buffer (100 ml)

Added 50 ml of 1M Tris-HCl, pH 6.8.

Added 4 ml of 10% (w/v) SDS.

Added distilled water to 100 ml.

3.7.4 5X Tris-Glycine buffer, pH 8.3 (1000 ml)

Dissolved 15 g of Tris-base in distilled water.

Dissolved 72 g of Glycine in distilled water.

Dissolved 5 g of SDS in distilled water.

Adjusted pH the pH to 8.3 and added distilled water to 1000 ml.

3.8 Preparation of gel for electrophoresis

3.8.1 Separating gel (10% acrylamide; 10 ml)

Dissolved 0.01 g of lyophilized *Micrococcus luteus* cells in 4.2 ml of distilled water.

Added 3.3 ml of 30% (w/v) acrylamide solution

Added 2.5 ml of 4X separating gel buffer.

Added 50 µl of 10% (w/v) ammonium persulfate.

Added 5 µl of TEMED.

3.8.2 Stacking gel

Added 0.67 ml of 30% (w/v) acrylamide solution.

Added 1 ml of 4X stacking gel buffer.

Added 2.3 ml of distilled water.

Added 30 µl of 10% (w/v) ammonium persulfate.

Added 5 µl of TEMED.

BIOGRAPHY

Name	Miss Sutanya Jongutsa
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