CHAPTER 4

RESULTS AND DISCUSSION

4.1 Passage Selection of Laboratory-Derived VISA and Drug Susceptibility Testing

Passage of VSSA on increasing concentrations of vancomycin in BHI liquid cultures resulted in laboratory-derived strains with decreased susceptibility to vancomycin. Table 4.1 and 4.2 show vancomycin MICs of VSSA strains (KY, SS, UH7, UH9, and UH35) and their laboratory-derived strains (KY-8, SS-8, UH7-8, UH9-8, UH35-8, KY-8-1, SS-8-1, UH7-8-1, UH9-8-1, and UH35-8-1). Before selection, all VSSA strains had vancomycin MICs of 1 μg/ml. The passaging on increasing concentrations of vancomycin from 0.5 to 1 μg/ml, the vancomycin MICs of the bacterial strains increased from 1 to 4 μg/ml. These strains were defined to be intermediate according to Clinical and Laboratory Standards Institute (CLSI) guideline that defines staphylococci for which the MICs of vancomycin are $\leq$ 2 μg/ml to be susceptible, while isolates for which the MICs are in a range of 4 to 8 μg/ml to be intermediate, and strains for which the MICs of vancomycin are $\geq$ 16 μg/ml are resistant. The laboratory-derived strains with vancomycin MICs of 4 μg/ml were characterized. Similar properties to those of their parental strains were observed. Thus the passaging was further continued on increasing concentratons of vancomycin ranging from 1, 1.5, 2, 2.5 and 3 μg/ml. Eventually, the laboratory-derived strains had vancomycin MICs of 7 μg/ml and were considered to be intermediate (CLSI, 2010).

TABLE 4.1

Vancomycin MICs of VSSA Strains

Bacterial Strains	Phenotypes	Vancomycin MICs (μg/ml)
KY	VSSA	1
SS	VSSA	1
UH7	hVISA	1
UH9	hVISA	1
UH35	hVISA	1
Mu3 (control)	hVISA	2

TABLE 4.2

Vancomycin MICs of Laboratory-Derived hVISA and VISA Strains

Bacterial Strains	Phenotypes	Vancomycin MICs (μg/ml)
KY-8	hVISA	4
SS-8	hVISA	4
UH7-8	hVISA	4
UH9-8	hVISA	4
UH35-8	hVISA	4
Mu3-8	hVISA	4
KY-8-1	VISA	7
SS-8-1	VISA	7
UH7-8-1	VISA	7
UH9-8-1	VISA	7
UH35-8-1	VISA	7
Mu3-8-1	VISA	7

4.2 Population Analysis Profiles of the Laboratory-Derived VISA Strains

All laboratory-derived strains with vancomycin MICs of 4 and 7 μg/ml were analyzed for heterogeneous vancomycin-resistant subpopulations. The area under concentration curve (AUC) of population analysis profile of each strain was calculated. The ratios of test strain AUC to that of Mu3, a control strain, were then determined (Liu and Chambers, 2003).

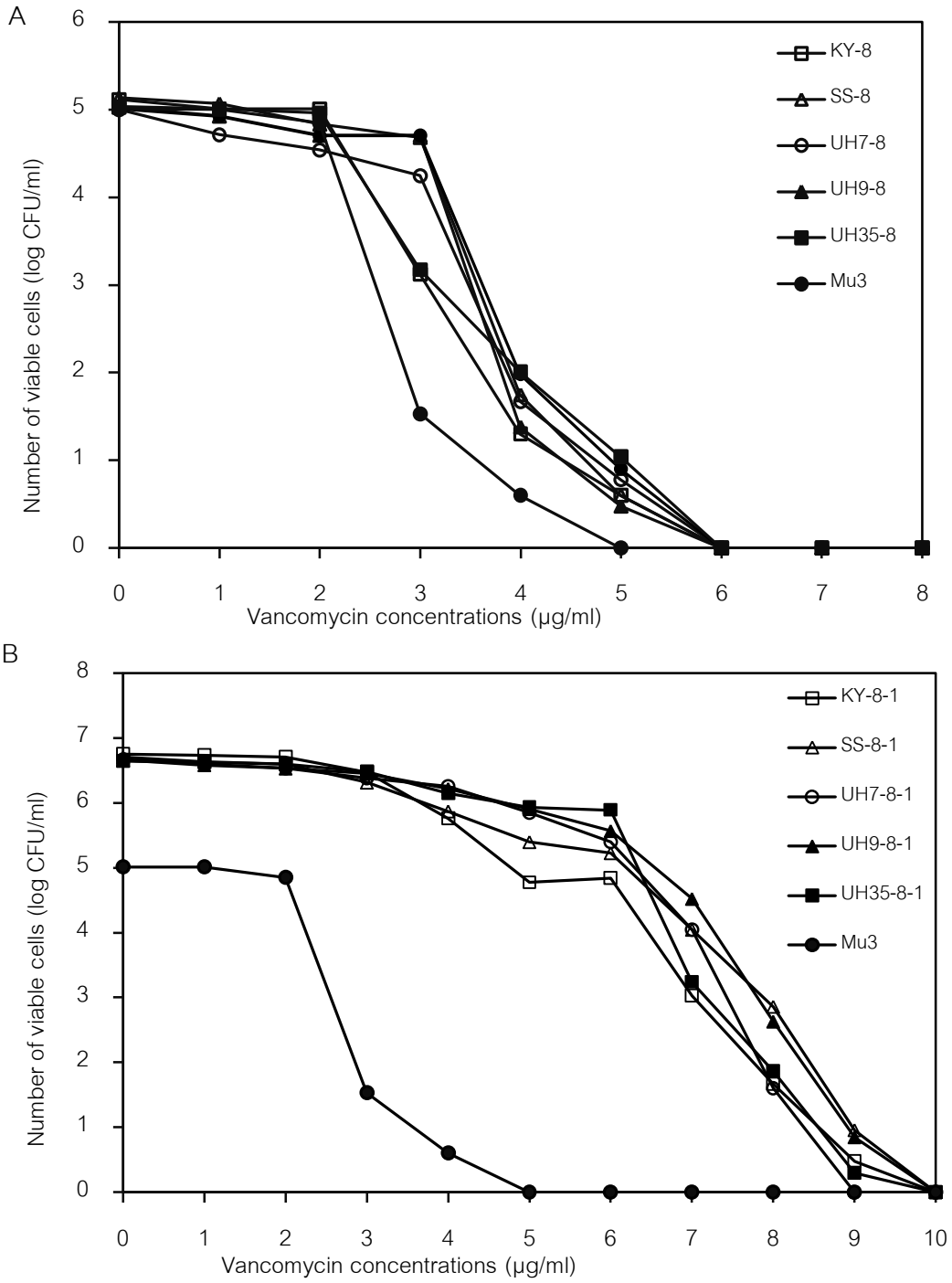
Figure 4.1 illustrates population analysis curves of two groups of the laboratory-derived strains with vancomycin MICs of 4 μg/ml and 7 μg/ml. Population analysis profiles of the bacterial strains with the MICs of 4 μg/ml (Fig. 4.1A) revealed that all strains but Mu3 contained large populations that were resistant to 3 μg/ml of vancomycin. All five strains tested contained subpopulations resisted to 4 to 5 μg/ml suggesting the presence of heterogeneous resistance of VISA strains. Table 4.3 shows the AUC ratios of all bacterial strains tested. The ratio of 0.9 to 1.3 is used to identify hVISA (Liu and Chambers, 2003). All the VISA strains with the MICs of 4 μg/ml showed the AUC ratios ranging from 1.22 to 1.33 indicating heterogeneous resistance. The bacterial strains contained subpopulations that were able to grow at various levels of

vancomycin concentrations higher than 4 μg/ml. Hence these bacterial strains are denoted as hVISA. It has been suggested that hVISA associated with treatment failure of vancomycin (Srinivasan *et al*., 2002; Liu and Chambers, 2003; Maor *et al*., 2007; Loomba *et al*., 2010). The failure was due to subpopulations of cells which resisted to various levels of vancomycin concentrations (Liu and Chambers, 2003). This was strongly supported by our results. However, the AUC ratios of hVISA were high. This suggests that the bacteria approached VISA stage for which the AUC ratio is > 1.3 (Liu and Chambers, 2003).

At the vancomycin MICs of 4 μg/ml, the passage-derived strains presented heterogeneous resistance to vancomycin. Increasing of the MICs to 7 μg/ml by further passaging resulted in subpopulations that were resistant to vancomycin at concentrations of 5 μg/ml and higher (Fig. 4.1B). All strains had almost 100% of population growing at 4 μg/ml of vancomycin and contained subpopulations that were resistant to 6 to 9 μg/ml of vancomycin. The results suggested that the population profiles are similar to uniform of VISA. VISA contains 100% of population growing at 4 μg/ml of vancomycin and subpopulations of cells growing at 8 μg/ml of vancomycin or higher (Liu and Chambers, 2003; Loomba *et al*., 2010). Thus, these derivatives are denoted as VISA and correlated to the AUC ratios which are not present in the range of hVISA (Table 4.3). Moreover, continued exposure to vancomycin of hVISA derivatives with MICs of 4 μg/ml favored outgrowth of resistant subpopulations in these strains. This may result in a rise of populations growing in 1 to 4 μg/ml and higher concentrations of vancomycin of VISA derivatives with MICs of 7 μg/ml. Thus, a uniformity of VISA phenotype was observed as shown in figure 4.1B. These suggested that long-term exposure to vancomycin of hVISA favors the development to VISA (Liu and Chambers, 2003; Maor *et al*., 2007).

FIGURE 4.1

Population Analysis Profiles of the Laboratory-Derived hVISA and VISA Strains



The population curves of hVISA strains with the vancomycin MICs of 4 µg/ml (A) and the VISA strains with vancomycin MICs of 7 µg/ml (B). Overnight cultures were standardized to 10^8 CFU/ml, diluted, and plated on various concentrations of vancomycin. Colonies were counted and colony forming units (CFUs) were then calculated. CFUs were plotted on a semi-log scale forming colonies on a given concentration of the drug.

TABLE 4.3

AUC Values of the Laboratory-Derived hVISA Strains (MICs of 4 μg/ml) and Laboratory-Derived VISA Strains (MICs of 7 μg/ml) and AUC Ratios

Bacterial Strains	AUC Values	AUCs of Tested Strains : AUC of Mu3 (14.5)
KY-8	17.61	1.22
SS-8	19.49	1.33
UH7-8	18.46	1.27
UH9-8	18.34	1.26
UH35-8	18.70	1.29
KY-8-1	43.80	3.02
SS-8-1	47.34	3.26
UH7-8-1	46.03	3.17
UH9-8-1	48.23	3.32
UH35-8-1	46.31	3.19

4.3 Whole Cell Autolytic Activity Profiles

One common characteristic of VISA strains is decreased autolytic activity comparing to those of vancomycin-susceptible *S. aureus* strains (Pfeltz *et al*., 2000; Koehl *et al*., 2004; Utaida *et al*., 2006). To investigate this property, whole cell autolysis of laboratory-derived VISA strains and their vancomycin-susceptible parental strains were performed. The bacterial cells were incubated in sodium phosphate buffer and autolysis was suggested as a decreased in an optical density presenting as a percentage of the initial OD_{600} determined after resuspension cells in the buffer.

Figures 4.2 to 4.6 show autolysis profiles of all laboratory-derived hVISA strains with vancomycin MICs of 4 μg/ml and VISA with the MICs of 7 μg/ml and their parental strains. In the group of hVISA (MICs of 4 μg/ml), a slight decreased whole cell autolytic activity was observed for KY-8 comparing to that of its parental strain KY (Fig. 4.2A). UH35-8 had decreased whole cell autolysis comparing to its parental UH35 (Fig. 4.6A). However, SS-8, UH7-8, and UH9-8

retained nearly all of autolytic activities of their parental strains (Fig. 4.3A to 4.5A). A slight difference observed may due to vancomycin resistant levels of hVISA derivatives are not high.

Increasing of the vancomycin MICs to 7 μg/ml resulted in more differences of whole cell autolytic activities between laboratory-derived strains and their parental strains as shown in Figures 4.2B to 4.6B. KY-8-1 had decreased whole cell autolytic activity comparing to that of its parental strain KY (Fig. 4.2B). It is obviously seen that SS-8-1, UH7-8-1, and UH9-8-1 had decreased whole cell autolysis when vancomycin MICs increased to 7 μg/ml (Fig. 4.3B, 4.4B, and 4.5B). These results were not observed at the MICs of 4 μg/ml for these three strains. Decreased whole cell autolysis was observed for UH35-8-1 when MIC increased to 7 μg/ml that was also observed at MICs of 4 μg/ml for this strain (Fig. 4.6B). These results are consistent with previous reports (Pfeltz *et al*., 2000; Koehl *et al*., 2004; Wootton *et al*., 2005; Utaida *et al*., 2006) in that VISA strains had reduced autolytic activities compared to that of vancomycin-susceptible strains. This reduced autolytic activity may associate with vancomycin resistance in that it results in cell wall turnover, which is the mechanism of degrading old peptidoglycan material by autolysin before making new peptidoglycan for cell growth, comes to be halted contributing to the accumulation of cell wall materials at outer surface of membrane and leads to limit the access of vancomycin to its true target at cytoplasmic membrane (Sieradzki *et al*., 1997; Pfeltz *et al*., 2000).

Figure 4.7A shows whole cell autolysis of all VSSA strains tested. The least whole cell autolytic activity was observed for strain UH9. Almost comparable rates of whole cell autolysis were observed for KY, SS, and UH7. Figure 4.8 and 4.9 show whole cell autolysis of all VISA tested with MICs of 4 μg/ml and 7 μg/ml, respectively. In the group of hVISA strains (MICs of 4 μg/ml), the lowest whole cell autolysis was observed for UH35-8 whereas other four hVISA strains had no significant differences (Fig. 4.8A). When MICs reduced to 7 μg/ml, UH9-8-1 had the lowest whole cell autolysis as shown in Figure 4.9A. Other four strains had almost comparable autolytic activities of whole cell to one another.

4.4 Effects of Vancomycin on Whole Cell Autolytic Activites

To examine the effects of vancomycin on whole cell autolytic activities of laboratory-derived VISA, the drug at concentrations of one-half of the MIC (2 μg/ml for strains with the MICs of 4 μg/ml and 3.5 μg/ml for strains with the MICs of 7 μg/ml) were added in the assay

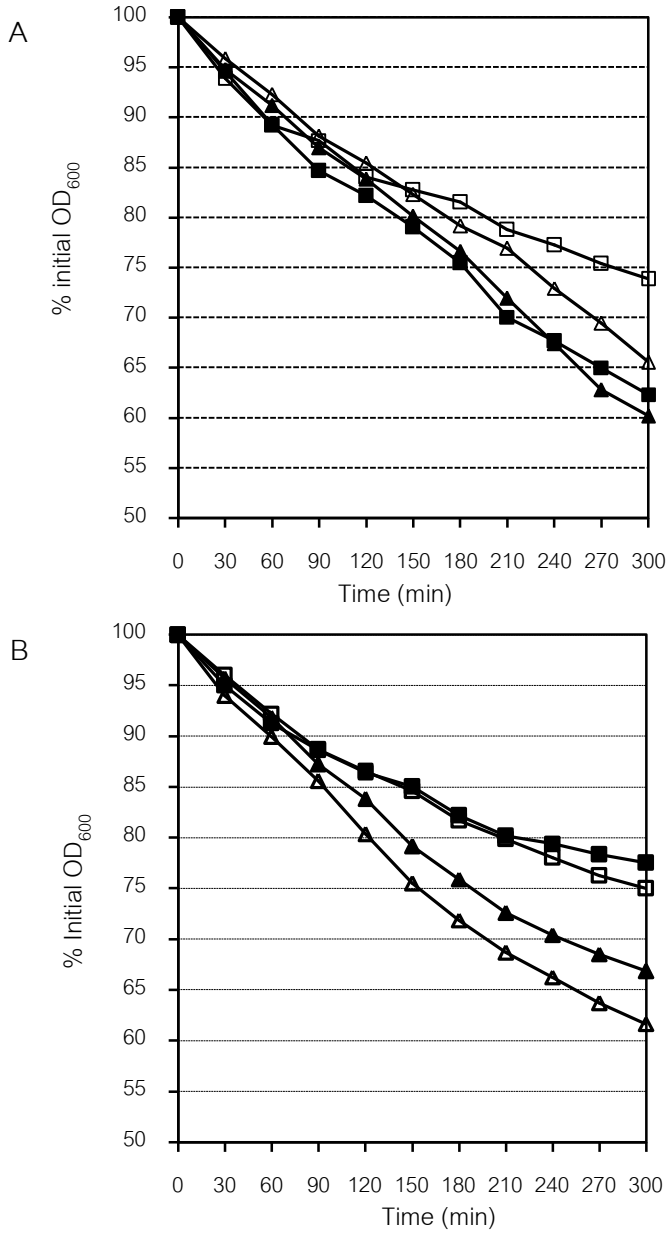
buffer before suspending the cells. Figures 4.2 to 4.6 show the effects of vancomycin on autolytic activities of hVISA with vancomycin MICs of 4 μg/ml and VISA with the MICs of 7 μg/ml and their susceptible parental strains. KY-8, with vancomycin MIC of 4 μg/ml had a slight increased autolytic activity when vancomycin was presented in the assay buffer (Fig. 4.2A). Similar result was observed for KY (Fig.4.2A). Similarly, hVISA UH35-8 had increased autolysis when vancomycin was included in the assay buffer (Fig. 4.6A). However, UH35 also retained nearly whole cell autolysis when vancomycin was included in the assay buffer (Fig. 4.6A). Almost comparable rates of autolysis were observed for SS-8, UH7-8, and UH9-8 as well as their parental strains when vancomycin was included in the assay buffer (Fig. 4.3A to 4.5A). The results suggest that vancomycin had no effects on autolytic activities of whole cells for these strains.

The group of VISA with vancomycin MICs of 7 μg/ml, the presence of the drug resulted in a slight reduced autolytic activity in KY (Fig. 4.2B). Similar results were observed for other VSSA tested (Fig. 4.3B to 4.6B). VISA strains SS-8-1 and UH7-8-1 had slight reduced autolytic activities when vancomycin was presented in the assay buffer (Fig. 4.3B and 4.4B) suggesting that vancomycin had slight effects on whole cell autolytic activity. These autolytic activity reductions are similar to other VISA strains that were reported by Pfeltz *et al*. (2000), Sieradzki and Tomasz (2006), and Utaida *et al*. (2006). This decreased whole cell autolysis may due to vancomycin inhibit autolytic system (Sieradzki and Tomasz, 2006). Cell wall materials, which accumulated at the outer surface of the membrane, trap for vancomycin molecules and block the access of autolysins to their targets resulting in inhibition of cell wall degradation (Sieradzki and Tomasz, 2006). However, no differences of autolytic activities were observed for other VISA strains tested when vancomycin was presented in the assay buffer (Fig. 4.2B, 4.5B, and 4.6B).

Figure 4.7B shows effect of vancomycin on whole cell autolysis of all parental strains. The least whole cell autolysis was observed for strain UH9. Strains KY, SS, and UH7 had almost comparable autolytic activities of whole cell to one another. Additionally, UH35-8 and UH9-8 had lower whole cell autolysis than other strains when vancomycin MIC reached 4 μg/ml (Fig. 4.8B). KY-8 had highest whole cell autolysis (Fig. 4.8B). When the MICs increased to 7 μg/ml, UH9-8-1 had lowest whole cell autolysis (Fig. 4.9B). Other four strains had almost comparable autolytic activities of whole cell to one another (Fig. 4.9B).

FIGURE 4.2

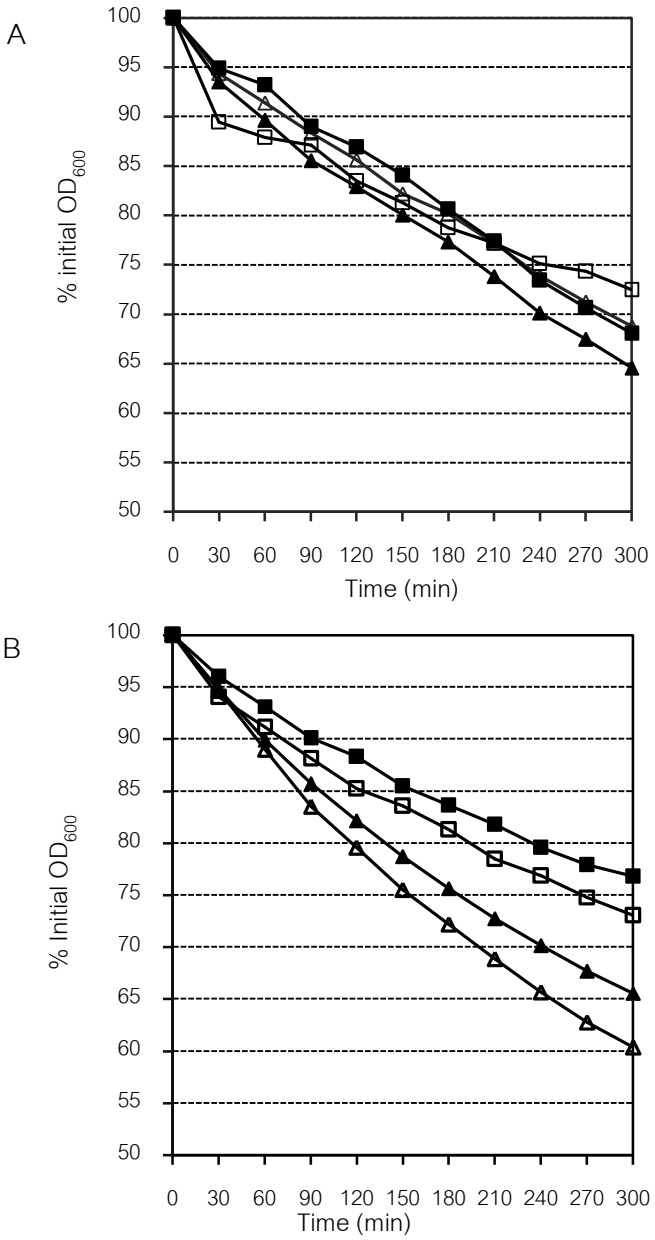
Whole Cell Autolytic Activity Profiles of Laboratory-Derived Strains KY-8, KY-8-1, and Their Parental Strain KY



Whole cell autolysis of parental KY (△) and hVISA strain KY-8 (□) with MIC of 4 μg/ml (A) and VISA strain KY-8-1 (□) with MIC of 7 μg/ml (B) in the absence (opened symbol) and presence (closed symbol) of vancomycin at concentrations of 2 μg/ml (for the strains with MIC 4 μg/ml) and 3.5 μg/ml (for the strains with MIC 7 μg/ml) in the assay buffer. Whole cell autolysis was monitored as reducing in OD_{600} and presented as a percentage of an initial OD_{600} which was taken after resuspension cells in the assay buffer.

FIGURE 4.3

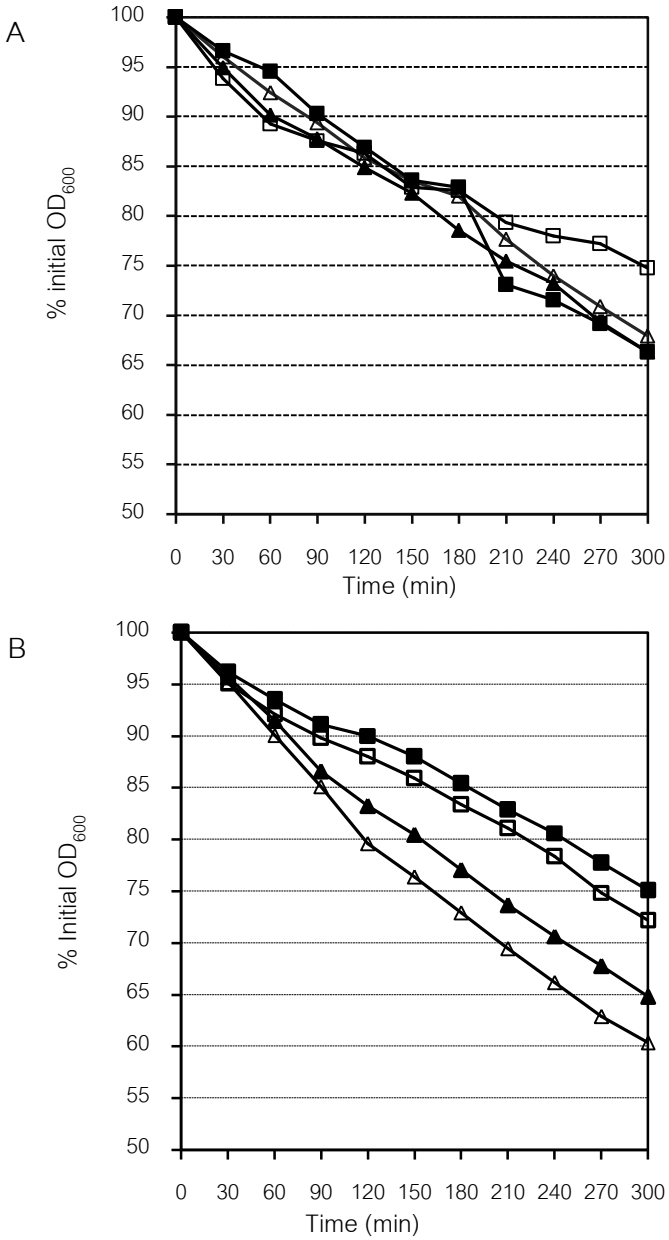
Whole Cell Autolytic Activity Profiles of Laboratory-Derived Strains SS-8, SS-8-1 and Their Parental Strain SS



Whole cell autolysis of parental SS (△) and hVISA strain SS-8 (□) with MIC of 4 μg/ml (A) and VISA strain SS-8-1 (□) with MIC of 7 μg/ml (B) in the absence (opened symbol) and presence (closed symbol) of vancomycin at concentrations of 2 μg/ml (for the strains with MIC 4 μg/ml) and 3.5 μg/ml (for the strains with MIC 7 μg/ml) in the assay buffer. Whole cell autolysis was monitored as reducing in OD_{600} and presented as a percentage of an initial OD_{600} which was taken after resuspension cell in the assay buffer.

FIGURE 4.4

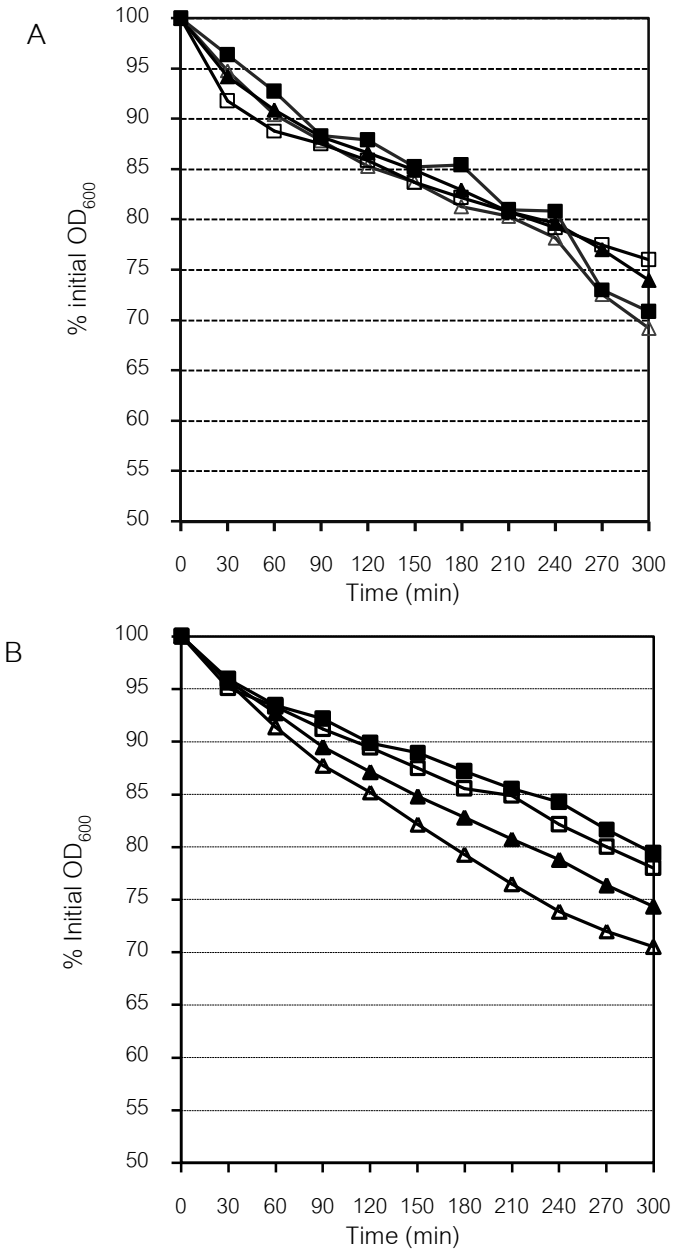
Whole Cell Autolytic Activity Profiles of Laboratory-Derived Strains UH7-8, UH7-8-1, and Their Parental Strain UH7



Whole cell autolysis of parental UH7 (△) and hVISA strain UH7-8 (□) with MIC of 4 μg/ml (A) and VISA strain UH7-8-1 (□) with MIC of 7 μg/ml (B) in the absence (opened symbol) and presence (closed symbol) of vancomycin at concentrations of 2 μg/ml (for the strains with MIC 4 μg/ml) and 3.5 μg/ml (for the strains with MIC 7 μg/ml) in the assay buffer. Whole cell autolysis was monitored as reducing in OD_{600} and presented as a percentage of an initial OD_{600} which was taken after resuspension cells in the assay buffer.

FIGURE 4.5

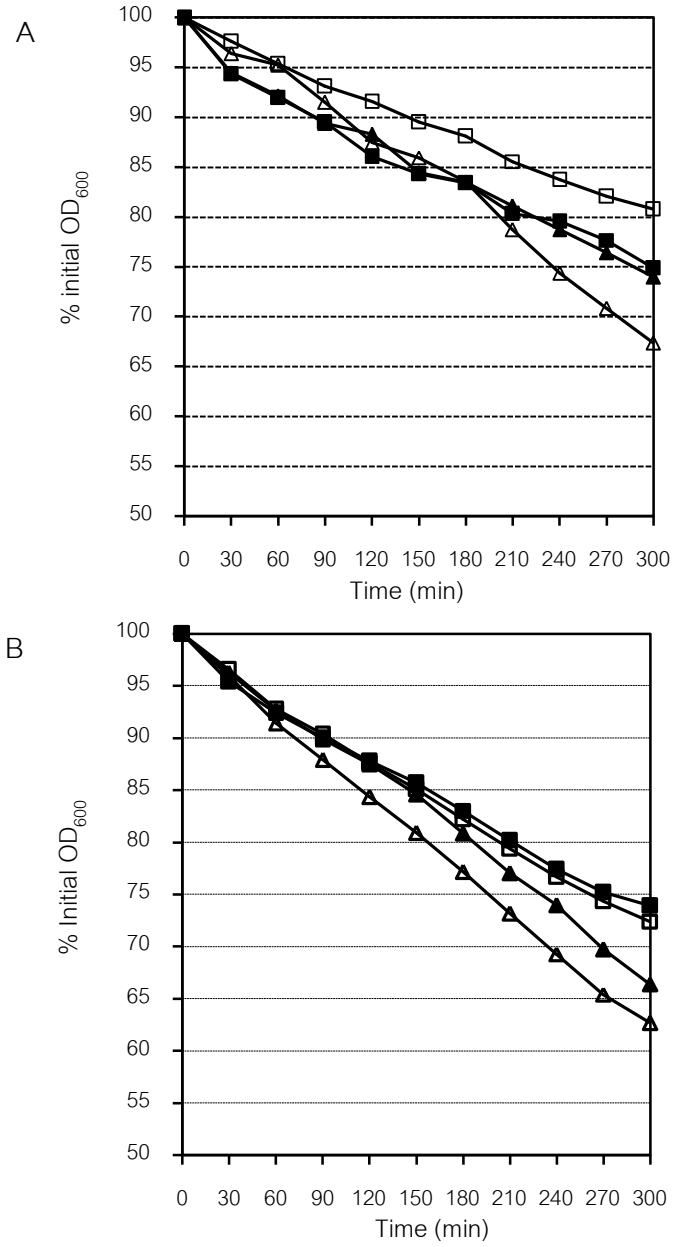
Whole Cell Autolytic Activity Profiles of Laboratory-Derived Strains UH9-8, UH9-8-1, and Their Parental Strain UH9



Whole cell autolysis of parental UH9 (△) and hVISA strain UH9-8 (□) with MIC of 4 μg/ml (A) and VISA strain UH9-8-1 (□) with MIC of 7 μg/ml (B) in the absence (opened symbol) and presence (closed symbol) of vancomycin at concentrations of 2 μg/ml (for the strains with MIC 4 μg/ml) and 3.5 μg/ml (for the strains with MIC 7 μg/ml) in the assay buffer. Whole cell autolysis was monitored as reducing in OD_{600} and presented as a percentage of an initial OD_{600} which was taken after resuspension cells in the assay buffer.

FIGURE 4.6

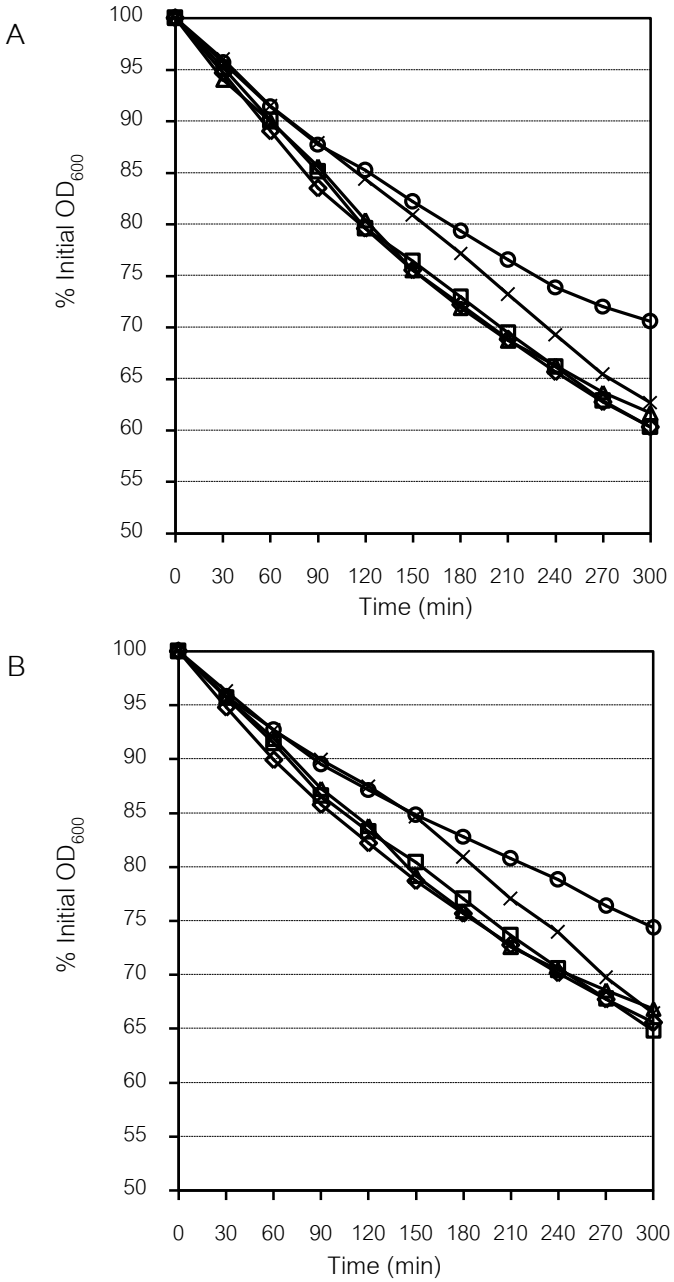
Whole Cell Autolytic Activity Profiles of Laboratory-Derived Strains UH35-8, UH35-8-1, and Their Parental Strain UH35



Whole cell autolysis of parental UH35 (△) and hVISA strain UH35-8 (□) with MIC of 4 μg/ml (A) and VISA strain UH35-8-1 (□) with MIC of 7 μg/ml (B) in the absence (opened symbol) and presence (closed symbol) of vancomycin at concentrations of 2 μg/ml (for the strains with MIC 4 μg/ml) and 3.5 μg/ml (for the strains with MIC 7 μg/ml) in the assay buffer. Whole cell autolysis was monitored as reducing in OD_{600} and presented as a percentage of an initial OD_{600} which was taken after resuspension cells in the assay buffer.

FIGURE 4.7

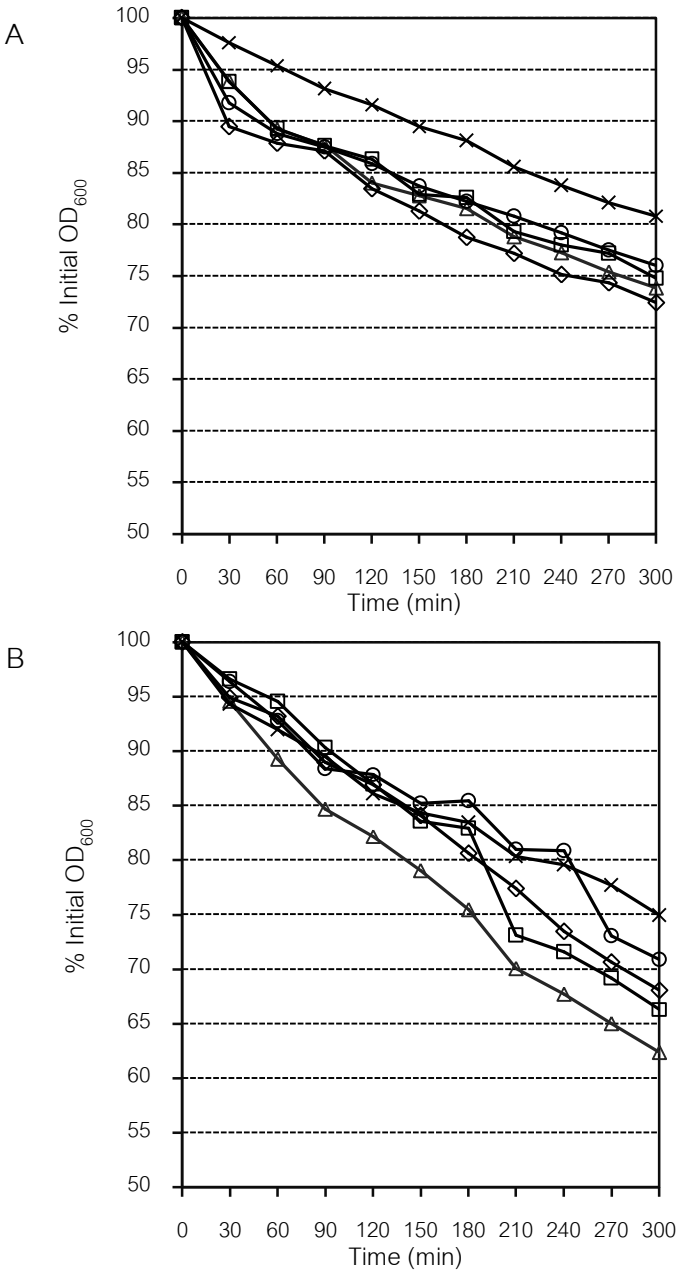
Whole Cell Autolytic Activity Profiles of VSSA in the Absence and Presence of Vancomycin



Whole cell autolysis of parental strains KY (△), SS (◇), UH7 (□), UH9 (○), and UH35 (x) in the absence (A) and presence (B) of vancomycin at concentration of 0.5 µg/ml in the assay buffer. Whole cell autolysis was monitored as reducing in OD_{600} and presented as a percentage of an initial OD_{600} which was taken after resuspension cells in the assay buffer.

FIGURE 4.8

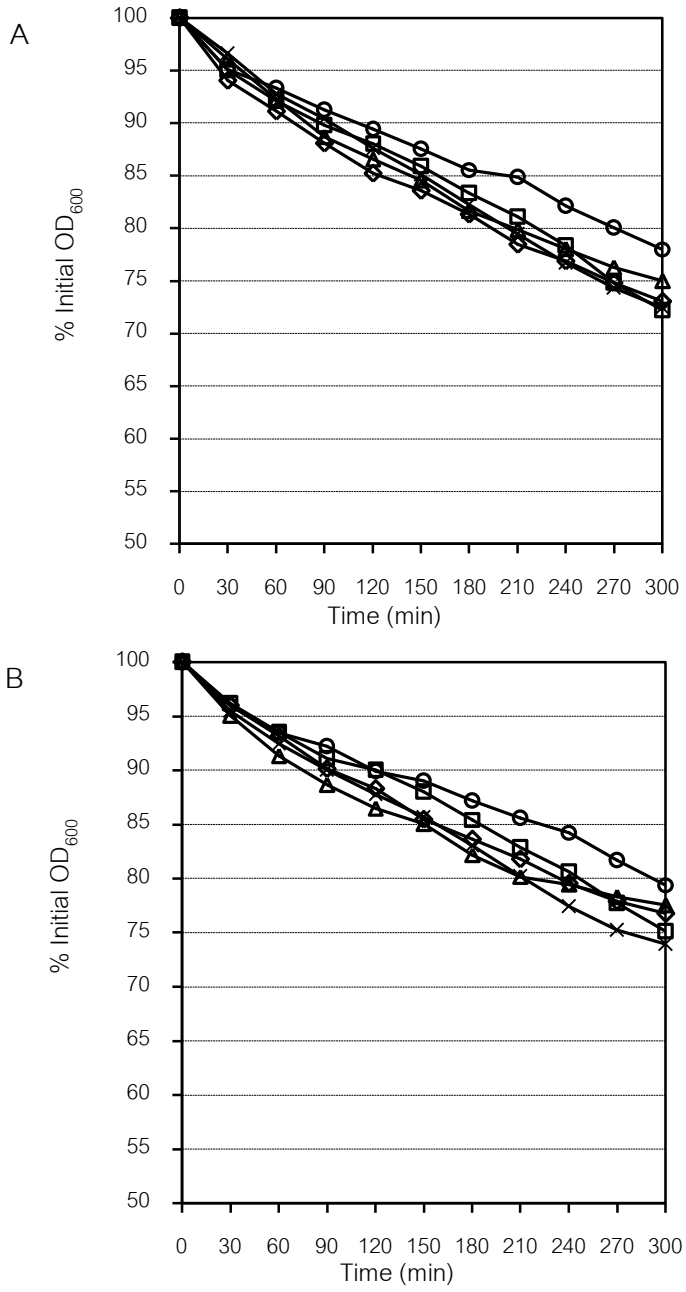
Whole Cell Autolytic Activity Profiles Laboratory-Derived hVISA (MICs of 4 μg/ml) in the Absence and Presence of Vancomycin



Whole cell autolysis of hVISA strains KY-8 (△), SS-8 (◇), UH7-8 (□), UH9-8 (○), and UH35-8 (x) with MICs of 4 μg/ml in the absence (A) and presence (B) of vancomycin at concentration of 2 μg/ml in the assay buffer. Whole cell autolysis was monitored as reducing in OD_{600} and presented as a percentage of an initial OD_{600} which was taken after resuspension cells in the assay buffer.

FIGURE 4.9

Whole Cell Autolytic Activity Profiles Laboratory-Derived VISA (MICs of 7 µg/ml) in the Absence and Presence of Vancomycin



Whole cell autolysis of VISA strains KY-8-1 (△), SS-8-1 (◇), UH7-8-1 (□), UH9-8-1 (○), and UH35-8-1 (x) with MICs of 7 µg/ml in the absence (A) and presence (B) of vancomycin at concentration of 3.5 µg/ml in the assay buffer. Whole cell autolysis was monitored as reducing in OD_{600} and presented as a percentage of an initial OD_{600} which was taken after resuspension cells in the assay buffer.