Autolysis of Heterogeneous Vancomycin-Intermediate *Staphylococcus Aureus*

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Abstract

Alterations of autolytic activities are always associated with vancomycin resistance in *S. aureus*. Two laboratory-derived heterogeneous vancomycin-intermediate *S. aureus* (hVISA) strains, KY-9 and UH7-9, had slight decreased whole cell autolysis. A slight increased whole cell autolytic activitiy was observed for KY-9 with the presence of vancomycin in the assay buffer. Similar result was achieved for its vancomycin susceptible parental strain, KY. Autolysin profiles in the absence and presence of vancomycin are varied among the bacterial strains tested, reflecting the genetic differences between the organisms and regulation of autolysins that may implicate the changes of autolysis.

Keywords: heterogeneous VISA, autolysin, vancomycin

1. Introduction

Heterogeneous vancomycin-inter mediate *Staphylococcus aureus* (hVISA) are those with minimum inhibitory concentrations (MICs) and appear to be in a susceptible range (≤2 μg/ml) but have a subpopulation that resists higher concentrations of vancomycin [1]. hVISA has become a significant clinical problem since it is associated with serious infections that can lead to treatment failure of glycopeptides. The first hVISA isolate, Mu3, was reported from Japan in 1997 [2]. Since then hVISA isolates have been increasingly reported worldwide including Southeast Asia [3-6]. In Thailand, Trakulsomboon *et al*. [4] has reported three hVISA isolates from three clinical patients with methicillin-resistant *S. aureus* (MRSA) infections. More recently, the first pediatric case of hVISA has also been reported from Thailand [7]. The clinical impact of hetero-resistant strains remains controversial [6, 8]. Nevertheless, the presence of hVISA may be a significant sign of the potential decline of vancomycin therapeutic effect against MRSA infections. This may represent an urgent warning for the possible emergence of vancomycin resistant strains in these regions.

Analysis of the mechanism of VISA resistance has been difficult because most characteristics of the VISA phenotype are not uniformly expressed in a consistent fashion among VISA strains. Alterations of autolytic activities are always associated with vancomycin resistance in *S. aureus* [9-13]. Sieradzki and Tomasz [12] showed that the level of resistance to vancomycin in VISA isolates is correlated with the decreasing autolytic rates of the bacteria. Reduction in autolytic activity may allow the cell to tolerate vancomycin that might otherwise cause lysis-inducing effects. This might be an important step in the

development of vancomycin resistance *in S. aureus* [9, 14]. In this study, the autolytic properties of laboratory-derived heterogeneous VSIA strains were investigated as an initial step to elucidate the molecular mechanism of vancomycin resistant in *S. aureus*.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Vancomycin-sensitive *S. aureus* (VSSA; university hospital), KY; SS; UH7; and UH9, and laboratory-derived hVISA strains obtained by exposure to those susceptible isolates to selected concentrations of vancomycin, KY-9; SS-9; UH7-9; and UH9-9, were used throughout the study. Strains in appropriate medium were stored at -80°C in 30 % (v/v) glycerol. Unless otherwise specified, three to five colonies of each bacterial strain were used to inoculate onto brain heart infusion (BHI; BD) broth to start overnight cultures and were grown at 37°C with shaking at 200 rpm for 16-20 h.

2.2 Whole cell autolysis assay

The procedure was carried out according to Hanaki *et al*. [15]. Cultures in 100 ml of trypticase soy broth (TSB; BD) starting with a 2% (v/v) inoculum from 5 ml of overnight culture were grown to an OD_{600} of 0.7 at 37°C with shaking at 200 rpm. Cells were harvested by centrifugation (13,800 g, 4°C, 15 min in a 250 ml centrifuge tube), washed once with cold saline (0.9% NaCl) and resuspended in 50 ml of 0.01 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.0 to an initial OD_{600} of about 0.8. The cell suspension was then incubated at 37°C with shaking (200 rpm) and subsequent readings were taken every at 30 min for 5 h. When needed, vancomycin a concentration of one-half of MIC was included in the assay buffer before suspending the cells.

2.3 Zymographic detection of peptide-glycan hydrolases

Extraction of autolytic enzyme was performed as described by Koehl *et al*. [14]. Cultures were grown in the presence or absence of vancomycin. A 2% (v/v) inoculum from an overnight culture was used to inoculate 100 ml of fresh TSB in a 250 ml flask and allowed to grow to an OD_{600} of 0.7 at 37°C with shaking at 200 rpm. The cells were then harvested by centrifugation (13,800g, 4°C, 15 min), washed once with cold distilled water and 0.01 M K_2HPO_4-KH_2PO_4 buffer, pH 7.0, and resuspended in 1 ml of the buffer. The cell suspension was subjected to three freeze-thaw cycles, i.e., -80°C for 1 h and then 37°C in a water-bath for 10 min. The suspension was then centrifuged (12,000 rpm, 4°C, 10 min) to remove the cells, retaining the supernatant containing the autolysins. The protein concentrations were determined using the Bradford dye-binding procedure [16], with bovine serum albumin as a standard.

The electrophoresis and visualization of autolytic activity of the autolysin were performed as described by Foster [17] and Sugai *et al*. [18] with some modifications. The crude autolysin extracts (3 µg) were separated on a 10% sodium dodecyl sulfate polyacrylamide gel [19] containing 0.2% (w/v) *Micrococcus luteus* (Sigma) lyophilized cells. Following electrophoresis, gels were washed in distilled water for 30 min at room temperature with gentle agitation. The gels were then transferred to renaturation buffer containing 25 mM Tris-HCl (pH 8.0), 1% Triton X-100, and 10 mM $MgCl_2$ and incubated for 20 h at 37°C. After incubation, the gels were rinsed with distilled water, stained in 1% methylene blue in 0.01% KOH for 15 min, and destained in distilled water. Autolytic activity bands were observed as a zone of clearing in the opaque gel.

3. Results

3.1 Whole cell autolysis

Figure 1 shows whole cell autolytic activities of all four hVISA strains used in this study. All of the bacterial strains had almost comparable whole cell autolytic rates, compared to each other. Similarly, all clinical VSSA isolates showed almost comparable whole cell autolytic activities to one another.

Generally, decreased whole cell autolysis was reported for VISA strains, compared to those of their vancomycin susceptible parental strains [9]. Strain KY-9 had a slight decreased autolytic activity compared to that of its parental strain KY (Figure 2A). Similar results were also observed for the UH7-9 (Figure 2C). No significant differences of whole cell autolysis were observed for strains SS-9, and UH9-9, compared to those of their parental strains, SS and UH9, respectively (Figure 2B and 2D).

To examine the effects of vancomycin on whole cell autolytic properties of laboratory-derived hVISA strains, the drug at a concentration of one half of MIC (2 µg/ml) was included in the assay buffer before the OD_{600} was taken. The KY-9 and its vancomycin susceptible parental strain KY had increased autolytic activities when vancomycin was present in the assay buffer (Figure 2A). The SS isolate showed a similar result to that of the KY strain when vancomycin was present (Figure 2B). Vancomycin, however, had no effect on whole cell autolytic activities of the other hVISA isolates (Figure 2B-2D). Similarly, the VSSA strains UH7 and UH9 showed no significant differences of autolysis when vancomycin was included in the assasy (Figure 2C-2D). Additionally, strain UH9 showed the lowest autolysis compared to those of the others (Figure 3). Strain KY had the highest autolytic activity when vancomycin was present (Figure 3). Similarly, strain UH9-9 had the least whole cell autolytic activities comparing to those of the others (Figure 4). The highest autolytic activities were observed for strain KY-9. Strains SS-9 and UH7-9 showed almost comparable whole cell autolysis to one another (Figure 4).

3.2 Autolysin profiles

Zymographic analysis was carried out in order to determine whether alterations of autolysis were related to changes in endogenous autolysin expression. Figure 5 shows peptidoglycan hydrolase profiles produced by hVISA and their VSSA parental strains with *M. luteus* cells as substrate. Two majors bands of about 120 and 50 kDa that may correspond to the intermediately processed ATL protein and the completely processes endo-*β*-*N*-acetylglucosaminidase (GL) [20], respectively, were identified. The weaker activity band of about 62 kDa that may represent *N*-acetylmuramyl-L-alanine amidase (AM) [20], one of the ATL processed form, was also identified (Figure 5A and B).

It is clearly seen that the fully processed form in all hVISA but SS-9 displayed activities almost equivalent to those of their vancomycin susceptible parental strains. The autolytic activity of the 50 kDa of SS-9 was less than that of its VSSA parental strain SS (Figure 5A). Similarly, less intensity of the band of the intermediately processed ATL protein was also observed for the SS-9, compared to that of the SS (Figure 5A). This suggests that there is smaller amount or less activity of cell wall-associated autolysin in the SS-9 strain.

It can be seen that the activity bands of the 50 and 120 kDa, the two major forms of ATL protein, of all bacterial strains tested showed decreased intensity of the bands when grown with vancomycin at concentrations of one-half of the MIC (Figure 5B). The 62 kDa form also had decreased activity in the presence of vancomycin. Surprisingly, the band of a molecular weight higher than that of 120

kDa (asterisk, Figure 5B), which may corresponding to the uncleaved ATL, the major autolysin encoded from the *atl* gene [21-22], was clearly detected when vancomycin was present in the culture medium.

4. Discussion

In this study, the laboratory-derived hVISA strains were determined for their autolytic properties. Two of four strains showed no differences of their autolytic activities to those of their parental VSSA isolates. A Slight decrease in whole cell autolytic activities was observed for strain KY-9 and UH7-9. This is consistent with those reported by Pfeltz *et al.* [9].

It has been shown that vancomycin inhibits whole cell autolysis of *S. aureus* strains with reduced susceptibility to the drug [9]. Reduced autolysis results in increased cell wall thickness and resistance to vancomycin. It was speculated that the thickened wall limits the access of vancomycin to its true target. Additionally, it has been reported that the level of resistance to vancomycin in VISA isolates is correlated with the decreasing autolytic rates of the bacteria [12]. Surprisingly, vancomycin, by unknown mechanism, induced autolysis of KY-9 and its parental VSSA strain, KY (Figure 2A). Nevertheless, the other laboratory-derived hVISA strains used in this study retained nearly all of the autolysis of their VSSA parental strains. This may reflect the genetic differences of the organisms. The differences then may be responsible for the level of resistance to vancomycin of the organisms.

The amount or activity of autolysins may be responsible for the alterations of whole cell autolysis. Surprisingly, zymographic analysis shows that all heterogeneous VISA strains but the SS-9 produced almost equivalent intensities of the bands comparing to those of their VSSA parental strains. The band that may corresponding to the glucosaminidase of SS-9 showed less autolytic activity against *M. luteus* cell than that of its parental SS isolate. It is clear that this major ATL form of all strains used in this study showed decreased activity against the *M. luteus* cells when vancomycin was present in the growth medium. Similar results were also observed for the intermediately processes ATL and the 62 kDa forms. This is inconsistent with whole cell autolytic activities of the bacteria.

Reduced autolytic activities are common characteristics reported for most VISA isolates [9]. However, autolysin profiles verywidely among VISA with reduced autolytic activities. It has been shown that some VISA strains produced active autolysins but were resistant to lysis by autolysins [11]. Changes in the cell wall may responsible for this resistance. Utaida *et al.* [13] have reported that decreased autolytic activity of VISA strain Mu50 was not likely due to either a defect in autolysin activity or changes in the cell walls. This may be due to a tight regulation of autolysins. Thus, in addition to genetic differences between organisms that may reflect the differences of autolysis of different strains, the regulator of autolysin or other factors that may be involved in the changes should not be neglected.

5. Conclusion

Two laboratory-derived hVISA strains, KY-9 and UH7-9, showed slightly decreased whole cell autolysis. Vancomycin at concentrations of one half of the MICs, induced whole cell autolytic activities of the KY-9 and its vancomycin susceptible parental strain KY. Autolysin profiles vary among the bacterial strains tested.

6. Acknowledgment

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7. References

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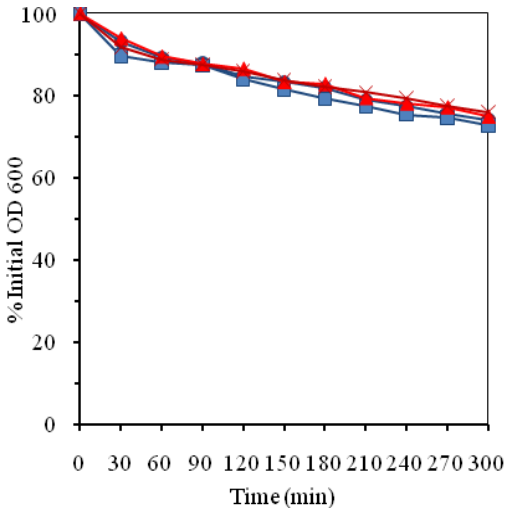

Figure 1 Whole cell autolytic activity profiles of laboratory-derived hVISA strains. Profiles of KY-9 (•); SS-9 (■); UH7-9 (▲); and UH9-9 (×) were determined. Autolysis was followed as a decrease in optical density presenting as a percentage of the initial OD_{600} determined directly after suspension in lysis buffer. The

data represent the means of three independent experiments in duplicate.

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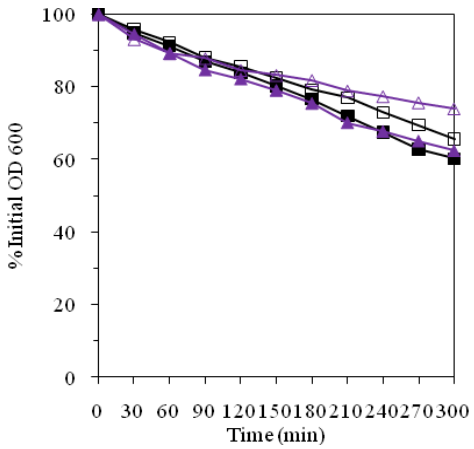

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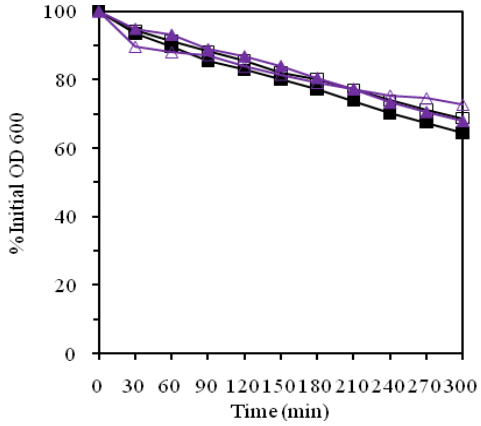

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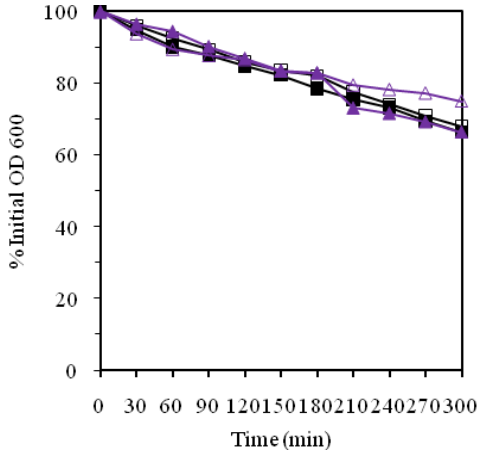

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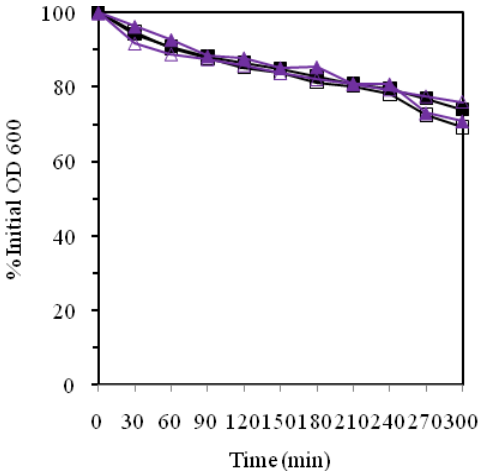

Figure 2 Whole cell autolytic activity profiles of *S. aureus* in the absence (open symbols) and presence (solid symbols) of vancomycin. Profiles of strains KY-9 (triangles) and its vancomycin susceptible parental strain KY (rectangles) (A); SS-9 (triangles) and its vancomycin susceptible parental strain SS (rectangles) (B); UH7-9 (triangles) and its vancomycin susceptible parental strain UH7 (rectangles) (C); and UH9-9 (triangles) and its vancomycin susceptible parental strain UH9 (rectangles) (D) were determined. Autolysis was followed as a decrease in optical density presenting as a percentage of the initial OD_{600} determined directly after suspension in lysis buffer. The data represent the means of three independent experiments in duplicate.

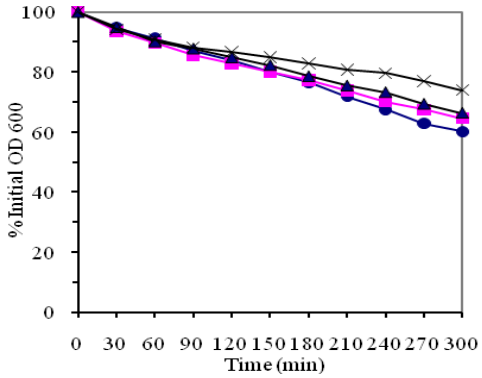

Figure 3 Whole cell autolytic activity

profiles of clinical VSSA isolates in the presence of one-half of MIC of vancomycin in the assay buffer. Profiles of KY (•); SS (■); UH7 (▲); and UH9 (×) were determined. Autolysis was followed as a decrease in optical density presenting as a percentage of the initial OD_{600} determined directly after suspension in lysis buffer. The data represent the means of three independent experiments in duplicate.

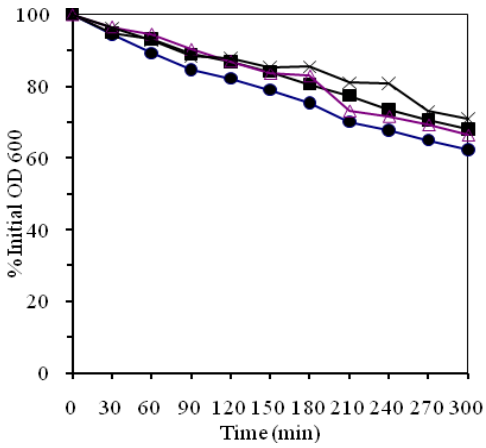

Figure 4 Whole cell autolytic activity profiles of laboratory-derived hVISA strains in the presence of one-half of MIC of vancomycin in the assay buffer. Profiles of KY-9 (•); SS-9 (■); UH7-9 (Δ); and UH9-9 (×) were determined. Autolysis was followed as a decrease in optical density presenting as a percentage of the initial OD_{600} determined directly after suspension in lysis buffer. The data represent the means of three independent experiments in duplicate.

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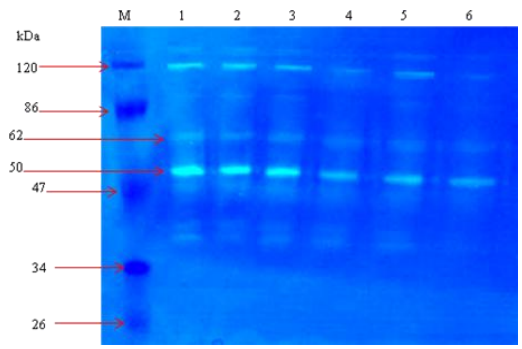

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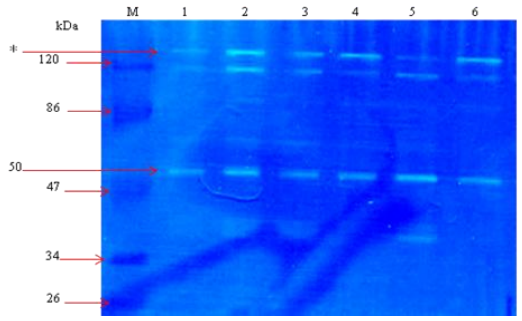

Figure 5 Autolytic enzymes produced by heterogeneous VISA and their VSSA parental strains. Autolysins were extracted from cells grown in the absence (A) and presence (B) of vancomycin at concentrations of one-half of MIC and assayed by SDS-PAGE zymogen method as described in Materials and Methods. Lanes: M, Fermentas prestained molecular size markers; 1, KY; 2, KY-9; 3, SS; 4, SS-9; 5, UH7; and 6, UH7-9. Bands with lytic activity were observed as clear zones in the opaque gel.