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Characteristics of Laboratory-Derived Vancomycin-Intermediate *Staphylococcus aureus* Strains

Sutanya Jongutsa[1, 2], Suwanna Trakulsomboon[3], Srisuda Pannanusorn[1], Ruthada Chanklan[1], Sudathip Chantorn[4], Worawat Surarit[4], Thrissawan Traijitt[4], and Sugunya Utaida[1*]

[1]Microbiology group, Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Rangsit Center, Pathum Thani 12121, Thailand
[2]Food and Drug Administration, Ministry of Health, Nonthaburi 11000, Thailand
[3]Faculty of Allied Health Sciences, Division of Medical Technology, Nakhonratchasima College, Nakhonratchasima 30000, Thailand
[4]Biochemistry and Biology group, Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Rangsit Center, Pathum Thani 12121, Thailand

*Corresponding author; E-mail: utaidas@gmail.com, sugunya9@tu.ac.th

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Abstract

Four clinical vancomycin susceptible *Staphylococcus aureus* (VSSA) isolates were subjected to selection for the vancomycin-intermediate *S. aureus* (VISA) phenotype using increasing concentrations of vancomycin. The vancomycin MIC achieved for VISA strains was 7 µg/mL. Population analysis profiles of the bacteria revealed that almost 100% of population growing at 4 µg/mL of vancomycin while some subpopulations were found to be resistant to concentrations ranging from 6 to 9 µg/mL of vancomycin. The results correlated with the AUC ratios for VISA, which ranged from 3.02-3.3 in this study. In the absence of vancomycin in the assay buffer, all the laboratory-derived VISA strains exhibited reduced whole cell autolysis compared to that of their VSSA counterparts. Examination of cell wall morphologies revealed that almost all the laboratory-derived VISA strains had thicker and rougher cell wall surfaces compared to their parental strains. Vancomycin had no remarkable effects on the thickness and roughness of the cell wall of all the laboratory-derived VISA derivatives.

Keywords**:** *vancomycin, VISA, whole cell autolysis, cell wall thickening*

1. Introduction

Staphylococcus aureus can cause a wide range of infections, from mild skin and eye infections to severe bacteremia (Ansari et al., 2019; Falcón et al., 2016; Kazimoto et al., 2018; Zhu et al., 2015). It is a member of the ESKAPE group, the most important bacterial group involved in infections and shows multidrug resistant characters (Mlynarczyk-Bonikowska et al., 2022). The emergence of infections caused by drug-resistant bacteria, especially methicillin-resistant *S. aureus* (MRSA) is a serious and growing global health concern (Gardete, & Tomasz, 2014; Shen et al., 2019; Vu et al., 2021). The glycopeptide vancomycin, first released in 1958 (Howden et al., 2010), has been recommended as a therapeutic agent for treating MRSA, which are often multi-drug-resistance (Ansari et al., 2019). In 1997, there were reports from Japan of *S. aureus* isolates displaying reduced susceptibility to vancomycin. These isolates are commonly known as vancomycin-intermediate *S. aureus* (VISA)

(Hiramatsu et al., 1997a) and heterogeneous VISA (hVISA) (Hiramatsu et al., 1997b). Since then, both hVISA and VISA isolates have been increasing reported around the world (Cui et al., 2021; Park et al., 2019; Shen et al., 2019; Zhu et al., 2015), including Southeast Asia (Ahmad et al., 2012; Lulitanond et al., 2009). There are many reports of phenotypic features of hVISA/VISA isolates. Cell wall thickening is the most common characteristic of hVISA/VISA strains that is associated with resistant to vancomycin of the bacteria (Cui et al., 2021; Falcón et al., 2016; Peng et al., 2017; Xu et al., 2018; Zhu et al., 2015). Additionally, altered autolytic activity is always associated with vancomycin resistance in *S. aureus*. Reduced autolysis of hVISA/VISA has been reported in several studies (Peng et al., 2017; Xu et al., 2018; Zhu et al., 2015). The emergence of hVISA/VISA infections threatens the efficacy of vancomycin therapy since vancomycin treatment of VISA infections often resulted in treatment failure (Gardete, & Tomasz, 2014). Accumulation of gene mutations may be involved in the evolution of these bacteria. Nevertheless, the molecular mechanism of VISA resistance remains elusive. To better clarify the mechanism of vancomycin resistance in *S. aureus*, it is very crucial to have both VISA and its isogenic vancomycin sensitive *Staphylococcus aureus* (VSSA) strains as the study model. Identification of the mutated genes in the VISA strains can then be performed by comparison between resistant and susceptible isolates. Hence, in this study, the laboratory derived VISA strains were generated, and their characteristics were then examined. The information achieved from this study can strongly support other available data regarding features of VISA isolates. Long term exposure of VSSA bacteria in the laboratory setting to vancomycin results in the development of an intermediate resistant phenotype over time. This is very challenging for long term clinical therapy. Lastly, future studies to uncover the exact molecular mechanism of the emergence of VISA will be plausible as more study models with same genetic backgrounds are available.

2. Objective

This study aims to investigate phenotypic characteristics of the laboratory-derived VISA strains obtained through passage selection of clinically vancomycin susceptible *S. aureus* (VSSA) isolates on increasing concentrations of vancomycin.

3. Materials and Methods

3.1 Bacterial strains and growth conditions

Four clinical isolates (KY, SS, UH7, and UH9) of vancomycin-susceptible *S. aureus* (VSSA) and their relevant VISA derivatives (KY-10, SS-10, UH7-10, and UH9-10) were used throughout the study. The bacterial strains were stored in 30% (v/v) glycerol containing brain heart infusion (BHI; BD) broth at -20°C. Unless otherwise stated, BHI broth was used for liquid cultures that were grown at 37°C with shaking at 200 rpm.

3.2 Passage selection for the laboratory-derived *S. aureus* strains with decreased susceptibility to vancomycin and population analysis profile

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 strain into 0.5 µg/mL of vancomycin (SIGMA) containing BHI broth 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 ranging from 0.5 ,1, 1.5, 2, 2.5, and 3 µg/mL. Minimum inhibitory concentrations (MICs) of vancomycin of the bacterial tested were examined employing a microdilution method (Pfeltz et al., 2000). Briefly, 96- well microtiter plates containing two-fold serial dilutions of vancomycin in BHI broth 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 as the MIC. Population analysis was performed as previously described (Pfeltz et al., 2000) by utilizing *S. aureus* strain Mu3 (Hiramatsu et al., 1997b) as a control. 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 to 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.

3.3 Whole-cell autolysis assay

The procedure was carried out as previously described (Utaida et al., 2006). Overnight cultures of bacterial cells in BHI broth were transferred into 100 mL of tryptic soy broth (TSB; BD) and 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), washed twice with ice-cold 0.9% NaCl, and resuspended in 50 mL of 0.01 M sodium phosphate buffer (Na_2HPO_4-NaH_2PO_4, pH 7.0) to an initial OD_{600} of about 0.8. The cell suspension was 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. When needed, vancomycin at concentration of one-half of the MICs was included in the assay buffer before suspending the cells.

3.4 Cell wall morphology by transmission electron microscopy

Samples were prepared for transmission electron microscopy as described by 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 (10,000 g, 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 left them to solidify. The water agar was 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 resuspended 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. Evaluation of cell wall thickness was performed by measurement from photographs of transmission electron microscope. Four cells of each strain with nearly equatorial cut surfaces were measured. The number of spots, which was measured of each cell, was eight spots.

4. Results and Discussion

4.1 Passage selection for the laboratory-derived VISA isolates and their population analysis profiles

Passage of four clinical VSSA isolates, namely KY, SS, UH7, and UH9 (vancomycin MICs of 1 µg/mL), on increasing concentrations of vancomycin in BHI liquid cultures resulted in *S. aureus* strains with reduced susceptibility to vancomycin. The passage, with increasing concentrations of vancomycin ranging from 0.5, 1, 1.5, 2, 2.5, to 3 µg/mL, led to an increase in vancomycin MICs from 1 to 7 µg/mL. These strains were defined to be intermediate resistance according to the Clinical and Laboratory Standards Institute (CLSI, 2021) guideline that defines staphylococci for which the MICs of vancomycin are ≤ 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 ≥ 16 µg/mL to be resistant.

All the laboratory-derived *S. aureus* with vancomycin MICs of 7 µg/mL were analyzed for heterogeneous vancomycin-resistant subpopulations by using population analysis. The area under concentration curve (AUC) of population analysis profile of each strain was calculated. The ratios of AUC of test strains to that of Mu3 (hVISA; vancomycin MIC value of 2 µg/mL), a control strain, were then determined (Ahmad et al., 2012; Holmes et al., 2012; Xu et al., 2018; Zhu et al., 2015). Increasing of the vancomycin MICs from 1 to 7 µg/mL resulted in subpopulations that were resistant to vancomycin at concentrations of 5 µg/mL and higher (Figure 1). All strains tested 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. Mu3, however, gave a different pattern of resistant subpopulation profile, which with smaller populations grew at vancomycin concentration of 4 µg/mL. The results suggested that the population profiles of the bacterial strains with the vancomycin MICs of 7 µg/mL are like to uniformity 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, & Chambers, 2003). Thus, these derivatives are

denoted as VISA and the result correlated with the area under the curve (AUC) ratios achieved, which were in the range of 3.02-3.32 (Table 1). Additionally, the values were much higher than that of hVISA range (0.9-1.3) (Ahmad et al., 2012; Holmes et al., 2012; Xu et al., 2018; Zhu et al., 2015). These suggested that long-term exposure to vancomycin of VSSA favors the development to VISA. Nevertheless, we cannot exclude that there may be a heterogeneous VISA developed during the passage selection for VISA. Gardete, & Tomasz (2014) has mentioned that hetero-VISA appears to be the stage that precedes the development of intermediate-level resistance in *S. aureus*. Most cells in cultures of heterogeneous VISA strains have low vancomycin MIC values, close to those of susceptible isolates. In the same cultures, however, low-frequency subpopulations of bacteria with increased MIC value of vancomycin are present as well. The homogeneously resistant VISA isolates, such as our VISA strains herein, contain populations with higher vancomycin MIC values than those of the hetero-VISA strains. The development of VISA may occur from mutations in genes encoding two-component systems (TCSs) such as *vraSR* (Chen et al., 2016; Gardete, & Tomasz, 2014) and *walKR* (Gajdiss et al., 2020; Gardete, & Tomasz, 2014; Howden et al., 2011; Peng et al., 2017). These genes function to control the transcription of genes involved in cell wall synthesis of *S. aureus*. Chen et al. (2016) demonstrated that *vraS*, a gene encoded for a histidine protein kinase VraS, was overexpressed in hVISA. VraS regulates the activity of VraR that functions to stimulate the gene expression required for cell wall synthesis. In addition, a study by Doddangoudar et al. (2012) has also shown that mutation in *vraS* appears to be essential for VISA development, with stop codons playing an important role in delaying non-susceptibility development and reversion. Interestingly, mutations in WalKR, that is essential for maintenance of cell wall metabolism, have been found frequently in clinical VISA isolates (Gardete, & Tomasz, 2014; Howden et al., 2011). It was also found that the WalK mutation in the laboratory-derived VISA strain influences vancomycin resistance (Shoji et al., 2011). Additionally, Peng et al. (2017) reported that the WalK mutation plays a key role in mediating vancomycin resistance in XN108, the first VISA isolate of the ST239 identified in mainland China.

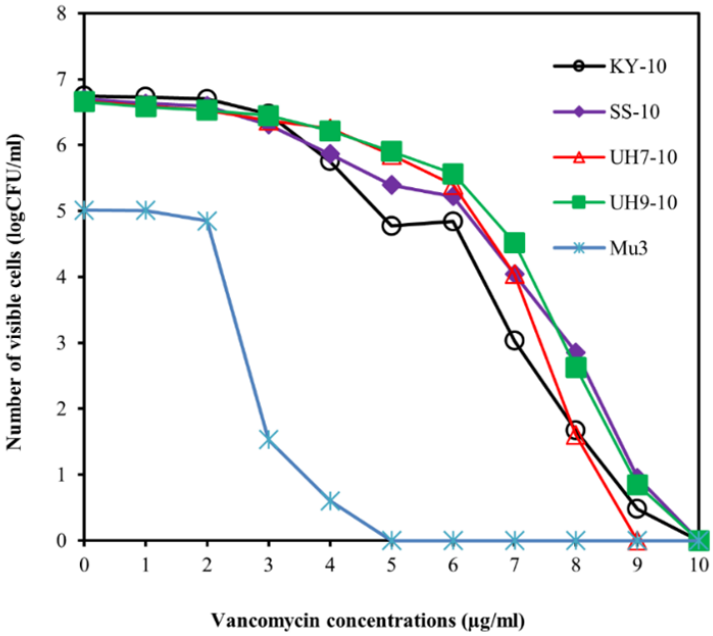

Figure 1 Population analysis profiles of the laboratory-derived VISA and Mu3 (control) strains

Table 1 AUC values of the laboratory-derived VISA strains and AUC ratios

Bacterial strains	AUC values	AUCs of tested strains: AUC of Mu3 (14.5)
KY-10	43.80	3.02
SS-10	47.34	3.26
UH7-10	46.03	3.17
UH9-10	48.23	3.32

4.2 Whole-cell autolytic activities

Figure 2 shows whole cell autolytic activities of all the laboratory-derived VISA isolates tested. Generally, decreased whole cell autolysis has been reported for VISA isolates compared to that of vancomycin susceptible isolates (Hu et al., 2013; Peng et al., 2017; Utaida et al., 2006; Xu et al., 2018; Zhu et al., 2015). Consistently, our results demonstrated herein that strain KY-10 had decreased whole cell autolysis compared to that of its vancomycin susceptible parental KY strain (Figure 2A). Similarly, the other three laboratory-derived VISA strains showed decreased whole cell autolytic activities in comparison to their respective parental isolates (Figures 2B-2D). Reduced autolytic activity is a common early phenotypic change in serial isolates obtained during persistent infection (Howden et al., 2010). Interestingly, it has been reported that a mutation in the WalK, which functions (with WalR) to control the transcription of cell wall lysing enzymes (Gajdiss et al., 2020), also confers this reduced autolytic activity phenotype of the VISA strain (Peng et al., 2017). Peng et al. (2017) have reported that the expression of genes involved in cell wall autolysis, such as *atlA*, *ssaA*, *isaA*, and *lytM* was significantly decreased in the VISA isolate XN108. The results were consistent with results from their autolysis assay. The authors implied that WalK(S221P) mutation in the XN108 plays a key role in the promotion of vancomycin resistance by decreasing the expression of autolysis genes resulting in reduced autolysis activity. Furthermore, Gajdiss et al. (2020) reported that depletion of YycH and YycI by antisense RNA-based approach caused impaired autolysis through direct modulation of WalRK phosphorylation activity. In the antisense *yycHI* strains, autolysis was reduced, and autolysin extracts exhibited less lysis of *Micrococcus luteus* cells in zymographic analysis. Similarly, reduced whole cell autolysis profiles observed for our VISA derivatives are supported by zymogram analyses employing *M. luteus* cells as substrates that revealed less intensities of the autolytic activity bands of the VISA strains compared to those of their parental counterparts (data not shown). Moreover, a study by Cameron et al. (2016) has also revealed that transcriptional profiling of *yycH* and *yycI* deletion mutants resulted in downregulation of the WalRK regulon including cell wall hydrolase genes *atlA* and *sle1*, with functional autolysis assays supporting these data by display an impaired autolytic phenotype for each deletion strain. The results highlighted a novel role for YycH and YycI in the development of reduced vancomycin susceptibility in *S. aureus.* Recently, Zhu et al. (2021) also demonstrated that the transcriptional levels of genes associated with autolysis, such as *atlA* and *sle1*, were significantly decreased in a novel mutation of WalK (I237T) VISA derivative.

We further examined the effect of vancomycin on whole cell autolytic activity of the laboratory-derived VISA strains by including the drug at concentration of one half of the MIC (3.5 µg/mL) in the assay buffer before the optical density was taken. It was observed that vancomycin had no detectable effects on whole cell autolysis of strain KY-10. Almost comparable rates of whole cell autolysis were observed for KY-10 in the presence and absence of vancomycin (Figure 2A). Similarly, whole cell autolysis activity of strain UH9-10 was not clearly changed in the presence of vancomycin as shown in Figure 2D. The laboratory-derived VISA strains SS-10 and UH7-10, however, showed a slight decrease in whole cell autolysis in the presence of vancomycin compared to those of the absence of vancomycin (Figure 2B and 2C). Decreased whole cell autolysis was also observed for all VSSA strains when vancomycin was presented in the assay buffer (Figure 2).

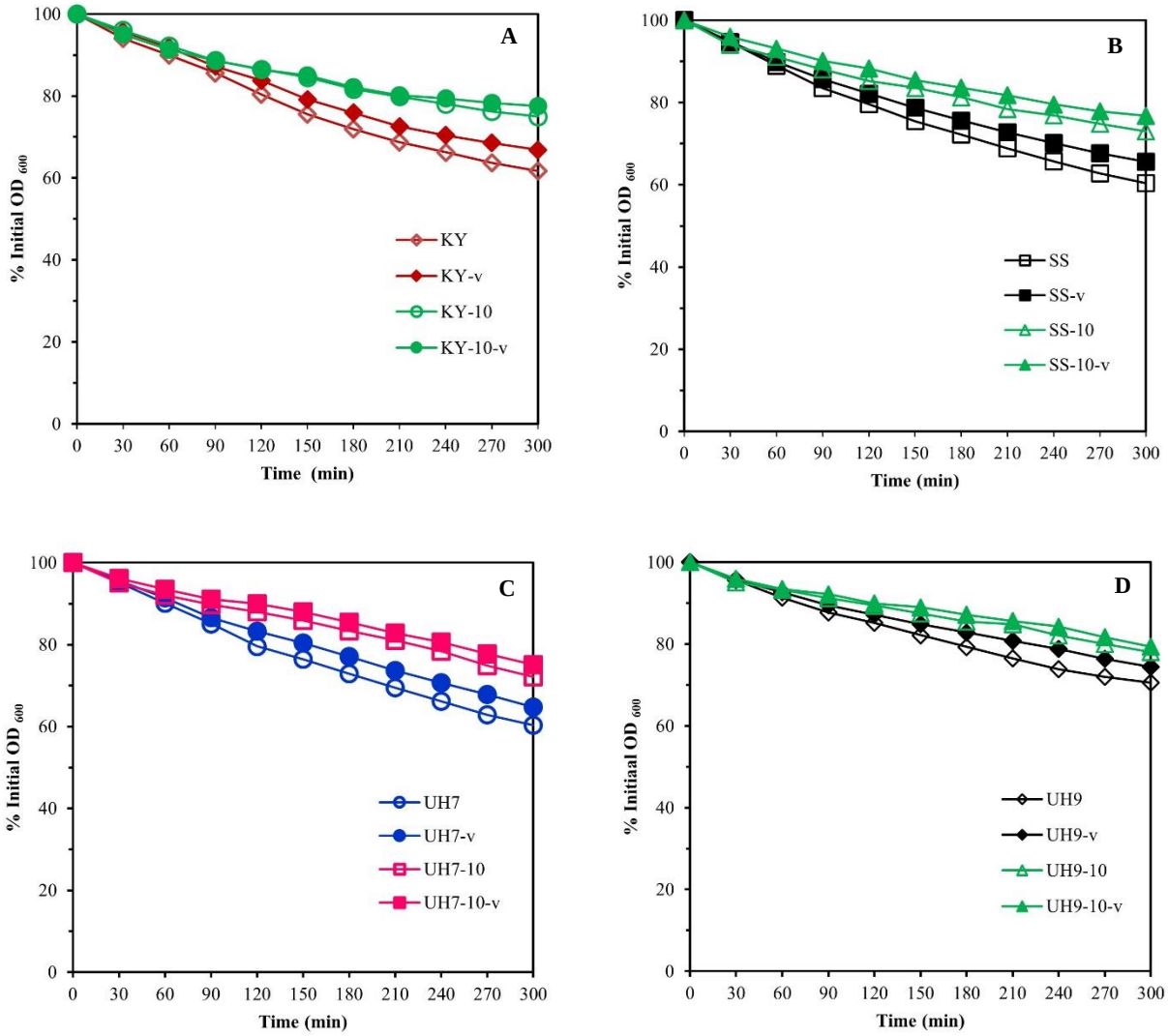

Figure 2 Whole cell autolytic activity profiles of VISAs and their respective parental VSSAs: KY-10 and KY (A), SS-10 and SS (B), UH7-10 and UH-7 (C), and UH9-10 and UH9 (D). Isolates in the absence (open symbol) and presence (closed symbol) of vancomycin (v) at concentrations of one half of MICs.

Pfeltz et al. (2000) reported that vancomycin inhibits whole cell autolysis of *S. aureus* strains with reduced susceptibility to the drug. It has been speculated that the inhibition of autolysis by vancomycin appears to be a general feature of *S. aureus* strains, reflecting the ubiquity of D-alanyl-D-alanine moieties in the cell wall of this bacterial species (Sieradzki, & Tomasz, 2006). In addition, it is clearly seen from our results herein that whole cell autolysis of different bacterial strains was affected differently by vancomycin. This may result from the different expression of genes associated with autolysis of the bacteria. It has been reported that in the presence of vancomycin at the subinhibitory concentrations, VISA and hVISA showed a very low transcription level of the *atl* gene with respect to the drug-free conditions. Moreover, *atl* expression ratio between the two strains was lower in VISA than hVISA (Cafiso et al., 2012). Similarly, Utaida et al. (2003) found that *atl* expression was reduced in *S. aureus* exposed to cell wall-active antibiotics. On the contrary, *atl* and *lytM* genes were upregulated in the vancomycin-resistant passage derivatives (Mongodin et al., 2003). Interestingly, it has been shown that sublethal doses of vancomycin induce biofilm

formation through an autolysis-dependent mechanism in vancomycin-non-susceptible *S. aureus* (Hsu et al., 2011). Additionally, *cid*/*lrg*, the genes that regulate autolysis in *S. aureus*, were found to be up-regulated in cells grown in the presence of vancomycin compared with unexposed controls. Vancomycin has poor tissue penetration. VISA strains or even vancomycin-susceptible and -tolerant organisms or heterogeneous VISA strains could survive and have the greatest possibility to enhance biofilm formation in sequestered sites leading to treatment failure (Hsu et al., 2011).

4.3 Cell wall morphologies of the laboratory-derived VISA derivatives

We also determined the cell wall morphologies of the laboratory-derived VISA derivatives by transmission electron microscopy. Table 2 shows the results of cell wall thickness measurement for VISA derivatives and their parental VSSA strains. The statistical significance of the data was evaluated at the significance level of 0.05. Almost all the VISA derivatives, KY-10, SS-10, and UH7-10, had significantly thicker cell walls than their parental strains (p-value = 0.001, 0.015, and 0.022, respectively). No significant difference in wall thickness was observed for UH9-10 and its parental strain (p-value = 0.091). Transmission electron micrographs of VISA derivatives and their parental strains are shown in Figures 3 to 6. Cell wall surfaces of KY-10, SS-10, and UH7-10 appeared to be more roughened than those of their parental strains (Figures 3 to 5). The roughened cell wall was not observed for UH9-10 (Figure 6). The results suggested that the thickened and roughened walls observed for VISA correlate with levels of vancomycin MICs. Cui et al. (2003) reported that *S. aureus* with reduced susceptibility to vancomycin had thickened cell wall and became thinner when bacteria decreased vancomycin MICs, indicating that cell wall thickening correlated with vancomycin resistance and was a common feature of VRSA or VISA. Peng et al. (2018) cured the mutations that contributed to the development of the VISA strain XN108 by using allelic replacement experiments with the native alleles derived from a vancomycin-susceptible *S. aureus* isolate DP65. They found that the isogenic mutant strains exhibited decreased vancomycin resistance (vancomycin MIC decreased from 12 to 4 μg/mL). These mutant strains with increased susceptibility to vancomycin had thinner walls compared to their parental VISA isolates.

Previous reports have revealed cell wall thickening in VISA strains (Peng et al., 2017; Peng et al., 2018; Pfeltz et al., 2000; Xu et al., 2018; Zhu et al., 2015). The production of an excess amount of peptidoglycan or reduced peptidoglycan turnover and cell lysis can affect cell wall synthesis in bacteria, influencing the mechanism of cell wall thickening (Cafiso et al., 2012; Cázares-Domínguez et al., 2015). In VISA isolates, the rate of arrival of vancomycin molecules to the sites of cell wall synthesis is delayed due to the presence of excess D-ala-D-ala residues, which could capture and slow down the progress of the antibiotic to the true site of cell wall biosynthesis at the bacterial septum (Gardete, & Tomasz, 2014). VISA strains use cell wall thickening as a vancomycin tolerant mechanism, suggesting that cell wall thickening is indispensable for resistance to the drug (Cázares-Domínguez et al., 2015). Additionally, thickened cell walls have been reported for macrolide-and gentamicin-resistant *S. aureus*, and it is a common ultrastructural characteristic of the bacteria (Fukutsuji et al., 2013; Hyo et al., 2013). Engrossingly, it has been reported that increased thickness of the cell wall of *Streptococcus anginosus* in a multispecies biofilm contributed to decreased susceptibility of the bacteria to vancomycin (Tavernier et al., 2018).

Additionally, we found that cells grown with vancomycin at one-half MICs for a transient period displayed no remarkable changes of cell wall morphologies. The thickness of the cell wall and wall surfaces of all VISA derivatives appeared to be no different between the absence and presence of vancomycin (Table 2 and Figures 3 to 6). Similarly, almost all parental strains had no differences in thickness and roughness when growing in the presence of vancomycin. All the laboratory-derived VISA isolates herein responded to vancomycin treatment in the same way as their parental VSSA strains, although UH7 had a slightly thickened cell wall in the presence of vancomycin (p-value = 0.031) (Table 2). Obviously, these responses are due to genetic background of each bacterial strain tested. Pfeltz et al. (2000) reported that VISA had thickened and roughened cell wall when grown in the presence of vancomycin. The authors suggested that genetic background of bacteria and the ratio of cell wall material to vancomycin molecules may play a role in roughening of the wall.

5. Conclusion

Passage of VSSA clinical isolates on increasing concentrations of vancomycin resulted in bacterial strains with reduced susceptibility to vancomycin from 1 to of 7 µg/mL. Population analysis profiles of the bacteria and their AUC ratio revealed the presence of VISA isolates. Decreased whole cell autolysis was observed for all the laboratory-derived VISA strains compared to their relevant VSSA isolates. Vancomycin affected differently on whole cell autolysis in each bacterial strain tested. Additionally, thicker walls and rougher surfaces were observed in the laboratory-derived VISA strains compared to their parental strains. When vancomycin was included in the medium, the thickness and roughness of cell wall of all VISA derivatives and almost all VSSA tested did not appear to differ between the bacteria grown in absence and presence of the drug. The data obtained from the present study can strongly support other available information regarding the features of VISA isolates. Long term exposure of the susceptible *S. aureus* strain to gradually increasing concentrations of vancomycin resulted in the emergence of bacterial isolates with intermediate resistance. In a clinical environment, this may lead to fewer options of antibiotics available for the treatment of infections caused by VISA. Lastly, further experiments such as comparative genome analysis between the VISA and its isogenic VSSA strain to determine for the altered genes, inactivation of the target genes, complementation studies, and transcriptome analysis; can then be performed to unveil the exact molecular mechanism of the emergence of VISA, as the study models with same genetic backgrounds are available.

Table 2 Cell wall thickness of VISA derivatives and their VSSA parental strains

Bacterial strains	**Cell Wall Thickness (nm ± SD)**	
	Bacteria grown in the absence of vancomycin	**Bacteria grown in the presence of vancomycin**
KY	38.48 ± 1.31	39.58 ± 2.40
KY-10	52.73 ± 3.31[a]	53.02 ± 2.76
SS	45.09 ± 4.01	46.35 ± 4.19
SS-10	54.64 ± 2.01[a]	56.16 ± 2.28
UH7	47.12 ± 2.57	51.45 ± 2.08[b]
UH7-10	56.21 ± 3.98[a]	56.41 ± 4.12
UH9	34.89 ± 2.62	37.49 ± 1.72
UH9-10	39.54 ± 1.78	42.64 ± 3.27

[a]The thickness of cell wall of VISA strains are significantly greater than those of their VSSA parental strains ($p < 0.05$).

[b]The thickness of cell wall of UH7 grown in the presence of vancomycin with concentration of one-half of its MIC is significantly greater than that grown in the absence of the drug ($p<0.05$).

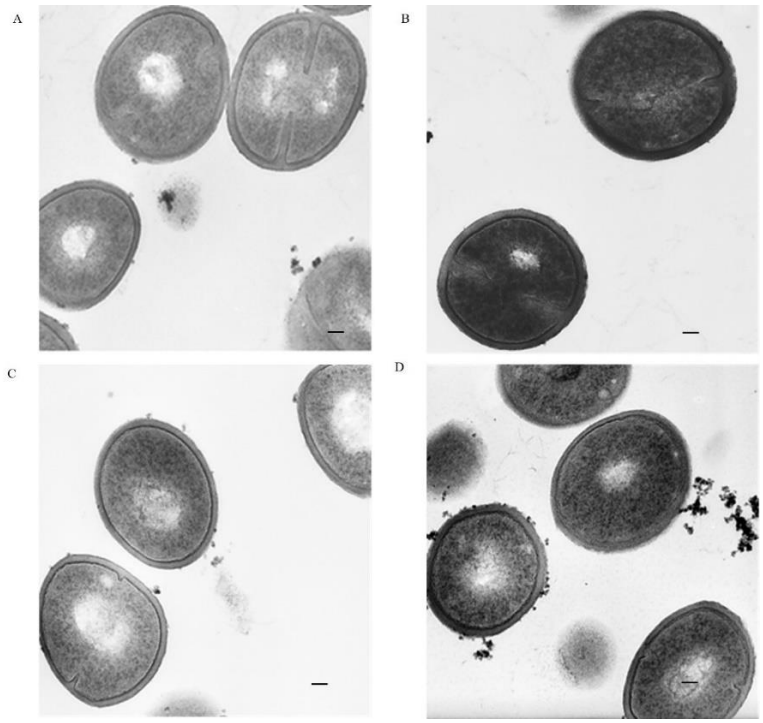

Figure 3 Transmission electron micrographs of VISA Derivative KY-10 and its parental strain KY. Magnification, x20,000. Bar= 100 nm. KY grown in the absence (A) and presence (C) of one-half of the MICs (0.5 µg/mL). KY-10 grown in the absence (B) and presence (D) of one-half of the MICs (3.5 µg/mL).

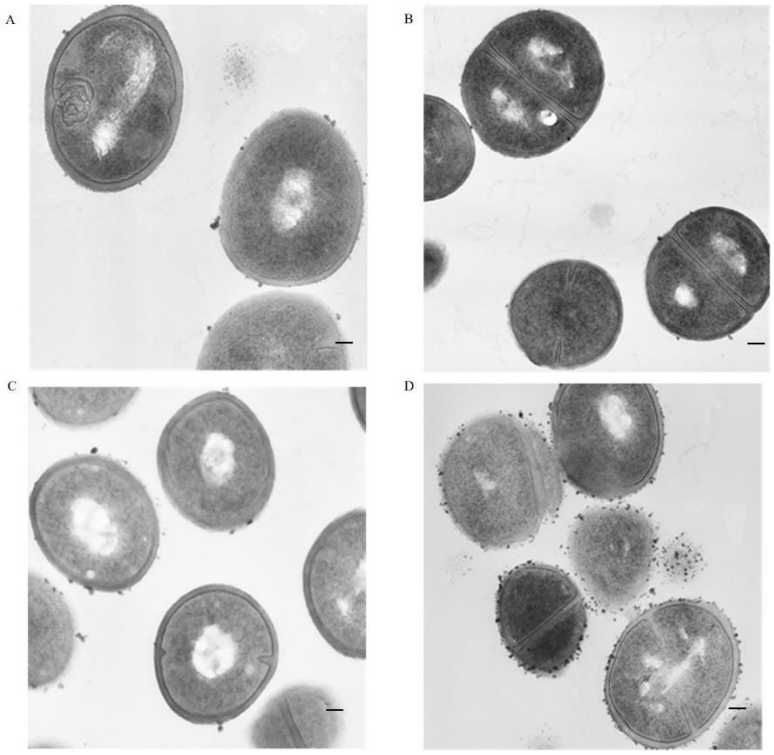

Figure 4 Transmission electron micrographs of VISA Derivative SS-10 and its parental strain SS. Magnification, x20,000. Bar = 100 nm. SS grown in the absence (A) and presence (C) of one-half of the MICs (0.5 µg/mL). SS-10 grown in the absence (B) and presence (D) of one-half of the MICs (3.5 µg/mL).

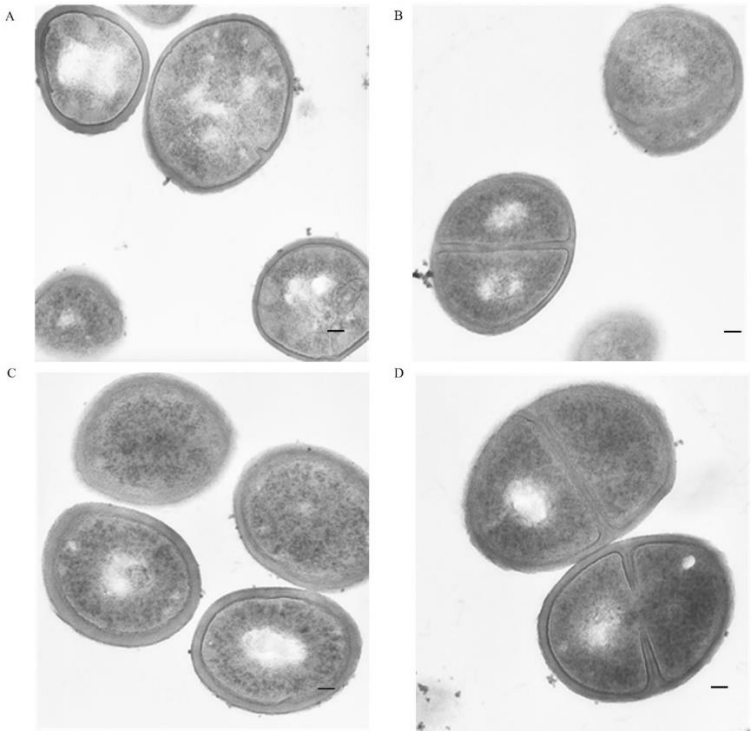

Figure 5 Transmission electron micrographs of VISA Derivative UH7-10 and its parental strain UH7 Magnification, x20,000. Bar = 100 nm. UH7 grown in the absence (A) and presence (C) of one-half of the MICs (0.5 µg/mL). UH7-10 grown in the absence (B) and presence (D) of one-half of the MICs (3.5 µg/mL).

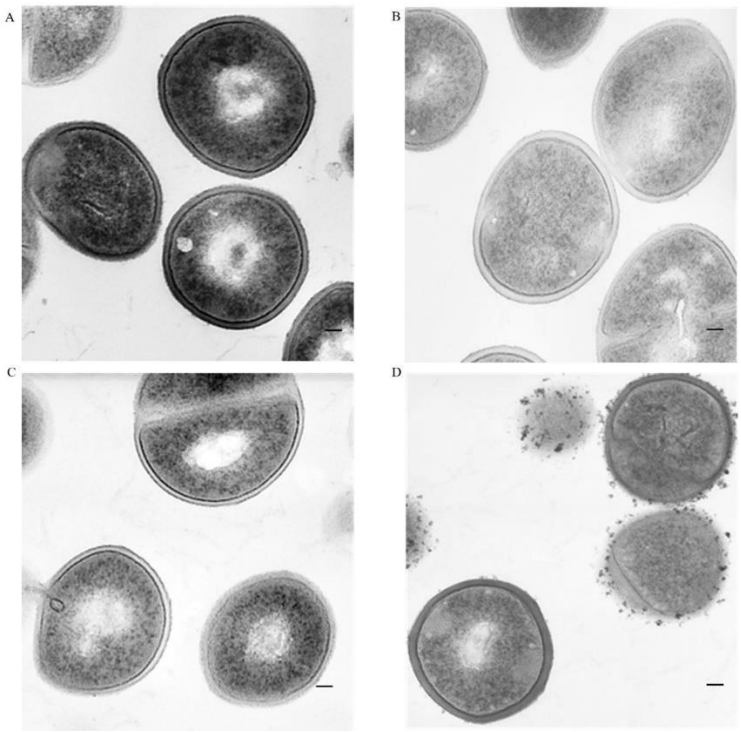

Figure 6 Transmission electron micrographs of VISA Derivative UH9-10 and its parental strain UH9 Magnification, x20,000. Bar= 100 nm. UH9 grown in the absence (A) and presence (C) of one-half of the MICs (0.5 µg/mL). UH9-10 grown in the absence (B) and presence (D) of one-half of the MICs (3.5 µg/mL).

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