Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Comparative proteomics profiling reveals down-regulation of *Staphylococcus aureus* virulence in achieving intermediate vancomycin resistance

Xin-Ee Tan[1], Hui-min Neoh[1,2]*, Mee-Lee Looi[1,3], Toh Leong Tan[4], Salasawati Hussin[5], Longzhu Cui[6], Keiichi Hiramatsu[2] and Rahman Jamal[1]

[1]UKM Medical Molecular Biology Institute (UMBI), Universiti Kebangsaan Malaysia, 56000 Cheras, Malaysia.
[2]Department of Bacteriology, School of Medicine, Juntendo University, Tokyo 113-8421, Japan.
[3]Taylor's University Lakeside Campus, School of Biosciences, 47500 Subang Jaya, Malaysia.
[4]Department of Emergency Medicine, Faculty of Medicine, Universiti Kebangsaan Malaysia, 56000 Cheras, Malaysia.
[5]Department of Medical Microbiology and Immunology, Faculty of Medicine, Universiti Kebangsaan Malaysia, 56000 Cheras, Malaysia.
[6]Division of Bacteriology, Department of Infection and Immunity, School of Medicine, Jichi Medical University, Tochigi 329-0498, Japan.
Email: hui-min@ppukm.ukm.edu.my

ABSTRACT

Aims: VraSR and GraSR were shown to be important in conferring intermediate vancomycin resistance in VISA. Nevertheless, the exact mechanism modulated by these systems leading to the development of VISA remains unclear. We employed a proteomic approach to determine the VraS and GraR regulons and subsequently derive the possible vancomycin resistance regulatory pathway(s) in the Mu50 lineage of *Staphylococcus aureus*.
Methodology and results: *Staphylococcus aureus* strains Mu50Ω, Mu50Ω-*vraS*m and Mu50Ω-*vraS*m-*graR*m are isogenic strains with ascending levels of vancomycin resistance. Total proteins were extracted from the 3 strains and trypsin digested prior to protein isolation and identification by LC-ESI MS/MS and PLGS 2.4. Expression profiles of resulting proteins were analyzed using Progenesis LC/MS software. Differential expression profiles revealed 3 regulons, each controlled by VraS (Mu50Ω-*vraS*m vs Mu50Ω), GraR (Mu50Ω-*vraS*m-*graR*m vs Mu50Ω-*vraS*m) and VraS-GraR (Mu50Ω-*vraS*m-*graR*m vs Mu50Ω), respectively. The regulon down-regulated by VraS in Mu50Ω-*vraS*m were proteins associated with virulence (MgrA, Rot, and SarA), while GraR up-regulated resistance-associated proteins (TpiA, ArcB and IsaA) in Mu50Ω-*vraS*m-*graR*m. The VraS-GraR regulon mediated both up-regulation of resistance-associated proteins (ArgF, ArcB, VraR and SerS) and down-regulation of virulence-associated protein GapB.
Conclusion, significance and impact of study: Down-regulation of virulence- in concert with up-regulation of resistance-associated proteins appears to be integral for development of intermediate-vancomycin resistance in the Mu50 lineage of *S. aureus*.

Keywords: vancomycin-intermediate, *Staphylococcus aureus*, virulence, proteomics profiling

INTRODUCTION

Medical attention on *Staphylococcus aureus* has increased ever since reports of strains having various levels of resistance towards vancomycin – "drug of last resort" for *S. aureus* infections – were published (CDC 1997; Hiramatsu *et al.*, 1997; Ploy *et al.*, 1998; Bierbaum *et al.*, 1999; Kim *et al.*, 2000; Oliveira *et al.*, 2001; CDC 2002; Denis *et al.*, 2002; Tiwari *et al.*, 2006; Aligholi *et al.*, 2008; Saha *et al.*, 2008; Azimian *et al.*, 2012). The mechanism behind complete vancomycin resistance exhibited in vancomycin-resistant *S. aureus* (VRSA) has been shown to be due to the transfer of a multi-resistant conjugative plasmid harboring the *vanA* operon from *Enterococcus faecalis* to *S. aureus* (Weigel *et al.*, 2003). However, genetic factor(s) leading to the development of intermediate vancomycin resistance in *S. aureus* (vancomycin-intermediate *S. aureus*, VISA) is still not well understood.

A number of genes (*vraS*, *graR*, *pbp4*, *mgrA*, *sarA*, *isdE*, *agrC*) have been reported to be associated with VISA (Finan *et al.*, 2001; Cui *et al.*, 2009; Trotonda *et al.*, 2009; Howden *et al.*, 2010). Among these, 2 two-component regulatory systems in *S. aureus*, VraSR (vancomycin-resistance associated sensor/regulator) and GraSR (glycopeptides-resistance sensor/regulator), were recently shown to be associated with intermediate vancomycin resistance in the Mu50 (the world's first reported VISA) lineage of *S. aureus* strains (Cui *et al.*, 2009). The report showed that the introduction of mutated

***Corresponding author**

ISSN (print): 1823-8262, ISSN (online): 2231-7538

vraS and *graR* in Mu50Ω, a susceptible isogenic strain of Mu50, resulted in a remarkable increase in its vancomycin resistance (Cui *et al.*, 2009) (Figure 1). Nevertheless, the pathway(s) regulated by these 2 two-component regulators in achieving *S. aureus* intermediate resistance remains obscured.

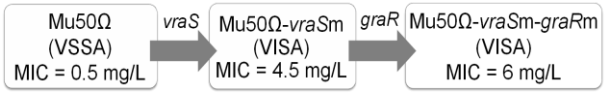

Figure 1: Conversion of vancomycin-susceptible *S. aureus* (VSSA) to a "beginner VISA" and later the Mu50-like VISA through stepwise acquisition of mutated *vraS* and *graR* genes (Cui *et al.*, 2009).

In this study, we employed a proteomic approach using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI MS/MS) technology to define probable regulons regulated by each of the VraSR and GraSR systems, and subsequently derived the possible vancomycin intermediate-resistance regulatory pathway in *S. aureus* strains of the Mu50 lineage.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Three isogenic strains of *S. aureus*, namely Mu50Ω, Mu50Ω-*vraS*m and Mu50Ω-*vraS*m-*graR*m (minimum inhibitory concentration (MIC) of vancomycin = 1 mg/L, 4.5 mg/L and 6 mg/L, respectively), used in this study have been described previously (Cui *et al.*, 2009). Briefly, Mu50Ω is a VSSA strain found at the same site where Mu50 was isolated. Chromosomal substitution of Mu50 *vraS* into Mu50Ω resulted in the strain Mu50Ω-*vraS*m, while Mu50 *graR* substitution in the chromosome of Mu50Ω-*vraS*m resulted in the strain Mu50Ω-*vraS*m-*graR*m. Strains were cultured in Brain Heart Infusion (BHI) broth (Becton Dickinson, USA) at 37 °C prior harvest at an optical density of 540 nm (OD_{540}) = 6.

Preparation of protein extracts

Fifty mL of bacterial culture for each tested strain was used for protein extraction. Cultures were pelleted and resuspended in 5 mL lysis buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$ and protease inhibitor (Roche, Switzerland) in phosphate buffered saline). Cells were then lysed with lysostaphin (Sigma Aldrich, USA) prior digestion with DNase I (Sigma Aldrich, USA). Cell debris was separated from proteins by centrifugation for 40 min at 4 °C and 8,000 *x g*. The proteins were precipitated by overnight incubation of the supernatant at −20 °C with ice cold acetone and collected via centrifugation for 40 min at 4 °C and 8,000 *x g*. The protein extracts were then air-dried and resolved in lysis buffer.

Protein clean-up and quantification

Extracted proteins were purified using Ettan™ 2-Dimensional (2D) Clean-Up Kit (Bio-Rad, Hercules CA, USA) according to manufacturer's instruction and resolved in a buffer solution containing 8 M urea and 100 mM Tris (pH 8.5). Protein concentrations were determined using Ettan™ 2-D Quant Kit (GE Healthcare Bio-Sciences Corp., USA) and absorbance was read at 480 nm using a spectrophotometer. Measurements were performed three times for each strain.

Protein identification using nanoLC-ESI MS/MS

nanoLC-ESI MS/MS analysis

Proteins were trypsin digested (Becher *et al.*, 2009) prior separation by reversed phase liquid chromatography and subsequent ESI tandem mass spectrometry. NanoLC-ESI MS/MS analysis was performed using a nanoACQUITY™ UPLC system (Waters, USA) coupled to a Q-Tof Premier™ mass spectrometer (Waters, USA). The analysis was repeated 3 times for each strain.

Peptides were loaded onto a trap column (nanoAcquity UPLC™ Trap Column, Symmetry® C18, 5 µm, 180 µm × 20 mm, Waters, USA). Following that, elutions were performed onto an analytical column (nanoAcquity UPLC™ column, BEH130 C18 1.7 µm, 75 µm x 200 mm, Waters, USA) by a binary gradient of buffer A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) over a period of 120 min with a flow rate of 0.3 µL/min. An electrospray was created from the Picotip™ EMITTER (SilicaTip™, FS360-20-10-N-20-C6.35CT, none coating, New Objective, USA) by the application of 2.5-3.0 kV.

A full scan in the Q-Tof (*m/z* 50-1990) with a resolution of 10,000 was performed for MS/MS analysis and the precursors were excluded for 1 s.

Protein identification

All MS/MS samples (*.raw files) were searched against a database composed of all *S. aureus* protein sequences extracted from UniProt using ProteinLynx Global Server (PLGS) 2.4 (Waters, USA) as the search engine. The samples were searched with trypsin as the primary digest reagent and allowing for 1 missed cleavage site. Resulting *.xml files were further analyzed for differential protein expression.

Differential protein expression analysis

Differential protein expression profiling was performed using Progenesis LC-MS software version 4.0 (Nonlinear Dynamics, UK). Peptides with charge states 1+ and ≥ 4+ were omitted, and those with score < 1 as well as hits equal to 1 were excluded. The resulting false positive rate for protein identification was set at ≤ 0.05%. Protein inventories generated were then compared between the 3 isogenic strains to identify the differentially expressed proteins. A fold change cutoff of ≥ 2 was applied.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

RESULTS

Comparative proteomic profiling of three tested strains (Mu50Ω, Mu50Ω-*vraS*m and Mu50Ω-*vraS*m-*graR*m) revealed only 21 differentially expressed proteins regulated by the 3 distinct regulons: VraS (comparative proteomic profiling between Mu50Ω and Mu50Ω-*vraS*m), GraR (Mu50Ω-*vraS*m vs Mu50Ω-*vraS*m-*graR*m) and VraS-GraR (Mu50Ω vs Mu50Ω-*vraS*m-*graR*m).

Table 1: Differentially expressed proteins in Mu50Ω-*vraS*m versus Mu50Ω with a fold change cutoff of ≥2.

Domain	ORF No.	Product/Function	Expression Changes	COG Functional Category
Information storage and processing	SAV1764	HTH type transcriptional regulator rot (Rot)	Down-regulated	K
	SAV0616	Transcriptional regulator sarA (SarA)	Down-regulated	K
	SAV0686	HTH type transcriptional regulator mgrA (MgrA)	Down-regulated	K
Cellular processes and signaling	SAV2569	Probable transglycosylase isaA (IsaA)	Down-regulated	M
	SAV0111	Immunoglobulin G binding protein A (Spa)	Down-regulated	M
	SAV0774	Triosephosphate isomerase (TpiA)	Down-regulated	G
Metabolism	SAV0605	Alcohol dehydrogenase (Adh)	Down-regulated	C
	SAV1553	Superoxide dismutase Mn Fe 1 (SodA)	Down-regulated	P
Poorly characterized	SAV1473	DNA binding protein HU (Hup)	Down-regulated	R

COG (cluster of orthologous groups) categories: K, transcription; M, cell wall/membrane/envelope biogenesis; G, carbohydrate transport and metabolism; C, energy production and conversion; P, inorganic ion transport and metabolism; R, general function prediction only.

Table 2: Differentially expressed proteins in Mu50Ω-*vraS*m-*graR*m versus Mu50Ω-*vraS*m with a fold change cutoff of ≥2.

Domain	ORF No.	Product/Function	Expression Changes	COG Functional Category
Metabolism	SAV0774	Triosephosphate isomerase (TpiA)	Up-regulated	G
	SAV2634	Ornithine carbamoyltransferase, catabolic (ArcB)	Up-regulated	E
Cellular processes and signaling	SAV2569	Probable transglycosylase isaA (IsaA)	Up-regulated	M

COG (cluster of orthologous groups) categories: G, carbohydrate transport and metabolism; E, amino acid transport and metabolism; M, cell wall/membrane/envelope biogenesis.

Differential protein expression regulated by VraS

Comparative proteomics revealed that all 9 differentially expressed proteins were down-regulated in Mu50Ω-*vraS*m compared to its parental Mu50Ω strain (Table 1). Among them, down-regulation of immunoglobulin G binding protein A (Spa) was the most significant, with a fold change of > 10. Interestingly, the SarA family proteins (transcriptional regulator SarA; HTH type transcriptional regulator, MgrA and Rot) constitute majority of the down-regulated proteins.

Differential protein expression regulated by GraR

On the other hand, there appears to be only 3 differentially expressed proteins regulated by GraR, and these proteins were unanimously up-regulated in Mu50Ω-*vraS*m-*graR*m compared to Mu50Ω-*vraS*m (Table 2).

Differential protein expression regulated by VraS-GraR

Interestingly, comparison between the protein inventories of Mu50Ω-*vraS*m-*graR*m with Mu50Ω (VraS-GraR regulon) generated a relatively divergent set of proteins, whereby the proteins regulated by VraS-GraR were almost, if not all, dissimilar with those regulated singly by VraS or GraR. Seven proteins were found to be up-regulated in Mu50Ω-*vraS*m-*graR*m strain, while only 2 proteins were down-regulated in comparison to Mu50Ω (Table 3).

DISCUSSION

Many reports have published on genetic determinants found to be responsible for intermediate vancomycin resistance in *S. aureus*. A larger proportion of these reports utilized gene expression studies to track the genetic changes responsible for the transformation of VSSA to VISA (Kuroda *et al.*, 2000; Mongodin *et al.*, 2003; Cui *et al.*, 2005; McAleese *et al.*, 2006), while reports using a proteomic approach have also been published in recent years (Pieper *et al.*, 2006; Scherl *et al.*, 2006).

Studies have shown that the proteins of a cell are continually adjusted to withstand harsh and sudden

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 3: Differentially expressed proteins in Mu50Ω-*vraS*m-*graR*m versus Mu50Ω with a fold change cutoff of ≥2.

Domain	ORF No.	Product/Function	Expression Changes	COG Functional Category
Information storage and processing	SAV0009	Seryl tRNA-synthetase (SerS)	Up-regulated	J
Cellular processes and signaling	SAV1423	Peptide methionine sulfoxide reductase MsrB (MsrB)	Up-regulated	O
	SAV1884	Response regulator protein vraR (VraR)	Up-regulated	T
Metabolism	SAV1169	Ornithine carbamoyltransferase (ArgF)	Up-regulated	E
	SAV2634	Ornithine carbamoyltransferase, catabolic (ArcB)	Up-regulated	E
	SAV1422	Glucose specific phosphotransferase enzyme IIA component (Crr)	Up-regulated	G
	SAV2688	Lactonase drp35 (Drp35)	Up-regulated	G
	SAV1687	Glyceraldehyde-3-phosphate dehydrogenase 2 (GapB)	Down-regulated	G
	SAS044	Probable tautomerase SA1195.1 (SAS044)	Down-regulated	Q

COG (cluster of orthologous groups) categories: J, translation, ribosomal structure and biogenesis; O, post-translational modification, protein turnover, chaperones; T, signal transduction mechanisms; E, amino acid transport and metabolism; G, carbohydrate transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism.

environmental changes (Renzone *et al.*, 2005). These differences in protein expression profiles of cells could be investigated by proteomic approaches (Hecker *et al.*, 2003). As a matter of fact, antimicrobial resistance-related proteins of many microorganisms have been explored via proteomics (Diniz *et al.*, 2004; Soualhine *et al.*, 2005; Pieper *et al.*, 2006; Bore *et al.*, 2007; Lis *et al.*, 2008; Fischer *et al.*, 2011). In addition, proteomic approaches have been applied to uncover molecular mechanisms responsible for bacterial drug resistance (Su *et al.*, 2010).

For this study, we used 3 isogenic strains which were specifically engineered to mimic the VSSA to VISA transformation in the Mu50 lineage of strains via the VraSR-GraSR pathway (Cui *et al.*, 2009). The lack of isogenic vancomycin-susceptible strains that could be considered the parental strains of VISA isolates has been a principal problem in mechanistic studies, hindering the possibility of attributing genotypic and phenotypic differences to a particular antibiotic susceptibility phenotype. The availability of Mu50Ω in this study, representing the isogenic susceptible counterpart of Mu50Ω-*vraS*m and Mu50Ω-*vraS*m-*graR*m strains, granted us an opportunity to determine changes in protein expression that are most likely associated with vancomycin intermediate resistance. Due to the isogenic nature of these 3 strains, they were very useful in tracking the proteomic changes in a VSSA (Mu50Ω) as it progresses to become a Mu50-like VISA (Mu50Ω-*vraS*m-*graR*m) via mutations in the *vraS* and *graR* genes. Using a proteomic approach to study the differential protein expression of the 3 isogenic strains, we demonstrated that acquisition of intermediate level of vancomycin resistance in Mu50 lineage of *S. aureus* strains seems to be accomplished in 2 phases. These include the initial down-regulation of genes involved in virulence regulated by VraSR, and subsequent up-regulation of cell wall metabolism-associated genes by GraSR.

Down-regulation of bacterial virulence appeared to be mediated by VraS through a complex regulatory network involving mainly SarA and SarA homologs (Rot, MgrA). SarA is a regulatory locus with a functional role in controlling the expression of a number of extracellular and cell-wall associated proteins (Cheung *et al.*, 1992). In addition, SarA (Rechtin *et al.*, 1999), as well as Rot (McNamara *et al.*, 2000), are both global regulators of virulence gene expression in *S. aureus*. Similarly, with the aid of a mice model, *mgrA* was shown to play an important role in the establishment and progression of septic arthritis and sepsis, indicating its role in virulence expression (Jonsson *et al.*, 2008). *mgrA* and *rot* are both reported to be positive regulators of *sarS* (Said-Salim *et al.*, 2003; Oscarsson *et al.*, 2005), where its up-regulation will ultimately lead to increased levels of *spa* expression (Cheung *et al.*, 2001). Inoculation of Spa^+ strains into mice were shown to cause higher mortality compared to infection by Spa^- strains (Patel *et al.*, 1987). Taking it all together, it appears that the down regulation of the network of SarA-Rot-MgrA-Spa virulence factors, via VraS regulation, will enable an initially vancomycin susceptible *S. aureus* (as represented by Mu50Ω, vancomycin MIC = 1 mg/L) to achieve the resistance level of a VISA (Mu50Ω-*vraS*m, vancomycin MIC = 4.5 mg/L).

While VraS appears to modulate many proteins which are generally involved in staphylococcal virulence to achieve vancomycin resistance, only 3 proteins (TpiA, ArcB and IsaA) were found to be differentially regulated by GraR to lead towards an increase of vancomycin MIC from 4.5 mg/L (Mu50Ω-*vraS*m) to 6 mg/L (Mu50Ω-*vraS*m-*graR*m). Interestingly, these 3 proteins are mainly associated with nutrient metabolism and cell wall biogenesis. Triosephosphate isomerase (TpiA) is needed by the cell for efficient carbohydrate metabolism (Gunsalus *et al.*, 1955), while the catabolic ornithine carbamoyltransferase (ArcB) is important for arginine

ISSN (print): 1823-8262, ISSN (online): 2231-7538

metabolism when the staphylococci are grown in anaerobic conditions (Cunin *et al.*, 1986). IsaA is a member of lytic transglycosylases, which acts by catalyzing the cleavage of β-1,4-glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine residues of peptidoglycan, leading to increased autolytic activity (Holtje *et al.*, 1975). Higher levels of IsaA in Mu50Ω-*vraS*m-*graR*m strain suggested that increased vancomycin resistance is accompanied by higher autolytic activity. These features are especially similar to those of Mu50 VISA isolates (Hanaki *et al.*, 1998). Despite the importance of GraR in acquisition of VISA phenotypes (Neoh *et al.*, 2008; Howden *et al.*, 2010), it appears that, on its own, this regulator will only mediate the regulation of minimal proteins, resulting in only an increase of 2 mg/L in vancomycin MIC. Nevertheless, even though only 3 proteins were regulated by GraR, these proteins may indicate the transition point of a "beginner VISA" (Mu50Ω-*vraS*m, vancomycin MIC = 4.5 mg/L) from virulence down-regulation to revealing resistance-enhancing characteristics.

We expected the comparison of differential protein profiles between Mu50Ω-*vraS*m-*graR*m with Mu50Ω to reveal a composite inventory made up of proteins differentially regulated singly by VraS and GraR. Intriguingly, this hypothesis was wrong. An almost completely different set of proteins appeared to be regulated by the VraS-GraR regulon, where this combined regulon could affect the jump in vancomycin MIC from 1 mg/L (Mu50Ω) to 6 mg/L (Mu50Ω-*vraS*m-*graR*m). Nevertheless, as vancomycin targets the cell wall, we noticed that even though the protein inventory was different from that of VraS and GraR, VraS-GraR still appears to regulate mostly cell wall metabolism-associated proteins.

We were curious to note that, ArcB (which was also up-regulated via GraR modulation) and also another enzyme involved in arginine metabolism, the ornithine carbamoyltransferase (ArgF), were up-regulated via the VraS-GraR regulon. Reports have shown the importance of glucose metabolism in *S. aureus* for the generation of D-fructose-6-phosphate and finally glucosamine-6-phosphate. These molecules are precursor metabolites for peptidoglycan, which are the building blocks needed for cell wall thickening, the salient VISA phenotype (Cui *et al.*, 2003). To this end, VISAs are predicted to shunt glucose molecules from the glycolytic pathway to the peptidoglycan biosynthesis pathway. This is a process which would likely disturb the energy metabolism of the cells (Cui *et al.*, 2000). Consequently, bacterial cells have to rely on alternative energy source(s) for survival. Studies have shown that arginine can serve as the sole energy source for *S. aureus* growth if glucose is not available (Makhlin *et al.*, 2007). In arginine biosynthesis, ArgF is involved in the formation of citrulline by catalyzing the transfer of carbamoyl moiety of carbamoylphosphate to 5-amino group of ornithine; whereas ArcB catalyzes the reverse reaction, which is the phosphorolysis of citrulline to yield ornithine and carbamoylphosphate. The latter reaction is part of bacterial arginine degradation process which converts arginine to ornithine, ammonia, and carbon dioxide, yielding 1 mol of ATP per mol of arginine consumed (Beenken *et al.*, 2004). We suspect that the increased expression of both ArcB and ArgF proteins in Mu50Ω-*vraS*m-*graR*m in this study serves to initiate the utilization of arginine as an energy source, compensating for reduced energy levels due to increased glucose metabolism for cell wall thickening. Moreover, elevated expression of ArcB and ArgF in our study indicates that arginine metabolism might play a role in this alternative pathway for cell wall synthesis in VISA as shown from the study.

Besides arginine, serine also appears to be an important amino acid in vancomycin resistance modulated via VraS-GraR regulon, as expression of the enzyme seryl tRNA-synthetase was also increased in Mu50Ω-*vraS*m-*graR*m. Increased cell wall biosynthesis is a key feature commonly found in *S. aureus* strains with reduced susceptibility to vancomycin (Howden *et al.*, 2010). Nascent peptidoglycans are cross-linked by inter-peptide bridge formed using aminoacyl-tRNAs as amino acid residues donors. Generally, the inter-peptide bridges constitute 5 glycine residues (Schneider *et al.*, 2004). However, altered peptidoglycan cross bridges, with serine residues in place of glycine, has been shown to contribute towards increased glycopeptide resistance (Billot-Klein *et al.*, 1996). Therefore, we postulated that the enhanced levels of seryl-tRNAs might be needed to mediate incorporation of serine residues during peptidoglycan biosynthesis in Mu50Ω-*vraS*m-*graR*m.

Hanaki *et al.* (1998) unraveled two important features of Mu50, that are the accelerated release of cell wall materials into the culture medium, in addition to increased autolysis, which IsaA probably plays a role. Increased cell wall turnover could bring about a great loss of resources for the bacteria if not recovered, since peptidoglycan comprised for more than 20% of the weight of Gram-positive cells (Reith *et al.*, 2011). In the case of Mu50Ω-*vraS*m-*graR*m, we deduced that the cell increases its glucose specific phosphotransferase enzyme IIA enzymes via VraS-GraR regulation, to recover the amino sugars *N*-acetylglucosamine and *N*-acetylmuramic acid which were lost during cell wall turnover.

Besides cell wall metabolism associated proteins, the VraS-GraR regulon also appears to regulate proteins involved in cellular processes and signaling, namely, VraR and peptide methionine sulfoxide reductase (MsrB) in Mu50Ω-*vraS*m-*graR*m. In our study, the VraR protein seems to be integral in down-regulating the SarA-Rot-MgrA-Spa virulence factors in allowing bacteria to achieve the "beginner" level of vancomycin intermediate-resistance. The up-regulation of VraR via the VraS-GraR regulon might have also further contributed to down-regulation of the SarA-Rot-MgrA-Spa network in the cell's process of resisting vancomycin.

MsrB is a bacterial Msr enzyme which protects the cell against oxidative damage by reduction of R-epimer of methionine sulfoxide (R-MetO) molecules. Vancomycin and some bactericidal antibiotics has been reported to induce oxidative stress as observed in a wild-type strain

ISSN (print): 1823-8262, ISSN (online): 2231-7538

of *S. aureus* that lethal concentration of this antibiotic could increase the production of hydroxyl radicals in the cells (Kohanski *et al.*, 2007). These highly reactive species will affect bacterial macromolecules leading to the oxidation of DNA, lipids and proteins (Clements *et al.*, 1999). It is interesting to observe that, in our study, even without vancomycin induction prior protein extraction of the strains, an intact Mu50 VraS-GraR regulon will result in increased MsrB expression, indicating the preparedness of the cell to counter any possible vancomycin/antibiotic attack.

CONCLUSION

In this study we attempted to reveal the proteomic changes which occur via the VraS, GraR, and VraS-GraR regulation in the generation of a VISA. We can summarize that the Mu50 lineage of VISAs appear to down-regulate virulence proteins (SarA and SarA homologues) to acquire "beginner" VISA phenotypes. This trade-off between bacterial resistance and virulence is mainly regulated by VraS. Upon evolvement into VISA strains, a different set of proteins responsible for vancomycin resistance are up-regulated. This is achieved only when both VraS and GraR are present, as GraR alone does not account for significant differential protein expression attributable to vancomycin resistance. However, our present study only revealed altered protein expressions that were exhibited in the absence of vancomycin induction. Further investigations are being carried out to study these strains under vancomycin challenge to identify a more comprehensive set of proteins responsible for vancomycin stress response. This will contribute to a better understanding of bacterial protein responses towards vancomycin and also towards the identification of new drug targets.

ACKNOWLEDGEMENT

The research was funded by the Ministry of Higher Education of Malaysia under the grant code FRGS/1/2011/SKK/UKM/03/15. We thank Zam Zureena Mohd Rani and Fara Zela Mohd Radin for their assistance and guidance in protein extraction and mass spectrometric identification of proteins. The authors are grateful to Lim Lay Cheng and Norshahida Abu Samah for helpful discussions in data analysis.

REFERENCES

Aligholi, M., Emaneini, M., Jabalameli, F., Shahsavan, S., Dabiri, H. and Sedaght, H. (2008). Emergence of high-level vancomycin-resistant *Staphylococcus aureus* in the Imam Khomeini Hospital in Tehran. *Medical Principles and Practice* **17, 432-434.**

Azimian, A., Havaei, S. A., Fazeli, H., Naderi, M., Ghazvini, K., Samiee, S. M., Soleimani, M. and Peerayeh, S. N. (2012). Genetic characterization of a vancomycin-resistant *Staphylococcus aureus* isolate from the respiratory tract of a patient in a university hospital in Northeastern Iran. *Journal of Clinical Microbiology* **50, 3581-3585.**

Becher, D., Hempel, K., Sievers, S., Zühlke, D., Pané-Farre, J., Otto, A., Fuchs, S., Albrecht, D., Bernhardt, J., Engelmann, S., Völker, U., van Dijl, J. M. and Hecker, M. (2009). A proteomic view of an important human pathogen - towards the quantification of the entire *Staphylococcus aureus* proteome. *Public Library of Science* **4, e8176-8187.**

Beenken, K. E., Dunman, P. M., McAleese, F., Macapagal, D., Murphy, E., Projan, S. J., Blevins, J. S. and Smeltzer, M. S. (2004). Global gene expression in *Staphylococcus aureus* biofilms. *Journal of Bacteriology* **186, 4665-4684.**

Bierbaum, G., Fuchs, K., Lenz, W., Szekat, C. and Sahl, H. -G. (1999). Presence of *Staphylococcus aureus* with reduced susceptibility to vancomycin in Germany. *European Journal of Clinical Microbiology* **18, 691-696.**

Billot-Klein, D., Gutmann, L., Bryant, D., Bell, D., van Heijenoort, J., Grewal, J. and Shlaes, D. M. (1996). Peptidoglycan synthesis and structure in *Staphylococcus haemolyticus* expressing increasing levels of resistance to glycopeptides antibiotics. *Journal of Bacteriology* **178, 4696-4703.**

Bore, E., Hébraud, M., Chafsey, I., Chambon, C., Skjæret, C., Moen, B., Møretrø, T., Langsrud, Ø., Rudi, K. and Langsrud, S. (2007). Adapted tolerance to benzalkonium chloride in *Escherichia coli* K-12 studied by transcriptome and proteome analyses. *Microbiology* **153, 935-946.**

Centers for Disease Control and Prevention (CDC) (1997). *Staphylococcus aureus* with reduced susceptibility to vancomycin - United States, 1997. Available from: https://www.cdc.gov/Mmwr/preview/mmwrhtml/00053311.htm. [Retrieved on: 22 April 2013].

Centers for Disease Control and Prevention (CDC) (2002). CDC reminds clinical laboratories and healthcare infection preventionists of their role in the search and containment of vancomycin-resistant *Staphylococcus aureus* (VRSA). Available from: https://www.cdc.gov/hai/settings/lab/vrsa_lab_search_containment.html. [Retrieved on: 16 August 2013].

Cheung, A. L., Koomey, J. M., Butler, C. A., Projan, S. J. and Fischetti, V. A. (1992). Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. *Proceedings of the National Academy of Sciences* **89, 6462-6466.**

Cheung, A. L., Schmidt, K., Bateman, B. and Manna, A. C. (2001). SarS, a SarA homolog repressible by *agr*, is an activator of protein A synthesis in *Staphylococcus aureus*. *Infection and Immunity* **69, 2448-2455.**

Clements, M. O. and Foster, S. J. (1999). Stress resistance in *Staphylococcus aureus*. *Trends in Microbiology* **7, 458-462.**

Cui, L., Lian, J. -Q., Neoh, H. -m., Reyes, E. and Hiramatsu, K. (2005). DNA microarray-based identification of genes associated with glycopeptide

ISSN (print): 1823-8262, ISSN (online): 2231-7538

resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **49, 3404-3413.**

Cui, L., Ma, X., Sato, K., Okuma, K., Tenover, F. C., Mamizuka, E. M., Gemmell, C. G., Kim, M. -N., Ploy, M. -C., El Solh, N., Ferraz, V. and Hiramatsu, K. (2003). Cell wall thickening is a common feature of vancomycin resistance in *Staphylococcus aureus*. *Journal of Clinical Microbiology* **41, 5-14.**

Cui, L., Murakami, H., Kuwahara-Arai, K., Hanaki, H. and Hiramatsu, K. (2000). Contribution of a thickened cell wall and its glutamine nonamidated component to the vancomycin resistance expressed by *Staphylococcus aureus* Mu50. *Antimicrobial Agents and Chemotherapy* **44, 2276-2285.**

Cui, L., Neoh, H. -m., Shoji, M. and Hiramatsu, K. (2009). Contribution of *vraSR* and *graSR* point mutations to vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **53, 1231-1234.**

Cunin, R., Glansdorff, N., Pierard, A. and Stalon, V. (1986). Biosynthesis and metabolism of arginine in bacteria. *Microbiological Reviews* **50, 314-352.**

Denis, O., Nonhoff, C., Byl, B., Knoop, C., Bobin-Dubreux, S. and Struelens, M. J. (2002). Emergence of vancomycin-intermediate *Staphylococcus aureus* in a Belgian hospital: Microbiological and clinical features. *Journal of Antimicrobial Chemotherapy* **50, 383-391.**

Diniz, C. G., Farias, L. M., Carvalho, M. A. R., Rocha, E. R. and Smith, C. J. (2004). Differential gene expression in a *Bacteroides fragilis* metronidazole-resistant mutant. *Journal of Antimicrobial Chemotherapy* **54, 100-108.**

Finan, J. E., Archer, G. L., Pucci, M. J. and Climo, M. W. (2001). Role of penicillin-binding protein 4 in expression of vancomycin resistance among clinical isolates of oxacillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **45, 3070-3075.**

Fischer, A., Yang, S. -J., Bayer, A. S., Vaezzadeh, A. R., Herzig, S., Stenz, L., Girard, M., Sakoulas, G., Scherl, A., Yeaman, M. R., Proctor, R. A., Schrenzel, J. and François, P. (2011). Daptomycin resistance mechanisms in clinically derived *Staphylococcus aureus* strains assessed by a combined transcriptomics and proteomics approach. *Journal of Antimicrobial Chemotherapy* **66, 1696-1711.**

Gunsalus, I. C., Horecker, B. L. and Wood, W. A. (1955). Pathways of carbohydrate metabolism in microorganisms. *Bacteriological Reviews* **19, 79-128.**

Hanaki, H., Kuwahara-Arai, K., Boyle-Vavra, S., Daum, R. S., Labischinski, H. and Hiramatsu, K. (1998). Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50. *Journal of Antimicrobial Chemotherapy* **42, 199-209.**

Hecker, M., Engelmann, S. and Cordwell, S. J. (2003). Proteomics of *Staphylococcus aureus* - current state and future challenges. *Journal of Chromatography B* **787, 179-195.**

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T. and Tenover, F. C. (1997). Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *Journal of Antimicrobial Chemotherapy* **40, 135-146.**

Holtje, J. V., Mirelman, D., Sharon, N. and Schwarz, U. (1975). Novel type of murein transglycosylase in *Escherichia coli*. *Journal of Bacteriology* **124, 1067-1076.**

Howden, B. P., Davies, J. K., Johnson, P. D. R., Stinear, T. P. and Grayson, M. L. (2010). Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: Resistance mechanisms, laboratory detection, and clinical implications. *Clinical Microbiology Reviews* **23, 99-139.**

Jonsson, I. -M., Lindholm, C., Luong, T. T., Lee, C. Y. and Tarkowski, A. (2008). *mgrA* regulates staphylococcal virulence important for induction and progression of septic arthritis and sepsis. *Microbes and Infection* **10, 1229-1235.**

Kim, M. -N., Pai, C. H., Woo, J. H., Ryu, J. S. and Hiramatsu, K. (2000). Vancomycin-intermediate *Staphylococcus aureus* in Korea. *Journal of Clinical Microbiology* **38, 3879-3881.**

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A. and Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130, 797-810.**

Kuroda, M., Kuwahara-Arai, K. and Hiramatsu, K. (2000). Identification of the up- and down-regulated genes in vancomycin-resistant *Staphylococcus aureus* strains Mu3 and Mu50 by cDNA differential hybridization method. *Biochemical and Biophysical Research Communications* **269, 485-490.**

Lis, M. and Bobek, L. A. (2008). Proteomic and metabolic characterization of a *Candida albicans* mutant resistant to the antimicrobial peptide MUC7 12-mer. *FEMS Immunology and Medical Microbiology* **54, 80-91.**

Makhlin, J., Kofman, T., Borovok, I., Kohler, C., Engelmann, S., Cohen, G. and Aharonowitz, Y. (2007). *Staphylococcus aureus* ArcR controls expression of the arginine deiminase operon. *Journal of Bacteriology* **189, 5976-5986.**

McAleese, F., Wu, S. W., Sieradzki, K., Dunman, P., Murphy, E., Projan, S. and Tomasz, A. (2006). Overexpression of genes of the cell wall stimulon in clinical isolates of *Staphylococcus aureus* exhibiting vancomycin-intermediate-*S. aureus*-type resistance to vancomycin. *Journal of Bacteriology* **188, 1120-1133.**

McNamara, P. J., Milligan-Monroe, K. C., Khalili, S. and Proctor, R. A. (2000). Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus. Journal of Bacteriology* **182, 3197-3203.**

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Mongodin, E., Finan, J., Climo, M. W., Rosato, A., Gill, S. and Archer, G. L. (2003). Microarray transcription analysis of clinical *Staphylococcus aureus* isolates resistant to vancomycin. *Journal of Bacteriology* **185, 4638-4643.**

Neoh, H-M., Cui, L., Yuzawa, H., Takeuchi, F., Matsuo, M. and Hiramatsu, K. (2008). Mutated response regulator *graR* is responsible for phenotypic conversion of *Staphylococcus aureus* from heterogeneous vancomycin-intermediate resistance to vancomycin-intermediate resistance. *Antimicrobial Agents and Chemotherapy* **52, 45-53.**

Oliveira, G. A., Dell'Aquila, A. M., Masiero, R. L., Levy, C. E., Gomes, M. S., Cui, L., Hiramatsu, K. and Mamizuka, E. M. (2001). Isolation in Brazil of nosocomial *Staphylococcus aureus* with reduced susceptibility to vancomycin. *Infection Control and Hospital Epidemiology* **22, 443-448.**

Oscarsson, J., Harlos, C. and Arvidson, S. (2005). Regulatory role of proteins binding to the *spa* (protein A) and *sarS* (staphylococcal accessory regulator) promoter regions in *Staphylococcus aureus* NTCC 8325-4. *International Journal of Medical Microbiology* **295, 253-266.**

Patel, A. H., Nowlan, P., Weavers, E. D. and Foster, T. (1987). Virulence of protein A-deficient and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement. *Infection and Immunity* **55, 3103-3110.**

Pieper, R., Gatlin-Bunai, C. L., Mongodin, E. F., Parmar, P. P., Huang, S. -T., Clark, D. J., Fleischmann, R. D., Gill, S. R. and Peterson, S. N. (2006). Comparative proteomic analysis of *Staphylococcus aureus* strains with differences in resistance to the cell wall-targeting antibiotic vancomycin. *Proteomics* **6, 4246-4258.**

Ploy, M. C., Grélaud, C., Martin, C., de Lumley, L. and Denis, F. (1998). First clinical isolate of vancomycin-intermediate *Staphylococcus aureus* in a French hospital. *Lancet* **351, 1212.**

Rechtin, T. M., Gillaspy, A. F., Schumacher, M. A., Brennan, R. G., Smeltzer, M. S. and Hurlburt, B. K. (1999). Characterization of the SarA virulence gene regulator of *Staphylococcus aureus*. *Molecular Microbiology* **33, 307-316.**

Reith, J. and Mayer, C. (2011). Peptidoglycan turnover and recycling in Gram-positive bacteria. *Applied Microbiology and Biotechnology* **92, 1-11.**

Renzone, G., D'Ambrosio, C., Arena, S., Rullo, R., Ledda, L., Ferrara, L. and Scaloni, A. (2005). Differential proteomic analysis in the study of prokaryotes stress resistance. *Annali dell'Istituto Superiore di Sanità* **41, 459-468.**

Saha, B., Singh, A. K., Ghosh, A. and Bal, M. (2008). Identification and characterization of a vancomycin-resistant *Staphylococcus aureus* isolated from Kolkata (South Asia). *Journal of Medical Microbiology* **57, 72-79.**

Saïd-Salim, B., Dunman, P. M., McAleese, F. M., Macapagal, D., Murphy, E., McNamara, P. J., Arvidson, S., Foster, T. J., Projan, S. J. and Kreiswirth, B. N. (2003). Global regulation of *Staphylococcus aureus* genes by Rot. *Journal of Bacteriology* **185, 610-619.**

Scherl, A., François, P., Charbonnier, Y., Deshusses, J. M., Koessler, T., Huyghe, A., Bento, M., Stahl-Zeng, J., Fischer, A., Masselot, A., Vaezzadeh, A., Gallé, F., Renzoni, A., Vaudaux, P., Lew, D., Zimmermann-Ivol, C. G., Binz, P. -A., Sanchez, J. -C., Hochstrasser, D. F. and Schrenzel, J. (2006). Exploring glycopeptide-resistance in *Staphylococcus aureus*: A combined proteomics and transcriptomics approach for the identification of resistance-related markers. *BMC Genomics* **7, 296-311.**

Schneider, T., Senn, M. M., Berger-Bächi, B., Tossi, A., Sahl, H. -G. and Wiedemann, I. (2004). *In vitro* assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly_5) of *Staphylococcus aureus*. *Molecular Microbiology* **53, 675-685.**

Soualhine, H., Brochu, V., Ménard, F., Papadopoulou, B., Weiss, K., Bergeron, M. G., Légaré, D., Drummelsmith, J. and Ouellette, M. (2005). A proteomic analysis of penicillin resistance in *Streptococcus pneumoniae* reveals a novel role for PstS, a subunit of the phosphate ABC transporter. *Molecular Microbiology* **58, 1430-1440.**

Su, H. -C., Ramkissoon, K., Doolittle, J., Clark, M., Khatun, J., Secrest, A., Wolfgang, M. C. and Giddings, M. C. (2010). The development of ciprofloxacin resistance in *Pseudomonas aeruginosa* involves multiple response stages and multiple proteins. *Antimicrobial Agents and Chemotherapy* **54, 4626-4635.**

Tiwari, H. K. and Sen, M. R. (2006). Emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) from a tertiary care hospital from northern part of India. *BMC Infectious Diseases* **6, 156-161.**

Trotonda, M. P., Xiong, Y. Q., Memmi, G., Bayer, A. S. and Cheung, A. L. (2009). Role of *mgrA* and *sarA* in methicillin-resistant *Staphylococcus aureus* autolysis and resistance to cell wall-active antibiotics. *The Journal of Infectious Diseases* **199, 209-218.**

Weigel, L. M., Clewell, D. B., Gill, S. R., Clark, N. C., McDougal, L. K., Flannagan, S. E., Kolonay, J. F., Shetty, J., Killgore, G. E. and Tenover, F. C. (2003). Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science* **302, 1569-1571.**

ISSN (print): 1823-8262, ISSN (online): 2231-7538