Activated ADI pathway: the initiator of intermediate vancomycin resistance in *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Canadian Journal of Microbiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>cjm-2016-0439.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Note</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>23-Sep-2016</td>
</tr>
</tbody>
</table>
| Complete List of Authors: | Tan, Xin Ee; The National University of Malaysia, UKM Medical Molecular Biology Institute  
Neoh, Hui-min; The National University of Malaysia, UKM Medical Molecular Biology Institute; School of Medicine, Juntendo University, Department of Bacteriology  
Looi, Mee-Lee; The National University of Malaysia, UKM Medical Molecular Biology Institute; Taylor’s University Lakeside Campus  
Chin, Siok Fong; The National University of Malaysia, UKM Medical Molecular Biology Institute  
Cui, Longzhu; School of Medicine, Jichi Medical University, Department of Infection and Immunity  
Hiramatsu, Keiichi; School of Medicine, Juntendo University, Department of Bacteriology  
Hussin, Salasawati; The National University of Malaysia, Department of Medical Microbiology and Immunology  
Jamal, Rahman; The National University of Malaysia, UKM Medical Molecular Biology Institute |
| Keyword:          | vancomycin-intermediate *Staphylococcus aureus*, arginine catabolism, ammonia, ATP, cell wall thickening |

https://mc06.manuscriptcentral.com/cjm-pubs
Activated ADI pathway: the initiator of intermediate vancomycin resistance in

*Staphylococcus aureus*

Xin-Ee Tan¹, Hui-min Neoh¹,²*, Mee-Lee Looi¹,³, Siok Fong Chin¹, Longzhu Cui⁴, Keiichi Hiramatsu², Salasawati Hussin⁵ and Rahman Jamal¹

¹UKM Medical Molecular Biology Institute (UMBI), Universiti Kebangsaan Malaysia, Malaysia; ²Department of Bacteriology, School of Medicine, Juntendo University, Japan; ³Taylor’s University Lakeside Campus, School of Biosciences, Malaysia; ⁴Division of Bacteriology, Department of Infection and Immunity, School of Medicine, Jichi Medical University, Japan; ⁵Department of Medical Microbiology and Immunology, Universiti Kebangsaan Malaysia, Malaysia.

Email addresses: Xin-Ee Tan - xinee2709@hotmail.com; Hui-min Neoh* - hui-min@ppukm.ukm.edu.my; Mee-Lee Looi - looimeelee@yahoo.com; Siok Fong Chin - chinsiokfong@ppukm.ukm.edu.my; Longzhu Cui - longzhu@jichi.ac.jp; Keiichi Hiramatsu - khiram06@juntendo.ac.jp; Salasawati Hussin - sawati@ppukm.ukm.edu.my; Rahman Jamal - rahman@ppukm.ukm.edu.my

*Corresponding author

Hui-min Neoh, Ph.D.,

UKM Medical Molecular Biology Institute (UMBI), UKM Medical Centre,

Jalan Yaacob Latif, 56000 Cheras, Kuala Lumpur, Malaysia.

Telephone: +60-3-9145-9062; Fax: +60-3-9171-7185

Email: hui-min@ppukm.ukm.edu.my
Abstract

Comparative proteomic profiling between two vancomycin intermediate *Staphylococcus aureus* (VISA) strains, Mu50Ω-*vraS* and Mu50Ω-*vraS*-*graR*, and vancomycin-susceptible *S. aureus* (VSSA) strain Mu50Ω, revealed up-regulated levels of catabolic ornithine carbamoyltransferase (ArcB) of the arginine catabolism pathway in VISA. Subsequent analyses showed that the VISA strains have higher levels of cellular ATP and ammonia, which are by-products of arginine catabolism, and displayed thicker cell walls. We postulate that elevated cytoplasmic ammonia and ATP molecules, resulting from activated arginine catabolism upon acquisition of *vraS* and *graR* mutations, are important requirements facilitating cell wall biosynthesis, thereby contributing to thickened cell wall and consequently reduced vancomycin susceptibility in VISA.

Keywords: vancomycin-intermediate *Staphylococcus aureus*, arginine catabolism, ammonia, ATP, cell wall thickening
Various genetic determinants have been reported to be associated with intermediate vancomycin resistance in *S. aureus* (Kuroda et al. 2003, Neoh et al. 2008, Shoji et al. 2011, Watanabe et al. 2011). In 2006, we published our discovery on higher *vraS* and *graR* gene expressions in VISA strain Mu50 compared to VSSA N315, and subsequently showed that genome engineering of mutated Mu50 *vraS* and *graR* into VSSA Mu50Ω converted it into a VISA (Cui et al. 2005, Cui et al. 2009). However, the exact mechanism regulated by VraS and GraR in contributing towards vancomycin intermediate resistance in the Mu50 lineage of strains is still unclear.

In this study, we employed a proteomic approach to elucidate the mechanism behind VraSR- and GraRS-mediated intermediate vancomycin resistance. Total proteins were extracted from Mu50Ω, a VSSA, and VISAs Mu50Ω-*vraSm* and Mu50Ω-*vraSm-graRm*. These VISA strains were generated via Mu50 *vraS* and *vraS-graR* substitution, respectively, into the chromosome of Mu50Ω, making the three strains genetically identical except for point mutations in *vraS* and *graR* (Cui et al. 2009) (Figure 1). Comparison of protein profiles between these three strains will enable further understanding of VraS and GraR contributions towards the strains’ difference in vancomycin resistance (vancomycin MICs: Mu50Ω = 0.5 mg/L, Mu50Ω-*vraSm* = 4 mg/L and Mu50Ω-*vraSm-graRm* = 8 mg/L).

The extracted proteins were digested and analysed using a nanoACQUITY™ UPLC system (Waters, USA) coupled to a Q-Tof Premier™ mass spectrometer (Waters, USA). Differential protein expression profiling was performed using Progenesis LC-MS software version 4.0 (Nonlinear Dynamics, UK). Comparative proteomic profiling of the 3 isogenic strains revealed 21 differentially expressed proteins regulated by 3 distinct regulons: VraS (comparative proteomic profiling between Mu50Ω and Mu50Ω-*vraSm*), GraR (Mu50Ω-
Interestingly, the results showed a consistent and significant up-regulation (fold change cutoff \( \geq 2 \)) of catabolic ornithine carbamoyltransferase (ArcB), which is part of the bacterial arginine degradation process (arginine deiminase, ADI pathway) (Cunin et al. 1986), in the VISA strains compared to Mu50Ω (Figure 2). This led us to suspect that arginine metabolism might play a role in conferring intermediate vancomycin resistance to the Mu50 lineage of \( S. \) aureus. Previously, Alexander et al. (2014) reported a link between urea cycle, tricarboxylic acid cycle, pentose phosphate pathway and vancomycin resistance, showing an association between cellular metabolic alterations and the VISA phenotype. In \( S. \) aureus, the ADI pathway is utilized for arginine metabolism, where its catabolism produces ammonia and adenosine triphosphate (ATP) (Cunin et al. 1986).

VISA strains such as Mu50 has been reported to have increased cell wall biosynthesis which is driven by the glucosamine-6-phosphate synthase (GlmS) pathway (Cui et al. 2000). The initial and rate-limiting step in this pathway requires ammonia generated by hydrolysis of L-glutamine into L-glutamate (Floquet et al. 2007). In bacteria, ammonia could be sourced from the urease pathway and also from arginine metabolism via the ADI pathway, which releases ammonia as a by-product (Beenken et al. 2004). As we did not observe differentially expressed urease pathway enzymes between the VISA and VSSA strains, we suspect that the ammonia needed for increased cell wall biosynthesis came from the activation of ArcB of the ADI operon, which was found to be over-expressed in the VISAs used in our study.

With that in mind, we determined the amount of cellular ammonia (a by-product of arginine catabolism) in the 3 tested strains using an Ammonia Assay Kit (Sigma Aldrich, Missouri, USA) according to manufacturer’s instructions. Interestingly, our results indicated
that both Mu50Ω-vraSm and Mu50Ω-vraSm-graRm had elevated levels of cellular ammonia, at 4.8156 ± 0.73306 µg/ml and 4.8622 ± 0.67307 µg/ml, respectively, compared to Mu50Ω (3.9933 ± 0.50210 µg/ml) (Figure 3). Even though the increment was not statistically significant, our results indicated parallel increased cellular ammonia concentration with reduced S. aureus vancomycin susceptibilities. The elevation of cellular ammonia in VISA is thought to directly facilitate GlmS pathway-driven cell wall biosynthesis. Alternatively, Badet et al. (1987) reported that the catalytic activity of GlmS is solely dependent on ammonia generated from hydrolysis of L-glutamine. Hence, increased cellular ammonia in VISA strains, as detected in our current study, could also provide L-glutamine supplies through enhancing glutamine synthetase (GS)-mediated assimilation of ammonia into L-glutamate. This ensures continuous functioning of the GlmS pathway and undisrupted cell wall biosynthesis in VISA.

Biosynthesis of the S. aureus peptidoglycan pentapeptide side chain is catalyzed by 4 mur ligases (murC, murD, murE, murF) which possess conserved ATP binding domains (Smith 2006). It has been shown that the activity of these ATP-dependent amide bond ligases is dependent on the hydrolysis of ATP (Lovering et al. 2012). Incidentally, ATP is also a by-product of the ADI pathway. We wondered if the ArcB activated ADI operon in our VISA strains will also result in an increased of their ATP levels.

We subsequently assayed the tested strains’ cellular ATP levels using Molecular Probe™ ATP Determination Kit (Invitrogen, Ltd., Paisley, UK). Luminescence was measured using a Varioskan LUX (Thermo Scientific, Massachusetts, USA) at 570 nm. The amount of cellular ATP was significantly higher in Mu50Ω-vraSm (65.77 ± 9.72471 nM, p < 0.01) and Mu50Ω-vraSm-graRm (88.36 ± 6.23821 nM, p < 0.001) compared to the isogenic
susceptible parental strain Mu50Ω (41.78 ± 9.25807 nM). There was also a significant difference between cellular ATP levels of Mu50Ω-vraSm and Mu50Ω-vraSm-graRm (p < 0.01) (Figure 4). In short, the amount of cellular ATP in the tested strains was shown to increase with vancomycin MIC. In concordance with these findings, we postulate that elevated ATP levels associated with the ADI pathway is important for catalytic mechanism of pentapeptide side chain formation during cell wall biosynthesis.

Although expression changes in determinants of energy production has been reported in S. aureus with reduced susceptibility to vancomycin via both transcriptomic (Mongodin et al. 2003, McAleese et al. 2006, Gardete et al. 2012) and proteomic (Pieper et al. 2006) approaches, our study has biochemically proven that cellular ATP molecules are produced in significantly higher amounts in VISA strains compared to their isogenic vancomycin susceptible counterpart. Elevated ATP levels in VISA strains will be crucial in helping these strains meet the demand of increased cell wall biosynthesis in response to the deleterious effect of vancomycin.

We proceeded to determine if the elevation of cellular ammonia and ATP levels in our tested strains accompanied cell wall thickening. Transmission electron microscopy (Tecnai™ Transmission Electron Microscope (TEM), FEI Corporate, Oregon, USA) of equatorial-cut cells of each strains was carried out to enable cell wall thickness measurement (Hanaki et al. 1998, Cui et al. 2003). Electron micrographs clearly depicted that Mu50Ω-vraSm-graRm had a significantly thicker cell wall (45.288 ± 8.7370 nm) compared to Mu50Ω (36.352 ± 5.8585 nm) and Mu50Ω-vraSm (37.985 ± 7.0362 nm) (both p < 0.001) (Figure 5).
Collectively, the results from our study suggests that activated arginine catabolism (via an activated ADI pathway) regulated by the VraSR and GraRS two two-component regulatory systems is responsible for supplying ammonia and ATP molecules to VISA strains of the Mu50 lineage to compensate for increased cell wall biosynthesis. The resulting thickened cell wall peptidoglycan will then act as the protective mechanism for these VISA strains against vancomycin (Figure 6). We are currently investigating if this finding also applies to VISA strains of different lineages.

Acknowledgement

The research was funded by the Ministry of Higher Education, Malaysia under the grant code FRGS/1/2014/SKK04/UKM/03/1. We would also like to thank Dr Ler Lian Wee and Miss Loo Sze Wan of Thermo Fisher Scientific for their kind permission to perform our ATP assay using their Varioskan Lux multimode Microplate Reader.

References


McAleese, F., Wu, S.W., Sieradzki, K., Dunman, P., Murphy, E., Projan, S., et al. 2006. Overexpression of genes of the cell wall stimulon in clinical isolates of Staphylococcus


Figure Caption

Figure 1: Stepwise transition of VSSA to VISA via direct engineering of Mu50 vraS and graR into Mu50Ω genome (Cui et al. 2009).

Figure 2: Mean abundances of ArcB in strains Mu50Ω, Mu50Ω-vraSm and Mu50Ω-vraSm-graRm. Data was generated from 5 independent experiments on each studied strain. ArcB protein was observed to be in significantly higher abundance in VISA strains († p < 0.001) compared to VSSA. The protein was not detected in Mu50Ω strain.

Figure 3: Cellular ammonia concentrations in strains Mu50Ω, Mu50Ω-vraSm and Mu50Ω-vraSm-graRm. Data was generated from experiment on 3 biological replicates of each tested strain. There was no significant difference in the concentrations of cellular ammonia among the studied strains.

Figure 4: Cellular ATP concentrations of strains Mu50Ω, Mu50Ω-vraSm and Mu50Ω-vraSm-graRm. Data was generated from experiment on 3 biological replicates of each tested strain. Significantly higher amount of cellular ATP was found in Mu50Ω-vraSm (* p < 0.01) and Mu50Ω-vraSm-graRm († p < 0.001) compared to Mu50Ω.

Figure 5: Transmission electron microscopic analysis of cell wall thickness in strains Mu50Ω, Mu50Ω-vraSm and Mu50Ω-vraSm-graRm. Data was generated from experiment on 3 biological replicates of each tested strain. (A) Morphometric evaluation of cross-sectional cell walls of Mu50Ω (panel 1), Mu50Ω-vraSm (panel 2) and Mu50Ω-vraSm-graRm (panel 3). Cell wall thickness measurements for each strain are presented in means ± standard
deviations under the corresponding panels. (B) Comparison of cell wall thickness among the 3 isogenic strains with computational-based statistical analysis. Mu50Ω-\textit{vraSm-graRm} was shown to have significantly thicker cell wall compared to Mu50Ω and Mu50Ω-\textit{vraSm}, with $p < 0.001$.

Figure 6: Overview of proposed mechanism facilitating cell wall thickening in VISA strains of Mu50 lineage.
A

1

2

3

36.352 ± 5.8585 nm

37.985 ± 7.0362 nm

45.288 ± 8.7370 nm