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Oxidative stress resistance and fitness-compensatory response in vancomycin-intermediate *Staphylococcus aureus* (VISA)

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Abstract

In this study, VISA cells carrying *vraS* and/or *graR* mutations were shown to be more resistant to oxidative stress. *Caenorhabditis elegans* infected with these strains in turn demonstrated lower survival. Altered regulation in oxidative stress response and virulence appears to be physiological adaptations associated with VISA phenotype in the Mu50 lineage.

Keywords: vancomycin-intermediate *Staphylococcus aureus*, oxidative stress, virulence, fitness cost

Bacterial antibiotic resistance has been reported to occur concurrently with changes in various cellular responses of the organism. In particular, altered virulence mechanism is common among antibiotic resistant strains. Acquisition of antibiotic resistance often imposes a fitness burden on bacterial cells (Beceiro et al. 2013); in most cases, increased resistance has been paralleled with decreased virulence, as reported in methicillin-resistant *Staphylococcus aureus* (Pozzi et al. 2012; Rudkin et al. 2012) and vancomycin-intermediate *S. aureus* (VISA) (Majcherczyk et al. 2008; Peleg et al. 2009). Apart from virulence, the association between antibiotic resistance and oxidative stress response has also been reported. Different classes of antibiotics, regardless of their primary targets, have been shown to induce lethality through generation of reactive oxygen species (ROS) (Kohanski et al. 2007; Brynildsen et al. 2013). In response, the bacteria will try to reduce antibiotic killing via reduction of cellular hydroxyl radical accumulation (Wang & Zhao 2009; Wang et al. 2010; Liu et al. 2012; Marathe et al. 2013; Dwyer et al. 2014).

We previously employed a proteomic approach to determine underlying regulatory pathway(s) mediating transition of vancomycin-susceptible *S. aureus* (VSSA, strain Mu50Ω) to VISA [strain Mu50Ω-*vraSm*, harbouring a *vraS* T700A mutation (Met234 → stop); and strain Mu50Ω-*vraSm-graRm*, harbouring both *vraS* T700A/*graR* A590G (Asn197 → Ser197) mutations compared to strain Mu50Ω] (Tan et al. 2016). In the study, unexpected features of up-regulated oxidized protein repair enzyme (MsrB) and down-regulated virulence-associated proteins (Spa, Rot, MgrA, SarA) in VISAs were observed. Consistent up-regulation of MsrB as well as down-regulation of virulence-associated proteins in VISA strains lead us to suspect possible interplay between oxidative stress response, virulence and antibiotic resistance in VISA strains of the Mu50 lineage.

Methionine sulfoxide reductases (Msr) are bacterial repair enzymes important for protection from oxidative killing (Atack & Kelly 2008; Lee et al. 2009; Zhao et al. 2010; Romsang et al. 2013). To determine if different MsrB expression levels in the 3 study strains affect their responses towards oxidative stress, Mu50Ω, Mu50Ω-*vraSm* and Mu50Ω-*vraSm-graRm* were treated with 3 oxidizing agents [cumene hydroperoxide, tert-butyl hydroperoxide and hydrogen peroxide (H₂O₂)] at various concentrations prior determination of viable cell counts. Interestingly, Mu50Ω-*vraSm* and Mu50Ω-*vraSm-graRm* were shown to have greater survival when challenged with cumene hydroperoxide (Figure 1A) and tert-butyl hydroperoxide (Figure 1B) compared to Mu50Ω, indicating that VISA strains (with up-regulated MsrB) exhibited higher resistance towards oxidative damage.

We postulate that in order to circumvent oxidative damage caused by vancomycin (Kohanski et al. 2007), VISA cells are primed to produce Msr proteins, which are the only known enzymes capable of reducing oxidized form of methionine, thereby restoring normal function of proteins (Oien & Moskovitz 2008). This cellular response is proposed to be mediated by *VraSR* system, since, in our previous study, up-regulation of MsrB proteins was identified in the *VraS* and *VraS-GraR* regulons, but not the *GraR* regulon (Tan et al. 2016). Accordingly, Pang et al.'s study demonstrated that complementation of *S. aureus vraSR* knockout mutant ($\Delta vraSR$) restored its *msrA1* expression to a higher level compared with $\Delta vraSR$ (Pang et al. 2014).

Nevertheless, a different survival trend was observed in H₂O₂-induced VISA cells. Although having lower MsrB expression, VSSA Mu50Ω displayed greater survival after H₂O₂ induction compared with VISA strains (Figure 1C). Increased susceptibility to H₂O₂ killing was previously reported to be associated with the lack of staphyloxanthin (carotenoid

pigmentation) (Liu et al. 2005; Liu et al. 2008). As suggested by Singh et al. (2015), *msrB* deletion reduced *S. aureus* susceptibility to H₂O₂, and this phenotype is accompanied by increased production of carotenoids in the mutant cells. In concordance, lower expression of MsrB in Mu50Ω might have resulted in higher levels of cellular carotenoids and subsequent resistance to H₂O₂.

In addition to increased resistance to oxidative stress, down-regulation of virulence-related proteins has also been observed in the Mu50Ω-*vraSm* and Mu50Ω-*vraSm-graRm* VISAs (Tan et al. 2016). We subsequently used a *Caenorhabditis elegans* survival assay to determine our study strains' virulence (Sifri et al. 2003). Forty L4 nematodes of *pos-1*-silenced *C. elegans* N2 strain were fed with the study strains of Mu50Ω, Mu50Ω-*vraSm* and Mu50Ω-*vraSm-graRm*, respectively; worm survival (quantity of live and dead worms) for every strain was then scored every 24 hours for 14 days and plotted on a Kaplan-Meier survival plot (Figure 2). The experiment showed that VISA strains exhibited higher nematocidal activity via complete killing of all 40 *C. elegans* on the 3rd (Mu50Ω-*vraSm-graRm*) and 8th (Mu50Ω-*vraSm*) day of the assay. On the other hand, killing of *C. elegans* fed with VSSA was gradual and surviving worms were still observed at the end of the 14-days assay.

C. elegans exhibits specific immune response towards different infective microorganisms; transcription profiles of *C. elegans* exposed to *Candida albicans* has been shown to be different from those infected with *Pseudomonas aeruginosa* or *S. aureus* (Pukkila-Worley et al. 2011). Both living and heat-killed *S. aureus* have been reported to be capable of triggering *C. elegans* responses (Irazoqui et al. 2010). These studies suggested that *C. elegans* distinguish infections from different pathogens via recognition of specific bacterial pathogen-associated molecular patterns (PAMPs). Spa, a *S. aureus* cell wall surface protein, has been

reported to be one of the PAMPs found in this Gram-positive bacterium (Bekeredjian-Ding et al. 2007). In our study, we postulate that down-regulation of Spa protein in VISA strains diminished the capability of *C. elegans* innate immune system to identify the bacteria, allowing VISA to achieve immune evasion. Consequently, VISA infections of *C. elegans* were found to be more lethal compared with VSSA. Even though *C. elegans* produces ROS in response to *S. aureus* infection (Chávez et al. 2007), as VISA strains in this study were found to be more resistant to oxidative killing presumably due to higher expression of MsrB enzymes, the strains had a survival edge from the ROS attack of *C. elegans* compared to VSSA. This allows VISA strains to bypass *C. elegans* defence mechanisms, resulting in expedited killing of the hosts. Nonetheless, these suggested interactions were confined to speculations deduced from our previous proteomics analysis and current phenotypic investigations. Further experiments will be required to confirm this hypothesis.

Taking into consideration the results from our previous (Tan et al. 2016; Tan et al. 2017) and current studies, we propose the interplay between cellular metabolism, oxidative stress response and virulence in VISA strains of Mu50 lineage (Figure 3). The *vraS* and *graR* gene mutations in VISA strains activate arginine catabolism to supply substrates for cell wall biosynthesis (Tan et al. 2017), while oxidative stress response was triggered to neutralize oxidative damages induced by vancomycin. These metabolic alterations subsequently impose a fitness burden on VISA cells, causing a trade-off between bacterial resistance and virulence. Further experiments will be important to consolidate these postulations.

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Figure Legends

Figure 1: Tolerance assessment of Mu50Ω, Mu50Ω-*vraSm* and Mu50Ω-*vraSm-graRm* to oxidative stress by determining cell survival post-treatment with different oxidizing agents. Both VISA strains showed higher resistance to oxidative stress induced by cumene hydroperoxide (panel A) and tert-butyl hydroperoxide (panel B) compared to VSSA. In contrast, VSSA strain was more resistant to oxidative killing by hydrogen peroxide (panel C) compared to VISA strains.

Figure 2: Kaplan-Meier survival plot for *C. elegans* fed with Mu50Ω, Mu50Ω-*vraSm* and Mu50Ω-*vraSm-graRm*. A significant decrease in survival of *C. elegans* infected with VISA strains Mu50Ω-*vraSm* ($p < 0.05$) and Mu50Ω-*vraSm-graRm* ($p < 0.05$) was observed compared to those infected with VSSA strain Mu50Ω. Pairwise comparison also demonstrated a significant reduction in the survival of Mu50Ω-*vraSm-graRm*-infected *C. elegans* compared with Mu50Ω-*vraSm*-infected worms ($p < 0.05$).

Figure 3: VraSR- and GraSR-mediated regulatory pathways associated with intermediate vancomycin resistance in *Staphylococcus aureus* of the Mu50 lineage: (1) contribution of arginine catabolism (arginine deiminase, ADI) pathway to cell wall thickening, (2) MsrB-associated oxidative stress resistance, and (3) fitness-compensatory response.

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