A key regulatory mechanism of antimicrobial resistance in pathogenic *Acinetobacter baumannii*

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***Acinetobacter baumannii* is a Gram-negative bacterial pathogen that has become a pressing global health issue in recent decades. Although virulence factors for this pathogen have been identified, details of how they are regulated are largely unknown. One widely employed regulatory mechanism that bacteria, such as *A. baumannii*, have adopted is through two component signal transduction systems (TCS). TCS consist of two proteins; a histidine kinase and response regulator. The histidine kinase allows the bacterium to sense alterations in the extracellular milieu, transmitting the information to the response regulator which prompts the cell to modify gene expression levels accordingly. Bacteria can encode multiple TCS, where each system can mediate specific responses to particular conditions or stressors. Identifying those conditions in which these TCS are expressed, and the genes they regulate known as their 'regulon', is vital for understanding how *A. baumannii* survives and persists within the hospital environment or the human host during infection. As we enter the post-antibiotic era, knowledge of TCS could prove to be invaluable, as they offer an alternative target for the treatment of multidrug resistant bacterial infections.**

Acinetobacter baumannii is a Gram-negative opportunistic 'superbug' that causes a diverse range of nosocomial infections, primarily in patients whom are immunocompromised, such as those within intensive care units[1]. The *Acinetobacter* species is one of the leading causes of bacterial pneumonia within the hospital environment[2,3] and have been responsible for numerous outbreaks of nosocomial infections, worldwide[4]. Within Australia, *A. baumannii* isolates are not only confined to hospitals but are also seen within community-based settings, primarily in tropical regions of Northern Australia[5,6]. These community-acquired infections have been shown to be predominant in individuals whom have underlying medical conditions such as diabetes mellitus or other risk factors including excessive alcohol consumption[6].

High levels of innate and acquired multidrug resistance mechanisms represent a leading factor in the pathogenic success of this organism. Intrinsic resistance mechanisms include low outer membrane permeability, production of chromosomally-encoded antibiotic inactivating enzymes and efflux pump systems[7,8]. Acquired mechanisms contributing to resistance include the uptake of foreign resistance determinants via horizontal gene transfer[9,10] and over-expression of resistance genes through introduction of insertional elements or mutations particularly within their regulators[11,12]. This ability of the bacterium to acquire and modulate expression of an array of antimicrobial resistance determinants provides a strong ecological advantage to survive the selective pressures typically found within the hospital setting, resulting in the growing emergence of multidrug resistant *A. baumannii* lineages[1].

Infections caused by multidrug resistant *A. baumannii* isolates, particularly those resistant to carbapenems, have been linked to increased morbidity and mortality, as well as a significant rise in hospital associated costs[13]. Recently, the World Health Organization recognised the impending threat of carbapenem resistant *A. baumannii* isolates, listing them as one of the top three critical priorities for research and development towards new therapeutic treatments for antibiotic-resistant bacterial species (excluding Mycobacteria)[14]. Despite the risk this bacterium poses to susceptible individuals and our healthcare facilities, virulence traits including antimicrobial resistance, are not completely understood. Furthermore, the regulatory mechanisms that modulate these characteristics are even more ill-defined.

One simple yet highly sophisticated mechanism that bacteria utilise to effectively regulate the expression of virulence factors employs TCS[15,16]. TCS consist of two proteins; a histidine kinase which

senses variations in the extracellular milieu and a response regulator which alters gene expression upon activation by its cognate histidine kinase[17] (Figure 1). TCS not only modulate virulence-associated mechanisms but also fundamental biological processes such as pathways involved in metabolism[18,19] and osmoregulation[20]. The proportion of genes coding for TCS within a bacterial genome is thought to be dependent on a range of factors, including genome size and the diversity of different environments the bacterium may encounter[21]. Generally, histidine kinase and response regulator genes are co-transcribed in an operon, but they can also exist in the genome separated from their cognate partner, and are defined as orphans[22]. In *A. baumannii*, genomic analyses have identified 12 response regulator genes in an avirulent isolate that increases to 16 to 19 in pathogenic isolates[23]. Of these, only a handful have been experimentally examined[24–29].

An aspect of my research has focused on the AdeRS TCS which was originally identified as a regulator of the AdeABC three component efflux system in many *A. baumannii* clinical isolates (Figure 2*a*). When overexpressed, AdeABC exports a broad range of structurally-unrelated antimicrobials including antibiotics, biocides and dyes[25,30–34]. Importantly, within this group of substrates are compounds from the carbapenem class of antibiotics as well as tigecycline, one of the last lines of defence against *A. baumannii*[35]. Analysis of many multidrug resistant *A. baumannii* isolates has shown a high incidence of mutations or the presence of insertional elements in the AdeRS regulatory system, deeming this TCS to be a significant contributor to the observed multidrug resistance phenotype[11,36,37]. At a genetic level, the *adeRS* genes lie adjacent to *adeABC*, but are divergently transcribed (Figure 2*b*). The AdeR response regulator protein binds to a 10 base-pair direct repeat DNA sequence and modulates *adeABC* expression[38]; however, the external signal(s) that interact with the AdeS histidine kinase remain unknown. Aside from changes in antimicrobial resistance, deletion of *adeRS* seen in some clinical isolates, has identified significant alterations in persistence strategies, such as

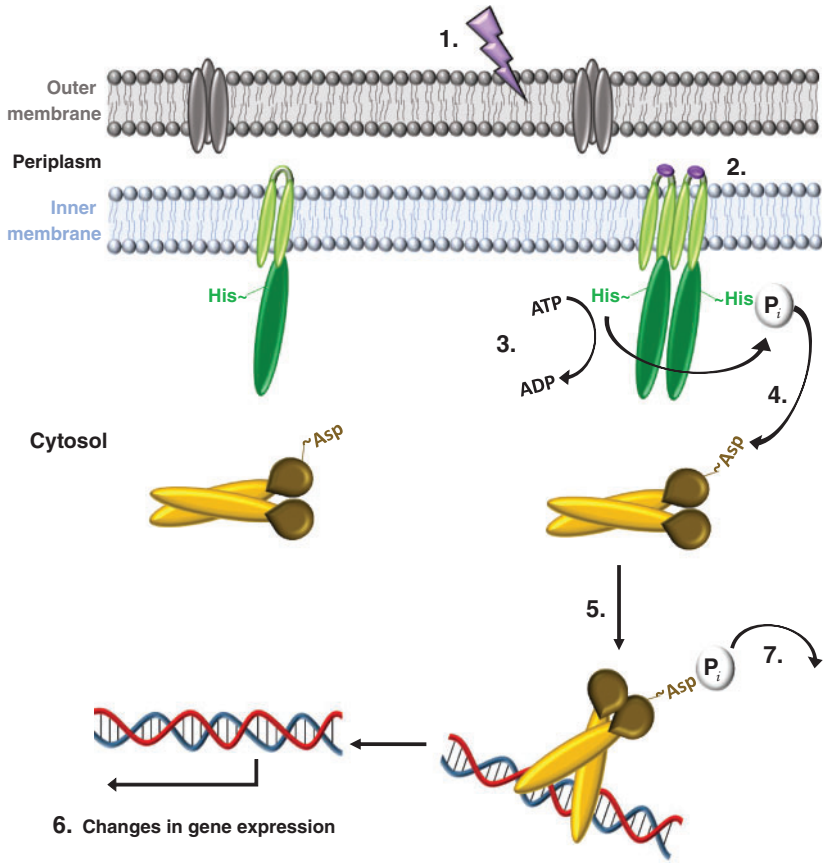

Figure 1. Schematic representation of a typical two component signal transduction system cascade in Gram-negative bacteria. Two component signal transductions systems consist of a histidine kinase (HK) and response regulator (RR) protein (green and yellow, respectively). HK proteins are generally localised in the inner membrane and possess a variable N-terminal sensing domain and a highly conserved C-terminal kinase region (light and dark green, respectively). In contrast, cytosolic RR proteins contain a highly conserved N-terminal domain and a variable C-terminal output domain (brown and yellow, respectively). The HK detects the presence of an external stimulus (1). Binding of the stimulating agent induces a conformational change in the HK (2) resulting in trans-autophosphorylation between HK homodimers whereby one monomer catalyses phosphorylation using ATP of the conserved histidine (His) residue in the second monomer (3). This phosphate (P_i) is subsequently transferred to the highly conserved aspartate (Asp) residue on the RR protein (4). Phosphorylation of the RR induces conformational changes that alters its DNA binding properties (5) modulating target gene expression (6). Resetting the system to pre-stimulus state is attained by de-phosphorylation of the RR (7) through phosphatase activity of the HK or by other phosphatase enzymes.

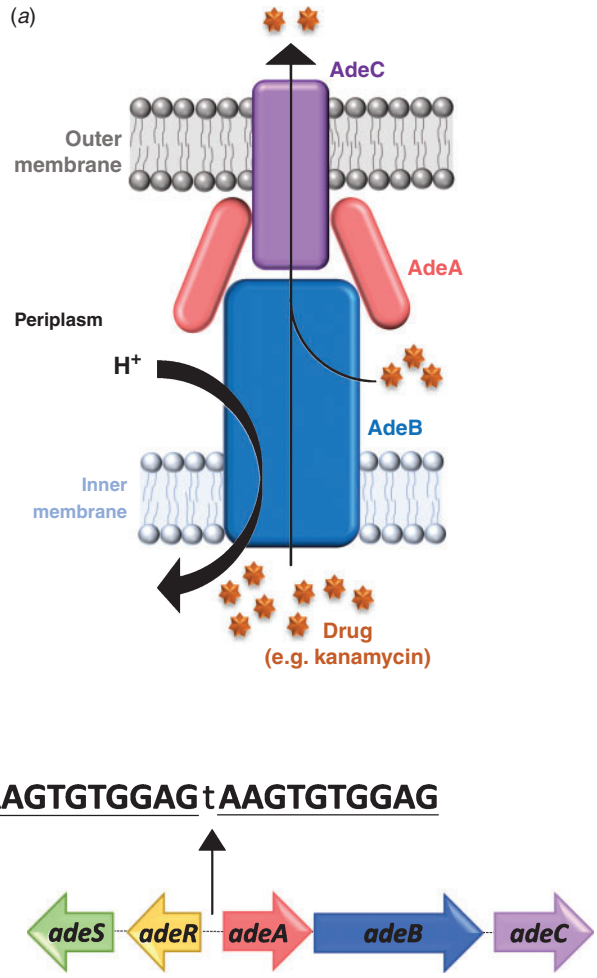

Figure 2. Composition of the AdeABC tripartite pump and genetic organisation of the *adeRS* and *adeABC* operons typically found in the *Acinetobacter baumannii* membrane and chromosome, respectively. (*a*) The pump is constituted of three proteins; AdeB the cytoplasmic membrane transporter (blue), AdeA the membrane fusion protein (pink) and AdeC the outer membrane protein (purple), that come together to form a functional complex. Activity of the AdeABC pump is coupled to the proton gradient (H^+), where substrates of the pump, e.g. kanamycin (orange stars), can be directly expelled into the external environment. (*b*) The *adeRS* genes lie adjacent to the *adeABC* genes and are divergently transcribed (arrows indicate the coding sequence and the direction of transcription). Located within the intergenic region between *adeR* and *adeA* is a 10 base-pair direct repeat, separated by a thymine nucleotide. This repeat is predicted to be where the AdeR response regulator binds to modulate *adeABC* gene expression.

the production of an extracellular protective matrix known as a biofilm[32,33].

Many clinical *A. baumannii* isolates harbour different genetic arrangements of the *adeRS* and *adeABC* operons[39]. Examination into one *A. baumannii* clinical isolate identified that insertional-inactivation of the outer membrane component of the pump (AdeC) did not affect resistance towards two previously identified AdeABC substrates[25]. It was suggested that in the absence of AdeC, AdeAB can utilise an alternative outer membrane protein to form a functional tripartite complex[25]. Given that AdeC may not be essential to confer antimicrobial resistance, and the diverse genetic arrangements of these operons across clinical *A. baumannii* isolates, a key aim of my studies was to (1) ascertain whether clinical isolates which naturally lack *adeC* also confer antimicrobial resistance and to (2) determine if the previously observed regulatory properties governed by AdeRS are also maintained. The well characterised *A. baumannii* 'type' strain ATCC 17978[40] isolated from an infant with fatal meningitis, which does not carry *adeC*, was chosen for genetic manipulation. Through double homologous recombination techniques, mutant derivatives targeting either *adeRS* or *adeAB* genes were generated and compared to the ATCC 17978 parent. Antibiogram analysis of the *adeAB* mutant identified changes in resistance to a subset of structurally related antimicrobials, including a commonly utilised clinical disinfectant. The role of the AdeRS TCS in modulating expression of *adeABC* is of current debate. As deletion of *adeRS* exhibited similar resistance levels to the *adeAB* deletion strain, my research supports the hypothesis that the AdeRS TCS activates expression of the *adeAB*(*C*) operon[25]. These research findings shed new light on the resistance capabilities of the AdeABC pump, questioning the views that AdeABC does not contribute towards the intrinsic resistance of *A. baumannii* and that antibacterial efflux can only occur when AdeABC is constitutively over-expressed[32].

To identify the effects of the deletion of *adeRS* on the global transcriptional landscape, RNA-sequencing methodologies were employed. Numerous changes in gene transcription levels were identified including expression of *adeAB*. Additionally, other genes known to be important for virulence, such as iron sequestering and pilus assembly operons were differentially expressed. AdeR has previously been found to bind to a 10 base-pair direct repeat only found within the intercistronic region between the *adeRS* and *adeABC* operons[38]. Genomic analyses within ATCC 17978 also support this finding. Therefore, in the *adeRS* deletion strain, aside from alterations in *adeAB* expression, the transcriptional changes in the aforementioned virulence associated genes are likely to be indirect. Interestingly, these direct/indirect transcriptional changes differed from an *adeRS* deletion mutant constructed in a different *A. baumannii* clinical isolate[33], emphasising that changes in the global transcriptional landscape are dependent on the isolate under investigation.

With a lack of currently effective antimicrobial treatments and a less than promising pipeline for the generation of new antibiotics, research into novel antimicrobial treatments is of significant interest. Histidine kinases of TCS are seen as attractive targets, primarily due to their presence in many pathogenic bacterial species[16] and the absence of homologues in higher eukaryotes, including humans[41]. A number of novel inhibitors towards some TCS regulatory cascades present across a number of clinically relevant

pathogenic bacterial species have been identified[42]; however, no inhibitors have been recognised for TCS found within *A. baumannii*. Results from this research area have made promising leads but progress is slow and many challenges still remain[42].

In recent decades, *A. baumannii* has fast become an extremely problematic hospital-acquired pathogen, propelled by its ability to flourish within hostile clinical environments and accrue resistance to the current armamentarium of therapeutic treatments. The AdeABC efflux system is a known contributor to the multidrug resistance phenotype displayed by this organism. My research into this system has identified that AdeAB in ATCC 17978 is functional despite the absence of AdeC and can provide intrinsic antimicrobial resistance, albeit to a limited substrate range. Antibacterial drug research efforts over recent decades have highlighted the eligibility of targeting TCS regulatory cascades for the development as an alternate therapy to treat bacterial infections. In light of this research, the AdeRS system holds particular interest due to its direct role in regulating a key aspect of multidrug resistance in many clinical *A. baumannii* isolates. Further examination into the AdeRS TCS is required, particularly identifying the activating stimuli of this system. This knowledge may be instrumental in the identification of novel inhibitors, which could aid in the future treatment of infections caused by this formidable pathogen.

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Biography

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