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The StkSR Two-Component System Influences Colistin Resistance in Acinetobacter baumannii

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Abstract: Acinetobacter baumannii is an opportunistic human pathogen responsible for numerous severe nosocomial infections. Genome analysis on the A. baumannii clinical isolate 04117201 revealed the presence of 13 two-component signal transduction systems (TCS). Of these, we examined the putative TCS named here as StkSR. The stkR response regulator was deleted via homologous recombination and its progeny, ∆stkR, was phenotypically characterized. Antibiogram analyses of ∆stkR cells revealed a two-fold increase in resistance to the clinically relevant polymyxins, colistin and polymyxin B, compared to wildtype. PAGE-separation of silver stained purified lipooligosaccharide isolated from ∆stkR and wildtype cells ruled out the complete loss of lipooligosaccharide as the mechanism of colistin resistance identified for ∆stkR. Hydrophobicity analysis identified a phenotypical change of the bacterial cells when exposed to colistin. Transcriptional profiling revealed a significant up-regulation of the pmrCAB operon in ∆stkR compared to the parent, associating these two TCS and colistin resistance. These results reveal that there are multiple levels of regulation affecting colistin resistance; the suggested ‘cross-talk’ between the StkSR and PmrAB two-component systems highlights the complexity of these systems.

Keywords: pmrCAB; hydrophobicity; lipid A; adherence; phosphoethanolamine; TCS; antibiotic resistance

1. Introduction

Acinetobacter baumannii is a Gram-negative nosocomial opportunistic pathogen that causes severe infections worldwide and is at the top of the World Health Organization’s (WHO) list of priority pathogens for the R&D of new antibiotics [1]. Pandemic outbreaks of A. baumannii are increasing in incidence and severity due to its multidrug and pandrug-resistant nature [2–5]. Patients presenting with these infections commonly rely on “last resort” antibiotics such as the polymyxin class of drugs. Polymyxin E (colistin) and polymyxin B are cationic peptides composed of a cyclic heptapeptide covalently attached to a fatty acyl chain [6,7]. Colistin is bactericidal with positively charged α,γ-diamonobutyric acid residues which interact by attaching and displacing the magnesium and calcium divalent cations of the negatively charged phosphate groups of lipid A. Lipid A stabilizes the lipooligosaccharide or lipopolysaccharide component of the bacterial membrane [8–10]. Displacement of these cations increases the permeability of the outer membrane causing uptake into the periplasm and perturbation and surface corrugation of the outer and inner membranes. This can result in the collapse of the outer membrane and leakage of the cellular content, a phenomenon known as blebbing [11,12].

For A. baumannii and other pathogenic bacteria, the ability to sense and respond to external and internal signals is critical for survival in the environment as well as within
the host. Two-component signal transduction systems (TCS) aid in the regulation of pathogenesis and thus are implicated in the circumvention of antibiotic treatments [5,8,13–15]. Regulation of these systems typically involves a membrane-bound histidine kinase and its cognate response regulator, however, ‘cross-talk’ between histidine kinases and non-cognate response regulators also occurs [16,17]. When a signal is received by a histidine kinase, the histidine kinase protein forms a dimer and is autophosphorylated at a conserved histidine residue. The phosphate molecule activates its cognate response regulator protein by being transferred to the conserved aspartate residue in the N-terminal region [5,18,19]. Activation of the response regulator typically produces a conformational change in the C-terminal or variable region of the response regulator protein [18], and in the case of DNA-binding response regulators, this allows for stimulation or repression of target genes thereby affecting cellular processes [19,20].

TCS are associated with alterations in antibiogram profiles; for example, in A. baumannii the PmrAB system is linked to increased polymyxin resistance, where mutations in pmrA and/or pmrB result in up-regulation of the pmrCAB operon leading to the modification of lipid A by the addition of phosphoethanolamine (pEtN) to either 4′ or 1′ phosphate lipid A [5,21–23]. Other two-component systems implicated in antibiotic resistance include the AdeRS system which regulates an efflux pump which significantly contributes to antibiotic resistance of aminoglycosides and tigecycline in A. baumannii [5,24]. Additionally, in other bacteria like Klebsiella pneumoniae, TCS cascades have been identified in the PhoPQ and the PmrAB systems involved in regulating colistin resistance via the pmrCAB operon [25,26].

Two main mechanisms for colistin resistance have been previously identified in A. baumannii strains. One is the complete loss of the lipid A portion of the lipooligosaccharide, which correlates with mutations in lpxA, lpxC, lpxD, lpsB, lpsD, and vacJ genes which encode acyltransferases essential to lipid A biosynthesis associated with permeability defects [8,9,27,28]. The second is the modification of lipooligosaccharide by the addition of pEtN or 4-amino-4-deoxy-l-arabinose to the phosphate groups of lipid A [2,8]. Two distinct pathways for this modification have been identified; the first requires pmrC encoded in an operon with the PmrAB two-component system [10,27–31]. The second is correlated to the insertion of an IS element (ISAbA125) in an H-NS-family transcriptional regulator, increasing expression of eptA which encodes a second pEtN transferase [32]. The increase in pEtN reduces the overall membrane electronegativity, thereby reducing its affinity to polymyxins [23]. Here we identified a novel regulator that influences the expression of pmrCAB elucidating that multiple layers of regulation can be employed by A. baumannii to confer colistin resistance.

2. Materials and Methods

2.1. Bacterial Strains and Media

The clinical A. baumannii bacterial strains used in this study have been described previously [33] and were cultured as previously described [34]. For maintenance, conjugation, cloning, and replicating plasmids, Escherichia coli DH5α, SM10, or JM109 cells were used [35–37]. The A. baumannii 04117201 isolate belongs to the International Clone type II lineage and was obtained from a tracheal aspirate sample at Flinders Private Hospital, SA, Australia.

2.2. Eukaryotic Cell Adherence Assay

Adherence of A. baumannii to human type 2 A549 pneumocytes was investigated as previously described [34]. The data collected for the adherence assays were obtained from at least three independent experiments and represent data points from each experiment in quadruplicate wells.

2.3. Minimum Inhibitory Concentration and Disk Diffusion Susceptibility

Minimum inhibitory concentrations (MIC) were determined using standard methods as previously described [38]. In brief, 96-well microtiter trays containing a 2-fold dilution
series of the compound of interest were prepared and cultures added 1:1 in Mueller-Hinton broth (MH) (Oxoid, ThermoFisher, Adelaide, SA, Australia) before incubating overnight at 37 °C with shaking (~50 rpm). Bacterial growth was examined by absorbance at OD\textsubscript{600} using a Multiskan EX (Adelab Scientific, Adelaide, SA, Australia). The standard agar disc diffusion technique was performed on MH 1% agar with bacterial strains diluted to the McFarland 0.5 standard. Results of the Kirby–Bauer disc diffusion were evaluated according to the Clinical and Laboratory Standards Institute guidelines [39,40].

2.4. Deletion Replacement Mutant Construction by Homologous Recombination

Construction of \textit{A. baumannii} 04117201 stalkR-inactivated derivatives was undertaken by homologous recombination as described previously [24] with the following modification. DNA fragments corresponding to 1.2 kb upstream and 1 kb downstream of stalkR were PCR amplified using VELOCITY\textsuperscript{TM} DNA polymerase (Bioline, Sydney, NSW, Australia) using specific oligonucleotides designed in PRIMER Biosoft NetPrimer (www.premiersoft.com, accessed on 22 September 2015) (Table S1). Amplified PCR products of the appropriate size were cloned into the suicide vector pEX18Tc via XbaI, BamHI, and SacI restriction sites and transformed into \textit{E. coli} DH5α cells. Following sequence confirmation, pEX18Tc clones were electroporated into freshly prepared electrocompetent \textit{A. baumannii} 04117201 bacterial cells and homologous recombination was undertaken as previously described [41]. Two ΔstalkR derivatives were generated, and the phenotypic analysis of one is presented here.

2.5. Cell Surface Hydrophobicity Tests

Cell surface hydrophobicity was examined as described previously [34,41]. The OD\textsubscript{600} of the cell suspension was determined before (OD\textsubscript{initial}) and after (OD\textsubscript{final}) the addition of xylene. The hydrophobicity index was calculated as \((\text{OD}_{\text{initial}} - \text{OD}_{\text{final}}/\text{OD}_{\text{initial}}) \times 100\). Quantitative data were collected from at least three experiments from three different days.

2.6. Transmission Electron Microscopy

Transmission electron microscopy (TEM) was undertaken as previously described [42] with the following modifications. \textit{A. baumannii} cells grown to log phase were treated for 1 h at 37 °C with shaking in the presence of colistin at 0, 3, or 12 µg/mL. Washed and fixed samples were dehydrated in an ethanol series and embedded in 1 mL EMbed 812/Araldite 502 resin (Emgrid, Adelaide, SA, Australia). Samples were cut with a diamond knife (Diatome, Philadelphia, PA, USA) and microtome (Leica EM UC6 Ultramicrotome) (Leica, Sydney, NSW, Australia) and examined using a TEM (FEI Tecnai G2 Spirit, New York, NY, USA). A magnification of 60,000× was used for bacterial cell analysis, whereas for determination of blebs per cell 30 TEM slides (~600 cells) at a magnification of 11,500× was used. A total of 30 independent fields, including a minimum of 10 cells per field of view, spanning three different slides, were used to calculate blebs/cell.

2.7. SDS-PAGE and Lipooligosaccharide Silver Staining

SDS-PAGE and lipooligosaccharide silver staining was undertaken as previously described [43,44]. In short, Proteinase K treated samples were resolved on an SDS-PAGE gel. Fixation was achieved using ethanol and glacial acetic acid and followed by oxidation using ethanol and glacial acetic acid and periodic acid. The gel was washed with MilliQ and stained with NaOH, NH\textsubscript{4}OH and AgNO\textsubscript{3}.

2.8. RNA Isolation

RNA was extracted as previously described [34] with the following modifications. Cells were grown in MH broth (Oxoid, ThermoFisher, Adelaide, SA, Australia) to an OD\textsubscript{600} of 0.6 and treated with colistin at 3 µg/mL for 1 h. Harvested bacterial cells were lysed and TRIzol and chloroform treated. Phases were separated by centrifugation and RNA extracted using the RNA Mini Kit II (Bioline, Australia) following the manufacturer’s recommendations before DNaseI treatment.
2.9. Quantitative Reverse Transcription PCR

The iScript reverse-transcriptase cDNA synthesis kit (Bio-Rad, Sydney, NSW, Australia) was used to synthesize cDNA following the manufacturer’s recommendations. Quantitative reverse transcription PCR was performed using iTaq™ Universal SYBR® green supermix (Bio-Rad, Sydney, NSW, Australia) in conjunction with a Rotor-Gene Q (Qiagen, Melbourne, VIC, Australia) with oligonucleotides designed in PRIMER Biosoft NetPrimer (www.premiersoft.com, accessed on 10 July 2016) (Table S1). A typical quantitative reverse transcription PCR run was 2 min at 95 °C followed by 40 cycles of 10 s at 95 °C, 15 s at 60 °C, and 20 s at 72 °C [45]. Transcriptional differences were calculated using the $2^{-\Delta\Delta CT}$ method [46] and the 16S rRNA (A1S_r01) and GAPDH (A1S_2501) transcription levels were used as a reference.

2.10. DNA Extraction

Bacterial cultures of $A. baumannii$ 04117201 and $\Delta stkR$ were grown in MH broth to an OD$_{600}$ of 0.6. DNA purification was conducted using a spin column purification kit (NucleoSpin tissue, Macherey-Nagel, Düren, Germany) following manufacturer’s instructions. Library preparation for sequencing on a MinION using a Flongle Flow cell and adaptor (Oxford Nanopour, Düren, UK) was undertaken following the SQK-RBK004 protocol as per the manufacturer’s instructions.

2.11. Library Quality Control and Bioinformatics

Base calling was achieved using guppy-basecaller converting fast5 files to fastq format, along with demultiplexing and adapter trimming. Filtlong v0.2.0 (https://github.com/rrwick/Filtlong, accessed on 15 January 2021) was used to remove reads less than 1000 bp and 5% of the lowest quality reads. Post quality control (QC), Nanopore reads were de novo assembled using Unicycler v0.4.8 (https://github.com/rrwick/Unicycler, accessed on 20 January 2021) [47] producing a complete chromosome for the 04117201 wild-type (WT) strain and the $\Delta stkR$ mutant strain. To correct the indels in the assembly, Pilon v1.23 [48] was run to polish the complete assemblies with RNAseq Illumina reads. To confirm the quality of the assembly improved, the pre pilon assembly and post pilon assembly was run through ideal (https://github.com/mw55309/ideel, accessed on 27 January 2021). Ideael calculates the ratio of the protein sequences predicted in the assemblies compared to the top protein hits they aligned against in the Uniprot database. Finally, Bandage was used to visualize the completed assembly [49]. To identify the variants between strains, reads from the 04117201 WT strain and two $\Delta stkR$ mutant strains (Section 2.4) were aligned against the complete 04117201 WT strain using Bowtie2 v2.3.5 [50]. The mapped reads were piled up against the reference 04117201 WT strain using SAMtools v1.9 [51]. VCFools v0.1.17 [52] was used to identify the SNPs observed in the alignment. To identify which genes the SNPs were found in, the assembled genomes were annotated using Prokka v1.14.6 [53] and validated using progressive Mauve (v2) and Artemis.

2.12. Statistical Analysis

The Graphpad Prism 7.0 (La Jolla, CA, USA) statistical package was used to analyze the results where appropriate. Assessments of skewness, kurtosis, and the Shapiro–Wilk normality test [54] were undertaken on all measures, and non-parametric tests were applied as needed.

2.13. Accession Numbers

Sanger sequencing of the DNA sequence of the two-component signal transduction system StkRS of $A. baumannii$ 04117201 has been deposited in Genbank ID 2135660.
3. Results

3.1. Selection of A. baumannii Isolate

The commonly used reference strain A. baumannii ATCC 17978 is not highly virulent in our hands, as seen in Figure 1 and Table 1, therefore we sought to identify a potentially virulent A. baumannii isolate. Within our collection, 11 clinical isolates were screened for two important clinical virulence-related phenotypes: cell adherence and antibiotic resistance [55]. Incubation with human A549 pneumocytes revealed the A. baumannii isolate 04117201 to be significantly more adherent than the reference ATCC 17978 strain ($p < 0.001$; Figure 1), and more adherent than the other clinical strains tested (Figure 1).

![Figure 1](image.png)

*Figure 1.* Cell adherence capacity of A. baumannii clinical strains to A549 pneumocytes. The CFU/well values of 11 A. baumannii strains were examined after exposure to A549 cells. Bars indicate the standard error of three separate experiments. Assessments of skewness, kurtosis and the Shapiro–Wilk normality test were undertaken. *** represents a $p$-value of $< 0.001$ as determined by an analysis of variance test, between ATCC 17978 and the clinical isolate.

Antimicrobial resistance screening of the 11 clinical isolates identified the 04117201 strain as more resistant to many of the compounds tested compared to the reference ATCC 17978 strain. However, it is sensitive to erythromycin (Table 1), allowing the use of this antibiotic as a selection marker for mutant construction. As previously published, we have shown that the A. baumannii 04117201 cells can form a well-established biofilm, which is an important virulence factor [56].

3.2. Construction of an A. baumannii 04117201 ΔstkR Derivative

The genome of the WT A. baumannii 04117201 strain was sequenced by Sanger sequencing at the Welcome Trust Sanger Institute and assembled using Velvet v1.2.03. Visual inspection of the sequence using RAST v2.0 identified 13 TCS response regulator proteins in the genome. A comparison of the 13 TCS identified in the clinical 04117201 strain was undertaken against those present in the A. baumannii SDF and ATCC 17978 strains identifying five response regulators proteins AdeR, BarA, StkR and two orphan response regulators NasT and RsbU not present in the SDF avirulent strain (Table 2). The TCS not present in A. baumannii SDF we named as StkSR ‘sticky’ due to the observed increased adherence to eukaryotic cells. The StkSR system identified in the clinical 04117201 strain encompasses a membrane bound hybrid histidine kinase and a cytosolic response regulator. The StkR response regulator has a conserved C-terminal helix-turn-helix motif placing it in
the LuxR family. To assess the role of the StkSR system in the clinical 04117201 strain, two independent \textit{A. baumannii} \( \Delta \text{stkR} \) mutants were constructed. One \( \Delta \text{stkR} \) mutant was selected and its growth kinetics compared to the WT in different media representing different types of environment conditions including, MH (laboratory), minimal M9 medium (desiccation on surfaces and ventilator tubing), lung medium (pneumonia) [57], and whole sheep blood (septicemia) (Figure S1). Growth of \( \Delta \text{stkR} \) was comparable to WT in MH, M9 and lung medium. However, bacterial growth was significantly reduced in whole sheep’s blood. Both the WT and \( \Delta \text{stkR} \) strains showed a 5-log decrease in colony forming units (CFU) in the first hour post exposure to whole sheep’s blood, after which the CFU of both strains did not change for 2 h. The \( \Delta \text{stkR} \) mutant strain showed limited recovery and reached \( 10^4 \) CFU by the 4-h time point, whereas the WT did not reach a \( 10^4 \) CFU for another hour; both the WT and \( \Delta \text{stkR} \) strains recovered to \( 10^6 \) CFU by 7 h. These data indicate that the deletion of the response regulator \( \text{stkR} \) does not result in a general decrease in bacterial cell fitness when grown in MH, M9 or lung media; however, there is a marked increase in bacteria cell CFU recovery of the \( \Delta \text{stkR} \) mutant strain compared to the WT strain after exposure to whole sheep’s blood.

Table 1. Antimicrobial resistance profiles of \textit{Acinetobacter baumannii} clinical strains.

| Compound \(^ab\) | ATCC 17978 | 04117201 | 1172312 | WM98 \(^c\) | 08315000 | 04397670 | 9030751 | 1077697 | 9038266 | 08325050 | 083217005 |
|-----------------|-------------|-----------|---------|------------|----------|----------|--------|--------|--------|----------|----------|
| KAN \(^c\) | R | R | R | S | S | R | R | S | R | S | R |
| ERY | S | S | S | S | R | S | S | I | S | I | R |
| TET | S | R | R | I | R | I | R | I | R | R | R |
| AMP | 250 | >500 | >500 | 250 | >500 | >500 | >500 | >500 | >500 | >500 | >500 |
| CIP | 3.25 | 30 | 30 | 3.25 | >240 | 60 | 60 | 60 | 60 | >240 | >240 |
| CST | 4 | 8 | 8 | 16 | 8 | 8 | 32 | 32 | 16 | 16 | 4 |
| TEL | 0.93 | 1.87 | 1.87 | 1.87 | 3.75 | 3.75 | 1.87 | 1.87 | 3.75 | 3.75 | 3.75 |
| GEN | 32 | 500 | 500 | 16 | 125 | 250 | 125 | 125 | 250 | 125 | 125 |
| PMB | 8 | 16 | 16 | 16 | 8 | 16 | 32 | 32 | 8 | 8 | 8 |
| RIF | 8 | 8 | 8 | 8 | >64 | 4 | 4 | 4 | 4 | 4 | 4 |
| \( \text{H}_2\text{O}_2 \) \(^d\) | 125 | 250 | 250 | 500 | 250 | 250 | 125 | 250 | 250 | 125 | 250 |

\(^a\) KAN, kanamycin; ERY, erythromycin; TET, tetracycline; AMP, ampicillin; CIP, ciprofloxacin; CST, colistin; TEL, tellurite; GEN, gentamicin; PMB, polymyxin B; RIF, rifampicin; \( \text{H}_2\text{O}_2 \), hydrogen peroxide. \(^b\) (\( \mu \text{g/mL} \)). \(^c\) R, Resistant; I, Intermediate; S, Susceptible; determined by Kirby Bauer against the CLSI standards. \(^d\) (nM).

Table 2. Response regulators identified in the clinical \textit{Acinetobacter baumannii} 04117201.

| Peg \(^a\) in 04117201 | Location in 04117201 | A1S \(^a\) in ATCC 17978 | Location in ATCC 17978 | Present in SDF | Response Regulator \(^a\) | Description |
|-----------------|------------------|------------------|------------------|----------------|-----------------|------------|
| 25 | 18192–18935 | A1S_1753 | 2038890–2044193 | N | AdeR | TCS, known as AdeRS, involved in antibiotic resistance and AdeABC efflux pump [58] |
| 95 | 40498–39824 | A1S_2751 | 3193938–3189264 | Y | PmrA | TCS, known as PmrAB involved in lipid A modification [59] |
| 226 | 180539–179853 | A1S_2883 | 3334601–333044 | Y | BaeR | TCS known as BaeRS involved in chemical transport and regulation of AdeABC and AdeJK pumps [60] |
| 519 | 97888–98604 | A1S_2137 | 2497340–2492622 | Y | KdpE | TCS, known as KdpED involved in potassium transport [61] |
| 838 | 118775–122230 | A1S_1394 | 1636507–1641464 | N | StkR | TCS known as GerE (renamed here as StkSR) |
Table 2. Cont.

| Peg # in 04117201 | Location in 04117201 | AIS + # in ATCC 17978 | Location in ATCC 17978 | Present in SDF | Response Regulator a | Description |
|-------------------|----------------------|-----------------------|------------------------|----------------|----------------------|-------------|
| 1104              | 21883–21119          | A1S_3229              | 3720752–3725517        | Y              | OmpR                 | TCS, EnvZ-OmpR, involved in osmotic stress [62] |
| 1270              | 84347–85009          | A1S_2288              | 2655031–2650369        | Y              | QseB                 | TCS, known as QseBC involved in biofilm formation [63] |
| 1649              | 134448–135158        | A1S_3375              | 3881425–3886134        | Y              | PhoB                 | TCS, known as PhoRB involved in phosphate stress and quorum sensing [64] |
| 1825              | 90896–91486          | A1S_2006              | 2319938–2324522        | N              | NasT                 | Orphan response regulator known as NasT; no histidine kinase identifiable up or down stream |
| 1962              | 98859–99575          | A1S_0748              | 887026–892617          | Y              | RstA                 | TCS, known as BfmR involved in biofilm formation [65] |
| 2189              | 37589–36954          | A1S_0233              | 259526–264163          | N              | PilR                 | TCS, known as PilR involved in Type 4 fimbriae expression [66] |
| 2213              | 62365–63105          | A1S_0261              | 284993–289733          | Y              | AlgB                 | TCS, known as AlgBZ involved in alginate biosynthesis [67] |
| 2465              | 1290–2258            | A1S_0621              | 670111–674210          | N              | RsbU                 | Orphan response regulator known as RsbU; no histidine kinase identifiable up or down stream [68] |

a Annotated by Rapid Annotation using subsystem technology (RAST) https://rast.nmpdr.org/ (accessed on 15 March 2015). b Locus tag number.

3.3. Assessment of the A. baumannii ΔstkR Mutant Strain Antibiogram

As TCS are known for regulating a variety of genes involved in antibiotic resistance, the ΔstkR and WT strains were assessed for their ability to resist a variety of antimicrobial compounds by MIC assays (Table 3). Five of the 16 compounds tested had altered antibiograms between the WT and ΔstkR strains. There was at least a two-fold increase in resistance to colistin and polymyxin B (both cationic antimicrobial compounds) for ΔstkR. Chloramphenicol and rifampicin showed a two-fold increase of the ΔstkR mutant compared to the WT strain and kanamycin resistance increased past the MIC threshold of detection in the ΔstkR mutant compared to the WT strain (Table 3).

Table 3. Antimicrobial resistance profile of the Acinetobacter baumannii 04117201 and ΔstkR mutant.

| Acinetobacter baumannii Strain | Compound          | WT (µg/mL) | ΔstkR (µg/mL) | Antibiotic Family Group |
|--------------------------------|-------------------|------------|---------------|------------------------|
| Novobiocin                     | 31                | 31         | Aminocoumaria |
| Amikacin                       | 10                | 10         | Aminoglycoside |
| Gentamicin                     | 500               | 500        | Aminoglycoside |
| Kanamycin                      | 3000              | >3000      | Aminoglycoside |
| Streptomycin                   | >300              | >300       | Aminoglycoside |
| Chloramphenicol;               | 5                 | 5–10       | Amphenicol    |
| Rifampicin                     | 4                 | 4–8        | Ansamycins    |
| Ampicillin                     | >500              | >500       | Beta-lactam    |
| Ciproflaxacin                  | 30                | 30         | Carboxy fluoroquinoline |
| Chlorhexidine                  | 7.5–15            | 15         | Chlorobenzenes |
| Triclosan                      | 0.15–0.65         | 0.31–0.65  | Diphenyl ethers |
| Tellurite                      | 1.87              | 1.87       | Metal         |
| Triton X100                    | 64                | 64         | Nonionic surfactant |
| Pentamidine                    | 125               | 125        | Phenol ether  |
| Colistin                       | 8                 | 16         | Polymyxin     |
| Polymyxin                      | 16                | 32         | Polymyxin     |
| Nalidixic acid                 | 1250              | 1250       | Quinolone     |
3.4. Examination of Cell Surface Hydrophobicity of the A. baumannii ΔstkR Mutant Strain When Exposed to Sub-MIC Levels of Colistin

Colistin is a cationic antimicrobial compound that acts by attaching and displacing the magnesium and calcium cations and thereby destabilizing the lipooligosaccharide in A. baumannii [69]. The hydrophobicity of the cell surface influences the affinity of colistin to the membrane [8,70]. Therefore, the cell surface hydrophobicity of the ΔstkR mutant strain was compared with the WT by its affinity to xylene with and without exposure to sub-MIC levels of colistin [41,71]. Under no colistin stress the ΔstkR mutant strain showed a higher cell surface hydrophobicity compared with the WT strain ($p < 0.001$; Figure 2), however, under sub-MIC levels of colistin, the ΔstkR mutant strain showed a significant decrease in cell surface hydrophobicity compared with the WT strain ($p < 0.01$; Figure 2). These results demonstrate the ability of the StkR response regulator to alter the bacterial cells surface hydrophobicity in response to external stressors.

![Graph showing cell surface hydrophobicity comparison](image)

**Figure 2.** Cell surface hydrophobicity of A. baumannii 04117201 and ΔstkR mutant. Cell surface hydrophobicity was assessed by the cells’ affiliation to xylene. A. baumannii strain 04117201 (WT) is without colistin exposure, WT + is with exposure to 3 µg/mL of colistin. A. baumannii 04117201 ΔstkR mutant is without colistin exposure, ΔstkR+ is with exposure to 3 µg/mL of colistin. Bars represent the standard deviation of three separate experiments. A Kruskal–Wallis test was performed to compare the means of the WT and ΔstkR mutant with and without colistin exposure. *** represents a $p$-value < 0.001 and ** represents a $p$-value < 0.01 as established by an analysis of variance test.

3.5. Visualizing the Effects of Colistin Stress on the Bacterial Cell Envelope

As the mode of action of colistin involves the disruption of the cell surface lipopolysaccharide [72], changes to the bacterial cell surface were assessed by TEM. Neither the WT nor ΔstkR mutant strain showed disruption of the bacterial cell outer membrane without colistin treatment (Figure 3A,B). However, after exposure to a sub-MIC level of colistin (3 µg/mL) (Figure 3C,D) significantly more protruding events per bacterial cell (blebs/cell) were observed in the WT strain (2.5 ± 0.3 blebs/cell) compared to the ΔstkR strain (0.7 ± 0.05 blebs/cell; $p < 0.0001$) [23,73]. Treatment of the WT and ΔstkR mutant strains with 12 µg/mL of colistin resulted in both strains exhibiting extensive membrane perturbation leading to a breakdown of the bacterial cell wall with partially disintegrated cells (Figure 3E,F).
Figure 3. Membrane perturbation of *A. baumannii* 04117201 (WT) and ΔstkR mutant after colistin stress. WT and ΔstkR mutant strains were subjected to sub-MIC and MIC levels of colistin (3 µg/mL and 12 µg/mL) and cross-sections were examined by TEM (60,000 × magnification); 200 nm scale is shown in white. Images are of (A) WT, (B) ΔstkR, (C) WT after exposure to 3 µg/mL colistin, (D) ΔstkR after exposure to 3 µg/mL of colistin, (E) WT after exposure to 12 µg/mL colistin, and (F) ΔstkR after exposure to 12 µg/mL colistin; arrows show membrane perturbation and/or leakage of cellular contents.

3.6. Visualizing the Lipooligosaccharide Composition of the *A. baumannii* ΔstkR Mutant and WT Strain

*A. baumannii* acquires resistance to colistin by two known mechanisms: one is the complete loss of the lipid A portion of lipooligosaccharide [70,74] and the other is through the modification of lipid A [75]. To see if disruption of *stkR* influences lipid A production, silver staining of the bacterial cell lipooligosaccharide was performed. The WT and ΔstkR bacterial strains were grown under normal conditions and their lipid A profiles assessed. Colistin resistance cannot be attributed to the loss of lipid A as both the WT and ΔstkR cells were visually observed to be identical (Figure 4).
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Figure 4. Lipooligosaccharide analysis of the 04117201 (WT) and ΔstkR mutant. The lipid A and O-antigen components of the strains’ membrane was resolved on a 15% polyacrylamide gel and silver stained. (A) control lipopolysaccharide from *Pseudomonas aeruginosa* PA01 showing the presence of O-antigen; (B) WT, and (C) ΔstkR. Brackets identify either the lipid A core or O-antigen regions.

### 3.7. Transcriptional Profiling of the *A. baumannii* ΔstkR Mutant and WT Strain Treated with Sub-MIC Concentrations of Colistin

The PmrAB TCS regulates *pmrC*, whose product is responsible for lipid A modification by the addition of pEtN leading to an increase in colistin resistance [74,75]. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of the *pmrA*, *pmrB* and *pmrC* genes identified an increase in transcription of these genes in the ΔstkR mutant strain when exposed to the sub-MIC of colistin, by 6.63-fold (*p* < 0.02), 8.22-fold (*p* < 0.0001) and 17.63-fold (*p* < 0.0003), respectively, compared to the WT strain. Increased transcription of *pmrCAB* can be related to point mutations in PmrAB itself [72]. A sequencing analysis was therefore undertaken on the ΔstkR mutant strain to identify if any mutations in the *pmrAB* genes were present. Sanger sequencing analysis of the 3.6 kb region encompassing the *pmrCAB* operon identified no point mutants in the ΔstkR mutant strain compared to the WT (data not shown). This finding suggests that deletion of the *stkR* gene influences the *pmrCAB* operon and is likely a new mechanism influencing colistin resistance.

### 3.8. Comparative Genomes

To determine whether any other mutations may be causing the phenotypes exhibited by the ΔstkR mutant strain, the WT *A. baumannii* strain 04117201 was resequenced and the ΔstkR mutant strain sequenced using a Nanopore MinION. Two independent ΔstkR mutant strains were compared with the WT strain, which identified 14 single nucleotide polymorphisms (SNPs) present in both of the ΔstkR strains assessed (Table 4). Out of the 14 SNPs identified, seven are in non-coding regions of the chromosome; two are in repeat regions including a hypothetical protein; two are synonymous and three are non-synonymous. The first of the non-synonymous SNPs is at position 1150782 within a gene annotated to a glutamine, therefore the open reading frame now encodes a fusion of two aldehyde dehydrogenase. This produces an amino acid change from a stop codon to a glutamine, therefore the open reading frame now encodes a fusion of two aldehyde dehydrogenase transcribed together and this mRNA is unlikely to form an active protein. Further analysis of the WT 04117201 identified six copies of aldehyde dehydrogenase in the chromosome. Aldehyde dehydrogenase is implicated in a broad range of metabolic pathways; however, as it is commonly found in multiple copies in the genome, this inactive gene is likely to have minimal, if any, impact on cellular regulation [76]. The second non-synonymous change is at position 3132365 and is annotated as the phenazine biosynthesis protein (PhzF), a secondary metabolite known to be produced in *Pseudomonas* spp. which correlates to the colour exhibited by the species [77]. Although there is a change from
a nonpolar (phenylalanine) to a polar amino acid (serine), this change is close to the C-terminal end of the protein and is unlikely to affect the secondary structure and function. To date, this gene has not been implicated in antibiotic resistance. The final non-synonymous change is at position 3445432, encoding an alcyol-CoA dehydrogenase, a class of enzymes that are associated with oxidation in the mitochondria and not involved in cellular regulation. “The non-coding regions harboring SNPs were also investigated. All were between 26 to 47 bps from the stop or start codon of any potential coding regions, predicting that these mutations would not influence gene transcription. The SNP at position 538323 results in a glutamine to a glycine change in the region annotated as a repeat hypothetical protein. This region is also upstream from an annotated tail protein and downstream of a major capsid protein suggesting that this region is potential a prophage. The other hypothetical protein containing a SNP is at position 3201037 placed downstream of a polymerase sigma factor RpoN and upstream of the outer membrane phospholipid-binding lipoprotein MlaA. No genetic factors have been described that indicate a link between these proteins”.

Table 4. Single nucleotide polymorphisms regions identified in the ΔstkR mutant compared to the WT strain.

| SNP Position | Codon in WT | Codon in ΔstkR | Amino Acid in WT | Amino Acid in ΔstkR | Annotation of Region |
|--------------|-------------|----------------|------------------|---------------------|---------------------|
| 176528       | -           | -              | -                | -                   | Non-coding region   |
| 538323       | GAG         | GGG            | Glu              | Gly                 | Hypothetical protein (Repeat region) |
| 1001055      | -           | -              | -                | -                   | Non-coding region   |
| 1058011      | -           | -              | -                | -                   | Non-coding region   |
| 1098529      | -           | -              | -                | -                   | Non-coding region   |
| 1150782      | TAA         | CAA            | Stop             | Gln                 | Aldehyde dehydrogenase |
| 1590889      | GCT         | GCC            | Ala              | Ala                 | Hypothetical protein |
| 2730447      | TTT         | TTC            | Phe              | Phe                 | Hypothetical protein |
| 2796261      | -           | -              | -                | -                   | Non-coding region   |
| 3132365      | TTC         | TCC            | Phe              | Ser                 | Phenazine biosynthesis protein (PhzF) |
| 3201037      | CCC         | TCC            | Pro              | Ser                 | Repeat region       |
| 3361708      | -           | -              | -                | -                   | Non-coding region   |
| 3370031      | -           | -              | -                | -                   | Non-coding region   |
| 3445432      | AAA         | GAA            | Lys              | Glu                 | Alcyol-CoA Dehydrogenase |

Table: SNP positions identified with SAMtools v1.9 and VCFools v0.11.17. Annotation of region undertaken using Prokka.

4. Discussion

Two-component systems have gained considerable attention due to their role in modulating a plethora of virulence mechanisms and multidrug resistance. In A. baumannii these systems include: BfmRS, which is involved in fitness, capsule production and biofilm formation; AdeRS, which regulates an efflux pump associated with antibiotic resistance; GacSA, which is involved with regulating the phenyl acetic acid catabolic pathway as well as attenuated virulence in a mouse infection model; the BaeSR system, which modulates efflux pumps and has been implicated in cross talk with the AdeRS systems, and the PmrAB system, which is involved in colistin resistance [4,5,26,66,78]. In this study the diverse nature of these systems was explored through the uncharacterized TCS, StkSR. We chose to investigate the role of this system in the clinical isolate 0411201 because of its potential involvement in influencing virulence (Figure 1) (Table 1) and its ability to form strong biofilms [56].

Comparative bioinformatics analysis of the genes encoding potential response regulator proteins in the WT 04117201 A. baumannii strain identified five TCS that are absent from the avirulent A. baumannii strain SDF and therefore are hypothesized to affect virulence potential (Table 2). These systems included two orphan response regulators, NasT and RsbU, and two previously characterized response regulators, AdeR, which is associated
with efflux pump regulation, and BarA, coordinating the control of type IV pili [24,66]. The novel hybrid TCS named here as StkSR was chosen for further phenotypic investigation.

A knockout mutant was constructed by replacing the stkR gene with an erythromycin resistance cassette giving rise to the ΔstkR mutant strain. This produced a significant increase in expression of the pmrC, pmrA and pmrB genes in the ΔstkR mutant after sub-MIC exposure to colistin, identifying the stkR gene as a potential repressor of the genes pmrA and pmrB either directly or indirectly. Thus, this result has identified a potential regulatory relationship between the StkSR and PmrAB TCS.

The increased spread of multidrug- and pan-resistant strains of A. baumannii is of global concern. As carbapenem resistance increases, clinicians are more reliant on colistin as a ‘last resort’ antibiotic. As a consequence of its use, colistin resistant strains are continuing to be prevalent, which is resulting in an increase in mortality rates [3,74]. Inactivation of the stkR response regulator in A. baumannii strain 04117201 resulted in a 2-fold increase in resistance to the polymyxins, colistin and polymyxin B (Table 3). Colistin requires unmodified lipooligosaccharide or lipopolysaccharide to be present to exert its antibacterial properties; to date two mechanisms of resistance to colistin have been described in A. baumannii, the complete loss of the lipooligosaccharide portion of the outer membrane and modification of phosphate groups of the lipid A by the addition of pEtN or 4-amino-4-deoxy-l-arabinose, both of which reduce the overall membrane electronegativity [2,8,73,79]. Silver staining of purified lipooligosaccharide from the ΔstkR mutant and its WT parent identified the presence of lipooligosaccharide and a similar lipid A composition. Therefore, unlike Henry et al. (2012), the increase in colistin resistance could not be attributed to the loss of lipid A or the gross modification of lipid A under normal conditions [80,81]. However, bacterial cells which have a reduced cell surface hydrophobicity also show an increase in colistin resistance [82]. Therefore, cell surface hydrophobicity was assessed in both ΔstkR and WT cells. This revealed a significant reduction in the cell surface hydrophobicity of the ΔstkR derivative compared to the WT when in the presence of colistin (Figure 2). This suggests that modification of the bacterial cell is decreasing the electronegative of the cell and thereby decreasing the cell surface hydrophobicity. This modification could be a result of the addition of pEtN to lipid A, correlating the altered expression of the pmrCAB operon with cell surface hydrophobicity. When there is no colistin stress on the ΔstkR and WT bacterial cells there is an increased cell surface hydrophobicity in ΔstkR compared to the WT parent, we suggest that modification of the cell surface is only occurring when the WT and ΔstkR mutant strains are stressed with sub-MIC concentrations of colistin, showing that the bacterial cell is actively responding to the antibiotic as suggested previously by Boinett et al. 2019 [28].

To visualize the physical alteration of the cell membrane when exposed to colistin, TEM analysis identified varying levels of membrane perforation, from partially disintegrated cells to complete lysis of the WT and ΔstkR mutant strains. As the cell surface hydrophobicity of the bacterial cell changes, so does the electronegativity and therefore the ability of colistin to attach and displace the cationic cations Ca2+ and Mg2+ causing blebbing events on the cell surface. Under sub-MIC treatment with colistin, the WT strain displayed a significant increase in membrane perturbation/protrusion known as blebbing events compared to the ΔstkR mutant strain (Figure 3C,D). These results are supported by other studies investigating the effect of colistin on the membrane showing envelope disruption and outer membrane collapse [23,78].

The increased substitution of lipid A by pEtN through transcription of pmrC, eptA a pmrC homologue, or the plasmid mediated mcr-1 gene, results in increased colistin resistance [31,35,36,85]. Plasmids carrying MCR-1, a pEtN transferase enzyme that contributes to colistin resistance, have been identified in E. coli, K. pneumoniae and the Acinetobacter species [79]. Genome analysis did not identify this plasmid in the A. baumannii strain 04117201 nor the ΔstkR derivative (data not shown). The pmrC homologue eptA has been associated with an IS element (ISAbu125) insertion into a H-NS regulator gene altering transcription of eptA. However, no IS element was found within H-NS identified in the ΔstkR
mutant strain, therefore we discounted these mechanisms as possible means of colistin resistance [83–88]. Similarly, an alternate means of colistin resistance is the complete loss of lipopolysaccharide/lipooligosaccharide, achieved by insertion elements affecting the transcription of the lpxA genes [89]. Whole genome sequencing confirmed that this mutation was not present and there were no other mutations likely to cause the phenotypes observed.

The pmrCAB system was examined by qRT-PCR, as it plays a major role in conferring colistin resistance by the modification of lipid A with pEtN. The PmrAB TCS can be regulated by other two-component systems as seen in Salmonella enterica and K. pneumoniae. In these cases, the PhoPQ system leads to the activation of a small polypeptide known as pmrD, which binds to pmrA and stabilizes it in its phosphorylated state leading to an increase in transcription of pmrC [30]. Even though a pmrD homologue was not identified in A. baumannii 04117201, the cross-regulation of pmrCAB is not limited to interactions with the PhoPQ system but can be further complicated by other TCS (e.g., preAB in S. enterica) [90]. This PreAB TCS is like the qseBC system found in E. coli and appears to be able to influence the expression of pmrCAB as well, suggesting that the pmrCAB system could be regulated by several TCS.

We showed that the levels of pmrA, pmrB, and pmrC were significantly increased in ΔstkR compared to the WT when exposed to colistin. Additionally, we demonstrated that the PmrAB TCS is a likely contributor to colistin resistance and that the StkSR two-component system is a probable influence on the regulation of the PmrAB system. Taken collectively, these data could explain the modified colistin resistance seen when stkR is inactivated [90,91]. Lastly, an increase in colistin resistance through the PmrAB TCS is reported to lead to a decrease in fitness [30,92]. However, we have yet to identify any decrease in fitness of the ΔstkR mutant.

5. Conclusions

This is the first study to describe the novel TCS StkSR identified in an A. baumannii strain isolated from an Australian hospital. We have demonstrated that the observed increase in colistin resistance seen for the ΔstkR derivative of this isolate is not due to the loss of lipid A but correlates to the increased transcription of the pmrCAB operon which is linked to the modification of lipid A. This modification decreases the overall membrane electronegativity and therefore decreases the cells affinity to the colistin compound. Colistin represents a “last resort” antibiotic used for the treatment of severe A. baumannii infections, and understanding the resistance mechanisms to this clinically important compound is vital for its continued application.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms10050985/s1, Figure S1: Growth analysis of A. baumannii 04117201 and ΔstkR mutant strains, Table S1: Oligonucleotides used in this study.

Author Contributions: Conceptualization, S.K.G., U.H.S. and M.H.B.; methodology, S.K.G., J.A.C.-J. and U.H.S.; software, B.P., M.R. and R.A.E.; validation, S.K.G., U.H.S., R.A.E. and M.H.B.; formal analysis, S.K.G., B.P.; investigation, S.K.G., U.H.S. and B.P.; resources, S.K.G. and M.H.B.; data curation, S.K.G., B.P., M.R. and R.A.E.; writing—original draft preparation, S.K.G., U.H.S., B.P. and M.H.B.; writing—review and editing, S.K.G., M.R., J.A.C.-J., R.A.E. and M.H.B.; visualization, S.K.G., U.H.S. and B.P.; supervision, R.A.E. and M.H.B.; project administration, S.K.G. and M.H.B.; funding acquisition, S.K.G., U.H.S. and M.H.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Health and Medical Research Council, Australia, grant number 535053 to M.H.B., S.K.G. was the recipient of a Flinders University Research Scholarship.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: We thank Ruth Hall from the University of Sydney for providing the initial sequence of A. baumannii strain 04117201, Sophie Leterme at Flinders University, for her contribution.
to the statistical analysis and David Oggunniyi Institute at the University of South Australia, for providing the A549 human pneumocytes cell line. Thank you to Felise Adams and Paul Radford for their critical editing of the paper.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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