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Different surface charge of colistin-susceptible and -resistant *Acinetobacter baumannii* cells measured with zeta potential as a function of growth phase and colistin treatment

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Objectives: Electrostatic forces mediate the initial interaction between cationic colistin and Gram-negative bacterial cells. Lipopolysaccharide (LPS) loss mediates colistin resistance in some *A. baumannii* strains. Our aim was to determine the surface charge of colistin-susceptible and –resistant *A. baumannii* as a function of growth phase and in response to polymyxin treatment.

Methods: The zeta potential of *A. baumannii* ATCC 19606 and 10 clinical multidrug-resistant strains (MICs 0.5–2 mg/L) was assessed. Colistin-resistant derivatives (MIC >128 mg/L) of wild-type strains were selected in the presence of 10 mg/L colistin, including the LPS-deficient *lpxA* mutant, ATCC 19606R. To determine the contribution of LPS to surface charge, two complemented ATCC 19606R derivatives were examined, namely ATCC 19606R+*lpxA* (containing an intact *lpxA* gene) and ATCC 19606R+V (containing empty vector). Investigations were conducted as a function of growth phase and polymyxin treatment (1, 4 and 8 mg/L).

Results: Wild-type cells exhibited a greater negative charge (-60.5 ± 2.36 to -26.2 ± 2.56 mV) than colistin-resistant cells (-49.2 ± 3.09 to -19.1 ± 2.80 mV) at mid-log phase (ANOVA, $P < 0.05$). Opposing growth-phase trends were observed for both phenotypes: wild-type cells displayed reduced negative charge and colistin-resistant cells displayed increased negative charge at stationary compared with mid-logarithmic phase. Polymyxin exposure resulted in a concentration-dependent increase in zeta potential. Examination of ATCC 19606R and complemented strains supported the importance of LPS in determining surface charge, suggesting a potential mechanism of colistin resistance.

Conclusions: Zeta potential differences between *A. baumannii* phenotypes probably reflect compositional outer-membrane variations that impact the electrostatic component of colistin activity.

Keywords: physicochemical properties, Gram-negative, polymyxin

Introduction

The increasing prevalence of multidrug-resistant (MDR) bacterial pathogens globally, coupled with a steady decline in the development of new antimicrobial agents[1] has the world facing a possible return to the 'pre-antibiotic' era.[2] The approval of several new antibiotics active against Gram-positive bacteria has provided some respite for those infections,[3] but unfortunately Gram-negative pathogens, such as *Acinetobacter baumannii*, are proving more problematic with predictions that the availability of a new antibiotic against these infections is still at least a decade away.[4]

The complex Gram-negative cell envelope presents an effective permeability barrier to the passage of many antibiotics.[5] This barrier can be largely attributed to anionic lipopolysaccharide (LPS) molecules that principally comprise the outer leaflet of the bi-layer membrane. The formation of a dense, negatively charged surface layer[6] is favourable to the action of polymyxins. Polymyxin antibiotics structurally comprise a cyclic heptapeptide

ring, attached to a tripeptide side chain, which is covalently linked to a fatty acyl tail.[7] At physiological pH, protonation of five primary amine groups together with the hydrophobic tail deliver a cationic amphipathic character that has been suggested to enable self-promoted uptake of the antibiotic through the outer membrane.[8] Initial electrostatic binding to negatively charged phosphates present on the lipid A portion of LPS displaces divalent cations that play a stabilizing role in bridging neighbouring LPS molecules.[6] Insertion of the *N*-terminal fatty-acyl tail of polymyxins into the external lipid sheet is thus enabled, resulting in membrane disruption.[8] Polymyxin B and colistin (polymyxin E) have a rapid bactericidal effect against many MDR Gram-negative species.[9] In an era of diminishing therapeutic options, these polymyxins have been revived as a last line of defence against MDR Gram-negative infections.

The Gram-negative pathogen *A. baumannii* has created considerable challenges for clinicians due to a high level of intrinsic antimicrobial resistance, as well as a remarkable ability to acquire numerous genetic mutations leading to the production of the MDR phenotype.[10] This dilemma is accentuated by the emergence of extremely drug-resistant strains, which are resistant to all antibiotics, including colistin.[11,12] Furthermore, the phenomenon of colistin heteroresistance describes strains that appear susceptible on the basis of MICs, but harbour highly colistin-resistant subpopulations.[13,14] These subpopulations may be amplified upon colistin exposure, potentially resulting in therapeutic failure.[14] Studies of polymyxin resistance in other Gram-negative species have highlighted LPS modifications involving alterations to lipid A phosphate groups by the addition of sugar moieties under the control of the two-component regulatory systems, PhoPQ and PmrAB.[15–17] These changes result in a reduction in the net negative charge of lipid A, effectively diminishing the electrostatic polymyxin–bacterium interaction. Importantly, we have recently shown that colistin resistance in the *A. baumannii* reference strain, ATCC 19606, can be mediated by complete loss of LPS. Sequence analysis of paired colistin-susceptible and –resistant ATCC 19606 strains revealed that spontaneous mutations in the key lipid A biosynthesis genes, *lpxA*, *lpxC* and *lpxD*, resulted in the arrest of lipid A biosynthesis and therefore LPS assembly.[18] While the genetic and molecular basis of polymyxin resistance in other *A. baumannii* strains remains to be fully established, it is likely that modification of the bacterial surface charge is involved.

Zeta potential measurements have been applied in bacteriological studies to address the hypothesis that the susceptibility of a bacterium to peptide antibiotics may be closely related to the charge exhibited on the cell surface.[19–23] No such studies have been conducted on *A. baumannii*. Furthermore, while the ongoing expression of membrane proteins, lipids and extracellular polymers throughout the bacterial growth cycle has been shown to affect surface physicochemical properties of *Escherichia coli*,[24,25] the influence of these potential modifications on the surface charge of *A. baumannii* cells and the resultant electrostatic interaction with colistin has yet to be addressed. Using the zeta potential as a measure of bacterial surface charge, the aims of this study were to compare the surface charge of colistin-susceptible and -resistant *A. baumannii* cells, to investigate the differences in the surface charge with respect to bacterial growth phase and finally, to determine the influence of colistin exposure on the surface charge of *A. baumannii* at different growth phases.

Materials and methods

Chemicals

Stock solutions (1 mg/mL) of colistin (20374 U/mg colistin sulphate; Zhejiang Shenghua Biok Biology Co. Ltd, China) and polymyxin B nonapeptide (PBN, Lot No. 088K4054; Sigma-Aldrich, Castle Hill, Australia) were prepared in Milli-Q™ water (Millipore, North Ryde, Australia) and filtered through 0.22 μm syringe filters (Sartorius, Melbourne, Australia). Both solutions were stored at 4°C for up to 1 month; conditions under which these antibiotics have proved to be stable.[26]

Bacterial strains

Thirteen strains of *A. baumannii* were employed in this study; these included six colistin-heteroresistant clinical MDR strains (clinical strains FADDI-AB016, FADDI-AB027, FADDI-AB032, FADDI-AB048, FADDI-AB050 and FADDI-AB052) and reference strain ATCC 19606 (from the ATCC, Manassas, VA, USA), as well as four colistin-susceptible clinical MDR strains (FADDI-AB013, FADDI-AB014, FADDI-AB035 and FADDI-AB040). The MICs of colistin for these strains ranged from 0.5 to 2 mg/L. From each of these wild-type strains, paired colistin-resistant derivatives were obtained;[13] these were designated as 'R' strains and were able to grow on nutrient agar plates containing 10 mg/L colistin (see below) (Medium Preparation Unit, Melbourne, Australia). The paired colistin-resistant strains had MICs of >128 mg/L. Additionally, the zeta potential of two complemented strains derived from ATCC 19606R was measured to examine the effect of LPS loss on the surface charge of *A. baumannii*: colistin-susceptible ATCC 19606R+*lpxA*, which contained an introduced intact *lpxA* gene (MIC 1 mg/L), and ATCC 19606R+V, a colistin-resistant control strain that was deficient in the *lpxA* gene but contained an empty vector (MIC >128 mg/L).[18] All strains were stored in tryptone soya broth (Oxoid, Adelaide, Australia) with 20% glycerol at −80°C.

Wild-type strains were subcultured onto nutrient agar plates before the experiments. Cation-adjusted Mueller–Hinton broth (Oxoid, Adelaide, Australia) was employed for overnight cultures, and subsequently used to prepare mid-logarithmic cells according to OD_{600} (0.4–0.6) by subculture into fresh broth (1 in 100 dilution). Stationary-phase cultures were grown overnight. All broth cultures were incubated at 37°C in a shaking water bath at 100 rpm. Growth of paired colistin-resistant strains was conducted in the same manner as for their parent wild-type strains; the initial subculture and overnight broth culture were grown in the presence of 10 mg/L colistin to maintain selective pressure. Mid-logarithmic and stationary-phase colistin-resistant cultures were grown in the absence of colistin to avoid potential structural modifications induced by the antibiotic. Identical sample preparation protocols were also employed for strains ATCC 19606R+*lpxA* and ATCC 19606R+V, with the addition that these complemented strains were grown in the presence of 100 mg/L ampicillin to maintain selection for the plasmids.[18]

Sample preparation for measurement of zeta potential

To achieve adequate cleansing of the bacterial surface prior to zeta potential measurements, cells were harvested from broth culture by centrifugation at 3000 **g** for 5 min at 25°C, and washed twice with Milli-Q™ water as described previously.[27] For all investigations, washed cells were resuspended in the test solution to prepare bacterial suspensions containing $\sim 1 \times 10^9$ cfu/mL, following which a 10-fold dilution was performed in the same medium immediately prior to zeta potential measurement. The resulting suspension was used to fill clear disposable

zeta cells (ATA Scientific, Australia) immediately prior to zeta potential measurement.

The influence of ionic strength and pH on the zeta potential of bacterial cells has been reported,[28–31] thus demonstrating the importance of determining the effect of these conditions on the zeta potential of *A. baumannii*. For this purpose, cultures were washed and suspended with potassium chloride (KCl) solutions over a range of pH values (5.0–8.0), and low (0.01–0.3 mM) and high (10–300 mM) ionic strengths. Adjustment of pH was achieved by drop-wise addition of HCl (10% v/v) or NaOH (10% w/v) solution. Ionic strength investigations were conducted at pH 5.5, while pH investigations were conducted in 0.01 mM KCl solution.

To examine the effect of polymyxin treatment on *A. baumannii* cells, colistin or PBN (1 mg/mL) was added to 5 mL of bacterial culture ($\sim 1 \times 10^9$ cfu/mL) to achieve final concentrations of 1, 4 and 8 mg/L. PBN (an inactive derivative of polymyxin B produced by proteolytic cleavage of the terminal diaminobutyric acid residue and *N*-fatty acyl chain) was used as a comparator for colistin. Bacterial broth cultures were then incubated in a shaking water bath (37°C, 100 rpm) for 20 min and prepared for zeta potential analysis as described above.

Zeta potential measurement

The electrophoretic mobility (EPM) of bacterial cells was measured with a zeta potential analyser at 150 V (Zetasizer Nano ZS™; Malvern Instruments Ltd, Malvern, UK) before being converted to zeta potentials using the Helmholtz–Smoluchowski theory.[32] Measurements were performed at 25°C in either Milli-Q™ water or KCl solutions of desired pH and ionic strength. Prior to sample analysis, polarization of the electrodes using the test solution was carried out in the absence of cells under identical conditions to establish consistent sample conductance readings.[33] EPM measurements were performed in triplicate on three separately prepared samples on at least two separate days to determine reproducibility of results. Between each measurement, electrodes were rinsed with copious amounts of ethanol and Milli-Q™ water, followed by the test bacterial suspension. Statistical analyses were conducted using the Student's *t*-test and analysis of variance (ANOVA) using GraphPad Prism V5.0 software (GraphPad Software, San Diego, CA, USA).

Results

Influence of ionic strength and pH on zeta potential of A. baumannii

The influence of ionic strength on the zeta potential and background conductivity of paired colistin-susceptible and -resistant *A. baumannii* ATCC 19606 cells is illustrated in Figure 1. Similar results were acquired for FADDI-AB016 and FADDI-AB016R strains (data not shown). At ionic strengths of <0.1 mM, the zeta potential was independent of ionic strength (Figure 1), and the colistin-resistant cells displayed less-negative zeta potentials in comparison with the colistin-susceptible cells. Above ionic strengths of 0.1 mM, the zeta potential of both phenotypes increased with ionic strength and the difference observed between the zeta potentials measured for colistin-susceptible and -resistant cells became almost negligible. A linear relationship between background conductivity and ionic strength was acquired across the entire range of ionic strengths tested (0.01–300 mM) in the presence of bacteria (data not shown). Analysis of variance revealed that alterations in pH across the range 5–8 did not significantly influence the zeta potential of colistin-susceptible and –resistant *A. baumannii* cells ($P > 0.05$; Figure 2). The change in the pH of Milli-Q™ water over time was examined in the present study, revealing a stable pH of ~5.8 over the course of 8 h at room temperature (data not shown). As this pH value of 5.8 is close to the pH of 6 included in the above-mentioned study (Figure 2), post-hoc analysis of the zeta potentials at pH 6 versus the most physiologically relevant pH of 7 was conducted, revealing no significant difference (*t*-test, $P > 0.05$). From these data, the decision was made to perform subsequent measurements in Milli-Q™ water, where the ionic strength is negligible, thereby allowing for a valid comparison between the zeta potential of colistin-susceptible and -resistant *A. baumannii*.

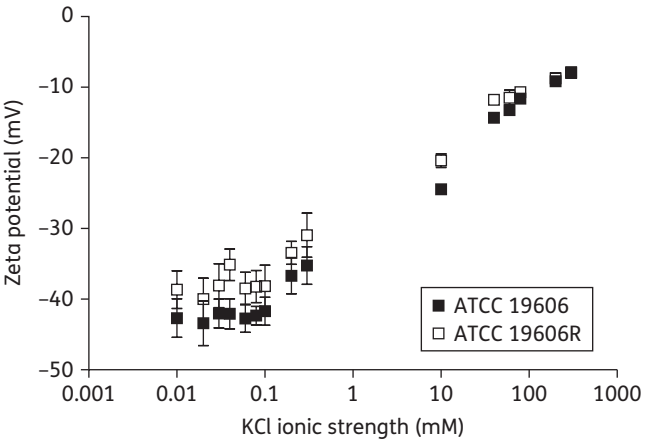

Figure 1. Zeta potential (mean ± SD) of *A. baumannii* ATCC 19606, and paired colistin-resistant strain ATCC 19606R, at mid-logarithmic phase as a function of KCl ionic strength at pH 5.5.

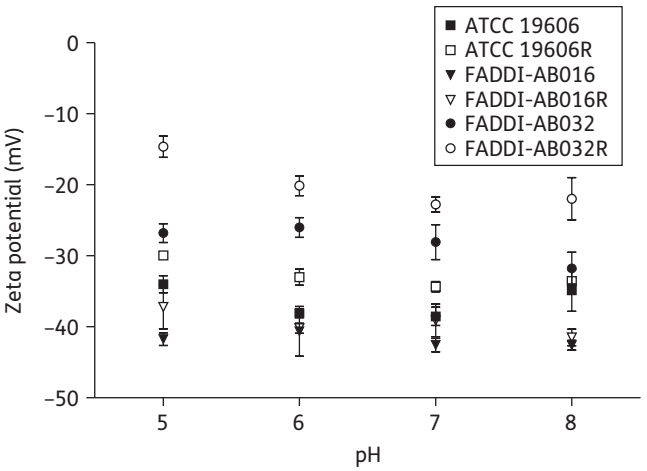

Figure 2. Zeta potential (mean ± SD) of paired colistin-susceptible and -resistant *A. baumannii* strains measured in 0.01 mM KCl as a function of pH.

Zeta potential of colistin-susceptible versus -resistant A. baumannii

Figure 3 shows the zeta potential of paired colistin-susceptible and -resistant *A. baumannii* cells at mid-logarithmic phase measured in Milli-Q™ water. A negative zeta potential was obtained for all tested strains. Colistin-susceptible *A. baumannii* strains exhibited

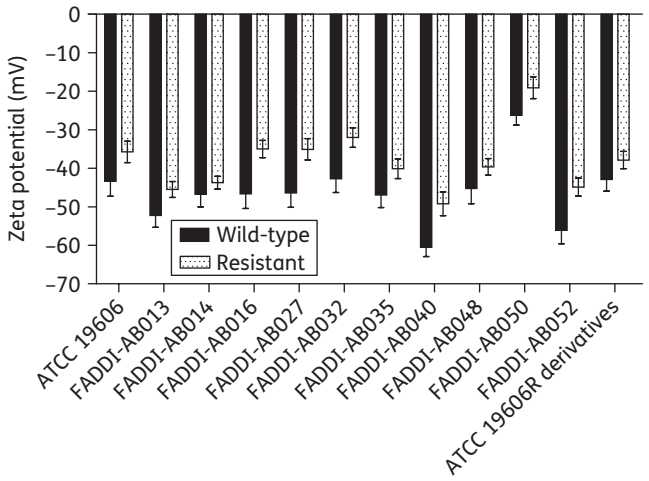

Figure 3. Zeta potential (mean ± SD) of mid-logarithmic paired colistin-susceptible and -resistant *A. baumannii* cells. 'ATCC 19606R derivatives' refers to colistin-susceptible ATCC 19606R+*lpxA* and colistin-resistant ATCC 19606R+V, which were both derived from ATCC 19606R.

zeta potentials ranging from −60.5 ± 2.36 to −26.2 ± 2.56 mV. The zeta potential values of colistin-resistant cells were significantly less negative overall than those of their colistin-susceptible parent strains, ranging from −49.2 ± 3.09 to −19.1 ± 2.80 (Figure 3; ANOVA, $P < 0.05$); post-hoc analysis revealed that the zeta potential of colistin-susceptible and -resistant cells was not statistically different for the FADDI-AB014 paired strains, nor for the complemented derivative strains (ATCC 19606R+*lpxA* versus ATCC 19606R+V strains), containing the *lpxA* gene and vector only, respectively (*t*-test, $P > 0.05$). Additionally, there was no statistically significant difference between the zeta potentials of the colistin-susceptible strains ATCC 19606 and ATCC 19606R+*lpxA*, or the colistin-resistant strains ATCC 19606R and ATCC 19606R+V (*t*-test, $P < 0.05$).

Influence of growth phase on the zeta potential of A. baumannii

Figure 4 illustrates the zeta potential of colistin-susceptible (Figure 4a) and -resistant (Figure 4b) *A. baumannii* cells, at both mid-logarithmic and stationary phases. All measurements were conducted in Milli-Q™ water. Analysis of variance revealed that overall, colistin-susceptible strains exhibited a significantly less negative surface charge at stationary phase in comparison with mid-logarithmic cells ($P < 0.05$); ATCC 19606R+*lpxA* exhibited the opposite trend to all other colistin-susceptible strains in that a greater negative surface charge was detected at stationary phase. Similarly, a greater negative surface charge was exhibited by all colistin-resistant strains, including ATCC 19606R+V, at stationary phase in comparison with mid-logarithmic phase (ANOVA, $P < 0.05$).

Effect of colistin treatment on the zeta potential of A. baumannii

The contribution of colistin and PBN to the background conductivity of the bacterial suspension was found to be negligible at

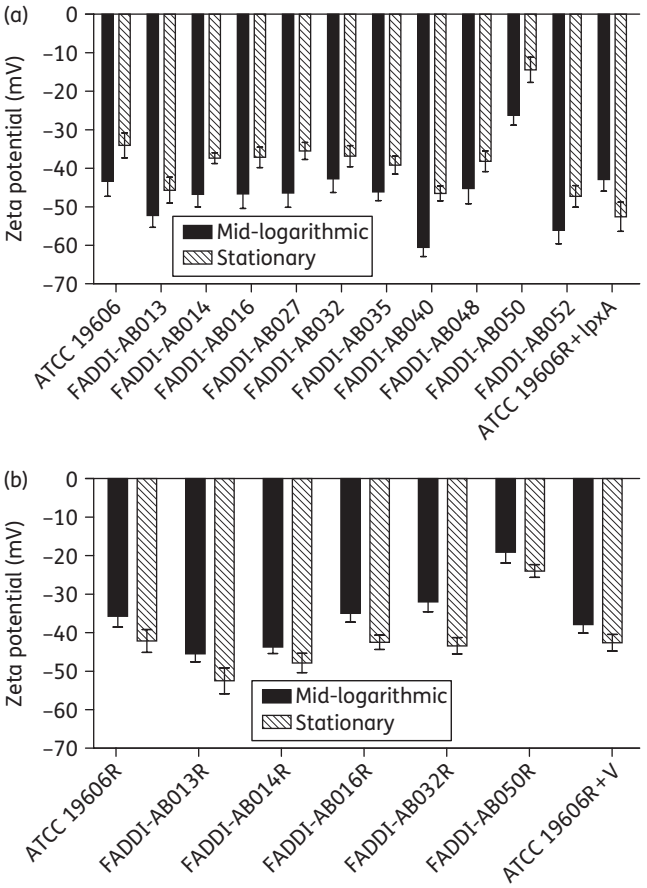

Figure 4. Zeta potential (mean ± SD) of colistin-susceptible (a) and -resistant (b) *A. baumannii* cells, including ATCC 19606R+*lpxA* and ATCC 19606R+V, at mid-logarithmic and stationary growth phases.

the highest tested concentration of 8 mg/L, with an increase of no greater than 0.004 mS/cm (data not shown). Figure 5(a) illustrates a substantial increase in zeta potential (i.e. less negative) upon colistin and PBN exposure (8 mg/L) for both colistin-susceptible and -resistant cells at mid-logarithmic phase, including strains ATCC 19606R+*lpxA* and ATCC 19606R+V. Colistin treatment of stationary-phase *A. baumannii* yielded similar observations to those observed with mid-logarithmic cells (data not shown). In all experiments, the surface charge neutralizing effect imparted by colistin and PBN was of similar magnitude. Concentration-dependent changes to *A. baumannii* zeta potential were demonstrated following treatment of two paired colistin-susceptible and -resistant strains (ATCC 19606 and FADDI-AB016) with colistin (Figure 5b) and PBN (Figure 5c) at concentrations of 1, 4 and 8 mg/L.

Discussion

Ionizable acidic and basic moieties present on phospholipids, lipoproteins and LPS, which comprise the outer glycolipid sheet of the asymmetrical outer membrane, collectively dictate the net surface charge on Gram-negative bacteria.[34] LPS represents the primary component of the outer leaflet, and consists of an outer *O*-polysaccharide side chain connected to a core

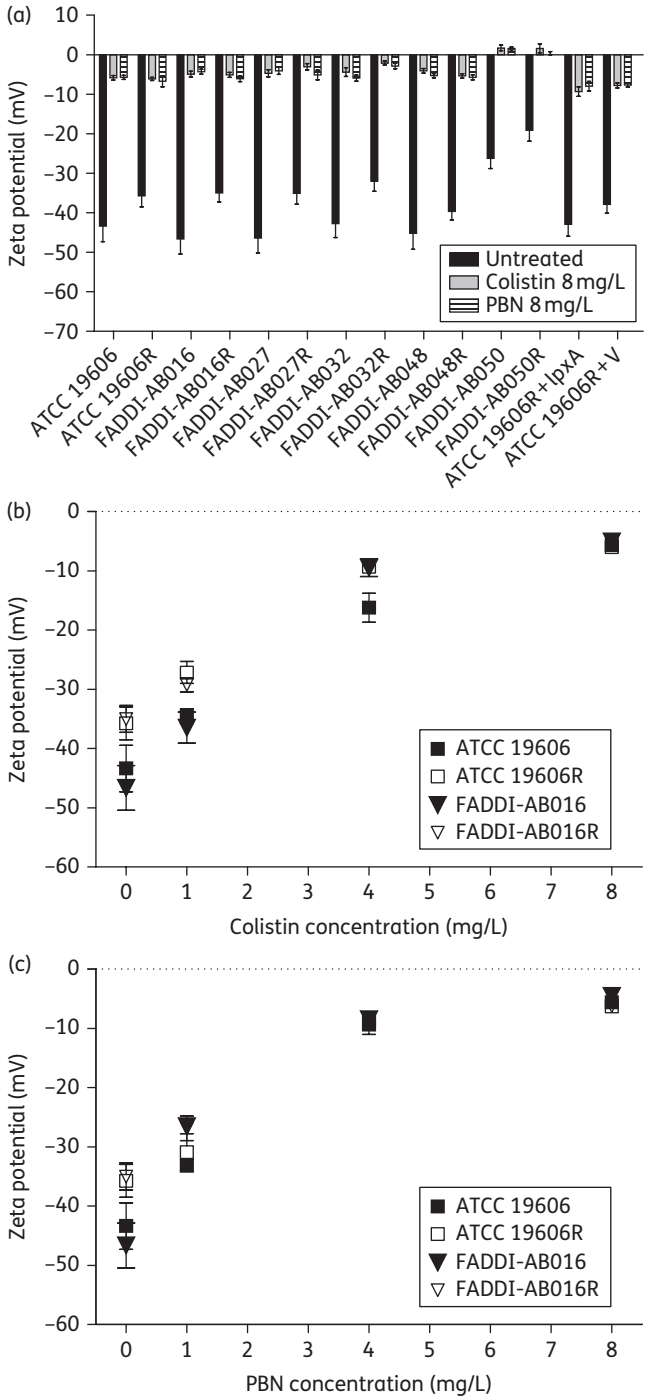

Figure 5. (a) The increase in mean zeta potential for a series of paired *A. baumannii* strains at mid-logarithmic phase following treatment with 8 mg/L colistin and PBN. (b and c) The effect of increasing concentrations of colistin and PBN, respectively, on the zeta potential of paired colistin-susceptible and -resistant *A. baumannii* ATCC 19606 and FADDI-AB016 at mid-logarithmic phase. Data shown represent the mean ± SD.

oligosaccharide, which is attached to lipid A; it is the lipid A portion that is incorporated within the membrane, forming an anchor upon which LPS can build. Anionic character is mainly afforded by carboxyl and phosphate moieties concentrated on lipid A as well as sugars within the core oligosaccharide.[35] Various forces work in concert to stabilize divalent cations that bind LPS molecules within the membrane, resulting in the formation of a dense negatively charged barrier, which poses a major hindrance to the passage of many hydrophobic antibiotics.[36] For polymyxins, which are cationic at physiological pH, antibacterial activity is initiated by electrostatic attraction to the anionic surface of Gram-negative bacterial cells.[8,37]

Bacterial surface charge has often been described by the zeta potential, which represents the potential at the shear plane of the electrical double layer encompassing a cell in solution. This charge is highly dependent on both the composition of the cell surface and the nature of the surrounding medium. Consideration of the background conductivity (salt concentration) and pH of the electrolyte in which cells are suspended is thus necessary, as these factors govern the adsorption of ions onto bacterial cells, and influence the degree of ionization of charged moieties on the cell surface.[29] The corresponding rise in zeta potential of *A. baumannii* cells towards neutrality observed with increasing ionic strength (Figure 1) may be explained by shielding of the actual surface charge by increased amounts of cations within the proximity of the negatively charged bacterial surface. The zeta potential of both colistin-susceptible and -resistant *A. baumannii* cells behaved independently of pH across the range 5–8 (ANOVA, $P > 0.05$; Figure 2). This has been observed for other Gram-negative species,[28–31] and represents a condition in which carboxyl, phosphate and amino functionalities on bacterial cell surfaces would be expected to be mostly ionized. In the present study, zeta potential measurements were performed immediately following sample preparation in Milli-Q™ water, which maintained a stable pH of ~5.8 over 8 h. Thus, the zeta potential measurements were performed at a physiologically relevant pH, and potentially confounding influences arising from variations in pH and ionic strength were minimized.

The negative zeta potentials observed in this study for *A. baumannii* cells at mid-logarithmic phase were of a magnitude comparable to those observed for other Gram-negative species under similar conditions.[28,30,31,38] The less-negative zeta potential exhibited by colistin-resistant cells in comparison with colistin-susceptible cells at mid-logarithmic phase (Figure 3), is most plausibly explained by alterations in the composition and structure of the outer membrane. This finding may also explain the greater tendency for colistin-resistant *A. baumannii* cells to aggregate in small chains or clusters[27] in comparison with their wild-type parent cells, and correlates with the understanding that the stability of colloid aggregate systems is enhanced with particles exhibiting a lower magnitude of charge owing to decreased electrostatic repulsion.[28] Charge shielding esterification of phosphates present on lipid A with 4-amino-4-deoxy-L-arabinose or 2-aminoethanol, has been noted in polymyxin-resistant strains of *E. coli*,[39] *Pseudomonas aeruginosa*,[15] *Salmonella typhimurium*[40] and *Yersinia pestis*.[16] We have recently demonstrated an additional mechanism of colistin resistance in *A. baumannii* that involves the complete loss of LPS from the outer membrane, due to mutations of any of the key lipid A biosynthesis genes, *lpxA*, *lpxC* or *lpxD*.[18] In accordance with this finding, the zeta potential of the LPS-deficient ATCC 19606R complemented with *lpxA* (i.e. *A. baumannii* ATCC

19606R+*lpxA*) was found to mirror that of wild-type ATCC 19606 (colistin-susceptible) (*t*-test, $P<0.05$); similarly, comparable results were obtained for ATCC 19606R+V (containing empty vector) and ATCC 19606R (colistin-resistant) (*t*-test, $P<0.05$). The modification[15,16,39,40] or complete loss of lipid A would be expected to lead to a less negatively charged Gram-negative outer membrane, as was observed with *A. baumannii* in this study (Figure 3). The residual negative charge on colistin-resistant cells (Figure 3) may be attributed to negatively charged functionalities present on phospholipids and proteins.[41]

Bacterial growth is known to influence the conformation and composition of outer-membrane lipids[42] and LPS,[43] which is likely to be reflected by alterations to surface charge. The reported effect of growth phase on bacterial surface charge varies depending on the species; while zeta potential changes have been noted for *E. coli* and *Lactobacillus rhamnosus*,[44–46] growth-phase effects have not been demonstrated for *P. aeruginosa* or other lactic acid bacterial strains.[38,47] Interest in examining the zeta potential of *A. baumannii* at different growth phases stemmed from findings that revealed morphological changes[27] and reduced colistin susceptibility of *A. baumannii* at stationary phase in comparison with mid-logarithmic phase.[48] In the present study, we report a less-negative zeta potential for the colistin-susceptible cells at stationary phase in comparison with mid-logarithmic phase (Figure 4a). This would theoretically reduce the initial electrostatic attraction between colistin and bacterial cells, and may thus be expected to decrease antibacterial activity as has been observed for colistin-susceptible *A. baumannii* strains.[48] For colistin-resistant strains (including ATCC 19606R+V) and colistin-susceptible ATCC 19606R+*lpxA*, the opposite trend was noted whereby cells exhibited a greater negative charge at stationary phase (Figure 4); reasons for this phenomenon are unknown and warrant further investigation.

To examine the relationship between surface charge and polymyxin activity, colistin and PBN treatment of paired *A. baumannii* strains was conducted. Measurements were performed in Milli-Q™ water in which the background conductivity was ~0.0015 mS/cm. At this low range, the zeta potential behaved independently of the electrolyte concentration (Figure 1). Hence, as increases in background conductivity detected following polymyxin addition in Milli-Q™ water were negligible (<0.004 mS/cm), comparisons between untreated and treated *A. baumannii* cells were possible. Structurally, colistin and polymyxin B differ in a single amino acid residue. PBN is a polymyxin B derivative produced by proteolytic cleavage of the terminal diaminobutyric amino acid residue and *N*-fatty acyl tail.[49] These deletions are accompanied by a loss of antibacterial activity, owing to the inability of the fatty acyl chain to penetrate the lipid barrier.[37,49] However, retention of the cationic character of PBN at physiological pH enables it to maintain electrostatic contacts with the outer membrane, thus sensitizing Gram-negative bacteria to hydrophobic antibiotics.[50] In keeping with this suggestion, colistin and PBN were both found to neutralize the charge on colistin-susceptible and -resistant cells to a similar extent at mid-logarithmic (Figure 5) and stationary growth phases (data not shown). This finding substantiates the importance of electrostatic forces in initiating polymyxin action; an observation that has also been reported for other cationic antimicrobial peptides.[19,23,51–53] It should be noted that these zeta potential alterations not only reflect the specific binding of colistin and PBN to LPS on colistin-susceptible cells, but also involve non-specific binding to other negatively charged moieties, such as proteins and phospholipids present on the surface of both phenotypes. Indeed, as ATCC 19606R produces no LPS, colistin binding to the surface of these cells must result entirely from binding to other surface molecules. Interestingly, while the amount of colistin required to neutralize the surface charge of both the ATCC 19606 parent strain and the LPS-deficient strain, ATCC 19606R, was very similar (Figure 5b), the colistin-resistant derivative is >100-fold more resistant to killing by colistin. This suggests that electrostatic binding of colistin is necessary but not sufficient for bacterial killing.

Commercially available instrumentation to determine the zeta potential employs micro-electrophoresis or electrophoretic light scattering methods. Calculations are based on the particle EPM using the Helmholtz–Smoluchowski equation,[32] which is a model developed for solid spherical particles. Complexities are seen with the approximation of bacterial charge using this theory, as the soft polyelectrolyte layer surrounding the cell is ion permeable.[54] Alternative models have been derived for soft particles;[55] however, deficiencies have similarly been identified in their application to biological samples. Consequently, the zeta potential popularly remains as an accepted predictor of bacterial surface charge. In view of that, the relative measurements obtained in this investigation provide a valid comparison between colistin-susceptible and -resistant *A. baumannii* at different growth phases, and in response to colistin treatment.

In summary, our study is the first to reveal a relationship between colistin susceptibility and net bacterial surface charge, in which a less negative charge was detected for colistin-resistant cells. The effect of growth phase on the zeta potential of colistin-susceptible and -resistant cells was also observed. These differences may be a manifestation of variations in the composition of the external leaflet of the outer membrane. Comparison between ATCC 19606 and ATCC 19606R complemented strains supported the involvement of LPS in surface charge, reflecting a potential mechanism of colistin resistance. In addition, treatment with both colistin and PBN neutralized the charge on both colistin-susceptible and -resistant *A. baumannii* cells. Overall, our study has reinforced the importance of the electrostatic interaction as a key component involved in the initiation of colistin antibacterial activity. Further investigations into the molecular basis for the interaction between colistin and *A. baumannii* cells will provide important information for the development of novel antibiotics.

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Transparency declarations

None to declare.

Disclaimer

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