A nanomechanical study of the effects of colistin on the *Klebsiella pneumoniae* AJ218 capsule

Anna Mularski^{1*}, Jonathan Wilksch², Eric Hanssen³, Jian Li⁴, Takehiro Tomita⁵, Sacha James
Pidot², Tim Stinear², Frances Separovic¹, Dick Strugnell²

¹ School of Chemistry, The University of Melbourne, VIC 3010, Australia

² Department of Microbiology and Immunology, The Peter Doherty Institute for Infection and Immunity, The
University of Melbourne, VIC 3010, Australia

³ Advanced Microscopy Unit, Bio21 Molecular Science and Biotechnology Institute, The University of
Melbourne, VIC 3010, Australia

⁴ Drug delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University,
381 Royal Parade, Parkville, VIC 3052, Australia

⁵ Microbiological Diagnostic Unit Public Health Laboratory, The Peter Doherty Institute for Infection and
Immunity, The University of Melbourne, VIC 3010, Australia

* Corresponding author: mularski.a@unimelb.edu.au

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Abbreviations:

AFM – atomic force microscopy
AMP – antimicrobial peptide
CFU – colony forming units
ESBL – extended-spectrum beta-lactamases
LB – Luria-Bertani
LPS – lipopolysaccharide
MBC – minimum bactericidal concentration
MIC – minimum inhibitory concentration
PEI – polyethylene imine

Abstract

Atomic force microscopy (AFM) measurements of capsule thickness presented revealed that that the wild-type *K. pneumoniae* AJ218 capsular polysaccharides were rearranged by exposure to colistin. The increase in capsule thickness measured near minimum inhibitory/bactericidal concentration (MIC/MBC) is consistent with the idea that colistin displaces the divalent cations that cross-bridge adjacent lipopolysaccharide (LPS) molecules through the capsule network. Cryo-electron microscopy demonstrated that the measured capsule thickness at near MIC/MBC of 1.2 μ M was inflated by the disrupted outer membrane, through which capsule is excreted and LPS is bound. Since wild-type and capsule-deficient strains of *K. pneumoniae* AJ218 have equivalent MICs and MBCs, the presence of the capsule appeared to confer no protection against colistin in AJ218. A spontaneously-arising colistin mutant showed a ten-fold increase in resistance to colistin; genetic analysis identified a single amino acid substitution (Q95P) in the PmrB sensor kinase in this colistin-resistant *K. pneumoniae* AJ218. Modification of the lipid A component of the LPS could result in a reduction of the net negative charge of the outer membrane, which could hinder binding of colistin to the outer membrane and displacement of the divalent cations that bridge adjacent LPS molecules throughout the capsular polysaccharide network. Retention of the cross-linking divalent cations may explain why measurements of capsule thickness did not change significantly in the colistin-resistant strain after colistin exposure. These results contrast with those for other *K. pneumoniae* strains that suggest the capsule confers colistin resistance.

1 Introduction

2
3 Increased resistance coupled with a marked reduction in the discovery and development of
4 new antibiotics approved by regulatory bodies internationally (Taubes, 2008) has led to the
5 re-evaluation of antibiotics once considered too toxic for use, including colistin (polymyxin
6 E). (Velkov et al. 2013) (Falagas et al. 2005). Colistin is a cyclic lipopeptide, with a narrow
7 antibacterial spectrum, mainly against Gram-negative bacteria (Li et al. 2006). It has been
8 proposed that colistin, which is amphipathic and cationic at physiological pH, binds
9 electrostatically to the anionic lipid A phosphodiester, the component of the
10 lipopolysaccharide (LPS) molecules anchored into the outer membrane of Gram-negative
11 bacteria. Colistin is believed to displace the divalent cations that cross-bridge adjacent LPS
12 molecules. The subsequent destabilisation of the outer membrane facilitates the insertion of
13 hydrophobic colistin domains, further disrupting the outer membrane and enabling self-
14 promoted uptake of colistin (Velkov et al. 2013). However, the work of Zhang et al. (2000)
15 argues against destabilisation of the outer membrane as the mechanism by which bacterial
16 cells are killed. The concentrations of polymyxin drugs that resulted in rapid killing of *P.*
17 *aeruginosa* resulted in minimal membrane permeabilisation, which suggests that the
18 formation of colistin induced membrane defects may simply be a consequence of colistin
19 exposure and not the driver of colistin efficacy.

20
21 To gain further insight into the mechanism of action of colistin with Gram-negative bacteria,
22 we used atomic force microscopy (AFM) to examine the physiological responses of the
23 bacterium, *Klebsiella pneumoniae* AJ218, when exposed to colistin. AFM has proven to be
24 useful for measuring the physical properties of live biological cells under physiologically-
25 relevant conditions that require little sample preparation and does not damage samples

(Dupres et al. 2005; Engel et al. 2000; Lower et al. 2001). Previous studies of live bacteria with cationic antimicrobial peptides (AMPs) (Kao et al. 2012; Shaw et al. 2008; Strauss et al. 2010). Gaboriaud *et al.* (2005) and Wang *et al.* (2013; 2015) have demonstrated that long-range electrostatic interaction, cell wall elasticity and cytoplasmic turgor pressure of bacteria can be determined using AFM.

K. pneumoniae are a source of shared antimicrobial resistance such as the extended-spectrum beta-lactamases (ESBLs) and, more recently, carbapenem-resistance (Kumarasamy et al. 2010; Centers for Disease Control and Prevention, 2013). An external loosely organised polysaccharide layer (known as the capsule) is typically assembled on the outside of the *K. pneumoniae* cell envelope. It grows up to several hundred nanometers in thickness and takes the form of a hydrated polyelectrolyte network (Dzul et al. 2011) The capsule provides protection from environmental stresses, including host immune defenses, antibiotics, desiccation and detergents (Struve et al. 2003). Through the formation of biofilms, the exopolysaccharide capsule also plays a role in bacterial adherence to surfaces (Schembri et al. 2005). Further, a direct association has been discovered between capsular size and pathogenicity of isolates in animal models (Highsmith et al. 1985). Since the LPS molecules embedded in the outer membrane radiate through the capsular polysaccharides, the role of the capsule in the mode of action of colistin warrants investigation.

Llobet et al. (2008) reported that purified capsular polysaccharides increased the resistance of a capsule-deficient *K. pneumoniae* mutant to polymyxin B, suggesting that the bacterial capsule acts as an effective countermeasure to resist AMPs. They also postulated that AMPs may trigger *in vivo* the release of capsular material, which in turn protects bacteria against AMPs. Also, when *K. pneumoniae* cells were exposed to a melittin derived peptide, the

1 association of the peptide with the membrane dominated rather than the interaction of the
2 cationic peptide and the negatively-charged capsule (Mularski et al. 2015). However, this
3 does not negate the idea that extracellular capsular polysaccharides play a role in protecting
4 bacterial cells from AMPs.

5
6 Formosa et al. (2015) conducted extensive genetic and AFM studies of colistin-susceptible
7 and resistant strains of *K. pneumoniae* (ATCC 700603 and ATCCm, respectively). They
8 observed that colistin removed the capsule from the susceptible strain but not from the
9 resistant strain. In addition, they identified a multilayered capsule architecture in the colistin-
10 resistant strain leading them to the hypothesis that capsular polysaccharides might be
11 implicated in the mechanism of colistin resistance in *K. pneumoniae*.

12
13 In this study, AFM capsule thickness measurements were used to show that interaction with
14 colistin resulted in a rearrangement of capsular polysaccharides in wild-type *K. pneumoniae*
15 AJ218. Cryo-electron microscopy was used to examine the effect of colistin on the outer
16 membrane, from which capsule and LPS emanate. Wild-type and capsule deficient strains of
17 *K. pneumoniae* AJ218 were compared with a colistin-resistant strain, to gain insight into how
18 resistance may be acquired.

19 20 **Materials and Methods**

21 22 1. Colistin

23 Colistin (sulfate) (95% purity) was purchased from Beta Pharma (Jiangsu, China). All
24 colistin sulfate solutions were prepared in 10 mM HEPES buffer (pH 7.4), stored at 4°C and
25 used within two weeks of preparation.

2. Bacterial strains, culture conditions and harvesting

Klebsiella pneumoniae AJ218 (capsule serotype K54) is a human urinary tract infection isolate, identified in the microbiological laboratory of the Alfred Hospital, Melbourne, Australia. (Jenney et al. 2006) All strains were cultured on Luria-Bertani (LB) agar at 37°C. LB broths inoculated with these cultures were grown for 16 h at 37°C while shaking (180 rpm). Stationary-phase cells were then harvested by centrifugation (10 min at $3500 \times g$ and 4 °C) and washed twice with Milli-Q™ water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$). The final concentration of cells in Milli-Q™ water was approximately 2×10^8 colony forming units per mL (CFU mL⁻¹).

A *wzc* mutant defective in the transporter that enables capsule polysaccharide export was isolated following random mini-Tn5Km2 transposon insertion mutagenesis (de Lorenzo et al. 1990) of *K. pneumoniae* AJ218. To confirm transposon insertion within the *wzc* gene, Y-linker ligation PCR and subsequent DNA sequencing analysis of the transposon-flanking region was performed to ensure correct location of the mutation (Kwon et al. 2000).

A colistin-resistant *K. pneumoniae* AJ218 strain was created by selecting spontaneous mutants grown on a colistin concentration gradient agar plate. Briefly, LB agar containing 10 µM colistin was poured into a petri dish at a gradient and allowed to solidify. This was then overlaid with LB agar without colistin. A diluted *K. pneumoniae* AJ218 culture was subsequently spread over the surface of the agar and incubated at 37°C overnight. Bacterial colonies that formed at the highest colistin concentration were selected for further analysis.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of colistin was determined for wild-type, capsule-deficient and colistin-resistant *K.*

pneumoniae strains using the microdilution broth method outlined by the Clinical and Laboratory Standards Institute (2003).

3. Bacterial sample conditions for AFM measurements

Polyethyleneimine (PEI) coated glass slides were used to immobilise wild-type bacteria. The PEI coating method is described by Wang et al. (2013). Weakly attached bacteria were removed by rinsing with 10 mM HEPES buffer (pH 7.4) and bacteria coated slides were stored in buffer prior to use. All mechanical measurements were performed within 2-3 h of removal of the bacteria from growth media. Bacteria-coated slides were placed in a fluid cell in either 10 mM HEPES buffer (pH 7.4) or 0.1 μ M peptide solution (in 10 mM HEPES buffer, pH 7.4) and kept stationary within the calibrated AFM for at least 40 min before measurements commenced. Note that, in comparable conditions, cells remain viable for the duration of the experiment as determined by live/dead cell fluorescence assays using a Molecular Probes Bacterial Viability Kit (Wang et al. 2013).

4. Atomic force microscopy and force measurements

AFM measurements were performed using an MFP-3D instrument (Asylum Research, Santa Barbara, CA). Silicon nitride cantilevers were purchased from Bruker (MLCT, Camarillo, CA) with a nominal spring constant of 0.01 Nm^{-1} and probe radius of 20 nm (according to the manufacturer's specifications). Cantilever spring constants were determined using the thermal tune method (Hutter et al. 1993) included in the MFP-3D software. Calibrated spring constants were within the range of 0.016-0.020 Nm^{-1} . All cantilevers used were from the same batch. All tips were cleaned in a BioForce UV/ozone cleaner (BioForce Nanosciences Inc, Ames, IA) before use. Photodetector sensitivity was measured on a clean silica slide in buffer prior to force measurements for each experiment (Velegol et al. 2002). The slope of

the constant compliance region of the force curves obtained was used to convert the deflection, d , in mV to nm. The cantilever deflection was then converted into a force, F , according to Hooke's law, $F = k \times d$, where k is the force constant of the cantilever (Radmacher et al. 1995).

Cells were imaged in contact mode at a scan rate of 1 Hz. Trace and retrace were monitored to locate the true apex of cells and ten force curves were measured at different locations along it. The cell apex was probed during force measurements rather than the cell periphery, which has a high degree of curvature that makes quantifying mechanical properties difficult (Gaboriaud et al. 2008). Imaging was repeated after each collection of ten force curves to ensure no change in cell morphology had occurred. Force curves were acquired at a loading rate of 600 nm s^{-1} . The maximum loading force was controlled in all experiments to avoid puncturing the bacterial cell wall with the AFM tip (Suo et al. 2009).

The force profiles acquired following the addition of colistin were measured on single wild-type cells over a 2 h time frame. At each time point, 10 force curves were measured along the apex of the cell. The data obtained showed excellent reproducibility from location to location along each cell apex and from cell to cell in all experimental conditions (Supporting Information, Figure S1). Force curves were overlaid on the same axis (as seen in Supporting Information Figure S1), and the median curve was selected for analysis. Experiments were performed at least five times to ensure reproducibility in the observed trends over time.

5. Bacterial cell indentation and force curve analysis

Wang et al. (2013) have demonstrated that the indentation of *K. pneumoniae* cells is comprised of four distinct stages when working at low electrolyte concentrations and that the

Pincus model (2001), Hertz model, (Hertz 1896; Radmacher 1997) and Hooke's law (Gaboriaud et al. 2008; Radmacher et al. 1995) can be used to extract biophysical parameters from experimental data. Exposure to colistin changed the form of force curves measured on wild-type cells (Figure 4) so that the method outlined by Wang et al. (2013), was not applicable (see Results and Discussion). Hence, force curves obtained from both wild-type and colistin-resistant cells for this work were analysed using Hooke's law:

$$F_{loading} = K_{bacterium} \delta \quad (1)$$

where $F_{loading}$ is the applied loading force, $K_{bacterium}$ is the bacterial spring constant, and δ is the indentation. The slope of this linear compliance region of the force profile yields $K_{bacterium}$, which is directly related to the relative turgor pressure of the bacterium. The indentation depth at which the linear portion of the force curve begins provides an estimate of the bacterial capsule thickness. The contact point, i.e., zero indentation, is defined as the onset of cantilever deflection since long-range non-contact double layer interactions are negligible in this study (electrolyte concentration is 10 mM which corresponds to a Debye length of ~ 3 nm).

6. Cell viability assays

Cell viability assays were performed using a LIVE/DEAD® BacLight Bacterial Viability Kit (Molecular Probes, Eugene OR). Wild-type *K. pneumoniae* cells were adhered to modified glass slides that were prepared in the same way as for AFM experiments (described above). The purpose was twofold: to confirm that the bacteria were viable at the commencement of an AFM experiment, and to determine the rate at which cells were compromised after exposure to the peptide. Cells were visualised and images captured using a Zeiss LSM 700 confocal microscope and Zeiss Zen imaging software. Green and red channel images were thresholded using ImageJ. Cells were counted using the 'Analyse particles' function.

Microscopy was performed at the Biological Optical Microscopy Platform, University of Melbourne.

7. Capsule extraction and uronic acid analysis

Cell-associated capsule was extracted from bacterial cultures grown at various time-points by a hot phenol/chloroform extraction, followed by ethanol precipitation (Campos et al. 2004). Quantification of the extracted capsule was determined by measuring the uronic acid content present in the polysaccharide (Blumenkrantz et al. 1973). Briefly, 1.2 mL of 12.5 mM sodium borohydride in concentrated sulfuric acid was added to 0.2 mL capsule extracts and boiled before addition of 20 μ L of 0.15% 3-hydroxydiphenol in 0.5% sodium hydroxide. Absorbance was measured at 520 nm. Standard curves were constructed with D-glucuronic acid.

8. Genome sequencing and analysis

Whole genome sequencing was performed on the NextSeq (Illumina) using 2 x 150 bp chemistry as per the manufacturer's recommendations. Prior to further analysis, reads were filtered to remove those containing ambiguous base calls, any reads less than 50 nucleotides in length, and containing only homopolymers. All reads were furthermore trimmed to remove residual ligated Nextera adaptors and low quality bases (less than Q10) at the 3' end. The DNA sequence of the *K. pneumoniae* AJ218 genome was *de novo* assembled using Spades (Bankevich et al. 2012). Fastq read files from both the wild-type and mutant were then subjected to read-mapping against the *de novo* assembled AJ218 genome using Snippy, a Python utility that uses the reads from each genome aligned to the core genome to construct a tally of putative differences at each nucleotide position (including substitutions, insertions, and deletions) (www.bioinformatics.net.au).

9. Cryo-electron microscopy

Bacteria were exposed to buffer or 1.2 μM colistin sulfate solution before being absorbed on glow discharge holey carbon grids and plunge frozen in liquid ethane. The resulting grids were observed on a FEI Tecnai F30 operating at 300 kV under low dose. Each micrograph was recorded on a Gatan Ultrascan 1000 with a total dose of $\sim 2,000 \text{ e}^-/\text{nm}^2$.

Results and Discussion

AFM imaging and optimization of conditions for force measurements

Bacterial cells adhered to either PEI coated slides were imaged in contact mode in HEPES buffer (pH 7.4). Figure 1a shows a typical $15 \times 15 \mu\text{m}$ AFM image of *K. pneumoniae* cells. The rod-shaped bacterial cells were well dispersed and the surfaces of the cells were topographically homogeneous on this scale, and on a scale with increased definition ($3 \times 3 \mu\text{m}$, Figure 1b).

Figure 1. 3D projections of AFM images: (a) $15 \times 15 \mu\text{m}$ image of topographically homogeneous, rod shaped *K. pneumoniae* cells; and (b) $3 \times 3 \mu\text{m}$ image of a single *K. pneumoniae* cell. All cells were imaged *in situ* (10 mM HEPES buffer, pH 7.4).

A prerequisite for AFM measurements of the kind reported here is firm adhesion of the bacterium to the PEI coated substrate for the duration of an experiment and dispersion on the substrate such that single cells can be probed during measurement (Figure 2a). Initial working concentrations of $1.2 \mu\text{M}$ resulted in considerable aggregation of cells and what appeared to be ‘extra’ material at the cell surface, interfering with the imaging process (Figure 2b and 2c). This phenomenon was reported by Soon et al. (2011) in their work with colistin and *A. baumannii*. Further, cells adhered to the PEI-coated surface often did not withstand more than 3 or 4 measurement cycles, where one cycle consists of ten replicate force curves along the apex of the cell followed by imaging to ensure no change had occurred in cell morphology or position on the PEI-coated surface. Imaging at the lower concentration of $0.1 \mu\text{M}$ (Figure 2d) revealed cells that were dispersed, and most cells adhered strongly enough to the surface to withstand multiple force measurements; hence a suitable concentration to begin experimentation.

Figure 2. Height images ($10 \times 10 \mu\text{m}$) of *K. pneumoniae* cells submerged in: (a) $10 \mu\text{M}$ HEPES buffer, (b) and (c) $1.2 \mu\text{M}$ colistin solution, and (d) $0.1 \mu\text{M}$ colistin solution. All imaging was performed between 40 to 60 min exposure time.

1 Analysis of force curves

2 In untreated wild-type cells, the non-linear portion of the force curves can be analysed using
3 various models, such as the Hertz (Hertz 1896; Radmacher 1997) and Pincus (Considine et
4 al. 2001) models to provide information about the stiffness and organisation of the capsule as
5 described in previous work (Mularski et al. 2015; Wang et al. 2013). However, in 0.1 μ M
6 colistin, the form of force curves collected on the apex of the same cell changed over time
7 (Figure 3). At low exposure times, force curves retained the form that was characteristic of
8 the indentation of an encapsulated cell, representative of an easily compressible material
9 (capsule). At high exposure times (Figure 4, blue), force curves resembled what would be
10 measured on a rigid surface, like those obtained from *K. pneumoniae* capsule deficient
11 mutant cells (Figure 4, purple) or glass (Figure 4, black). These significant changes in the
12 capsule over the time course of experiments as a result of colistin exposure precluded
13 determination of the polymer brush length and Young's modulus using previous
14 methodology (Mularski et al. 2015; Wang et al. 2013). For the work presented here, a
15 simplified version of the model was used (Figure 4a) that allowed us to analyse all force
16 curves collected in this work, regardless of solution conditions. The mechanical model of *K.*
17 *pneumoniae* cell indentation is comprised of three stages: (i) long-range double layer
18 interaction between charged tip and surface, (ii) indentation of capsular polysaccharide, and
19 (iii) indentation of cytoplasm.

Figure 3. Typical force profiles measured on single *K. pneumoniae* cells displaying median capsule loss in: (a) 0.1 μM colistin solution at increasing exposure times (red – blue) demonstrating the transition from easily indented to rigid surface; and (b) in buffer only. Force profiles of capsule deficient *K. pneumoniae* mutant (purple) and glass surface (black) in buffer are included for comparison.

Figure 4b shows a typical force profile (red) for the indentation of a *K. pneumoniae* cell in buffer with Hooke's law fit (blue) applied to the linear portion of the force curve. The onset of non-zero force corresponds to the point at which contact was first made between the tip and the sample since long-range non-contact double layer interactions are negligible in this study (as electrolyte concentration is 10 mM which corresponds to a Debye length of ~ 3 nm). The indentation of capsular polysaccharides continues until the loading force on the cell is sufficient such that the turgor pressure is the dominant opposing force, corresponding to the linear constant compliance region. The gradient of the linear Hooke's law fit to this region provides an estimate of the cell's turgor pressure. The turgor pressure of a bacterial cell is a

measure of the osmotic stability and viability of the cell (Eq. 1). Control experiments revealed that the bacterial spring constant, which is related to the turgor pressure, of wild-type *K. pneumoniae* cells in 10 mM HEPES buffer was 118 ± 40 mN/m (data not shown), in good agreement with previous work in similar conditions (Mularski et al. 2016; Mularski et al. 2015; Wang et al. 2013). The indentation depth at which the linear portion of the force curve begins provides an estimate of the bacterial capsule thickness where the contact point, i.e., zero indentation, is defined as the point at which contact was first made between the tip and the sample.

Figure 4. (a) Mechanical model of *K. pneumoniae* cell indentation: (i) long-range double layer interaction between charged tip and surface (in the experimental conditions used here, 10 mM HEPES buffer at pH 7.4, the expected Debye length is ~ 3 nm, the long range double layer interactions can be safely neglected); (ii) indentation of capsular polysaccharide; and (iii) indentation of cytoplasm. (b) Typical force profile of *K. pneumoniae* cell in HEPES buffer (red) with Hooke's law fit (blue) and derived parameters, bacterial spring constant,

$K_{bacterium} = 97 \text{ mN/m}$, and capsule thickness = 340 nm. The gradient of the Hooke's law fit gives the bacterial spring constant which is related to the turgor pressure of the cell.

Force measurements

The analysis of force curves in Figure 3(a) yielded the capsule thickness and turgor pressure over the duration of the experiment. Typical of cells exposed to 0.1 μM colistin, a significant reduction in capsule thickness occurred, from the average measured capsule thickness of cells in buffer, 320 nm, to a low of 128 nm at 63 min exposure to colistin. Coupled with the changing nature of the force curves, from those for easily compressible to rigid surfaces, a reduction in capsule thickness suggests either loss of capsular polysaccharides or a rearrangement of the capsule after exposure to 0.1 μM colistin. The duration of these low concentration experiments was shorter (~60-90 min) than for those in previous work (~120 min) (Mularski et al. 2015) due to the observed effect of colistin exposure: the reduction in measured capsule thickness coincided with reduced adhesion to the PEI coated slides. After 2-3 cycles of force curve collection where force curves indicated a rigid surface, the cell was swept away by the movement of the AFM tip while raster scanning the surface, indicating a difference in cell adhesive properties. This is in good agreement with a study by Soon et al. (2011) that demonstrated that colistin-induced change in the adhesive properties of *A. baumannii*. Successive force curves collected on a wild-type cell in buffer are shown for contrast in Figure 3(b) showing a smaller loss in capsule thickness, due to the scanning action of the AFM tip.

The turgor pressures, based on $K_{bacterium}$, measured after exposure to 0.1 μM colistin were not substantially different to those of bacteria in buffer. Fluorescent dye-exclusion assays confirmed the viability of colistin-treated (0.1 μM) *K. pneumoniae* cells following exposure

1 to the antibiotic (0, 30, 60, 90, 120 min). At 0.1 μ M colistin, a maximum of 4% of bacterial
2 cells had compromised membranes, even after 120 min, (Supporting Information, Figure S3).
3 This indicates that while low concentration (0.1 μ M) colistin has a significant effect on the
4 capsular polysaccharide, it did not result in membrane lysis.

5
6 AFM of cells treated with 1.2 μ M colistin indicated that lysis had occurred in those bacteria
7 that were analysed. The turgor pressures or bacterial spring constant determined from force
8 curves on 10 single cells were 15 ± 4 mN/m (Figure 5) characteristic of fully lysed cells
9 (Mularski et al., 2016). The MIC and MBC for wild-type *K. pneumoniae* AJ218 for colistin
10 was determined to be 2 μ M, consistent with the loss of turgor pressure at 1.2 μ M colistin. All
11 AFM measurements at 1.2 μ M colistin resulted in turgor pressures indicative of lysis having
12 already occurred before measurements commenced. Fluorescent dye exclusion assays were
13 conducted on *K. pneumoniae* cells treated with 1.2 μ M colistin after various exposure times
14 (0, 30, 60, 90, 120 min). After 120 min exposure to colistin, 83% of bacterial cells had
15 compromised membranes (Supporting Information, Figure S3). Experiments conducted at the
16 median concentration of colistin at 0.6 μ M did not reveal any cells with turgor pressures
17 substantially different to those of bacteria in buffer or 0.1 μ M colistin (Figure 5).
18 Fluorescence assays were conducted on colistin-treated cells (0.6 μ M) to determine viability
19 of *K. pneumoniae* cells at each exposure time (0, 30, 60, 90, 120 min). By 120 min, 45% of
20 bacterial cells had compromised membranes (Supporting Information, Figure S3) suggesting
21 that at 0.6 μ M colistin, cells were undergoing lysis during the experimental window.

Figure 5. Average change in $K_{bacterium}$, bacterial spring constants (proportional to turgor pressure) of wild-type cells (blue) and colistin-resistant cells (red) in buffer, 0.1 μM, 0.6 μM (wild-type only) and 1.2 μM colistin solution. The wild-type cells were lysed at 1.2 μM colistin.

To investigate the role of the capsule in colistin interaction with *K. pneumoniae* AJ218, MIC and MBC were determined for a capsule-deficient *K. pneumoniae* mutant. The defined (Wza-Wzc) capsule deficient mutant had an MIC and MBC equal to that of the wild-type, suggesting that the capsular interaction with colistin plays no significant role in protecting the bacterial cell. This is in keeping with the view that the site of interaction for colistin is within the LPS as the capsule deficient mutant retains the LPS component of the cell envelope. The effect of colistin on the wild-type and colistin-resistant capsules at each concentration is summarised by Figure 6 where box plots describe the variation in rate of change in the capsule thickness at each concentration. Cells were subjected to repeated force curve collection cycles of at least 20 min. For comparison, capsule thickness measurements collected on wild-type cells in buffer are shown, indicating that a small amount of capsular polysaccharide (-2.8 nm/min) is lost by virtue of subjecting the cell to repeated force curve collection cycles. As stated earlier, at 0.1 μM colistin, all single cell AFM experiments

revealed a reduction in capsule thickness which occurred at an average rate of -7.4 nm/min. At 0.6 μ M colistin, the measured reduction in capsule thickness of capsule was reduced (to -4.8 nm/min). At 1.2 μ M colistin, there was an increase in successive capsule thickness measurements. The average rate of change was +9 nm/min, corresponding to an average increase in capsule thickness of 92 nm for the seven single cells experiments, although the spread of the data was even larger at this concentration. The measured increase in thickness at 1.2 μ M suggests that either there is further rearrangement of the capsule taking place or that the bacteria have increased capsule excretion in response to colistin exposure.

Figure 6. Rate of capsule thickness change for wild-type cells (blue) and colistin-resistant cells (red). The increase in rate and scatter with concentration suggest a rearrangement of the capsular polysaccharides for wild-type cells in response to colistin exposure.

Experiments were performed on a colistin-resistant *K. pneumoniae* isolate. Expectedly, the MIC and MBC of 31 μ M for the colistin-resistant isolate were an order of magnitude higher than for the wild-type. AFM measurements determined that the turgor pressure of the colistin-resistant isolate was comparable to the wild-type (Figure 5) in buffer (bacterial spring constant 111 ± 36 mN/m) and in 0.1 μ M (127 ± 43 mN/m). From the rate of capsule thickness change data plotted alongside that of the wild-type (Figure 6), it is apparent that no increase in capsule thickness is observed in the colistin-resistant mutant. Successive force curves

collected on a colistin-resistant cell in 0.1 μM colistin solution are shown in contrast to a wild-type cell in buffer and in 0.1 μM colistin solution (Supporting Information, Figure S2). Since the wild-type and capsule-deficient cells have the same MIC and MBC, the capsule did not appear to confer advantage. However, the colistin-resistant strain had a 10-fold increased MIC and MBC and the capsule responded differently to colistin exposure, suggesting that the capsule may play a role in their ability to withstand greater amounts of colistin.

The response of wild type cells to 0.1 μM and 1.2 μM colistin could also be explained by the loss or increased production of capsular polysaccharide, respectively. To examine this hypothesis, *K. pneumoniae* cell suspensions in buffer or colistin solutions (0.1 μM or 1.2 μM) were harvested after 80 and 180 min time-points, after which the cell-associated capsule polysaccharides were extracted and quantified by measuring uronic acid (a sugar present in the polysaccharide). Results revealed no difference between the amount of uronic acid for wild-type and colistin-resistant cells in buffer and after exposure to both 0.1 and 1.2 μM colistin solution for both 80 and 180 min (Supporting Information, Figure S4). Thus, the amount of cell-associated capsular polysaccharide remains constant after colistin exposure for the duration of AFM experiments for both wild-type and colistin-resistant cells. These results imply that the effect of colistin exposure on the capsule is one of rearrangement: at 0.1 μM we see polymer collapse in the capsule, due to electrostatic interaction of colistin with the capsule and the LPS; and at 1.2 μM , the concentration at which cells are lysed, we see an unravelling of the capsular polysaccharides, perhaps brought about by the displacement of the divalent cation LPS cross-linkers through which the capsule is extruded. Unravelling of the capsular polysaccharides at 1.2 μM explains the poor imaging quality in Figure 2(b) and 2(c) where cells appear to have ‘extra’ material at the cell surface.

Genome sequencing

The genomes of wild-type *K. pneumoniae* AJ218 and colistin-resistant strain were sequenced in an effort to identify the genetic change(s) responsible for the resistance to colistin and to determine the implications on this change for encapsulation. The colistin-resistant mutant was found to have an amino acid substitution (Q95P) in the PmrB sensor kinase as well as a guanine nucleotide insertion in an open reading frame that encodes a hypothetical protein that displays some homology to other proteins of the LexA/signal peptidase superfamily (locus ref: WP_032443403). Mutations at other sites in PmrB (L82R, T157P) have also been found to be related to colistin resistance in *K. pneumoniae*, possibly due to increased activation of the cognate response regulator PmrA, leading to modification of the lipid A component of the LPS and a reduction in the net negative charge of the outer membrane. (Cannatelli et al. 2014; Choi et al. 2014; Jayol et al. 2014) The reduction of net negative charge of the outer membrane enables bacterial cells to avoid the initial electrostatic attraction of colistin to the outer membrane. This is considered the most common mechanism of colistin resistance in *K. pneumoniae* (Velkov et al. 2013). These data are in good agreement with our AFM observations of the capsule. The colistin-resistant mutant, with reduced net negative charge on its LPS, has less interaction with colistin-molecules. As a result, fewer divalent cation cross-linkers are displaced and the capsule is retained. In our wild-type cells, these cross linkers are displaced and the capsular polysaccharides unravel.

The results of our genetic studies contrast with those of Formosa et al. (2015) with colistin-susceptible and resistant strains of *K. pneumoniae* (ATCC 700603 and ATCCm, respectively). Their studies found no variation in the genes associated with LPS synthesis. They did identify an insertional inactivation/mutation in the *mgrB* gene of their colistin-resistant strain, which is considered a key target for acquired resistance in colistin in *K.*

1 *pneumoniae* (Poirel et al., 2015). They linked the inactivation of the *mgrB* gene to the
2 multilayered capsule architecture they observe in their colistin-resistant strain, and made the
3 hypothesis that capsular polysaccharides might be implicated in the mechanism of colistin
4 resistance in *K. pneumoniae* ATCCm. Such capsular architecture was not present in colistin-
5 resistant *K. pneumoniae* AJ218.

6
7 Formosa et al. (2015) also observed that at 0.1 μ M colistin, the capsule was entirely removed
8 from their colistin-susceptible strain. While the MIC of their strain (0.5 μ M) was lower than
9 ours (2 μ M), it was grown in colistin-containing media for 24 h. Their observation, however,
10 is consistent with the decreasing capsule thickness measurements we report here in wild-type
11 AJ218 at the same concentration. Uronic acid assays (Supporting Information, Figure S4)
12 confirmed that, in the absence of AFM raster scanning, capsular polysaccharide remains
13 associated with AJ218 cells, which suggests a more complex interaction between the outer
14 membrane, LPS and capsule in *K. pneumoniae* AJ218 than for the colistin-susceptible *K.*
15 *pneumoniae* ATCC.

16 17 Cryo-electron microscopy

18 To investigate whether the measured increase in capsule thickness in wild-type cells at 1.2
19 μ M may be a phenomenon associated with lysis, cells treated with colistin were visualised
20 using cryo-electron microscopy. Figure 7a shows a single *K. pneumoniae* cell treated with
21 buffer only. Cytoplasmic membrane, peptidoglycan layer, outer membrane and fimbriae are
22 all clearly visible. Figures 7(b-d) show damage that occurred after 2 h exposure to colistin
23 before commencing microscopy. What appear to be outer membrane blebs were visible in 26
24 of 28 cells imaged (Figure 7c and 7d). Similar phenomena were reported by Liu et al. (2014),
25 Mortensen et al. (2009) and Lounatmaa et al. (1976; 1976) in their work studying the

interaction of colistin with *A. baumannii*, *P. aeruginosa* and *Salmonella enterica* var Typhimurium, respectively. Liu et al. (2014) have reported SEM and TEM images of similar protrusions on the outer membranes of colistin treated *K. pneumoniae* cells. They also showed significant cytoplasm shrinkage in these cells due to the dehydrating sample preparation required for these techniques having a greater effect on compromised cells. The ultrastructure modifying preparation may not yield a true reflection of what would occur to cells *in vivo*, as shown by cryo-electron microscopy where flash freezing of cells in equilibrium with their surrounds occurs.

Figure 7. Cryo-electron micrographs of *K. pneumoniae* cells in: (a) HEPES buffer; and (b-d) in 1.2 μM colistin. Membrane blebs, peptidoglycan discontinuity, outer and inner membrane ruptures are shown as labelled.

Visible damages to at least one of the cytoplasmic membrane (Figure 7d), peptidoglycan layer (Figure 7c) and outer membrane (Figure 7b) were apparent in all 28 cells imaged. Damage of the kind shown in Figure 7d, where the distance between the outer and cytoplasmic membranes increased (from ~20 nm in Figure 7a) to ~80 nm was observed in 18 of 28 cells imaged. A similar observation was made by Liu et al. (2014) in their focused ion beam tomography study of colistin treated *K. pneumoniae*. It seems that, coupled with the unravelling of capsular polysaccharides at 1.2 μ M, the damaged outer membrane, being a less taut barrier, contributed to the greater capsule thicknesses found when force measurements were conducted on lysed cells. The latter is an important consideration when interpreting the results of these kinds of AFM measurements.

Conclusions

AFM capsule thickness measurements as presented here demonstrated that the wild-type *K. pneumoniae* AJ218 capsular polysaccharides are rearranged by exposure to colistin. The increase in capsule thickness measured near MIC/MBC is consistent with an unravelling of the capsular polysaccharides, perhaps brought about by the displacement of the divalent cation LPS cross-linkers through which the capsule is extruded. At concentrations near the MIC/MBC, the lysed outer membrane from which capsule and LPS emanate most probably augmented the measured capsule thickness.

The 10-fold increase in resistance in the colistin-resistant *K. pneumoniae* AJ218 derivative was identified as a substitution (Q95P) in the PrmB sensor kinase. This could result in a reduction of the net negative charge of the outer membrane due to modification of the lipid A component of the LPS. Less electrostatic interaction of colistin with the LPS would result in

fewer divalent cation LPS cross linkers being displaced and may, therefore, retain the capsule, explaining why the capsule thickness of the colistin-resistant strain did not increase following colistin exposure. Wild-type and capsule-deficient strains of *K. pneumoniae* AJ218 had equivalent MIC and MBC, which suggests that the presence of the capsule confers little protection against colistin.

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Supporting information

S1. Reproducibility of force curves measured on a single: a) wild-type *K. pneumoniae* cell in buffer; b) wild-type *K. pneumoniae* cell in 0.1 μM colistin solution (time); c) wild-type *K. pneumoniae* cell in 0.6 μM colistin solution (time); d) wild-type *K. pneumoniae* cell in 1.2

1 μM colistin solution (time); e) capsule deficient *K. pneumoniae* cell in buffer; f) capsule
2 deficient *K. pneumoniae* cell in 0.1 μM colistin solution (time); g) colistin-resistant deficient
3 *K. pneumoniae* cell in buffer; h) colistin-resistant deficient *K. pneumoniae* cell in 0.1 μM
4 colistin solution (time); and i) colistin-resistant deficient *K. pneumoniae* cell in 1.2 μM
5 colistin solution (time).

6
7 S2. Five successive force curves, measured approximately 5 min apart on a typical single a)
8 wild-type cell in 0.1 μM colistin solution; b) wild-type cell in buffer; and c) colistin-resistant
9 cell in 0.1 μM colistin solution.

10
11 S3. Cell viability assays performed on: a) Wild-type cells in 0.1 μM colistin solution; b)
12 Wild-type cells in 0.6 μM colistin solution; c) Wild-type cells in 1.2 μM colistin solution; d)
13 Colistin-resistant cells in 0.1 μM colistin solution; e) Colistin-resistant cells in 1.2 μM
14 colistin solution.

15
16 S4. Amount of uronic acid (μg) per 10^{10} colony forming units (CFU) for wild-type (blue),
17 colistin-resistant (red) and capsule deficient (red) *K. pneumoniae* AJ218. Data for all cell
18 types shows no significant difference in amount of uronic acid as a function of exposure time.

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