Colistin requires *de novo* lipopolysaccharide biosynthesis for activity

Akshay Sabnis[1], Anna Klöckner[1,2], Michele Becce[3], Lindsay E. Evans[1,4], Molly M. Stevens[2,3] and Andrew M. Edwards[1#]

[1] MRC Centre for Molecular Bacteriology and Infection, Imperial College London, Armstrong Rd, London, SW7 2AZ, UK.

[2] Department of Bioengineering, Imperial College London, Imperial College London Rd, London, SW7 2AZ, UK.

[3] Department of Materials, Imperial College London, Imperial College London Rd, London, SW7 2AZ, UK.

[4] Department of Chemistry, Imperial College London, Molecular Sciences Research Hub, White City Campus, Wood Lane, London, W12 OBZ, UK.

[#] For correspondence:

Tel: 0044 (0)207 594 2072

Fax: 0044 (0)207 594 3096

a.edwards@imperial.ac.uk

Keywords: *Pseudomonas* / *E. coli* / *Klebsiella* / Colistin / lipopolysaccharide / Phospholipid / LPS

Running title: Colistin mode of action

Colistin is a last resort antibiotic for infections caused by highly drug-resistant Gram-negative pathogens such as *Pseudomonas aeruginosa* [1,2]. Unfortunately, treatment failure is common despite low rates of resistance, and efforts to address this are compromised by our poor understanding of colistin's mode of action [3-6]. Here, we show that colistin causes dose-dependent membrane disruption and killing of *P. aeruginosa* via a process that requires *de novo* lipopolysaccharide (LPS) biosynthesis. Colistin binds to LPS in the outer membrane (OM), leading to disruption of the cation-bridges that stabilise the lipopolysaccharide molecules. Due to the weakening of these bridges, *de novo* synthesis of LPS results in the release of lipopolysaccharide from the OM. The subsequent loss of membrane integrity enables colistin to access the cytoplasmic membrane (CM), leading to permeabilisation and bacterial lysis. Inhibition of LPS biosynthesis in *Escherichia coli* and *Klebsiella pneumoniae* also inhibited the bactericidal activity of colistin, suggesting a conserved mechanism across clinically-relevant pathogens. Together, these findings reveal the mechanism by which colistin kills bacteria and provide the foundations for the development of new approaches to enhance treatment outcomes.

The emergence of multi-drug resistant Gram-negative pathogens has led to a dramatic increase in the use of cyclic lipopeptide antibiotics such as colistin (polymyxin E) and polymyxin B as therapeutics of last resort [7,8]. Despite this, our understanding of the mechanism by which these drugs kill bacteria is poor. The currently proposed mode of action of colistin begins with binding of the antibiotic to LPS, which leads to disruption of the OM via the displacement of cations that form stabilising bridges between LPS molecules (Fig. 1a) [9-11]. It is hypothesised that colistin then migrates across the OM via a process termed 'self-directed uptake', although this has never been demonstrated experimentally [11-13]. This crucial step enables colistin to access the CM target, which it disrupts, leading to bacterial lysis and death [11-13]. Furthermore, the existence of colistin-tolerant persister cells suggests that bacterial processes contribute to the lethality of the antibiotic, although none have yet been identified [14-16]. Given these gaps in our understanding of this increasingly important class of antibiotics, the aim of this work was to determine the mechanism by which polymyxins kill bacteria.

57 To test the current model of colistin activity, we employed a panel of assays to investigate
58 membrane damage, cell lysis and bacterial viability during exposure of *P. aeruginosa* PA14 to clinically-
59 relevant concentrations of the antibiotic [17,18]. Colistin caused immediate and dose-dependent
60 disruption of the OM, as determined by uptake of the normally membrane-impermeant dye *N*-phenyl-
61 1-naphthylamine (NPN) (Fig. 1b, Supplementary Fig. 1). Within 30 min of colistin exposure, *P.*
62 *aeruginosa* began to release periplasmic β-lactamase into the culture supernatant, indicative of severe
63 OM damage (Fig. 1c). This was followed by disruption of the CM by 60 min, as detected by staining of
64 cells with the membrane-impermeant DNA-reactive fluorophore propidium iodide (Fig. 1d,
65 Supplementary Fig. 2). Subsequently, bacterial lysis induced by colistin was observed with a decrease
66 in OD_{595nm} from 90 min, and there was a corresponding drop in c.f.u. counts by 2 h (Fig. 1ef). These
67 findings provide a timeline for the current model of polymyxin-mediated bacterial killing, whilst the
68 survival of a sub-population of bacteria exposed to the antibiotic confirmed the existence of colistin-
69 tolerant persister cells within *P. aeruginosa* populations (Fig. 1f, Supplementary Fig. 3).
70 To further investigate colistin-mediated membrane disruption, we measured the release of
71 LPS and phospholipids into the extracellular space. *P. aeruginosa* released large quantities of
72 phospholipids, most likely in the form of OMVs, in the absence of colistin, but this was almost
73 completely blocked by colistin at bactericidal concentrations (Fig. 1g) [19]. By contrast, colistin
74 exposure resulted in the time-dependent release of LPS into the culture supernatant (Fig. 1h,
75 Supplementary Fig. 4).
76 As reported previously, released LPS completely inhibited the antibacterial activity of colistin
77 at 2 μg ml^{-1} (Supplementary Fig. 5) [20]. However, we also considered the possibility that colistin-
78 induced LPS release contributed to the bactericidal activity of the antibiotic. This is because the loss
79 of LPS from the OM results in lipid asymmetry and a loss of integrity of the OM, which if uncorrected
80 culminates in cell death in most bacteria [21]. To counteract lipid asymmetry, bacteria increase the
81 production and trafficking of LPS to the OM [22]. Therefore, we hypothesised that colistin activity
82 would be enhanced by blocking LPS biosynthesis, thereby preventing *P. aeruginosa* from restoring the

83 composition of the outer leaflet of the OM. To test this, we reduced the ability of *P. aeruginosa* to
84 restore LPS to the OM of colistin-treated cells by exposing bacteria to the polymyxin antibiotic in the
85 presence of sub-lethal concentrations of the LPS biosynthesis inhibitors CHIR-090 or cerulenin
86 (Supplementary Table 2, Supplementary Fig. 6) [23-25].

87 As described above (Fig. 1f), colistin alone caused rapid killing of *P. aeruginosa* (Fig. 2a).
88 However, in contradiction to our hypothesis, the presence of either inhibitor of LPS biosynthesis
89 almost completely blocked killing of *P. aeruginosa* by colistin (Fig. 2ab). To ensure that the protective
90 effect of LPS biosynthesis inhibition wasn't due to reduced LPS on the bacterial cell surface, we
91 exposed *P. aeruginosa* to colistin for 2 h and then added CHIR-090 or cerulenin. The addition of either
92 LPS biosynthesis inhibitor paused or greatly slowed colistin-mediated killing of *P. aeruginosa*,
93 confirming that *de novo* LPS biosynthesis is needed for the continuous bactericidal activity of the
94 polymyxin antibiotic (Fig. 2c).

95 Scanning electron microscopy (SEM) supported the data showing that colistin activity was
96 dependent on *de novo* biosynthesis of LPS. Exposure to the polymyxin antibiotic alone caused
97 significant morphological changes to *P. aeruginosa* consistent with a loss of membrane integrity (Fig.
98 2d). However, bacteria exposed to colistin in the presence of CHIR-090 or cerulenin had a similar
99 appearance to untreated cells (Fig. 2efghi). The protective effects of CHIR-090 and cerulenin against
100 colistin-mediated killing extended to clinical isolates of *P. aeruginosa*, *K. pneumoniae* and *E. coli*,
101 suggesting the polymyxin antibiotic has a broadly conserved mechanism against Gram-negative
102 pathogens (Supplementary Fig. 7). By contrast to the LPS biosynthesis inhibitors, neither sub-
103 inhibitory nor bacteriostatic concentrations of the protein synthesis inhibitor tetracycline prevented
104 colistin-mediated killing of *P. aeruginosa* (Supplementary Fig. 8). Therefore, colistin requires *de novo*
105 LPS but not protein biosynthesis for activity, and functions in the absence of bacterial replication.
106 However, *de novo* LPS biosynthesis was not required for the bactericidal activity of EDTA, which also
107 disrupts cation bridges in the LPS monolayer, indicating that the requirement for *de novo* LPS
108 biosynthesis is specific to polymyxin-mediated killing (Supplementary Fig. 9).

109 Next, we determined which of the steps in the current model of action of colistin were
110 dependent on *de novo* LPS biosynthesis. Using NPN, we found that colistin rapidly permeabilised the
111 OM, and this was unaffected by the presence of CHIR-090 or cerulenin (Fig. 3a). Therefore, *de novo*
112 LPS biosynthesis is not required for the initial interactions of the polymyxin antibiotic with the bacterial
113 surface. However, colistin-mediated release of β-lactamase from *P. aeruginosa* was blocked by
114 cerulenin, indicating that *de novo* LPS biosynthesis is required for severe damage to the OM (Fig. 3b).
115 This assay could not be repeated using CHIR-090 as it reacted with the chromogenic substrate used to
116 detect and quantify β-lactamase. Nonetheless, the presence of cerulenin or CHIR-090 prevented
117 colistin-mediated CM damage and lysis of *P. aeruginosa* (Fig. 3cd). This demonstrated that *de novo*
118 LPS biosynthesis is required for colistin to cross the OM and provided a direct link between the degree
119 of OM damage and the ability of colistin to access the CM. Colistin-mediated disruption of the CM and
120 subsequent lysis of clinical *P. aeruginosa*, *K. pneumoniae* and *E. coli* isolates were also reduced or
121 blocked when LPS biosynthesis was inhibited, showing that the dependency on LPS biosynthesis for
122 colistin activity is conserved (Supplementary Fig. 10, 11).

123 To determine how *de novo* LPS biosynthesis modulated the degree of colistin-mediated
124 damage to the OM, we tested whether CHIR-090 or cerulenin affected LPS release caused by the
125 polymyxin antibiotic. Both inhibitors blocked colistin-induced LPS release, demonstrating that this
126 process, which results in severe damage to the OM, was dependent upon *de novo* LPS biosynthesis
127 (Fig. 3e, Supplementary Fig. 12-14). Therefore, *de novo* LPS biosynthesis triggers the loss of LPS from
128 the cell surface, and thus enables colistin to cross the OM.

129 The data shown above confirmed that neither CHIR-090 nor cerulenin inhibited the initial
130 interactions of colistin with the surface of *P. aeruginosa* (Fig. 2c, 3a). However, we hypothesised that
131 the severe OM damage mediated by the combination of colistin and *de novo* LPS biosynthesis causing
132 LPS release would increase binding of the antibiotic to *P. aeruginosa* by exposing the CM target. To
133 test this, we measured the binding of colistin to bacterial cells in the absence or presence of CHIR-090
134 or cerulenin using colistin labelled with a BoDipy fluorophore (Supplementary Fig. 15, 16). Exposure

135 of *P. aeruginosa* to BoDipy-colistin resulted in strong binding of the antibiotic to the bacterial cells
136 during the first two hours, which was maintained over the rest of the incubation period (Fig. 3f). By
137 contrast, when the experiment was repeated in the presence of CHIR-090 or cerulenin, binding to
138 bacteria was significantly reduced, with a slow accumulation of BoDipy-colistin over the course of the
139 assay (Fig. 3f).

140 Taken together, the data described in Figure 3 demonstrate that *de novo* LPS biosynthesis
141 enables colistin to exert its bactericidal activity by causing LPS release and increasing the severity of
142 the OM damage induced by the antibiotic. This provides colistin with access to the CM, which it
143 disrupts, leading to lysis and bacterial killing.

144 Polymyxin antibiotics consist of a positively-charged peptide ring and a hydrophobic lipid tail
145 [1,11,26]. Previous work demonstrated that the polymyxin peptide ring displaces cations which act as
146 bridges between LPS molecules [27]. This leads to sufficient permeabilisation of the OM to allow the
147 passage of small molecules such as NPN [11,13]. However, there is evidence that the lipid tail is
148 required for CM damage and bacterial killing [28]. Therefore, we hypothesised that the lipid tail of
149 colistin and *de novo* LPS biosynthesis act in concert to cause the severe OM damage required to enable
150 the polymyxin antibiotic to access the CM.

151 To test this, we exposed *P. aeruginosa* to polymyxin B (PMB), which is structurally and
152 functionally homologous to colistin, or a variant of PMB that consists of the cyclic peptide ring but not
153 the lipid tail, known as polymyxin B nonapeptide (PMBN) (Supplementary Fig. 17). Both PMB and
154 PMBN caused sufficient OM disruption to allow NPN to cross the LPS monolayer, albeit at a slower
155 rate and to a lesser degree for PMBN than for PMB (Fig. 4ab). As with colistin, inhibition of LPS
156 biosynthesis did not affect NPN staining in response to either PMB or PMBN (Fig. 4ab). This showed
157 that *de novo* LPS biosynthesis was not required for minor OM disruption caused by the initial
158 interaction of the polymyxin peptide ring with the bacterial surface. However, whilst PMB severely
159 damaged the OM of *P. aeruginosa* cells, as determined by the release of periplasmic β-lactamase into
160 the culture supernatant, this did not occur with PMBN (Fig. 4cd). Furthermore, in addition to the

161 requirement for the lipid tail of the polymyxin, the PMB-mediated release of β-lactamase was also
162 inhibited by cerulenin, confirming the synchronous role of *de novo* LPS biosynthesis in this process
163 (Fig. 4c). These findings are supported by previous work using scanning electron microscopy that
164 demonstrated PMB caused much greater OM damage than PMBN [29].

165 The severe nature of the OM damage mediated by the polymyxin lipid tail appeared crucial
166 for the bactericidal activity of the antibiotic, since PMB, but not PMBN caused permeabilisation of the
167 CM, cell lysis and bacterial death, via a mechanism that was dependent upon *de novo* LPS biosynthesis
168 (Fig. 4efghij). Therefore, whilst the peptide ring of the polymyxin antibiotics weakens the OM
169 sufficiently to allow the uptake of small molecules, the lipid tail is required, in conjunction with *de*
170 *novo* LPS biosynthesis, for the severe damage to the OM that enables PMB to exert its bactericidal
171 effects.

172 Combined, the findings described above, together with other studies, enable the construction
173 of a novel model for the mechanism of action of colistin (Fig. 4k). Firstly, colistin interacts with LPS on
174 the bacterial cell surface via the peptide ring of the antibiotic [11]. This displaces the cations that
175 stabilise LPS molecules in the monolayer, and thus results in minor disruption of the OM [27]. As LPS
176 is synthesised and transported to the cell surface, colistin-induced weakening of the lateral forces
177 between LPS molecules causes LPS to be pushed out and released from the outer leaflet of the OM.
178 The loss of LPS leads to severe OM damage, which is additionally dependent upon the lipid tail of the
179 polymyxin antibiotic. This perturbation to the OM enables the release of periplasmic macromolecules
180 and also facilitates access of the relatively large colistin molecules to the phospholipids in the CM,
181 which the antibiotic disrupts via its detergent-like properties [28]. Once the OM and CM are
182 compromised, the bacteria lyse and die.

183 This revised model addresses key gaps in our understanding of the mechanism of action of
184 polymyxin antibiotics by revealing how these antibiotics cross the OM, explaining why the polymyxin
185 acyl tail is required for bactericidal activity and providing a direct link between OM disruption and the
186 bactericidal activity of the antibiotic. Such insight is needed because colistin is an increasingly

187 important antibiotic for the treatment of infections caused by multi-drug resistant pathogens, but has
188 a treatment failure rate of >70% [3-6]. This study provides two explanations for the extremely high
189 rate of treatment failure. Firstly, bacteria release LPS in response to colistin, which sequesters and
190 inactivates the antibiotic [20]. Secondly, we identify LPS biosynthesis as a bacterial process that could,
191 when decreased, confer colistin tolerance to persister cells with reduced metabolic activity [14,15].
192 To address the high rate of polymyxin treatment failure, there is increasing interest in the use
193 of synergistic antibiotic combinations. For example, it has been shown that novobiocin enhances the
194 activity of polymyxin B by promoting the transport of LPS to the OM and causing LPS release [30].
195 However, the reason why enhanced LPS transport conferred this increased susceptibility to the
196 polymyxin antibiotic was unknown [30]. The data presented in this report provide a mechanistic
197 explanation for this finding by showing that *de novo* LPS production and release from the OM is
198 required for polymyxin activity. Thus, it is anticipated that a greater understanding of the mode of
199 action of polymyxin antibiotics including colistin, combined with our growing appreciation of the
200 processes of LPS biosynthesis and transport, will provide the necessary platform for the identification
201 and development of therapeutic approaches to improve treatment efficacy.
202
203

204 **Methods**

205 **Bacterial strains and growth conditions**

206 The bacterial strains used in this study are listed in Supplementary Table 1. For each experiment,
207 bacteria were grown for 18 h to stationary-phase at 37°C with shaking (180 r.p.m.). All strains were
208 grown in Luria broth (LB; Thermo Fisher Scientific, USA), with the exception of *P. aeruginosa* strain
209 A23, which was cultured in Tryptic Soy Broth (TSB; BD Biosciences, USA), and *K. pneumoniae* strain
210 KPC, which was grown in Brain Heart Infusion Broth (BHI; VWR International, USA). For routine culture
211 of bacteria on solid media, strains were grown on the relevant media as described above
212 supplemented with 1.5% technical agar (BD Biosciences). Growth media were supplemented with
213 chloramphenicol (25 µg ml^{-1}) where required. Enumeration of bacterial c.f.u. was done by plating 10-
214 fold serial dilutions of bacterial cultures on to Mueller-Hinton agar (MHA; Thermo Fisher Scientific)
215 plates. Inoculated agar plates were incubated statically for 18 h in air at 37°C.

216 **Determination of minimum inhibitory concentrations of antibiotics**

217 The minimum inhibitory concentration (MIC) of colistin, polymyxin B, ciprofloxacin, tetracycline,
218 cerulenin (all from Sigma-Aldrich, USA) and CHIR-090 (Axon Medchem, Netherlands) for bacterial
219 strains was determined by the broth microdilution protocol [31]. A microtitre plate was used to
220 prepare a range of antibiotic concentrations in 200 µl Mueller-Hinton broth (MHB) by two-fold serial
221 dilutions. For certain experiments, the media were supplemented with purified LPS from *E. coli* (Sigma-
222 Aldrich). Stationary-phase bacteria were diluted 1000-fold in fresh MHB, and seeded into each well of
223 the microtitre plate to a final concentration of 5 x 10^5 c.f.u. ml^{-1}. The microtitre plates were then
224 incubated statically at 37°C for 18 h in air, after which point the MIC was defined as the lowest
225 antibiotic concentration at which there was no visible growth of bacteria.

226

227

228 **OM disruption assay**

229 To detect damage to the OM of bacteria, the well-established NPN uptake assay was used [32].
230 Stationary-phase bacterial cells were washed in fresh MHB and diluted to an optical density (OD_{600nm})
231 of 0.5 in 5 mM pH 7.2 HEPES buffer (Sigma-Aldrich). This bacterial suspension was added to wells
232 containing the relevant antibiotics in HEPES buffer, as well as the fluorescent probe *N*-phenyl-1-
233 naphthylamine (NPN; Acros Organics, USA) at a final concentration of 10 µM. Samples were placed in
234 a black microtitre plate with clear-bottomed wells and fluorescence measured immediately in a Tecan
235 Infinite 200 Pro multiwell plate reader (Tecan Group Ltd., Switzerland) using an excitation wavelength
236 of 355 nm and an emission wavelength of 405 nm. Fluorescence measurements were obtained every
237 30 seconds for a total period of 60 min.

238 **β-lactamase release assay**

239 *P. aeruginosa* cells were grown overnight with imipenem (Fresenius Kabi, Germany; 2 µg ml^{-1}) to
240 induce expression of the endogenous β-lactamase AmpC [33]. Stationary-phase cells were washed
241 twice by centrifugation (12,300 x *g*, 3 min) followed by resuspension in MHB, and added to 3 ml MHB
242 containing the relevant antibiotics to a final inoculum of 10^8 c.f.u. ml^{-1}. Cultures were then incubated
243 at 37°C with shaking (180 r.p.m.) for 60 min. Every 15 min, aliquots were obtained and bacteria
244 removed by centrifugation (12,300 x *g*, 3 min). The resulting supernatant was then recovered and 200
245 µl added to the wells of a microtitre plate. Nitrocefin (Abcam, UK) was added to each well to a final
246 concentration of 250 µM and the mixture incubated for 2.5 h at room temperature. Absorbance was
247 measured at 490 nm in a Bio-Rad iMark microplate absorbance reader (Bio-Rad Laboratories, USA).

248 **CM disruption assay**

249 As described above for the β-lactamase release assay, stationary-phase bacterial cultures were
250 washed and inoculated into 3 ml MHB containing relevant antibiotics. Cultures were incubated at 37°C
251 with shaking (180 r.p.m.) for 4 h, and every 30 min, aliquots (200 µl) were taken and bacteria isolated

252 by centrifugation (12,300 x *g*, 3 min). Cells were then washed in sterile phosphate-buffered saline
253 (PBS; VWR International), before being added to the wells of a black-walled microtitre plate, and
254 propidium iodide (Sigma-Aldrich) was added to each well at a final concentration of 2.5 µM.
255 Fluorescence was measured immediately in a Tecan Infinite 200 Pro multiwell plate reader (excitation
256 at 535 nm and emission at 617 nm). To account for differences in fluorescence values arising from
257 variations in cell number due to growth inhibition by antibiotics, relative fluorescence unit (r.f.u.)
258 measurements were corrected for OD at 600 nm.

259 **Determination of bacterial lysis**

260 Washed stationary-phase bacteria were inoculated into 3 ml MHB containing the relevant antibiotics,
261 as described above. Cultures were then placed in a shaking incubator (37°C, 180 r.p.m.) for 8 h, and
262 every 30 min, samples (200 µl) were transferred to a microtitre plate, where OD_{595nm} measurements
263 were obtained using a Bio-Rad iMark microplate absorbance reader.

264 **Determination of bactericidal activity of antibiotics**

265 Stationary-phase bacteria were washed and added to a final inoculum of 10^8 c.f.u. ml^{-1} in 3 ml MHB
266 containing the relevant antibiotics. Cultures were incubated with shaking (37°C, 180 r.p.m.) for 8 h.
267 Bacterial survival was determined after 2, 4, 6 and 8 h through enumeration of c.f.u. counts by serial
268 dilution in 10-fold steps of cultures in 200 µl sterile PBS.

269 **Emergence of colistin resistance assay**

270 Bacteria in stationary-phase were washed and added to 3 ml MHB containing colistin (2 µg ml^{-1}), and
271 survival was determined every 2 h up to 8 h as described above. Following 8 h incubation, the MIC of
272 colistin against the recovered bacterial population was determined by the broth microdilution method
273 as stated above. The recovered bacteria were washed by centrifugation (12,300 x *g*, 3 min) and
274 resuspension in fresh LB, before being inoculated into 3 ml LB and grown for 18 h to stationary-phase
275 in a shaking incubator (37°C, 180 r.p.m.). This population was then exposed to colistin (2 µg ml^{-1}) for a

276 second time, with bacterial viability again measured over 8 h. After the second colistin exposure, the
277 MIC of colistin was again determined, before the bacteria were exposed to colistin for a third time,
278 with associated measurements of survival and the colistin MIC of the recovered population obtained.

279 **Determination of membrane lipid release from bacteria**

280 The supernatant from bacterial cultures exposed to colistin was recovered every 2 h by centrifugation
281 (12,300 x *g*, 3 min). Recovered supernatants were mixed with 5 µl of FM-4-64 styryl dye (Thermo Fisher
282 Scientific) at a final concentration of 5 µg ml^{-1} in the wells of a black-walled microtitre plate.
283 Fluorescence was measured using a Tecan Infinite 200 Pro multiwell plate reader (excitation at 565
284 nm, emission at 600 nm) to quantify the phospholipid released into the supernatant by bacteria
285 exposed, or not, to colistin.

286 **Determination of LPS release from bacteria**

287 The chromogenic Limulus Amebocyte Lysate (LAL) assay (all reagents from Thermo Fisher Scientific)
288 was used to detect and quantify the LPS released from bacteria into the culture supernatant as
289 described previously [34]. Samples of cell-free culture supernatant (50 µl) were equilibrated to 37°C
290 and loaded into the wells of a microtitre plate at the same temperature. Limulus amebocyte lysate
291 reagent (50 µl) was added to each well, and the mixture incubated at 37°C for 10 min. Chromogenic
292 substrate solution (100 µl, 2 mM) was subsequently added to each well and the microtitre plate was
293 incubated for a further 6 min at 37°C. The enzymatic reaction was stopped by adding 50 µl of 25%
294 acetic acid to each well, and the presence of LPS was determined by measuring absorbance at 405 nm
295 in a Tecan Infinite 200 Pro multiwell plate reader. A standard curve was generated using an *E. coli*
296 endotoxin standard stock solution, which enabled the conversion of A_{405nm} values into concentrations
297 of LPS in the supernatant.

298

299

300 **Extraction of LPS and visualisation by silver staining**

301 LPS was extracted from the supernatant of bacterial cultures as previously described, and the ultrafast
302 silver staining method was used for visualising LPS on 12% SDS-PAGE gels [35,36]. Stationary-phase
303 bacteria (1 ml) were washed and inoculated to a final concentration of 10^8 c.f.u. ml^{-1} into 9 ml MHB
304 containing the relevant antibiotics. Bacterial cultures were incubated with shaking (37°C, 180 r.p.m.)
305 for up to 8 h, and 9 ml of supernatant was recovered following centrifugation (3270 x *g*, 30 min) and
306 filter sterilisation (0.2 µm filter) to remove bacterial cells. Absolute ethanol (30 ml) was added to the
307 supernatant and the samples were stored at -20°C for 30 min. The precipitate was pelleted by
308 centrifugation at 3270 x *g* for 30 min and resuspended in 500 µl Laemmli buffer. Precipitated protein
309 was removed by digesting with proteinase K (50 µg) overnight, before 25 µl was run on an acrylamide
310 mini-gel system. For certain experiments, the concentration of LPS in these samples was determined
311 using the LAL assay described above. Electrophoresis was carried out at 12 mA in the stacking gel and
312 25 mA in the separating gel.

313 LPS preparations subjected to SDS-PAGE were visualised by first oxidising the gel with 200 ml 40%
314 ethanol-5% acetic acid containing 0.7% sodium periodate for 20 min at room temperature. The gel
315 was washed three times with distilled water for 5 min each time. Silver staining solution was freshly
316 prepared by first adding concentrated ammonium hydroxide (4 ml) to 0.1 M sodium hydroxide (56 ml)
317 before adding 200 ml of distilled water. Silver nitrate (20% w/v, 10 ml) was added in drops whilst
318 stirring, before the addition of 30 ml of distilled water. This solution was used to stain the gel for 10
319 min, which was subsequently washed with distilled water for 5 min three times. The silver stain was
320 developed with 200 ml of water containing formaldehyde (100 µl of a 37% w/v solution) and 10 mg
321 citric acid. Development was stopped using 10% acetic acid before the gel was washed in distilled
322 water and then imaged using a Bio-Rad Gel Doc EZ Imager (Bio-Rad Laboratories).

323

324

325 **Determination of colistin activity**

326 The activity of colistin during incubation with *P. aeruginosa* was determined using an established zone
327 of inhibition assay [37]. Bacterial cultures exposed to colistin were centrifuged (12,300 x *g*, 3 min), and
328 the supernatant recovered. Stationary-phase *P. aeruginosa* was diluted in MHB to a concentration of
329 10^6 c.f.u. ml^{-1}, before 60 µl of this bacterial culture was spread across the surface of an MHA plate and
330 allowed to air dry. Wells measuring 10 mm in diameter were made in the agar plate and filled with
331 130 µl of the culture supernatant recovered from colistin-treated bacterial populations. Agar plates
332 were incubated in air statically at 37°C for 16 h and the zone of growth inhibition around each well
333 was measured at four perpendicular points. A standard plot was generated showing a linear
334 relationship between the size of the zone of inhibition and colistin concentration, which enabled
335 inhibitory zone size to be converted to percentage colistin activity. Colistin activity was also measured
336 after 4 h incubation (37°C, end-over-end rotation) in the presence of purified LPS, or in MHB alone
337 over the course of 8 h in a shaking incubator (37°C, 180 r.p.m.).

338 **Determination of colistin binding to bacterial cells**

339 Colistin was labelled with the fluorophore BoDipy FL SE D2184 (Thermo Fisher Scientific) by incubating
340 100 µl of the BoDipy NHS ester compound (10 mg ml^{-1} in dimethyl sulfoxide, DMSO) with 250 µl colistin
341 (10 mg ml^{-1} in water) and 650 µl sodium bicarbonate (0.2 M, pH 8.5) for 2 h at 37°C. BoDipy molecules
342 that had not bound to colistin were removed by dialysis using a Float-A-Lyser G2 dialysis device
343 (Spectrum Laboratories, USA) that had a molecular weight cut-off of 0.5 kDa. Dialysis was carried out
344 at 4°C against sterile distilled water, which was changed four times during the course of the 24 h
345 dialysis period. Time of flight mass spectrometry analysis confirmed successful labelling with the
346 fluorophore, and the antibiotic activity of BoDipy-labelled colistin was assessed using MIC and
347 bacterial survival assays, as described above.

348 Stationary-phase bacterial cultures were washed and added to 3 ml MHB containing the relevant
349 antibiotics at an inoculum of 10^8 c.f.u. ml^{-1}, as stated above. Cultures were incubated for 8 h (37°C,

350 180 r.p.m.), and every 2 h bacteria were recovered by centrifugation (12,300 x *g*, 3 min). The pelleted
351 cells were washed thoroughly by four rounds of centrifugation (12,300 x *g*, 3 min) and resuspension
352 in fresh MHB in order to remove any BoDipy-colistin that was not attached to the bacteria. BoDipy-
353 colistin bound to the bacterial cells was quantified by measuring fluorescence in a Tecan Infinite 200
354 Pro multiwell plate reader (excitation at 490 nm, emission at 525 nm) using a black-walled clear-
355 bottomed microtitre plate. The relative fluorescence values of samples containing cells were corrected
356 using OD_{600nm} measurements.

357 **Bacterial growth assay**

358 Stationary-phase bacteria were diluted 1000-fold in fresh MHB, and 4 µl was seeded into the wells of
359 a microtitre plate containing 200 µl MHB and the relevant antibiotics at a final density of 5 x 10^5 c.f.u.
360 ml^{-1}. The microtitre plate was incubated with shaking (180 r.p.m.) at 37°C in a Tecan Infinite 200 Pro
361 multiwell plate reader and optical density measurements were taken at 600 nm every 15 min over 14
362 h.

363 **Scanning electron microscope (SEM) imaging**

364 Stationary-phase bacterial cells were washed and inoculated into 3 ml MHB containing the relevant
365 antibiotics as described above. After 2 h incubation (37°C, 180 r.p.m.), 25 µl of the cultures was spotted
366 onto a silicon chip (University Wafer, USA) and allowed to air-dry. The sample was fixed using
367 glutaraldehyde (2.5% in 0.01 M PBS) for 30 min at room temperature, before the chip was washed
368 three times in PBS (0.01 M). The sample was dehydrated using increasing concentrations of ethanol
369 (10%, 30%, 50%, 70%, 90% and 100%) for 5 min each time, before a coating of 15 nm chromium
370 (Quorum Technologies, UK) was applied. Representative images of bacterial cells were obtained using
371 a LEO Gemini 1525 field emission gun scanning electron microscope (FEG SEM; Carl Zeiss Microscopy
372 GmbH, Germany).

373

374 **Statistical analyses**

375 Experiments were performed on at least three independent occasions, and resulting data are
376 presented as the arithmetic mean of these biological repeats, unless stated otherwise. Error bars
377 represent the standard deviation of the mean. For single comparisons, a two-tailed unpaired Student's
378 *t*-test was used to analyse the data. For multiple comparisons at a single time point or concentration,
379 data were analysed using either a one-way analysis of variance (ANOVA) for parametric data, or a
380 Kruskal-Wallis test for non-parametric data. Where data were obtained at several different time points
381 or concentrations, a two-way ANOVA was used for statistical analysis. Appropriate post-hoc tests
382 (Dunnett's, Welch's, Sidak's, Holm-Sidak) were carried out to correct for multiple comparisons, with
383 details provided in the figure legends. Asterisks on graphs indicate significant differences between
384 data, and the corresponding p-values are reported in the figure legend. All statistical analyses were
385 performed using GraphPad Prism 7 software (GraphPad Software Inc., USA).

386 **Data availability**

387 The data that support the findings of this study are available from the corresponding author upon
388 reasonable request.

389

390

Acknowledgements

Alain Filloux, Aishwarya Krishna (both Imperial College London) and Alireza Abdolrasouli (Imperial College Healthcare NHS Trust) are gratefully acknowledged for providing bacterial strains. Lisa Haigh (Department of Chemistry Mass Spectrometry Facility, Imperial College London) is thanked for processing and analysing mass spectrometry samples. A.S. is supported by a PhD studentship funded by a Medical Research Council Doctoral Training Award to Imperial College London (MR/N014103/1). A.K. is funded by the DFG German Research Foundation (KL3191/1-1). M.B. and M.M.S gratefully acknowledge support from the Rosetrees Trust (M300-CD1). L.E.E. and A.M.E. acknowledge funding from the Wellcome Trust (204337/Z/16/Z). A.M.E. also acknowledges support from the National Institute for Health Research (NIHR) Imperial Biomedical Research Centre (BRC), and declares funding from Shionogi & Co., Ltd for an unrelated project.

Author contributions

A.S. and A.M.E. designed the experiments. A.S., A.K. and M.B. performed experiments. All authors analysed data and contributed to writing of the manuscript.

Competing interests

The authors declare no competing financial interests.

411 **References**

412 1. Biswas, S., Brunel, J. M., Dubus, J. C., Reynaud-Gaubert, M., Rolain, J. M. Colistin: an update on the
413 antibiotic of the 21st century. *Expert Rev. Anti. Infect. Ther.* **10(8),** 917-934 (2012).
414 2. Nation, R. L. *et al.* Framework for optimisation of the clinical use of colistin and polymyxin B: the
415 Prato polymyxin consensus. *Lancet Infect. Dis.* **15(2),** 225-234 (2015).
416 3. Linden, P.K. *et al.* Use of parenteral colistin for the treatment of serious infection due to
417 antimicrobial-resistant *Pseudomonas aeruginosa*. *Clin. Infect. Dis.* **37(11),** e154-160 (2003).
418 4. Falagas, M. E. *et al.* Colistin therapy for microbiologically documented multidrug-resistant Gram-
419 negative bacterial infections: a retrospective cohort study of 258 patients. *Int. J. Antimicrob. Agents.*
420 **35(2),** 194-199 (2010).
421 5. Gales, A. C., Jones, R. N., Sader, H. S. Contemporary activity of colistin and polymyxin B against a
422 worldwide collection of Gram-negative pathogens: results from the SENTRY Antimicrobial
423 Surveillance Program (2006-09). *J. Antimicrob. Chemother.* **66(9),** 2070-2074 (2011).
424 6. Paul, M. *et al.* Colistin alone versus colistin plus meropenem for treatment of severe infections
425 caused by carbapenem-resistant Gram-negative bacteria: an open-label, randomised controlled
426 trial. *Lancet Infect. Dis.* **18(4),** 391-400 (2018).
427 7. Falagas, M. E., Kasiakou, S. K. Colistin: the revival of polymyxins for the management of multidrug-
428 resistant gram-negative bacterial infections. *Clin. Infect. Dis.* **40(9),** 1333-1341 (2005).
429 8. Tamma, P. D. *et al.* The use of intravenous colistin among children in the United States: results from
430 a multicenter, case series. *Pediatr. Infect. Dis. J.* **32(1),** 17-22 (2013).
431 9. Vaara, M., Vaara, T. Sensitization of Gram-negative bacteria to antibiotics and complement by a
432 nontoxic oligopeptide. *Nature.* **303(5917),** 526-528 (1983).
433 10. Moore, R. A., Bates, N. C., Hancock, R. E. Interaction of polycationic antibiotics with *Pseudomonas*
434 *aeruginosa* lipopolysaccharide and lipid A studied by using dansyl-polymyxin. *Antimicrob. Agents*
435 *Chemother.* **29(3),** 496-500 (1986).

436 11. Velkov, T., Thompson, P. E., Nation, R. L., Li, J. Structure--activity relationships of polymyxin
437 antibiotics. *J. Med. Chem.* **53(5),** 1898-916 (2010).
438 12. Hancock, R. E., Bell, A. Antibiotic uptake into gram-negative bacteria. *Eur. J. Clin. Microbiol. Infect.*
439 *Dis.* **7(6),** 713-720 (1988).
440 13. MacNair, C.R. *et al.* Overcoming mcr-1 mediated colistin resistance with colistin in combination with
441 other antibiotics. *Nat. Commun.* **9(1),** 458 (2018).
442 14. Mulcahy, L. R., Burns, J. L., Lory, S., Lewis, K. Emergence of *Pseudomonas aeruginosa* strains
443 producing high levels of persister cells in patients with cystic fibrosis. *J. Bacteriol.* **192(23),** 6191-
444 6199 (2010).
445 15. Chung, E. S., Wi, Y. M., Ko, K. S. Variation in formation of persister cells against colistin in
446 *Acinetobacter baumannii* isolates and its relationship with treatment failure. *J. Antimicrob.*
447 *Chemother.* **72(7),** 2133-2135 (2017).
448 16. Fisher, R. A., Gollan, B., Helaine, S. Persistent bacterial infections and persister cells. *Nat. Rev.*
449 *Microbiol.* **15(8),** 453-464 (2017).
450 17. Markou, N. *et al.* Colistin serum concentrations after intravenous administration in critically ill
451 patients with serious multidrug-resistant, gram-negative bacilli infections: a prospective, open-label,
452 uncontrolled study. *Clin. Ther.* **30(1),** 143-151 (2008).
453 18. Garonzik, S. M. *et al.* Population pharmacokinetics of colistin methanesulfonate and formed colistin
454 in critically ill patients from a multicenter study provide dosing suggestions for various categories of
455 patients. *Antimicrob. Agents Chemother.* **55(7),** 3284-3294 (2011).
456 19. Toyofuku, M., Nomura, N., Eberl, L. Types and origins of bacterial membrane vesicles. *Nat. Rev.*
457 *Microbiol.* (2018).
458 20. Yokota, S. I. *et al.* Release of large amounts of lipopolysaccharides from *Pseudomonas aeruginosa*
459 cells reduces their susceptibility to colistin. *Int. J. Antimicrob. Agents.* **51(6),** 888-896 (2018).
460 21. Henderson, J. C. *et al.* The Power of Asymmetry: Architecture and Assembly of the Gram-Negative
461 Outer Membrane Lipid Bilayer. *Annu. Rev. Microbiol.* **70,** 255-278 (2016).

462 22. May, K.L., Silhavy, T. J. The *Escherichia coli* Phospholipase PldA Regulates Outer Membrane
463 Homeostasis via Lipid Signaling. *MBio.* **9(2),** (2018).
464 23. Ried, G., Hindennach, I., Henning, U. Role of lipopolysaccharide in assembly of *Escherichia coli* outer
465 membrane proteins OmpA, OmpC, and OmpF. *J. Bacteriol.* **172(10),** 6048–6053 (1990).
466 24. McClerren, A. L. *et al.* A slow, tight-binding inhibitor of the zinc-dependent deacetylase LpxC of lipid
467 A biosynthesis with antibiotic activity comparable to ciprofloxacin. *Biochemistry.* **44(50),** 16574-
468 16583 (2005).
469 25. Sutterlin, H. A. *et al.* Disruption of lipid homeostasis in the Gram-negative cell envelope activates a
470 novel cell death pathway. *Proc. Natl. Acad. Sci. U. S. A.* **113(11),** e1565-1574 (2016).
471 26. Pristovsek, P., Kidric, J. Solution structure of polymyxins B and E and effect of binding to
472 lipopolysaccharide: an NMR and molecular modeling study. *J. Med. Chem.* **42(22),** 4604-4613 (1999).
473 27. Schindler, M., Osborn, M. J. Interaction of divalent cations and polymyxin B with lipopolysaccharide.
474 *Biochemistry.* **18(20),** 4425-4430 (1979).
475 28. Naoki, S. *et al.* The Contribution of the N-Terminal Structure of Polymyxin B Peptides to Antimicrobial
476 and Lipopolysaccharide Binding Activity. *Bull. Chem. Soc. Jpn.* **77(10),** (2004).
477 29. Dixon, R. A., Chopra, I. Leakage of periplasmic proteins from *Escherichia coli* mediated by polymyxin
478 B nonapeptide. *Antimicrob. Agents Chemother.* **29(5),** 781-788 (1986).
479 30. Mandler, M. D. *et al.* Novobiocin Enhances Polymyxin Activity by Stimulating Lipopolysaccharide
480 Transport. *J. Am. Chem. Soc.* **140(22),** 6749-6753 (2018).
481 31. Wiegand, I., Hilpert, K., Hancock, R. E. Agar and broth dilution methods to determine the minimal
482 inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* **3(2),** 163-175 (2008).
483 32. Helander, I. M., Mattila-Sandholm, T. Fluorometric assessment of gram-negative bacterial
484 permeabilization. *J. Appl. Microbiol.* **88(2),** 213-219 (2000).
485 33. Bagge, N. *et al. Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global
486 gene expression and beta-lactamase and alginate production. *Antimicrob. Agents Chemother.* **48(4),**
487 1175-1187 (2004).

488 34. Roslansky, P. F., Novitsky, T. J. Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans.
489 *J. Clin. Microbiol.* **29(11),** 2477-2483 (1991).
490 35. Crosby, H. A., Bion, J. F., Penn, C. W., Elliott, T. S. Antibiotic-induced release of endotoxin from
491 bacteria *in vitro*. *J. Med. Microbiol.* **40(1),** 23-30 (1994).
492 36. Fomsgaard, A., Freudenberg, M. A., Galanos, C. Modification of the silver staining technique to
493 detect lipopolysaccharide in polyacrylamide gels. *J. Clin. Microbiol.* **28(12),** 2627-2631 (1990).
494 37. Pader, V. *et al. Staphylococcus aureus* inactivates daptomycin by releasing membrane
495 phospholipids. *Nat. Microbiol.* **2,** 16194 (2016).
496

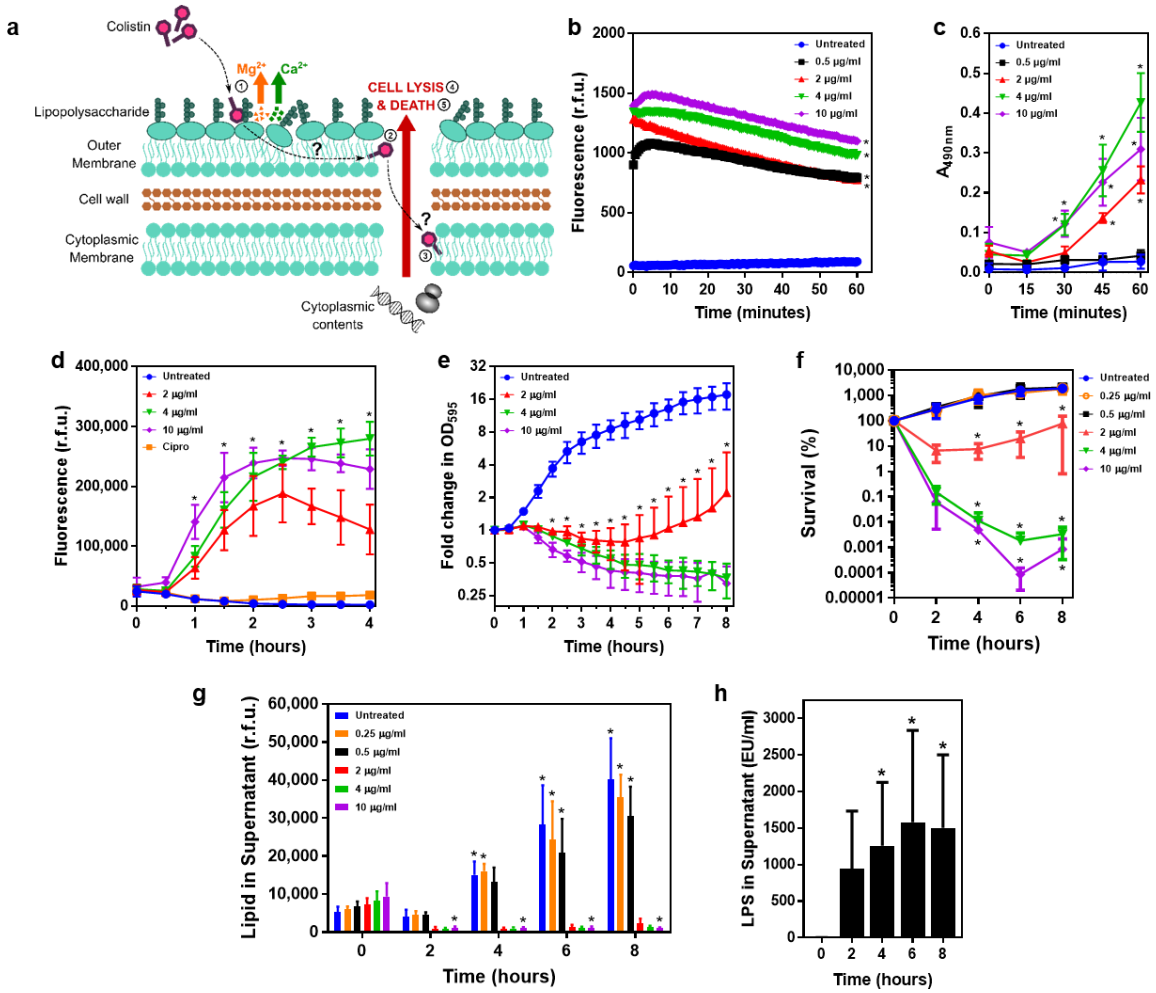

Figure 1. Colistin-mediated membrane damage causes the release of LPS but not membrane lipid from the surface of *P. aeruginosa*.

a, Diagrammatic representation of the current hypothesised mechanism of action of colistin: colistin binds to LPS on the OM, causing displacement of cations that form bridges between LPS molecules and leading to membrane disruption (1). The antibiotic then crosses the OM via 'self-directed uptake' (2). Colistin subsequently disrupts the CM (3), resulting in cell lysis (4) and bacterial death (5). **b,** Relative fluorescence of *P. aeruginosa* PA14 cells incubated with the indicated concentrations of colistin and the phospholipid-reactive membrane impermeant dye NPN (10 µM). Disruption of the LPS monolayer enables NPN to access phospholipids in the inner leaflet of the OM, resulting in fluorescence (n=4; *p<0.05 compared to untreated bacteria, error bars are omitted for clarity). **c,** Release of periplasmic β-lactamase into the culture supernatant in response to colistin as determined using the chromogenic substrate nitrocefin (250 µM), which produces a product detectable at A_{490nm}

510 (n=4; *p<0.001 compared to untreated bacteria). **d,** Colistin-mediated permeabilisation of the CM of
511 *P. aeruginosa* PA14 as determined by the binding of the membrane impermeant dye propidium iodide
512 (2.5 µM) to DNA, which results in fluorescence (n=3 in duplicate; *p<0.01 compared to untreated cells
513 for all colistin-exposed populations). **e,** Lysis or growth of *P. aeruginosa* PA14 exposed, or not, to
514 colistin over time as determined by OD_{595mn} measurements (n=4; *p<0.05 compared to untreated
515 bacteria for all colistin-exposed populations). **f,** Killing or replication of *P. aeruginosa* PA14 exposed,
516 or not, to colistin over time, as determined by c.f.u. counts (n=4; *p<0.01 compared to untreated
517 cells). **g,** The concentration of phospholipids released from the surface of *P. aeruginosa* PA14 over
518 time in the absence or presence of colistin, as determined by reactivity with the fluorescent probe
519 FM-4-64 (n=4; *p<0.05 compared to values at 0 hours). **h,** The presence of LPS in the culture
520 supernatant following exposure of *P. aeruginosa* PA14 to colistin (2 µg ml^{-1}), as determined using the
521 Limulus Amebocyte Lysate assay (n=3; *p<0.05 compared to 0 hours). Data in **a-g** were analysed by a
522 two-way ANOVA with Dunnett's post-hoc test. Data in **h** were analysed by a Kruskal-Wallis test with
523 Dunn's post-hoc test. Data are presented as the arithmetic mean, and error bars, where shown,
524 represent the standard deviation of the mean. r.f.u.: relative fluorescence units; OD: optical density;
525 EU: endotoxin units; OM: outer membrane; CM: cytoplasmic membrane; NPN: *N*-phenyl-1-
526 naphthylamine.
527
528

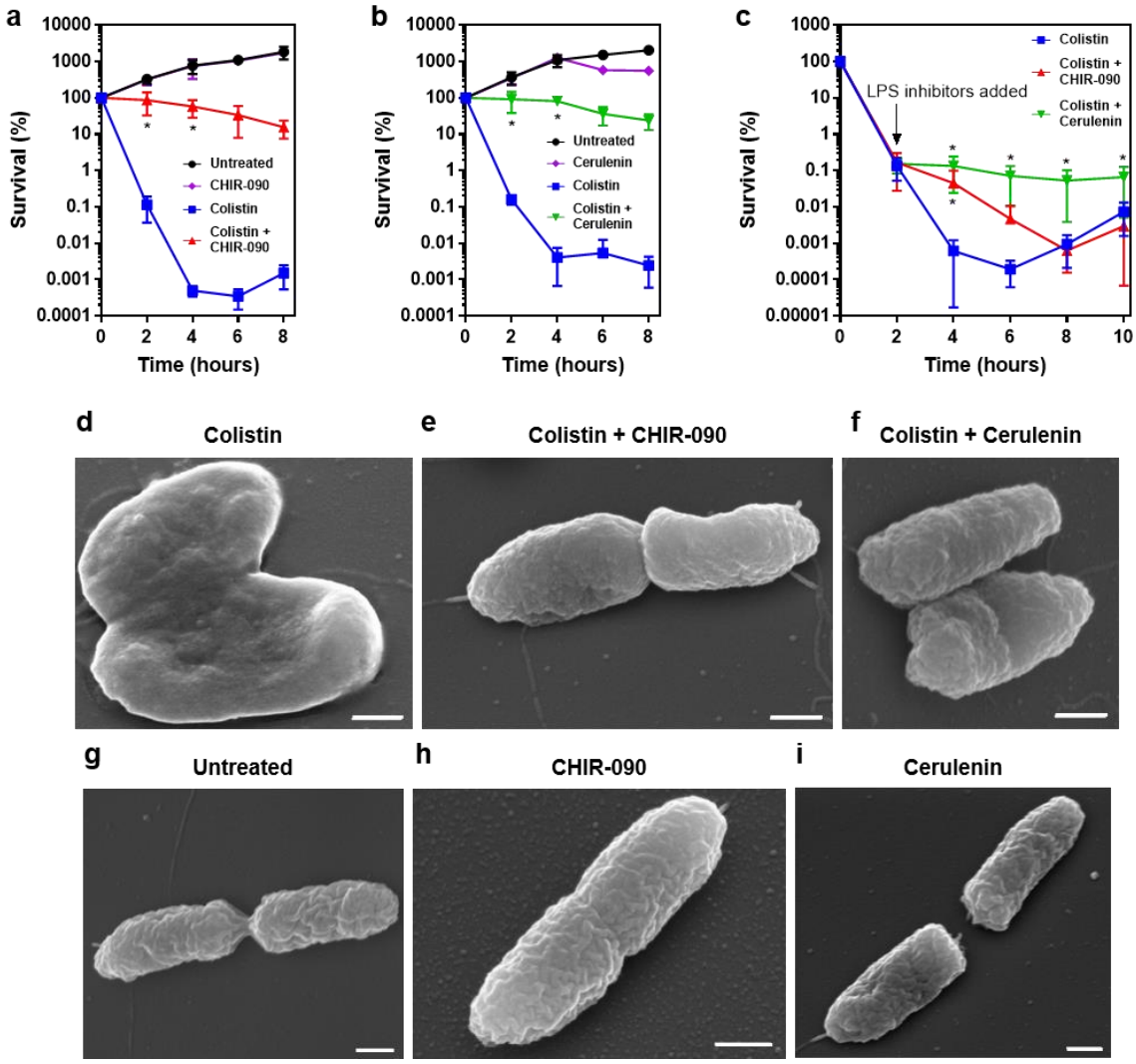

Figure 2. The bactericidal activity of colistin requires *de novo* LPS biosynthesis.

a, Survival over time of *P. aeruginosa* PA14 exposed to colistin (4 µg ml^{-1}), the LPS biosynthesis inhibitor CHIR-090 (0.125 µg ml^{-1}), both antibiotics or neither (n=4; *p<0.01 compared to colistin alone). Values for CHIR-090 alone and untreated overlap. **b,** Survival of *P. aeruginosa* PA14 during exposure to colistin (4 µg ml^{-1}), the LPS biosynthesis inhibitor cerulenin (32 µg ml^{-1}), both antibiotics or neither (n=4; *p<0.0001 compared to colistin alone). **c,** Survival of *P. aeruginosa* PA14 exposed to colistin (4 µg ml^{-1}) alone for 2 hours followed by no further treatment, or the addition of either CHIR-090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) (n=3 in duplicate; *p<0.05 compared to colistin alone). **d-i,** Representative scanning electron microscopy (SEM) images of *P. aeruginosa* PA14 exposed to colistin (4 µg ml^{-1}) only **(d)**, colistin (4 µg ml^{-1}) and CHIR-090 (0.125 µg ml^{-1}) **(e)**, colistin (4 µg ml^{-1}) and

541 cerulenin (32 µg ml^{-1}) **(f)**, in the absence of antibiotics **(g)**, exposed to CHIR-090 (0.125 µg ml^{-1}) only

542 **(h)**, or cerulenin (32 µg ml^{-1}) only **(i)**, for 2 hours in each case (Scale bars: 200 nm). Data in **a-c** were

543 analysed by a two-way ANOVA with Sidak's **(a, b)** or Holm-Sidak's **(c)** post-hoc tests. For panels **a, b,**

544 and **c,** data are presented as the arithmetic mean, and error bars represent the standard deviation of

545 the mean.

546

547

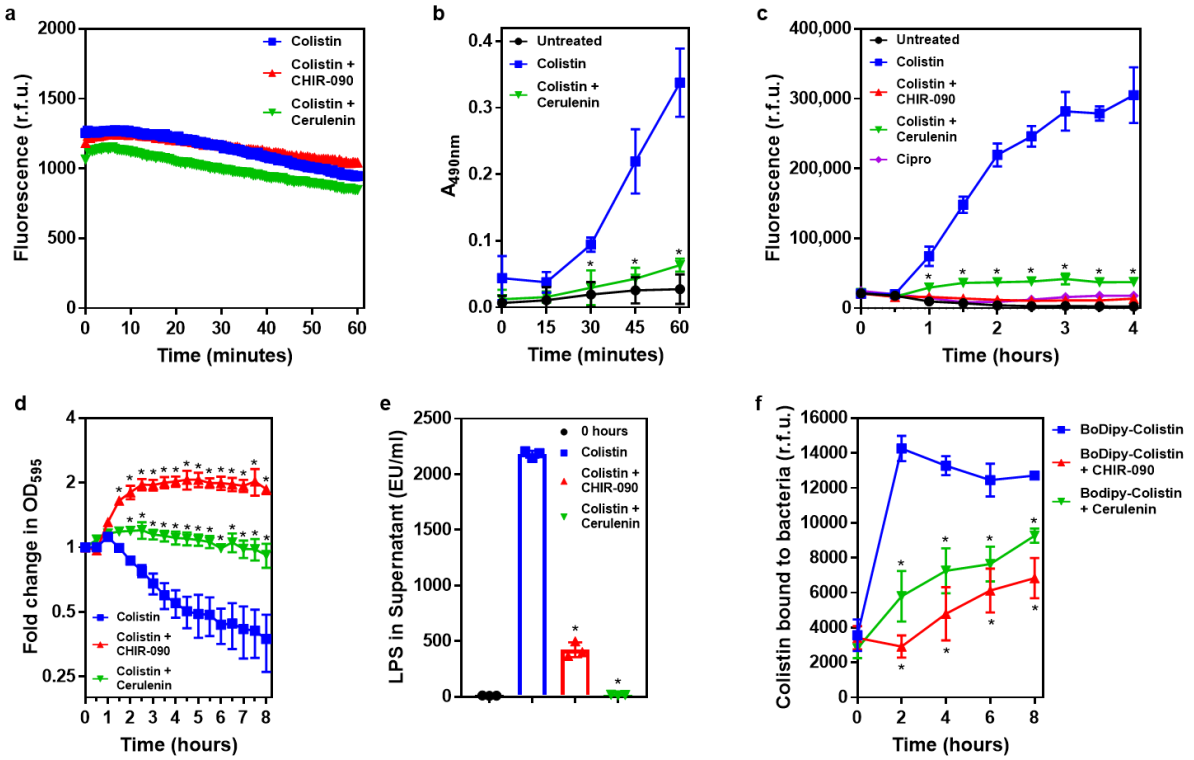

Figure 3. Colistin-mediated LPS release requires *de novo* LPS biosynthesis and enables the antibiotic to access the cytoplasmic membrane.

a, OM disruption of *P. aeruginosa* PA14 cells during exposure to colistin (4 µg ml^{-1}) in the absence or presence of CHIR-090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) as assessed by fluorescence generated with NPN dye (10 µM) (n=4; no significant differences ($p>0.05$) between datasets, error bars are omitted for clarity). **b,** Release of β-lactamase from the periplasm of *P. aeruginosa* PA14 cells exposed to colistin (4 µg ml^{-1}) in the absence or presence of cerulenin (32 µg ml^{-1}) as detected using 250 µM nitrocefin (n=4; *$p<0.01$ compared to colistin alone). **c,** Permeabilisation of the CM of *P. aeruginosa* PA14 cells during incubation with colistin (4 µg ml^{-1}) in the absence or presence of CHIR-090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}), or with a bactericidal concentration of ciprofloxacin (4 µg ml^{-1}), as determined using 2.5 µM propidium iodide (n=3 in duplicate; *$p<0.0001$ compared to colistin alone for all populations below the asterisks). **d,** Lysis of *P. aeruginosa* PA14 cells during exposure to colistin (4 µg ml^{-1}) in the absence or presence of CHIR-090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) as measured using OD_{595nm} readings (n=4; *$p<0.05$ compared to colistin alone). **e,** Binding of BoDipy-labelled colistin (5 µg ml^{-1}) to *P. aeruginosa* PA14 cells in the absence or presence of CHIR-090 (0.125 µg ml^{-1}) or

564 cerulenin (32 µg ml^{-1}) (n=4; *p<0.0001 compared to BoDipy-colistin alone). **f,** Concentration of LPS in
565 the supernatant of cultures of *P. aeruginosa* PA14 exposed for 4 hours to colistin (4 µg ml^{-1}) in the
566 absence or presence of CHIR-090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) as determined with the
567 Limulus Amebocyte Lysate assay (n=3; *p<0.0001 compared to colistin alone). Data in **a-d, f** were
568 analysed by a two-way ANOVA with Dunnett's post-hoc test. Data in **e** were analysed using a one-way
569 ANOVA with Dunnett's post-hoc test. Data are presented as the arithmetic mean, and error bars,
570 where shown, represent the standard deviation of the mean. r.f.u.: relative fluorescence units; OD:
571 optical density; EU: endotoxin units; OM: outer membrane; NPN: *N*-phenyl-1-naphthylamine; CM:
572 cytoplasmic membrane.
573
574

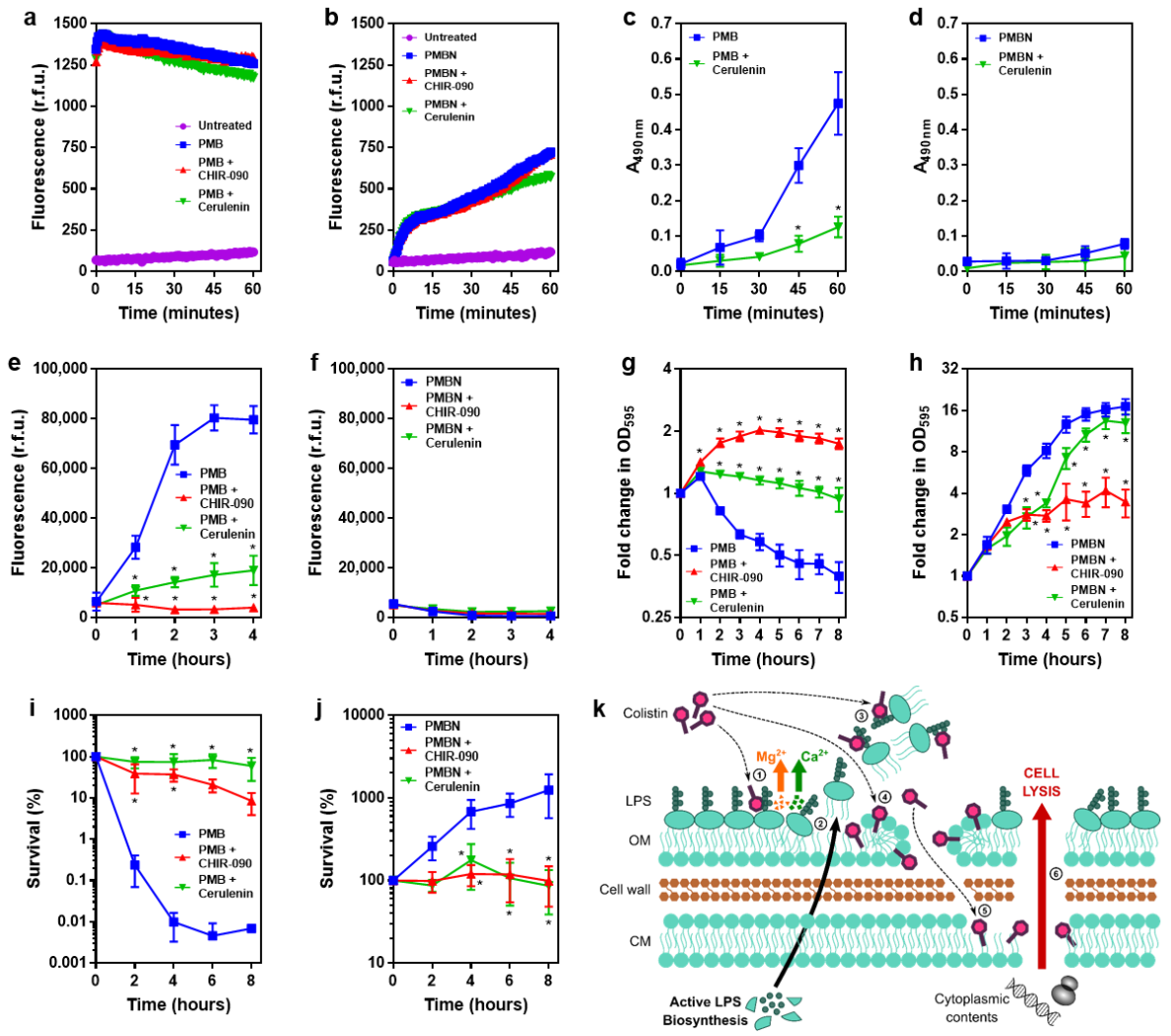

Figure 4. The polymyxin acyl tail is required for damage of both the outer and cytoplasmic membranes.

a, b, Permeability of the OM of *P. aeruginosa* PA14 during exposure to 4 µg ml^{-1} PMB **(a)** or an equivalent molar concentration (2.8 µg ml^{-1}) of PMBN **(b)**, in the absence or presence of CHIR-090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}), as detected using 10 µM NPN dye (n=4; all data with LPS biosynthesis inhibitors not significantly different (p>0.05) compared to PMB or PMBN alone, error bars are omitted for clarity). **c, d,** Release of periplasmic β-lactamase from *P. aeruginosa* PA14 incubated with 4 µg ml^{-1} PMB **(c)** or 2.8 µg ml^{-1} PMBN, in the absence or presence of cerulenin (32 µg ml^{-1}), as determined with 250 µM nitrocefin (n=4; *p<0.0001 compared to PMB or PMBN alone). **e, f,** Disruption to the CM of *P. aeruginosa* PA14 caused by 4 µg ml^{-1} PMB **(e)** or 2.8 µg ml^{-1} of PMBN **(f)**, in

587 the absence or presence of CHIR-090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}), quantified using 2.5 µM
588 propidium iodide (n=4; *p<0.0001 compared to PMB or PMBN alone). **g, h,** Lysis or growth of *P.*
589 *aeruginosa* PA14 exposed to 4 µg ml^{-1} PMB **(g)** or 2.8 µg ml^{-1} PMBN **(h)**, in the absence or presence of
590 CHIR-090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}), as assessed by OD_{595nm} readings (n=4; *p<0.001
591 compared to PMB or PMBN alone). **i, j,** Survival of *P. aeruginosa* PA14 exposed to 4 µg ml^{-1} PMB **(i)** or
592 2.8 µg ml^{-1} PMBN **(j)**, in the absence or presence of CHIR-090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1})
593 (n=4; *p<0.05 compared to PMB or PMBN alone). **k,** Diagrammatic representation of the proposed
594 mechanism of action of colistin based on the findings described here: colistin binds to LPS on the OM,
595 leading to the displacement of cations that form bridges between LPS molecules and causing minor
596 membrane disruption (1). The weakening of the intermolecular bridges in the LPS monolayer means
597 that as *de novo* LPS is synthesised and transported to the cell surface, LPS molecules are forced out
598 and released by the bacteria (2). This released LPS sequesters and inactivates free colistin
599 extracellularly (3). Loss of LPS from the cell surface also results in significant damage to the OM via the
600 action of the polymyxin lipid tail (4), and thus facilitates the access of colistin to the periplasm. This
601 enables colistin to bind to the CM (5), which it disrupts, culminating in the loss of cytoplasmic contents,
602 cell lysis and bacterial death (6). Data in **a, b, e-j** were analysed by a two-way ANOVA with Dunnett's
603 post-hoc test. Data in **c, d** were analysed by a two-way ANOVA with Sidak's post-hoc test. Data are
604 presented as the arithmetic mean, and error bars, where shown, represent the standard deviation of
605 the mean. r.f.u.: relative fluorescence units; OD: optical density; OM: outer membrane; PMB:
606 polymyxin B; PMBN: polymyxin B nonapeptide; NPN: *N*-phenyl-1-naphthylamine; CM: cytoplasmic
607 membrane.
608